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Influence of hormonal status, ageing and smoking on autonomic cardiovascular control and endothelial function

By

Etain Tansey B.A. Mod.

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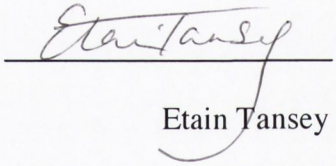
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I declare that this thesis is entirely my own work except for the plasma analysis of c-Reactive Protein using Particle-Enhanced Immunoturbidimetric Assay, which was performed by Pat Kelly at the Adelaide and Meath Hospital, Tallaght, Co. Dublin and is acknowledged within this thesis. This work has not been previously submitted as an exercise for a degree to this or any other University. I give my permission to the library to lend or copy this thesis.



Etain Tansey

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ABSTRACT

Ageing, hypertension and smoking are known risk factors for cardiovascular disease. By contrast female sex hormones are known to have cardioprotective qualities; however, their underlying mechanisms of action have yet to be fully elucidated. The primary objective of this thesis was to investigate the interactive effects of hormonal status, ageing, hypertension and smoking on the autonomic nervous system and endothelial function, two areas where deficits in normal function can have potentially adverse consequences as regards cardiovascular mortality. The methods employed included the use of three well-known physiological stressors and the assessment of baroreflex sensitivity (BRS). Endothelial health was assessed via blood flow responses to 3 minutes arterial occlusion and standard markers of endothelial function.

Resting blood pressure in young women was not effected by hormonal status. Postmenopausal women exhibited elevated resting blood pressure when compared with younger subjects. This increase was due solely to the ageing process and unrelated to hormonal milieu since postmenopausal women taking hormone replacement therapy (HRT) had similar pressure values. Postmenopausal women exhibited an elevation in systolic blood pressure relative to premenopausal women during the stressors, except for the cold pressor test, however younger subjects reacted with elevated total peripheral resistance (TPR) during a sustained handgrip and elevated diastolic pressure during mental stress. The elevation in systolic pressure in postmenopausal women during these tests was most likely due to their greater wave reflectance, indicating arterial stiffening.

Premenopausal and postmenopausal women did not exhibit significant differences in forearm vasodilator responses to 3 minutes arterial occlusion, which is compatible with a hormone- and age-independent response. This is supported by the fact that endothelium-dependent vasodilation was not different between menses and ovulation, oral contraceptive use and withdrawal or with the intake of HRT. In agreement with normal vasodilatory capacity of postmenopausal women no significant differences in plasma markers of endothelial function (von Willebrand factor (vWF), thrombomodulin (TM), and c-reactive protein (CRP)) or circulating endothelial cell (CEC) number were evident between young and middle-aged women.

Young female smokers had elevated resting blood pressure when compared with non-smokers at ovulation only. This is consistent with a modulatory effect of

cigarette smoke on oestrogen levels at ovulation. When comparing middle-aged smokers and non-smokers no differences in any resting cardiovascular parameters were evident although cardiac index was lower in the smoking group. Middle-aged smokers had elevated blood pressure responses to mental stress and a blunted vasodilatory reserve when compared with younger smokers and middle-aged non-smokers. This was probably due to prolonged exposure of the endothelium to toxic tobacco products. There was also a positive correlation between the percentage increase of blood flow from rest following arterial occlusion and number of cigarettes smoked per day. Smoking was associated with an elevation in CECs in both middle-aged and young subjects suggesting the elevation was not reflective of endothelial damage. In fact similar levels of plasma vWF, TM or CRP were evident in smokers and non-smokers.

Having assessed the role of ovarian hormones and exogenous female sex hormone administration on cardiovascular control in women it was decided to investigate the role of long-term hormonal manipulation on autonomic nervous system control and endothelial function. Using the genetically hypertensive (GH) rat, BRS and endothelial turnover rates were investigated, as assessment of these parameters is not easily performed in humans. Baroreflex function was found to be lower in female normotensive animals than in males however ovariectomy had no influence. Hypertension was associated with impaired reflex bradycardia and responses to the same doses of vasoactive substances were consistently greater in GH animals. Although CEC numbers were elevated in GH when compared with normotensive rats this was not reflective of endothelial damage since plasma vWF levels were unaffected. The detachment of these cells from the basal lamina appears unrelated to endothelial cell death via apoptosis or proliferation via mitosis since these rates were not different between the strains.

In conclusion, beneficial effects of female sex hormones such as sympathetic nervous system blunting, improved endothelium-dependent vasodilation and efficient baroreflex functioning were not observed in this thesis. Long-term smoking was associated with attenuated endothelium-dependent vasodilation in middle-aged smokers and elevated CECs in both young and middle-aged smokers. Further study is therefore required to evaluate the beneficial effect of female sex hormones on reducing cardiovascular risks and on the dislodging of CECs from the basal lamina in disease.

LIST OF ABBREVIATIONS

ACh	Acetylcholine
BMI	Body mass index
BRS	Baroreflex sensitivity
BSA	Body surface area
CEC	Circulating endothelial cell
cGMP	Cyclic guanosine monophosphate
CNS	Central nervous system
CRP	c-reactive protein
CVD	Cardiovascular disease
ecNOS	Endothelial constitutive nitric oxide synthase
EDCF	Endothelial-derived constricting factor
EDRF	Endothelial-derived relaxing factor
ELISA	Enzyme linked immunoabsorbant assay
FBF	Forearm blood flow
FMD	Flow-mediated dilation
HDL	High density lipoprotein
HRT	Hormone replacement therapy
LDL	Low density lipoprotein
L-NAME	N^G-nitro-L-arginine methyl ester
L-NMMA	N^G-monomethyl-L-arginine
MAP	Mean arterial pressure
NA	Noradrenaline
NO	Nitric oxide
NOS	Nitric oxide synthase
O^{2-}	Superoxide radical
PE	Phenylephrine
SBP	Systolic blood pressure
SHR	Spontaneously hypertensive rat
SHRSP	Spontaneously hypertensive rat (stroke-prone)
SMC	Smooth muscle cell
SNA	Sympathetic nervous activity
SNP	Sodium nitroprusside

SNS	Sympathetic nervous system
TM	Thrombomodulin
TPR	Total peripheral resistance
vWF	von Willebrand factor
WKY	Wistar Kyoto

PUBLICATIONS ARISING FROM THIS THESIS

- E. Tansey and C. Bell. Interactions of hormonal status, age and smoking on cardiovascular function in women. *J Human Hyper* (in review).
- E. Tansey. (2004). Effects of hormonal status, age and smoking on cardiovascular function in women. *J Physiol (London)* **554.P**, C74.
- E.A. Tansey and C. Bell. (2003). Does smoking affect endothelial function in young women? *Eur J Clin Invest* **33**, (Suppl. I), 2.
- E. Tansey and C. Bell. (2002). Interactions of hormonal status and smoking on cardiovascular reactivity to laboratory stress in women. *J Physiol (London)* **539**, 56P-57P.

Chapter 1 'Introduction'

Cardiovascular disease (CVD) risk varies markedly with regard to gender and age. One of the most important risk factors for CVD is male sex, with a male-to-female death ratio of 4:1 before the age of 50 (Kalin and Zumoffi, 1990). Men, in general, develop the signs of CVD 10 years before women. Premenopausal women have a lower CVD risk than postmenopausal women and men (Simon and Jaillon, 2000). However, after menopause the incidences of hypertension, atherosclerosis, angina, myocardial infarction, and stroke increase rapidly (Wenger *et al.*, 1993) and CVD becomes the leading cause of death in women (Sullivan and Fowlkes, 1996). Hypertension is one of the first determinants of CVD and there are gender differences in blood pressure, which may be a primary cause of the differing disease profiles. It is known that blood pressure increases with age in both men and women (Wiinber *et al.*, 1995) and pressure measurements are higher in men than in women of a similar age until sometime after menopause. Postmenopausal women also have elevated blood pressure relative to premenopausal women (Calhoun and Oparil, 1998). Premenopausal women are hence protected against the risk of CVD until menopausal age. Nevertheless at menopause this risk increases to equal or surpass that of age-matched males. Research has therefore focused on the cardioprotection derived from female sex hormones.

1.1 Ovarian hormones

1.1.1 Oestrogens

The vasoprotective effects of oestrogens include alterations in lipid metabolism, endothelial function, smooth muscle cell (SMC) proliferation, and associated extracellular matrix formation, and vascular reactivity (Oparil *et al.*, 1996). Oestrogen exerts a favourable profibrinolytic effect by decreasing fibrinogen, plasminogen activator inhibitor-1 and tissue plasminogen activator and increasing plasminogen and insulin sensitivity (Scarabin *et al.*, 1997). Long-term oestrogen administration is associated with lower circulating concentrations of renin, angiotensin-converting enzyme and endothelin-1 (Schunkert *et al.*, 1997). Oestrogen inhibits vasoconstriction and platelet activation on healthy vessel walls. This is

achieved by means of nitric oxide (NO) and prostacycline production with concurrent antagonism of endothelin synthesis. Oestrogen decreases collagen and elastin synthesis in animal models and in cell culture (Fischer, 1972). Isolated rabbit arteries previously contracted with endothelin-1 relax in an endothelium-dependent manner by the action of 17β-oestradiol (Mendelsohn and Karas, 1999) and the onset of atherosclerosis in castrated or apolipoprotein E-deficient male animals is prevented by oestrogen (Mendelsohn and Karas, 1999).

Oestrogen has a generalised impact on the female cardiovascular system with a direct beneficial effect on the vessel wall. Oestrogen either binds to the endothelium, potentiating endothelium-derived vasodilator release, and/or affects SMCs directly to modulate vascular tone (Ruehlmann and Mann, 1997; Dubey *et al.*, 2002). Oestrogen receptors have been demonstrated in smooth muscle (Losordo *et al.*, 1994) and oestrogens can directly inhibit vascular smooth muscle cell (SMC) contraction via endothelium-dependent (McNeill *et al.*, 1996) as well as endothelium-independent (Mügge *et al.*, 1993) mechanisms. They can modulate ion channel activity by inhibiting Ca^{2+} influx, which results in vascular SMC relaxation (see Ruehlmann and Mann, 1997). At physiological concentrations, oestrogens stimulate the opening of Ca^{2+}-activated K^+ channels by means of NO release and cGMP activation causing SMC hyperpolarisation (Mendelsohn and Karas, 1999).

Oestrogens decrease the expression of adhesion molecules involved in monocyte adhesion to endothelial cells and cause a decrease in certain chemokines involved in monocyte migration into the subendothelial space. Oestrogen is an antioxidant and decreases oxygen free radicals in the arterial wall. It reduces the size of carotid and aortic lesions and inhibits the proliferation of SMCs (Mendelsohn and Karas, 1999).

1.1.2 Progestins

Progesterone receptors have been identified in the endothelium and SMCs of arterial walls in animals (Lin *et al.*, 1986) and humans (Ingegno *et al.*, 1988). The effects of progestins on the cardiovascular system are more complex and less well studied than oestrogens. Progestins are added to oestrogen preparations in order to protect against the unwanted side-effect of endometrial hyperplasia. However, they have a number of potentially adverse effects. These include a reduction in HDL levels (Lobo, 1991), reduced blood flow, arterial size and compliance and decreased

NO production (as reflected in serum nitrite and nitrate levels) (Rosselli *et al.*, 1995). They also have adverse effects on thrombotic factors including increased plasminogen activator inhibitor-1 levels (Gebara *et al.*, 1995). Progestins counteract oestrogen's beneficial effects on carotid and aortic lesions, by negating the ability of oestradiol to decrease intimal plaque size and inhibit cellular proliferation, which prevents the re-growth of endothelial cells after denudation and disrupts oestrogen's effects on SMC proliferation (Hanke *et al.*, 1996; Mendelsohn and Karas, 1999).

Progesterone inhibits endothelial cell adhesion, migration, proliferation and angiogenesis (Iruela-Arispe *et al.*, 1996). In ovariectomised rats oestrogen replacement preserved endothelial reactivity, whereas combined oestrogen and progesterone treatment led to vascular responses similar to those seen in endothelium-denuded aortic rings (Rudd and Loscalzo, 1986). Progesterone was shown to inhibit oestradiol-induced endothelium-dependent relaxation of coronary arteries of ovariectomised dogs (Miller and Vanhoutte, 1991). Natural progesterone reduces regional arterial flow in menopausal women, due to an increase in vascular resistance, however this effect may be dose-dependent (Mercuro *et al.*, 1999) as μM concentrations have been reported to induce endothelium-dependent relaxation of rabbit coronary arteries (Jiang *et al.*, 1992). Natural progesterone administration at pharmacological doses to menopausal women has been shown to decrease forearm maximal venous volume significantly (Mercuro *et al.*, 1999) suggesting that as well as in arteries, progestins may also elicit an increase in venous wall tone.

1.2 Oral contraceptive use

Importantly, the effects of oestradiol on coagulation and fibrinolytic factors are not the same as those of the oral contraceptive ethinyl oestradiol (Helmerhorst *et al.*, 1998). Even though ethinyl oestradiol is administered at a much lower dose than is used for estrogen replacement therapy, it increases coagulation factors and decreases antithrombin levels and the sensitivity to activated protein C, a process that could increase the risk of thrombosis. Moreover, ethinyl oestradiol has a greater ability to induce hepatic synthesis of angiotensinogen than does oestradiol, and ethinyl oestradiol tends to cause sodium and fluid retention (Rosenthal and Oparil, 2000).

Oral contraceptives induce disorders of coagulation and increase blood pressure, whereas oestradiol when used for hormone replacement therapy tends to lower blood pressure and have neutral effects on the coagulation system. In this context, following the early reports that blood pressure is elevated in women on oral contraceptives (Tsung and Loh, 1979), many clinical studies have provided evidence that contraceptive oestrogens increase blood pressure (Chasan-Taber *et al.*, 1996; WHO, 1997). In a study of 83 women using oral contraceptives for 3 years, increases in both systolic and diastolic blood pressure (9.2 and 5.0 mmHg, respectively) were observed. In a subgroup of these subjects, the mean increases in systolic and diastolic blood pressure were 14.2 and 8.5 mmHg, respectively (Weir, 1974). Moreover, the blood pressure returned to pretreatment levels within 3 months of stopping contraceptive use (Weir, 1974). In a longitudinal study of 13358 women on oral contraceptives, a significant rise in blood pressure was observed, and this increase could be reversed by discontinuing oral contraceptive use (Fisch and Frank, 1977). The adverse effects of contraceptive oestrogens in the past can be attributed to the high doses (150 mg) of contraceptive oestrogens used. Indeed, with the current regimen of 30 mg, the blood pressure elevating effects of contraceptive oestrogens have been considerably reduced (Rosenthal and Oparil, 2000). Briggs and Briggs (1977) reported no rise in blood pressure in women taking 30 mg contraceptive oestrogens for 3 years, while there was an increase in women taking 50 mg contraceptive oestrogens. However oral contraceptive users, even those on low dose regimens (<50 mg oestradiol) have increased risks of thromboembolism (Lidegaard, 1993; WHO, 1995), stroke (WHO, 1996) but not myocardial infarction (Sidney *et al.*, 1996). These results however may depend on the progesterone preparation used in combination with oestrogen.

1.3 The endothelium

1.3.1 Endothelial cell dysfunction

The endothelium is considered a complex endocrine and paracrine organ responsible for a variety of functions including: vasoregulation, vascular remodelling, and anti/pro-thrombogenic processes however it is often subject to dysfunction and sometimes failure (see Goligorsky *et al.*, 2000). In 1980, Furchgott and Zawadzki demonstrated that the endothelium releases one or more local dilating factors, which work with constricting factors to regulate circulatory

haemodynamics. The predominant relaxing factor is NO, synthesised from the amino acid L-arginine. Other relaxing factors include prostacyclin and hyperpolarizing factor. The major constricting factors are endothelin-1, thromboxane, and prostaglandin H2.

Endothelial cell dysfunction is characterised by attenuated production and release of endothelium-derived relaxing factors (EDRFs) (Furchgott and Zawadzki, 1980; Vanhoutte, 1997), such as NO (Palmer *et al.*, 1987), enhanced synthesis of endothelium-derived constricting factors (EDCFs) (Vanhoutte, 1997), both of these or decreased endothelium-dependent hyperpolarization (Kähönen *et al.*, 1995). It has been shown that the production of EDCF closely parallels the increase in blood pressure in the spontaneously hypertensive rat (SHR) (Iwama *et al.*, 1992). This imbalance has been associated with impaired endothelium-dependent vasodilatation in the blood vessels of adult SHR (Vanhoutte, 1997) and in patients at risk for developing atherosclerosis (Dzau *et al.*, 1997; Bell *et al.*, 1998). Reduced cardiovascular event risk has been shown to improve endothelial function (Celermajer, 1997; Vogel, 1997). For example, most cholesterol-lowering trials have shown improved brachial or coronary endothelial function (Vogel, 1997). It seems likely therefore that endothelial function serves as an index of overall CVD risk. The constricting factor released by the endothelium in hypertension and therefore potentially in other CVD is probably a vasoconstrictor prostanoid and/or superoxide anion, because the impairment of endothelium-dependent relaxation is restored by cyclo-oxygenase inhibition (Takase *et al.*, 1994), and by superoxide dismutase (Nakazono *et al.*, 1991).

Endothelial cell dysfunction is prevalent in various pathological conditions, including stroke, myocardial infarction, hypertension and atherosclerosis. In addition to promoting vasodilation, normal endothelium has antiatherosclerotic and antithrombotic functions. It inhibits platelet aggregation (through NO synthesis), monocyte adhesion, vascular SMC proliferation and thrombosis (see Figure 1.1), all of which are important factors in the development of atherosclerosis and plaque disruption (Celermajer, 1997; Vogel, 1997). In contrast, dysfunctional endothelium upregulates chemotactic and adhesion molecules for monoytes and T lymphocytes and secretes colony-stimulating factors that induce differentiation of monocytes into macrophages as part of the atherosclerotic process. Increased production of oxygen free radicals has been reported in damaged endothelial cells, which combine with

and deactivate NO (Flavahan, 1992). In atherosclerosis there is an inverse correlation of NO availability and disease development (Hamon *et al.*, 1994).

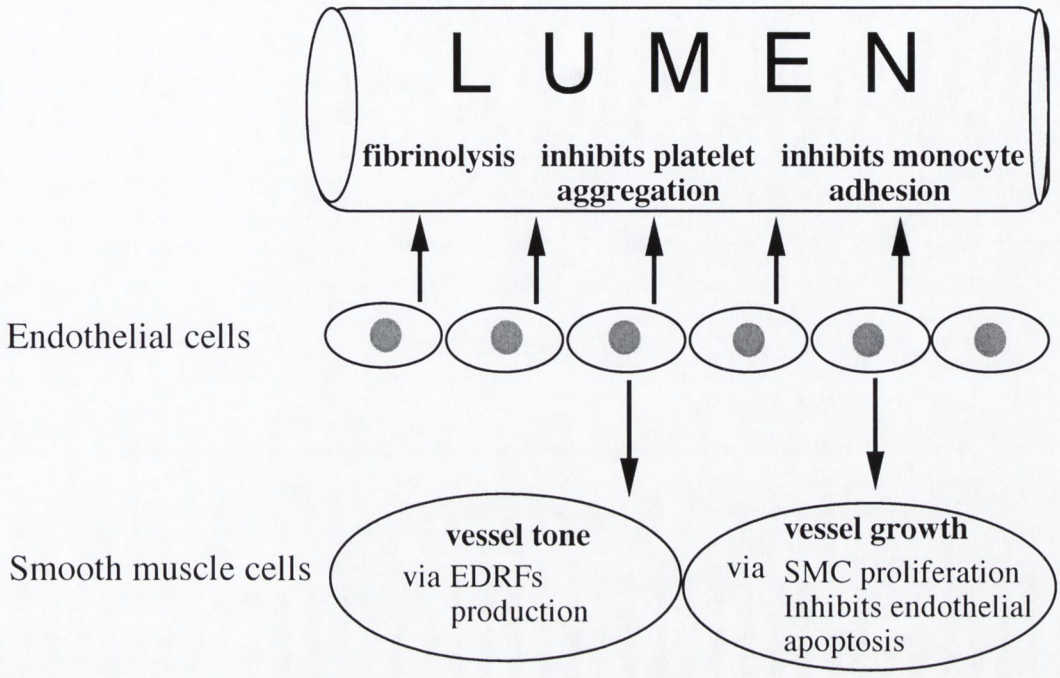


Figure 1.1 Functions of normal endothelium.

Factors secreted into the lumen of the blood vessels include: tissue plasminogen activator and its inhibitor, prostacyclin, prostaglandins and NO. Various cell surface adhesion molecules regulate haemostasis. Factors secreted luminally may effect vessel tone and growth.

1.3.2 Ovarian hormones and endothelial dysfunction

There is evidence for sex differences in endothelial cell dysfunction in hypercholesterolaemic humans (Chowienczyk *et al.*, 1994) and animal models of genetic hypertension (Kauser and Rubanyi, 1995). It has been suggested that sex differences in endothelial function are due to increased NO release in females, perhaps related to sex hormones (Chowienczyk *et al.*, 1994; Kauser and Rubanyi, 1995). Polycystic ovary syndrome is associated with endothelial cell dysfunction (Paradisi *et al.*, 2001) and this appears to be associated with elevated androgen levels. Oestrogen may have a beneficial effect on endothelial function (Costa *et al.*, 1996) and has been implicated in animal (Williams *et al.*, 1988, Huang *et al.*, 1997) and human (Taddei *et al.*, 1996) models of hypertension. Endothelial cell damage

has been found to be less severe in female than in male SHR, which could contribute to the gender differences observed in the extent and rate of progression of hypertension in SHR. Although female sex hormones may attenuate endothelial dysfunction in SHR by increasing EDRFs the influence of ovarian hormones on the generation of EDCF remains unknown. Oestrogen administration lowers LDL cholesterol and raises HDL cholesterol, both of which are associated with improved endothelial function (see Guetta and Cannon, 1996). Oestrogen also lowers lipoprotein (a) and increases VLDL and triglycerides, but these are thought to have lesser effects on endothelial function (see Guetta and Cannon, 1996). In experimental models, progestins tend to oppose the effects of oestrogen on endothelial function (Williams *et al.*, 1994).

1.3.3 Oestrogen and the endothelium

The vascular endothelium is oestrogen-sensitive (see Cid *et al.*, 2002; Koh, 2002 for reviews) and contains oestrogen receptors (Vencov *et al.*, 1996). The interaction between oestrogens and the endothelium is of fundamental importance in the cardiovascular protective effect observed in premenopausal women and it is thought that 17β-oestradiol may protect against the vascular injury response by stimulating the re-endothelisation of damaged blood vessels (White *et al.*, 1997). Oestrogen restores endothelial integrity after arterial injury or denudation by up-regulating endothelial cell growth and by inhibiting endothelial cell apoptosis (Krasinski *et al.*, 1997; Mendelsohn and Karas, 1999).

Premenopausal women have increased endothelium-dependent vasodilatation compared with men (Seiler *et al.*, 1993; Celermajer *et al.*, 1994) and postmenopausal women (Mercuro *et al.*, 1999) and the same is true in the case of female and male experimental animals (Farhat *et al.*, 1996). Endothelial function (measured as brachial artery flow-mediated vasodilation) begins to decline after 50 to 55 years of age in females compared with 40 years of age in men (Celermajer *et al.*, 1994). This effect has been attributed to differences in the functioning of the L-arginine-NO pathway. Oestradiol has been shown to increase endothelial constitutive NO synthase (ecNOS) expression (Hishikawa *et al.*, 1995) and enhanced basal NO release in the forearm vasculature has been noted (Sudhir *et al.*, 1996). At physiological concentrations 17β-oestradiol was found to rapidly stimulate NO release from human internal thoracic artery endothelium and human

arterial endothelium in culture (Stefano *et al.*, 2000). Previous reports correlate oestrogen with increased levels of basal or stimulated release of endothelium-derived NO (William *et al.*, 1988; Goetz *et al.*, 1994; Kauser and Rubanyi, 1995) and animals deprived of the gene coding the α-oestrogen receptor are characterised by lower NO levels in the aortic wall (Mercuro *et al.*, 2000). Considerable evidence supports a reduction of NO activity in conditions such as hypercholesterolaemia, hypertension, ageing and cigarette smoking, all of which predispose to atherosclerosis, a disease in which the endothelium is thought to play an important role. Furthermore, a sex-related difference has been observed. Although some studies have not shown an effect of oestrogen in men, trials of long-term oestrogen administration in high doses to transsexual men demonstrated increases in brachial artery flow-mediated vasodilation (McCrohon *et al.*, 1997; New *et al.*, 1997). Interestingly, increases in endothelium-dependent vasodilatation in men corresponded to decreases in testosterone levels.

Oestrogen administration increases acetylcholine (ACh)-mediated coronary vasodilation in primates (Adams *et al.*, 1997), postmenopausal women (Herrington *et al.*, 1994) and in patients affected by microvascular angina (Roque *et al.*, 1998). It has been shown to improve endothelium-dependent flow-mediated vasodilation to reactive hyperaemia and ACh, with oestrogen administered orally, intravenously and intra-arterially to postmenopausal women (Gilligan *et al.*, 1994; Lieberman *et al.*, 1994; Reis *et al.*, 1994). Physiological levels of 17β-oestradiol have been reported to attenuate ACh-induced coronary constriction in women with coronary heart disease (Collins *et al.*, 1995), and produce direct endothelium-independent relaxation of human coronary arteries *in vitro* (Mügge *et al.*, 1993). In ovariectomised female cynomolgus monkeys, fed with an atherogenic diet in order to produce a coronary lesion, oestrogens were able to restore the damaged endothelial function to normal by stimulating NO release, whereas this effect is absent in males (Williams *et al.*, 1990; Williams *et al.*, 1992).

At comparable LDL levels, hypercholesterolaemic males are characterised by a higher impairment of ACh-induced flow than premenopausal women (Chowienczyk *et al.*, 1994). Changes in endothelial function occur within an hour of either oestrogen administration or cholesterol lowering (Gilligan *et al.*, 1994; Reis *et al.*, 1994; Tamai *et al.*, 1997). Plasma levels of noradrenaline (NA) are higher in postmenopausal than in premenopausal women and correlate with the degree of

peripheral vasodilator reserve (Mercuro *et al.*, 1999). Increased sympathetic activity may therefore play a role in endothelial dysfunction in women.

1.4 Ovarian hormones and haemodynamic effects

Reproductive hormones are known to play an important physiological role in systemic and local vascular flow and resistance. The first evidence of a vascular effect of oestrogens was revealed in studies by Magness and Rosenfeld (1989) who found an increase in arterial flow velocity, with a reduction of vascular resistance and systemic blood pressure, after acute administration of 17β-oestradiol in ovariectomised ewes. A more recent study has confirmed this observation in humans, with oestrogen causing decreased local vascular resistance (Mercuro *et al.*, 1997) and blood pressure in women with mild postmenopausal hypertension (Mercuro *et al.*, 1998). Gilligan *et al.* (1994) however, did not find any appreciable haemodynamic effect of oestrogens in normotensive women without cardiovascular risk factors. Administered chronically, 17ß-oestradiol reportedly decreases mean arterial pressure (MAP) (Ueda *et al.*, 1986) or leaves it unchanged (Williams *et al.*, 1994) and decreases total peripheral resistance (TPR) and increases cardiac output in ovariectomised sheep (Magness *et al.*, 1993) and intact monkeys (Williams *et al.*, 1994). In ovariectomised rats with heart failure 17ß-oestradiol decreases TPR and left ventricular end diastolic pressure indicating a decrease in preload and afterload (Nekooeian and Pang, 1998). Oestradiol administration produces an increase in heart rate (Hernández *et al.*, 1998). This is probably due to a baroreceptor-mediated tachycardia, because MAP tends to fall. Another possibility is that oestrogen may have a direct effect on the sino-atrial node.

Peripheral vascular resistance in young women is closely related to hormonal changes during the menstrual cycle (Bartelink *et al.*, 1990), and oestrogen therapy increases aortic blood flow and cardiac output in postmenopausal women (Bartelink *et al.*, 1990; Pines *et al.*, 1991). The variability exhibited by forearm muscle blood flow during the menstrual cycle has been attributed to changes in plasma 17β-oestradiol concentrations and to oestrogen-progesterone balance (for review see Mercuro *et al.*, 2000). A comparative study of forearm blood flow in healthy pre- and postmenopausal women revealed that resting blood flow was lower in postmenopausal women and that the dilator capacity of the peripheral vasculature

and plasma oestradiol levels were positively correlated in postmenopausal women (Mercuro *et al.*, 1999). It has been found that oestrogen replacement therapy in postmenopausal women increases blood flow in the aorta (Pines *et al.*, 1992) and in cerebral (Penotti *et al.*, 1993), uterine (Ziegler *et al.*, 1991) and forearm (Volterrani *et al.*, 1995) vessels. It has also been shown that oestrogen increases coronary blood flow and dilates epicardial coronary arteries in humans (Gilligan *et al.*, 1994, Rosano *et al.*, 1995) and attenuates ACh-induced constriction in coronary conduit arteries in postmenopausal women (Reis *et al.*, 1994).

Compared to men and postmenopausal women, premenopausal women exhibit increased arterial elasticity of large arteries and an increase in agonist-stimulated endothelium-dependent vascular reactivity. Celermajer *et al.* (1994) noted increases in brachial artery diameter in response to endothelium-dependent and endothelium-independent stimulation in men and women before and after menopause. Endothelium-dependent vasodilatation deteriorated in both men and women with age, however the onset in women usually occurred later and after menopause. Similar findings were reported by New *et al.* (1997) who studied male-to-female transsexuals receiving large doses of oestrogen. In men, intravenous administration causes an approximate 32% increase in coronary blood flow (Blumethal *et al.*, 1997). Similar studies, using forearm blood flow, reveal that intravenous administration of oestrogens to postmenopausal women augments forearm vasodilator responses to ACh and substance P (Tagawa *et al.*, 1997). Oestrogen also enhances endothelium-dependent dilator responses in vessels of various other species, including canine coronary arteries, (Miller and Vanhoutte, 1991), rat aorta (Kauser and Rubanyi, 1995) and rabbit femoral artery (Gisclard *et al.*, 1988).

Venous vasomotion appears to be influenced by ovarian hormones. During pregnancy, the activity of oestrogen on the venous wall is particularly evident, inducing an increase in vascular compliance to contain the increased blood volume (Mercuro *et al.*, 2000). Also increases in venous maximal volume and compliance in healthy postmenopausal women have been observed after 17β-oestradiol administration (Mercuro *et al.*, 1997).

1.4.1 Ovarian hormones and blood pressure

Gender differences in blood pressure occur. Wiinber *et al.* (1995) found that blood pressure is higher in men than in women of a similar age. A similar pattern emerges when looking at animals. There is evidence from animal models of genetic hypertension that sex hormones are important in the development of this disease (Iams and Wexler, 1979; Ganten *et al.*, 1989; Turner *et al.*, 1991). In the majority of rat models of hypertension males have higher pressures than females. The SHR has been widely studied. Male SHR have higher blood pressures than females of a similar age (Ganten *et al.*, 1989). The onset of hypertension in SHR is delayed and less severe in females than in males (Dahl *et al.*, 1975; Ouchi *et al.*, 1987). Gender differences have also been found in Dahl salt-sensitive rats (Crofton *et al.*, 1993), deoxycorticosterone-salt hypertensive rats (Ouchi *et al.*, 1987) and the New Zealand genetically hypertensive rat (Ashton and Balment, 1991).

Many studies have looked at the effect of female sex hormones, particularly oestrogens on blood pressure regulation. The results are still somewhat confusing. Authors have reported that oestrogen exerts no influence on blood pressure (Akkad, *et al.*, 1997; Pripp *et al.*, 1999); that blood pressure is only minimally affected (van Ittersum *et al.*, 1998; Seely *et al.*, 1999); or that the reduction in blood pressure is only evident at night or in normotensive individuals (Beljic *et al.*, 1998; Seely *et al.*, 1999). There is no doubt however that there are gender differences in blood pressure. A greater release of endothelium-derived NO, caused by the chronic presence of oestrogen, may be responsible for the differences in peripheral resistance and blood pressure between female and male SHR. Chronic oestrogen treatment has been shown to lower blood pressure in some genetic and non-genetic models of hypertension in rats (Crofton and Share, 1997; Li *et al.*, 1997). A reduction in oestrogen levels after ovariectomy increases the MAP in conscious SHR (Costa *et al.*, 1996; Dantas *et al.*, 1999) but oestrogen treatment corrects this (Dantas *et al.*, 1999). In pregnant SHR, a significant reduction in blood pressure and a reversal of impaired endothelium-dependent responses have been observed (Ahokas *et al.*, 1991). Furthermore in women with postmenopausal mild-to-moderate hypertension oestrogen has been shown to reduce 24-h systolic and diastolic blood pressure (Mercuro *et al.*, 1997) and blood pressure responses to exercise (Pines *et al.*, 1996). Similar findings have been made in animal studies. After menopause, the incidence of arterial hypertension progressively increases in

women compared with men (Bradshaw, 1994) and hypertensive females are four times more likely than age-matched normotensive subjects to develop CVD (Kitler, 1992).

Although the oestrogen-dependent mechanisms contributing to the regulation of arterial pressure remain unclear, some evidence indicates a possible relationship between oestrogen, endothelial function, and the renin-angiotensin system. Plasma renin activity has been shown to be 27% higher in men than women regardless of age (Kaplan *et al.*, 1976; James *et al.*, 1986). Other similar studies have shown that plasma renin activity is higher in postmenopausal than premenopausal women but that they still do not reach the values of men of a similar age (Schunkert *et al.*, 1997). In animals the case is similar (Reckelhoff *et al.*, 2000).

1.5 Endothelium-derived relaxing factor (NO) and gender specificity

NO accounts for the activity of EDRF (Ignarro *et al.*, 1987; Palmer *et al.*, 1987), see Figure 1.2. It is a potent vasodilator and regulates tone in normal vessels and causes renal vasodilatation with consequent diuresis and natriuresis (Salom *et al.*, 1986). These actions would tend to lower blood pressure so a NO deficiency could potentially contribute to hypertension. NO possesses many anti-atherogenic properties (Vallance *et al.*, 1989; Cooke and Tsao, 1994). NO decreases platelet aggregation and adhesion (Radomski *et al.*, 1990; Radomski *et al.*, 1999), limits vascular SMC proliferation (Garg and Hasid, 1989), inhibits neo-intima formation (Vermeersch *et al.*, 2001), prevents monocyte chemotaxis (Bath *et al.*, 1991) and inhibits leukocyte adhesion to the endothelium (Kubes *et al.*, 1991).

A deficiency in NO may be responsible for the increased vascular resistance associated with human essential hypertension and that seen in animal models of hypertension. Studies of NO release in human essential hypertension (Panza *et al.*, 1990; Calver *et al.*, 1992; Cockcroft *et al.*, 1994) and in genetically hypertensive animals (Malinski *et al.*, 1993; Rees *et al.*, 1996) have been contradictory. An alternative hypothesis is that NO may be released in normal or even greater amounts in humans or animals with apparent endothelial cell dysfunction, but the NO may be scavenged before it can activate guanylyl cyclase. One of the known scavengers is the superoxide radical (O^{2-}), which reacts with NO to form peroxynitrite ($ONOO^-$) (Rubanyi and Vanhoutte, 1986). There is evidence that the apparently reduced NO

availability demonstrated in the SHRSP (stroke-prone) can be exactly accounted for by increased levels of O^{2-} (Grunfeld *et al.*, 1995). There is also evidence that O^{2-} influences the vascular reactivity of human vessels (Hamilton *et al.*, 1997) and that it may contribute to hypertension and endothelial cell dysfunction (Nakazono *et al.*, 1991).

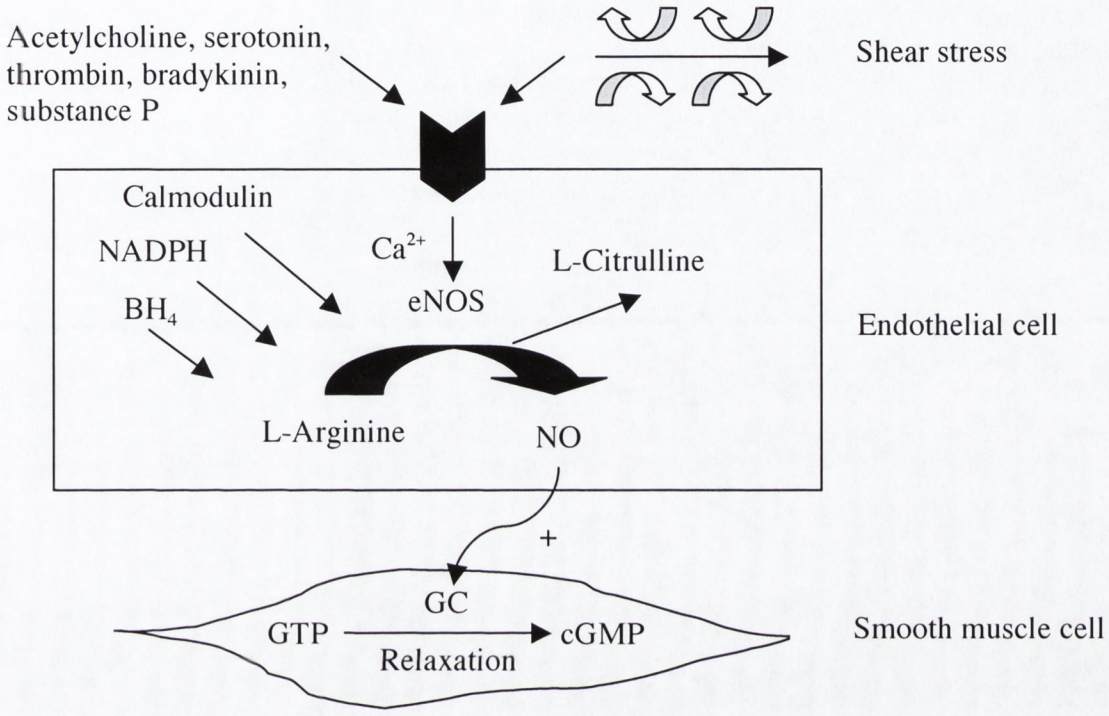


Figure 1.2 The nitric oxide pathway

Nitric oxide (NO) is produced from its precursor, L-arginine, by the enzyme nitric oxide synthase (NOS) in association with several cofactors. NOS can be activated by a large number of agonists, including acetylcholine (ACh), serotonin, bradykinin, and thrombin, as well as increased flow-induced shear stress. BH_4 = tetrahydrobiopterin; Ca^{2+} = calcium ion; cGMP = cyclic guanosine monophosphate; eNOS = endothelial nitric oxide synthase; GC = guanylate cyclase; GTP = guanosine triphosphate; NADPH = nicotinamide adenine dinucleotide phosphate. Adapted from Ganz (2002).

Many reports have related the protective effect of oestrogen on endothelial function to its antioxidant property (Yen *et al.*, 2001). The antioxidant effect of oestrogen may be attributed to its phenolic structure, which scavenges oxygen free radicals (Halliwell and Grootveld, 1987). It may also exert its antioxidant action by regulating the action of antioxidant enzymes such as superoxide dismutase (Kenaley

and Ji, 1991). A role for O^{2-} in the impaired endothelial function after ovariectomy has been suggested (Dantas *et al.*, 1999). Thus, the less pronounced endothelial dysfunction observed in oestrus females may reflect a decreased generation of vasoconstrictor prostaglandins and O^{2-}.

In porcine uterine arteries and cardiac and skeletal muscle, oestrogen treatment stimulates NOS activity by increasing the amount of mRNA for NOS isozymes (Weiner *et al.*, 1994). 17β-oestradiol has been shown to increase eNOS mRNA in cultured human endothelial cells (Hishikawa *et al.*, 1995), in rat aortas (Goetz *et al.*, 1994) and skeletal muscle arterioles in SHR (Huang *et al.*, 2000). This would lead to reduced peripheral vascular tone and endothelium-derived factors, among them NO, tend to counteract pressure-induced myogenic tone in skeletal muscle microvessels in normotensive (Kaley *et al.*, 1992; Sun *et al.*, 1994) but not hypertensive rats (Boegehold, 1992; Huang *et al.*, 1993).

Studies of NO production as determined by conversion of L-arginine to nitrite, demonstrate decreased whole body NO production in young to middle-aged healthy males compared to premenopausal females (Forte *et al.*, 1988). Plasma 17ß-oestradiol levels correlate positively with plasma concentrations of NO metabolites (White *et al.*, 1997). Premenstrual status is associated with increased basal vascular NO action in the human forearm arterial vasculature compared to that in males and postmenopausal females. There are decreased constrictor responses to an NOS inhibitor in postmenopausal women and males compared to premenopausal women, indicating a decrease in basal NO activity associated with menopause (Majmudar *et al.*, 2000). Two weeks of oestrogen replacement therapy in postmenopausal women restores basal vascular NO activity to levels seen in premenopausal women (Majmudar *et al.*, 2000). A link between the plasma levels of oestrogen and NO in perimenopausal and postmenopausal women (Gilligan *et al.*, 1994; Sudhir *et al.*, 1996) has been demonstrated, whereas in pre-eclampsia, a lower circulating oestrogen level is associated with reduced release of NO (Cockell and Poston, 1997). *In vivo* studies in humans (Rosselli *et al.*, 1995) and in animals (Brosnihan *et al.*, 1994; Rosselli *et al.*, 1994) show that oestrogen administration can increase circulating levels of nitrite and nitrate (the metabolites of NO) in serum. This suggests an enhancement of NO release into the circulation.

Van Buren *et al.* (1992) found in animal studies that local uterine artery injection of L-NAME suppressed, in a dose-dependent manner, the oestrogen-

induced increase in uterine blood flow. Dilation in response to substance P, a response mediated exclusively by NO, was greatly reduced in male SHR (Huang and Koller, 1996). In arterioles of female SHR and oestradiol-treated ovariectomised SHR, dilations to all concentrations of substance P were significantly greater than in arterioles of male SHR and ovariectomised female SHR. The similar responses of arterioles of all SHR groups to sodium nitroprusside, which affects smooth muscle directly, rules out the possibility of an altered reactivity of arteriolar smooth muscle to NO (Huang *et al.*, 1997).

1.6 Nitric oxide and hypertension

Female SHR exhibit a greater dilation to flow than male SHR (Koller and Huang, 1994) and the same is true of NO-mediated dilations to agonists (Huang *et al.*, 1996; 1997). Endothelial NO-mediated responses are impaired in arterioles of male SHR, but are still preserved in female SHR. Flow-dependent dilation of arterioles is mediated by endothelial NO and prostaglandins (Koller *et al.*, 1994) and in male SHR, the NO-mediated portion of the response is absent (Koller *et al.*, 1994, Koller and Huang, 1995). The arterioles of ovariectomised females without oestrogen-replacement respond in a similar fashion to those of males, but arterioles of ovariectomised rats given oestrogen therapy show a significantly greater dilation to flow than those of male and ovariectomised females (Huang *et al.*, 1998). Female SHR have less pressure-induced tone of arterioles than male SHR. The difference is due to the greater basal release of NO in arterioles of female SHR (Huang *et al.*, 1997). L-NG-Nitroarginine, an inhibitor of NOS, eliminates the differences in arteriolar tone in male and female SHR by decreasing the diameter of arterioles to an identical level (Huang *et al.*, 1997).

The superoxide radical has been implicated in models of experimental hypertension. The reduced availability of basal and stimulated NO in hypertension may be due to reduced production, increased scavenging, or both. In fact there is evidence for increased NOS activity in SHRSP compared with Wistar Kyoto rats (WKY), suggesting that NO production is increased rather than decreased in the hypertensive animals (McIntyre *et al.*, 1997). However this compensatory increase in NOS activity seems to be insufficient to correct the NO/O^{2-} imbalance. It therefore seems that the reduced NO availability in SHRSP is due to increased

scavenging. Increased NO availability may therefore contribute to the relative cardiovascular protection in females.

Human essential hypertension and several animal models of hypertension are associated with increased peripheral vascular resistance (Shepherd, 1983). The continuous release of NO is very important in the regulation of arteriolar tone and hence vascular resistance in both normotensive and hypertensive conditions and arteriolar tone is an important determinant of peripheral resistance. Studies show an enhanced arteriolar tone (Huang *et al*, 1993) and a reduced release of endothelium-derived vasodilators in hypertension, (Boegehold *et al.*, 1992; Huang *et al.*, 1993; Huang *et al.*, 1996) which could contribute to the elevated peripheral resistance. NO may be responsible for the delayed onset and/or severe development of hypertension in females via sex hormone modulation of peripheral resistance.

1.7 Oestrogen and nervous system control

1.7.1 Oestrogen and the autonomic nervous system

The long-term regulation of blood pressure may be contributed to by hormonal modulation of sympathetic activity. It has been shown that a group of hormones for example vasopressin, angiotensin II, atrial natriuretic peptide, and endothelin, can modulate sympathetic activity via direct effects on the CNS (Johnson and Gross, 1993; Osborn, 1997). He *et al.* (1998) showed that oestradiol can prevent the effects of vasopressin on baroreflex function in response to rapid changes in arterial pressure and decreases in endothelin and angiotensin have been found with oestrogen administration (Schunkter *et al.*, 1997). 17ß-oestradiol has been found to inhibit sympathetic outflow in various animal species (He *et al.*, 1998). This effect may be enhanced by signals from the baroreceptors. Baroreflex-modulated sympathetic activity is a major factor in the moment-to-moment maintenance of blood pressure. Under normal circumstances, the inhibition of sympathetic activity may not be sufficient to affect arterial blood pressure. When blood pressure is raised, however, an increase in baroreceptor input may enhance the inhibitory effect of oestrogen on sympathetic outflow and facilitate the return of blood pressure to relatively normal levels. This is supported by the fact that treatment of animals with 17ß-oestradiol has little or no effect on basal arterial blood pressure but markedly attenuates the pressor response to several vasoconstricting pressor agents e.g. angiotensin II (Yoshimura *et al.*, 1984; Naden and Rosenfeld,

1985), adrenergic stimuli (Naden and Rosenfeld, 1985; Shan *et al.*, 1994) and vasopressin (Toba *et al.*, 1991).

The inhibitory effect of 17ß-oestradiol on sympathetic outflow may persist during the chronic administration of oestrogen, since in women chronically treated with oestrogen, circulating levels of NA and heart rate are low (Lindheim *et al.*, 1992). The withdrawal of sympathetic outflow not only reduces vasomotor tone but also decreases trophic effects on vascular and cardiac muscle (Mark, 1996). This effect may also contribute to the attenuation of the pressor response to vasoconstrictor stimuli and the protection of cardiovascular system by oestrogen. There are a number of reports that oestrogen can act directly on the blood vessel to modify responses to vasoactive agents. The nature of this effect is controversial as some report an enhancement of the vasoconstrictor response to catecholamines with 17ß-oestradiol treatment (Yoshimura *et al.*, 1984; Chu and Beilin, 1997) and others report an attenuation (Shan *et al.*, 1994; Meyer *et al.*, 1997; Paredes-Carbajal *et al.*, 1995).

1.7.2 The arterial baroreflex

The arterial baroreceptors are located in the wall of the carotid sinus and aortic arch and are the mechanosensitive nerve endings of afferent fibres that run in the glossopharyngeal and vagal nerves. They are important in the short-term regulation of blood pressure and are strategically located to provide critical information about the pressure of the blood leading to the brain (carotid sinus) and the rest of the body (aortic arch). They are activated by stretch and signal changes in arterial pressure over a wide range from approximately 50 to 150 mmHg (Kirchheim, 1976). The baroreflex is a reflex loop with cardiac, vascular and cerebral components. The arterial baroreflex mediates reflex changes in heart rate by altering both cardiac parasympathetic and sympathetic activity. Cardiac parasympathetic activation and cardiac sympathetic inhibition are elicited in response to an increase in arterial pressure. Vasodilation and a decrease in cardiac output are also apparent. A decrease in arterial pressure elicits cardiac parasympathetic inhibition and cardiac sympathetic excitation along with vasoconstriction and an increase in cardiac output. Baroreflex-mediated heart rate response to temporary change in blood pressure is a well-known method of assessing arterial baroreflex function. In general baroreflex sensitivity (BRS) is

measured by relating the maximum changes in heart rate to changes in blood pressure after pressure manipulation (Coleman, 1980). It has been well documented that reflex bradycardia evoked by intravenous injection of phenylephrine is a reliable measure of parasympathetic tone while the sodium-nitroprusside-induced reflex tachycardia is an indicator of sympathetic tone (Pickering *et al.*, 1972; Coleman, 1980). Changes in the magnitude of the phenylephrine-induced bradycardia or the sodium-nitroprusside-induced tachycardia are indicative of altered sympatho-vagal balance.

1.7.3 Baroreflex sensitivity in hypertension

The baroreflex heart rate response to changes in blood pressure reflects the capacity of reflex autonomic regulation. It has long been established that defective cardiovascular autonomic control can lead to lower levels of baroreflex gain which are associated with an increased risk of CVD mortality (LaRovere *et al.*, 1998) and may contribute to the development of hypertension. It is not clear whether the diminished baroreflex function is the cause or effect of hypertension. However it seems to be positively associated with the severity of this disease (Mussalo *et al.*, 2002) and the reversal or prevention of hypertension improves its functioning (Cheng *et al.*, 1989; Edmunds *et al.*, 1990).

The arterial baroreflex response to changes in arterial pressure is dependent on the baseline level of cardiac parasympathetic and sympathetic tone. Increased heart rate has long been identified as a marker of sympathetic predominance in hypertension and is related to a higher mortality (Gillman *et al.*, 1993). There is increased sympathetic activity and decreased vagal activity in hypertension (Grassi, 1998), which is accompanied by diminished baroreflex function in both animals (Lantelme *et al.*, 1994) and humans (Siche *et al.*, 1995) and may be a cause of the resetting of the baroreflex to a higher blood pressure level. An imbalance in autonomic tone such as that seen in hypertension increases an individual's risk of sudden death (Barron and Lesh, 1996). In contrast an elevated parasympathetic tone negates this effect (Sleight, 1997).

There are a number of ways hypertension may alter the baroreflex. Hypertension is associated with altered renin-angiotensin-aldosterone system functioning (Unger *et al.*, 2003) as well as levels of vasopressin (Yang and Coote, 2003) and endothelin-1 (McNeill, 2003) all of which have been implicated in

impaired baroreflex function. Cardiac hypertrophy can also modulate the baroreflex gain at the efferent level in SHRs (Minami and Head, 1993).

Gender differences occur in the pathophysiology of hypertension. In several animal models of hypertension e.g. deoxycorticosterone-salt hypertension in the rat, SHR, and the Dahl salt-sensitive rat, the hypertension develops more rapidly in males than in females (Dahl *et al.*, 1975; Iams and Wexler, 1979; Ouchi *et al.*, 1987). Sympathetic nervous system control may be regulated differently between the genders. Also chronic oestrogen treatment lowers blood pressure in some genetic and non-genetic models of hypertension in rats (Croften and Share, 1997; Li *et al*, 1997) and pregnant SHR exhibit reductions in blood pressure (Ahokas *et al*, 1991). This may be due to more effective baroreceptor functioning possibly due to the presence of female sex hormones.

1.7.4 Baroreflex sensitivity and female sex hormones

Oestrogen receptors have been found in brain centres associated with cardiovascular regulation. The nucleus of the solitary tract, ventrolateral medulla and area postrema are CNS elements in the baroreceptor reflex arc, in which oestrogen receptors are located. This suggests that oestrogen may influence baroreceptor function (Saleh and Connell, 2000). Experiments in male rats show that direct injection of oestrogen into cardiovascular and autonomic nuclei in the brain stem elicits significant increases in parasympathetic tone and BRS, conversely sympathetic tone is significantly decreased (Saleh *et al.*, 2000). Oestrogen's effect on the cardiovascular nuclei therefore is to produce a shift in sympatho-vagal balance towards the parasympathetic limb. This results in an enhancement of baroreflex function, which has been shown to provide antifibrillatory protection in the heart (Esler, 1992).

The influence of gender on cardiovagal baroreflex gain in humans is controversial: both similar (Luzier *et al.*, 1998), lower (Abdel-Rahman *et al.*, 1994; Laitinen *et al.*, 1998) as well as higher (Du *et al.*, 1995; Huikuri *et al.*, 1996) levels have been reported in premenopausal women compared with postmenopausal women and men. The sensitivity of the cardiac baroreflex has been shown to change with fluctuations in serum oestrogen levels such as might occur during varying stages of the menstrual cycle (Minson *et al.*, 2000), following ovariectomy (He *et al.*, 1998; 1999) or during early or late pregnancy (Leduc *et al.*, 1991). Results are

contradictory. Cardiovagal baroreflex gain has been shown to remain unchanged during the luteal phase of the cycle whereas the sympathetic baroreflex increases (Minson *et al.*, 2000) and young women using oral contraceptives have lower sympathetic and vagal baroreflex gains during the high oestrogen phase than in the withdrawal phase (Minson *et al.*, 2000). Cooke *et al.* (2002) however, found no change in carotid-cardiac baroreflex gain over the duration of the menstrual cycle. Studies in pregnant women and animals suggest chronic exposure to elevated oestrogen levels may have a negative effect on baroreflex function (Brooks *et al.*, 1995). BRS can decrease by as much as 50% in normotensive pregnant women (Blake *et al.*, 2000). Some studies suggest BRS is enhanced after oestrogen therapy (Huikuri *et al.*, 1996; De Meersman *et al.*, 1998), whereas other studies suggest baroreflex function is unaffected (Virtanen *et al.*, 1999). Hunt *et al.* (2001) found that long-term oestrogen replacement in menopausal women had no effect on cardiovagal baroreflex gain but improved sympathetic baroreflex gain. Enhanced BRS has been seen in conscious ovariectomised female rats following peripheral oestrogen administration (El-Mas and Abdel-Rahman, 1998; He *et al.*, 1998, 1999). Progesterone on the other hand may cause an attenuation of BRS (Heesch & Rogers, 1995). Only the vagal arm of the baroreflex is examined in most studies, and therefore not much can be deduced about the sympathetic limb. Nevertheless the effects of female sex hormones on baroreceptor function and the sympathetic nervous system are likely to have important implications for cardiovascular regulation.

Oestrogen increases the density and enhances the function of presynaptic and central α_2-adrenoceptors (Johnson *et al.*, 1985), which are known to facilitate baroreflex function (Sved *et al.*, 1992). This results in a significant attenuation of NA-induced responses (Morita *et al.*, 1987) and a lowered plasma level in females compared with males (Davidson *et al.*, 1984; Luzier *et al.*, 1998), which can lead to a decrease in sympathetic tone. The activity of the baroreceptors is modulated by paracrine factors released from vascular endothelial cells. Their activity is enhanced by prostacyclin (Chen *et al.*, 1990, Xie *et al.*, 1990) and reduced by endothelin (Chapleau *et al.*, 1992). It has been reported that oestrogen increases prostacyclin and decreases endothelin production in vascular endothelium (Akishita *et al.*, 1996; Mikkola *et al.*, 1996). The responses of supraoptic vasopressin neurons to changes in blood pressure induced by intravenous injection of phenylephrine or nitroprusside

are modified by oestrogen in ovariectomised rats (Akaishi and Homma, 1996). Thus it is also possible that oestrogen modulates the effect of vasopressin on BRS peripherally via paracrine factors. Oestrogen may act centrally on parasympathetic preganglionic neurons to modulate vagal output while causing peripheral vasodilation. Another possibility is that continuous circulating oestrogen modulates autonomic tone through a potentiation of cholineacetyltransferase activity at the sino-atrial node thereby prolonging cardiac parasympathetic responses (Du *et al.*, 1995).

1.7.5 Baroreflex sensitivity and testosterone

An association between androgen deprivation and enhanced endothelial function in older men consistent with a deleterious effect of androgens on vascular reactivity has been found (Herman *et al.*, 1997). Reports have indicated that androgens may contribute to the development of hypertension in SHR through sustained enhancement of tyrosine hydroxylase activity, which leads to increased NA levels in blood vessels (Kumai *et al.*, 1994; 1995) and hence increased vascular tone. Castration of SHR and Dahl salt-sensitive male rats at a young age (3-5 weeks) attenuates the development of hypertension (Masubuchi *et al.*, 1982; Ouchi *et al.*, 1987; Ganten *et al.*, 1989; Ashton and Balment, 1991) and in castrated SHR renal α_2-adrenergic receptor density decreases by 60%, (Gong *et al.*, 1994) whereas similar treatment at 25 weeks has no effect (Ganten *et al.*, 1989). In SHR and normotensive WKY, castration decreases renal angiotensinogen mRNA and chronic testosterone increases its levels (Ellison *et al.*, 1989; Chen *et al.*, 1992).

A reduction in BRS in both male and female deoxycorticosterone-salt hypertensive rats has been found but to a greater extent in males (Ouchi *et al.*, 1988). Castration has been shown to attenuate baroreceptor control of reflex bradycardia but has no effect on reflex tachycardia (El-Mas *et al.*, 2001). The latter results have since been reproduced by the same group (El-Mas *et al.*, 2002). Testosterone therefore appears to enhance cardiac vagal efferent activity. Differences between the genders may also be due to the fact that males have stiffer arteries than their female counterparts (Ahimastos *et al.*, 2003). Nevertheless it must be remembered that studies in this area are sparse and care is needed in the interpretation of results.

1.8 Cigarette smoking

The association between smoking and CVD was first realised in the 1950s (Hammond and Horn, 1958). Smoking is now the single largest most preventable risk factor for mortality in the developed world. Approximately 1.1 thousand million people smoke world-wide and it is estimated that every 10 seconds another person dies from smoking-related illnesses causing about 5 million deaths per year (Ezzati and Lopez, 2003). Due to the large increase in the consumption of tobacco products, particularly in developing countries it has been estimated that 10 million people will die world-wide each year by 2020-2030 (Mackay and Crofton, 1996).

Smoking increases cardiovascular risk probably by a number of mechanisms including: (1) endothelial dysfunction, (2) coronary vasoconstriction, (3) procoagulation (4) increased catecholamine release, (5) elevated myocardial work and (6) reduced oxygen-carrying capacity of the blood due to carbon monoxide poisoning (Benowitz and Gourlay, 1997). There are over 5000 chemicals in tobacco smoke (Stedman, 1968). Nicotine and carbon monoxide are two elements, which are thought to account for the adverse effects of smoking (U.S. Department of Health and Human Services, 1990). Cigarette smoke contains high concentrations of pro-oxidants, such as hydroquinone, catechol, acetaldehyde, free oxygen radicals and peroxynitrites (Freed *et al.*, 2001).

1.8.1 Smoking and its pressor response

Cigarette smoking is associated with a marked and prolonged increase in heart rate and blood pressure (Groppelli *et al.*, 1992; Gerhardt *et al.*, 1999) and reflex cardiac control is altered by smoking (Mancia *et al.*, 1997; Gerhard *et al.*, 1999). It is not known exactly how smoking increases blood pressure and sympathetic outflow. Blood pressure and heart rate variations among smokers may be due to plasma nicotine levels (Kurihara, 1995). The effects on blood pressure and heart rate and the catecholamine concentrations after smoking a cigarette are similar after intravenous administration of nicotine (Malliani *et al.*, 1991). Tachycardia during smoking plays a role in the increase in blood pressure but α_1-adrenoceptor-mediated vasoconstriction (Cryer *et al.*, 1976), vasopressin release (Waeber *et al.*, 1995) and endothelial cell dysfunction (Celermajer *et al.*, 1993) may be involved. Sympathetic activation and vagal withdrawal have been reported after nicotine patch

administration (Lucini *et al.*, 1998). Inactivation of the parasympathetic nervous system could lead to alterations in sympathovagal cardiovascular control and prolonged tachycardic and pressor effects (Groppelli *et al.*, 1992; Grassi *et al.*, 1994).

1.8.2 Smoking and the autonomic nervous system

An increase in renal sympathetic nerve activity (SNA) occurs with smoking (Nakamura and Hayashida, 1992; Peterson *et al.*, 1983) and nicotine may have a direct effect on heart rate via stimulation of intracardiac sympathetic nerves (Kilpatrick *et al.*, 1981). Nicotine consumption has been found to increase heart rate maximally 5-10 minutes post-intake (Benowitz, 1988) and appears to have greater effects in women (Adan and Sánchez-Turet, 1995). α- and β-blockade diminish smoking's pressor responses and are associated with an increase in plasma adrenaline and NA (Cryer *et al.*, 1976). It has therefore been suggested that cigarette smoking is responsible for sympathetic activation (Levin *et al.*, 1992). Sympathetic activation by smoking may depend on an increased release and/or reduced clearance of catecholamines at the neuroeffector junctions (Grassi *et al.*, 1994). This same study found that sympathetic activation during smoking was mainly peripheral and central activity was inhibited probably by the baroreflex.

Smoking increases blood pressure (Groppelli *et al.*, 1992; Niedermaier *et al.*, 1993; Grassi *et al.*, 1994) and inhibits muscle SNA (Niedermaier *et al.*, 1993; Grassi *et al.*, 1994). This decrease in muscle SNA might be secondary to baroreflex responses to smoking that obscure a sympathetic excitatory effect of smoking and has been cited as evidence that smoking does not elicit central stimulation of efferent sympathetic nerve traffic (Haass and Kübler, 1996). Narkiewicz *et al.* (1998) found that cigarette smoking increases sympathetic outflow to the skin and muscle blood vessels. This group found that when blood pressure increases in response to smoking were counteracted by sodium nitroprusside infusion, SNA was increased as much as 3-fold more than baseline levels. Tachycardia may be also be observed during this period sometimes by as much as 37 beats per minute. The explanation for this is that sympathetic activity may not show due to baroreflex adjustments. Smoking has a strong tachycardic effect (Narkiewicz *et al.*, 1998), which could be due to an increased sympathetic drive to the heart rather than to peripheral vessels. Smoking has been shown to impair baroreflex functioning

(Mancia *et al.*, 1997), probably due to a decrease in arterial distensibility (Failla *et al.*, 1997). Arterial baroreflexes may be trying to exert a protective role during smoking by inhibiting sympathetic activation. In patients with impaired baroreflex functioning for example in hypertension (Matsukawa *et al.*, 1991) and heart failure (Ferguson *et al.*, 1992), sympathetic activation could be increased greatly and might not be readily controlled. It has already been shown that smoking and its subsequent increase in blood pressure is augmented in elderly hypertensive individuals (Narkiewicz *et al.*, 1995).

1.8.3 Cigarette smoking and Endothelial Cell Dysfunction

Cigarette smoking has many adverse effects on the endothelium which include increased oxidative stress (Church and Pryor, 1985; Heitzer *et al.*, 1996) decreased levels of vitamin C (Heitzer *et al.*, 1996), impaired production of prostacyclin (Reinders *et al.*, 1986), increased von Willebrand factor (Blann and McCollum, 1993), increased endothelial-monocyte adherence (Weber *et al.*, 1996) and diminished endothelial NO activity (Kiowski *et al.*, 1994; Zeiher *et al.*, 1995).

Smoking together with hypercholesterolaemia and hypertension is one of the most important risk factors for the development of atherosclerosis and CVD. Smoking has been found to influence lipid profiles (Djousse *et al.*, 2000) although this is not the only mechanism involved in its effects. Smoking is associated with increased intima-media thickness (Howard *et al.*, 1994; Poredos *et al.*, 1999) and endothelial cell dysfunction (Lekakis *et al.*, 1998; Pepine *et al.*, 1998). Endothelial cell dysfunction in the brachial and coronary arteries of healthy chronic and acute smokers as well as passive smokers has been described (Celermajer *et al.*, 1993; Zeiher *et al.*, 1995; Celermajer *et al.*, 1996; Lekakis *et al.*, 1997) and may be a direct result of increased degradation of NO as a result of the formation of reactive oxygen species (Heitzer *et al.*, 1996; Raij *et al.*, 2001) or a reduction of endothelial NOS (Higman *et al.*, 1996). It has been found that responses to reactive hyperaemia, an endothelium-dependent functional test, are impaired in smokers, which might indicate asymptomatic CVD (Celermajer *et al.*, 1994). Endothelial cell dysfunction as a result of smoking may also be involved in thrombosis causing increased fibrinogen levels and as well as increased platelet aggregation (Nowak *et al.*, 1987; Newby *et al.*, 1999).

1.8.4 Smoking and gender

Not much is known about gender differences in cardiovascular health in response to smoking. Smoking may influence the path of CVD in a sex-dependent way. It is known that female steroid hormones have a beneficial effect on the cardiovascular system but surprisingly it seems women rather than men are affected much more detrimentally by smoking. In young to middle-aged adults there is a 6-fold increase in the incidence of myocardial infarction in female smokers and a 3-fold increase in males who smoke approximately 20 cigarettes day^{-1} compared with those who never smoked (Njølstad *et al.*, 1996). This same study found that women who currently smoke are more at risk than their male counterparts (relative risk 3.3 vs 1.9) and this disparity is increased even more when women under 45 years are considered. In young women cigarette smoking is the most important risk factor for sudden cardiac death (Oparil, 1998).

Little information is available on the effects of smoking on young individuals particularly females. Population age (Njølstad *et al.*, 1996), sex differences in smoking habits and smoking cessation (Jousilahti *et al.*, 1999) and contraceptive use (Rosenberg *et al.*, 1985) may be responsible for some of the discrepancies observed. For example, although a dose-dependent relationship between smoking and blood pressure has been found in men (Berglund and Wilhelmsen, 1975; Handa *et al.*, 1990), it has not clearly been established in women (Gofin *et al.*, 1982; Brischetto *et al.*, 1983; Green *et al.*, 1986). Results are conflicting. No relationship (Brischetto *et al.*, 1983), a negative dose-dependent relationship independent of gender (Savdie *et al.*, 1984) and in middle-aged women a negative relationship (Gofin *et al.*, 1982) between cigarette smoking and blood pressure have been demonstrated. Menopausal status may affect cardiovascular mortality rates in female smokers. It has been found that smoking can destabilise atheromatic plaques and cause sudden coronary death in women that may vary with hormonal status (Burke *et al.*, 1998). Green *et al.* (1986) found that blood pressure was significantly lower in older but not in younger female smokers. Imamura *et al.* (2000) found that smoking in young females was associated with lower blood pressure and this was also the case for ex-smokers.

There is a greater risk of myocardial infarction in female smokers when compared with non-smokers and this risk increases with the number of cigarettes consumed (Nyboe *et al.*, 1991; Prescott *et al.*, 1998). However this risk elevation is

not seen in male smokers. The number of cigarettes smoked per day is positively associated with the risk of fatal coronary heart disease, myocardial infarction and angina pectoris (Willett *et al.*, 1987). Per 10 cigarettes per day the relative risk of coronary death is 1.8 in women and 1.2 in men (Tverdal *et al.*, 1993). In individuals with thrombocythaemia 60% of female smokers and 15% of non-smokers experience thrombosis but there is no difference in male smokers and non-smokers (Jantunen *et al.*, 2001). Smoking presents an increased risk in females who suffer with symptomatic peripheral arterial disease (Ness *et al.*, 2000) and the risk of diabetes mellitus is escalated by 74% in women and 45% in men, who smoke more than 2 packs day^{-1}, when compared to those who never smoked (Will *et al.*, 2001). However, there are discrepancies between results. The Framingham study failed to find a positive correlation between smoking and coronary heart disease among women (Seltzer *et al.*, 1989) and coronary mortality among smokers has been found not to be different between the genders (Hammond, 1966; Doll and Peto, 1976; Doll *et al.*, 1980).

1.8.5 Smoking and oestrogen

Smoking has anti-oestrogenic effects and alters oestrogen metabolism, which causes increased formation of inactive catechols (Baron *et al.*, 1990). These detrimental effects on oestrogen may elucidate, in part, the increased risk of CVD in female than in male smokers (see Figure 1.3). Also women receiving oral oestrogen replacement therapy have plasma oestrogen levels that are 40-70% lower than in non-smoking women (Geisler *et al.*, 1999). Smoking has differential effects on lipid metabolism and among women who smoke there is a 1.5-fold increase in the LDL/HDL cholesterol ratio compared to male smokers (Cullen *et al.*, 1998). HDL reduction in females could be linked to female steroid hormone concentrations.

Smoking, as well as having anti-oestrogenic effects, produces adverse outcomes on the female reproductive system. Women who smoke experience menopause at an earlier age than non-smokers (McKinlay *et al.*, 1985). Smoking can impair ovulatory function and causes variations in menstrual cycle function, it depletes oöcytes, impairs plantation, causes prematurity, increases spontaneous abortion rates and increases the risk of ectopic pregnancy 2-fold (ACOG, 1997; Windham *et al.*, 1999). Women taking oral contraceptives, particularly those over 35 years of age, have an increased risk of cardiovascular side-effects such as

thrombosis, myocardial infarction and cerebrovascular incidents when they smoke (Mishell, 1991). So although women are protected from CVD by their sex hormones it must be realised that this beneficial effect could be negated by the anti-oestrogenic and lipid-changing effects of cigarette smoking.

1.8.6 Smoking cessation in women

There are few studies that look at smoking cessation and its effects in women. However, a 24% reduction in mortality from coronary heart disease is evident in women within 2 years of cessation and after 10-14 years this risk is similar to that of non-smokers (Kawachi *et al.*, 1993). It has also been found that the risk of first myocardial infarction in women who had not smoked for at least 3 years was similar to that of women who were life-long non-smokers (Rosenberg *et al.*, 1990).

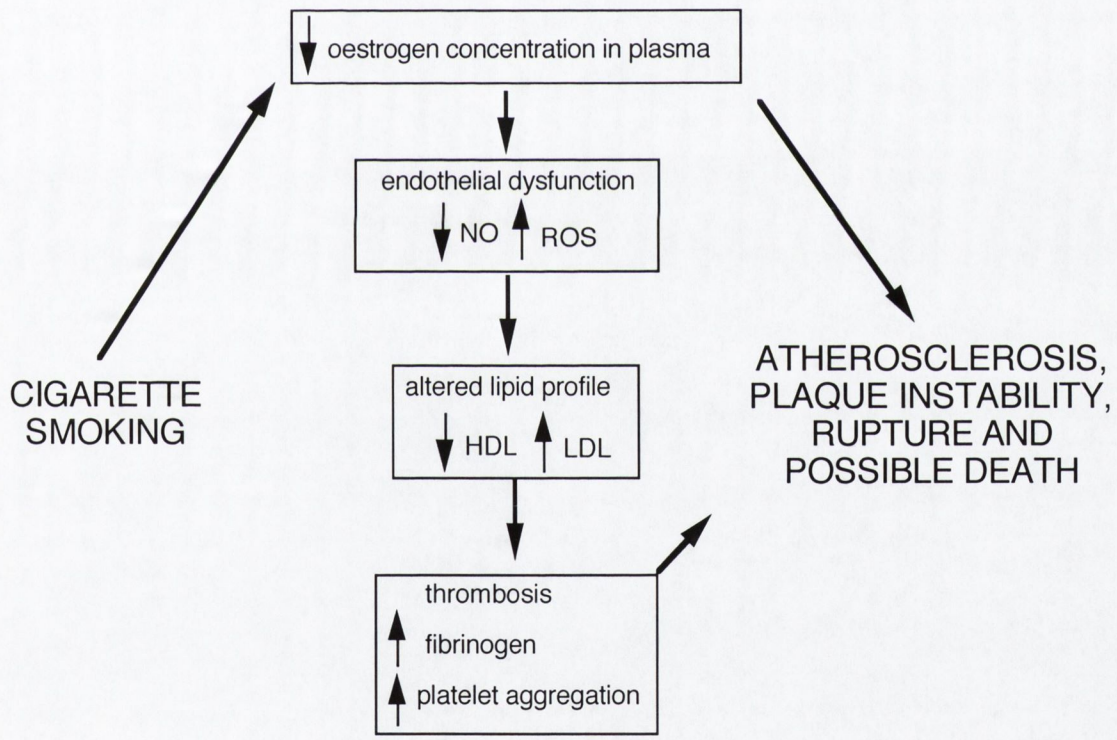


Figure 1.3. The anti-oestrogenic effect of smoking

Smoking decreases the oestrogen concentration in the plasma, which leads to: a decrease in NO activity, increased reactive oxygen species production, alterations in lipids and haemostasis, which can potentially lead to cardiovascular disease and possible death.

1.9 Methods of assessing cardiovascular reactivity

Cardiovascular reactivity is defined as the magnitude and pattern of cardiovascular responses to stress/challenge from baseline levels at rest to task performance or psychological stressor initiation (Andreassi, 1997). Various stressor states such as exercise, mental exertion and thermal stimuli are accompanied by reproducible changes in peripheral and central cardiovascular functioning. The autonomic nervous system is responsible for the co-ordination and integration of cardiovascular responses to stress and is controlled via the brainstem and cerebellum by afferent inputs from cortical and subcortical regions.

The cardiovascular changes that occur during physiological stress enable the body to cope with the demands placed upon it. These changes are primarily due to increases in sympathetic nervous system (SNS) activity and also by endocrine stimulation as reflected by NA and adrenaline levels in the plasma (Cryer, 1980). Both NA and adrenaline increase blood pressure due to vasoconstriction caused by NA and an increase in heart rate in the case of adrenaline (LeBlanc *et al.*, 1979). Plasma NA and adrenaline levels are in general, significantly correlated with heart rate, systolic pressure, cardiac work, and cardiac output (Halter *et al.*, 1984). Other responses to stress include increased skeletal muscle and decreased renal and splanchic blood flow (Brod, 1970), which allows the redirection of blood to the appropriate systems of the body such as heart, brain and skeletal muscle.

Stress tests are easily performed methods of assessing vasoreactivity, correlate with coronary risk factors and provide simple, non-invasive methods for evaluating risk for CVD (Barnett *et al.*, 1997; Minami *et al.*, 1999). The stress tests chosen in this present study allow for the simple and rapid evaluation of central and peripheral sympathetic activation.

1.9.1 Sustained isometric exercise

The sustained handgrip test is representative of static or isometric exercise and is characterised by sustained force production at a constant muscle length. When the muscle contracts at 40-60% of maximum voluntary contraction the intramuscular pressure exceeds the muscle perfusion pressure and blood flow becomes impeded (Bonde-Petersen *et al.*, 1975). Blood pressure increases up until

this point so as to preserve perfusion pressure. When blood flow is decreased to a threshold level the muscle chemoreflex is induced and this increases SNA. Vascular resistance increases in isometric exercise (Mitchell and Victor, 1996) as well as both systolic blood pressure and a proportional increase in diastolic pressure. This results in large increases in MAP.

Isometric handgrip is a potent α-adrenergic stimulus and the physiological responses seen with isometric exercise include increases in plasma adrenaline, heart rate, blood pressure, and myocardial contractility and cardiac output (Halter *et al.*, 1984). Blood pressure increases via SNS activation in parallel with skeletal muscle afferents, which may evoke sympathoexcitation, and this mechanism is initiated by central command (Mitchell, 1985; Rowell and O'Leary, 1990). Further increases are due to increases in SNA to the heart (Kluess *et al.*, 2000), which increase heart rate and cardiac output. Thereafter both mechanical and chemical stimuli trigger the exercise pressor reflex (Mitchell *et al.*, 1983; Rowell and O'Leary, 1990) by activating muscle nerve fibre endings, which also contribute to the increase in blood pressure (Rowell and O'Leary, 1990). Heart rate is raised by central command via vagal withdrawal with some contribution by mechanoreflexes. Chemical stimulation plays a primary role and is in the form of an increase in metabolite concentrations such as lactic acid, H^+, adenosine, bradykinin and others (Kaufman *et al.*, 1982; Rotto and Kaaufman, 1988; Rotto *et al.*, 1989; Costa and Biaggioni, 1994). These metabolites reflexly increase blood pressure by activating chemosensitive muscle afferents located within the interstitium of the muscle (Coote *et al.*, 1971) and also evoke an increase in muscle SNA (Mark *et al.*, 1985; Victor *et al.*, 1988). Sympathetic nerve discharge is coupled to increments in muscle cell H^+ activity (Victor *et al.*, 1988) and similar increases in muscle SNA, blood pressure and electromyogram activity have been noted during isometric exercise (Saito *et al.*, 1986).

Isometric handgrip is a vasoconstriction-inducing stimulus and an increase in sympathoexcitatory responses to the arm results in pronounced vasoconstriction in the contralateral non-exercising forearm (Cotzias and Marshall, 1993). However, work by Eklund and Kaijser (1976; 1978) and others reveals transient ß-adrenergic vasodilation in resting skeletal muscle at the onset of isometric handgrip, which may be due to unintentional contractions in the resting limb (Joyner and Halliwill, 2000).

Isometric exercise is therefore an important means of investigating the effects of both central command and muscle chemo- and mechanoreflexes on the circulation both in conjunction with each other and separately.

1.9.2 Mental Arithmetic

The mental stress test produces psychophysiological arousal and elicits a cardiovascular pattern characterised by increased heart rate, cardiac output, stroke volume, and blood pressure (Kaji *et al.*, 1989). The cardiovascular response to psychological stress is mediated primarily by an increase in ß-adrenergic activity (Eisenhofer *et al.*, 1985). Heart rate increases during mental stress primarily by an increase in cardiac sympathetic activity and it is this heart rate increase that is responsible for the increase in blood pressure (Freyschuss *et al.*, 1988).

During mental stress most vascular beds and veins undergo vasoconstriction, with the exception of skeletal muscle where vasodilatation occurs (Brod, 1970). Muscle SNA increases in the leg (Anderson *et al.*, 1987) but in fact there is a withdrawal of sympathetic activity to the forearm during mental stress (Halliwill *et al.*, 1997). This causes a decrease in forearm vascular resistance and so forearm blood flow increases during mental stress (Brod, 1970). Forearm vasodilator responses to mental stress still persist after nerve and stellate ganglion blockade (Linqvist *et al.*, 1996; Halliwill *et al.*, 1997). ß-adrenergic blockade attenuates the vasodilation observed during mental stress (Halliwill *et al.*, 1997) and so ß-mediated vasodilation has been proposed as a result of circulating adrenaline. Dietz and colleagues in 1994 noted that NOS inhibitors reduced this response by about 70%. They also confirmed that the dilator response was blunted by atropine administration but not so much as by the NOS inhibitor N(G)-Monomethyl-L-Arginine (L-NMMA). Activation of sympathetic-cholinergic innervation to skeletal muscle may be involved during mental stress and may stimulate the vascular endothelium to release NO. It is also possible that increases in blood pressure and heart rate may mechanically stimulate NO release from the endothelium.

The mechanisms involved in the forearm vasodilator responses to mental stress therefore include sympathetic withdrawal, stimulation of $ß_2$-receptors on vascular SMCs and endothelial cells via circulating adrenaline in combination with locally mediated NO release (Dawes *et al.*, 1997; Reed *et al.*, 2000).

1.9.3 The cold pressor test

Cold as well as the pain associated with it results in an increased catecholamine release from the adrenergic nerve endings and the adrenal medulla (Robertson *et al.*, 1979). The cold pressor test induces increases in plasma adrenaline, NA and adenosine levels (Halter *et al.*, 1984; Pasini *et al.*, 1999), systolic pressure, cardiac output (Halter *et al.*, 1984) and muscle SNA (Victor *et al*, 1987). Cold initiates a vasopressor response, which is mediated in large part by increased α-adrenergic activity (Abbound and Eckstein, 1966). α_2-receptor stimulation in the coronary and peripheral vasculature mediates vasoconstriction and an increase in peripheral vascular resistance and thereby an increase in systolic pressure and MAP (Madsen *et al.*, 2000). Further increases in systolic pressure and cardiac work occur as a result of myocardial $ß_1$-receptor stimulation of myocardial contractility and heart rate (Zeiher *et al.*, 1989). Increases in sympathetic neural outflow to the skeletal muscle vasculature as recorded in the peroneal nerve have also been observed (Victor *et al.*, 1987) due to activation of the SNS. Sympathetic tone increases following a complex series of events, including the involvement of voluntary central command and the activation of local mechanisms in the ischaemic area (Mark *et al.*, 1985).

The cold pressor test elicits systemic physiologic responses, which are endothelium-dependent. It is dependent on the capacity of the endothelium to reduce the vasoconstricting influences of sympathetic outflow and respond to humoral effects upon the vascular bed, including local NO release, which results in local endothelial-dependent vasodilatation (Zeiher *et al.*, 1989; Corretti *et al.*, 1995). Dilation seems to be due to ß-adrenoreceptor activation and perhaps flow-mediated endothelial dilation by α_2-adrenergic activity. Increases in shear stress cause endothelial cells to become stimulated, which results in increased synthesis and release of NO (Tousoulis *et al.*, 1997). Tousoulis *et al.* (1997) found that L-NMMA inhibited the cold-induced vasodilatation of normal arteries and suggested that vasoconstrictor stimuli were overcome by endothelium-derived NO.

1.9.4 Summary

These three tests as examples of exercise, mental and thermal stress allow for the rapid and non-invasive evaluation of central and peripheral sympathetic

activation on the cardiovascular system and also the effects of vasoconstrictor and vasodilatory stimuli on forearm blood flow and vascular resistance.

1.10 Methods of assessing endothelial function

To determine the effects of smoking and hormonal status on the endothelial cell layer, reactive hyperaemia, circulating endothelial cell number (CECs), thrombomodulin (TM) and von Willebrand factor (vWF) levels were measured. C-reactive protein (CRP) was measured as a marker of endothelial activation and inflammation.

1.10.1 Reactive hyperaemia

Endothelial function is most commonly assessed as a vasodilatory response to pharmacological or mechanical stimuli (Ludmer *et al.*, 1986). Endothelium-dependent vasodilatation evoked by intra-arterial infusion of vasoactive agents has been largely substituted by vascular responses to reactive hyperaemia, a non-invasive method. Increased blood flow shear is a mechanical means to stimulate vasodilation (Celermajer, 1997; Vogel, 1997) and change in blood flow is used as an index of resistance vessel function. Reactive hyperaemia is characterised by marked vasodilation and a dramatic increase in blood flow after a period of limb ischaemia caused by arterial occlusion. This increase in blood flow peaks immediately and decays exponentially (Patterson and Whelan, 1955). Blood flow to the forearm or calf is usually 2-5 $ml.100ml^{-1}.min^{-1}$ (Detry *et al.*, 1972) however, during reactive hyperaemia of 3-5 minutes in duration the peak blood flow response increases to an average of 20-30 $ml.100ml^{-1}.min^{-1}$ (Patterson and Whelan, 1955; Blair *et al.*, 1959). Perfusion pressure changes very little with increases in flow and so most of this increased flow is due to vasodilation (Joyner and Dietz, 1997). Maximal forearm blood flow (FBF) after ischaemia is considered a crude but reliable index for the overall dilatory capacity of a given vascular bed (Carlsson *et al.*, 1987).

The reasons for reactive hyperaemia are not fully understood. No oxygen-related or metabolic 'debt' occurs in the tissue (Blair *et al.*, 1959). The hyperaemic response is confined to the previously ischaemic vascular bed and persists even when denervation occurs (Duff and Shepherd, 1953). Myogenic relaxation of the vessels, which reduces vascular tone, during the ischaemia appears to account for a

portion of this response (Shepherd, 1983; Koller and Kaley, 1990). The vascular endothelium by releasing EDRFs (Koller and Kaley, 1990) or by stimulation of ATP-sensitive K^+ channels contributes to vascular SMC relaxation (Aversano *et al.*, 1991). The remaining vasodilation has been attributed to local release of mediators, for example, adenosine, prostaglandins, H^+, osmolarity, carbon dioxide, catecholamines, and ischaemic metabolites (Shepherd, 1983; Carlsson *et al.*, 1987; Aversano *et al.*, 1991).

Prostaglandins seem to play an important role (Kilbom and Wennmalm, 1976). Inhibition of cyclo-oxygenase with non-steroidal anti-inflammatory drugs has no effect on resting blood flow but can reduce peak flow responses by 15-30% (Carlsson *et al.*, 1987; Engelke *et al.*, 1996). Studies on adenosine's role show that responses are reduced by the adenosine receptor antagonist theophylline, which is thought to account for 30% of the response (Carlsson *et al.*, 1987) and endothelium-derived hyperpolarising factor may also contribute. Endothelial NO release can be stimulated by physiological changes, including hypoxia and increases in shear stress, both features of reactive hyperaemia (Pohl and Busse, 1989; LaMontagne *et al.*, 1992). Following periods of ischaemia ranging from 3-10 minutes minimal effects on peak flow immediately after ischaemia have been found with prior NOS inhibition by L-NMMA (Tagawa *et al.*, 1994; Engelke *et al.*, 1996). This is consistent with the concept that peak hyperaemic flow is mediated primarily by a myogenic mechanism (Koller and Kaley, 1990) although more recent evidence suggests both peak and maintenance of reactive hyperaemia involve NO production (Duffy *et al.*, 1999). Nevertheless in combination, cyclooxygenase and NOS inhibition cause a marked reduction in total excess flow after forearm ischaemia whereas the effects of each alone are minimal (Engelke *et al.*, 1996). However, L-arginine, the precursor for NO synthesis, improves endothelium-dependent vasodilatation in some patients with endothelial dysfunction (Drexler *et al.*, 1991; Dubois-Randé *et al.*, 1992; Imaizumi *et al.*, 1992; Koifman *et al.*, 1995) and increases in brachial artery diameter of 5-15% during reactive hyperemia have been found. This dilation is thought to be a result of increased brachial artery flow evoking release of NO (Celermajer *et al.*, 1994; Lieberman *et al.*, 1996).

The degree of peak flow following reactive hyperaemia is critically affected by morphological alterations of the vascular wall (Mulvany, 1991). Vasodilator and blood flow responses after reactive hyperameia and ACh as well as the increase in

brachial diameter after ischaemia are blunted in patients with essential hypertension (Lieberman *et al.*, 1996). Other vascular diseases also demonstrate abnormal endothelial responses (Chowcienczyk *et al.*, 1992; Johnstone *et al.*, 1993; Celermajer *et al.*, 1994). Reactive hyperaemia is therefore an easily performed method of assessing vasoreactivity and it correlates with coronary endothelial function among other cardiovascular risk factors (Celermajer *et al.*, 1994).

1.10.2 Circulating Endothelial Cells

Endothelial cells that have become detached from the blood vessel at the basal lamina and are detectable in the peripheral blood are known as circulating endothelial cells (CECs). The endothelium is activated by substances such as proinflammatory cytokines, growth factor, infectious agents, lipoproteins and oxidative stress. Increased or prolonged activation can lead to dysfunction and to the irreversible loss of integrity with cell detachment, apoptosis and necrosis. Programmed cell death i.e. apoptosis is an important mechanism underlying the changes seen in hypertension, inflammation and atherosclerosis (Karsan and Harlan, 1996; Bennett, 1997) and it can regulate endothelial cell number. The death of the endothelium can promote endothelial denudation and favour the development of atherosclerosis (Davies *et al.*, 1988). Apoptosis plays a key role in tissue and organ development and plays a role in physiological hypertrophy and hypertension (Hamet *et al.*, 1995; Teiger *et al.*, 1996). It is characterised by cell shrinkage, the formation of cytoplasmic vacuoles, plasma and nuclear membrane blebbing and chromatin condensation (Wyllie *et al.*, 1980, see Figure 1.3).

Endothelial dysfunction is often the first step in the onset of vascular disorders (Cines *et al.*, 1999). Reports of CECs first emerged in the 1970s (Bouvier *et al.*, 1970; Hladovec, 1978) and their study may be an important tool in the direct and non-invasive study of the endothelium. CECs are subject to the same physiological conditions, such as blood-borne activating factors, as the endothelium and more recently have been reported to be an *ex vivo* indicator of vascular injury (Dignat-Gorge and Sampol, 2000).

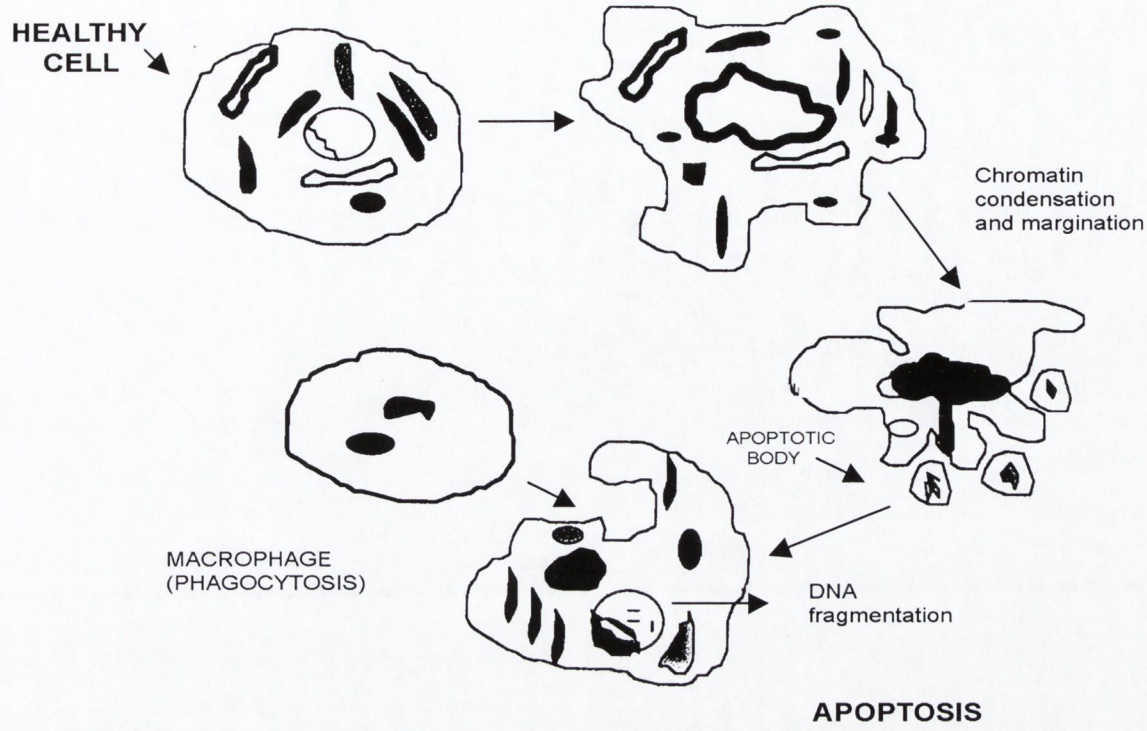


Figure 1.3 The apoptotic process

Apoptosis is a form of cell death where morphological changes observed include chromatin condensation and margination, cell volume shrinkage and budding of nuclear and plasma membranes to produce apoptotic bodies. Finally apoptotic cells are phagocytosed by macrophages. Adapted from Lopez-Farre *et al.* (1998).

CEC numbers increase in vascular disorders such as acute myocardial infarction, angina (Mutin *et al.*, 1999), peripheral vascular disease (Sinzinger *et al.*, 1986), sickle cell anaemia (Solovey *et al.*, 1997), hypertension (Hladovec and Prevovsky, 1989; Zhu, 1991), hypercholesterolaemia (Weber *et al.*, 1979) and with atherogenic stimuli such as nicotine (Hladovec, 1978a; 1978b) (see Table 1.1). CECs are raised in smokers (Sinzinger *et al.*, 1986). Increases in CEC number can be reversed by therapy in most cases including antihypertensive therapy (Zhu, 1991). CEC number and endothelial perturbation are linked to an increase in several plasma markers of endothelial cell dysfunction including vWF, soluble TM and endothelial cell adhesion molecules (Blei *et al.*, 1994; Dignat-Gorge and Sampol, 2000), IL-1 and CRP (Embury *et al.*, 1994), although this can depend on the disease (Mutin *et al.*, 1999). This could mean that detachment of endothelial cells is related to the amount of endothelium activation. The mechanisms by which CECs detach from the blood vessel wall are unclear, however various hypotheses include:

defective binding to the anchoring matrix (Visser *et al.*, 1989), integrin activation and apoptosis (Rüegg *et al.*, 1998), absence of growth factor (Solovey *et al.*, 1999), plaque rupture and subsequent mechanical dislodgement of cells, prolonged ischaemia of the heart muscle, oxidative burst after blood repurfusion and plasminogen activation (Mutin *et al.*, 1999).

Table 1.1 Number of CECs in various pathological conditions

Reference (yr.)	Condition	Mean No. of CEC in Affected Individuals/ml	Mean no. CECs in Healthy Individuals/ml
Mutunga *et al.* (2001)	Septic shock	30±3	2±1
Clancy (2000)	Systemic lupus	4-Fold ↑	
Solovey *et al.* (1997)	Sickle cell anaemia	23±18	3±2
O'Sullivan (2003)	Fit individuals	75±13	41±5
Woywodt *et al.* (2003)	Small vessel vasculitis	252	4
Mutin *et al.* (1999)	Acute myocardial infarction	15.5	0
George *et al.* (1991)	Angioplasty	11±3	0-1
George *et al.* (1993)	*Rickettsia conorii* infection	162±454	0
Drancourt *et al.* (1992)	*Rickettsia conorii* infection	27±30	
Camoin-Jau *et al.* (2000)	Cerebral thrombophlebitis	27	1.6±0.6
Lefevre *et al.* (1993)	Thrombotic thrombo-cytopaenic purpura	5-37	1
Sinzinger *et al.* (1986)	Smoking	393 ±20.3	2.4±2.3 (SD)
Janssens *et al.* (1999)	Chronic venous insufficiency	90% ↑	514±82 (SD)

Values are means ± S.E.M. CECs: circulating endothelial cells; ↑: increase; SD: standard deviation. Adapted from Segal *et al.* (2002).

CECs are flat, polygonal cells approx 30–50 μm in diameter and <1 μm in thickness (Hladovec and Rossman, 1973). Bouvier *et al.* (1970) showed that CECs are anucleated carcasses of endothelial cells or dead endothelial cells. However, some endothelial cells have a central space, which may be indicative of an extruded nucleus (Hladovec and Rossman, 1973). The nucleus may be lost by the centrifugation process or apoptosis or necrosis. CECs show the same features as the

endothelium and can easily be recognised by the granular cytoplasmic distribution of vWF (Mutin *et al.*, 1999). CECs can be found in their rounded form or because they are so thin they can be rolled into spindle-shaped cells or found in endothelial sheets (Mutin *et al.*, 1999). Circulating endothelial sheets originate from endocardial endothelium (Johnson *et al.*, 1979). CECs can be classified loosely depending on the site of origin as CD36 positive or negative. The CD36 antigen is primarily associated with the microvascular origin of endothelial cells (Dignat-Gorge and Sampol, 2000). Using this criterion it has been found that CECs from sickle cell patients originate from the microvessels and those isolated from acute coronary syndromes are predominantly derived from the macrovasculature (Solovey *et al.*, 1997; Mutin *et al.*, 1999).

Little is known about the half-life of circulating endothelial cells. George *et al.* (1992) reported the half-life to be no less than 24 hours in patients undergoing coronary angioplasty and Mutin *et al.* (1999) found that in the case of patients with acute myocardial infarction or unstable angina, CECs were still present in the blood in some patients up to 42 hours after the onset of chest pain.

1.10.3 Plasma markers of endothelial damage

The most frequently used markers of endothelial abnormality are plasma soluble substances such as vWF, TM, endothelin, tissue plasminogen activator and its inhibitor, or soluble adhesion molecules. Detection is usually by a direct and easily performed method for quantifying functional alterations caused by abnormal endothelial cell activation. CRP, on the other hand, is an acute phase reactant, which is a marker of low-grade inflammation and increased levels are found in certain CVDs.

Von Willebrand Factor (vWF)

vWF, also known as factor VIII-related antigen, is the carrier and stabiliser of procoagulant factor VIII, required for the adhesion of platelets to the endothelium (Houdijk *et al.*, 1986) and platelet-platelet interaction (Ruggeri, 1999). It is found in plasma (directly secreted by endothelial cells), platelet α-granules, Weibel-Palade bodies of endothelial cells (only secreted upon proper stimulation of the cell), and the subendothelium (Wagner 1990; van Mourik and de Wit, 2001). Plasma vWF is the largest protein in plasma and stems from the endothelium rather than from

platelet stores or megakaryocytes (Nichols *et al.*, 1995). Injured endothelial cells release increased levels of vWF, which has been described in hypertension (Pedrinelli *et al.*, 1994), diabetes (Jensen, 1989), intermittent claudication (Edwards *et al.*, 1994), coronary heart disease (Schumacher *et al.*, 2002) and rheumatoid arthritis (Farrell *et al.*, 1992) and may predict myocardial infarction, angina and mortality (Jansson *et al.*, 1991; Thompson *et al.*, 1995). vWF is an independent risk factor for stroke (Folsom *et al.*, 1999). It is also associated with risk factors for atherosclerosis e.g. hypercholesterolaemia and smoking (Badimon *et al.*, 1993; Blann, 1993). However contradictory as it may seem, vWF is an acute plasma reactant that increases in a variety of clinical conditions not necessarily associated with endothelial damage (Mannucci, 1998). Risk factors that increase levels of fibrinogen (e.g. use of oral contraceptives) and decrease fibrinolysis (e.g. smoking decreases plasminogen activator inhibitor) may have a synergistic effect with vWF (Tietjen *et al.*, 2001). Elevated concentrations have been found after exercise in healthy subjects, which could be considered an adrenergic stress response (Cohen *et al.*, 1968). Sympathoadrenal stimuli increase plasma vWF levels (Jern *et al.*, 1989; von Känel and Dimsdale, 2000). As well as genetic variation in vWF levels, which account for 60% of the variability with 30% of this attributed to ABO blood group (Gill *et al.*, 1987; Levy and Ginsburg, 2001) environmental factors also play a key role. Age, stress, thyroid hormone and oestrogens have been shown to influence VWF levels (see Levy and Ginsburg, 2001). The circulating half-life of vWF is >12 hours (Borchiellini *et al.*, 1996).

Thrombomodulin (TM)

TM is an endothelial membrane glycoprotein. TM plays a role in the anticoagulant pathway by acting as a thrombin receptor and thereby facilitating Protein C activation. The majority of TM is associated with the cell membrane but some cleaved TM fragments exist in the blood as soluble TM (sTM) (Ishii and Majerus, 1985). It is unclear what role sTM plays physiologically but it has come to be considered as a marker of endothelial damage (Ishii *et al.*, 1991). Most studies investigating sTM have examined peripheral artery disease but results are conflicting. It has been found that patients with peripheral artery disease and also those with atherosclerosis have higher sTM levels than age and sex-matched controls (Blann *et al.*, 1997). However the same group more recently found no

correlation between ankle-brachial index, as a measurement of peripheral artery disease, and TM (Blann *et al.*, 2000). Other groups support both arguments. A positive correlation between sTM concentration and peripheral artery disease (Seigneur *et al.*, 1993; Blann *et al.*, 1998) and no correlation whatsoever (Sernau *et al.*, 1995; Salomaa *et al.*, 2001) have been found. This latter group concluded that TM is a marker for microvascular but not macrovascular endothelial cell damage. Although a minimal amount of TM is present in the cytoplasm of platelets (Suzuki *et al.*, 1988), sTM in plasma is largely derived from membrane TM on the endothelium, suggesting a more specific marker for endothelial damage. Plasma levels of TM are increased in disorders such as diabetes mellitus, lupus erythematosus and cancer (Blann and Seigneur, 1996).

c-Reactive Protein (CRP)

As well as the conventional risk factors for CVD, factors such as infection are able to activate cytoplasmic transcription factors, which in turn lead to the expression of cell surface adhesion molecules, activation of cytokines and chemokines, precipitation of vascular inflammation and alteration in endothelial cell function (Cotran and Mayadas-Norton, 1998; Kinlay *et al.*, 1998). Mediators of endothelial activation and inflammation such as CRP and IL-6 can be measured in the circulation and are indicators of increased risk of future cardiovascular events.

Plasma CRP is an acute phase protein that is induced in response to inflammatory cytokines during chronic inflammation or infection and has now been accepted as an independent risk factor for CVD (Ridker *et al.*, 1997). CRP increases rapidy during inflammatory processes and it is the most sensitive of the acute phase reactants. There is considerable evidence to suggest that inflammation within large arteries contributes importantly to the development, progression and clinical expression of atherosclerosis (Ross, 1999) and it has been suggested that this may be mirrored by circulating biochemical markers such as CRP (Danesh *et al.*, 1998; Lagrand *et al.*, 1999). CRP has been implicated in the facilitating of LDL uptake by macrophages, thus contributing to foam-cell formation and hence atherogenesis (Zwaka *et al.*, 2001).

Levels of CRP are elevated in association with cardiovascular risk factors such as smoking, diabetes, hypertension, hyperlipidaemia, and are also elevated during acute coronary syndromes (see Blake and Ridker, 2002). CRP has been

positively correlated with cardiovascular risk in healthy men and women (Ridker *et al.*, 1997; Rifai *et al.*, 2002). CRP may be released from the liver and other sources include macrophages and SMCs of atherosclerotic plaques (Yasojima *et al.*, 2001). Increases in CRP levels are associated with an increase in myocardial infarction as well as ischaemic stroke (Ridker *et al.*, 1997). Thompson *et al.* (1995) found that subjects whose fibrinogen and CRP were low had an almost negligible risk for subsequent coronary events even with high cholesterol. On stimulation by cytokines, such as IL-6, CRP has been found to induce synthesis of other cytokines, cell adhesion molecules, and tissue factor in monocytes and endothelial cells (Cermak *et al.*, 1993; Pasceri *et al.*, 2000) and may well be a risk factor of thromboembolism. Hepatocyte production of IL-6 appears to be one of the most important stimulants of acute phase proteins, such as fibrinogen and CRP, from chronic inflammation and infection (Castell *et al.*, 1989).

1.10.4 Summary

Using the above markers the effects of hormonal status and smoking on the endothelial layer can be demonstrated in terms of functional (reactive hyperaemia) and structural (CECs, vWF, TM and CRP) integrity.

1.11 Objectives

The objectives of the work described in this thesis were to investigate the interactive effects of hormonal status, smoking and ageing on SNS activation and endothelial function. Tests of sympathetic activation and endothelial integrity were examined in response to differing hormonal milieus and compared in smokers and non-smokers. Initial studies were carried out using young premenopausal women and results from these studies were compared with middle-aged postmenopausal women. In order to assess the long-term effects of hormonal manipulation on BRS and endothelial turnover rates, techniques that are not easily employed in humans were required, and a rat model was used.

2.1 Materials

The full list of materials and names and addresses of sources listed below are given in Appendix I.

2.2 Subject Recruitment (Human studies)

Young female subjects were recruited from the staff and student body at Trinity College, and in the case of middle-aged postmenopausal subjects, from Trinity College staff and interested members of the public. All subjects were reasonably sedentary in that they did not participate in any regular form of physical activity. After initial recruitment all subjects visited the laboratory on two or more occasions. On the first visit, subjects underwent a full medical examination to ensure their ability to complete maximal exercise (see Appendix II). The subjects gave written informed consent to participate in the study and the objectives and methods were explained clearly (see Appendix III and IV). Subject height and body mass were measured and Body Mass Index (BMI) was recorded as mass (kg) divided by height (m) squared. Body Surface Area (BSA) was calculated using the equation (body mass $(kg)^{0.425}$ x height $(m)^{0.723}$ x 0.007184). Three blood samples were then taken from the median cubital vein in the ante-cubital fossa of the arm. A 3 ml blood sample was taken for haematological analysis of haemoglobin, haematocrit, and red and white blood cell counts using a Coulter T-890 counter machine (Coulter Electronics Ltd., Bedfordshire) and two 6 ml samples were taken for analysis of endothelial cell number and also for biochemical analysis of markers of endothelial function. Exclusion criteria included diabetes, hypertension and atherosclerosis. No subjects were taking anti-hypertensive or inflammatory medication including aspirin and in middle-aged subjects total blood cholesterol was measured to rule out subjects with hypercholesteraemia.

Smokers were allowed to follow their usual smoking habits on the day of testing but were required to abstain from smoking for at least 1 hour prior to

experimentation. This amounted to an abstention period of 2.0 ± 0.39 h for young smokers and 4.7 ± 1.4 h for older smokers. For brands of cigarettes smoked by participating subjects see Appendix V. Non-smokers confirmed they had not been exposed to smoke for twelve hours prior to their visit to the laboratory.

All young premenopausal women were between the ages 18-30 and had normal regular menstrual cycles. They presented themselves at the laboratory at least twice corresponding to two stages during their menstrual cycle: days 1-5 (the menstrual phase) and days 12-14 (ovulation). These times were selected in an effort to study the effects of high (days 12-14) and low (day 1-5) concentrations of oestrogen and to minimise the potential opposing effects of progesterone. Testing sequence was randomised. Menses was defined as the period from bleeding start to end and ovulation was determined by use of a commercial luteinising hormone detection kit (First Response ® Ovulation Predictor test, Carter-Wallace. Inc.). Subjects came to the laboratory within two days of obtaining a positive result using this test. All subjects in the oral contraceptive group were taking combined oestrogen and progesterone oral contraception (see Appendix VI) and were tested during the week of withdrawal from oral contraceptive use and the second or third week of oral contraceptive use. Testing sequence was randomised.

Postmenopause was defined as not having had a period within the last six months. All postmenopausal women were between 45–69 years. They were required to attend the laboratory for testing at least once as were postmenopausal women taking Hormone Replacement Therapy (HRT). 7 postmenopausal women were taking HRT: 2 were using a transdermal oestrogen preparation, 2 were using cyclic tablets containing oestrogen and norgestrel and their measurements were taken during the oestrogen phase, 2 were taking a continuous tablet consisting of conjugated oestrogen and medroxyprogesterone acetate and 1 was taking a conjugated equine oestrogen preparation (see Appendix VII). One postmenopausal smoker was on HRT while the other six were not taking any form of hormone replacement.

All procedures received ethical approval from The Federated Dublin Voluntary Hospitals and St. James' Hospital Research Ethics Committee.

2.3 Experimental protocol

2.3.1 Maximal fitness testing

On visit one each subject performed a standard incremental fitness test on a Monark 824E cycle ergometer to measure peak oxygen uptake ($V_{O_2}peak$). All tests were performed in a temperature and humidity controlled climatic chamber (temperature 18°C and humidity 40%). Subjects were informed about the exercise procedure and were instructed to continue until exhaustion. Subjects older than 45 years were subject to 12-lead electrocardiography prior to fitness testing and to continuous electrocardiographic and visual monitoring during the test by a registered medical practitioner. They were excluded and the test discontinued if, before or during fitness testing, ECG indicated: significant (>2mV) ST segment depression, ST segment elevation, T-wave inversion, abnormal Q-waves, arrhythmia or dysrhythmia, consecutive premature ventricular contractions, and heart rate exceeding calculated maximum (220-age beat.min^{-1}) for five minutes. The testing was discontinued in subjects of this age group if they experienced chest, left arm or jaw pain, excessive shortness of breath, dizziness or light-headedness, exhaustion or nausea, developed cyanosis or requested exercise cessation.

All subjects wore loose fitting sports clothes. Saddle height was adjusted so as the subject's leg was almost fully extended when the pedal was at its lowest point. Handle-bars were adjusted by the subject to obtain optimal positioning. Heart rate was measured using a Polar Favour heart rate monitor (Polar Electro, Finland). This method consists of a transmitting belt, which is placed around the upper abdomen and a receiving watch unit, which was out of view of the subject. Metabolic parameters were measured continuously using a Metalyser gas analysis system (CORTEX Biophysik GmbH, Germany), which was calibrated daily. Subjects were fitted with a moulded silicone rubber facemask into which the metalyser volume transducer and gas sensor was fastened. The facemask was strapped to the head by a Hans Rudolph head-piece with adjustable elastic straps. Three sizes of facemasks were available to accommodate different head size. The headstraps were used to tighten the mask to the face so no air could escape. The integrity of the seal was checked by blocking the inspiratory valve and asking the subject to breathe in deeply to ensure no leaks were felt.

The test began with a 60 watt workload, which was increased incrementally at 3 minute intervals by 30 watts with the addition of a 0.5 kg weight to the weight

pan of the ergometer. The subject was required to sustain a cycling speed of 60 revolutions per minute. Heart rate was measured during the last minute of the workload. Subjects cycled until volitional exhaustion. Once the test was completed, the weights were removed and the subjects were instructed to cycle at a moderate pace to warm down.

2.3.2 Sympathetic activation testing

All sympathetic activation testing took place in the afternoon between 1300-1800 hrs at 19-21°C. The subjects were asked to abstain from tea, coffee, alcohol and exercise on the day of testing and to only eat a light lunch. Subjects were seated comfortably for 10 minutes prior to testing so as to get resting baseline values. The tests used to elicit sympathetic stimulation were: an isometric handgrip, mental arithmetic and the cold pressor test. Time was left in between tests to ensure pre-test values were similar to resting baseline measurements. During each test the following parameters were measured and recorded via a Maclab data acquisition system: blood pressure, heart rate, stroke volume and forearm blood flow (see Appendix VIII).

2.3.3 Blood pressure measurement

Blood pressure measurements were made by applanation tonometry using the Colin CBM-7000 continuous blood pressure monitor. This method has previously been validated (Sato *et al.*, 1993). The principle underlying tonometry is that intra-arterial pressure can be measured by pressure transducers centred over the flattened portion of the radial arterial wall because any force exerted on these transducers can be opposed by an equivalent force in order to establish its magnitude.

The pressure cuff was wrapped around the bare upper arm of the subject. A wrist brace was then place on the same arm with the subject's thumb strapped down so as to prevent movement artifacts. The wrist was left facing upwards and the arm was rested at the level of the heart. The radial artery was located via pulsation and the Colin sensor was placed in the location of the strongest pulse. When the sensor was switched on an oscillometric cuff measurement for calibration was performed automatically. Measurement was only begun when the tonogram was symmetrical, the signal strength was close to 100% and the sensor hold down less than 120. The Colin was recalibrated between tests.

MAP was calculated as diastolic pressure + 1/3(pulse pressure) and pulse pressure was defined as systolic pressure–diastolic pressure. Rate pressure product, as an estimate of cardiac work, was calculated by multiplying systolic blood pressure by heart rate and dividing by 100.

2.3.4 Heart rate measurement

A standard three lead frontal plane ECG was recorded continuously and displayed together with a beat-to-beat ratemeter triggered from each QRS complex of the ECG recording.

2.3.5 Stroke volume measurement

Impedance cardiography was used to permit noninvasive monitoring of stroke volume. The impedance cardiograph (built in-house) was used in conjunction with an 8 surface electrodes (3M Health care, Germany) configuration to record impedance, dZ/dt, and Z signals. The four outer electrodes were used for feeding the constant current through the tissue, while the four inner electrodes were used to detect the voltage changes, which result from the change in impedance. One pair of detection electrodes was placed in the midaxillary lines, one left and one right at the level of the xyphoid. The second pair of detection electrodes was placed at either side of the base of the neck. The third and fourth pairs of current feeding electrodes were placed approximately 3 cm above and below the detection electrodes. This technique relies on the fact that caudo-rostral movement of the blood into the aorta during cardiac ejection alters the electrical impedance of the thorax.

Stroke volume was calculated using the Kubicek equation (Kubicek *et al*, 1966):

$$SV\ (ml) = L^2/Z^2.\ dZ/dt.\ t.\ 135,$$

where the measured parameters are the vertical distance between the detection electrodes in cm (L), the basal impedance in $ohm.cm^{-1}$ (Z), the left ventricular ejection time in s (t) and the rate of change of impedance during systolic ejection in $ohm.s^{-1}$ (dZ/dt). The constant 135 is the resistivity of the blood in ohms.

The average of three stroke volumes was taken at each measurement time point. Stroke volume index was calculated as stroke volume divided by BSA. Cardiac output was then calculated as the product of stroke volume and heart rate in a given cardiac cycle. Cardiac index was obtained by dividing cardiac output by

BSA. Total peripheral resistance was calculated as mean arterial pressure divided by cardiac output.

2.3.6 Forearm blood flow measurement

Forearm blood flow was measured by venous occlusion plethysmography. The principle behind this technique is that an occluding cuff is placed around the upper arm and inflated to 40 mmHg. Complete cessation of the venous return from the forearm occurs but the arterial inflow of blood continues so that the arm begins to fill up with blood. This type of plethysmography measures the change in forearm circumference, which is linearly proportional to the volume change. It is known that the initial rate of volume change of the tissue is equal to that of the arterial inflow (Whitney, 1953). In this present study an indium/gallium silastic rubber strain gauge was positioned around the upper part of the forearm, which was raised on either side of the strain gauge to prevent pressure being exerted upon it. The indium/gallium column was stretched and narrowed as the forearm circumference increased and this caused its electrical resistance to increase. The electrical resistance was then measured and converted to change in arm circumference. Output from the strain gauge passed through a model 270-A Plethysmograph amplifier (Parks Electronics Laboratory, USA) and the data were collected via a MacLab data acquisition system. The cuff was inflated for 4 s every 10 s.

Blood flow was expressed in millilitres per minute per 100 ml forearm tissue. Forearm vascular resistance was calculated by dividing MAP by forearm blood flow. Forearm vascular conductance was calculated as 1/vascular resistance.

2.3.7 Isometric handgrip

Maximum handgrip strength was tested with a calibrated handgrip dynamometer (Stoelting, U.S.A.) to obtain the maximum voluntary contraction resulting in the maximum force. Subjects were then instructed to sustain contraction at 50% of the predetermined maximum force. This was maintained until volitional fatigue. The subjects could see the assigned target force to assist in the maintenance of the appropriate contraction. Results of the handgrip are expressed as percentages of the time to fatigue with 100% fatigue taken as the point where subjects were no longer able to sustain 50% maximum voluntary contraction.

2.3.8 Mental Arithmetic

Subjects were instructed to subtract the number seven from a three-digit number as quickly and accurately as possible for a two-minute time period. For those who came back for retesting a different 3-digit starting point was selected.

3.3.9 Cold pressor test

The right foot was immersed up to the ankle in a basin of iced water (2-3 °C) for 2 minutes.

2.3.10 Reactive hyperaemia

Endothelial dysfunction was investigated by forearm reactive hyperaemia. This technique involves the inflation of a cuff past systolic pressure so as to occlude the vessels underneath and impede blood flow to the limb. The cuff was inflated for 3 minutes after which time the pressure was released and blood flow was measured for a period of 1 minute. The pre- and post-cuff inflation blood flow values were measured by venous occlusion plethysmography using a indium/gallium silastic rubber strain gauge. A sample trace may be seen in Figure 2.1.

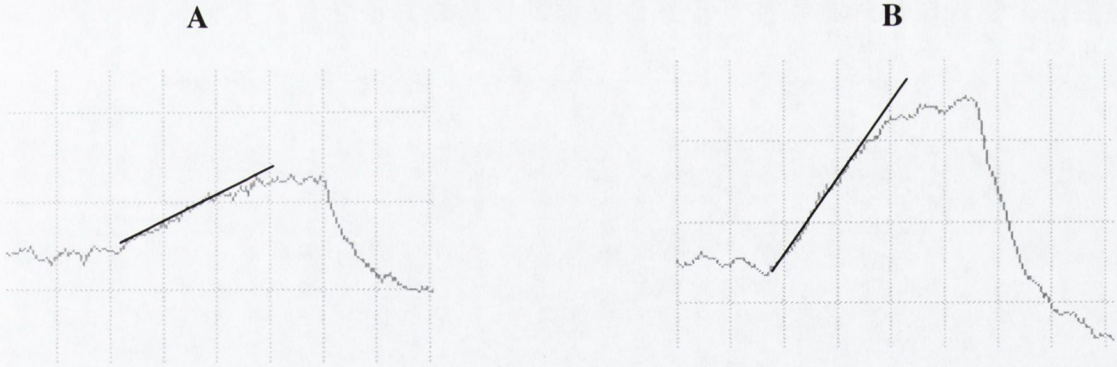


Figure 2.1 Blood flow in response to reactive hyperaemia.

Sample trace of blood flow measured by venous occlusion plethysmography pre- (A) and post- (B) reactive hyperaemia. The slope of the curve represents the rate of arterial inflow. Care was taken to obtain the slope from the linear portion of the curve, which represented blood flow increase immediately after cuff release.

2.4 Blood Studies (Human)

2.4.1 Samples

Subjects were asked to give a venous blood sample on their first visit. A 3 ml sample was collected in an EDTA coated tube and analysed for haematological abnormalities, a 6 ml sample was collected in a heparinised tube and analysed for structural damage of endothelial cells via circulating endothelial cell (CEC) number, and a second 6 ml sample was collected in a heparinised tube and used for biochemical analysis of various markers of endothelial function. The two 6 ml blood samples were immediately stored on ice after collection.

2.4.2 Isolation of plasma for markers of endothelial cell function

The blood sample was centrifuged on the same day as collection at 2500 x g in a Sigma 2K15C laboratory centrifuge. The plasma was then stored at –80°C until required.

2.4.3 Isolation and quantification of CECs

Isolation of CECs was performed on the same day as sample collection and was similar to that previously described by other groups (Hladovec and Rossman, 1973; Sinzinger *et al.*, 1986). An initial centrifugation of 500 rpm at 4°C for 20 minutes produced a platelet-rich plasma. 1ml of supernatant was aspirated, mixed with 200 μl adenosine-5-diphosphate (Boehringer Mannheim, Germany) and mechanically shaken for 10 minutes at room temperature to induce platelet aggregation. The supernatant was then centrifuged again at 500 rpm at 4°C for 10 minutes to remove platelet aggregates. The endothelial cell pellet was obtained by a final centrifugation at 2000 rpm at 4°C for 20 minutes. The sediment was resuspended in 50 μl of normal saline for analysis.

The endothelial solution was transferred to gelatin subbed slides in 10 μl aliquots and smears were made. Slides were left to dry overnight in a 37°C oven. Slides were fixed in 4% paraformaldehyde for 5 minutes and stained with Cresyl Violet. The preparations were then dehydrated through increasing strengths of alcohol, mounted in DPX and examined using a light microscope. The number of endothelial cells on 5 slides was counted and the average taken as the mean CEC count of that subject.

The isolated cells were known to be endothelial in origin from the result of a previous study in this laboratory, which stained for vWF (O'Sullivan, 2001).

2.4.4 Plasma analysis of von Willebrand Factor using ELISA

The Enzyme Linked ImmunoSorbent Assay (ELISA) technique (Kordia Laboratory Supplies, Netherlands) using paired antibodies was used for the detection of vWF in plasma. The plate wells (Nunc-Immuno^{TM} Plate, Denmark) were coated with capture antibody (goat anti-vWF IgG, 100 μl), diluted 1:100 in coating buffer (50mM Carbonate, composition: 1.59 g Na_2CO_3, 2.93 g $NaHCO_3$ made up to 1 litre in distilled water, pH 9.6) and incubated overnight at 4°C. The plate contents were emptied and incubated with 150 μl of blocking buffer (composition: 2.0 g NaCl, 0.73 g $Na_2HPO_4.12H_2O$, 0.05 g KH_2PO_4, 0.05 g KCl, 5 g Bovine Serum Albumin up to 250ml with distilled water, pH 7.4) for 60 min at room temperature. Plates were washed four times with PBS-Tween (8.0 g NaCl, 2.9 g $Na_2HPO_4.12H_2O$, 0.2 g KH_2PO_4, 0.2 g KCl, 1.0 ml Tween-20 up to 1 litre with purified water, pH 7.4).

A standard curve was obtained using a Haemostasis Reference Plasma (Biopool International, California, USA), which was reconstituted with 1 ml purified water to contain a reference range of von Willebrand protein from 99-135%. Reference plasma was diluted 1:100 (100%) and then serially down to 1:3200 (3.25%) using a diluent buffer (HBS-Tween-BSA: 5.95 g HEPES, 1.46 g NaCl, 2.5 g Bovine Serum Albumin, 0.25ml Tween-20 made up to 250 ml distilled water, pH 7.2). Plasma samples were diluted 1:300 using the HBS-BSA-Tween diluent buffer. Standard and samples (100 μl) were added to each well and incubated for 90 minutes at room temperature (22°C). Plates were then washed four times in PBS-Tween.

The secondary antibody (peroxidase conjugated goat anti-vWF, 100 μl) was diluted in HBS-BSA-Tween, 100μl was applied to each well and the plate was incubated for 90 mins at room temperature (22°C). After washing the plate four times with PBS-Tween the bound antibody conjugate activity was expressed by incubation with ortho-phenylenediamine (OPD) (Sigma Steinheim, Germany). OPD was prepared by dissolving one 5 mg OPD tablet in 12 ml substrate buffer (constituents: 2.6 g citric acid, 6.9 g Na_2HPO_4 made up to 500 ml with distilled water) and then adding 12 μl 30% H_2O_2 immediately before use. 100 μl of freshly

prepared OPD substrate was added to every well. The colour was allowed to develop for 15 minutes and then the colour reaction was stopped with the addition of 50 μl per well of 2.5 M H_2SO_4. The colour generated was proportional to the concentration of vWF initially present in the sample. The absorbance values were determined by reading the plate at a wavelength of 490 nm within 30 minutes. vWF plasma concentrations were then calculated from the standard curve constructed by plotting the mean absorbance for each standard against the standards concentration, and reading unknown absorbances from this line (r=1, Y=(195.11)X + 1.32). Concentrations are expressed in units, which indicate the percentage of vWF given that the standard is 100%.

2.4.5 Plasma analysis of Thrombomodulin using ELISA

The detection of plasma TM (CD141) was by means of a commercially available ELISA kit (sCD141 ELISA Kit, Diaclone Research, France). A monoclonal antibody specific for sCD141 had been coated onto the wells of the plates provided. Standard plasma was reconstituted with standard buffer diluent (supplied by the manufacturer) to give a concentration of 20 ng/ml sCD141. Serial dilutions to 0.625 ng/ml using the standard diluent were then made. Plasma samples were tested undiluted. 100μl of the standard and plasma samples were then added to the wells. The biotinylated anti-sCD141 antibody was diluted (1:27.5) with the biotinylated antibody diluent and 50 μl was added to each well. The plate was covered and allowed to incubate for 60 minutes at room temperature. The plate was washed three times with a wash buffer that was supplied (1:200 in distilled water). 100 μl of streptavidin-HRP solution (1:6666, diluted with HRP diluent, supplied) was added to each well, the cover was replaced and the plate was incubated at room temperature for 30 minutes. The plates were emptied and washed three times, as before. 100 μl of ready-to-use chromogen TMB substrate solution was added to each well and incubated in the dark for 12-15 minutes at room temperature. The enzyme-substrate reaction was stopped by addition of 100 μl 1.8 M sulphuric acid to each well. The results were read immediately. The absorbance of each well was read at a wavelength of 450 nm on a spectrophotometer. Plasma TM concentrations were calculated from the standard curve constructed by plotting the mean absorbance for each standard against the standards concentration, and reading unknown absorbances from this line (r=1; Y=(13.98)X + 0.32).

2.4.6 Plasma analysis of c-Reactive Protein using Particle-Enhanced Immuno-turbidimetric Assay (work by Pat Kelly at the Adelaide and Meath Hospital).

The principle behind this assay is that anti-CRP antibodies coupled to latex microparticles react with antigen in the sample to form an antigen/antibody complex, which can be measured turbidimetrically following agglutination. Supplied in the CRP kit are Buffer, 2x15ml (Tris buffer: 16mmol/l, pH 7.4; preservative) and Anti-CRP antibody-latex/buffer, 2x15 ml (Latex particles coated with anti-CRP mouse monoclonal antibodies: 0.1%; glycine buffer: 50 mmol/l, pH 8.0; preservative). The samples were mixed and centrifuged at 3000g for 5 minutes before the beginning of the assay to remove precipitate. Reference plasma was used in the calibration to produce a 2-point curve, where CRP concentration could be ascertained (Roche/Hitachi analyzer model 917) against optical density or absorbance. The first point was at a concentration of 0 and the second at 11.2 mg/L. The raw data were printed and concentration values were given in mg/L. Expected values are in the range < 5.

2.5 New Zealand Genetically Hypertensive (GH) Rat (Animal studies)

2.5.1 Animal characteristics

All rats used in the experiments in the following chapters were age-matched adult male and females (12-18 weeks) bred in the Trinity College BioResources unit from stock derived from the Wellcome Medical Research Institute, University of Otago (New Zealand). The selection consisted of normotensive controls and GH animals from the colony that produced the original GH strain (Phelan, 1968). Animals were housed usually 3-4 to a cage. All animals were allowed free access to laboratory chow and tap water in a room with a controlled temperature-regulated environment (20-22°C) and lighting (10-h light-dark cycle). GH rats do not appear to have any special dietary needs. Animals were cared for in accordance with the guidelines set out by the Department of Health and Children based on the Cruelty to Animals Act, 1876 and the European Community Directive, 86/609/EC.

2.5.2 Tail cuff measurements

Before each set of experiments the blood pressure integrity of each animal was confirmed by performing tail-cuff plethysmography (Life Science Instruments,

IITC Inc., USA) so as to establish a systolic arterial blood pressure of <145 mmHg in control animals and >170 mmHg in GH animals.

All measurements took place at a temperature of 30°C so as to dilate the blood vessels of the rat's tail. The rats were left to acclimatise at this temperature for 15 minutes. After this period they were placed in a restraining container in order to obtain clear readings without movement artifacts. Each rat was familiarised with the restraining procedure before measurements were made. The tail cuff was then placed on the upper part of the tail and the pressure was set to 300 mmHg for GH rats and 180 mmHg for normotensive rats. The pressure valve was released slowly and the systolic pressure at which discrete individual pulses could be seen on the recording was taken as the systolic pressure of that animal. All recordings were displayed and stored using a PowerLab system (AD Instruments) (see Appendix IX).

2.5.3 Surgery

Animals were anaesthetised with urethane (1.25 g/kg i.p.). Depth of anaesthesia was assessed by a paw retraction test. All animals were placed in a supine position on a stainless steel operating table maintained at 34°C (Palmer Bioscience, U.K.). A tracheal tube was inserted into the trachea just below the larynx to maintain airway patency. The right femoral artery was cannulated with polythene tubing for recording of intra-arterial pressure. A polythene catheter was placed in the right internal jugular vein for infusion of vasoactive substances.

A period of 15 minutes was allowed before recording took place for stabilisation of blood pressure and heart rate. All parameters were displayed and stored using a PowerLab system (AD Instruments). Where possible animals from each strain were studied on the same day to minimise external influences.

2.5.4 Heart rate measurement

A three-lead surface ECG was recorded continuously via transcutaneous needle electrodes and displayed together with a beat-to-beat ratemeter triggered from each QRS complex.

2.5.5 Blood pressure measurement

Blood pressure was measured continuously from the abdominal aorta via the femoral artery by a blood pressure transducer (AD Instruments). The baseline was set to zero after each set of infusions.

2.5.6 Drugs

Phenylephrine (PE) (Sigma) is a sympathomimetic with direct effects on predominantly α-adrenergic receptors. It does not significantly stimulate the CNS. It constricts resistance and capacitance blood vessels, increasing TPR, resulting in increased systolic and diastolic blood pressure. Venous return to the heart may be decreased by its actions. It causes reflex bradycardia, which may be blocked with atropine.

Sodium nitroprusside (SNP) (M & B Laboratory Chemicals) is a nitrovasodilator and is metabolised to NO, which dilates both arterioles and venules but regional distribution of blood flow is minimally affected by the agent. It acts on vascular SMCs independent of the endothelium (Sudhir *et al*, 1996).

A stock solution of phenylephrine (10 mg/ml) was prepared using saline (0.9%) and was further diluted to 140 μg/ml with saline. Sodium nitroprusside was dissolved in saline to give a 200 mM stock solution and was subsequently diluted to 0.5 mM with saline.

2.5.7 Drug Infusions

Infusions were controlled by a slow injection apparatus (Palmer, England) designed to deliver fixed volumes per minute. A preliminary trial was designed so as to find the optimum infusion rates for each drug. The drugs were infused initially at 6 different rates (see Appendix X). The two rates chosen were those that demonstrated peak blood pressure changes that were approximately twice that of the minimum (see Table 2.1) and were located on the linear portion of the curve. For a sample chart of blood pressure and heart rate responses to the maximum infusion rate of each drug see Appendix XI.

Table 2.1 Rate of infusions and concentrations of each substance delivered

Infusion rate ($ml.min^{-1}$)	[Phenylephrine] ($mM.min^{-1}$)	[Sodium Nitroprusside] ($mM.min^{-1}$)
0.2	1.506×10^{-4}	1×10^{-4}
0.05	3.765×10^{-5}	_____
0.025	_____	1.25×10^{-5}

2.6 Blood studies (Animal)

2.6.1 Samples

Animals were anaesthetised with urethane (1.25 g/kg i.p.). A 5 ml blood sample was collected by cardiac puncture. Blood was withdrawn slowly and the animal was sacrificed by cervical dislocation. Blood samples were separated into 2ml and 3ml aliquots. The 2ml sample was centrifuged to obtain plasma for analysis of vWF (via ELISA as previously described, however instead of a 1:300 dilution as with the human plasma a 1:100 dilution of rat plasma was found to give optimum results). The 3ml blood sample was required for the isolation of CECs under light microscropy (as previously described).

2.6.2 Isolation of the aortic endothelial cell layer via the *en face* method

The aortic endothelial cell layer was isolated by a method similar to that described by Warren in 1965. Briefly, the thoracic aorta was removed, cleaned of any remnants of fat or fibrous tissue still adhering to its outer surface and opened longitudinally. It was then pinned out and cut into 10 mm sections. The sections were then placed on polylysine-coated slides with the endothelial surface downward. The slides were put on a metal block and cooled at –20°C. Care was taken to ensure no air or water bubbles remained between the endothelium and the slide. At the first signs of freezing and beginning at one corner the muscle layers of the wall were immediately rolled back so as to leave the endothelial cell layer stuck to the slide. The slides were then frozen at –80°C for further analysis.

2.6.3 Cell proliferation

All animals were injected intraperitioneally with BrdU (1 ml/kg body weight) 1 hour before sacrifice. Newly formed DNA incorporating BrdU was

identified by immunostaining using a commercially available kit (5-Bromo-2′-deoxy-uridine Labeling and Detection Kit II, Roche Diagnostics, Germany). BrdU staining was performed on *en face* preparations.

Cells were fixed with ethanol fixative (50 mM glycine solution to 70 ml ethanol to get 100 ml fixative, pH 2.0) for 20 min at –20°C. The preparation was then washed 3 times in washing buffer (1:10 with redistilled water). Cells were incubated with anti-BrdU monoclonal antibody (1:10 with incubation buffer, both supplied) for 30 min at 37°C in a humidified chamber. After washing three times the cells were covered with anti-mouse-Ig-AP solution (1:10 with PBS) and incubated at 37°C for 30 mins. The preparation was then washed three times and covered with a colour-substrate solution (13 µl NBT-solution and 10 µl X-phosphate-solution were added to 100 nM Tris HCL-buffer, 100 mM NaCl, 50 mM $MgCl_2$, pH 9.5) for 15-30 mins. After washing 3 times the slide was counterstained using 1% methyl green for 10 mins at room temperature. Preparations were then mounted in aqueous mounting solution and viewed under a light microscope x40. The nuclei of positively stained cells stain blue. The percentage of BrdU-positive cells was obtained from a range of 6-10 adjacent x40 objective fields of the arterial segment and cells were counted until a minimum of 500 was observed.

2.6.4 Cell apoptosis

Apoptotic cell death was detected using a commercially available kit - DeadEnd Colorimetric Apoptosis Detection System (Promega Corporation, Madison, USA). TUNEL was performed on *en face* preparations.

Cells were firstly washed with PBS for 5 min at room temperature. They were then fixed in 4% paraformaldehyde for 15 mins and washed 2 times. The cells were permeabilised using proteinase K (1:500 in PBS) for 10 min at RT. The cells were then washed and re-fixed in 4% paraformaldehyde for 5 mins followed by washing x 2. An equilibration buffer (200 mM potassium cacodylate, 25 mM Tris-HCl, pH 6.6, 0.2 mM DTT, 2.5 mM $CoCl_6.(H_20)_6$, 0.25 mg/ml BSA supplied) was added to the cells for 10 min. A reaction buffer (1µl biotinylated nucleotide mix (250 µM biotinylated nucleotide mix, 10 mM Tris-HCl, pH7.6, 1 mM EDTA), 1 µl TdT enzyme and 98µl equilibrium buffer) was used to incorporate the 3′-OH DNA ends to fragmented DNA strands. The negative control was the same but instead of TdT enzyme 1 µl of deionised water was added. The cells were covered with

reaction mix and then incubated in a humidified chamber for 60 min. The reaction was terminated by adding 20X SSC (1:10 with deionised water) for 15 mins. The preparation was washed with PBS. Endogenous peroxidase was blocked with 0.3% H_2O_2 for 3-5 mins followed by washing x 3 with PBS. A 1:500 solution of horseradish-peroxidase-labelled streptavidin in PBS was then bound to the biotinylated nucleotide (30 min at room temperature) and this was detected using the chromogen diaminobenzidine (DAB) (50μl DAB substrate buffer to 950μl deionised water and add 50 μl DAB chromogen and 50μl hydrogen peroxide) for 10 mins. When a light brown background developed the cells were rinsed several times with deionised water. The cells were counterstained using 1% methyl green for 10 mins at room temperature. Preparations were then mounted using 100% glycerol and viewed under a light microscope x 40. The nuclei of positively stained cells were stained brown. The percentage of TUNEL-positive cells was obtained from a range of 6-10 adjacent x40 objective fields of the arterial segment and cells were counted until a minimum of 500 was observed.

2.6.5 Endothelial nitric oxide production

NOS was inhibited using N^G-nitro-L-arginine methyl ester (L-NAME, 30 mg/kg i.v., Sigma). L-NAME is a more soluble analog of arginine and a competitive inhibitor of eNOS. The magnitude of the increase in MAP to LNAME infusion was noted and expressed as a percentage of the basal MAP.

2.7 Statistical Analysis

A paired or unpaired Student's *t*-test or a One-way Analysis of Variance followed by a Student Newman Keuls post-hoc test was applied where appropriate to determine statistical significance. Simple regression analysis was performed by calculating Pearson's correlation coefficient. Scatter plots were used to display individual data within a group. Statistical and graphics software used were: InStat and Prism Graphpad software. Results are expressed as the mean ± standard error of the mean (S.E.M) unless otherwise stated and S.E.M values are rounded off to mean values plus one decimal place for accuracy. $P < 0.05$ was considered statistically significant. The term 'trend' refers to a difference in data that although not significant had a statistically calculated P value of between 0.05-0.1.

Chapter 3 'Effects of menstrual cycle phase, oral contraceptive use and smoking on sympathetic activation in young healthy females'

3.1 Introduction

After menopause the rate of CVD progression increases rapidly among women and the rate of incidence of arterial hypertension increases past that of age-matched men. This is presumably related to reduced levels of endogenous female sex hormones, with concurrent metabolic and haemodynamic modifications. The increased cardiovascular morbidity and mortality after menopause cannot be fully explained by changes in metabolic parameters. It appears rather that oestrogenic deprivation has a widespread impact on the cardiovascular system including the blood vessel wall. Other factors that have a detrimental impact on the blood vessel wall include hypertension, ageing, elevated LDL levels and lifestyle factors such as being unfit and smoking. Postmenopausal women demonstrate vascular hyperreactivity and a poor vasodilatory reserve (Mercuro *et al.*, 2001) and postmenopause CVD becomes the number one cause of death among women. In order to under stand the situation postmenopause it is important to look at the hormonal profiles of premenopausal women and determine what gives them cardioprotection.

As already stated menopause is associated with cardiovascular hyper-reactivity. Results from studies investigating the effects of menstrual cycle phase on cardiovascular reactivity to stress have proved inconsistent (Little and Zahn, 1974; Strauss *et al.*, 1983; Stoney *et al.*, 1986, 1990). Collins *et al.* (1985) found no difference in blood pressure reactivity to mental stress while Hastrup and Light (1984) concluded that systolic blood pressure increased during the luteal phase compared with the follicular in response to a reaction time test. In another study systolic blood pressure was elevated in the follicular phase as compared with the luteal phase when performing behavioural tasks (Polefrone and Manuck, 1988) while Girdler *et al.* (1993) found no difference in cardiovascular reactivity with cycle phase. The reasons for these inconsistencies are unknown but the majority of

studies do not take into account confounding lifestyle factors such as oral contraceptive use and smoking, both of which are known to alter cardiovascular reactivity. It has been reported that oral contraceptive users exhibit greater blood pressure responses to public speaking than non-users (Davis and Matthews, 1990) and smoking is a major cardiovascular risk factor in young women (Willett *et al.*, 1987). Ward *et al.* (1994) found that after smoking cessation a decrease in heart rate reactivity to stress was evident. However there are studies that suggest smokers do not demonstrate exaggerated responses to haemodynamic stress. For example Girdler *et al.* (1997) found no differences in reactivity between male smokers and non-smokers. In a study investigating the effects of smoking and gender on stress-induced heart rate variability, Adan and Sánchez-Turet (2001) found that smokers did not have greater reactivity than non-smokers.

The cardioprotective effects of oestrogen have been widely studied. However it is important to remember that due to smoking's anti-oestrogenic effects and its induction of unfavourable lipid profiles women may lose their natural protection to CVD. Smoking appears to produce greater detrimental outcomes in women than in men (Bolego *et al.*, 2002). A lack of endogenous oestrogen at menopause or changes in thrombogenic factors, such as in women taking oral contraceptives, combined with smoking may leave women at greater risk of cardiovascular mortality.

Smoking has been shown to alter cardiovascular control including baroreflex function (Mancia *et al.*, 1997), possibly due to a decrease in arterial distensibility (Failla *et al.*, 1997). BRS is enhanced after oestrogen therapy in postmenopausal women (Huikuri *et al.*, 1996). Recent evidence has shown that BRS is modulated during the menstrual cycle. Minson *et al.* (2000) found that BRS increased around mid-cycle when oestrogen levels are high while progesterone seemed to antagonise this effect. Rajkumar *et al.* (1997) found that HRT increases arterial compliance in postmenopausal women while smoking has been found to decrease this parameter (Giannattasio *et al.*, 1994). Other deleterious effects of smoking on the cardiovascular system include alterations in parasympathetic and sympathetic nervous balance (Laustiola *et al.*, 1988; Kotamaki, 1995), alterations in the renin-angiotensin-aldosterone system (Kotamaki, 1995) and diminished NO production (Barua *et al.*, 2001). In postmenopausal women who smoke there may be a beneficial effect of long-term oestrogen therapy on arterial structure and function in relation to CVD (Teede *et al.*, 1999).

The aim of this study therefore was two-fold. Firstly to determine whether differing hormonal profiles affect responses to cardiovascular stress in young premenopausal oral contraceptive users and non-users. The second aim was to determine whether smoking affects the cardiovascular response to stress in premenopausal women with normal regular menstrual cycles.

3.2 Methods

3.2.1 Subjects

The subjects were separated into three groups depending on hormonal and/or smoking status. Young healthy female non-smokers (n=10) were recruited into the premenopausal non-smokers group. Young healthy female smokers (n=7) were recruited into the premenopausal smokers group and young healthy women taking oral contraceptives (n=10) were recruited into the oral contraceptive user group.

3.2.2 Experimental protocol

Subjects were required to attend the laboratory on three separate occasions. Session one involved a medical examination followed by a maximal oxygen consumption incremental exercise test to quantify fitness levels. On this occasion subjects were then familiarised with the experimental procedure for the stress tests. The second and third sessions involved each subject reporting to the laboratory during the menstrual or the ovulatory period of the menstrual cycle or for oral contraceptive users during withdrawal and oral contraceptive use.

3.2.3 Maximal fitness testing

Subjects performed a standard incremental fitness test on a cycle ergometer to establish maximum oxygen consumption.

3.2.4 Sympathetic activation testing

Each subject was seated comfortably in an upright position and performed a sustained handgrip to fatigue, a 2 minute mental arithmetic test and a 2 minute cold pressor test (described in detail in Chapter 2). During the tests heart rate was measured using a three lead frontal ECG, blood pressure was measured continuously using applanation tonometry, stroke volume was assessed using impedance cardiography and forearm blood flow was measured by venous occlusion plethysmography. From these parameters, MAP, pulse pressure, cardiac index, stroke index, rate-pressure product, TPR and forearm conductance were calculated.

3.2.5 Statistical analysis

Measurements were compared within groups (menses versus ovulation, oral contraceptive use versus withdrawal and between groups (non-smokers versus smokers, oral contraceptive users versus non-users) using paired and unpaired Student's *t*-tests, respectively. $P < 0.05$ was considered significant.

3.3 Results

3.3.1 Subject characteristics of premenopausal smokers and non-smokers

Physical characteristics of both smokers and non-smokers are shown in Table 3.1. Subjects in both groups were evenly matched as there were no appreciable differences in age, height, body mass or BMI. Subjects had similar menstrual cycle length and flow duration and were of similar physical fitness. Smokers smoked 17 ± 1.5 cigarettes.day^{-1} for an average of 8 ± 1.3 years, which was a mean of 8 ± 1.6 pack years.

3.3.2 Resting cardiovascular parameters of premenopausal smokers and non-smokers

There was no significant difference between smokers and non-smokers when considering the resting cardiovascular parameters of pulse pressure, heart rate, stroke index, cardiac index, rate-pressure product, forearm blood flow (FBF), forearm conductance or TPR at menses or at ovulation (see Table 3.2). The cardiac index and TPR figures in this study were taken as an average result of 9 premenopausal non-smokers, 6 premenopausal smokers and 9 oral contraceptive users. This was due to difficulties with the impedance cardiograph.

Resting MAP, SBP and DBP were significantly higher in smokers than in non-smokers at ovulation (see Figure 3.1) whereas there was no difference in the measurements taken at menses between the two groups (see Figure 3.2).

MAP, SBP and DBP were not changed by hormonal status in non-smokers (see Figure 3.3). However, smokers had significantly higher resting SBP at ovulation when compared with menses, the difference in resting MAP did not quite reach significance (P=0.07) and DBP remained unaffected, see Figure 3.4.

3.3.3 Cardiovascular reactivity

Sustained handgrip

There was no significant difference in the time to fatigue for the sustained handgrip test between smokers and non-smokers or between menses and ovulation (at menses: 101 ± 20.0 cf 88 ± 7.6 s and at ovulation: 93 ± 16.0 cf 86 ± 7.4 s).

Sustained handgrip elicited a typical pressor response causing MAP, heart rate and TPR to increase throughout the duration of the test. Graphs of these

responses may be seen in Figure 3.5, which demonstrates examples of cardiovascular responses in the non-smoking cohort at menses. The increase in MAP from rest during the sustained handgrip was visible in both smokers and non-smokers at the two phases of the menstrual cycle (see Table 3.3 and Figure 3.6). The absolute magnitude of elevation tended to be greater at ovulation than at menses in non-smokers (18 ± 2.3 cf 24 ± 4.4 Δ mmHg, P=0.09) and greater in non-smokers at ovulation than in smokers (24 ± 4.4 cf 11 ± 3.7 Δ mmHg, P=0.051). MAP was elevated due to an increase in both DBP and SBP (see Table 3.3). Pulse pressure was not altered by sustained handgrip in either group (see Table 3.3).

Heart rate was significantly increased in non-smokers and smokers at menses and at ovulation (see Figure 3.7 A). The magnitude of change in heart rate during a sustained handgrip tended to be greater at ovulation than at menses in non-smokers (23 ± 4.2 cf 15 ± 5.0 Δ bpm, P=0.09) and greater in non-smokers at ovulation than in smokers (23 ± 4.2 cf 10 ± 3.8 Δ bpm, P=0.06).

Conductance measurements, which reflect vasodilatation occurring in the forearm itself and are not a result of transient changes in arterial pressure, demonstrated no change in either group at menses but at ovulation both groups showed a decrease (see Figure 3.7 B). There was no difference between conductance values at menses and at ovulation in either group.

Sustained handgrip increased TPR in both smokers and non-smokers at menses and at ovulation (see Table 3.3 and Figure 3.7 C). No difference between the absolute change in TPR values at menses or at ovulation were found in either smokers or non-smokers (non-smokers: 11.1 ± 1.82 cf 18.1 ± 4.65 Δ $(L.min^{-1}).mmHg$ and smokers: 16.0 ± 7.70 cf 7.8 ± 3.40 Δ $(L.min^{-1}).mmHg$). Cardiac index was similar for both smokers and non-smokers during a sustained handgrip (see Table 3.3).

Mental Arithmetic

An example of the response to mental arithmetic is shown in Figure 3.8, which depicts the response of non-smokers at menses. MAP increased significantly during the first minute, heart rate increased throughout the two minute time period, TPR tended to decrease and cardiac index was elevated throughout.

Blood pressure in increased by 60 seconds (see Table 3.4 and Figure 3.9 A) and thereafter no change was evident. Incremental increases in MAP during the test

were due to an increase in both SBP and DBP (see Table 3.4). There was no difference between the absolute magnitude of MAP responses at menses and at ovulation in either group: non-smokers: 4 ± 1.9 cf 7 ± 1.9 Δ mmHg, smokers: 8 ± 6.1 cf 5 ± 0.9 Δ mmHg. DBP and SBP responses were similar for smokers during menses and ovulation. In non-smokers a greater increase in systolic pressure was evident at ovulation than at menses (12 ± 3.3 cf 3 ± 2.0 Δ mmHg, $P<0.05$). Pulse pressure did not change in either group (see Table 3.4).

Heart rate was elevated during mental stress in both non-smokers and smokers. Non-smokers showed increased heart rate at all time points during the two minute period while smokers demonstrated a significant increase at most time points and a trend for an increase at others (see Figure 3.10 A). The absolute magnitude of the response was similar between non-smokers and smokers at menses (18 ± 3.3 cf 11 ± 4.8 Δ bpm) but was greater in non-smokers at ovulation than in smokers (15 ± 3.6 cf 4 ± 1.6 Δ bpm, $P<0.05$). No difference between values obtained at menses and ovulation were observed.

Both non-smokers and smokers demonstrated negligible changes in forearm vascular conductance during mental arithmetic (see Table 3.4). TPR showed a tendency towards a decrease in both smokers and non-smokers at menses (see Table 3.4). All non-smokers and smokers showed an individual decrease in TPR at ovulation although the changes were not statistically significant. The absolute change in TPR was not different between non-smokers and smokers or between menses and ovulation (non-smokers –7.1 ± 3.30 cf –5.6 ± 3.17 Δ $(L.min^{-1}).mmHg^{-1}$, smokers: -5.4 ± 2.20 cf 0.9 ± 2.22 Δ $(L.min^{-1}).mmHg^{-1}$). Mental stress was accompanied by an increase in cardiac index in both groups at menses and at ovulation. Absolute differences at menses and ovulation were: non-smokers: 0.6 ± 0.31 cf 0.5 ± 0.20 Δ $L.min.m^{-2}$ and smokers: 0.4 ± 0.14 cf 0.1 ± 0.18 Δ $L.min.m^{-2}$. Peak increases from rest occurred by 60 seconds (see Table 3.4 and Figure 3.10).

Cold pressor test

The cardiovascular responses at menses to the cold pressor test are shown in Figure 3.11. In smokers and non-smokers MAP was not changed at menses by cold exposure. At ovulation an increase was seen in non-smokers and smokers at 60 s (see Table 3.5 and Figure 3.12 A). This was due to an increase in SBP at this time point (see Table 3.5). Non-smokers showed a difference in responsiveness to cold at

menses and ovulation at 30 and 60 seconds, with a greater increase at ovulation than at menses (see Figure 3.12 D). The absolute change in MAP was - at menses: 1 ± 0.7 cf at ovulation: 9 ± 3.1 Δ mmHg, $P<0.05$). This was due to an increase in SBP and DBP (see Table 3.5). The increase in diastolic pressure was significantly higher at ovulation in non-smokers than in smokers (19 ± 4.6 cf 5 ± 2.82 Δ mmHg, $P<0.05$). Pulse pressure was increased in non-smokers during the first minute of the cold pressor test. Smokers exhibited an increase in pulse pressure during the first minute of the cold pressor test at ovulation only. No difference in pulse pressure at menses or at ovulation or between the two groups was found (see Table 3.5).

Cold stress at menses and ovulation increased heart rate in non-smokers but not in smokers (see Figure 3.13 A). There was no difference in the magnitude of change in heart rate between menses and ovulation or between smokers and non-smokers (smokers: -0.7 ± 3.16 cf 2.0 ± 3.0 Δ bpm, non-smokers: 8 ± 3.8 cf 6 ± 1.5 Δ bpm). Forearm conductance was unaffected by cold exposure as was TPR (see Table 3.5).

The cold pressor test did not affect cardiac index at menses or at ovulation. When comparing menses and ovulation no difference was found in the response of non-smokers to this test but values were greater at ovulation in smokers (see Figure 3.13 B).

3.3.4 Subject characteristics of oral contraceptive users

Physical characteristics of oral contraceptive (OC) users are shown in Table 3.6.

3.3.5 Resting cardiovascular parameters

There were no significant differences between resting cardiovascular parameters (heart rate, diastolic pressure, systolic pressure, MAP, pulse pressure, stroke index, forearm blood flow and forearm conductance) measured during OC use and at withdrawal except for TPR, which was elevated during the withdrawal period and cardiac index, which was lower during this period (see Table 3.7).

3.3.6 Cardiovascular reactivity in oral contraceptive users

Sustained handgrip

There was no significant difference in the time to fatigue for the sustained handgrip test during OC use or during the withdrawal period (85 ± 11.7 and 67 ± 12.4 s, respectively).

Sustained handgrip elicited a pressor response, which included an elevation of MAP and heart rate throughout the duration of the test and this response was not different between OC use and withdrawal (See Figure 3.14 A and B, respectively). MAP was elevated due to an increase in both DBP and SBP (see Table 3.8). No significant difference between the magnitude of MAP responses to this test during withdrawal and OC use was observed (20 ± 4.8 cf 16 ± 4.2 Δ mmHg). Pulse pressure was not changed by sustained handgrip (see Table 3.8).

Heart rate increased by a similar amount at both withdrawal and OC use (22 ± 3.0 cf 18 ± 3.5 Δ bpm). TPR was increased at variable time points during OC use and withdrawal (see Figure 3.13 C) and the absolute magnitude of increase was similar during withdrawal and at OC use (9.9 ± 2.87 cf 4.5 ± 2.50 Δ $(L.min^{-1}).mmHg^{-1}$).

Conductance measurements were unaltered during the sustained handgrip (see Table 3.8) and cardiac index responses to sustained handgrip were similar at withdrawal and during OC use (see Table 3.8).

Mental Arithmetic

Mental arithmetic produced typical pressor responses. MAP tended to increase during the first 60 s (see Figure 3.15 A). The magnitude of change in MAP was similar at withdrawal and OC use (3 ± 1.8 cf 5 ± 2.7 Δ mmHg). No change in pulse pressure was obvious (see Table 3.9). There was a tendency for systolic and diastolic pressure to increase during the first minute of mental stress (see Table 3.9) and heart rate was increased throughout the two minute time period (see Figure 3.15 B). The absolute change in heart rate observed with mental stress was similar at withdrawal and during oral contraceptive use (11 ± 2.2 cf 17 ± 4.6 Δ bpm).

TPR was unchanged by mental stress in OC users (see Table 3.9) while conductance values were elevated, particularly during the second minute (see Figure 3.15 C). The absolute magnitude of change in TPR from rest was not different at withdrawal or during OC use (-2.6 ± 1.74 cf –3.6 ± 1.74 Δ $(L.min^{-1}).mmHg^{-1}$. There

was no change in cardiac index during OC use with mental stress however at withdrawal a significant increase was noted (see Table 3.9). Nevertheless the absolute differences in cardiac index from rest were unchanged (during OC use: -0.4 ± 0.30 cf at withdrawal: 0.4 ± 0.20 Δ $L.min^{-1}.m^{-2}$).

Cold pressor test

MAP was increased during the first 60 s of exposure to the cold pressor test during OC use and at withdrawal (see Figure 3.16 A). This increase was due to an elevation in both systolic and diastolic pressure at these time points (see Table 3.10). The absolute magnitude of change in MAP was not different at withdrawal or during OC use (12 ± 3.5 cf 12 ± 3.7 Δ mmHg). Pulse pressure was unchanged from rest during the cold pressor test, see Table 3.10. Heart rate was elevated throughout testing during OC use and withdrawal (see Figure 3.16 B). The elevation of heart rate was not different at withdrawal or OC use (14 ± 7.9 cf 14 ± 5.5 Δ bpm).

TPR was unchanged by the cold pressor test (see Table 3.10). Cardiac index responses were also unchanged (see Table 3.10). Forearm conductance values were not altered by the cold pressor test and were not affected during OC use (see Table 3.10).

3.3.7 Comparison of responses to sympathetic activation in young healthy women with normal menstrual cycles and those using oral contraceptives

In order to compare measurements made in oral contraceptive users with those of cycling women results at menses were compared with those at withdrawal and those at ovulation (oestrogen only) were compared with those taken during oral contraceptive use (oestrogen plus progesterone).

There were no differences in the physical characteristics of women with normal regular menstrual cycles and oral contraceptive users. There was a trend however for oral contraceptive users to have a greater body mass and BMI than cycling women (P=0.06 in each case, see Table 3.1 and 3.6). Resting cardiovascular variables were similar between the groups (see Table 3.2 and 3.7) although TPR was greater and cardiac index was lower during ovulation than during oral contraceptive use.

Having looked at the parameters that produced the most consistent changes within the groups it was decided to compare only these. For the sustained handgrip

these were MAP, heart rate and TPR, for mental arithmetic these were MAP, heart rate, TPR and cardiac index and for the cold pressor test these were MAP and heart rate.

The absolute magnitude of MAP change during the sustained handgrip was not different in cycling women or those on the oral contraceptive pill (menses: 18 ± 2.3 cf withdrawal: 20 ± 4.8 Δ mmHg and ovulation: 24 ± 4.4 cf OC use: 16 ± 4.2 Δ mmHg). Change in heart rate during this test was not altered by oral contraceptive use (menses: 15 ± 5.0 cf withdrawal: 22 ± 3.0 Δ bpm and ovulation 23 ± 4.2 cf OC use: 18 ± 3.5 Δ bpm). However although the absolute increase in TPR was not different between menses and withdrawal (11 ± 1.8 cf withdrawal: 10 ± 2.9 Δ $(L.min^{-1}).mmHg^{-1}$, respectively) a greater increase was seen at ovulation than during OC use (19 ± 4.7 cf 5 ± 2.5 Δ $(L.min^{-1}).mmHg^{-1}$).

There was a similar increase in MAP during mental stress at menses and at withdrawal (4 ± 1.9 cf 3 ± 1.8 Δ mmHg) and at ovulation and during OC use (7 ± 1.9 cf 5 ± 2.7 Δ mmHg). A greater increase in systolic blood pressure was evident at ovulation when compared with oral contraceptive use (12 ± 3.3 cf 2 ± 2.4 Δ mmHg). Heart rate change was not different in the withdrawal and menses groups (18 ± 3.3 cf 11 ± 2.2 Δ bpm). Neither the magnitude of change in TPR (-7.1 ± 3.3 cf –2.6 ± 1.7 Δ $(L.min^{-1}).mmHg^{-1}$) nor cardiac index (0.6 ± 0.3 cf 0.4 ± 0.2 Δ $L.min^{-1}.m^{-2}$) was different between the menstrual cycle group at menses and the oral contraceptive users at withdrawal.

Oral contraceptive users demonstrated a significantly greater increase in MAP at withdrawal during the cold pressor test than the menstrual cycle group at menses (12 ± 3.5 cf 1 ± 0.7 Δ mmHg, $P< 0.01$). The magnitude of heart rate elevation was not dissimilar between the groups (menses: 8 ± 3.8 cf withdrawal: 14 ± 7.6 Δ bpm and ovulation: 6 ± 1.5 cf during OC use: 14 ± 5.5 Δ bpm, $P=0.09$).

Table 3.1 Physical characteristics of subjects.

Non-smokers n=10, smokers n=7. Data are expressed as mean ± S.E.M.

	Non-smokers	**Smokers**
Age (Years)	23 ± 1.1	24 ± 1.2
Cycle length (days)	30 ± 0.8	28 ± 0.5
Menses duration (days)	6 ± 0.3	5 ± 0.1
Height (cm)	168 ± 2	164 ± 1.8
Body Mass (kg)	69 ± 4.1	61 ± 3.6
BMI ($kg.m^{-2}$)	24 ± 1.1	23 ± 1.4
VO_2peak ($(ml.min^{-1}).kg^{-1}$)	35 ± 3.2	32 ± 3.3

Table 3.2 Resting cardiovascular characteristics of subjects.

Non-smokers n=10, smokers n=7. Data are expressed as mean ± S.E.M.

	Non-smokers at menses	Smokers at menses
Heart Rate ($beat.min^{-1}$)	72 ± 3.3	80 ± 5.8
Diastolic pressure (mmHg)	61 ± 1.9	63 ± 3.1
Systolic pressure (mmHg)	106 ± 1.8	108 ± 2.9
MAP (mmHg)	76 ± 1.6	78 ± 2.8
Pulse pressure (mmHg)	45 ± 2.0	45 ± 2.1
TPR $(L.min^{-1}).mmHg^{-1}$	23.9 ± 3.90	26.0 ± 2.80
Stroke index $(ml.m^{-2})$	33 ± 6	23 ± 1
Cardiac index $((l.min^{-1}).m^{-2})$	2.2 ± 0.30	1.8 ± 0.20
RPP $((mmHg.beats.min^{-1})^{-2})$	74 ± 5.0	86 ± 8.0
Forearm conductance $((ml.min^{-1}).100ml^{-1}.mmHg^{-1})$	0.07 ± 0.020	0.08 ± 0.040

	Non-smokers at ovulation	Smokers at ovulation
Heart Rate ($beat.min^{-1}$)	75 ± 4.3	84 ± 3.5
Diastolic pressure (mmHg)	62 ± 1.9 *	69 ± 2.3
Systolic pressure (mmHg)	108 ± 2.9 *	117 ± 3.2 §
MAP (mmHg)	77 ± 2.2 *	85 ± 2.3
Pulse pressure (mmHg)	46 ± 1.7	49 ± 2.6
TPR $(L.min^{-1}).mmHg^{-1}$	26.6 ± 3.40	26.5 ± 3.10
Stroke index $(ml.m^{-2})$	26 ± 3.9	16 ± 4.3
Cardiac index $((l.min^{-1}).m^{-2})$	1.6 ± 0.29	2.1 ± 0.26
RPP$((mmHg.beats.min^{-1})^{-2})$	81 ± 6.0	98 ± 5.7
Forearm conductance $((ml.min^{-1}).100ml^{-1}.mmHg^{-1})$	0.05 ± 0.010	0.05 ± 0.010

* denotes a significant difference ($P<0.05$) between values at menses or ovulation in smokers and non-smokers. § denotes a significant difference ($P<0.05$) between values at menses and ovulation within the one group.

Table 3.3 Cardiovascular parameters before and during sustained handgrip (100% fatigue). Non-smokers n=10; smokers n=7. Data are expressed as mean ± S.E.M.

	Non-smokers at menses		**Smokers at menses**	
	before	during	before	during
MAP (mmHg)	76±1.6	95±3.0***	78±2.8	96±5.7*
DBP (mmHg)	61±1.9	79±3.3***	63±3.1	82±5.5*
SBP (mmHg)	106±1.8	124±4.3**	108±2.9	122±6.4
Pulse pressure (mmHg)	45±2.0	47±5.8	45±2.1	41±2.7*
Heart rate (bpm)	72±3.3	87±5.5*	80±5.8	90±5.8*
Stroke index ($ml.m^{-2}$)	23±4.1	33±6.1	17±2.1	24±1.5*
Cardiac index ($(l.min^{-1}).m^{-2}$)	2.2±0.30	1.8±0.30	1.8±0.20	1.6±0.20
RPP ($(mmHg.beats.min^{-1})^{-2}$)	74±5.0	107±10.0	86±8.0	109±6.0
TPR ($mmHg.min.l^{-1}$)	23.9±3.90	35±4.90***	26.0± 2.80	42.0±8.80
Forearm vascular conductance ($(ml.min^{-1}).100\ ml^{-1}.mmHg^{-1}$)	0.07±0.019	0.05±0.015	0.08±0.038	0.07±0.025

	Non-smokers at ovulation		**Smokers at ovulation**	
	before	during	before	during
MAP (mmHg)	77±2.2	102±4.4***	85±2.3	98±2.8*
DBP (mmHg)	62±1.9	87±3.7***	69±2.3	86±5.7
SBP (mmHg)	108±2.9	131±5.9**	117±3.2	130±2.6*
Pulse Pressure (mmHg)	46±1.7	44±3.0	49±2.6	55±7.4
Heart rate (bpm)	75±4.3	98±6.5***	84±3.5	94± 4.1
Stroke index ($ml.m^{-2}$)	26±3.9	13±1.9	16±4.3	12±3.2
Cardiac index ($(l.min^{-1}).m^{-2}$)	1.9±0.30	1.1±0.24	2.1±0.30	1.8±0.22
RPP ($(mmHg.beats.min^{-1})^{-2}$)	81±6.0	129±10.8***	98±5.7	119±6.7*
TPR ($mmHg.min.l^{-1}$)	26.6±3.41	37.7±3.33**	26.5±3.15	36.3±5.22
Forearm vascular conductance ($(ml.min^{-1}).100\ ml^{-1}.mmHg^{-1}$)	0.05±0.007	0.04±0.008***	0.05±0.005	0.04±0.007

* denotes a significant difference ($P<0.05$) between before and during values (** $P<0.01$, *** $P< 0.001$).

Table 3.4 Cardiovascular parameters before and during mental stress (60 s).

Non-smokers n=10; smokers n=7. Data are expressed as mean ± S.E.M.

	Non-smokers at menses		**Smokers at menses**	
	before	during	before	during
MAP (mmHg)	76±1.5	80±2.8*	77±1.9	85±7.7
DBP (mmHg)	61±1.8	65±2.9*	61±2.1	67±7.4
SBP (mmHg)	107±1.6	111±3.3	110±1.9	120±8.6
Pulse pressure (mmHg)	46±1.7	47±2.7	49±1.8	53±2.8
Heart rate (bpm)	69±3.7	88±4.5***	74±5.6	85±7.3
Stroke index ($ml.m^{-2}$)	29±6.3	31±5.9	22±2.3	23±1.9
Cardiac index ($(l.min^{-1}).m^{-2}$)	2.0±0.34	2.9±0.57*	1.8±0.2	2.2±0.3*
RPP ($(mmHg.beats.min^{-1})^{-2}$)	74±4.1	97±6.7**	81±6.7	103±13.1
TPR ($mmHg.min.l^{-1}$)	27.8±4.10	20.7±2.71	31.8±3.21	26.3±3.42
Forearm vascular conductance ($(ml.min^{-1}).100\ ml^{-1}.mmHg^{-1}$)	0.05±0.011	0.05±0.011	0.05±0.007	0.04±0.009

	Non-smokers at ovulation		**Smokers at ovulation**	
	before	during	before	during
MAP (mmHg)	78±2.2	85±2.0**	83±1.8	88±2.4**
DBP (mmHg)	66±1.9	74±2.5*	67±1.6	71±3.5*
SBP (mmHg)	114±3.4	122±2.9**	115±3.2	123±4.0**
Pulse Pressure (mmHg)	47±2.2	53±2.4	48±3.0	51±4.6
Heart rate (bpm)	76±4.7	91±4.3**	79±3.9	83±4.2
Stroke index ($ml.m^{-2}$)	22±3.1	23±3.1	23±1.3	22±2.1
Cardiac index ($(l.min^{-1}).m^{-2}$)	1.6±0.22	2.1±0.31*	1.8±0.20	1.9±0.24
RPP ($(mmHg.beats.min^{-1})^{-2}$)	82±5.7	107±5.9***	91±5.1	102±7.3**
TPR ($mmHg.min.l^{-1}$)	32.5±5.11	27.0±3.72	29.7±3.32	30.5±3.60
Forearm vascular conductance ($(ml.min^{-1}).100\ ml^{-1}.mmHg^{-1}$)	0.06±0.004	0.06±0.008	0.04±0.006	0.05±0.007

* denotes a significant difference ($P<0.05$) between before and during values (** $P<0.01$, *** $P< 0.001$).

Table 3.5 Cardiovascular parameters before and during cold pressor test (60s).

Non-smokers n=10; smokers n=7. Data are expressed as mean ± S.E.M.

	Non-smokers at menses		**Smokers at menses**	
	before	during	before	during
MAP (mmHg)	76±1.7	77±2.0	78±2.4	81±6.6
DBP (mmHg)	61±1.7	61±2.3	61±2.5	63±6.2
SBP (mmHg)	105±2.0	109±2.4*	111±2.8	116±7.8*
Pulse pressure (mmHg)	44±2.2	49±3.1*	50±2	53±3.7
Heart rate (bpm)	69±4.1	77±5.9	75±5.6	74±3.6
Stroke index ($ml.m^{-2}$)	32±7.3	27±4.3	23±3.3	21±3.2
Cardiac index (($l.min^{-1}$).m^{-2})	2.1±0.41	1.9±0.22	1.6±0.23	1.6±0.26
RPP (($mmHg.beats.min^{-1}$)$^{-2}$)	73±5.3	85±6.9*	83±7.2	87±8.7
TPR ($mmHg.min.l^{-1}$)	25.4±4.33	25.3±3.53	29.8±3.42	34.3±6.71
Forearm vascular conductance (($ml.min^{-1}$).100 $ml^{-1}.mmHg^{-1}$)	0.06±0.022	0.06±0.014	0.04±0.005	0.04±0.007

	Non-smokers at ovulation		**Smokers at ovulation**	
	before	during	before	during
MAP (mmHg)	78±1.6	87±3.5*	83±2.1	90±4.0*
DBP (mmHg)	66±2.1	71±3.2	67±2.5	72±4.3
SBP (mmHg)	109±2.4	121±3.9**	117±1.8	128±3.7**
Pulse Pressure (mmHg)	42±2.2	49±3.1*	50±2.5	56±2.4**
Heart rate (bpm)	72±3.2	79±3.7**	79±2.9	81±3.1
Stroke index ($ml.m^{-2}$)	19±2.8	18±2.8	26±5.7	25±2.7
Cardiac index (($l.min^{-1}$).m^{-2})	1.4±0.21	1.3±0.32	2.1±0.55	2.0±0.38
RPP (($mmHg.beats.min^{-1}$)$^{-2}$)	77±3.7	95±6.0***	92±3.8	103±5.2
TPR ($mmHg.min.l^{-1}$)	36.3±4.43	39.9±6.01	28.8±4.82	28.4±4.26
Forearm vascular conductance (($ml.min^{-1}$).100 $ml^{-1}.mmHg^{-1}$)	0.05±0.007	0.04±0.006	0.04±0.09	0.04±0.016

* denotes a significant difference between before and during values ($P<0.05$, ** $P<0.01$, *** $P< 0.001$).

Table 3.6 Physical characteristics of subjects.

n=10. Data are expressed as mean ± S.E.M.

	Oral contraceptive users
Age (years)	23 ± 0.8
Height (cm)	166 ± 1.4
Body Mass (kg)	60 ± 1.3
BMI ($kg.m^{-2}$)	22 ± 0.5
VO_2peak ($(ml.min^{-1}).kg^{-1}$)	37 ± 2.5

Table 3.7 Resting cardiovascular characteristics of subjects.

n=10. Data are expressed as mean ± S.E.M.

	OC users during use	OC users at withdrawal
Heart Rate ($beat.min^{-1}$)	73 ± 3.6	71 ±3.3
Diastolic pressure (mmHg)	61 ± 1.9	59 ± 1.6
Systolic pressure (mmHg)	110 ± 2.0	107 ± 2.1
MAP (mmHg)	77 ± 1.6	75 ± 1.8
Pulse pressure (mmHg)	49 ± 2.5	49 ± 2.1
TPR $(L.min^{-1}).mmHg^{-1}$	18.4 ± 1.63	23.4 ± 2.62*
Stroke index ($ml.m^{-2}$)	38 ± 3.9	33 ± 6.0
Cardiac index ($[l.min^{-1}].m^{-2}$)	2.7 ± 0.33	2.2 ± 0.34*
RPP ($[mmHg.beats.min^{-1}]^{-2}$)	81 ± 4.9	76 ± 3.8
Forearm blood flow $(ml.min^{-1}).100ml^{-1}$	7.2 ± 2.42	4.6 ± 0.84
Forearm conductance $((ml.min^{-1}).100ml^{-1}.mmHg^{-1})$	0.09 ± 0.031	0.06 ± 0.012

* denotes a significant difference between oral contraceptive users during use and at withdrawal ($P<0.05$).

Table 3.8 Cardiovascular parameters before and during sustained handgrip (100% fatigue). n=10. Data are expressed as mean ± S.E.M.

	OC use		Withdrawal from OC use	
	before	during	before	during
MAP (mmHg)	77±1.6	95±5.4**	75±1.8	95±5.7**
DBP (mmHg)	61±1.9	78±4.5***	59±1.6	80±4.8***
SBP (mmHg)	110±2.0	128±8.0*	107±2.1	127±7.9**
Pulse pressure (mmHg)	49±2.5	50±5.1	49±2.1	49±3.7
Heart rate (bpm)	73±3.6	91±4.0***	71±3.3	92±3.5***
Stroke index ($ml.m^{-2}$)	38±3.9	33±5.3	33±6.0	22±2.5*
Cardiac index ($(l.min^{-1}).m^{-2}$)	2.7±0.31	2.8±0.42	2.2±0.37	2.0±0.24
RPP ($(mmHg.beats.min^{-1})^{-2}$)	81±4.9	118±11.7**	76±3.8	117±8.8***
TPR ($mmHg.min.l^{-1}$)	18.4±1.61	23.9±3.23	23.4±2.67	33.3±4.42
Forearm vascular conductance ($(ml.min^{-1}).100\ ml^{-1}.mmHg^{-1}$)	0.09±0.029	0.07±0.021	0.06±0.011	0.05±0.011

* denotes a significant difference between before and during values ($P<0.05$, ** $P<0.01$, *** $P< 0.001$).

Table 3.9 Cardiovascular parameters before and during mental stress (60s).

n=10. Data are expressed as mean ± S.E.M.

	OC use		Withdrawal from OC use	
	before	during	before	during
MAP (mmHg)	78±1.2	81±2.4	78±2.3	82±3.5
DBP (mmHg)	67±5.1	71±5.4	63±2.3	67±2.8
SBP (mmHg)	110±2.3	112±2.8	107±2.7	113±3.6
Pulse pressure (mmHg)	49±2.4	48±2.4	44±1.6	47±1.6
Heart rate (bpm)	71±3.3	88±5.5**	70±3.7	78±4.0***
Stroke index ($ml.m^{-2}$)	36±3.5	32±4.3	32±5.1	33±4.5
Cardiac index (($l.min^{-1}$).m^{-2})	2.5±0.21	2.7±0.43	2.1±0.33	2.6±0.35*
RPP (($mmHg.beats.min^{-1})^{-2}$)	78±5.0	96±6.9*	73±5.1	90±5.7***
TPR ($mmHg.min.l^{-1}$)	20.5±2.07	18.4±2.71	25.1±3.05	21.5±2.65
Forearm vascular conductance (($ml.min^{-1}$).100 $ml^{-1}.mmHg^{-1}$)	0.05±0.005	0.05±0.006	0.04±0.005	0.06±0.010*

* denotes a significant difference between before and during values ($P<0.05$, ** $P<0.01$, *** $P< 0.001$).

Table 3.10 Cardiovascular parameters before and during the cold pressor test (60s). n=10. Data are expressed as mean ± S.E.M.

	OC use		Withdrawal from OC use	
	before	during	before	during
MAP (mmHg)	80±1.8	92±3.7**	79±2.2	91±5.2**
DBP (mmHg)	63±1.4	75±2.8**	62±2.4	72±4.2*
SBP (mmHg)	113±3.6	127±5.8*	111±2.8	126±6.2*
Pulse pressure (mmHg)	50±3.5	52±3.2	49±1.9	53±3.7
Heart rate (bpm)	72±3.6	86±3.9*	70±4.5	84±6.9
Stroke index ($ml.m^{-2}$)	34±3.9	31±4.0	34±5.5	31±4.5
Cardiac index ($(l.min^{-1}).m^{-2}$)	1.6±0.21	2.1±0.55	2.0±0.32	2.4±0.36
RPP ($(mmHg.beats.min^{-1})^{-2}$)	81±5.9	110±10.2*	76±5.9	102±9.4***
TPR ($mmHg.min.l^{-1}$)	22.7±3.35	22.8±2.04	23.0±3.39	25.6±3.65
Forearm vascular conductance ($(ml.min^{-1}).100\ ml^{-1}.mmHg^{-1}$)	0.04±0.006	0.03±0.004	0.07±0.018	0.06±0.026

* denotes a significant difference between before and during values ($P<0.05$, ** $P<0.01$, *** $P< 0.001$).

Figure 3.1 Effect of smoking on resting blood pressures at ovulation.

A. Systolic blood pressure is elevated in smokers at ovulation when compared with non-smokers. B. Diastolic blood pressure is higher in smokers than in non-smokers at ovulation. C. MAP at ovulation is higher in smokers than in non-smokers. (non-smokers: n=10, smokers n=7; * $P<0.05$, Student's *t*-test). Data represents mean ± S.E.M.

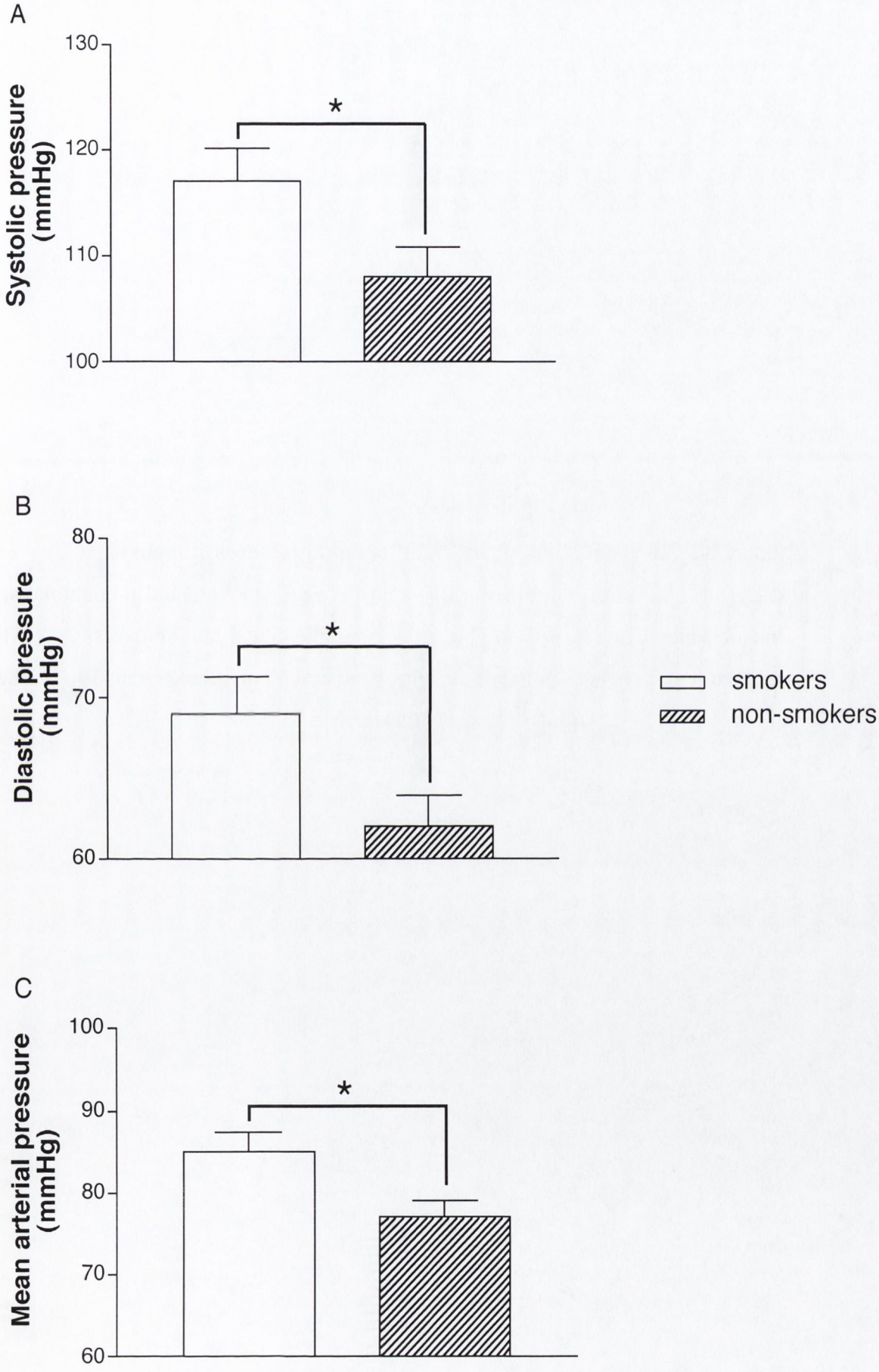

A
Systolic pressure (mmHg)
130
120
110
100
*
B
Diastolic pressure (mmHg)
80
70
60
*
smokers
non-smokers
C
Mean arterial pressure (mmHg)
100
90
80
70
60
*

Figure 3.2 Effects of smoking on resting blood pressures at menses.

A. Systolic blood pressure is not different between smokers and non-smokers. B. Diastolic blood pressure is similar between smokers and non-smokers. C. MAP is not different between smokers and non-smokers. (non-smokers: n=14, smokers: n=7, Student's *t*-test). Data represents mean ± S.E.M.

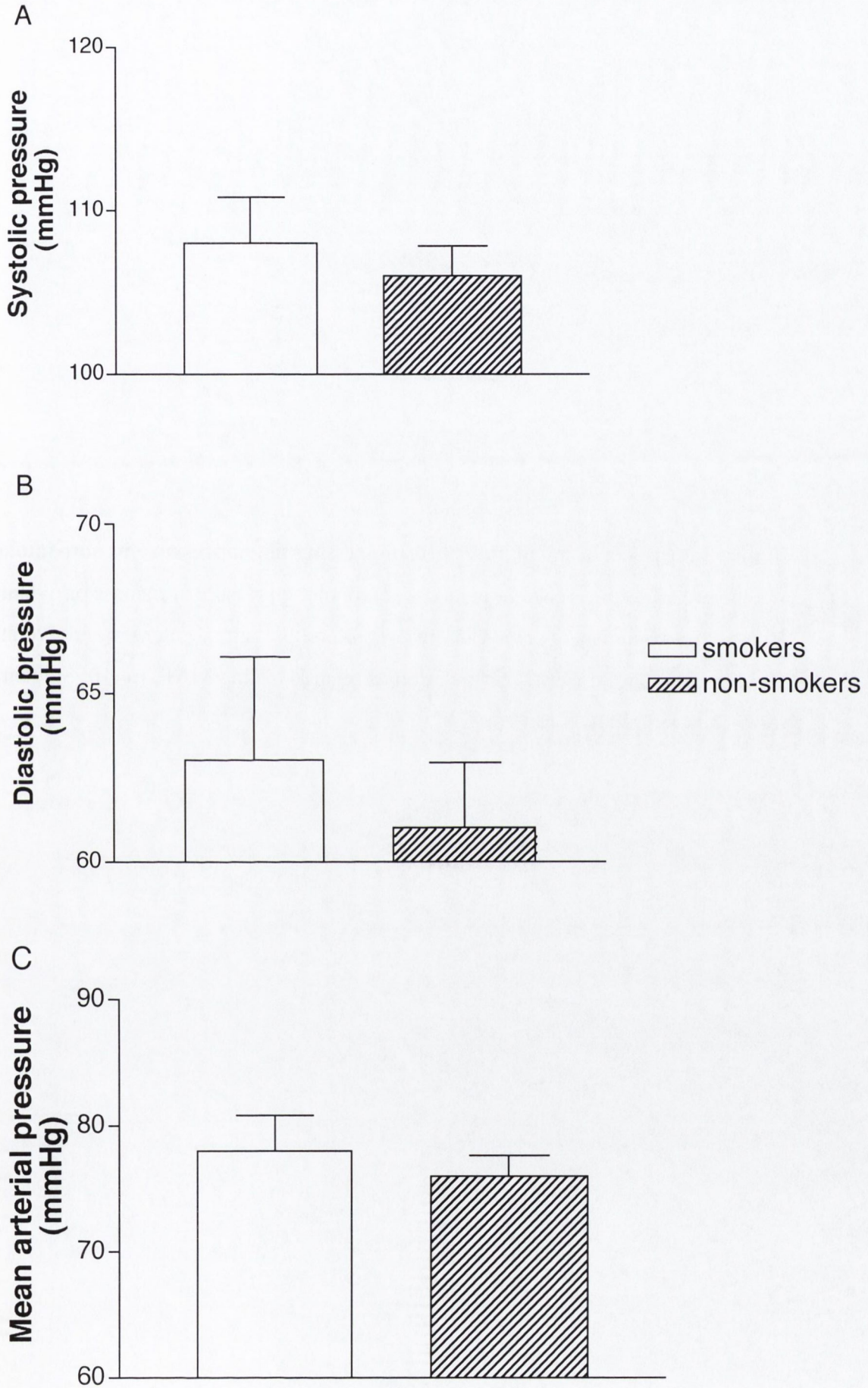

A
120
110
100
Systolic pressure (mmHg)
B
70
65
60
Diastolic pressure (mmHg)
smokers
non-smokers
C
90
80
70
60
Mean arterial pressure (mmHg)

Figure 3.3 Effects of hormonal status on resting blood pressure in non-smokers.

A. There was no difference in resting systolic blood pressure at menses or ovulation.

B. No difference was observed in diastolic blood pressure at menses and ovulation.

C. Menses did not differ from ovulation as regards resting MAP. (n=10, Student's *t*-test). Data represents mean ± S.E.M.

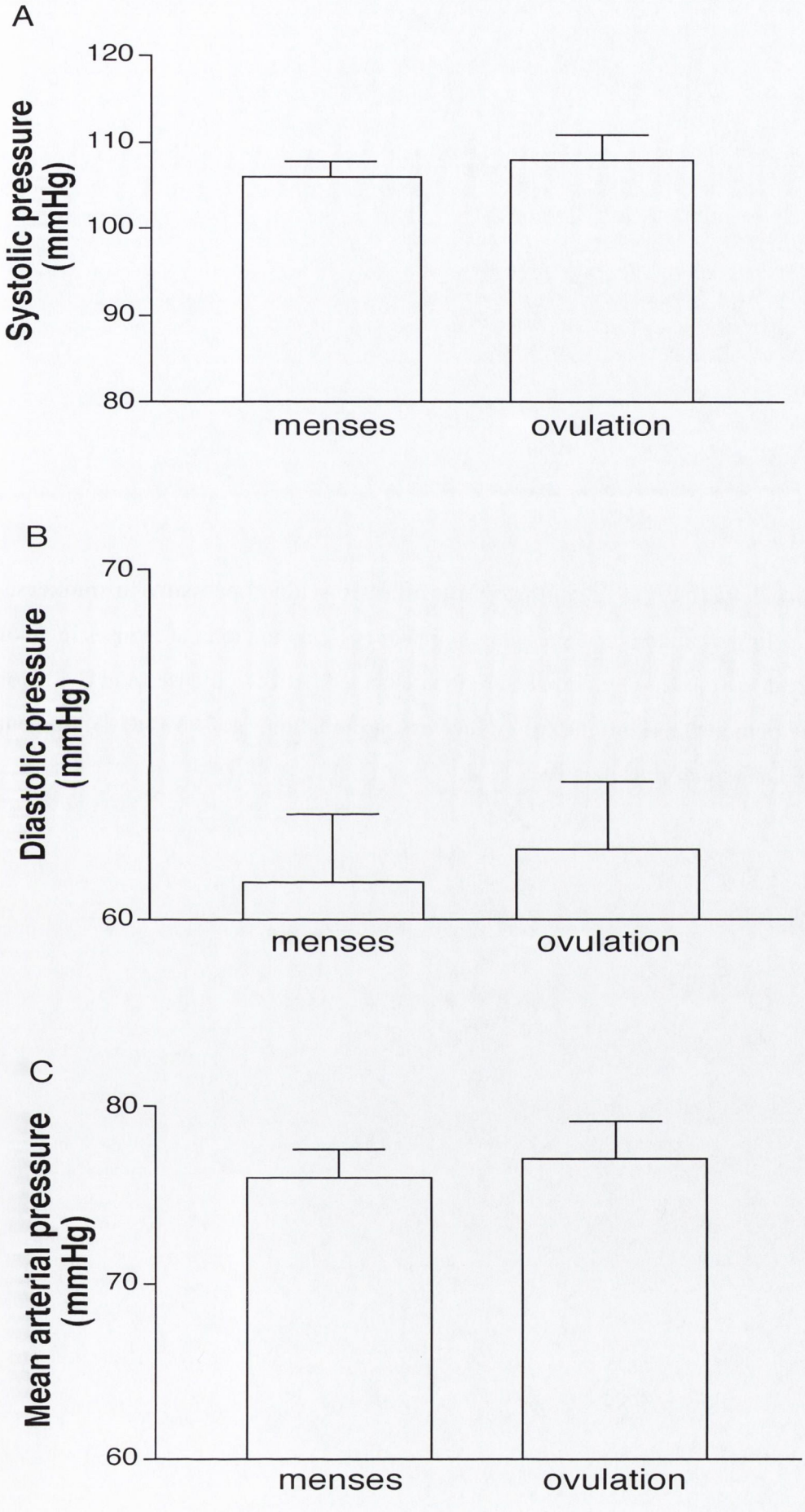

A
120
110
100
90
80
Systolic pressure (mmHg)
menses
ovulation
B
70
60
Diastolic pressure (mmHg)
menses
ovulation
C
80
70
60
Mean arterial pressure (mmHg)
menses
ovulation

Figure 3.4 Effects of hormonal status on resting blood pressure in smokers.

A. Resting systolic blood pressure is greater at ovulation than at menses in smokers. B. Resting diastolic blood pressure is unchanged between menses and ovulation. C. MAP demonstrates a trend for an increase at ovulation. (n=7; * $P<0.05$, Student's *t*-test). Data represents mean ± S.E.M.

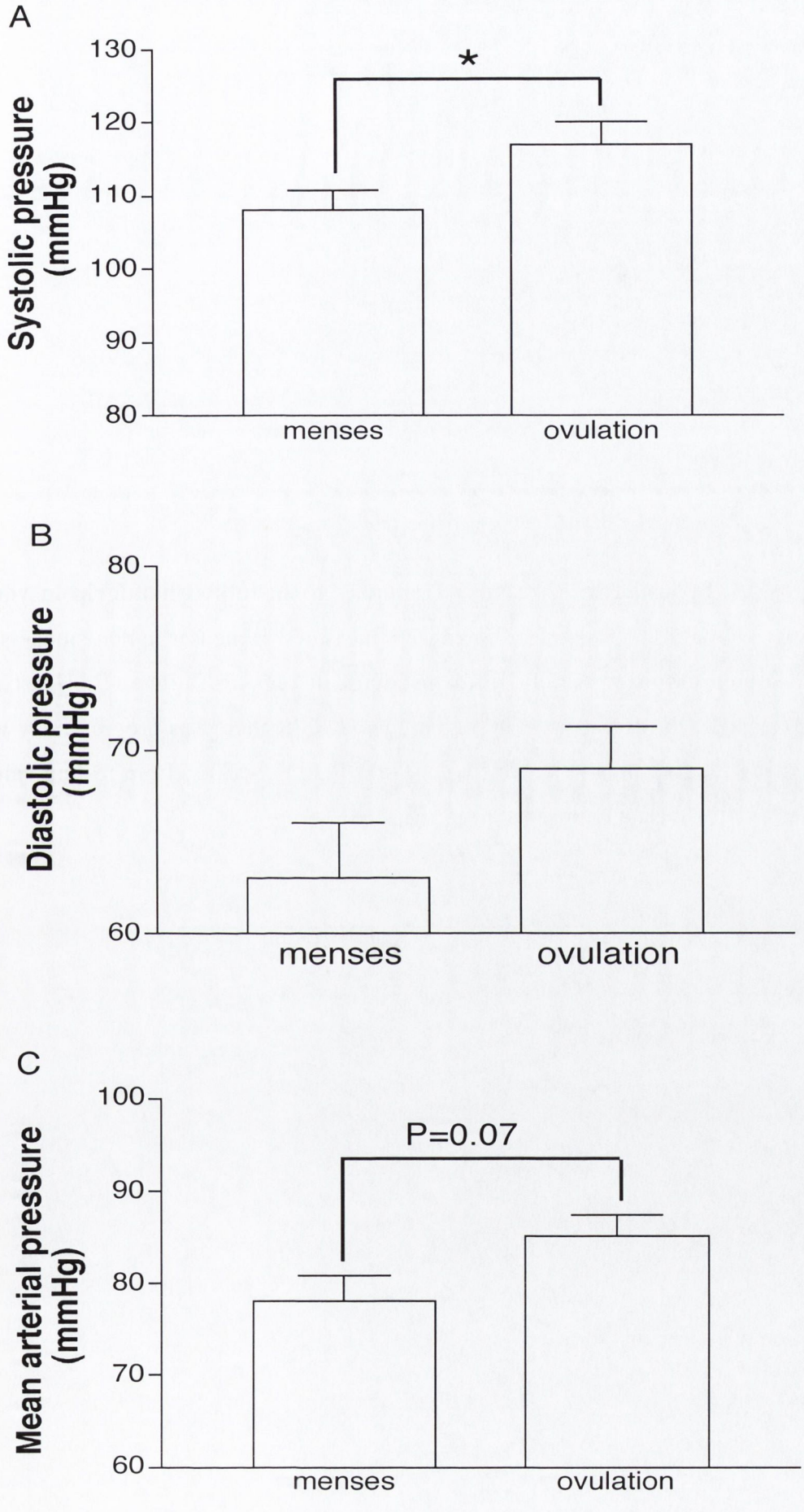

A
Systolic pressure (mmHg)
130
120
110
100
90
80
*
menses
ovulation
B
Diastolic pressure (mmHg)
80
70
60
menses
ovulation
C
Mean arterial pressure (mmHg)
100
90
80
70
60
P=0.07
menses
ovulation

Figure 3.5 Typical cardiovascular responses to sustained handgrip in young healthy women. The examples shown were measured at menses in non-smokers. A. MAP increases progressively from rest with increasing fatigue. B. Heart rate increases at each increasing level of fatigue. C. TPR increases progressively with increasing fatigue. (n=14; * $P<0.05$, ** $P<0.01$, *** $P<0.001$ from resting values, Student's *t*-test). Data represents mean ± S.E.M.

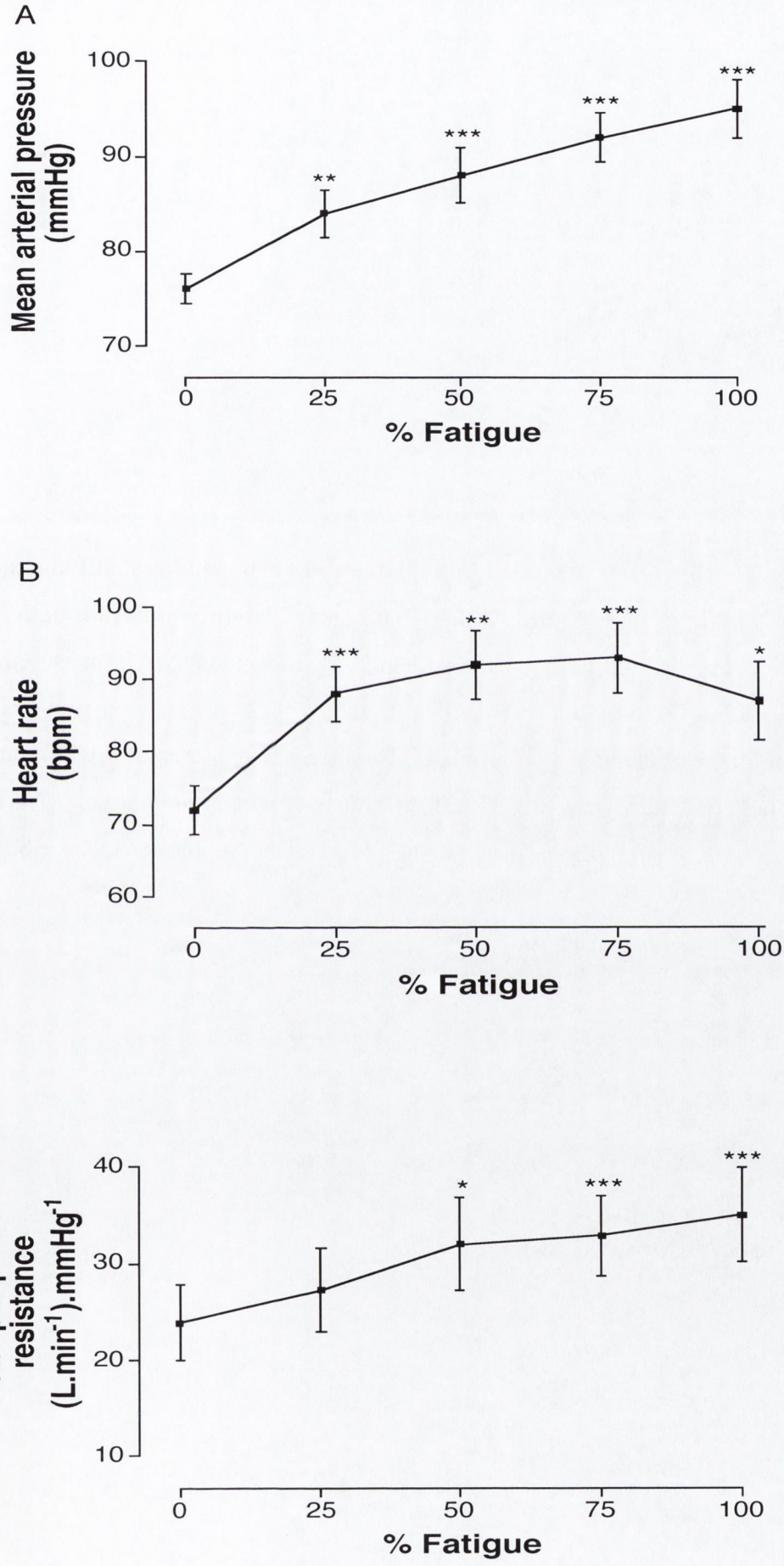

A
Mean arterial pressure (mmHg)
100
90
80
70
**
***
***
***
0
25
50
75
100
% Fatigue
B
Heart rate (bpm)
100
90
80
70
60
***
**
***
*
0
25
50
75
100
% Fatigue
C
Total peripheral resistance (L.min-1).mmHg-1
40
30
20
10
*
***
***
0
25
50
75
100
% Fatigue

Figure 3.6 Effect of a sustained handgrip on MAP in smokers and non-smokers at menses and ovulation. A. MAP increased significantly in both groups independent of smoking or hormonal status. B. Individual MAP measurements in non-smokers. C. Individual MAP measurements in smokers. (non-smokers: n=10, smokers: n=7; * denotes a significant difference between rest and 100% fatigue in non-smokers, § denotes a significant difference from rest in smokers (*/§ $P<0.05$, **/§§ $P<0.01$, ***/§§§ $P<0.001$), Student's *t*-test). Data represents mean ± S.E.M.

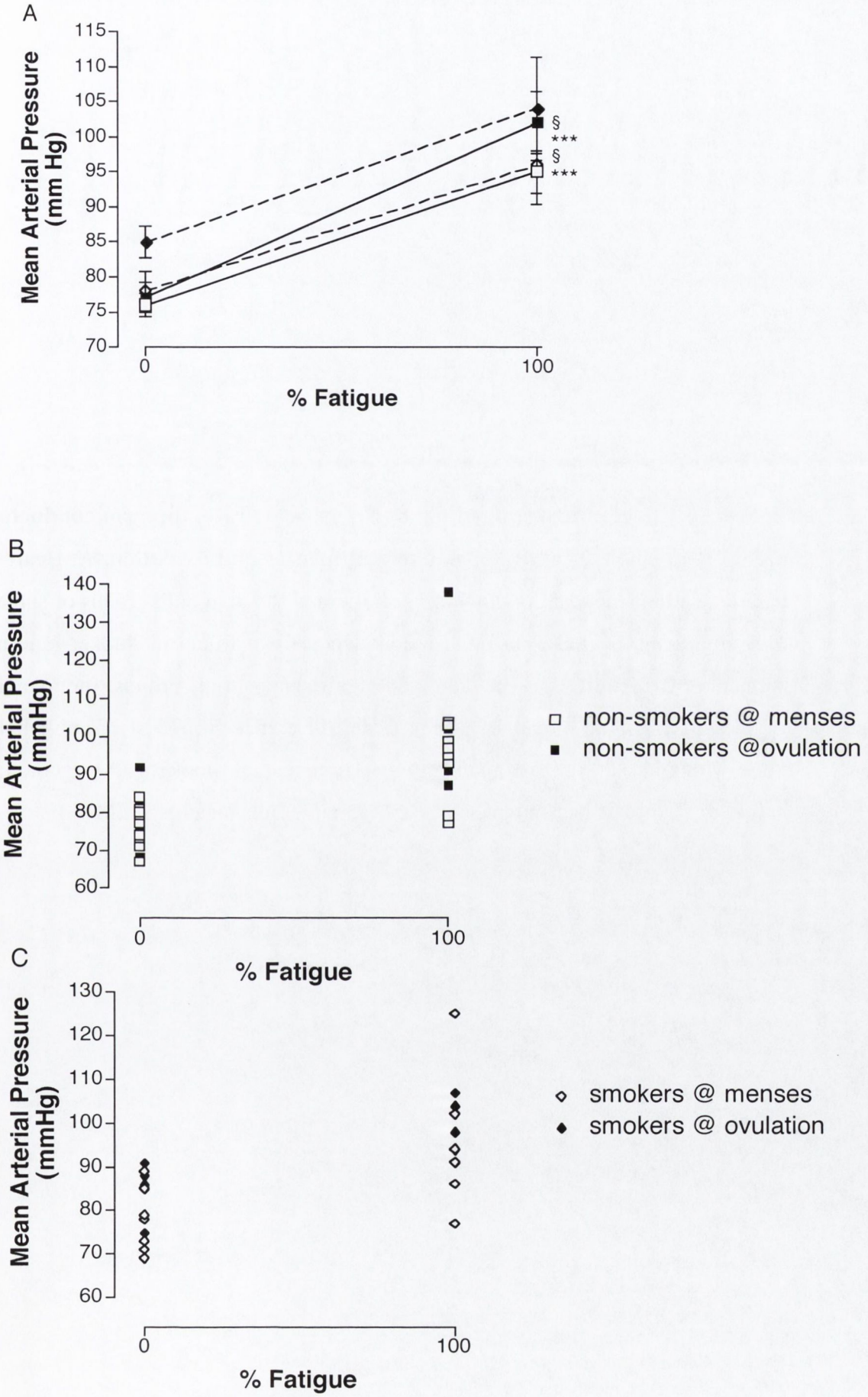

A
Mean Arterial Pressure (mm Hg)
115
110
105
100
95
90
85
80
75
70
0
100
% Fatigue
§
***
§
***
B
Mean Arterial Pressure (mmHg)
140
130
120
110
100
90
80
70
60
0
100
% Fatigue
non-smokers @ menses
non-smokers @ovulation
C
Mean Arterial Pressure (mmHg)
130
120
110
100
90
80
70
60
0
100
% Fatigue
smokers @ menses
smokers @ ovulation

Figure 3.7 Effect of a sustained handgrip on heart rate, forearm conductance and TPR in smokers and non-smokers at menses and ovulation. A. Heart rate was elevated independent of smoking or hormonal status in both groups of subjects. B. A significant decrease in forearm conductance was found in smokers and non-smokers at ovulation. C. TPR increased significantly in all groups. (non-smokers n=10, smokers n=5; * denotes a significant difference from rest in non-smokers while § denotes a significant difference from rest in smokers (*/§ $P<0.05$; ** $P<0.01$; *** $P<0.001$), Student's *t*-test*)*. Data represents mean ± S.E.M.

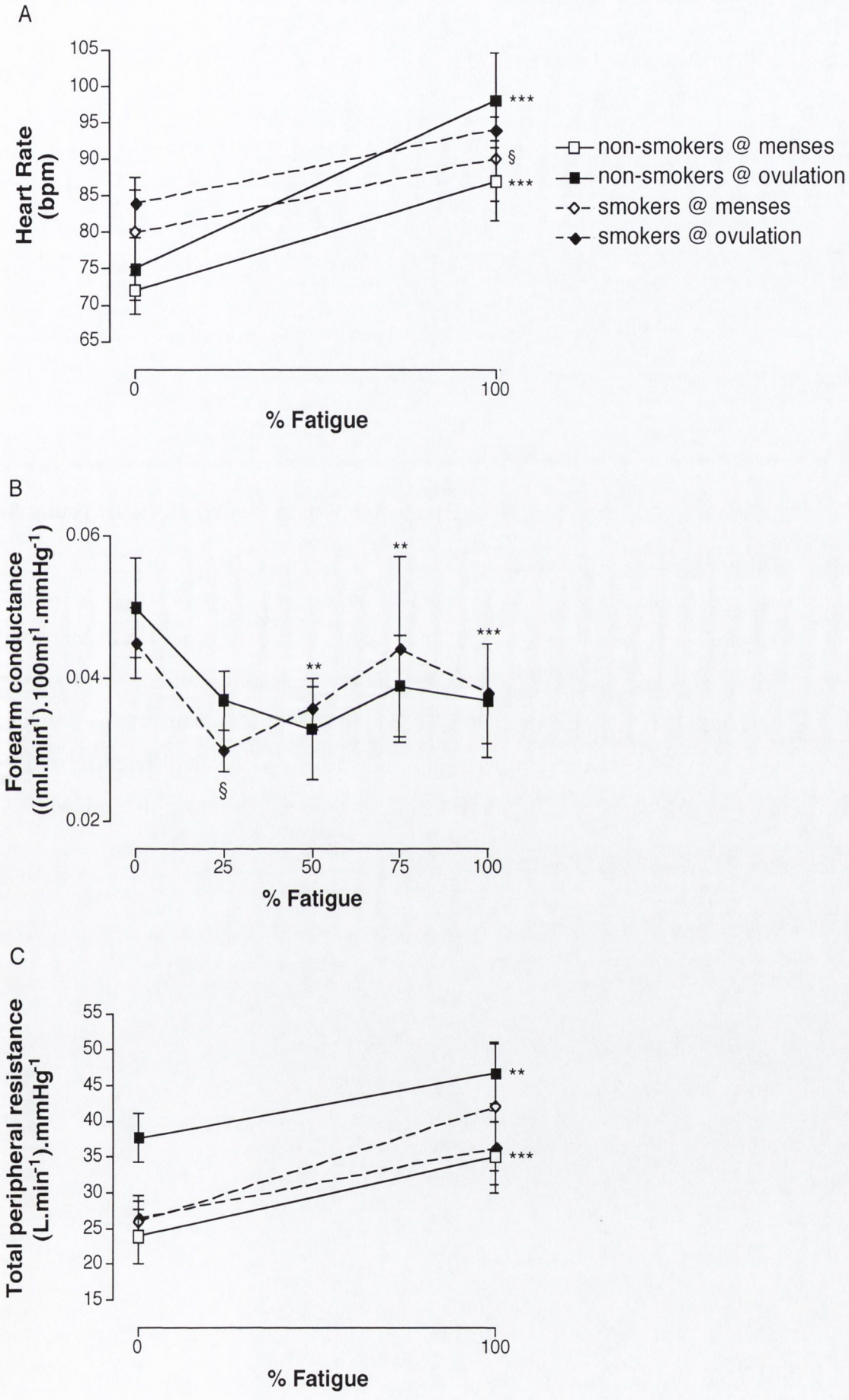

A
Heart Rate (bpm)
105
100
95
90
85
80
75
70
65
***
§
***
0
100
% Fatigue
non-smokers @ menses
non-smokers @ ovulation
smokers @ menses
smokers @ ovulation
B
Forearm conductance ((ml.min-1).100ml-1.mmHg-1)
0.06
0.04
0.02
**
**
***
§
0
25
50
75
100
% Fatigue
C
Total peripheral resistance (L.min-1).mmHg-1
55
50
45
40
35
30
25
20
15
**
***
0
100
% Fatigue

Figure 3.8 Typical cardiovascular responses to mental stress in young healthy women.

The examples shown were measured at menses in non-smokers. A. MAP increases significantly during the first minute of mental stress. B. Heart rate is increased at all time progressions. C. TPR decreased at 30 seconds and a trend was visible thereafter. D. Cardiac index was increased at all time points during mental stress. (non-smokers: n=10, smokers: n=7; * denotes a significant difference from $P<0.05$; ** $P<0.01$; *** $P<0.001$, Student's *t*-test). Data represents mean ± S.E.M.

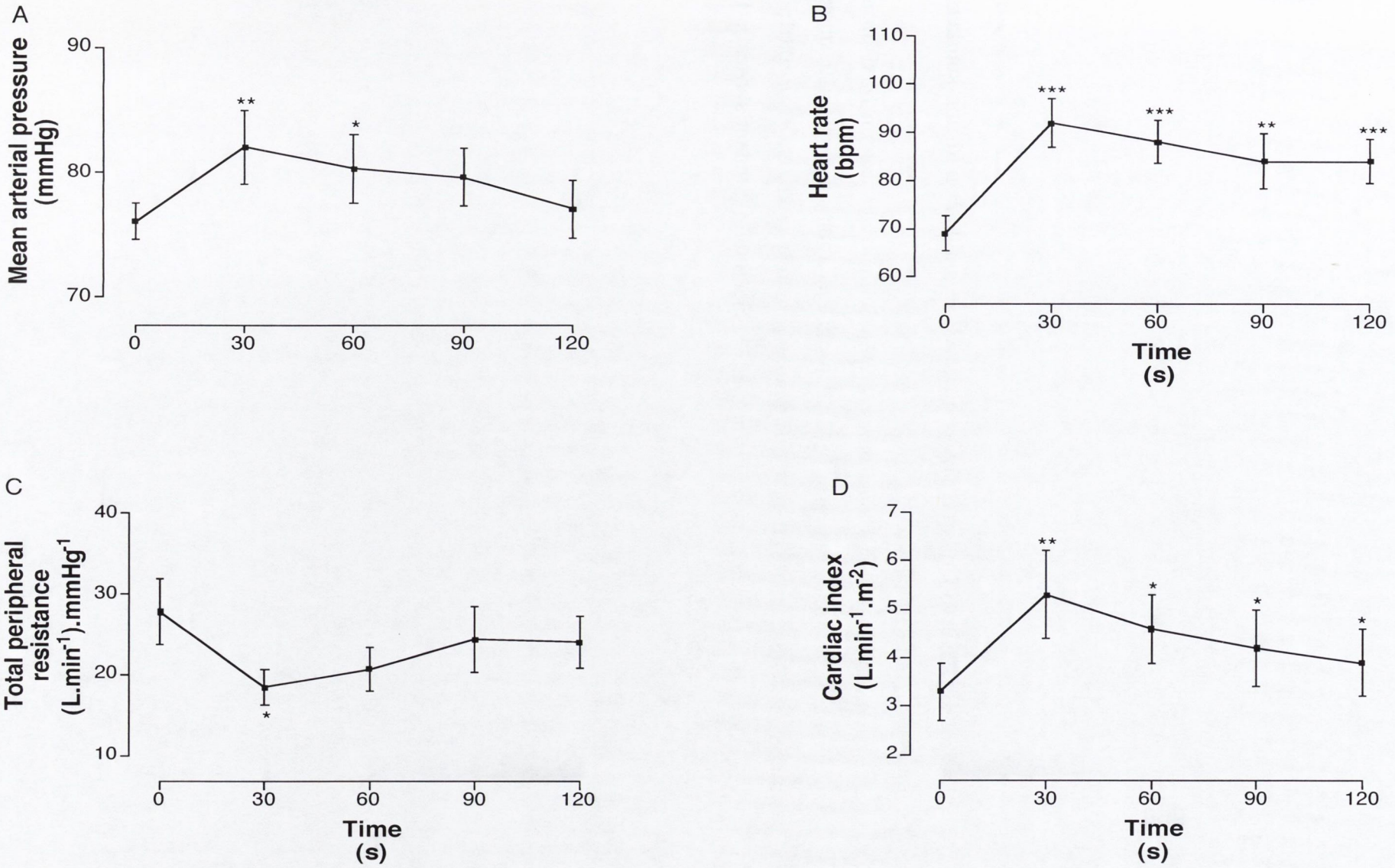

A
Mean arterial pressure (mmHg)
90
80
70
**
*
0
30
60
90
120
B
Heart rate (bpm)
110
100
90
80
70
60
***
***
**
***
0
30
60
90
120
Time (s)
C
Total peripheral resistance (L.min-1).mmHg-1
40
30
20
10
*
0
30
60
90
120
Time (s)
D
Cardiac index (L.min-1.m-2)
7
6
5
4
3
2
**
*
*
*
0
30
60
90
120
Time (s)

Figure 3.9 Effect of mental stress on MAP in smokers and non-smokers at menses and at ovulation.

A. MAP increased with mental stress. B. Individual increases in MAP in non-smokers. C. Individual increases in MAP in smokers. (non-smokers: n=10, smokers: n=7; */§ denotes a significant difference from rest ($P<0.05$; ** $P<0.01$; *** $P<0.001$) in non-smokers and smokers, respectively, Student's *t*-test). Data represents mean ± S.E.M.

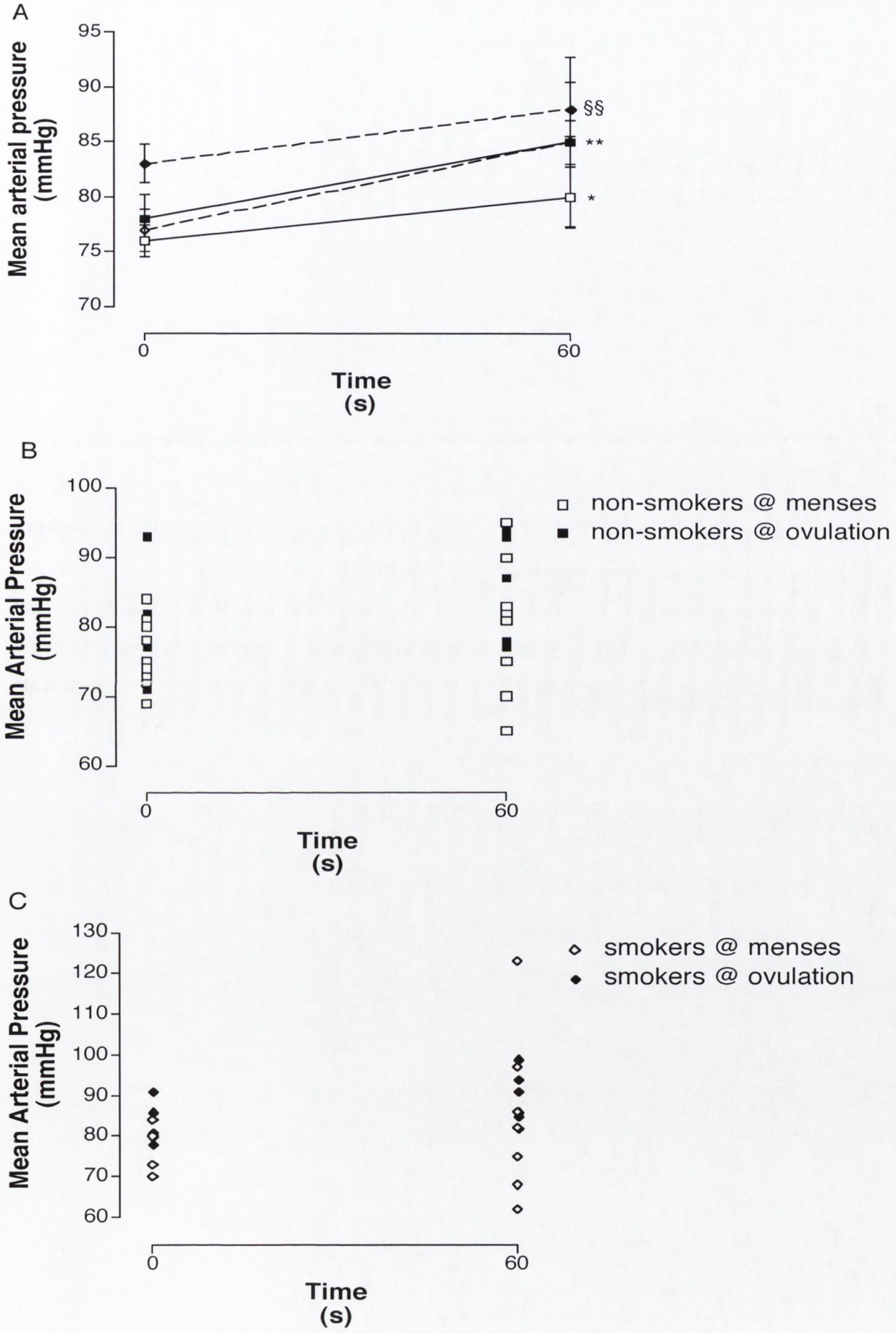

A
Mean arterial pressure (mmHg)
95
90
85
80
75
70
§§
**
*
0
60
Time (s)
B
Mean Arterial Pressure (mmHg)
100
90
80
70
60
non-smokers @ menses
non-smokers @ ovulation
0
60
Time (s)
C
Mean Arterial Pressure (mmHg)
130
120
110
100
90
80
70
60
smokers @ menses
smokers @ ovulation
0
60
Time (s)

Figure 3.10 Effect of mental stress on heart rate and cardiac index in smokers and non-smokers at menses and at ovulation.

A. Increased heart rate was observed with mental stress. B. Cardiac index was elevated during mental stress. (non-smokers: n=10, smokers: n=7; */§ denotes a significant difference from rest ($P<0.05$; ** $P<0.01$; *** $P<0.001$) in non-smokers and smokers, respectively, Student's *t*-test). Data represents mean ± S.E.M.

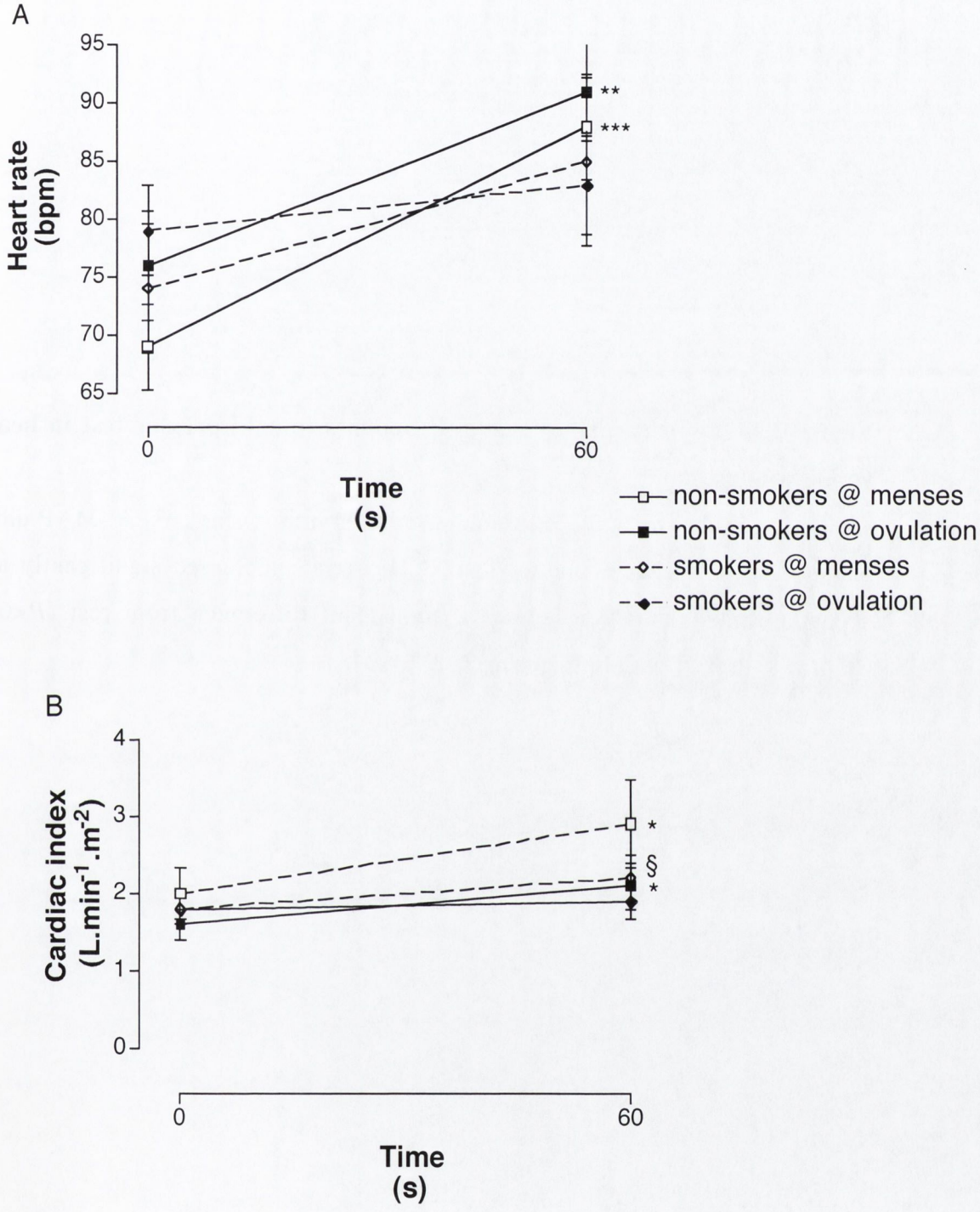

A
Heart rate (bpm)
95
90
85
80
75
70
65
0
60
Time (s)
**
***
non-smokers @ menses
non-smokers @ ovulation
smokers @ menses
smokers @ ovulation
B
Cardiac index (L.min-1.m-2)
4
3
2
1
0
0
60
Time (s)
*
§
*

Figure 3.11 Typical cardiovascular responses to cold pressor test in healthy young women.

The examples shown were measured at menses in non-smokers. A. MAP did not change during the cold pressor test. B Heart rate increased significantly at 90 seconds alone. (n=10; * denotes a significant difference from rest ($P<0.05$), Student's *t*-test). Data represents mean ± S.E.M.

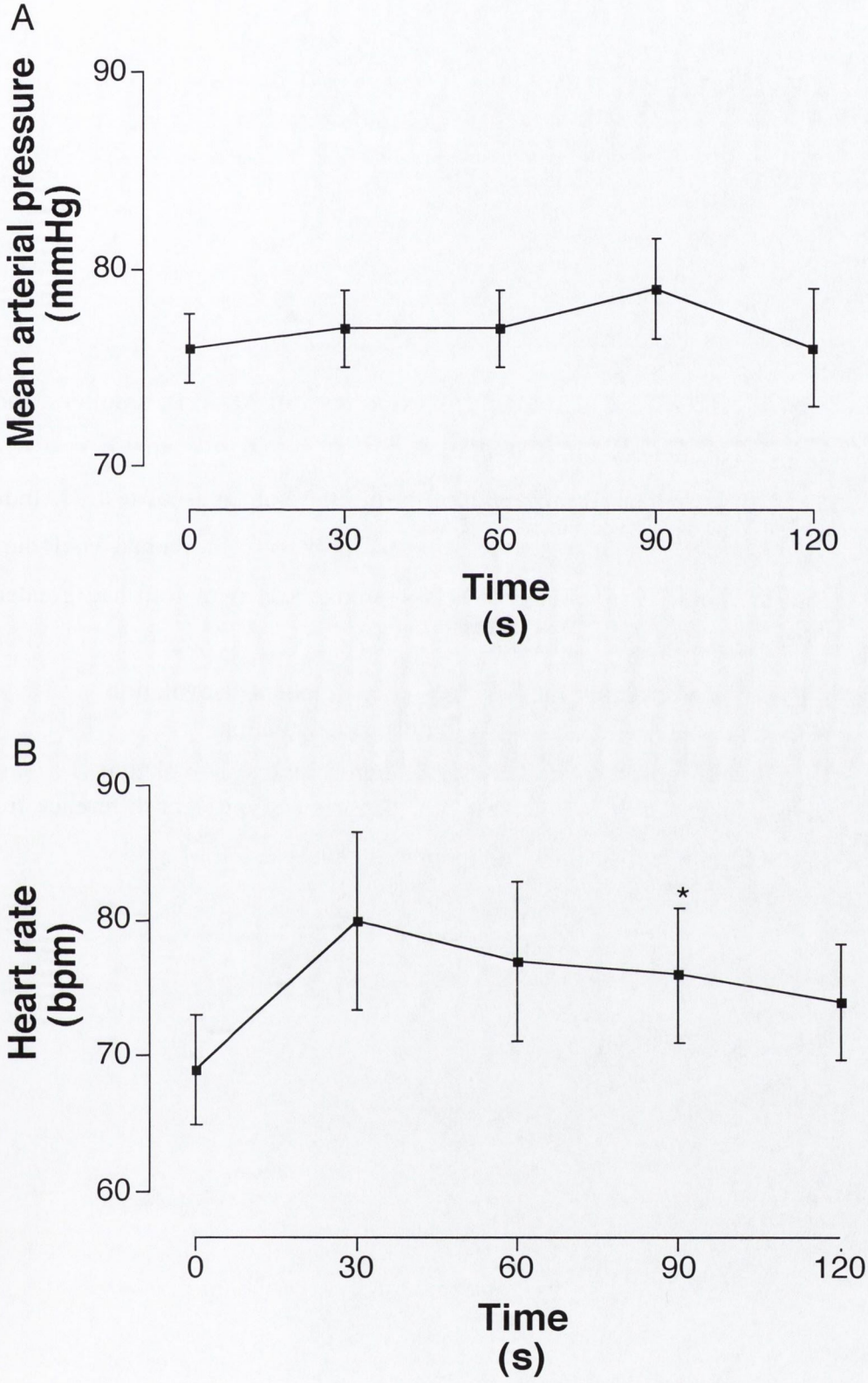

A
Mean arterial pressure (mmHg)
90
80
70
0
30
60
90
120
Time (s)
B
Heart rate (bpm)
90
80
70
60
*
0
30
60
90
120
Time (s)

Figure 3.12 Effect of the cold pressor test on MAP in smokers and non-smokers at menses and at ovulation.

A. MAP is increased at ovulation during the cold pressor test. B. Individual MAP during the cold pressor test in non-smokers. C. Individual MAP during the cold pressor test in smokers. D. Non-smokers at ovulation had greater MAP responses to the test than at menses.

—□— non-smokers @ menses —■— non-smokers @ ovulation
—◇— smokers @ menses —◆— smokers @ovulation
▭ non-smokers @ menses ▨ non-smokers @ ovulation
(non-smokers: n=9; smokers: n=7; * denotes a significant difference from rest ($P<0.05$), Student's *t*-test). Data represents mean ± S.E.M.

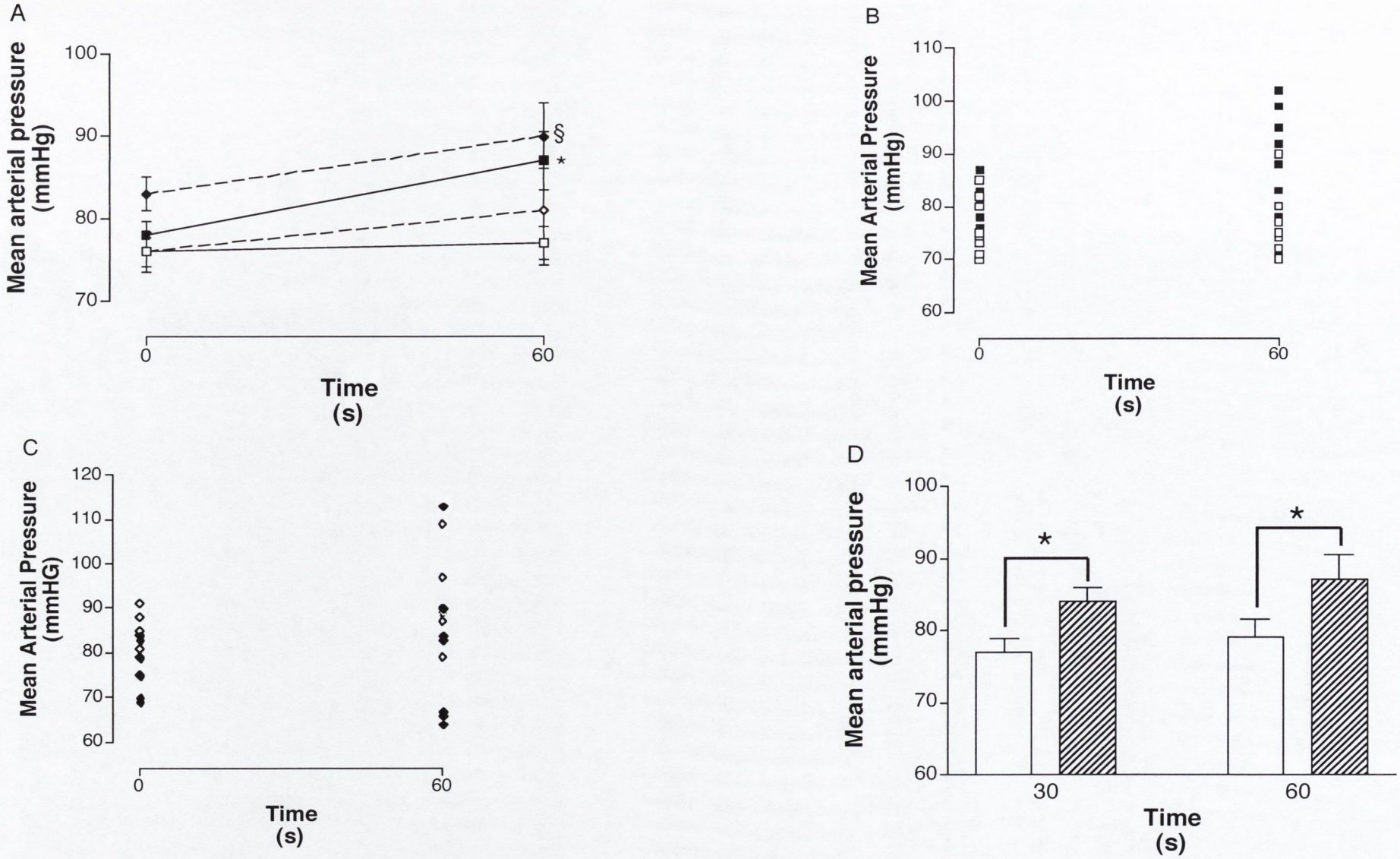

A
Mean arterial pressure (mmHg)
100
90
80
70
§
*
0
60
Time (s)
B
Mean Arterial Pressure (mmHg)
110
100
90
80
70
60
0
60
Time (s)
C
Mean Arterial Pressure (mmHG)
120
110
100
90
80
70
60
0
60
Time (s)
D
Mean arterial pressure (mmHg)
100
90
80
70
60
*
*
30
60
Time (s)

Figure 3.13 Effect of the cold pressor test on heart rate and cardiac index.
A. At ovulation heart rate increased at most time points in non-smokers and at 90 seconds at menses. B. Cardiac index responses increased significantly at ovulation when compared to menses in smokers during the cold pressor test. (non-smokers: n=9, smokers: n=7; * $P<0.05$, ** $P<0.01$, Student's *t*-test). Data represents mean ± S.E.M.

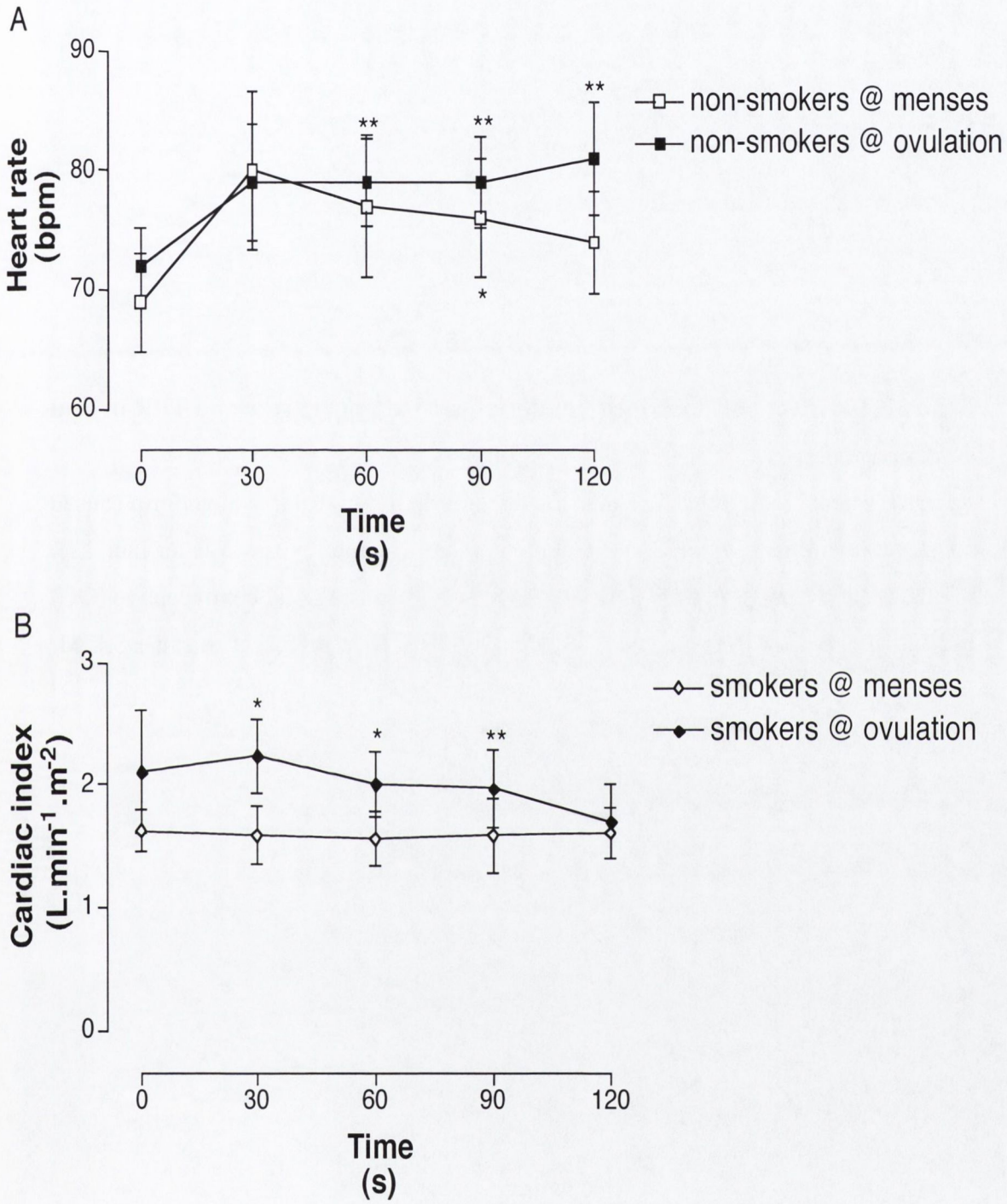

A
90
80
70
60
Heart rate (bpm)
**
**
**
*
non-smokers @ menses
non-smokers @ ovulation
0
30
60
90
120
Time (s)
B
3
2
1
0
Cardiac index (L.min-1.m-2)
*
*
**
smokers @ menses
smokers @ ovulation
0
30
60
90
120
Time (s)

Figure 3.14 Effect of a sustained handgrip on MAP, heart rate and TPR in oral contraceptive users.

A. MAP was increased during a sustained handgrip. B. A sustained handgrip caused an elevation in heart rate. C. TPR demonstrated a tendency towards an increase. (n=10; */§ $P< 0.05$, **/§§ $P< 0.01$, ***/§§§ $P<0.001$ during oral contraceptive (OC) use and at withdrawal, respectively, Student's *t*-test). Data represents mean ± S.E.M.

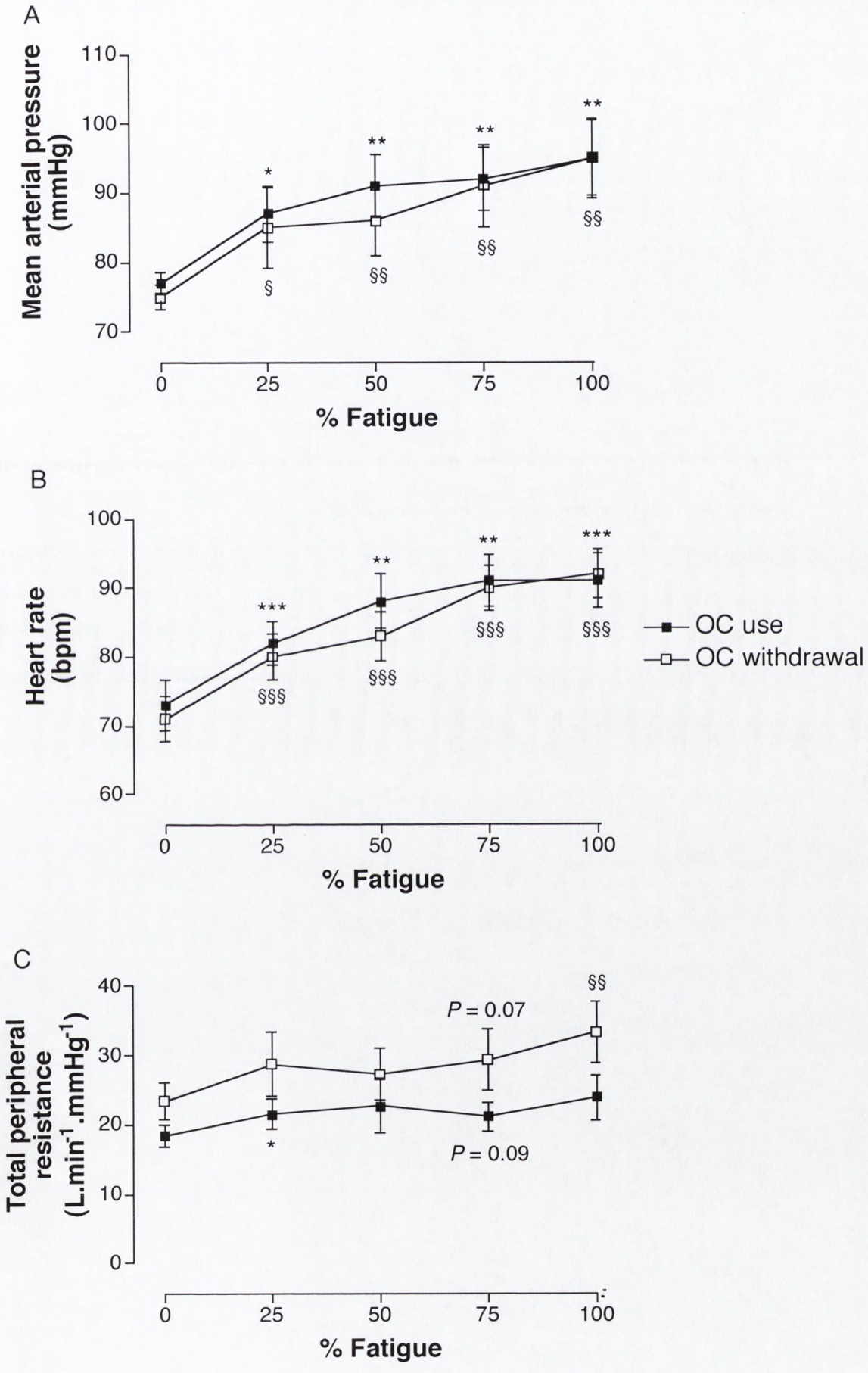

A
Mean arterial pressure (mmHg)
110
100
90
80
70
*
**
**
**
§
§§
§§
§§
0
25
50
75
100
% Fatigue
B
Heart rate (bpm)
100
90
80
70
60
***
**
**
***
§§§
§§§
§§§
§§§
0
25
50
75
100
% Fatigue
OC use
OC withdrawal
C
Total peripheral resistance (L.min-1.mmHg-1)
40
30
20
10
0
*
§§
P = 0.07
P = 0.09
0
25
50
75
100
% Fatigue

Figure 3.15 Effects of mental stress on MAP, heart rate and forearm vascular conductance in oral contraceptive users.

A. MAP was unchanged by mental stress. B. An elevation in heart rate was observed during mental stress. C. Forearm vascular conductance increased during mental stress measurements taken at withdrawal. (n=10; */§ $P<0.05$, **/§§ $P<0.01$, ***/§§§ $P<0.001$ during oral contraceptive (OC) use and at withdrawal, respectively, Student's *t*-test). Data represents mean ± S.E.M.

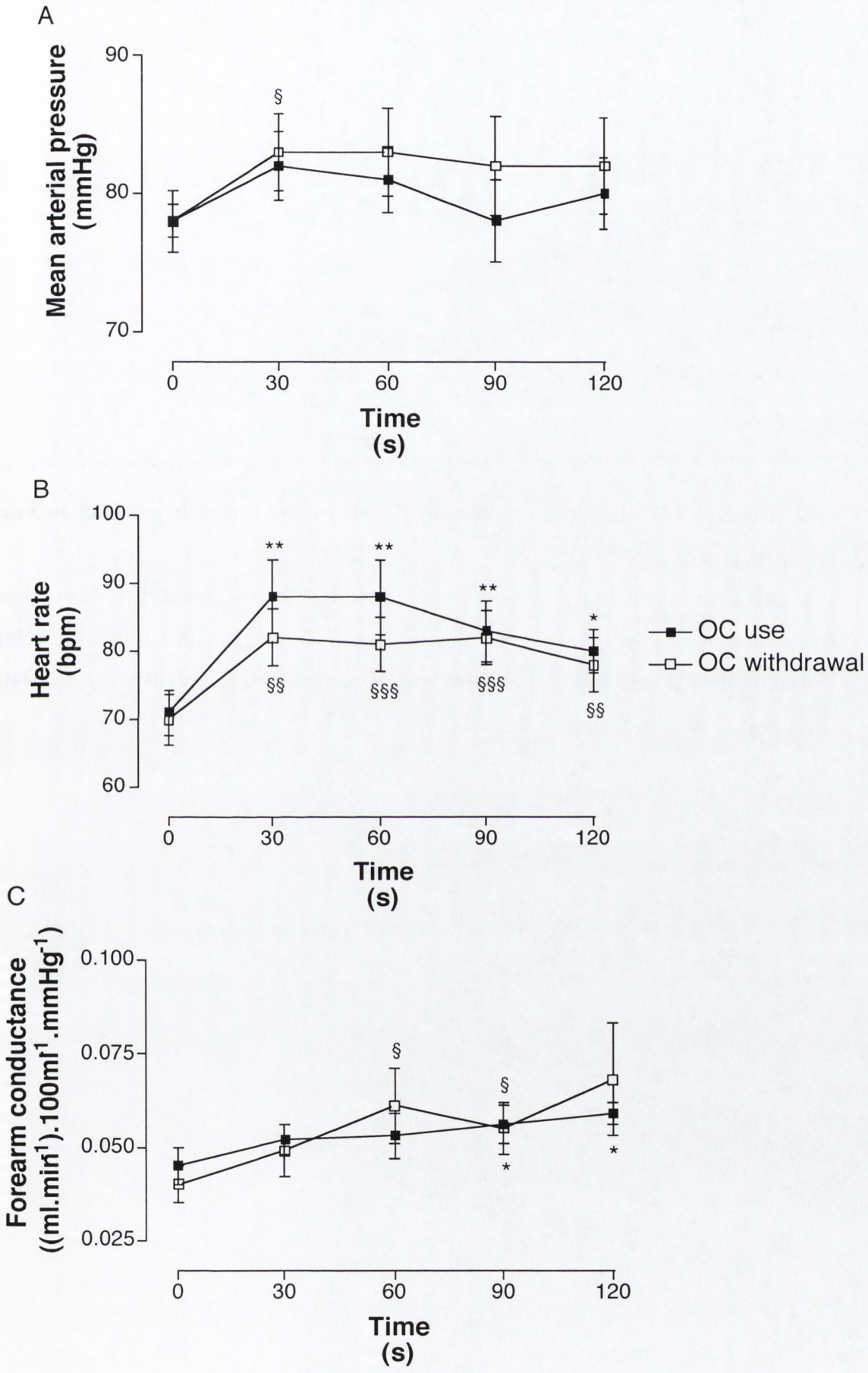
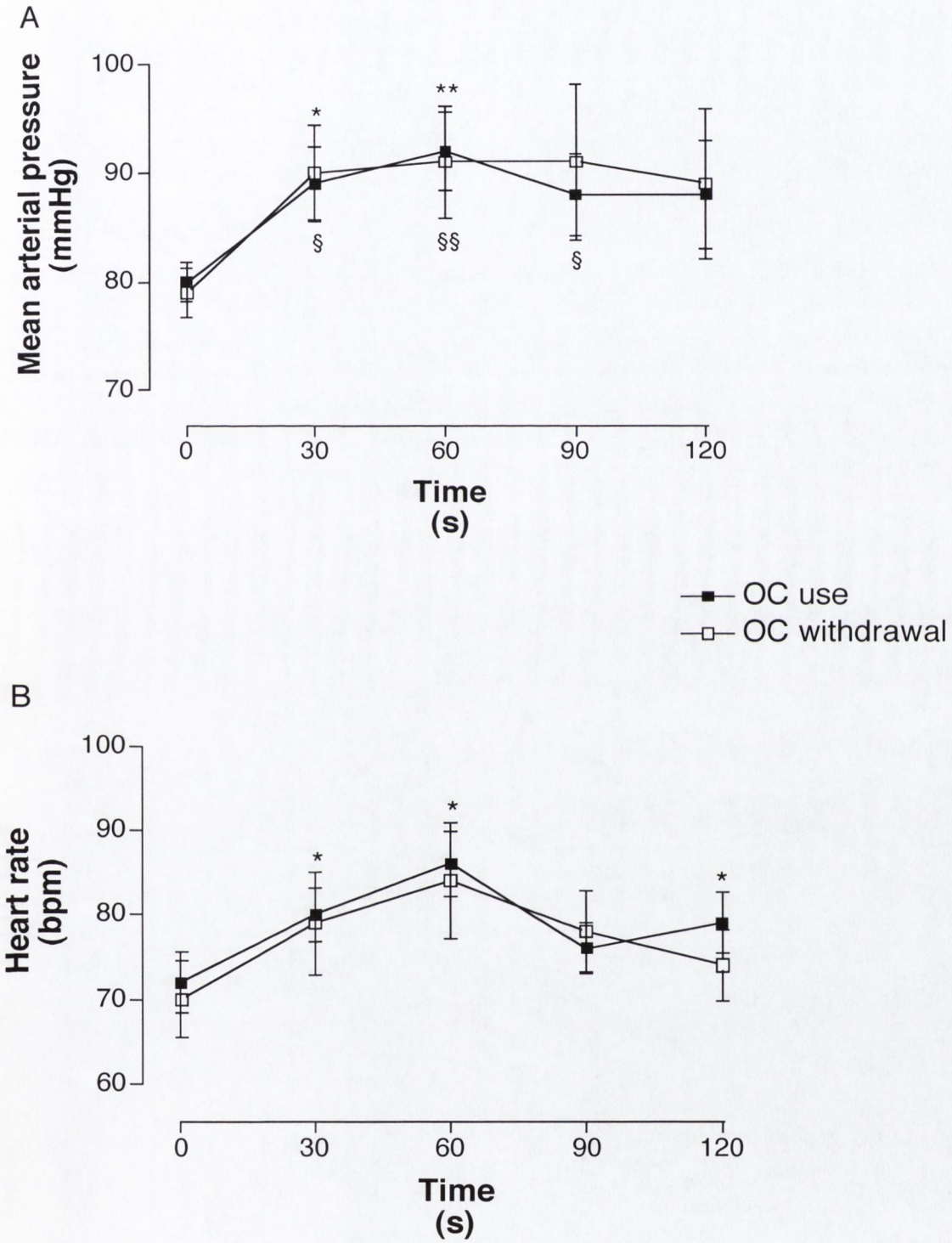

A
Mean arterial pressure (mmHg)
90
80
70
§
0
30
60
90
120
Time (s)
B
Heart rate (bpm)
100
90
80
70
60
**
**
**
*
§§
§§§
§§§
§§
0
30
60
90
120
Time (s)
OC use
OC withdrawal
C
Forearm conductance ((ml.min-1).100ml-1.mmHg-1)
0.100
0.075
0.050
0.025
§
§
*
*
0
30
60
90
120
Time (s)

Figure 3.16 Effect of the cold pressor test on MAP and heart rate in oral contraceptive users.

A. MAP was elevated by the cold pressor test. B. The cold pressor test caused an elevation in heart rate during oral contraceptive (OC) use. (n=10; */§ $P<0.05$, **/§§ $P<0.01$ during OC use and at withdrawal, respectively, Student's t-test). Data represents mean ± S.E.M.


A
Mean arterial pressure (mmHg)
100
90
80
70
*
**
§
§§
§
0
30
60
90
120
Time (s)
OC use
OC withdrawal
B
Heart rate (bpm)
100
90
80
70
60
*
*
*
0
30
60
90
120
Time (s)

3.4 Discussion

There was no variation in cardiovascular reactivity to a sustained handgrip during the menstrual cycle in this study. TPR responses to a sustained handgrip were less during OC use than at ovulation (5 ± 2.5 cf 19 ± 4.7 Δ $(L.min^{-1}).mmHg^{-1}$). indicating a blunting of reactivity with the presence of progesterone. Systolic blood pressure in response to mental stress was elevated in non-smokers at ovulation when compared with menses (12 ± 3.3 cf 3 ± 2.0 Δ mmHg) and ovulation was accompanied by a greater increase in systolic blood pressure (12 ± 3.3 cf 2 ± 2.4 Δ mmHg) than oral contraceptive use. A trend towards greater blood pressure and heart rate reactivity (see Table 3.4) in response to mental stress was evident at ovulation in non-smokers when compared with smokers. However this trend was not significant. The cold pressor test seems to be affected by menstrual cycle phase in both smokers and non-smokers as elevations in MAP were present at ovulation only (see Table 3.5). Elevations in diastolic pressure were greater in response to this test at ovulation in non-smokers than in smokers.

3.4.1 Resting blood pressure and heart rate

Resting cardiovascular parameters measured in this study in premenopausal non-smokers did not differ at menses or at ovulation. Studies investigating the effects of the menstrual cycle on blood pressure and heart rate have produced varied results. Prior studies have demonstrated that oestrogen increases, decreases, or does not influence blood pressure (Crane *et al.*, 1971; Sudhir *et al.*, 1997). Investigators have found evidence of higher systolic pressure (Kelleher *et al.*, 1986; Dunne *et al.*, 1991) and diastolic pressure (Dunne *et al.*, 1991) at menses than at most other stages of the cycle. Other groups suggest that resting systolic pressure is significantly higher at ovulation than at the follicular or luteal phase but resting diastolic blood pressure is not different (Moran *et al.* 2000). Freedman (1974) found both systolic and diastolic pressure were elevated during and just after mid-cycle. Similarly to the results of the present study Chan *et al.* (2001) found there was no difference between resting blood pressure at menses or at ovulation. These latter results concur with previous findings (Green *et al.*, 1986; Karpanou *et al.* 1993; Hashimoto *et al.*, 1995; Ettinger *et al.*, 1998) that found no change in blood pressure throughout the

menstrual cycle. Heart rate values were not different between the two phases of the menstrual cycle in non-smokers or smokers in the present study and are therefore similar to other results in the literature (Girdler *et al.*, 1993; Ettinger *et al.*, 1998; Sato *et al.*, 1998). The fact that resting cardiovascular parameters including heart rate do not differ between the period of oral contraceptive use and withdrawal and that oral contraceptive users and non-users are comparable in the various parameters measured suggests that the presence of oestrogen with progesterone does not effect resting cardiovascular variables. The result of this present study would therefore support the hypothesis that blood pressure and heart rate values in premenopausal non-smokers do not depend on cycle phase or oral contraceptive use.

Most studies on menstrual cycle phase and cardiovascular haemodynamics do not take smoking status into account or deal with non-smokers alone. Those that have looked at menstrual cyle phase, smoking and cardiovascular reactivity are discussed later. In the present study resting systolic pressure was significantly higher at ovulation than at menses in the smoking group and a similar trend was observed for MAP. Although studies have found no difference (Green *et al.*, 1986; Barry and Bell, 2002), significantly lower (Imamura *et al.*, 2002) as well as higher (Morris and Bell, 2002) blood pressures in male smokers than in non-smokers, no study, as far as I am aware, has investigated the effect of menstrual cycle phase on blood pressure in young healthy female smokers. The present study found that female smokers have significantly elevated systolic, diastolic and MAP at ovulation when compared with non-smokers. This would suggest that there is some interaction between smoking and oestrogen concentrations in females.

Smoking is known to alter oestrogen metabolism and possess generalised anti-oestrogenic effects (Baron *et al.*, 1990). Smoking reduces urinary levels of oestrogens in premenopausal women (MacMahon *et al.*, 1982). It has already been shown that women who smoke and receive oral oestrogen replacement therapy have plasma oestrogen levels 40-70% lower than non-smoking women (Geisler *et al.*, 1999). Similarly, female smokers at ovulation may have diminished oestrogen levels when compared with non-smokers and a lack of oestrogen is known to elevate blood pressure (Beljic *et al.*, 1998; Dantas *et al.*, 1999).

Another important factor that may explain this difference is that renin levels are higher at rest in smokers than in non-smokers indicating an increase in sympathetic activity and the activation of the renin-angiotensin-aldosterone system

(Laustiola *et al.*, 1988; Kotamaki, 1995). The renin-angiotensin-aldosterone system could enhance peripheral resistance by causing the blood vessels to stiffen and thus increase blood pressure. Also during the follicular and luteal phases aldosterone correlates with oestradiol concentration in women (Chidambaram *et al.*, 2002). Smoking has also been shown to increase aldosterone levels (Garcia Calzado *et al.*, 1990). Tersman *et al.* (1991) found that in the follicular phase of the menstrual cycle aldosterone is correlated with oestradiol in smoking women but not in non-smoking women. So although there is no difference in resting blood pressure in non-smokers at menses and ovulation, smokers could exhibit differences due to the additional effect of smoke on aldosterone levels. However the fact that TPR was not different between the groups in the present study (see Table 3.2) would suggest an alteration in vascular resistance is not the principal factor responsible.

Adrenergic receptor number can alter depending on oestrogen and/or smoking status. Healthy women during the menstrual cycle show a decrease in α_2-adrenoreceptor number with increasing plasma oestrogen levels (Brodde, 1983). α_2-adrenoreceptors are more prominent than α_1-adrenoreceptors in human resistance arteries (Flavahan *et al.*, 1987). Increased SNA has been shown to increase α_2-adrenergic responsiveness (Freedman *et al.*, 1992). On the other hand Kneale *et al.* (2000) found the $ß_2$-adrenergic receptor agonist, albuterol, is a more potent vasodilator in women than in men and affects TPR. Smoking decreases ß-adrenoreceptors (Laustiola *et al.*, 1988) and its cessation increases $ß_2$-adrenoreceptor density (Laustiola *et al.*, 1991). Therefore smoking could diminish $ß_2$-receptor vasodilation and cause an increase in blood pressure at ovulation. In non-smokers at ovulation α-adrenergic vasoconstriction may have decreased and $ß_2$-adrenergic activity would then have proceeded unabated.

It is also a possibility that a decrease in basal NO production (Barua *et al.*, 2001) and bioavailability in cigarette smokers (Kugiyama *et al.*, 1996) could account for this difference. *In vivo* studies in humans (Rosselli *et al.*, 1995) and animals (Brosnihan *et al.*, 1994) show that oestrogen administration can increase circulating levels of nitrite and nitrate in serum suggesting an enhancement of NO release into the circulation. Perhaps, therefore, the normal regulatory action of oestrogen on varied aspects of cardiovascular control was impaired by smoking in my study resulting in elevated diastolic, systolic and MAP at rest.

3.4.2 Cardiovascular reactivity to stress

Results from cardiovascular reactivity studies during the menstrual cycle are often contradictory and depend on the form of stress applied. Differences could be due to differences in design and methodology such as different times of testing, not controlling adequately for menstrual cycle phase or not taking into account lifestyle factors such as smoking.

Static exercise usually elicits a pressor response characterised by elevated heart rate, blood pressure and myocardial contractility (Victor *et al.*, 1989). In isometric handgrip the pressor response is due to muscle chemoreflex activity, which increases SNA and central command, which elevate heart rate and cardiac output (Rowell and O'Leary, 1990). Heart rate in this experiment was most probably increased via vagal withdrawal (Victor *et al.*, 1989) and blood pressure by SNA increase and central command. Oral contraceptive users in the present study displayed a blunted increase in TPR during oral contraceptive use than at ovulation in non-users. This could suggest a difference in the influence of oestrogen and oestrogen plus progesterone on vascular resistance. In fact combination HRT (oestrogen plus progesterone) has been shown to reduce TPR and increase vagal activity whereas oestrogen treatment alone has produced no such effect (Farag *et al.*, 2002). However it must be remembered that the oestrogens and progestins used in oral contraceptives are different to those present during the normal menstrual cycle.

In the present study there was a tendency for the absolute increase in MAP and heart rate to be higher in response to a sustained handgrip during ovulation than menses in non-smokers. This increase also tended to be greater in non-smokers than smokers at ovulation. The magnitude of response to a sustained handgrip can be related to the degree of sympathetic activation. However since no statistically conclusive differences were observed between the different phases it does not seem likely that sympathetic activation was substantial. In agreement with the present findings Ettinger *et al.* (1998) and Petrofsky *et al.* (1976) found that heart rate and blood pressure responses are independent of cycle phase when performing ischaemic isometric exercise. Ettinger found that throughout the exercise MAP and heart rate changes were not different between menses and the follicular phase. However, further study is warranted.

A decrease in conductance values occurred in both smokers and non-smokers at ovulation. Isometric handgrip is a vasoconstriction-inducing stimulus

and an increase in sympathoexcitatory responses to the arm results in pronounced vasoconstriction in the contralateral forearm (Cotzias and Marshall, 1993). The increase in SNA during a sustained handgrip probably caused the vasoconstriction observed in this study. Why this did not happen at menses remains unclear although the majority of subjects had their first session at menses and some vasodilation can occur during isometric handgrip due to unintentional contractions in the contralateral arm (Cotzias and Marshall, 1993). TPR however tended to increase in all groups in response to a sustained handgrip.

Mental arithmetic produced typical psycho-physiological arousal (Folkow, 1991), which included elevated MAP, systolic and diastolic pressure, heart rate and cardiac output (see Table 3.4). These findings are consistent with those of other investigators (Jern *et al.*, 1991; Yoshiuchi *et al.*, 1997; Critchley *et al.*, 2000) and were for the most part independent of hormonal status. Systolic pressure was elevated in non-smokers at ovulation when compared with menses, which suggests a difference in cardiac reactivity during the menstrual cycle in relation to this form of stress. MAP responses were maximal by 60 seconds in both groups. Non-smokers had greater heart rate responses to mental stress at ovulation than smokers. This suggests that at ovulation smokers have blunted heart rate reactivity relative to non-smokers. This result is in accordance with results from Girdler *et al.* (1997). Mental arithmetic in this present study primarily caused sympathetic activation of the myocardium. Heart rate and cardiac index were seen to increase (see Figure 3.10) and these changes may be responsible for the blood pressure changes observed in each group. Men also show increases in heart rate with mental stress however their cardiac outputs remain unaltered (O'Sullivan and Bell, 2001), this would concur with previous work that females are myocardial hyperreactors when compared with men. The fact that systolic pressure and heart rate responses to mental stress were greater at ovulation than during oral contraceptive use indicates a difference in the effects of sex hormones on the heart. Contrary to the general assertion that oral contraceptive use increases blood pressure it seems likely that the presence of progesterone blunts the pressor effects of oestrogen alone. This corresponds with data from animal studies: Eikelis and van den Buuse (2000) found that female SHR had greater pressor and tachycardic responses to an open-field test at dioestrus than at any other phase of the oestrus cycle. Oestrogen administration had no effect on

this stress response while oestrogen and progesterone administration attenuated the blood pressure and heart rate elevation.

West *et al.* (2001) compared cardiovascular reactivity to smoking as well as oral contraceptive use in healthy females during a mental stress task. The results showed that in oral contraceptive non-users there was no effect of smoking status or nicotine administration on the response. However among oral contraceptive users non-smokers had greater heart rate and diastolic pressure reactivity to stress. It has also been found in response to mental stress smokers have lower heart rates than non-smokers (Straneva *et al.*, 2000). This concurs with the lower myocardial reactivity observed in smokers in the present study (see Tables 3.3-3.5). It has also been found that greater blood pressure and TPR responses to stress occur in women who smoke and use oral contraceptives than smokers who do not use oral contraceptives (Davis, 1999). Emmons and Weidner (1988) found that oral contraceptive users produced similar increases in heart rate and diastolic blood pressure in response to mental stress when compared with non-users and that systolic pressure was marginally elevated in this group. In the present study the magnitude of increase in MAP in response to mental stress was similar in oral contraceptive users and non-users. Oral contraceptive non-users at menses however had elevated heart rate responses to mental stress when compared with oral contraceptive users at withdrawal.

TPR although demonstrating a trend towards a decrease remained on the whole unchanged during mental stress (see Table 3.4). There was no difference in the magnitude of response between smokers and non-smokers. This is consistent with the fact that most of the vasculature undergoes vasoconstriction except for the skeletal muscle where vasodilatation occurs (Brod, 1970). This provides limited evidence that vasodilation via adrenaline release from the adrenal medulla on SNA could cause skeletal muscle vasodilation by binding with β_2 adrenoreceptors (Joyner and Halliwill, 2000). FBF has previously been found to increase during mental stress (Brod, 1970) via sympathetic withdrawal and ß-adrenergic vasodilation (Halliwill *et al.*, 1997).

While mental stress produces β-adrenergic receptor activity the cold pressor test primarily evokes α-adrenergic receptor activity (Girdler *et al.*, 1993). Cold pressor testing has been found to increase plasma adrenaline, heart rate, systolic pressure and cardiac output (Halter *et al.*, 1984). In the present study there was no

difference in MAP during the cold pressor test in either group at menses but at ovulation an increase was observed by 60 s (see Figure 3.12). When comparing values obtained from males to those of females at menses the following was the case (male data from O' Sullivan and Bell, 2001) (Males: 85 ± 1.7 cf 103 ± 4.2 mmHg (n=8; mean ± SEM); female non-smokers: 76 ± 1.7 cf 77 ± 2 mmHg, smokers: 78 ± 2.4 cf 81 ± 6.6 mmHg). These data show that there is a significant elevation in MAP in healthy young men in response to the cold pressor test, which is not evident in females at menses. Whether this difference is due to the presence of sex hormones or differences in thermoregulation between the genders is unclear. Thermoregulation in response to cold will be discussd more fully in the General Discussion. Systolic pressure was increased by the cold pressor test and this appears to be similar to other studies where responses in females and males were analysed (LeBlanc *et al.*, 1978). LeBlanc and colleagues found in that study that while systolic pressure in males increases throughout 2 minutes of cold pressor testing in females the response was at its maximum at 1 minute. The results of the present study would concur.

There was an increase in MAP at ovulation in both groups in the present study indicating that blood pressure responses to the cold pressor test are dependent on cycle phase (see Figure 3.12 D). When looking at the magnitude of MAP increase the elevation in non-smokers was significantly greater at ovulation than menses but did not reach statistical significance for smokers. This result is in contradiction to those of Hastrup and Light (1984) who found that responses elicited by the cold pressor test are independent of cycle phase. Ettinger *et al.* (1998) found no difference between muscle SNA responses during the cold pressor test at either menses or ovulation. Tersman *et al.* (1991) found however that the blood pressure response to the cold pressor test was elevated at the luteal phase when compared with the follicular phase. This could be due to elevated oestrogen levels during the luteal phase. Of course the possibility of an oestrogen-progesterone interaction cannot be ruled out. Women have been found to have greater α_1-adrenergic vasoconstriction during the luteal than the follicular phase and increased α_2 vasoconstriction during the follicular phase (Freedman and Girgis, 2000). In the present study an increase in blood pressure was found at ovulation only and perhaps is due to changes in α-adrenergic receptor density around this time. Also oestrogens and progesterones alter thermoregulation in humans (Carpenter and Nunneley, 1988; Brooks *et al.*, 1997), which will be discussed more fully in the General Discussion.

Oral contraceptive users however demonstrated increased MAP at withdrawal and during OC use. However no difference in the absolute magnitude of increase was observed between the groups. This is in agreement with the results of Emmons and Weidner (1988) who found no difference in the response of oral contraceptive users and non-users to the cold pressor test.

Heart rate was seen to increase in non-smokers at both stages of the cycle, which was not the case in smokers indicating increased cardiac reactivity as compared with smokers. This is in line with females being cardiac hyperreactors and if smoking is anti-oestrogenic female smokers may react similarly to males. Johansson and Hjalmarson (1988) found that the myocardium could have greater adrenergic activity in females than males. Menstrual cycle phase has been shown to have an effect on reactivity to the cold pressor test in smokers during the follicular and luteal phases (Tersman *et al.*, 1991). However in the present study the absolute magnitude of change in heart rate was not significantly different between the groups. LeBlanc *et al.* (1978) found that there was a reduction in the responses to the cold pressor test in physically trained individuals but this could not play a part in this study since both groups had similar VO_{2peak}.

Conclusions

Resting blood pressure in young women is elevated by moderate cigarette consumption and is most marked at ovulation indicating a possible interaction between the anti-oestrogenic effect of smoking and blood pressure. Female smokers and oral contraceptive users display somewhat blunted cardiovascular responsiveness to stress when compared with premenopausal non-smokers. Menstrual cycle phase, oral contraceptive use and smoking habits should be taken into account when assessing sympathetic activation.

Chapter 4 'Effects of hormonal status, age and smoking on sympathetic activation in healthy females'

4.1 Introduction

Hypertension is one of the leading causes of death in women. At menopause the incidence of hypertension is dramatically increased suggesting a lowering effect of female sex hormones on blood pressure. Menopause is associated with dramatic changes in haemodynamic variables including increased blood pressure and TPR and decreased cardiac output (Staessen *et al.*, 1998). Responses to these changes involve various adaptations in the cardiovascular system partly via the autonomic nervous system. Parasympathetic as well as SNA is modulated by oestrogen (Saleh and Connell, 2000).

Studies concerning the effects of female sex hormone replacement on CVD have produced varied results. Some studies indicate that oestrogen replacement therapy decreases the risk of cardiovascular death in postmenopausal women (Grodstein *et al.*, 2000). In the Framingham study, early menopause was associated with a 4-fold increase in CVD (Wilson *et al.*, 1985). A 2-fold increase in the risk of coronary heart disease has been found in young women who undergo surgical menopause without using HRT, however this is not the case in women using hormones (Colditz *et al*, 1987). Some evidence suggests that oestrogen replacement therapy can improve coronary risk factors (Barrett-Connor *et al.*, 1997). On the contrary the Heart Estrogen/Progestin Replacement Study (HERS) found no significant evidence of a clinical benefit of hormonal therapy in women with established coronary artery disease (Hulley *et al.*, 1998). Recent data from the Women's Health Initiative (Rossouw *et al.*, 2002) support the HERS trial by indicating increased rates of stroke, myocardial infarction, coronary heart disease and thrombosis among women taking HRT as compared with placebo.

The influence of female hormones on autonomic nervous system control could elucidate the relationship between CVD and the menopause. Some investigators have reported oestrogen-induced reductions in basal and stress-induced sympathetic tone (Rosano *et al.*, 1997; Del Rio *et al.*, 1998) whereas others have reported no effect on cardiovascular responses to stress (Snabes *et al.*, 1996).

Elevated cardiovascular and neuroendocrine responses to stress have been found in postmenopausal women relative to premenopausal women (Saab *et al.*, 1989; Owens *et al.*, 1993) while those using HRT have lower systolic pressure and pulse pressure responses to stress (Matthews *et al.*, 2001). HRT has been found to reduce cardiovascular responsiveness to mental stress in postmenopausal women with risk factors for coronary heart disease (McCubbin *et al.*, 2002) and decreased vascular resistance and MAP has been observed with treatment in postmenopausal women (Light *et al.*, 2001; Meyer *et al.*, 2001). Menozzi *et al.* (2000) found that oestradiol administration to postmenopausal women blunted cardiovascular responsiveness to the cold pressor test.

It has also been reported that smokers have a higher sympathetic activation to stress than non-smokers (Pomerleau and Pomerleau, 1989) and cigarette smoking is an independent risk factor for CVD. Smoking appears to be a greater cardiovascular risk factor in women than in men (Njølstad *et al.*, 1996) and has been implicated in various female-specific diseases (Seltzer, 2003). Smoking has anti-oestrogenic effects and HRT in postmenopausal women has been reported to attenuate the deleterious effects on arterial compliance and plasma lipids that accompany smoking (Teede *et al.*, 1999). Women who smoke have earlier menopause onset than non-smokers (McKinlay *et al.*, 1985). This means female smokers could develop CVDs including hypertension earlier than non-smokers. In addition it seems likely that the deleterious effects of smoking could be due in part to the fact that smoking itself elevates blood pressure. This involves several factors, including increased sympathetic vasomotor and cardiac tone (Cryer *et al.*, 1976; Narkiewicz *et al.*, 1998; Benck *et al.*, 1999), decreased BRS (Gerhardt *et al.*, 1999) and loss of endothelium-dependent vasodilator function due to endothelial damage (Celermajer *et al.*, 1993; Zeiher *et al.*, 1995; Lind *et al.*, 2003).

Ageing also has various implications for CVD. This is likely a result of age-related changes in the structure of the cardiovascular vessels as well as cardiovascular autonomic control. In white men, MAP rises evenly over the first four decades of adulthood but, in white women, the rate of rise increases markedly after the age of 40 years (Burt, 1995), suggesting that hormonal status may contribute to blood pressure stability. Ageing is associated with reduced vagal responsiveness and a tendency towards reliance on sympathetic control of cardiac responses (Uchino *et al.*, 1999) and this situation appears to be similar in men and

women (Uchino *et al.*, 1999). Increased pressor responses to mental stress have been found with age (Becker *et al.*, 1996) and Jennings *et al.* (1997) found that cardiac and vascular reactivity to stress was elevated with increasing age and hypertension.

With this in mind the aim of the present study was to investigate whether ageing, HRT and smoking in postmenopausal women can influence the cardiovascular system by exerting effects on the sympathoadrenal response to stress.

4.2 Methods

4.2.1 Subjects

Healthy non-smoking postmenopausal women (n=10), postmenopausal women on Hormone Replacement Therapy (HRT) (n=7) and postmenopausal smokers (n=7) were recruited into the study.

4.2.2 Experimental protocol

Subjects were required to attend the laboratory on two separate occasions. Session one involved a medical examination, including the establishment of cholesterol levels, followed by a maximal oxygen consumption incremental exercise test to quantify fitness levels. On this same occasion subjects were then familiarised with the experimental procedure for the stress tests. The second session involved each subject reporting to the laboratory for sympathetic activation testing.

4.2.3 Maximal fitness testing

Subjects performed a standard incremental fitness test on a cycle ergometer to establish peak oxygen consumption. All subjects (> 45 years) were subject to 12-lead electrocardiography prior to fitness testing and to continuous electrocardiographic and visual monitoring during the test by a medical doctor.

4.2.4 Sympathetic activation testing

Each subject was seated comfortably in an upright position and performed a sustained handgrip to fatigue, a 2 minute mental arithmetic test and a 2 minute cold pressor test (described in detail in Chapter 2). During the tests heart rate was measured using a three lead frontal ECG, blood pressure was measured continuously using applanation tonometry, stroke volume was assessed using impedance cardiography and FBF was measured by venous occlusion plethysmography. From these parameters, MAP, pulse pressure, stroke index, cardiac index, rate-pressure product, TPR and forearm conductance were calculated.

4.2.5 Statistical analysis

Paired and unpaired Student's *t*-tests were used in the analyses of within group and between group results, respectively. All data are expressed as mean ± S.E.M. $P< 0.05$ was considered significant.

4.3 Results

4.3.1 Subject characteristics

Physical characteristics of postmenopausal women, postmenopausal women taking HRT and postmenopausal smokers are shown in Table 4.1. Subjects in each group were similar as there were no significant differences in age, height, body mass or BMI. All groups had similar cholesterol and fitness levels. The women were postmenopausal for a similar duration of time. Smokers smoked 23 ± 5.2 cigarettes.day^{-1} with a range of 8-40 day^{-1} for an average of 30 ± 3.9 years, which was a mean of 39 ± 12.2 pack years.

4.3.2 Resting cardiovascular parameters

There were no significant differences in resting cardiovascular parameters (heart rate, diastolic pressure, systolic pressure, MAP, pulse pressure, stroke index, rate-pressure product, FBF, forearm conductance or TPR) between postmenopausal non-smokers and smokers nor between postmenopausal HRT users and non-users (see Table 4.2). Cardiac index was significantly lower in postmenopausal smokers than in non-smokers (see Table 4.2).

4.3.3 Cardiovascular reactivity

Sustained handgrip

There was no significant difference in the time to fatigue for the sustained handgrip test between postmenopausal women, postmenopausal women on HRT or postmenopausal smokers (106 ± 11.8, 104 ± 9 and 110 ± 11.2 s, respectively).

The MAP, pulse pressure and diastolic and systolic blood pressure responses to a sustained handgrip were similar between postmenopausal smokers and non-smokers (see Figure 4.1 A and Table 4.3). Blood pressure increased at all time points throughout the exercise. There was no difference between the magnitudes of blood pressure increase between the two groups (25 ± 7.0 cf 26 ± 7.1 Δ mmHg).

At all fatigue percentages heart rate was increased in both postmenopausal smokers and non-smokers (see Figure 4.1 B). The absolute magnitude of change in heart rate was similar between the groups (8 ± 2.8 cf 8 ± 1.8 Δ bpm). Cardiac index was elevated by a sustained handgrip in both groups but only significantly so in smokers (see Table 4.3). Forearm vascular conductance was decreased in non-

smokers whereas smokers showed no difference in forearm conductance values during the test (see Figure 4.1 C). However no significant differences were found between the groups. TPR was unaltered by sustained handgrip in both groups (see Table 4.3).

There was no difference in MAP, diastolic pressure or systolic pressure responses to a sustained handgrip in postmenopausal HRT users and non-users (see Figure 4.2 A and Table 4.3). Both groups demonstrated increased pressures at all fatigue percentages. The absolute magnitude of change in MAP was 26 ± 7.1 cf 23 ± 6.2 Δ mmHg in postmenopausal women and those taking HRT, respectively. However while non-users demonstrated an increase in pulse pressure at each fatigue percentage, women on HRT did not show any alteration in pulse pressure throughout the duration of the test (see Table 4.3). Heart rate was elevated at all fatigue percentages in non-smokers and increased until 50% fatigue in HRT users (see Figure 4.2 B). The absolute magnitude of change in heart rate with a sustained handgrip was not different between the postmenopausal HRT users and non-users (13 ± 5.9 cf 8 ± 1.8 Δ bpm). Cardiac index was not affected by a sustained handgrip and did not change from resting values in either HRT users or non-users (see Table 4.3 and Figure 4.2 C). Forearm vascular conductance was unchanged by sustained handgrip in HRT users, as was TPR (see Table 4.3). However when looking at the absolute change in TPR postmenopausal HRT users demonstrated a tendency towards lower responses than postmenopausal HRT non-users (-4.9 ± 4.83 cf 7.2 ± 4.40 Δ $(L.min^{-1}).mmHg^{-1}$, $P= 0.09$).

Mental Arithmetic

Mental arithmetic was accompanied by an increase in MAP in both non-smokers and smokers (see Figure 4.3 A). When observing the absolute magnitude of change in MAP smokers had a significantly greater responsiveness than did non-smokers (20 ± 5.3 cf 7 ± 3.3 Δ mmHg, $P<0.05$). Although there was a trend towards an increase in pulse pressure in both groups this increase did not quite reach significance (see Table 4.4). Systolic pressure was elevated significantly by mental arithmetic in both smokers and non-smokers (see Table 4.4). In non-smokers a trend towards an elevation of diastolic pressure was evident but did not quite reach significance while in smokers a similar trend was evident (see Table 4.4). When looking at the absolute magnitude of change in diastolic pressure in response to

mental stress older smokers showed a significantly greater increase in pressure than did non-smokers 6 ± 2.6 cf 17 ± 4.6 Δ mmHg. Systolic pressure followed a similar trend which was not quite significantly different: 11 ± 4.6 cf 26 ± 7.5 Δ mmHg, P=0.086. Heart rate was elevated in both groups (see Figure 3.3 B). No difference between the magnitude of response was observed between non-smokers and smokers (7 ± 2.6 cf 8 ± 2.4 Δ bpm). Cardiac index was elevated during the test (Figure 4.3 C). There was no difference in the change in cardiac index from rest between smokers and non-smokers (0.5 ± 0.16 cf 0.4 ± 0.12 Δ $L.min^{-1}.m^{-2}$). Mental arithmetic did not alter forearm vascular conductance (see Table 4.4). A trend towards a decrease in TPR was evident in non-smokers and smokers (see Figure 4.3 D). There was no difference between the absolute change in TPR between the groups (-5.0 ± 2.63 cf -1.2 ± 1.96 Δ $(L.min^{-1}).mmHg^{-1}$).

MAP and systolic blood pressure were increased during mental arithmetic in HRT users and postmenopausal HRT non-users showed a similar pattern (see Figure 4.4 A and Table 4.4). The magnitude of change in MAP from rest was not different between the groups (16 ± 6.3 cf 7 ± 3.3 Δ mmHg). An increase in diastolic blood pressure was evident in HRT users (see Table 4.4) and heart rate was also elevated in both groups (see Figure 4.4 B). There was no difference in the absolute change in heart rate between postmenopausal HRT users and non-users (10 ± 0.9 cf 7 ± 2.6 Δ bpm). Pulse pressure was increased significantly by mental stress in HRT users (see Table 4.4). While cardiac index was increased in postmenopausal non-HRT users no such increase was evident in HRT users (see Table 4.4 and Figure 4.4 C). However no difference was observed in the magnitude of cardiac index increase between the groups (0.2 ± 0.27 cf 0.4 ± 0.12 Δ $L.min^{-1}.m^{-2}$). Forearm vascular conductance was unaffected by mental arithmetic in both groups (see Table 4.4). Mental stress had no effect on TPR in the HRT cohort although a trend was visible for a reduction in this parameter in the postmenopausal HRT non-users (see Figure 4.4 D). No difference was observed however between the absolute magnitude of change between the two groups (-0.7 ± 2.27 cf –5.0 ± 2.63 Δ $(L.min^{-1}).mmHg^{-1}$).

Cold pressor test

MAP, pulse pressure, systolic and diastolic pressure were unaffected by exposure to the cold pressor test in smokers (see Figure 4.5 A and Table 4.5). Non-smokers however demonstrated an increase in MAP at 30 s exposure while pulse

pressure and systolic pressure demonstrated a trend towards an increase (see Figure 4.5 A and Table 4.5). No difference was observed between the absolute magnitude of MAP change in smokers and non-smokers (4 ± 3.8 cf 3 ± 2.4 Δ mmHg). Heart rate was unaltered by this test (see Table 4.5). Forearm conductance, TPR and cardiac index (see Table 4.5) were unaffected by cold exposure and responses were not different between the groups.

There were no significant differences between the increased MAP in response to the cold pressor test in either the HRT users or postmenopausal non-HRT users (see Figure 4.5 B). Absolute changes in the postmenopausal HRT non-users and postmenopausal HRT users were 3 ± 2.4 cf 13 ± 6.0 Δ mmHg. While pulse pressure was increased in the non-HRT users, women on HRT showed no change in this parameter (see Table 4.5). Heart rate and cardiac index were unchanged by cold exposure (see Table 4.5). While forearm vascular conductance and TPR were unchanged in postmenopausal non-HRT users, HRT users demonstrated a significant decrease in conductance at 60 s (see Table 4.5).

Table 4.1 Physical characteristics of subjects.

Postmenopausal women n=10, postmenopausal women taking HRT n=7, postmenopausal smokers n=7. Data are expressed as mean ± S.E.M.

	Postmenopausal	Postmenopausal + HRT	Postmenopausal smokers
Age (years)	57 ± 1.9	58 ± 2.0	53 ± 2.7
Years postmenopause	8 ± 1.6	10 ± 1.1	10 ± 2.9
Years on HRT	-	10 ± 1.4	-
Height (cm)	164 ± 2.3	161 ± 3.2	165 ± 2.4
Body Mass (kg)	70 ± 2.7	63 ± 4.6	72 ± 6.6
BMI ($kg.m^{-2}$)	26 ± 1.3	24 ± 1.3	26 ± 1.7
Cholesterol ($mmol.l^{-1}$)	5.4 ± 0.38	5.3 ± 0.49	5.6 ± 0.59
VO_2peak ($(ml.min^{-1}).kg^{-1}$)	21 ± 1.5	24 ± 2.9	22 ± 3.3

Table 4.2 Resting cardiovascular characteristics of subjects.

Postmenopausal women n=10, postmenopausal women taking HRT n=7, postmenopausal smokers n=7. Data are expressed as mean ± S.E.M.

	Postmenopausal	Postmenopausal + HRT	Postmenopausal smokers
Heart Rate ($beat.min^{-1}$)	83 ± 4.3	74 ± 3.7	74 ± 3.3
Diastolic pressure (mmHg)	74 ± 4.2	69 ± 3.1	68 ± 2.9
Systolic pressure (mmHg)	126 ± 5.5	125 ± 3.1	112 ± 2.6
MAP (mmHg)	91 ± 4.0	88 ± 2.5	82 ± 2.6
Pulse pressure (mmHg)	52 ± 4.8	56 ± 3.9	44 ± 1.9
TPR $(L.min^{-1}).mmHg^{-1}$	31.6 ± 3.66	32.2 ± 4.05	36.6 ± 3.43
Stroke index $(ml.m^{-2})$	23 ± 3.7	27 ± 2.9	22 ± 2.9
Cardiac index $((l.min^{-1}).m^{-2})$	1.8 ± 0.22	1.9 ± 0.35	1.3 ± 0.19*
RPP $((mmHg.beats.min^{-1})^{-2})$	106 ± 10.0	93 ± 4.3	83 ± 3.2
Forearm blood flow $(ml.min^{-1}).100ml^{-1}$	5.4 ± 1.72	3.6 ± 0.64	4.7 ± 0.77
Forearm conductance $((ml.min^{-1}).100ml^{-1}.mmHg^{-1})$	0.06 ± 0.021	0.04 ± 0.012	0.06 ± 0.011

* denotes a significant difference between postmenopausal non-smokers and smokers ($P<0.05$).

Table 4.3 Cardiovascular parameters before and during sustained handgrip (100% fatigue). Postmenopausal women n=10, postmenopausal women taking HRT n=7, postmenopausal smokers n=7. Data are expressed as mean ± S.E.M.

	Postmenopausal		Postmenopausal + HRT		Postmenopausal smokers	
	before	during	before	during	before	during
MAP (mmHg)	91±4.0	118±9.8**	88±2.5	111±4.6*	82±2.6	107±6.4*
DBP (mmHg)	74±4.2	95±9.3**	69±3.1	91±4.8*	68±2.9	87±6.0*
SBP (mmHg)	126±6.2	163±12.1**	125±3.1	152±6.0***	112±3.5	146±8.2**
Pulse pressure (mmHg)	52±4.8	68±6.4*	56±3.9	62±5.2	44±1.9	58±4.4*
Heart rate (bpm)	83±4.3	91±5.2**	74±3.2	87±5.1	74±2.3	82±3.8*
Stroke index ($ml.m^{-2}$)	23±3.7	23±2.5	27±2.9	17±1.7	22±2.9	25±3.0
Cardiac index ($(l.min^{-1}).m^{-2}$)	1.8±0.21	2.1±0.32	1.9±0.33	2.4±0.31	1.3±0.11	1.8±0.21*
RPP ($(mmHg.beats.min^{-1})^{-2}$)	103±8.1	155±18.2*	93±4.3	134±11.4*	83±3.3	113±10.4*
TPR ($mmHg.min.l^{-1}$)	32.2±3.61	39.2±6.36	32.3±4.02	27.2±3.93	37.4±3.41	36.5±4.90
FVC ($(ml.min^{-1}).100ml^{-1}.mmHg^{-1}$)	0.06±0.02	0.07±0.04	0.04±0.01	0.03±0.01	0.06±0.01	0.05±0.02

* denotes a significant difference between before and during values within the one group ($P<0.05$, $**P<0.01$, $***P<0.001$).

Table 4.4 Cardiovascular parameters before and during mental stress (60s). Postmenopausal women n=10, postmenopausal women taking HRT n=7, postmenopausal smokers n=7. Data are expressed as mean ± S.E.M.

	Postmenopausal		Postmenopausal + HRT		Postmenopausal smokers	
	before	during	before	during	before	during
MAP (mmHg)	92±5.0	99±4.8	87±2.8	103±5.2*	83±4.3	102±5.2**
DBP (mmHg)	76±5.0	81±5.1	68±3.2	80±4.2	66±4.6	82±4.9*
SBP (mmHg)	124±6.0	134±5.7*	125±3.3	149±7.8*	117±4.0	143±7.6*
Pulse pressure (mmHg)	48±4.1	53±4.9	57±3.7	69±4.8*	51±2.3	60±6.2
Heart rate (bpm)	79±3.0	85±3.6*	71±3.4	80±3.5***	71±1.5	80±2.8*
Stroke index ($ml.m^{-2}$)	22±1.8	18±1.0	25±3.4	28±2.6	24±1.4	20±0.8
Cardiac index ($(l.min^{-1}).m^{-2}$)	1.4±0.13	1.9±0.24*	1.9±0.26	2.1±0.47	1.5±0.14	1.9±0.13*
RPP ($(mmHg.beats.min^{-1})^{-2}$)	98±8.0	115±8.2	88±4.6	119±6.1*	83±3.3	115±9.3*
TPR ($mmHg.min.l^{-1}$)	37±1.6	32±2.7	28±7.1	28±5.6	31±2.1	30±3.1
FVC ($(ml.min^{-1}).100\ ml^{-1}.mmHg^{-1}$)	0.05±0.01	0.05±0.01	0.05±0.01	0.05±0.01	0.06±0.02	0.05±0.01

* denotes a significant difference between before and during values within the one group ($P<0.05$, $**P<0.01$, $***P<0.001$).

Table 4.5 Cardiovascular parameters before and during the cold pressor test (60s). Postmenopausal women n=10, postmenopausal women taking HRT n=7, postmenopausal smokers n=7. Data are expressed as mean ± S.E.M.

	Postmenopausal		Postmenopausal + HRT		Postmenopausal smokers	
	before	during	before	during	before	during
MAP (mmHg)	88±4.5	90±3.4	88±3.5	101±5.8	84±2.4	88±2.3
DBP (mmHg)	71±4.5	72±3.1	67±4.0	78±4.7	68±3.0	71±2.0
SBP (mmHg)	122±5.8	128±5.9	133±5.0	146±8.6	114±1.3	121±4.0
Pulse pressure (mmHg)	51±4.3	57±5.5*	67±5.7	68±4.9	46±1.9	49±3.5
Heart rate (bpm)	77±2.7	77±1.3	70±3.8	74±2.8	74±3.1	72±2.0
Stroke index ($ml.m^{-2}$)	13±0.9	14±1.3	16±2.7	13±2.0	13±1.9	14±1.9
Cardiac index ($(l.min^{-1}).m^{-2}$)	1.6±0.12	1.8±0.23	2.1±0.41	1.9±0.32	1.5±0.22	1.6±0.22
RPP ($(mmHg.beats.min^{-1})^{-2}$)	95±6.3	99±4.7	93±5.3	108±7.4	84±3.4	87±3.6
TPR ($mmHg.min.l^{-1}$)	32.1±1.6	31.3±2.7	26.4±3.4	34.3±4.5	37.2±6.7	34.1±4.1
FVC ($(ml.min^{-1}).100\ ml^{-1}.mmHg^{-1}$)	0.04±0.01	0.04±0.01	0.04±0.01	0.03±0.01*	0.05±0.01	0.03±0.01

* denotes a significant difference between before and during values within the one group ($P<0.05$).

Figure 4.1 Effect of a sustained handgrip on MAP, heart rate and forearm vascular conductance in postmenopausal smokers and non-smokers.

A. MAP was elevated during a sustained handgrip in postmenopausal smokers and non-smokers. B. A sustained handgrip elevated heart rate in both postmenopausal smokers and non-smokers. C. Forearm vascular conductance was decreased in non-smokers during a sustained handgrip. (non-smokers: n=10, smokers n=7; */§ $P<0.05$, **/§§ $P<0.01$ in non-smokers and smokers, respectively, Student's *t*-test). Data represents mean ± S.E.M.

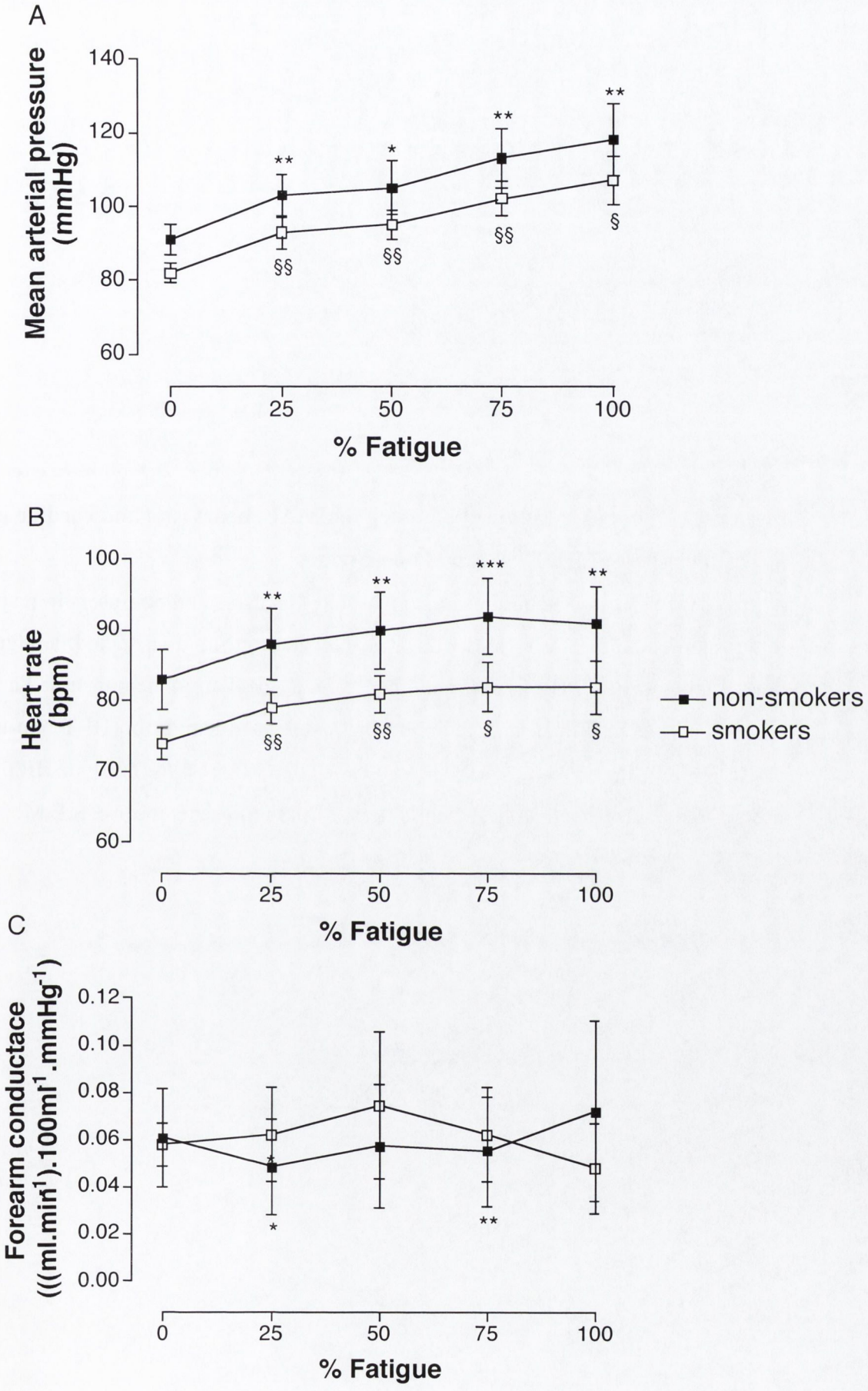

A
Mean arterial pressure (mmHg)
140
120
100
80
60
**
*
**
**
§§
§§
§§
§
0
25
50
75
100
% Fatigue
B
Heart rate (bpm)
100
90
80
70
60
**
**
***
**
§§
§§
§
§
non-smokers
smokers
0
25
50
75
100
% Fatigue
C
Forearm conductace (((ml.min-1).100ml-1.mmHg-1)
0.12
0.10
0.08
0.06
0.04
0.02
0.00
*
**
0
25
50
75
100
% Fatigue

Figure 4.2 Effect of a sustained handgrip on MAP, heart rate and cardiac index in postmenopausal HRT users and non-users.

A. MAP was elevated in both postmenopausal HRT users and non-users in response to a sustained handgrip. B. Heart rate was elevated during a sustained handgrip in both postmenopausal HRT users and non-users. C. HRT users demonstrated a trend towards an increase in cardiac index during a sustained handgrip. (HRT non-users: n=10, HRT users n=7; */§ $P<0.05$, **/§§ $P<0.01$, ***/§§§ $P<0.001$ in HRT non-users and users, respectively, Student's *t*-test). Data represents mean ± S.E.M.

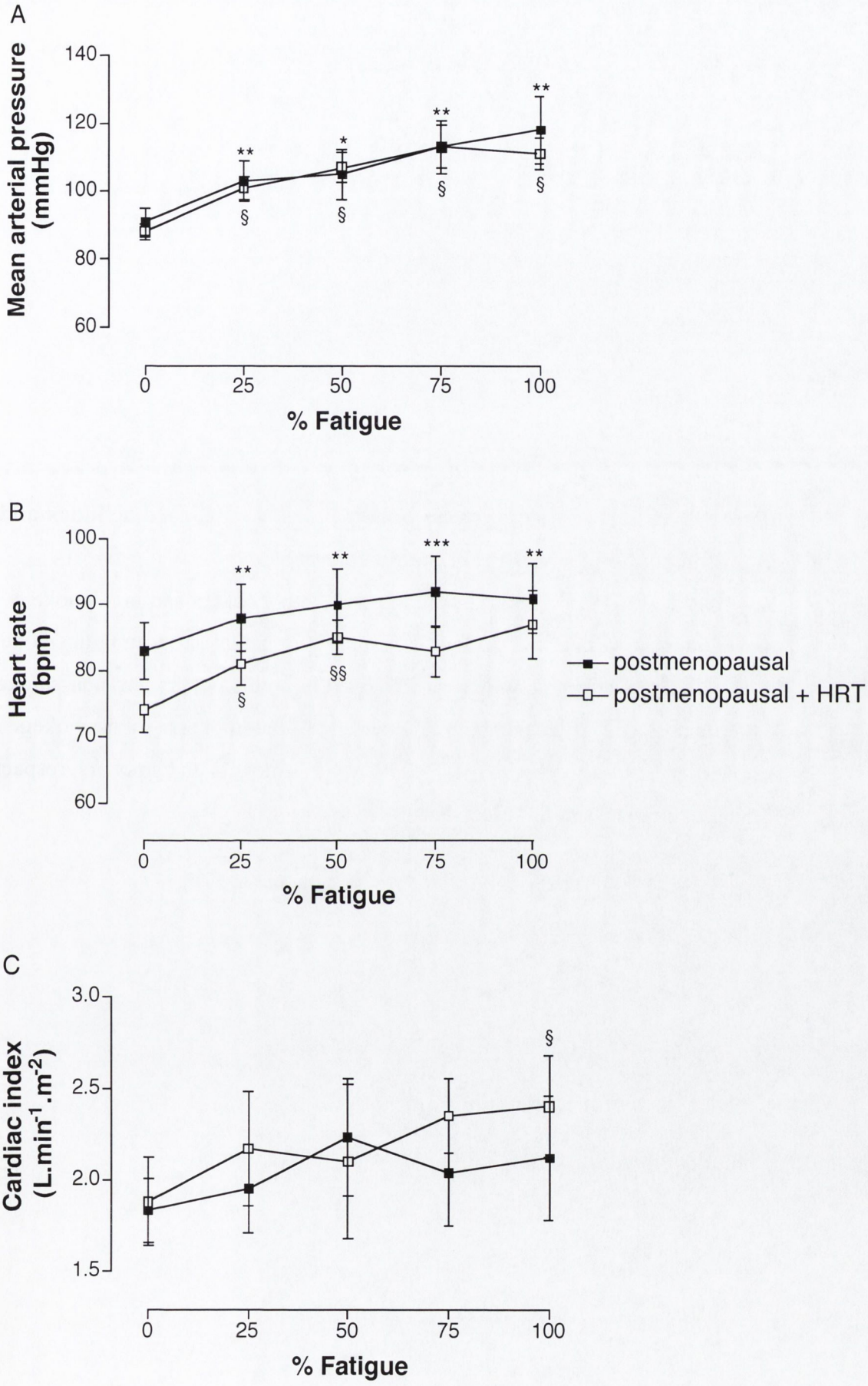

A
Mean arterial pressure (mmHg)
140
120
100
80
60
**
*
**
**
§
§
§
§
0
25
50
75
100
% Fatigue
B
Heart rate (bpm)
100
90
80
70
60
**
**
***
**
§
§§
0
25
50
75
100
% Fatigue
postmenopausal
postmenopausal + HRT
C
Cardiac index (L.min-1.m-2)
3.0
2.5
2.0
1.5
§
0
25
50
75
100
% Fatigue

Figure 4.3 Effect of mental stress on MAP, heart rate, cardiac index and TPR in postmenopausal smokers and non-smokers.

A. MAP was increased with mental stress in both smokers and non-smokers. B. An elevation in heart rate was evident in response to mental stress in both smokers and non-smokers. C. Cardiac index was elevated in both smokers and non-smokers. D. TPR tended to decrease in both smokers and non-smokers. (non-smokers: n=10, smokers n=7; */§ $P<0.05$, **/§§ $P<0.01$ in non-smokers and smokers, respectively, Student's *t*-test). Data represents mean ± S.E.M.

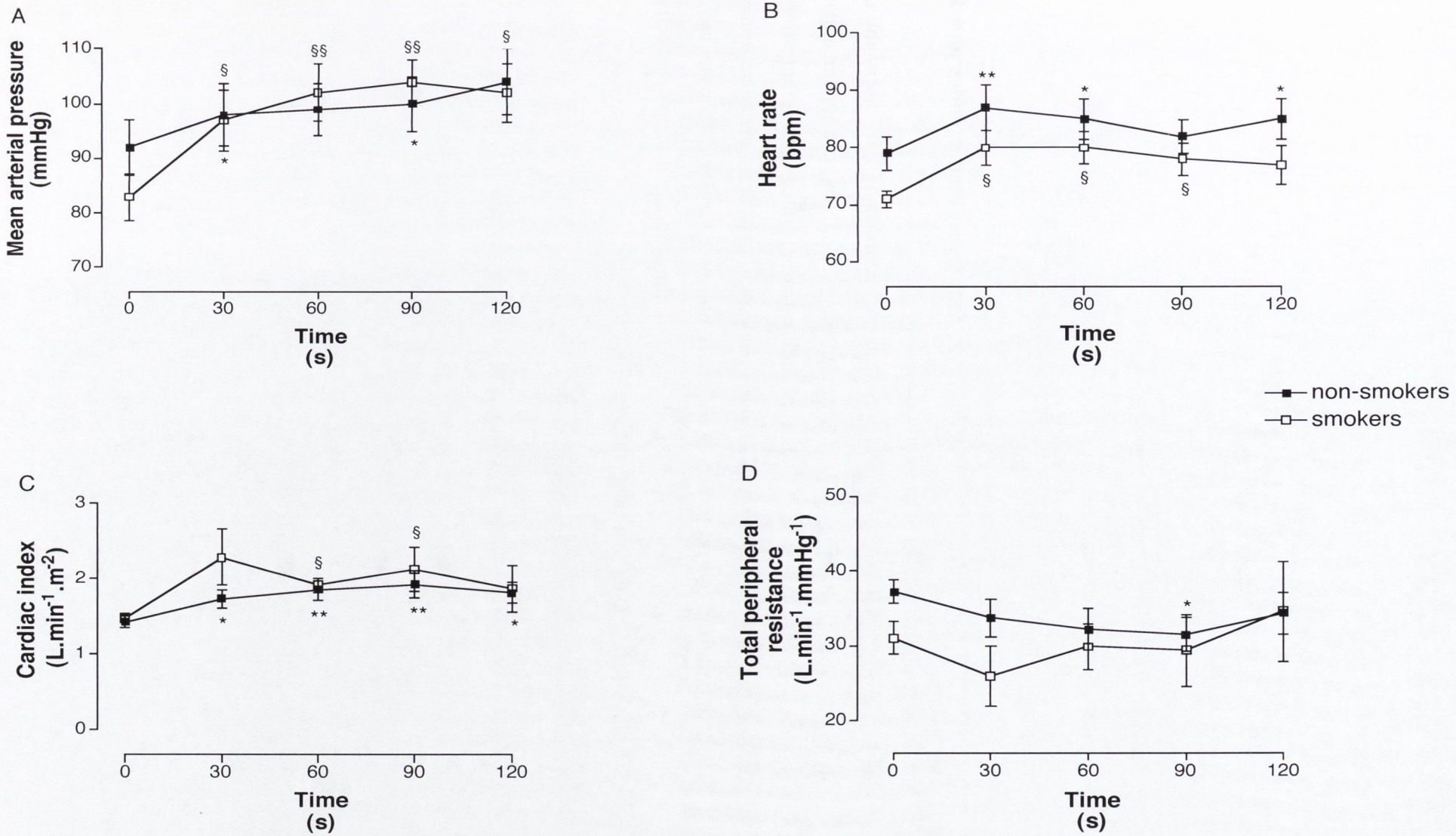

A
Mean arterial pressure (mmHg)
110
100
90
80
70
§
§§
§§
§
*
*
0
30
60
90
120
Time (s)
B
Heart rate (bpm)
100
90
80
70
60
**
*
*
§
§
§
0
30
60
90
120
Time (s)
non-smokers
smokers
C
Cardiac index (L.min-1.m-2)
3
2
1
0
§
§
*
**
**
*
0
30
60
90
120
Time (s)
D
Total peripheral resistance (L.min-1.mmHg-1)
50
40
30
20
*
0
30
60
90
120
Time (s)

Figure 4.4 Effect of mental stress on MAP, heart rate, cardiac index and TPR in postmenopausal HRT users and non-users.

A. MAP was significantly increased in both postmenopausal HRT users and non-users. B. Mental stress caused an elevation in heart rate in both postmenopausal HRT users and non-users. C. Cardiac index was elevated by mental stress in postmenopausal non-HRT users. D. TPR tended to decrease in response to mental stress in both HRT users and non-users. (HRT non-users: n=10, HRT users n=7; */§ $P<0.05$, */§ $P<0.01$, ***/§§§ $P<0.001$ in HRT non-users and users, respectively, Student's *t*-test). Data represents mean ± S.E.M.

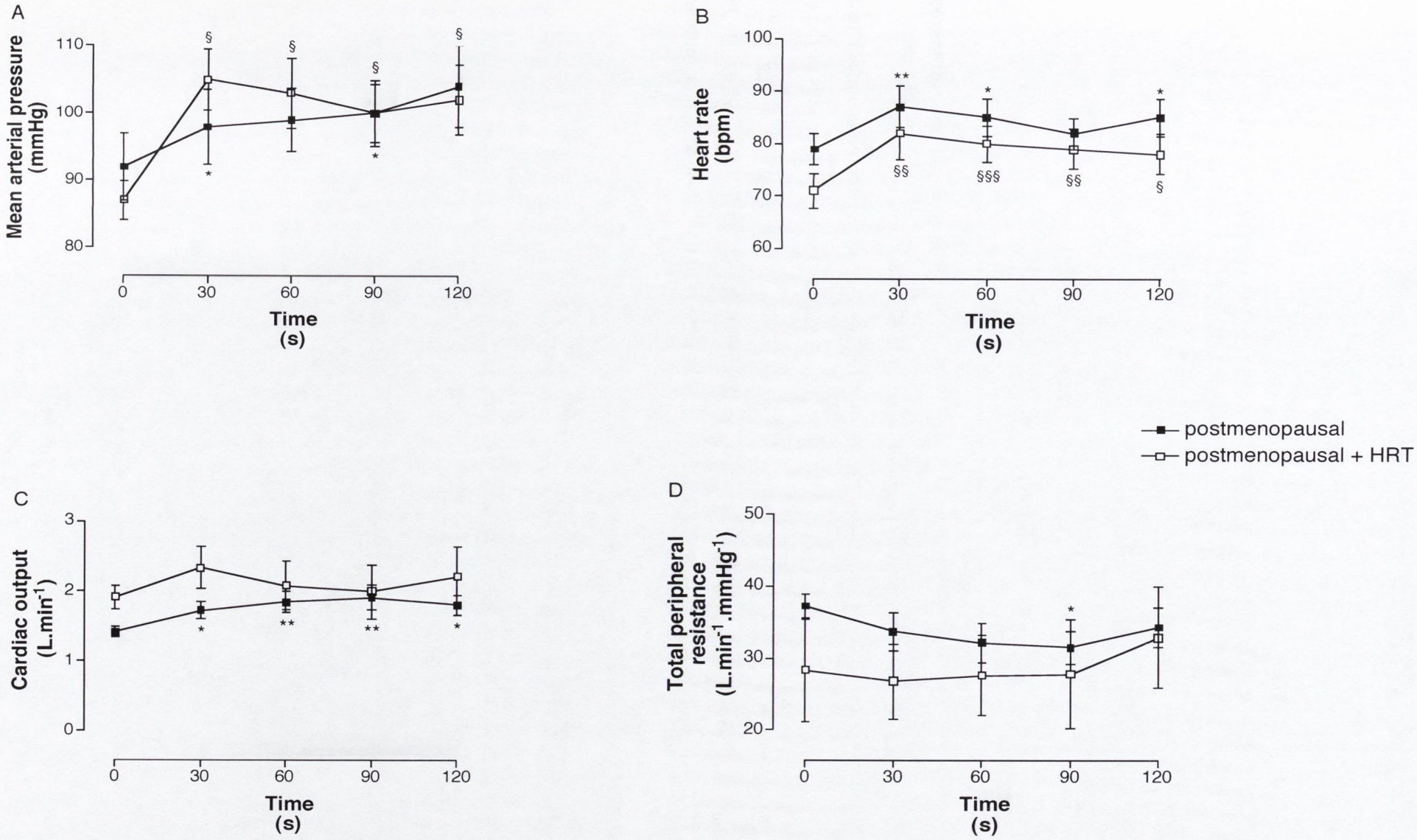
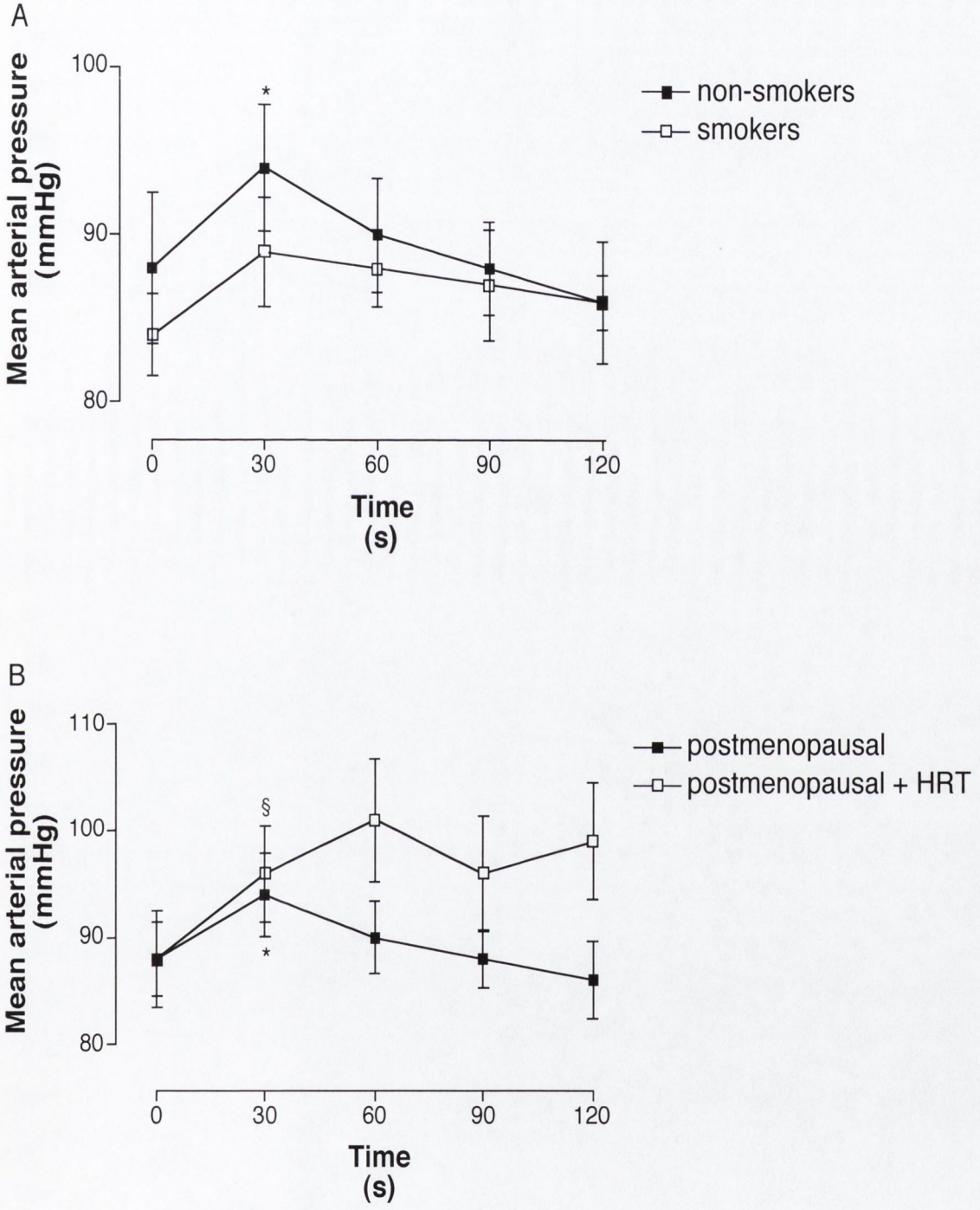

A
Mean arterial pressure (mmHg)
110
100
90
80
§
§
§
§
*
*
0
30
60
90
120
Time (s)
B
Heart rate (bpm)
100
90
80
70
60
**
*
*
§§
§§§
§§
§
0
30
60
90
120
Time (s)
postmenopausal
postmenopausal + HRT
C
Cardiac output (L.min-1)
3
2
1
0
*
**
**
*
0
30
60
90
120
Time (s)
D
Total peripheral resistance (L.min-1.mmHg-1)
50
40
30
20
*
0
30
60
90
120
Time (s)

Figure 4.5 Effect of the cold pressor test on MAP in postmenopausal smokers and non-smokers and in postmenopausal HRT users and non-users.

A. MAP tended to increase during the first 30 seconds of the cold pressor test in smokers and non-smokers. B. MAP increased during the first 30 seconds of the cold pressor test in postmenopausal HRT users and non-users. (non-smokers: n=10, smokers: n=7; HRT non-users: n=10, HRT users: n=7 */§ $P<0.05$ in non-smokers/smokers and HRT users, respectively, Student's *t*-test). Data represents mean ± S.E.M.


A
Mean arterial pressure (mmHg)
100
90
80
*
0
30
60
90
120
Time (s)
non-smokers
smokers
B
Mean arterial pressure (mmHg)
110
100
90
80
§
*
0
30
60
90
120
Time (s)
postmenopausal
postmenopausal + HRT

4.4 Discussion

The present study found no difference in resting cardiovascular parameters between HRT users and non-users nor between postmenopausal smokers and non-smokers (see Table 4.2). Stress reactivity in middle-aged female smokers and non-smokers was not different in response to physiological stress except for mental stress where greater blood pressure responses were noted in smokers (diastolic pressure: 17 ± 4.6 cf 6 ± 2.6 Δ mmHg; systolic pressure followed a similar trend: 26 ± 7.5 cf 11 ± 4.6 Δ mmHg). Postmenopausal HRT users tended to have lesser TPR responses to the SHG than non-users (seeTable 4.3), while the cold pressor test did not elicit any differences between HRT users and non-users and postmenopausal smokers and non-smokers (see Table 4.5).

4.4.1 HRT use and cardiovascular reactivity

The fact that HRT did not have any effect on resting blood pressure in the present study may be due to differences in oestrogen preparation (Wren and Routledge, 1983), route of administration, dosage (Harvey *et al.*, 1999), the presence of progesterone (Hulley *et al.*, 1998) or the duration of use. The PEPI trials (The PEPI Writing Group, 1995) found no difference in blood pressure with oral oestrogen replacement therapy. Some studies using oral oestrogens have demonstrated an increase in aldosterone and plasma renin levels (Oelkers *et al.*, 1976; Crane and Harris, 1978) both of which can lead to hypertension. Transdermal oestrogen, on the contrary, has been shown to have no effect on plasma renin or aldosterone levels (Pang *et al.*, 1993) and to decrease MAP (Seely *et al*, 1999). Oral oestrogens have been found to have favourable effects on concentrations of LDLs and HDLs, but transdermal oestradiol had no effect (Chetkowski *et al.*, 1986). Vongpatanasin *et al.* (2001) found that in the same group of women transdermal oestrogen replacement decreased SNA and MAP while oral oestrogen did not. Subjects in the present study were using a variety of oestrogen only, combination, transdermal and oral preparations, which may have prevented any differences being observed.

Little is known about the effect of gender on responses to stress and even less is known about the effects of the menopause. Postmenopausal women are reported to have increased adrenergic responsiveness to mental stress when

compared with premenopausal women (Blumenthal *et al.*, 1991) and transdermal oestrogen has been shown to reduce adrenergic responsiveness to mental stress in men (Del Rio *et al.*, 1994). In this latter study low concentrations of oestradiol caused an attenuation in the secretion of adrenaline during mental stress, which resulted in smaller increases in heart rate, blood pressure, plasma free fatty acids and ketones. This would indicate a weaker activation of the SNS and adrenal medulla. Del Rio *et al.* (1998) found that systolic pressure increased more with placebo treatment than with oestradiol or progesterone. This group however found no difference in the response to mental stress of diastolic pressure, heart rate, NA and cortisol levels with either oestrogen or progesterone. Ceresini *et al.* (2000) on the other hand found that diastolic pressure responses to mental stress were blunted by oestradiol treatment.

There are a number of studies that have found no difference in cardiovascular reactivity between HRT users and non-users. The results of the present study agree with those of Matthews *et al.* (2001) in so far as diastolic pressure, cardiac output and TPR responses to mental stress were unaffected by HRT use. Similarly to the present study Matthews and colleagues (2001) used subjects taking various HRT regimens and doses. However when they tried to standardise the HRT regimens they found 6-8 weeks of transdermal oestrogen replacement had no effect on cardiovascular reactivity to mental stress. In another study suppression of ovarian hormones in premenopausal women did not have any effect on cardiovascular responses to stress (Matthews *et al.*, 1998). Moreover Collins *et al.* (1982) found oestrogen plus progesterone did not affect the response of postmenopausal women to mental stress as no changes in blood pressure or heart rate were observed with treatment. In fact Burleson *et al.* (1998) reported that HRT users had greater heart rate reactivity than non-users. Combined HRT is associated with increased adrenaline levels in response to stressors when compared with oestrogen treatment alone (Burleson *et al.*, 1998). It has also been found that oestrogen blunted stressor responses to a speech task and the cold pressor test when compared to placebo and oestrogen plus progesterone (Lindheim *et al.*, 1994). In this same study plasma NA levels were blunted with oestrogen treatment while they increased with the addition of progesterone and with placebo indicating that the presence of progesterone may be a confounding factor. It would appear therefore that due to the differences in hormone regimens in the present study no clear

conclusion can be drawn. The fact that TPR responses tended not to be as great during the sustained handgrip in HRT users than in non-users suggests that perhaps female sex hormones blunt vascular as opposed to cardiac responses to this form of stress.

An interesting observation was that during all the sympathetic activation tests in the present study pulse pressure increased in postmenopausal non-HRT users. Except for mental stress no such significant increase was observed in HRT users. Large stress-induced responses in pulse pressure are a measure of carotid atherosclerosis measured two years later in postmenopausal women (Matthews *et al.*, 1998). Pulse pressure increases with age and is associated with decreased arterial distensibility (Bots *et al.*, 1993) and arterial distensibility decreases with menopause (Westendorp *et al.*, 1999). Postmenopausal women that elect not to use HRT may therefore be more susceptible to age-related changes in vascular distensibilty, however HRT use does not appear to have any discernible effects on the cardiovascular response to stress.

4.4.2 Smoking and cardiovascular reactivity

The resting cardiovascular characteristics were not different between middle-aged smokers and non-smokers in the present study. Smoking status did not affect responses to either the sustained handgrip or the cold pressor test. However middle-aged smokers demonstrated greater MAP and diastolic pressure and a trend towards greater systolic blood pressure responses to mental stress than non-smokers indicating greater cardiovascular reactivity in older smokers. This is in contrast to the situation in younger smokers and non-smokers (see data Chapter 3) where no difference was observed between the groups. This difference could partially be a result of ageing itself since diastolic responses to mental stress were significantly greater in non-smoking postmenopausal as compared with premenopausal women.

4.4.3 Comparison of young versus older responses to cardiovascular stress

At rest MAP, systolic pressure and diastolic blood pressure were elevated in postmenopausal when compared with premenopausal women ($P<0.05$) (see Chapter 3 for young data). Heart rate was not quite significantly different between the groups but tended to be greater in postmenopausal rather than premenopausal women ($P=0.06$) indicating a trend toward sympathetic dominance in the postmenopausal

group. TPR and rate pressure product tended to be elevated in the postmenopausal group relative to their younger counterparts. These changes were not reversed by HRT.

During the sustained handgrip there was an elevation in MAP, involving both systolic and diastolic blood pressure, heart rate and rate pressure product in both the young and middle-aged groups. During this test TPR rose only in the young group not in the postmenopausal group. Peak systolic pressure was greater for both the sustained handgrip and mental arithmetic in the postmenopausal when compared with the premenopausal group and diastolic pressure was greater for mental stress. It has previously been found that healthy postmenopausal women have exaggerated blood pressure responses to mental stress when compared with premenopausal women (Lindheim *et al.*, 1992; Owens *et al.*, 1993; Bairey Merz *et al.*, 1998; Sung *et al.*, 1999). In response to mental stress premenopausal women had greater cardiac reactivity when compared with postmenopausal women as evinced by greater heart rate responses. This appears to correspond with what happens during ageing (Faucheux *et al.*, 1989). These observations are however, not supportive of enhanced sympathetic reactivity in postmenopausal women. The differences in resting blood pressure and hence blood pressure responses to stress could have been affected by vascular compliance. To obtain an index of arterial stiffness the interval between the first pressure peak and the peak of the reflected pressure wave on the pulsatile blood pressure record was measured (Millasseau *et al.*, 2002). This confirmed that there was significantly greater reflectance in the older group (inter-peak time young: 0.274 ± 0.007 s, postmenopausal: 0.231 ± 0.007 s, $P<0.01$).

In response to the cold pressor test diastolic blood pressure responses were greater in premenopausal subjects. Systolic blood pressure and heart rate followed a similar pattern, which was not quite significant ($P=0.07$ and 0.09, respectively). The fact that responses to the cold pressor test were greater in pre- than in postmenopausal women may in part be due to adaptation to the cold since 4 postmenopausal women were self-reported regular sea swimmers. However it is also quite likely that there is a diminished response of adrenergic receptors to cold with ageing. This will be discussed further in the General Discussion. Blumental *et al.* (1991) however found no difference in blood pressure, heart rate or catecholamine responses to the cold pressor test between pre and postmenopausal women. This group used an ice-on-forehead stressor challenge, which cannot be directly

compared with either foot or hand testing. LeBlanc *et al.* (1978) found a decreased heart rate response to the cold pressor test in middle-aged subjects however no difference in blood pressure responses between young and middle-aged subjects was observed. In a recent study Cankar and Finderle (2003) found that when comparing premenopausal responses to local cooling, females responded with greater SNS reactivity than did their male counterparts. In the present study a trend towards lesser heart rate reactivity was observed in the middle-aged subjects (P=0.09). Dishman *et al.* (2002) found that during the cold pressor test increases in systolic pressure were inversely related to fitness among women. The present result directly contradicts that assertion as postmenopausal women were less fit than their premenopausal counterparts. However this same groups assertion that physical fitness can affect heart rate responses to mental stress may still hold true.

Conclusions

There is no appreciable alteration in cardiovascular sympathetic reactivity to stressors between premenopause and postmenopause and HRT does not appear to alter the sympathetic response to physiological and psychological stress. The elevation in resting blood pressure, which occurs at menopause is linked to age rather than hormonal changes. Except for increased blood pressure responses to mental stress in postmenopausal smokers the present results would fail to support the concept that stress-induced SNS stimulation becomes exaggerated with age, hormone loss at menopause or smoking.

Chapter 5 'Interactions of hormonal status, age and smoking on markers of endothelial cell function in women'

5.1 Introduction

The deleterious effects of smoking on the cardiovascular system seem likely to be secondary to the fact that smoking itself elevates blood pressure. This involves several factors, including increased sympathetic vasomotor and cardiac tone (Narkiewicz *et al.*, 1998), decreased BRS (Gerhardt *et al.*, 1999) and loss of endothelium-dependent vasodilator function due to endothelial damage (Blann and McCollum, 1993; Celermajer *et al.*, 1993; Zeiher *et al.*, 1995). The increase in cardiovascular risk associated with smoking is reportedly greater in women than in men (Bolego *et al.*, 2002). This probably reflects the relative protection from CVD that is normally conferred by circulating oestrogen in premenopausal women, since smoking alters oestrogen metabolism so as to increase inactive catechol oestrogens and lower effective plasma oestrogen levels (Baron *et al.*, 1990; Bolego *et al.*, 2002). Also, HRT in postmenopausal women has been reported to attenuate the deleterious effects on arterial compliance and plasma lipids that accompany smoking (Teede *et al.*, 1999).

Evidence suggests that at menopause oestrogen deprivation can increase the risk of CVD in women (Barrett-Connor and Bush, 1991). Part of the beneficial effects of oestrogen prior to menopause may be due to favourable changes in lipid metabolism (see Samsioe *et al.*, 1998), the coagulation system (Scarabin *et al.*, 1997) and blood flow (Volterrani *et al.*, 1995). It is generally accepted that the regulatory actions of oestrogen on the vessel wall, particularly its interactions with the endothelium, are a primary mechanism by which oestrogen exerts its effect. Oestrogen receptors have been identified on vascular SMCs and endothelial cells (Karas *et al.*, 1994; Vencov *et al.*, 1996) and can therefore affect the vasculature directly.

The endothelium plays an important role in local regulation of vascular activity by synthesising a variety of vasodilating and vasoconstricting substances including prostacyclin, prostaglandins, nitric oxide and endothelin-1 (Katz, 1995).

Impaired endothelium-dependent vasodilatation is associated with diseases such as essential and secondary hypertension (Park *et al.*, 2001), atherosclerosis (for review see Mombouli and Vanhoutte, 1999), hypercholesterolaemia (Drexler, 1997), smoking (Zeiher *et al.*, 1995) and ageing (Taddei *et al.*, 1995) all of which increase the risk of CVD. The majority of these conditions are characterised by decreased NO synthesis or availability (see Vapaatalo and Merraala, 2001 for review). An important means by which oestrogen may offer cardiovascular protection to women is by improving the function of the vascular endothelium, specifically by increasing NO bioavailability. To support this hypothesis the shear-stress dependent vasodilatatory reserve in the forearm declines 10 years later in women than in men (Celermajer *et al.*, 1994).

Both the acute and long-term vasodilator effects of oestradiol are mediated in part via generation of endothelium-derived NO and are attenuated by NO synthesis inhibitors (Caulin-Glaser *et al.*, 1997; Chen *et al.*, 1999). The treatment of postmenopausal women with oral oestradiol has been shown to increase NO levels (Imthurn *et al.*, 1997). Oestradiol induces an increase in intracellular free calcium concentration in endothelial cells (Stefano *et al.*, 2000), which could contribute to the increase in endothelial-derived NO. Oestrogen also stimulates prostaglandin synthesis (Chang *et al.*, 1980) and decreases endothelin-1 production (Ylikorkala *et al.*, 1995).

Unopposed oestrogen therapy has been shown to cause rapid dilation of coronary arteries in cholesterol-fed ovariectomised monkeys (Williams *et al.*, 1992) and to have favourable effects on the endothelium of postmenopausal women (Lieberman *et al.*, 1994) and transsexual men (New *et al.*, 1997). Information on the effects of combined hormone therapy preparations on CVD risk and endothelial function is scarce. The progestagens added to oestrogen in combined HRT may attenuate the positive effects of oestrogen. Progestagens are added to oestrogen preparations so as to prevent endometrial hyperplasia and endometrial cancer. However the literature in relation to the role of combined HRT in CVD prevention is unclear. Some studies have found a positive effect of combined HRT on endothelial function (Herrington *et al.*, 1994; McCrohon *et al.*, 1996; Gerhard *et al.*, 1998; Vehkavaara *et al.*, 2000) while others have found no significant effect at all (Sorensen *et al.*, 1998; Van Baal *et al.*, 1999; Teede *et al.*, 2001). The vasodilator effect of oestradiol replacement therapy has been found to be diminished by co-

administration of synthetic progestins such as medroxyprogesterone and cyproterone acetate (Rosselli *et al.*, 1995; Imthurn *et al.*, 1997). Interestingly, in the studies that have found no beneficial effects of combined HRT the treatment has been long-term: 15 months (Van Baal *et al.*, 1999), 2.9 ± 0.5 years (Sorensen *et al.*, 1998) and 2 years (Teede *et al.*, 2001). Those that have found diminished effects of oestrogen replacement therapy on addition of a progestagen have also been long-term studies: 12 months (Imthurn *et al.*, 1997) and up to 24 months (Rosselli *et al.*, 1995). The maximum duration of HRT use in the other studies as far as I am aware is 14 weeks.

In addition to concerns over HRT use premenopausal oral contraceptive use is associated with an increased cardiovascular risk. Unlike natural oestrogens, ethinyl oestradiol increases triglycerides and total cholesterol and is associated with impaired glucose tolerance (Rosenthal and Oparil, 2000). Apart from the blood pressure elevating effects of contraceptive oestrogens (Chasan-Taber *et al.*, 1996), clinical studies provide evidence that oral contraceptives can induce procoagulant effects, hypercholesterolaemia and have unfavourable effects on plasma lipoprotein - HDL and LDL levels (Rosenthal and Oparil, 2000). Although the lower doses used in more recent studies may be safer, contraceptive oestrogens can elevate blood pressure and oral contraceptive-induced hypertension is still the most frequent form of secondary hypertension in younger women (Calhoun and Oparil, 1998). High, but not low, doses of oral contraceptive oestrogens are associated with increased risk of ischaemic stroke and venous thromboembolism (Rosenthal and Oparil, 2000). However despite this apparent increased CVD risk with oral contraceptive use it has been found that these women have similar FBF responses to ACh infusion compared with non-users and that basal NO production is also enhanced by oral contraceptive use (John *et al.*, 2000). Others have found that 3-6 months of combined oral contraceptive use does not have any effect on NO levels (Merki-Feld *et al.* 2002).

It remains uncertain how hormonal status or age itself, interact with the effects of smoking on endothelial function in women. The present study was designed to clarify this situation further.

5.2 Methods

5.2.1 Subjects

The subjects were separated into three groups depending on hormonal and/or smoking status. Young healthy female non-smokers (n=10) and smokers (n=7) with regular menstrual cycles were recruited into the premenopausal group. Young healthy women taking combined oestrogen and progesterone oral contraception (n=10) were recruited into the oral contraceptive user group. Healthy middle-aged postmenopausal women (n=10), postmenopausal HRT users (n=7) and postmenopausal smokers (n=7) were recruited into the middle-aged postmenopausal group.

5.2.2 Experimental protocol

Subjects were required to attend the laboratory on up to three occasions. Session one involved a medical examination to exclude subjects taking anti-hypertensive medication or those unsuitable to perform maximal exercise. Subjects gave a 15 ml blood sample (1 x 3 ml, 2 x 6 ml vacutainers) prior to exercise. The first 3 ml was for haematological analysis while the second two samples were for quantification of CECs and plasma markers of endothelial function. Blood samples were taken from the subjects at random when they arrived for their medical on the first day. For premenopausal non-smokers this resulted in 5 samples taken during the menstrual, 2 during the follicular and 3 during the luteal phases. For premenopausal smokers this resulted in 3 during the menstrual, 1 during the follicular and 2 during the luteal phase. For the oral contraceptive group all samples were taken during the last two weeks of oral contraceptive use.

A maximal incremental exercise test on a cycle ergometer (see Chapter 2) was then performed to measure peak oxygen consumption (VO_2peak) so as to quantify fitness levels. On session two, FBF response to 3 min arterial occlusion (reactive hyperaemia) was measured. Blood flow was measured by venous occlusion plethysmography. For the younger subjects this session occurred twice corresponding to menses and ovulation or withdrawal and oral contraceptive use.

5.2.3 Blood studies

CECs were isolated as described in Chapter 2. CEC smears were made on the same day as blood collection. The average CEC number from five slides was

used to derive CEC counts. Plasma samples were analysed for plasma concentrations of vWF, TM and CRP as described in detail in Chapter 2.

5.2.4 Reactive hyperaemia

Subjects were seated comfortably in an upright position with their arm rested at the level of the heart. A 10-minute rest period was allowed before the start of testing in which time resting cardiovascular parameters were measured. These included heart rate via a standard 3-lead ECG, MAP via applanation tonometry, TPR as derived from MAP/cardiac output, forearm blood flow via venous occlusion plethysmography and forearm vascular conductance. FBF was then measured continuously throughout the testing session. The pressure cuff was placed around the subject's upper arm and was inflated to a supra-systolic pressure for 3 min to induce forearm ischaemia. Following pressure release, FBF was recorded for one minute. A sample trace may be seen in Figure 2.1. Since the magnitude of the hyperaemic response varied in both amplitude and duration between different subject groups, it was necessary to incorporate both these parameters into a quantitative evaluation of the response. To evaluate the duration of hyperaemia, the time elapsing between peak hyperaemia and 2/3 recovery was calculated. The FBF values of each individual were logarithmically transformed and plotted versus time. The product of peak blood flow change and 2/3 recovery time was used as the index of hyperaemia. This was used as an estimate of the area under the curve. The 2/3 recovery time was chosen having viewed the time-course of blood flow return to baseline.

5.2.5 Statistical analysis

Data were expressed as mean ± S.E.M. and were analysed with unpaired (young smokers and non-smokers, oral contraceptive users and non-users, postmenopausal HRT users and non-users, postmenopausal smokers and non-smokers, young and middle-aged smokers and young and middle-aged non-smokers) and paired (menses and ovulation and withdrawal and oral contraceptive use) Student *t*-tests. Pearson's correlation coefficient was used to determine any relationship between variables. $P<0.05$ was considered significant.

5.3 Results

5.3.1 Subject characteristics of smokers and non-smokers with regular menstrual cycles

Physical characteristics of both young smokers and non-smokers are shown in Table 5.1. Subjects in both groups were evenly matched as there was no appreciable differences in age, height, body mass or BMI. Subjects had similar cycle length and flow duration and were of similar physical fitness levels. Smokers smoked 17 ± 1.5 cigarettes.day^{-1} for an average of 8 ± 1.3 years, which was a mean of 8 ± 1.6 pack years.

5.3.2 Resting cardiovascular characteristics of smokers and non-smokers with regular menstrual cycles

At menses no significant differences in resting cardiovascular parameters (heart rate, MAP, TPR, forearm blood flow and forearm conductance) were evident between the groups. However smokers had an elevated MAP at ovulation when compared with non-smokers while the other parameters were not different (see Table 5.4).

5.3.3 Tests of endothelial function during the menstrual cycle in smokers and non-smokers

Reactive hyperaemia

FBF was measured at rest and compared with that observed immediately post occlusion for a duration of 1 min (see Figure 5.1 A). All subjects showed a significant increase in FBF on release of the inflated cuff (see Chapter 2 Figure 2.1 for a sample trace). An increase in slope represented an increase in arterial inflow of blood. Each individual produced a response, which was approximately 3 times the resting FBF value. This magnitude of increased flow has been previously found after three minutes of arterial occlusion (Carlsson *et al.*, 1987). Following cuff release the FBF increase above baseline levels was 241 ± 45.8 % in non-smokers at menses, 162 ± 36.8% at ovulation and in smokers 198 ± 24.8% at menses and 201 ± 40.5% during ovulation (see Figure 5.1 B). There were no differences in the percentage increase in blood flow between the groups or at the different phases of the menstrual cycle.

FBF increased appreciably immediately after release of the occlusive cuff in both groups at menses and at ovulation but returned to baseline values by 30 seconds post-occlusion (see Figure 5.1 A). There was no difference in hyperaemic index between smokers (at menses: 205 ± 58.6 $((ml.min^{-1}).100ml^{-1}).s$, at ovulation: 168 ± 54.2 $((ml.min^{-1}).100ml^{-1}).s$ and non-smokers (at menses: 185 ± 54.0 ((ml.min-1).$100ml^{-1}$).s, at ovulation: 110 ± 29.1 $((ml.min^{-1}).100ml^{-1}).s$ or at the two phases of the menstrual cycle.

Blood markers of endothelial function

See Table 5.7 and Figures 5.8 A, 5.9 A, B and C.

5.3.4 Subject characteristics of young women on the oral contraceptive pill

Physical characteristics of oral contraceptive users are shown in Table 5.2.

5.3.5 Resting cardiovascular characteristics of young women on the oral contraceptive pill

There were no significant differences between resting cardiovascular parameters (heart rate, MAP, TPR, forearm blood flow and forearm conductance) measured during oral contraceptive use and at withdrawal except for TPR, which was elevated during the withdrawal period (see Table 5.5).

5.3.6 Tests of endothelial function during oral contraceptive use and the withdrawal period

Reactive hyperaemia

All subjects showed a significant increase in FBF on release of the inflated cuff. Each individual produced a response, which was approximately 2.5-3 times the resting FBF value. This amounted to a 219 ± 40.8% increase during oral contraceptive use and 320 ± 72% increase during withdrawal (see Figure 5.2 B). The percentage increase was not dependent on oral contraceptive phase.

FBF increased appreciably immediately after release of the occlusive cuff during oral contraceptive use and at withdrawal (see Figure 5.2 A). During oral contraceptive use this elevation returned to baseline levels by 40 s post occlusion. At withdrawal the increase in FBF was evident again until 40 s. There was no

difference observed in the hyperaemic index at withdrawal or during OC use (215 ± 52.4 cf 171 ± 31.3 ((ml.min^{-1}).100ml^{-1}).s, respectively.

Blood markers of endothelial function

See Table 5.8.

5.3.7 Comparison of endothelial function in young healthy women with normal regular menstrual cycles and those on oral contraceptives

There was no difference observed in the responses of young cycling women and women on the oral contraceptive pill to arterial occlusion. Because an elevation in FBF persisted in the oral contraception group when it had returned to baseline levels in the menstrual cycle group a difference in blood flow decay time was a possibility. The 2/3 recovery time was noted. However there was no difference in this period when it was compared between the groups: at menses 22 ± 4.8 s, at withdrawal 34 ± 6.1 s and at ovulation 20 ± 4.7 s, during oral contraceptive use 28 ± 4.4 s. No differences in hyperaemic index values were noted between the groups at menses: 185 ± 54.0 ((ml.min^{-1}).100ml^{-1}).s, at withdrawal: 215 ± 52.4 ((ml.min^{-1}).100ml^{-1}).s and at ovulation: 110 ± 29.1 ((ml.min^{-1}).100ml^{-1}).s, during OC use: 171 ± 31.3 ((ml.min^{-1}).100ml^{-1}).s.

As regard the plasma markers of endothelial function no differences were found between oral contraceptive users and non-users except for CRP, which was greater in oral contraceptive users (menstrual cycle group: 1.0 ± 0.3, oral contraceptive group: 2.4 ± 0.5 mg/L^{-1}, see Figure 5.12 B). No difference in CEC number was noted between the groups.

5.3.8 Subject characteristics of middle-aged women

Physical characteristics of postmenopausal women, postmenopausal women taking HRT and postmenopausal smokers are shown in Table 5.3. Subjects in each group were comparable as there were no significant differences in age, height, body mass or BMI. All groups had similar cholesterol and fitness levels. The women were postmenopausal for a similar duration of time. Smokers smoked 23 ± 5.2 cigarettes.day^{-1} with a range of 8 - 40 day^{-1} for an average of 30 ± 3.9 years, which was a mean of 39 ± 12.2 pack years.

5.3.9 Resting cardiovascular parameters of middle-aged subjects

There were no significant differences between resting cardiovascular parameters (heart rate, MAP, pulse pressure, forearm blood flow and forearm conductance) between postmenopausal smokers and non-smokers and postmenopausal HRT users and non-users (see Table 5.6).

5.3.10 Tests of endothelial function in postmenopausal women, postmenopausal HRT users and postmenopausal smokers

Reactive hyperaemia

All subjects demonstrated an increase in FBF after the release of the occluding cuff. An average increase of 135 ± 26.8% was observed in postmenopausal non-HRT users, 260 ± 110% in postmenopausal HRT users and 118± 27.5% in postmenopausal smokers. There was no difference in the percentage increase between HRT users and non-user or between smokers and non-smokers (see Figure 5.3 B). In postmenopausal HRT users and non-users blood flow returned to baseline levels by 30 s post occlusion (see Figure 5.3 A). In smokers however blood flow returned to baseline values by 20 s (see Figure 5.3 A). When comparing the 2/3 recovery time postmenopausal HRT users and non-users demonstrated no significant difference in this parameter (postmenopausal: 20 ± 3.2 s, postmenopausal + HRT: 17 ± 2.5 s). However smokers had a tendency for a shorter recovery time when compared with postmenopausal non-smokers (12 ± 2.3 s cf 20 ± 3.2 s, P=0.07). When the hyperaemic index was calculated a significantly greater value was found in postmenopausal non-smokers than smokers (116 ± 27.3 cf 36 ± 7.0 $((ml.min^{-1}).100ml^{-1}).s$, see Figure 5.3 C) while the value for the HRT group was similar to the non-using group (119 ± 26.7 $((ml.min^{-1}).100ml^{-1}).s$.

Blood markers of endothelial function

See Table 5.9 and Figures 5.8 B, 5.10 A, B and C.

5.3.11 Comparison of endothelial function in pre- and postmenopausal women

Considering the fact that there were no appreciable differences between the menstrual cycle and oral contraceptive groups and that menses has a similar hormonal profile when compared with the postmenopausal state, FBF values in

response to reactive hyperaemia at menses were taken as an example of the typical response to this test in young females.

A graph of responses to reactive hyperaemia for both groups may be seen in Figure 5.4 A. There was a tendency for a lower percentage increase in FBF after release of the occlusive cuff in postmenopausal women than in premenopausal women, however this trend was not quite significant (135 ± 26.8 cf 241 ± 45.8 %, P=0.07, see Figure 5.4 B). When investigating the 2/3 recovery time no difference was noted between the groups. A regression line of age versus percentage increase in blood flow from rest after release of the occlusive cuff did not show any relationship between the two variables ($r^2 = 0.18$, see Figure 5.5).

Plasma markers of endothelial function were not different between pre- and postmenopausal women in any of the markers however CRP demonstrated a distinct trend for increased levels in postmenopausal women when compared with young women (5.5 ± 2.08 cf 1.0 ± 0.30, see Figure 5.12 A). Plasma CRP levels did not significantly correlate with age in these groups ($r^2 = 0.19$, see Figure 5.13 A). No difference in CEC number was noted between the groups.

5.3.12 Comparison of endothelial function in pre- and postmenopausal smokers

Responses to reactive hyperaemia were compared between young and middle-aged smokers using menses as a reference in premenopausal women (see Figure 5.6 A). There was a tendency for a lower percentage increase in FBF from resting levels directly after release of the cuff in postmenopausal smokers than in premenopausal smokers, this trend was not quite significant (118 ± 27.5 cf 198 ± 24.8 %, P=0.057, see Figure 5.6 B). However the hyperaemic index was found to be significantly lower in postmenopausal than premenopausal smokers (218 ± 69.8 cf 36 ± 7.0 $((ml.min^{-1}).100ml^{-1}).s$, see Figure 5.6 C). The 2/3 recovery time was delayed in younger smokers (27 ± 4.3 cf 12 ± 2.3, P<0.05, see Figure 5.6 C). There was a significant inverse regression relationship between number of cigarettes $smoked.day^{-1}$ and percentage increase in blood flow from resting values (see Figure 5.7 A) and between years smoking and percentage increase in blood flow from resting values (see Figure 5.7 B) when both young and middle-aged smokers were grouped. This was possibly the reason why there was also a significant inverse correlation between age and percentage increase in blood flow from resting values in smokers. When looking at the relationship between pack years and percentage

increase in blood flow from resting values a distinct inverse relationship was noted however this was not deemed significant (see Figure 5.7 C).

Older smokers demonstrated lower TM levels than their younger counterparts (3.8 ± 0.92 cf 8.9 ± 0.74, see Figure 5.11 A) and plasma TM levels correlated inversely with age in these groups (r^2 = 0.55, see Figure 5.13 C). There was a tendency for CRP levels to be higher in middle-aged smokers (0.6 ± 0.14 cf 3.3 ± 1.34 units, see Figure 5.11 B) and plasma CRP levels were positively correlated with age in smokers (r^2 = 0.39, see Figure 5.13 B). CEC number was not different between the two groups.

5.3.13 VO_2peak

Premenopausal women had significantly greater VO_{2peak} than postmenopausal women (35 ± 3.2 cf 21 ± 1.2 ($(ml.min^{-1}).kg^{-1}$), $P<0.001$) and premenopausal smokers showed a tendency for greater VO_{2peak} when compared with postmenopausal smokers (32 ± 3.3 cf 22 ± 3.3 ($(ml.min^{-1}).kg^{-1}$), $P=0.076$). In order to investigate whether physical fitness influenced the increased reactive hyperaemia a regression graph of Vo_2peak and percentage change of blood flow from rest was plotted (see Figure 5.14). No relationship between the variables was observed.

Table 5.1 Physical characteristics of subjects with regular menstrual cycles

Non-smokers n=10, smokers n=7. Data are expressed as mean ± S.E.M.

	Non-smokers	Smokers
Age (Years)	23 ± 1.1	24 ± 1.2
Cycle length (days)	30 ± 0.8	28 ± 0.5
Menses duration (days)	6 ± 0.3	5 ± 0.1
Height (cm)	168 ± 2	164 ±1.8
Body Mass (kg)	68 ± 3.7	61 ± 3.6
BMI ($kg.m^{-2}$)	24 ± 1.1	23 ± 1.4
VO_2peak ($(ml.min^{-1}).kg^{-1}$)	35 ± 3.2	32 ± 3.3

Table 5.2 Physical characteristics of subjects using oral contraception

n=10. Data are expressed as mean ± S.E.M.

	Oral contraceptive users
Age (years)	23 ± 0.8
Height (cm)	166 ± 1.4
Body Mass (kg)	60 ± 1 3
BMI ($kg.m^{-2}$)	22± 0.5
VO_2peak ($(ml.min^{-1}).kg^{-1}$)	37 ± 2.5

Table 5.3 Physical characteristics of middle-aged women

Postmenopausal women n=10, postmenopausal HRT users n=7, postmenopausal smokers n=7. Data are expressed as mean ± S.E.M.

	Postmenopausal	Postmenopausal + HRT	Postmenopausal smokers
Age (years)	57 ±1.9	58 ± 2.0	53 ± 2.7
Years postmenopause	8 ± 1.6	10 ± 1.1	7 ± 2.6
Years on HRT	-	10 ± 1.4	-
Height (cm)	164 ± 2.3	161 ± 3.2	165 ± 2.4
Body Mass (kg)	70 ± 2.7	63 ± 4.6	72 ± 6.6
BMI ($kg.m^{-2}$)	26 ± 1.3	24 ± 1.3	26 ± 1.7
Cholesterol ($mmol.l^{-1}$)	5.4 ± 0.38	5.3 ± 0.50	5.6 ± 0.59
VO_2peak ($(ml.min^{-1}).kg^{-1}$)	21 ± 1.5	24 ± 2.9	22 ± 3.3

* denotes a significant difference between smokers and non-smokers (P <0.05)

Table 5.4 Resting cardiovascular characteristics of subjects with regular menstrual cycles

Non-smokers n=10, smokers n=7. Data are expressed as mean ± S.E.M.

	Non-smokers at menses	Smokers at menses
Heart Rate ($beat.min^{-1}$)	72 ± 3.3	80 ± 5.8
MAP (mmHg)	76 ± 1.6	78 ± 2.8
TPR ($L.min^{-1}$).$mmHg^{-1}$	23.9 ± 3.90	26.0 ± 2.80
Forearm blood flow ($ml.min^{-1}$).$100ml^{-1}$	5.2 ± 1.61	5.0 ± 2.22
Forearm conductance (($ml.min^{-1}$).$100ml^{-1}$.$mmHg^{-1}$)	0.07 ± 0.020	0.08 ± 0.040

	Non-smokers at ovulation	Smokers at ovulation
Heart Rate ($beat.min^{-1}$)	75 ± 4.3	84 ± 3.5
MAP (mmHg)	77 ± 2.2	85 ± 2.3*
TPR ($L.min^{-1}$).$mmHg^{-1}$	26.6 ± 3.40	26.5 ± 3.10
Forearm blood flow ($ml.min^{-1}$).$100ml^{-1}$	3.7 ± 0.51	3.6 ± 0.42
Forearm conductance (($ml.min^{-1}$).$100ml^{-1}$.$mmHg^{-1}$)	0.05 ± 0.010	0.05 ± 0.010

* denotes a significant difference between non-smokers and smokers at ovulation and ($P < 0.05$)

Table 5.5 Resting cardiovascular characteristics of subjects using oral contraception

n=10. Data are expressed as mean ± S.E.M.

	OC users during use	OC users at withdrawal
Heart Rate (beat.min^{-1})	73 ± 3.6	71 ± 3.3
MAP (mmHg)	77 ± 1.6	75 ± 1.8
TPR (L.min^{-1}).mmHg^{-1}	18.4 ±1.63	23.4 ± 2.62*
Forearm blood flow (ml.min^{-1}).100ml^{-1}	7.2 ± 2.42	4.6 ± 0.84
Forearm conductance ((ml.min^{-1}).100ml^{-1}.mmHg^{-1})	0.09 ±0.031	0.06 ± 0.012

OC – oral contraceptive

* denotes a significant difference between oral contraceptive users during use and at withdrawal (P<0.05).

Table 5.6 Resting cardiovascular characteristics of middle-aged women.

Postmenopausal women n=10, postmenopausal HRT users n=7, postmenopausal smokers n=7. Data are expressed as mean ± S.E.M.

	Postmenopausal	Postmenopausal + HRT	Postmenopausal smokers
Heart Rate (beat.min^{-1})	83 ± 4.3	74 ± 3.2	74 ± 2.3
MAP (mmHg)	91 ± 4.0	88 ± 2.5	82 ± 2.6
TPR (L.min^{-1}).mmHg^{-1}	31.6 ± 3.66	32.2 ± 4.05	36.6 ± 3.43
Forearm blood flow (ml.min^{-1}).100ml^{-1}	4.3 ± 0.72	4.2 ± 1.24	4.1 ± 0.87
Forearm conductance ((ml.min^{-1}).100ml^{-1}.mmHg^{-1})	0.06 ± 0.021	0.04 ± 0.012	0.06 ± 0.021

Table 5.7 CEC number, plasma vWF concentration, plasma TM concentration and plasma CRP concentration in subjects with regular menstrual cycles.

Non-smokers n=10, smokers n=7. Data are expressed as mean ± S.E.M.

	Non-smokers	Smokers
CECs (cells.ml^{-1}blood)	59 ± 10.6	117 ± 22.1*
vWF (units)	70 ± 25.0	72 ± 34.9
TM (ng.ml^{-1})	7 ± 0.8	8 ± 0.7
CRP (mg.L^{-1})	1 ± 0.3	0.6 ± 0.14

* denotes a significant difference between non-smokers and smokers

Table 5.8 CEC number, plasma vWF concentration, plasma TM concentration and plasma CRP concentration in young oral contraceptive users

n=10. Data are expressed as mean ± S.E.M.

	Oral contraceptive users
CECs (cells.ml^{-1}blood)	85 ± 10.8
vWF (units)	59 ± 21.5
TM (ng.ml^{-1})	5 ± 0.9
CRP (mg.L^{-1})	2 ± 0.5

Table 5.9 CEC number, plasma vWF concentration, plasma TM concentration and plasma CRP concentration in middle-aged women.

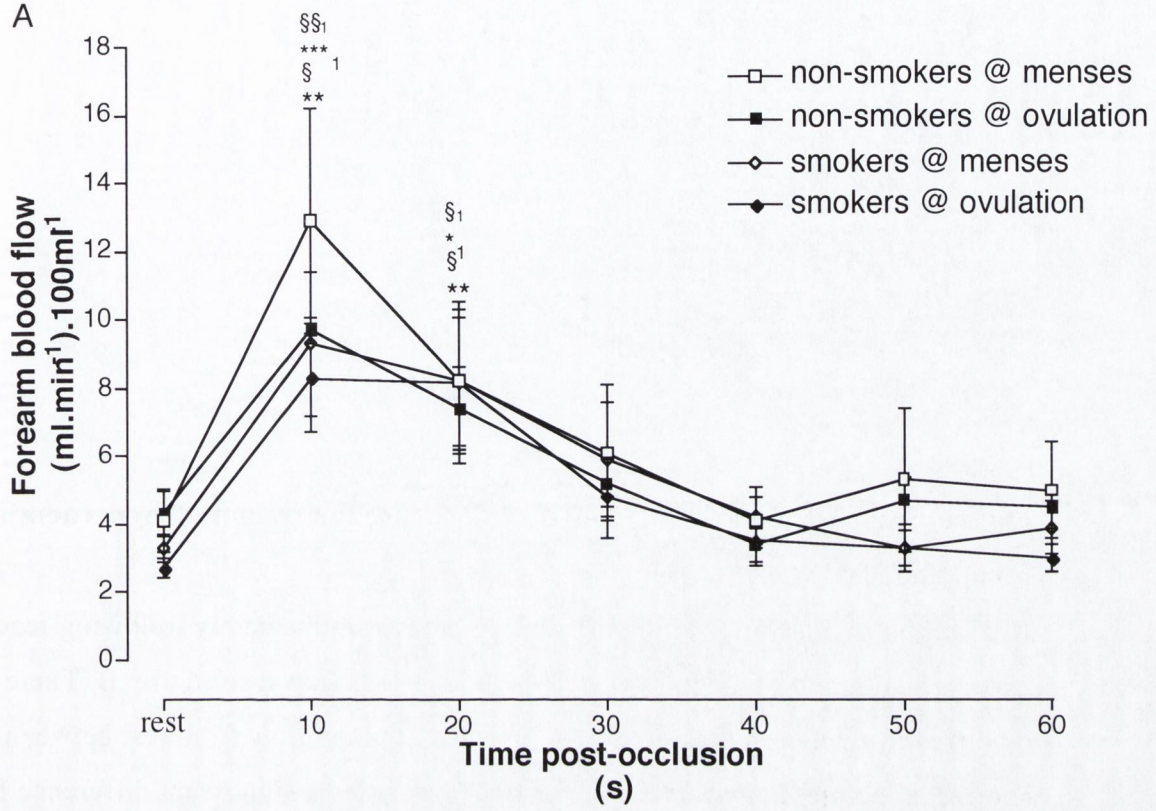
Postmenopausal women n=10, postmenopausal HRT users n=7, postmenopausal smokers n=7. Data are expressed as mean ± S.E.M.

	Postmenopausal	Postmenopausal + HRT	Postmenopausal smokers
CECs (cells.ml^{-1}blood)	54 ± 11.2	65 ± 21.3	106 ± 18.7*
vWF (units)	62 ± 10.1	25 ± 9.5§	67 ± 22.2
TM (ng.ml^{-1})	7 ± 1.2	5 ± 0.8	4 ± 0.9
CRP (mg.L^{-1})	6 ± 2.1	2 ± 0.7	3 ± 1.3

* denotes a significant difference between smokers and non-smokers, § denotes a significant difference between postmenopausal HRT users and non-users

Figure 5.1 Effects of smoking and menstrual cycle phase on reactive hyperaemia in premenopausal women.

A. Forearm blood flow was significantly increased immediately following reactive hyperaemia in both smokers and non-smokers independent of cycle stage. Blood flow returned to baseline levels by 30 seconds. B. There was no difference in the percentage increase in blood flow from rest after reactive hyperaemia between smokers or non-smokers or with hormonal status. (non-smokers: n=10, smokers: n=7; */§ denotes a significant difference from resting values: * represents non-smokers at menses, *1 non-smokers at ovulation, § smokers at menses and §1 smokers at ovulation (*/§ $P<0.05$, **/§§ $P<0.01$, ***/§§§ $P<0.001$), Student's *t*-test). Data represents mean ± S.E.M.


A
Forearm blood flow (ml.min-1).100ml-1
Time post-occlusion (s)
rest
10
20
30
40
50
60
non-smokers @ menses
non-smokers @ ovulation
smokers @ menses
smokers @ ovulation

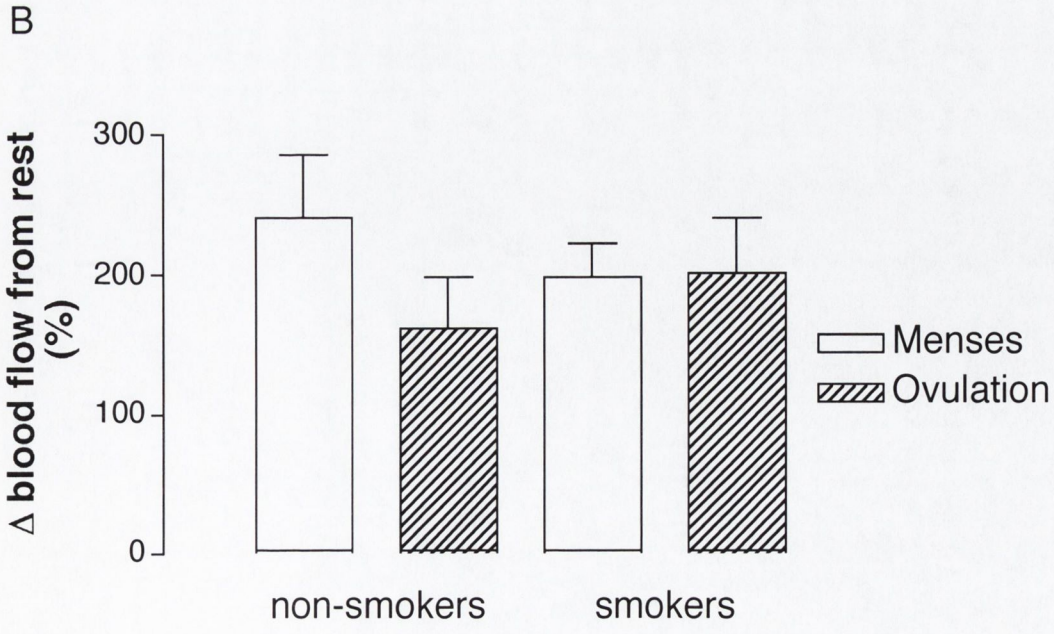
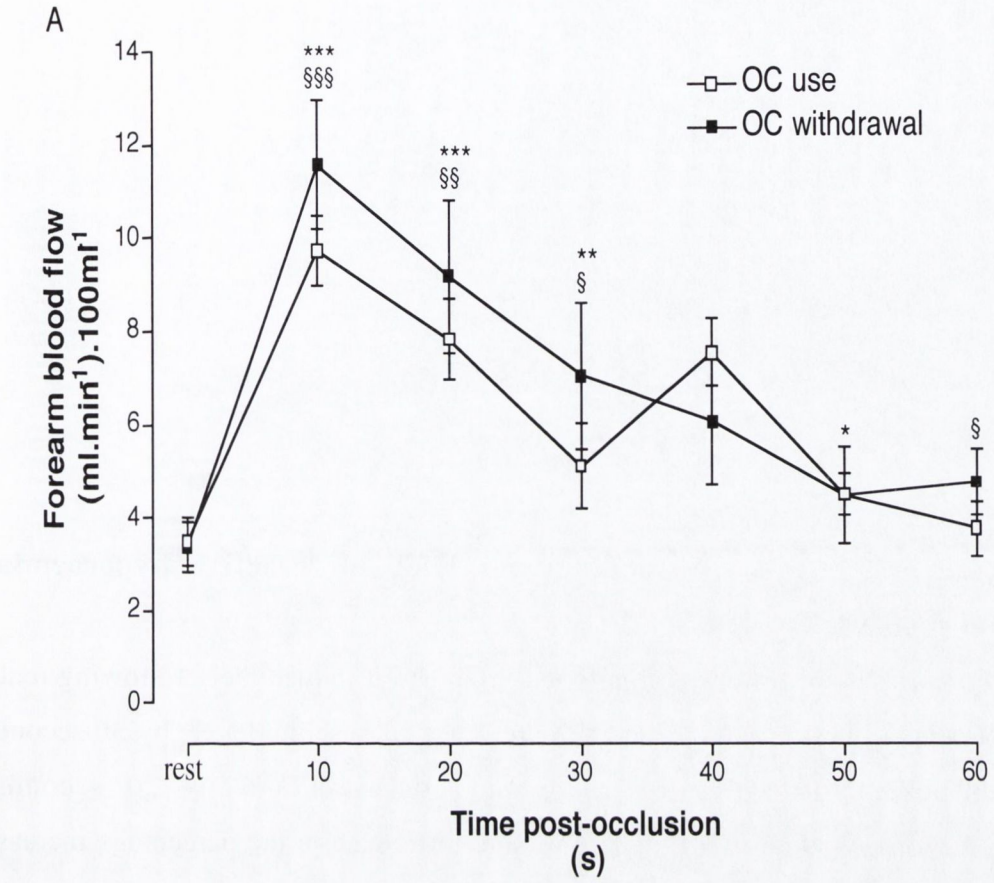

B
Δ blood flow from rest (%)
non-smokers
smokers
Menses
Ovulation

Figure 5.2 Effects of oral contraceptive use on reactive hyperaemia in premenopausal women.

A. Forearm blood flow was significantly increased immediately following reactive hyperaemia during oral contraceptive use and at withdrawal from use. B. There was no difference between the percentage increase in blood flow from rest between oral contraceptive use and withdrawal. (n=10, */§ denotes a significant difference from resting values during oral contraceptive use/withdrawal from use respectively. (*/§ $P<0.05$, **/§§ $P<0.01$, ***/§§§ $P<0.001$), Student's *t*-test). Data represents mean ± S.E.M.


A
OC use
OC withdrawal
Forearm blood flow
(ml.min-1).100ml-1
14
12
10
8
6
4
2
0
***
§§§
***
§§
**
§
*
§
rest
10
20
30
40
50
60
Time post-occlusion
(s)

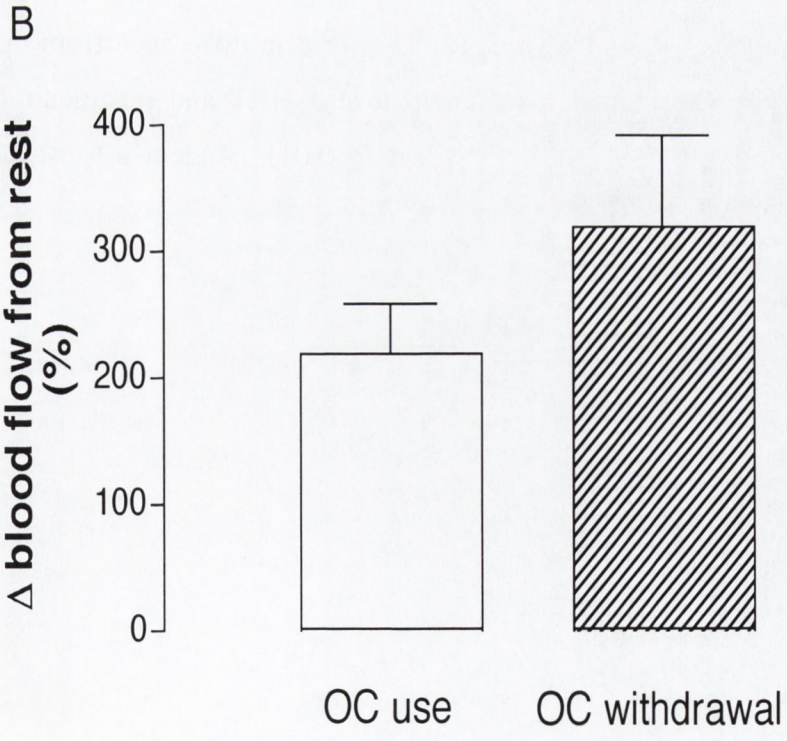

B
Δ blood flow from rest
(%)
400
300
200
100
0
OC use
OC withdrawal

Figure 5.3 Effects of smoking and HRT on reactive hyperaemia in postmenopausal women.

A. Forearm blood flow was significantly increased immediately following reactive hyperaemia in all groups. Blood flow returned to baseline levels by 30 seconds in both postmenopausal HRT users and non-users and by 20 seconds in postmenopausal smokers. B. There was no difference in the percentage increase of blood flow from rest between HRT users and non-users or smokers and non-smokers. (postmenopausal women: n=10, postmenopausal women + HRT n=7, postmenopausal smokers: n=7). */§/‡ denotes a significant difference from resting values in postmenopausal women, postmenopausal + HRT and postmenopausal smokers, respectively. (*/§/‡ $P<0.05$, **/§§/‡‡ $P<0.01$), Student's *t*-test). Data represents mean ± S.E.M.

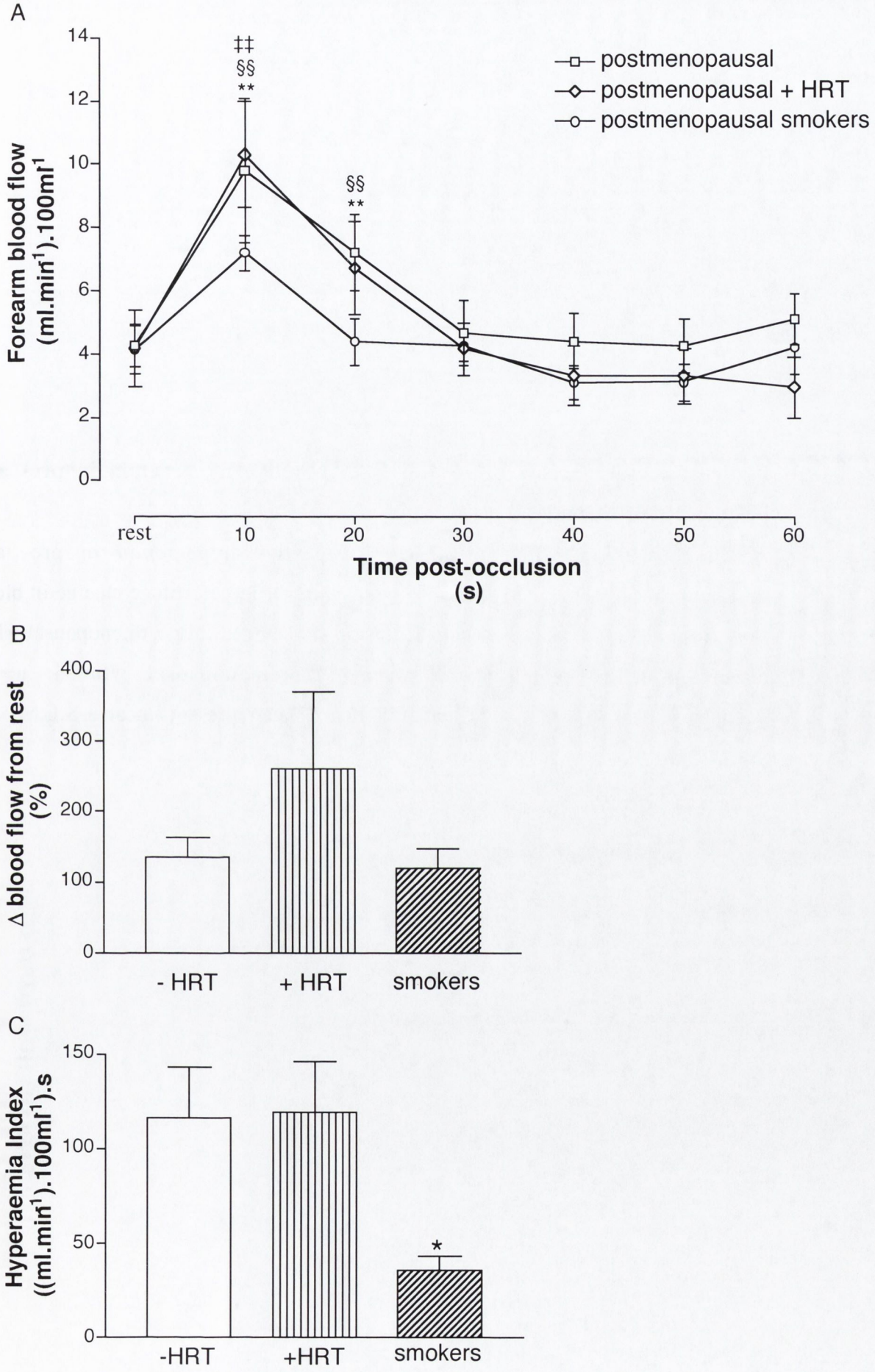
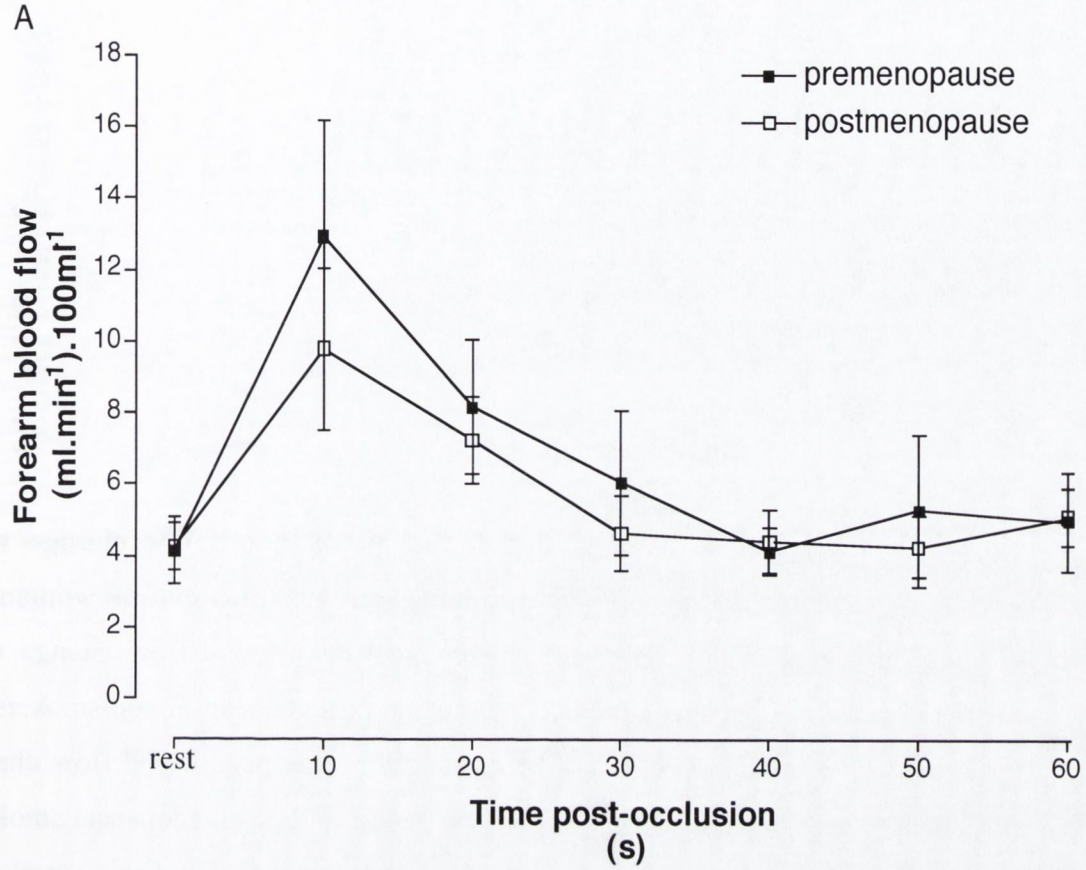

A
Forearm blood flow (ml.min-1).100ml-1
14
12
10
8
6
4
2
0
‡‡
§§
**
§§
**
postmenopausal
postmenopausal + HRT
postmenopausal smokers
rest
10
20
30
40
50
60
Time post-occlusion (s)
B
Δ blood flow from rest (%)
400
300
200
100
0
- HRT
+ HRT
smokers
C
Hyperaemia Index ((ml.min-1).100ml-1).s
150
100
50
0
*
-HRT
+HRT
smokers

Figure 5.4 Comparison of responses to reactive hyperaemia in pre- and postmenopausal women.

A. Forearm blood flow responses to reactive hyperaemia in pre- and postmenopausal women. B. A trend towards a lower in percentage change in blood flow from rest after release of cuff occlusion was evident in postmenopausal when compared with premenopausal women. (premenopausal women: n=10, postmenopausal women: n=10; Student's *t*-test). Data represents mean ± S.E.M.


A
premenopause
postmenopause
Forearm blood flow
(ml.min-1).100ml-1
18
16
14
12
10
8
6
4
2
0
rest
10
20
30
40
50
60
Time post-occlusion
(s)

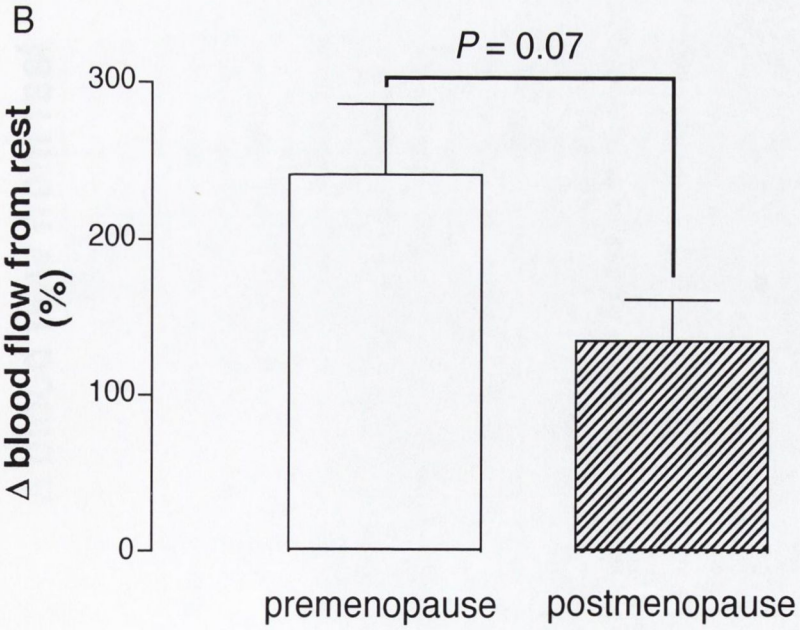
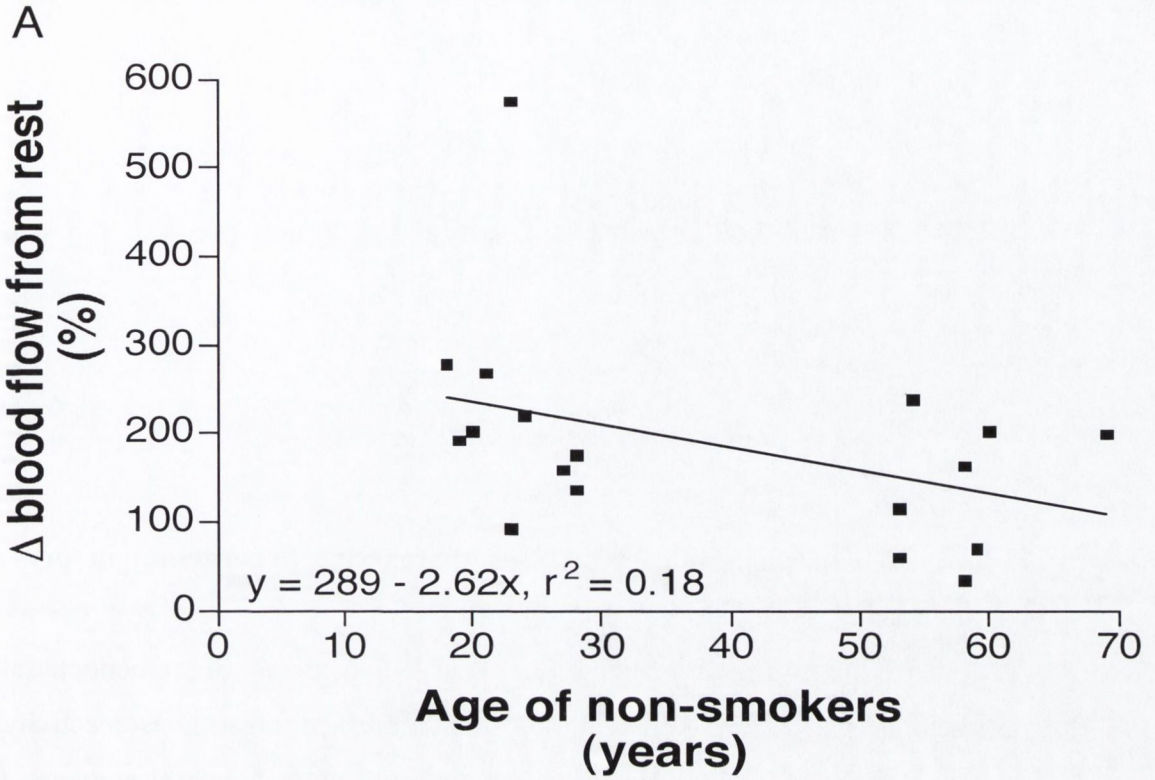

B
P = 0.07
Δ blood flow from rest
(%)
300
200
100
0
premenopause
postmenopause

Figure 5.5 Correlation between percentage peak blood flow change with reactive hyperaemia and age in premenopausal and postmenopausal women.

A. There was no correlation between percentage peak blood flow change with reactive hyperaemia and age in premenopausal and postmenopausal non-smokers. B. A significant correlation was found between percentage peak blood flow change with reactive hyperaemia and age in premenopausal and postmenopausal smokers. (premenopausal smokers: n=7, postmenopausal smokers: n=7, Pearson's correlation coefficient).


A
Δ blood flow from rest (%)
600
500
400
300
200
100
0
y = 289 - 2.62x, r 2 = 0.18
0
10
20
30
40
50
60
70
Age of non-smokers (years)

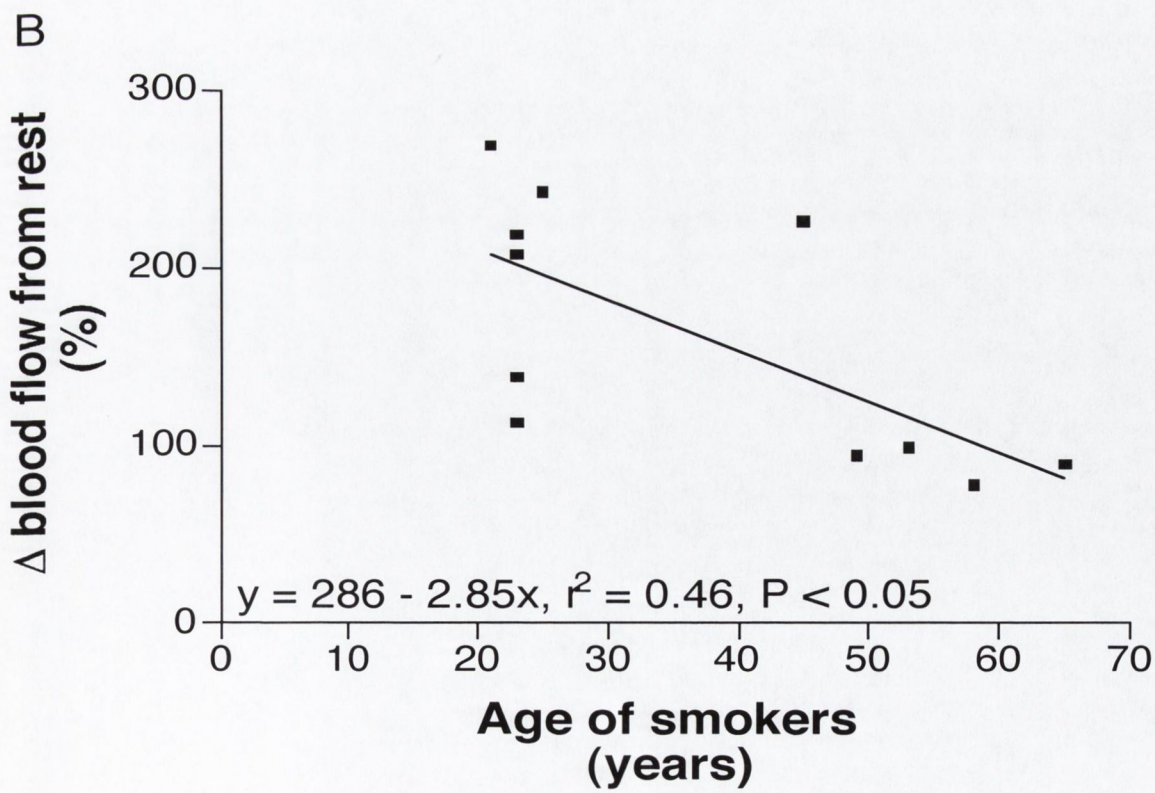
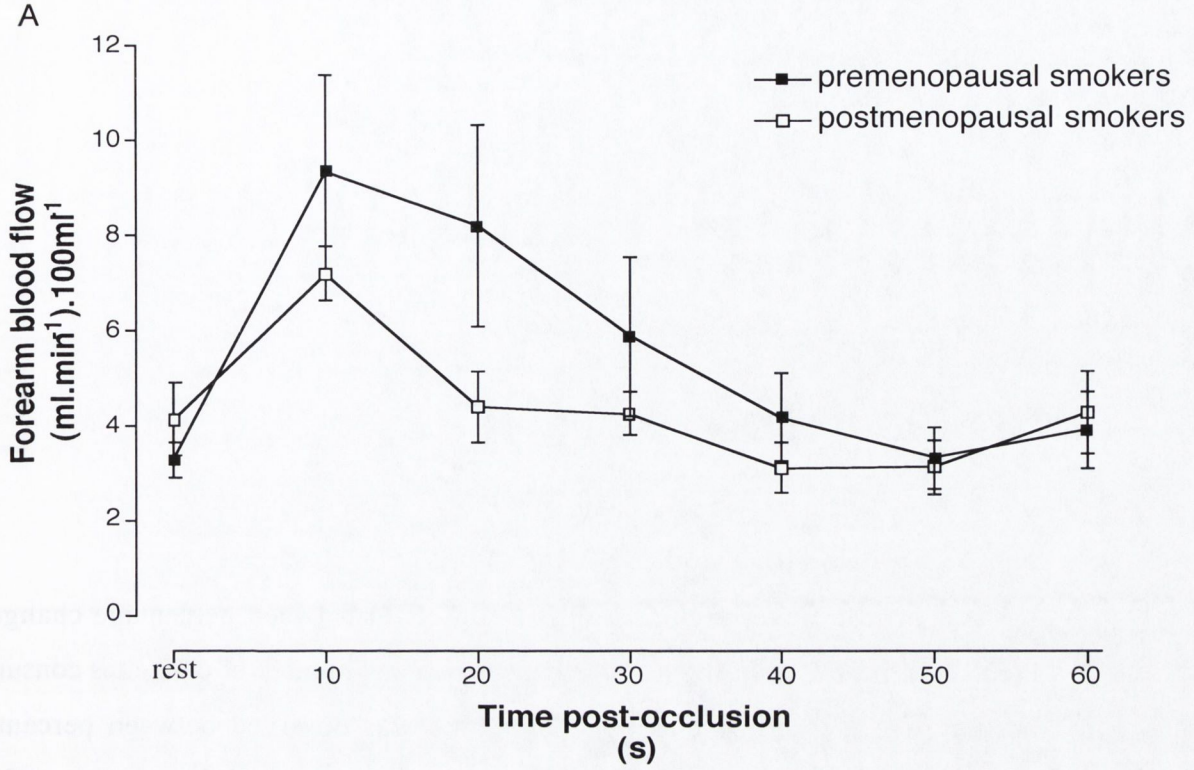
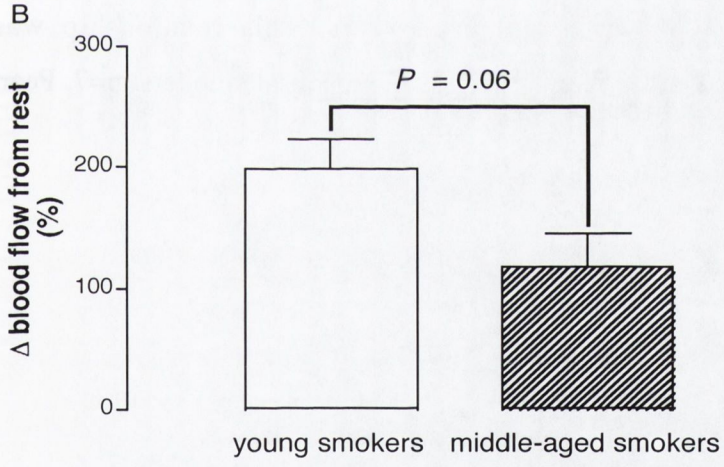

B
Δ blood flow from rest (%)
300
200
100
0
y = 286 - 2.85x, r2 = 0.46, P < 0.05
0
10
20
30
40
50
60
70
Age of smokers (years)

Figure 5.6 Comparison of responses to reactive hyperaemia in pre- and postmenopausal smokers.

A. Forearm blood flow in responses to reactive hyperaemia in premenopausal and postmenopausal women. B. A trend towards diminished forearm vasoreactivity was observed in postmenopausal when compared with premenopausal smokers. C. A trend towards decreased time to half peak blood flow was observed in postmenopausal when compared with premenopausal smokers. (premenopausal smokers: n=7, postmenopausal smokers: n=7, Student's *t*-test). Data represents mean ± S.E.M.


A
premenopausal smokers
postmenopausal smokers
Forearm blood flow (ml.min-1).100ml-1
0
2
4
6
8
10
12
rest
10
20
30
40
50
60
Time post-occlusion (s)


B
P = 0.06
Δ blood flow from rest (%)
0
100
200
300
young smokers
middle-aged smokers

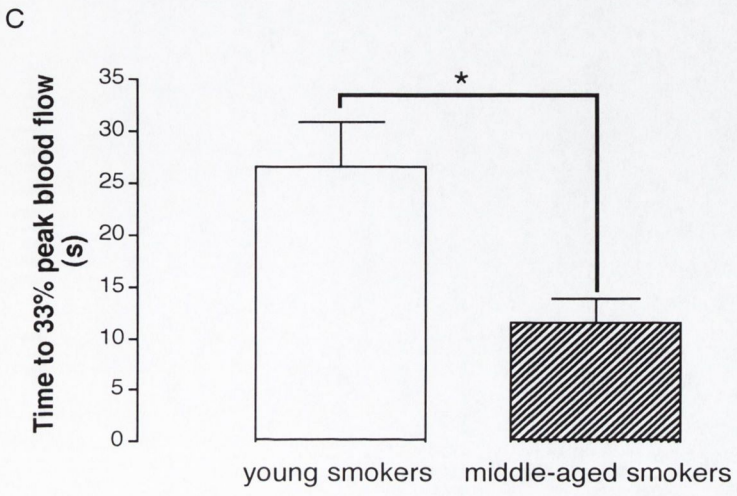

C
*
Time to 33% peak blood flow (s)
0
5
10
15
20
25
30
35
young smokers
middle-aged smokers

Figure 5.7 Correlation studies

A. A significant inverse correlation was observed between percentage change in blood flow from rest during reactive hyperaemia and number of cigarettes consumed per day. B. A significant inverse correlation was observed between percentage change in blood flow from rest during reactive hyperaemia and time smoking. C. An inverse correlation was observed between the percentage change in blood flow from rest during reactive hyperaemia and pack years. This relationship was not significant. (premenopausal smokers: n=7, postmenopausal smokers: n=7, Pearson's correlation coefficient).

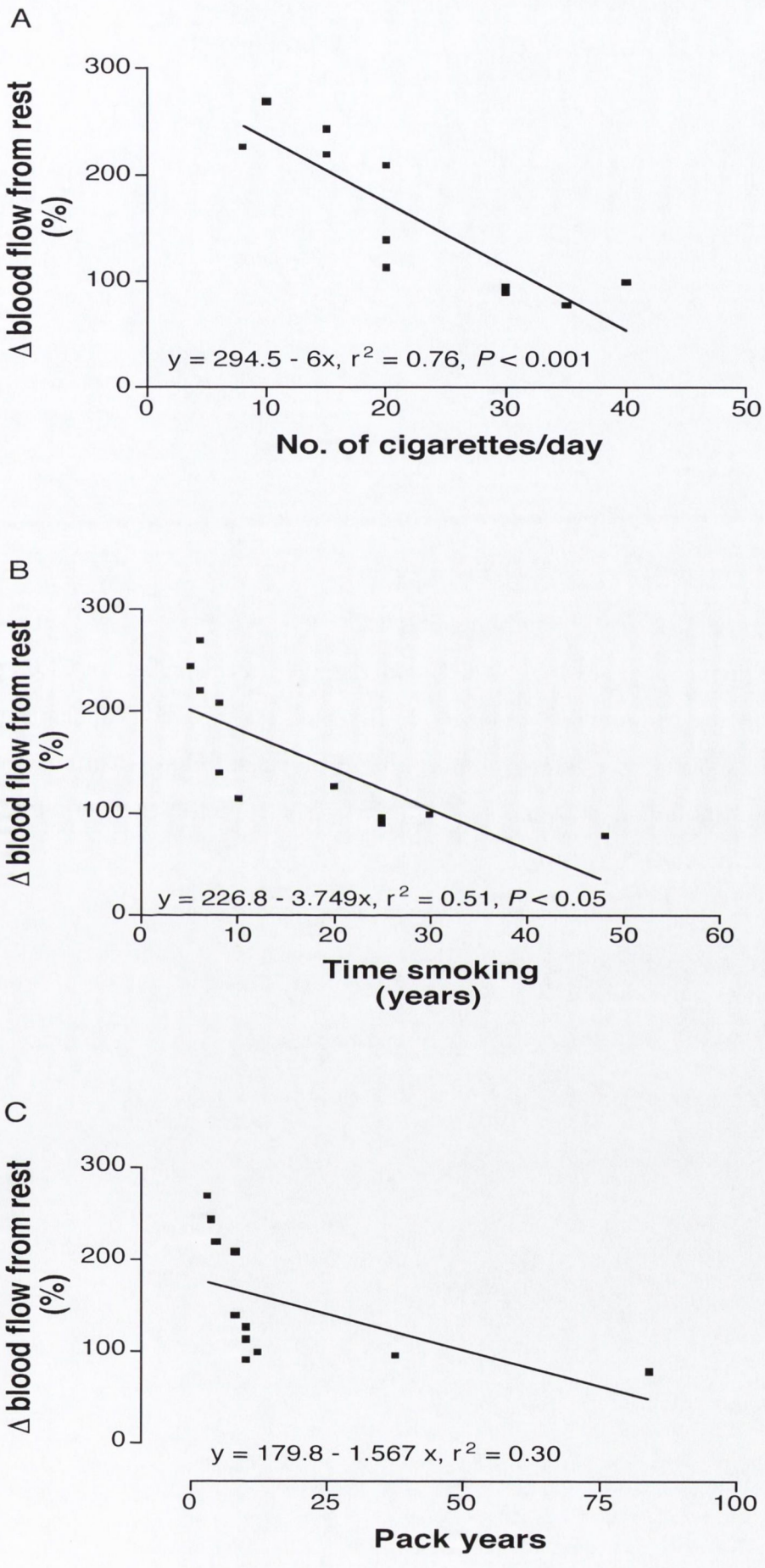
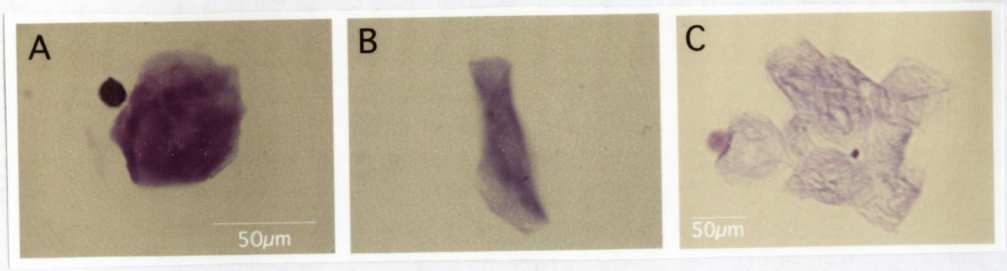

A
Δ blood flow from rest (%)
300
200
100
0
y = 294.5 - 6x, r² = 0.76, P < 0.001
0
10
20
30
40
50
No. of cigarettes/day
B
Δ blood flow from rest (%)
300
200
100
0
y = 226.8 - 3.749x, r² = 0.51, P < 0.05
0
10
20
30
40
50
60
Time smoking (years)
C
Δ blood flow from rest (%)
300
200
100
0
y = 179.8 - 1.567 x, r² = 0.30
0
25
50
75
100
Pack years

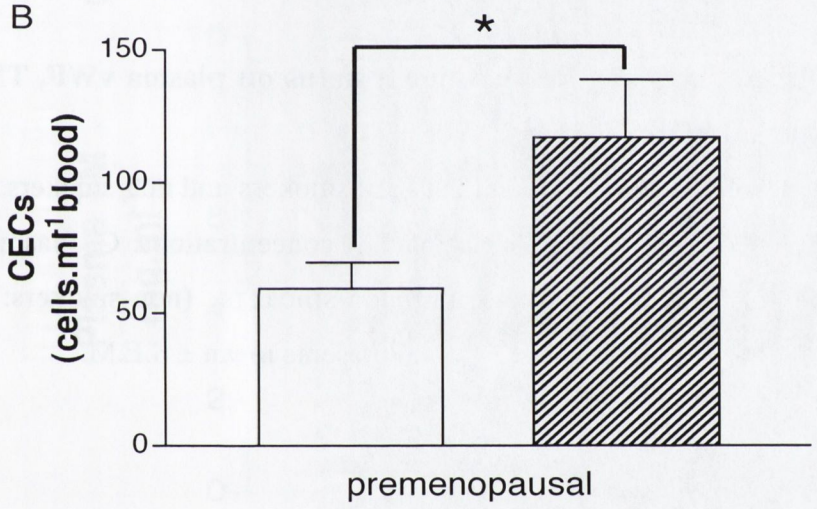
Figure 5.8 Effects of smoking on CECs.

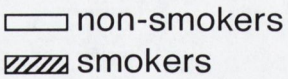
A. Premenopausal smokers had significantly greater numbers of CECs per ml blood than non-smokers (non-smokers: n=10, smokers: n=6). B. Postmenopausal smokers had significantly greater numbers of CECs per ml blood than non-smokers (non-smokers: n=10, smokers: n=7, * $P<0.05$, Student's *t*-test). Data represents mean ± S.E.M.

A


A
50μm
B
C
50μm

B


*
150
100
50
0
CECs
(cells.ml-1 blood)
premenopausal


non-smokers
smokers

C

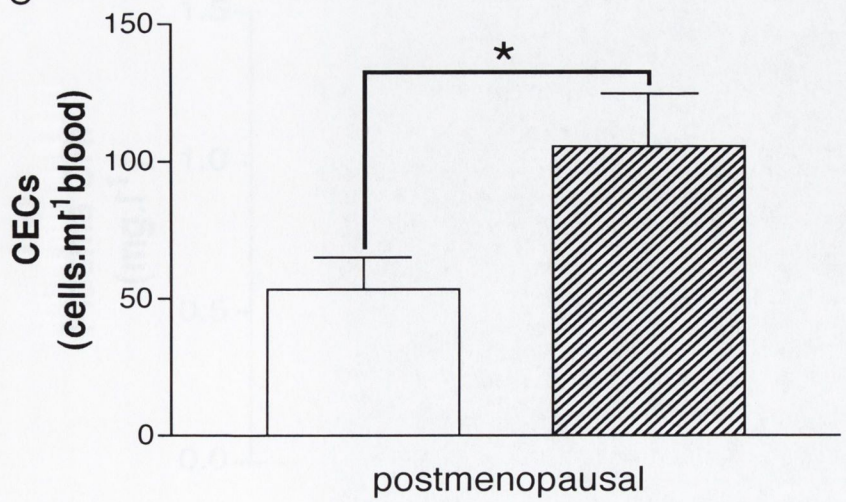

*
150
100
50
0
CECs
(cells.ml-1 blood)
postmenopausal

Figure 5.9 Effects of smoking and hormonal status on plasma vWF, TM and CRP in premenopausal women.

A. Plasma vWF levels were not different between smokers and non-smokers. B. No significant differences were found in plasma TM concentrations. C. Plasma CRP were not different between smokers and non-smokers. (non-smokers: n=10, smokers: n=6, $P<0.05$, Student's *t*-test). Data represents mean ± S.E.M.

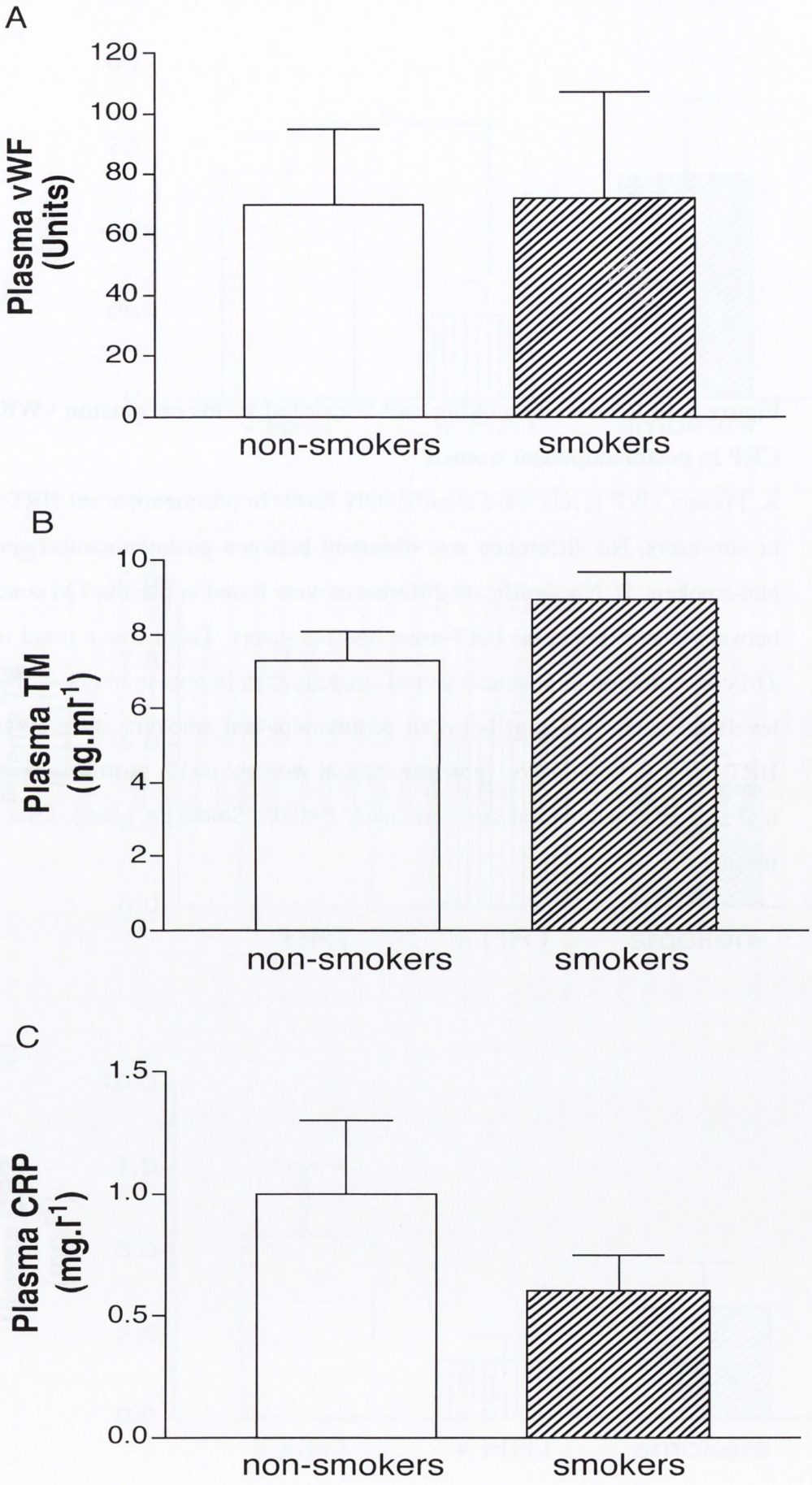

A
Plasma vWF (Units)
120
100
80
60
40
20
0
non-smokers
smokers
B
Plasma TM (ng.ml-1)
10
8
6
4
2
0
non-smokers
smokers
C
Plasma CRP (mg.l-1)
1.5
1.0
0.5
0.0
non-smokers
smokers

Figure 5.10 Effects of smoking and hormonal status on plasma vWF, TM and CRP in postmenopausal women

A. Plasma vWF levels were significantly lower in postmenopausal HRT users than in non-users. No difference was observed between postmenopausal smokers and non-smokers. B. No significant differences were found in plasma TM concentrations between postmenopausal HRT users and non-users. There was a trend for a lower TM concentration in postmenopausal smokers than in non-smokers. C. Plasma CRP levels were not different between postmenopausal smokers and non-smokers or HRT users and non-users. (postmenopausal women: n=10, postmenopausal + HRT: n=7 and postmenopausal smokers: n=7; $P<0.05$, Student's *t*-test). Data represents mean ± S.E.M.

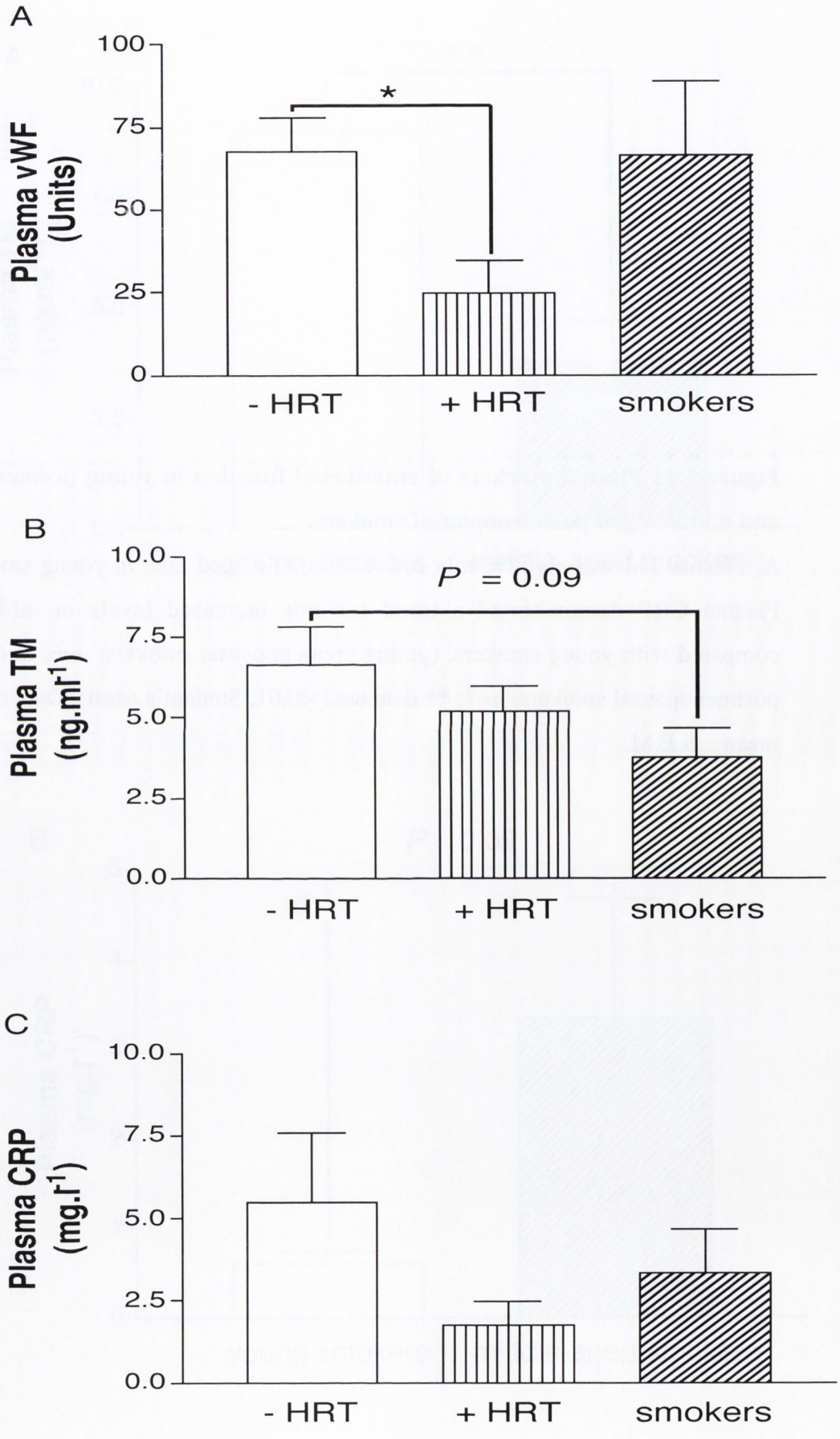

A
Plasma vWF (Units)
100
75
50
25
0
*
- HRT
+ HRT
smokers
B
Plasma TM (ng.ml-1)
10.0
7.5
5.0
2.5
0.0
P = 0.09
- HRT
+ HRT
smokers
C
Plasma CRP (mg.l-1)
10.0
7.5
5.0
2.5
0.0
- HRT
+ HRT
smokers

Figure 5.11 Plasma markers of endothelial function in young premenopausal and middle-aged postmenopausal smokers.

A. Plasma TM was significantly lower in middle-aged than in young smokers. B. Plasma CRP demonstrated a trend towards increased levels in middle-aged compared with young smokers. (young premenopausal smokers: n=6, middle-aged postmenopausal smokers: n=7; ** denotes $P<0.01$, Student's *t*-test). Data represents mean ± S.E.M.


A
**
10.0
7.5
5.0
2.5
0.0
Plasma TM
(ng.ml-1)
young smokers
middle-aged smokers


B
P = 0.09
5
4
3
2
1
0
Plasma CRP
(mg.l-1)
young smokers
middle-aged smokers

Figure 5.12 Plasma CRP levels.

A. CRP levels demonstrated a trend towards an increase in postmenopausal when compared to premenopausal women. B. Plasma CRP levels were increased with oral contraceptive use. (premenopausal women: n=10, postmenopausal women: n=10, oral contraceptive users n=10; * denotes $P<0.05$, Student's *t*-test). Data represents mean ± S.E.M.

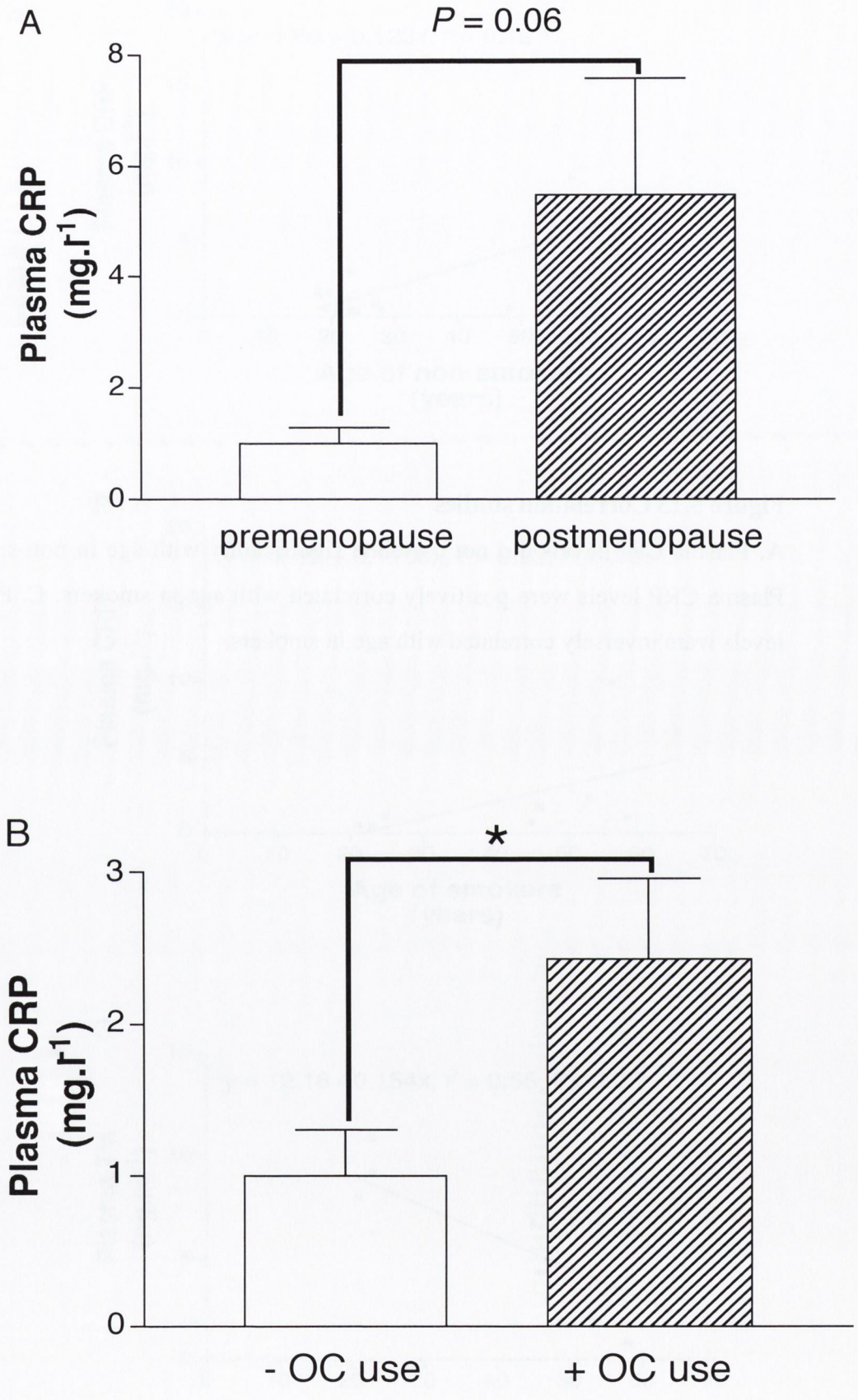

A
P = 0.06
Plasma CRP (mg.l-1)
8
6
4
2
0
premenopause
postmenopause
B
*
Plasma CRP (mg.l-1)
3
2
1
0
- OC use
+ OC use

Figure 5.13 Correlation studies

A. Plasma CRP levels did not correlate significantly with age in non-smokers. B. Plasma CRP levels were positively correlated with age in smokers. C. Plasma TM levels were inversely correlated with age in smokers.

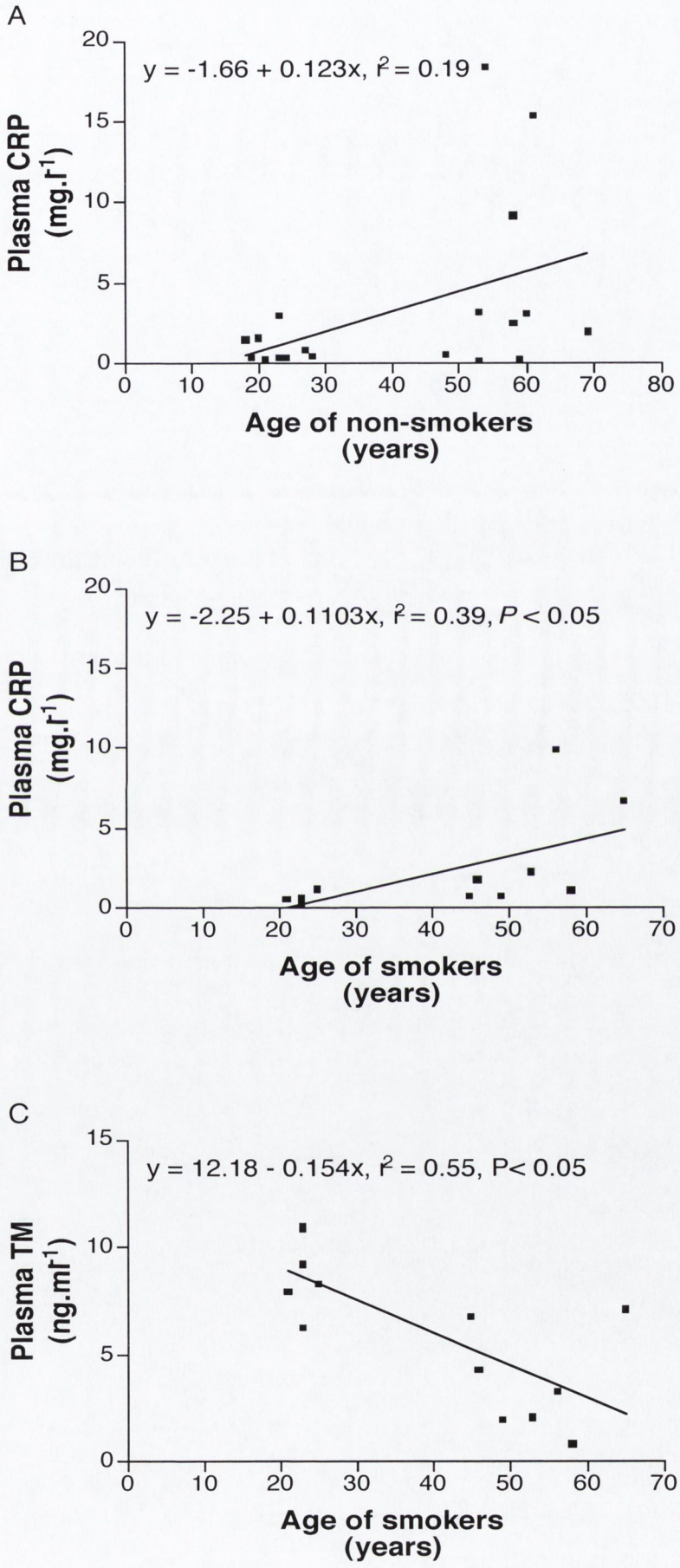
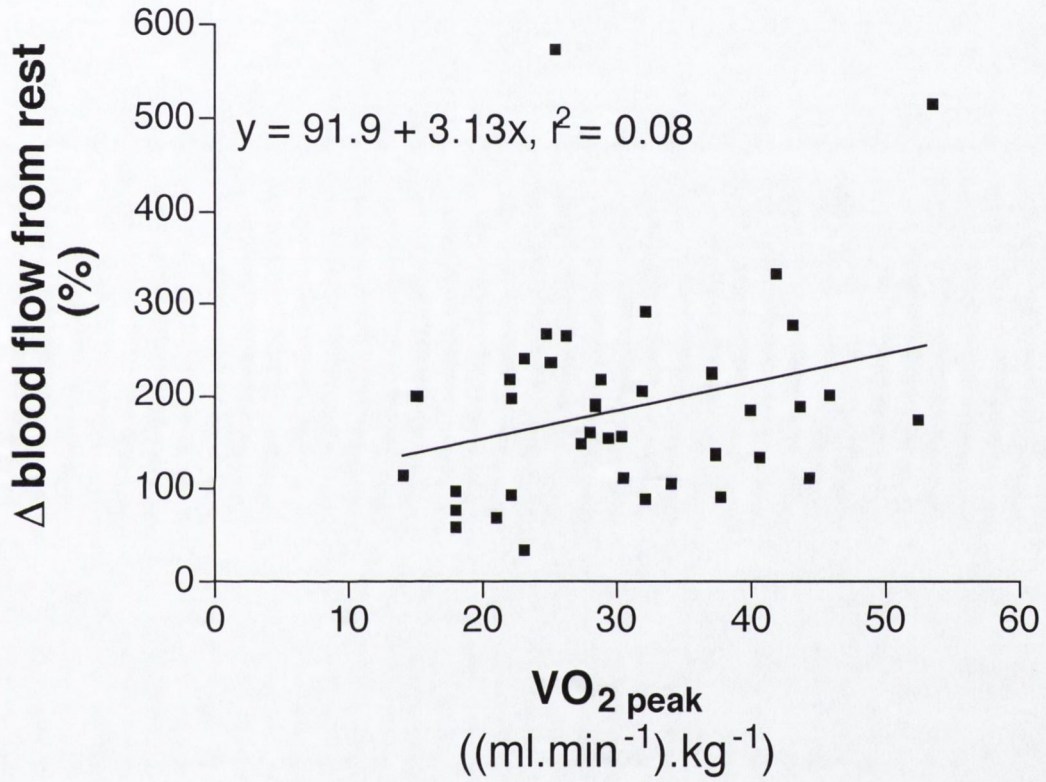

A
y = -1.66 + 0.123x, r² = 0.19
Plasma CRP (mg.l⁻¹)
Age of non-smokers (years)
B
y = -2.25 + 0.1103x, r² = 0.39, P < 0.05
Plasma CRP (mg.l⁻¹)
Age of smokers (years)
C
y = 12.18 - 0.154x, r² = 0.55, P< 0.05
Plasma TM (ng.ml⁻¹)
Age of smokers (years)

Figure 5.14 Correlation between % change in blood flow post occlusion and VO_{2peak}

VO_{2peak} did not have any effect on the magnitude of forearm blood flow increase in response to arterial occlusion.


Δ blood flow from rest (%)
600
500
400
300
200
100
0
y = 91.9 + 3.13x, r² = 0.08
0
10
20
30
40
50
60
VO2 peak
((ml.min-1).kg-1)

5.4 Discussion

The results of this study indicate that neither menstrual cycle phase nor oral contraceptive use alters the endothelium-dependent response to temporary arterial occlusion in young healthy women. No difference was observed in this response when comparing premenopausal and postmenopausal women, although a trend towards a lower percentage increase in flow after release of occlusion was evident in the postmenopausal cohort. HRT use did not influence the endothelium-dependent response in postmenopausal women. There was therefore virtually no alteration in endothelial dilator capacity with either ageing or hormonal withdrawal. In young smokers brachial artery vasoreactivity was unaltered by smoking status. Postmenopausal smokers however demonstrated a diminished vascular forearm reserve when compared with postmenopausal non-smokers and premenopausal smokers. Both young and older smokers presented with a 2-fold increase in CECs when compared with non-smokers but no differences in vWF levels were evident between the groups. This indicates a disparity between functional and biochemical markers of endothelial function in female smokers.

5.4.1 Endothelial responses to temporary arterial occlusion in young and middle-aged women

Other studies have found that endothelium-dependent vasodilatation of the brachial artery is modulated by menstrual cycle phase. Changes in brachial artery diameter in response to endothelial-dependent stimulants have been found to be greater during the follicular and luteal phases of the ovarian cycle compared with the menstrual phase (Hashimoto *et al.*, 1995; Kawano *et al.*, 1996; Arora *et al.*, 1998; Chan *et al.*, 2001). FBF in response to arterial occlusion has been found to correlate with serum oestradiol (Dietz, 1999) as have responses to endothelium-dependent and -independent vasodilators (Dietz, 1999). It was therefore suprising to see no difference in endothelium-dependent vasodilatation between the two phases of the menstrual cycle in the present study. This result cannot easily be explained. It is likely that differences in methodology are responsible. The majority of studies investigating vasoreactivity and its association with hormonal status look at brachial artery diameter using ultrasound and/or laser Doppler imaging as a measure of endothelium-dependent vasodilatation (Hashimoto *et al.*, 1995; Kawano *et al.*, 1996;

Arora *et al.*, 1998). Interestingly Kawano *et al.* (1996) although finding increased brachial artery diameter during the follicular and luteal phases of the menstrual cycle when compared with menses did not find any corresponding change in blood flow responses to reactive hyperaemia. An increase in brachial artery diameter therefore may not necessitate a corresponding increase in arterial blood flow. In fact Malik *et al.* (2001) found that peak blood flow response was more reproducible than FMD because of considerably lower intra-subject variability. Furthermore not all studies use reactive hyperaemia as a method of assessing endothelium-dependent vasodilatation. For example Arora *et al.* (1998) used ACh infusion and Chan *et al.* (2001) used bradykinin infusion. Kawano *et al.* (1996) used 5 mins reactive hyperaemia.

It is not clear whether NO levels change throughout the menstrual cycle. Some studies suggest that NO levels are unaltered by menstrual cycle phase (Merki-Feld *et al.*, 2000) while others have shown greater NO levels around ovulation than at menses (Taguchi *et al.*, 2000). 3-6 months of combined oral contraceptive use has been found to have no effect on NO levels (Merki-Feld *et al.* 2000). Few studies have been conducted into endothelium-dependent vasodilatation in response to oral contraceptive use however John *et al.* (2000) found that there was no difference in FBF responses to ACh infusion as measured by venous occlusion plethysmography in oral contraceptive users and non-users. This supports the results seen in the present study. Interestingly John *et al.* (2000) found that basal NO levels were enhanced in the oral contraceptive group.

Although no statistical difference between premenopausal and postmenopausal responses to arterial occlusion was observed in the present study there was a distinct trend (P=0.07) for postmenopausal women to have a lower percentage increase in FBF from rest than premenopausal women. Perhaps with a larger subject number a more conclusive result would have been obtained. Ageing itself is associated with structural and functional changes at the level of the endothelium, SMC and extracellular matrix (Matz and Andriantsitohaina, 2003). Ageing and menopause are associated with a decline in endothelium-dependent vasodilatation (Sarabi *et al.*, 1999). In the course of ageing, there is an alteration in the balance between relaxing and constricting factors released by the endothelium (Matz and Andriantsitohaina, 2003). A reduction in vasorelaxation occurs due to decreased NO and endothelium-derived hyperpolarising factor. This is associated

with increased vasoconstriction mediated by cyclooxygenase products such as thromboxane A_2. Also, the endothelin-1 and angiotensin II pathways as well as enhanced oxidative stress may play a role in age-related endothelial dysfunction (Matz and Andriantsitohaina, 2003).

The majority of studies that report a beneficial effect of oestrogen on cardiovascular risk profiles of middle-aged women use oestrogen-only preparations. Not many studies have looked at a combination of oestrogen and progesterone because of the infrequent use of combined therapy until recent years. Oral oestrogen therapy for 9 weeks enhances flow-mediated brachial artery dilator responses to hyperaemia (Lieberman *et al.*, 1994) and oestrogens have been shown to modulate endothelium-dependent vascular responses in the short-term (Williams *et al.*, 1992; Gilligan *et al.*, 1994). However chronic progesterone has been found to attenuate oestrogen-induced endothelium-dependent relaxation in canine coronary arteries (Miller and Vanhoutte, 1991). Progestins have been shown to inhibit oestradiol-induced synthesis of endothelium-derived NO (Rosano *et al.*, 2000; Xiao *et al.*, 2001). Combined oestrogen and progestin administration to postmenopausal women has been found not to increase serum nitrite and nitrate levels in contrast to increases in these products of NO measured in the same women when taking oestrogen alone (Rosselli *et al.*, 1995). In the present study there was considerable variation in the HRT regimens used by the participants and 4 of these were using combined oestrogen and progesterone HRT. Lower plasma levels of oestrogen during chronic administration of HRT as compared with acute infusion may add to discrepancies within the literature. The results of this present study correspond with others that found no difference in the long-term treatment of postmenopausal women with combined HRT (Sorensen *et al.*, 1998; Van Baal *et al.*, 1999; Teede *et al.*, 2001). Whether the HRT induced a transient initial effect on endothelium-dependent vasodilatation that was not discernible in the long-term is not known. Another possibility is that the women taking part in this study were more motivated compared to women that did not attend. Thus women who attended the screening, independent of hormonal status, were probably at lower cardiovascular risk.

It is a possibility that the effect of HRT on endothelial function is influenced by the route of administration. In the present study 2 out of 7 women were receiving transdermal oestrogen replacement therapy. Three weeks of oestrogen therapy via a transdermal patch has been shown not to have any effect on baseline blood flow

levels or on responses to ACh (Gilligan *et al.*, 1995). Vehkavaara *et al.* (2000) found that oral oestrogen replacement increased endothelium-dependent vasodilatation whereas transdermal oestradiol did not affect this response. Similarly Cagnacci *et al.* (1999) in a study using transdermal oestrogen over 8 weeks reported no significant changes in FMD. Jensen-Urstad and Johansson (2001) found no difference between those taking oral oestrogen replacement and those that were not and Haines *et al.* (2001) found a similar result in postmenopausal women with angina pectoris who were given combined HRT therapy or placebo.

5.4.2 Endothelial responses to temporary arterial occlusion in smokers

The aim of the second part of this study was to investigate the effects of smoking on endothelial function in women. Short-term smoking enhances endothelin-1-induced vasoconstriction (Kiowski *et al.*, 1994) and smoking can cause increases in arterial wall stiffness, which can potentially lead to hypertension, atherosclerosis and ischaemia (Kool *et al.*, 1993). Cigarette smoking has been found to impair (Celermajer *et al.*, 1993; Barry and Bell, 2002) but also to have no effect (Jacobs *et al.*, 1993) on endothelium-dependent vasodilatation in healthy young adults. Data are not widely available on the effects of smoking in women. Most studies use male subjects however it is not valid to compare responses in men with those in women. In the present study forearm vasoreactivity was comparable in young smokers and non-smokers consistent with the fact that smoking does not appear to result in permanent endothelial dysfunction in the forearm of young healthy women.

Although young non-smokers and smokers demonstrated no differences in FBF responses to temporary arrest of arterial blood flow a diminished forearm vascular reserve was evident when comparing middle-aged non-smokers and smokers. This could suggest that duration of smoking is important for the deleterious effects of smoking on endothelial vasodilatory function to become evident since older smokers were smoking for 4 times as long as young smokers although their daily cigarette intake was not dissimilar. It is also probable that the presence of female ovarian hormones in the premenopausal women protected them since a trend for diminished vascular reserve was seen in postmenopausal when compared with premenopausal non-smokers. To support this hypothesis smokers are known to reach menopause at an earlier age than do non-smokers (Willett *et al.*,

1983) and as already stated cardiovascular mortality risk is lower for women with a late menopause (DeKleijn *et al.*, 2002).

5.4.3 Blood markers of endothelial function

CECs are classified as *ex vivo* indicators of vascular injury (Dignat-Gorge and Sampol, 2000). CECs were found in preparations from all subjects in the present study. For examples of CECs isolated from the subjects in this study see Figure 6.8 A. The majority of cells showed no visible nucleus. Cells were often very weakly stained, which may mean that the cells were at the later stages of cell death. Patches of more darkly and of lightly stained areas were visible on many cells and may be evidence of the presence of vacuoles (Yu *et al.*, 1990).

There was no difference in CEC number between premenopausal and oral contraceptive users, pre-and postmenopausal women or HRT users and non-users. Ageing and loss of endogenous oestrogens at menopause did not therefore affect endothelial cell desquamation. This is supported by the fact that functionally endothelium-dependent vasodilatation was not very different between the groups.

In this study, smokers were found to have twice as many CECs as non-smokers. It was however striking that the absolute levels of CECs were no higher in women who had smoked for 30 years than in those who had smoked for only several years. No association existed between CEC concentration and functional evidence of endothelial integrity as reflected by reactive hyperaemia. Thus in young smokers there was elevation of CECs without any decrement in dilator responsiveness, while in older smokers the severe decline in dilator capacity was not accompanied by any further rise in CEC number over that seen in the young group. These facts suggest that the rise in CECs associated with smoking may not reflect endothelial damage.

Elevated numbers of CECs in smokers concurs with the literature (Sinzinger *et al.*, 1986; Sowemimo-Coker *et al.*, 1989). The values for endothelial cell number do seem to differ in the literature (see Table 1.1). This could be due to differences in methodology. Previous studies have used specialised cell counters and immunocapturing techniques, which may not detect older CEC carcasses.

The mechanism behind the increase in CEC number in this study is unclear. There are a number of mechanisms that may lead to cell death and desquamation. An increase in endothelial cell apoptosis is likely as the endothelial synthesis of NO is diminished in smokers while NO inhibits apoptosis (see Dimmeler and Zeiher

(1999) for review). Vayssier-Taussat *et al.* (2001) found that on exposure to tobacco smoke endothelial cells *in vitro* react via a stress response leading to an early step in the apoptotic pathway towards cell death. This group attributed this response to oxidative stress imposed by smoke. Another explanation is that smokers may have increased resting sympathetic activation than in non-smokers. SNS activation has been shown to increase endothelial cell death as measured by IgG while ß-adrenergic receptor inhibition abolishes this effect (Pettersson *et al.*, 1990). However smokers and non-smokers did not show any appreciable differences in resting cardiovascular parameters such as heart rate. Since cigarette smoking is a mediator of inflammation perhaps it damages the endothelium in this way. Alternatively increased CEC number could arise due to an increased endothelial cell turnover rate caused by direct stimulation of cell proliferation rather than as a consequence of cell death. Recently an *in vitro* study of cultured arterial tissue found that the smoke constituents nicotine and cotinine both upregulate endothelial growth factor expression (Conklin *et al.*, 2002).

While CEC number was found to increase in young smokers vWF, TM or CRP did not increase in this group. vWF levels are increased in hypertension (Blann *et al.*, 1993) and also with smoking (Blann, 1991; Smith *et al.*, 1993). However Blann (1991) found that this occurred only in cases where more than 20 cigarettes/day were consumed. In my study none of the young and not all of the middle-aged subjects smoked more than 20 cigarettes/day. There have been some investigations into the variations of vWF concentration during the menstrual cycle in women, however, a recent study with a large subject cohort found no cyclic variation (Önundarson *et al.*, 2001). HRT use decreased substantially the vWF concentration in the present study and this is in accordance with other studies that found decreases with transdermal oestrogen and combined HRT (van Baal *et al.*, 1999; Lip *et al*, 1997; Seljeflot *et al.*, 2000). The results would suggest that effects of sex hormones on the level of vWF do not necessarily reflect changes in endothelial cell function.

Baseline TM levels have been found to be higher (Markuljak *et al.*, 1995) and lower (Blann *et al.*, 1997) in smokers than in non-smokers however, Markuljak *et al.* (1995) found that immediately after smoking levels remain unchanged. sTM levels were lower in older smokers than in younger smokers and a trend for decreased TM levels in postmenopausal smokers than in non-smokers was found

(P=0.09). Elevated plasma TM levels are thought to reflect endothelial injury (Seigneur *et al.*, 1993). However there are some discrepancies in the literature. Recently it was shown that plasma TM levels were significantly lower in subjects who developed coronary artery disease than in controls (Salomaa *et al.*, 1999) and that at increased TM levels coronary artery disease risk decreased. TM could potentially protect against undesirable coagulation. Also oxidised LDL-induced down-regulation of TM expression has been found (Ishii *et al.*, 1996) and smoking increases oxidised LDL (Tsiara *et al.*, 2003). TNFα and IL-1 are thought to down-regulate TM *in vitro* through transcription inhibition and internalisation and degradation by lysosomes (Hirokawa and Aoki, 1991). Whether this occurs *in vivo* is not known.

CRP is an acute phase reactant induced by cytokines such as IL-1, IL-6 and TNF-α, which are released from macrophages and other activated cells within the vessel wall during an inflammatory process. CRP can induce other cytokines, cell adhesion molecules, and tissue factor in monocytes and endothelial cells (Cermak *et al.*, 1993; Gabay and Kushner, 1999; Pasceri *et al.*, 2000). CRP may contribute to atherogenesis by facilitating uptake of LDL by macrophages, thus accelerating foam cell formation (Zwaka *et al.*, 2001). CRP has a direct link with endothelial dysfunction (Cleland *et al.*, 2000) and has been shown to be an independent predictor of reduced vasodilatory capacity in coronary artery disease (Fichtlscherer *et al.*, 2000). It has been found that women who develop cardiovascular events have higher baseline CRP levels than controls (Ridker *et al.*, 1998). Although most studies suggest a link between smoking and CRP levels (de Ferranti and Rifai, 2002) some contradict this assertion (Ridker *et al.*, 1997). Tracy *et al.* (1997) found that CRP levels were correlated with pack years of smoking and this would correspond to the present study where no difference was found between CRP levels in young smokers and non-smokers while a trend towards greater CRP levels in middle-aged women was found. This is supported by the fact that there again was a trend toward greater CRP levels in postmenopausal than premenopausal smokers. It is still not clear whether the effects of smoking on CRP are due to the smoke directly or to smoking-induced inflammation. Many studies have found increased CRP levels in women taking HRT when compared to those that were not (Cushman *et al.*, 1999; Ridker *et al.*, 1999; Herrington *et al.*, 2001). In contrast transdermal administration of oestrogen replacement therapy significantly lowered CRP levels (Modena *et al.*,

2002; Sattar *et al.*, 1999) or left it unaltered (Vehkavaara *et al.*, 2001) in postmenopausal women. Van Baal *et al.* (1999) found no difference in CRP levels with combined HRT. No difference in CRP levels was found in the present study perhaps because of the variation in HRT regimen used. There was however an increase in CRP levels with oral contraceptive use. Oral contraceptive use is associated with an increase in the incidence of inflammatory disease (Swan *et al.*, 1981) and although not many studies have looked at CRP levels in users, Kay *et al.* (1971) found an increase in CRP with oral contraception. In a recent study plasma CRP levels were found to be twice as high in oral contraceptive users as in non-users (Dreon *et al.*, 2003). A similar situation was found in the present study. Inflammation markers also have been recently shown to be important and independent risk factors for CVD in postmenopausal women (Ridker *et al.*, 2000). Little information is available on menopause-related differences in inflammatory markers however one recent study found no difference between CRP levels in postmenopausal and premenopausal women (Sites *et al.*, 2002). The fact that there was a trend towards increased levels in postmenopausal women in the present study (P=0.06) suggests that they have increased cardiovascular risk. However CRP elevation may not always indicate an inflammatory process. CRP levels can be increased by obesity, low levels of physical activity and ageing (Kushner, 2001).

Conclusions

Neither menstrual cycle phase nor oral contraceptive use influences endothelial responses to temporary arterial occlusion in young females. A trend towards diminished endothelium-dependent peak FBF is evident in postmenopausal when compared with premenopausal women possibly due to a deficit of ovarian hormones and ageing itself. Long-term HRT does not affect vasoreactivity and vascular changes seen with HRT may depend on the types of steroids used, the dose and the route of administration. It is possible that the addition of a progestin, although protecting the uterus form the harmful effects of unopposed oestrogen, might negate some of the beneficial cardiovascular effects of oestrogen.

Long-term smoking attenuates endothelium-dependent vasodilatation in middle-aged smokers when compared with middle-aged non-smokers and younger female smokers. Ageing, number of cigarettes consumed per day and duration of

smoking may play an important role in this decline. CECs are increased by cigarette smoke possibly due to increased cell turnover, cell apoptosis or desquamation. While CRP appears to be increased with smoking TM is lowered and vWF is unaffected. There is therefore dissociation between changes in endothelium-dependent dilator capacity and changes in any of a range of plasma markers that are generally accepted as reflecting endothelial dysfunction.

Chapter 6 'Effects of hypertension and gender on baroreflex sensitivity in the rat'

6.1 Introduction

In the previous Chapters the role of female sex steroids on SNS activity and endothelial function was investigated. To elucidate whether long-term hormonal manipulation of male and female sex hormones contributes to gender differences in the maintenance of blood pressure, BRS was measured. BRS is not easily assessed in humans so a rat model was used. Since both a normal and a genetically hypertensive strain of rat were available, it was also possible to look at the effects of hypertension on BRS.

By the 1950s the possibility of hereditary transmission of hypertension from parents to off-spring was recognised. However no animal strains were available as a model of this type of hypertension. A result was the breeding of normotensive rats with above average blood pressures, which gave rise to the first genetically hypertensive rats (Phelan, 1968). These rats originated from the Wellcome Medical Research Institute, University of Otago, New Zealand and hence are officially referred to as New Zealand Genetically Hypertensive (GH) rats. These rats are born exclusively from the mating of male and female littermates with no random breeding. They are different from the widely studied SHR strain in so far as the hypertensive trait in the SHR is carried only by a small number of genes whereas that in the GH rat involves many genes (Phelan, 1968; Tanase *et al.*, 1970; Okamoto *et al.*, 1972). The GH rat strain may be extremely useful in investigating human hypertension as human hypertension also seems to be carried by a large number of genes (Dominiczak *et al.*, 2000).

Defective cardiovascular autonomic control can lead to lower levels of baroreflex gain, which are associated with hypertension. Baroreceptor reflex regulation has been shown to reset towards a higher blood pressure level and to operate with reduced sensitivity in hypertension (Mussalo *et al.*, 2002). Reduced BRS may contribute to the persistence of blood pressure elevation or indeed may be a secondary consequence of hypertension. However the degree of baroreflex impairment has been shown to be related to severity of hypertension (Mussalo *et al.*,

2002). Decreased BRS is also associated with an increased risk of CVD mortality (LaRovere *et al.*, 1998). Those with a familial history of hypertension have lower BRS than those without a family history, regardless of blood pressure level (Parmer *et al.*, 1992). These results suggest that depression of BRS may be genetically determined. BRS is lower in SHR than in WKY before the onset of hypertension (Andresen *et al.*, 1980). Attenuated baroreflex gain has also been demonstrated in the Lyon genetically hypertensive rat (Lantelme *et al.*, 1998) and in the New Zealand genetically hypertensive strain (Brady *et al.*, (2002).

An important influencing factor on blood pressure regulation is gender with males having higher blood pressures than age-matched females (Wiinber *et al.*, 1995). The gender-associated differences in blood pressure observed in humans have also been documented in various animal models. Male SHR (Ganten *et al.*, 1989), Dahl salt-sensitive rats (Croften *et al.*, 1993), deoxycorticosterone-salt hypertensive rats (Ouchi *et al.*, 1987) and the New Zealand GH rat (Ashton and Balment, 1991) have elevated blood pressure compared with females. There therefore may be gender differences in the pathophysiology of hypertension. The importance of gender as a determinant of autonomic cardiovascular control has led investigators to look at the effect of hypertension and gender on baroreflex control of blood pressure.

Some studies suggest that oestrogen administration improves BRS. A decrease in sympathetic tone, an attenuated pressor response to phenylephrine and an enhanced depressor response to sodium nitroprusside have been observed in animals injected with oestrogen (Saleh and Connell, 2000). Saleh *et al.* (2000) found that in oestrogen-replaced animals administration of oestrogen into the central autonomic nuclei in the brain caused a decrease in baseline MAP and renal SNA. Further decreases in MAP and renal SNA were also noted in these animals following phenylephrine infusion when compared with controls. Phenylephrine-evoked changes in heart rate and vagal parasympathetic nerve activity have been found to be enhanced (Saleh and Connell, 2000) while sodium nitroprusside produces attenuated tachycardic responses with oestrogen administration (Saleh *et al.*, 2000). Changes in cardiovascular responses to vasoactive agents may at least in part, be due to increased BRS since during phenylephrine infusion, the ratios of reductions in heart rate and SNA to increases in MAP are greater in oestrogen-treated animals than in controls (He *et al*, 1998).

However, there continues to be some debate as to the exact role of oestrogen in relation to the baroreflex. Results are somewhat conflicting since some studies show that young women exhibit smaller heart rate decreases on administration of phenylephrine compared with men (Abdel-Rahman *et al.*, 1994). Similarly a lower reflex bradycardic response has been observed in middle-aged women compared with age-matched men (Huikuri *et al.*, 1996) and there is a large body of evidence to suggest that BRS is reduced in women (Abdel-Rahman *et al.*, 1994; Huikuri *et al.*, 1996; Convertino, 1998; Laitinen *et al.*, 1998). Some experimental evidence indicates that baroreflex function is attenuated in female compared with male rats (El-Mas and Abdel-Rahman, 1998; Abdel-Rahman, 1999). Testosterone has been shown to enhance baroreflex function in male rats while castration diminishes this reflex (El-Mas *et al.*, 2001). Sevre *et al.* (2001) found that BRS was diminished in hypertensive subjects when compared with normotensive subjects and that hypertensive female subjects had comparably lower BRS than hypertensive men. This group found a positive correlation between systolic pressure and BRS in the female hypertensive group only. Studies therefore suggest that autonomic dysfunction may play a more important role in female than in male hypertension.

The reason for the sex differences observed in BRS and whether it involves neural, hormonal or developmental factors are unclear. Often only the vagal limb of the baroreflex is investigated and so not much is known about the sympathetic limb. The disparity within the literature is immense and cannot easily be explained. However the effects of oestrogen on baroreceptor function and the SNS are likely to have important implications for cardiovascular regulation.

With this in mind the objective of this present study was to determine whether BRS is altered differentially by hypertension and/or gender and whether the absence of gonadal hormones via gonadectomy influences the baroreflex.

6.2 Methods

6.2.1 Animals used

Age-matched adult male and female (14-18 weeks) normotensive (N) and New Zealand genetically hypertensive (GH) Wistar rats (n=12 per group) were used in the study. N (n=8 per group) and GH (n=12 per group) rats were either gonadectomised at three-four weeks or left intact. The control animals and GH groups were fed a normal laboratory chow diet. However dietary phyto-oestrogens have recently been shown to have an impact on cardioprotection in oestrogen-depleted females (Fang *et al.*, 2001). Therefore the control and gonadectomised normotensive animals in this study were fed a diet designed to reduce phyto-oestrogens and excluded soybean and alfalfa ingredients (GEFCO UK, Lillico Ltd., Surrey).

6.2.2 Surgery

Male GH and N rats were castrated. Briefly, the rat was anaesthetised (i.p.) with ketamine hydrochloride 50 mg/kg (Parke-Davis veterinary) and xylazine 10 mg/kg (Bayer AG). A single 1 cm incision was made in the scrotum. The testes were isolated and removed. The skin was sutured. Female GH and N rats were bilaterally ovariectomised. The rat was anaesthetised with ketamine hydrochloride 50 mg/kg and xylazine 10 mg/kg. A 1-2cm incision was made in the lateral abdominal muscles and a further incision was made in the muscles overlying the ovaries on both sides and the ovaries were isolated and removed.

An antibiotic powder (Cicatrin, Wellcome) was put on all post-surgery wounds and each rat received a subcutaneous injection of flunixin (Schering-Plough Animal Health) (2.5 mg/kg) to control pain.

6.2.3 Experimental protocol

The experimental protocol used was described in detail in Chapter 2. Briefly, the blood pressure integrity of the animals was measured using tail-cuff plethysmography. All N animals had systolic blood pressures < 145 mmHg and GH animals > 170 mmHg. Animals were anaesthetised with urethane (1.25 g/kg i.p.) and placed in a supine position on a stainless-steel operating table (Palmer Bioscience, U.K.) maintained at 34°C. A tracheal tube was inserted into the trachea

just below the larynx to maintain airway patency. The right femoral artery was cannulated with polythene tubing for recording of intra-arterial pressure. A polythene catheter was placed in the right internal jugular vein for infusion of vasoactive substances.

A three-lead surface ECG was recorded continuously via transcutaneous needle electrodes and displayed together with a beat-to-beat ratemeter triggered from each QRS complex. Blood pressure was measured continuously from the abdominal aorta via the femoral artery by a blood pressure transducer (AD Instruments).

A period of 15 minutes was allowed before recording for stabilisation of blood pressure and heart rate measurements. All parameters were displayed and stored using a PowerLab system (AD Instruments).

6.2.4 Drugs

A stock solution of phenylephrine (10 mg/ml) was prepared and further diluted to 140 μg/ml with saline (0.9%). Sodium nitroprusside (200 mM stock solution) was diluted to 0.5 mM with saline.

6.2.5 Baroreflex testing

BRS was evaluated by intravenous infusions of two separate doses of sodium nitroprusside (1.25×10^{-5} and 1×10^{-4} mmol.min^{-1}) and phenylephrine (3.77×10^{-5} and 1.51×10^{-4} mmol.min^{-1}) to elicit depressor and pressor responses. Subsequent infusions were made only after blood pressure returned to pre-infusion values, which usually took 5 minutes. A subset of rats was given infusions of saline at the same rates as that used for sodium nitroprusside and phenylephrine to ensure changes in blood volume, which could potentially have effects on venous return and hence atrial and ventricular pressure, did not influence the baroreflex gain. There was no difference in blood pressure (BP) or heart rate (HR) before or after infusion (pre-infusion BP: 134 ± 8.2 cf post: 135 ± 8.4 mmHg and pre-infusion HR: 386 ± 10.0 cf post: 385 ± 9.9 bpm).

Each vasoactive substance was infused until a stable plateau in blood pressure and heart rate was observed. This occurred within 30 s of the start of infusion. The catheter was flushed with heparinised saline (0.1 ml) between infusions to prevent contamination and blood clotting within the catheter.

Continuous infusion of either sodium nitroprusside or phenylephrine allowed the assessment of the effect of decreasing and increasing intra-arterial pressure, respectively, on heart rate. Baroreflex gain was assessed in terms of the peak change in heart rate per unit change in mean arterial pressure (ΔHR and ΔMAP) independent of dose.

6.2.6 Statistical analysis

The relationship between changes in blood pressure and heart rate was assessed by regression analysis. The slope of the regression line expressed as beats per minute per millimeters of mercury was taken as an indication of reflex control of heart rate i.e. BRS. Actual values in the barcharts are those obtained by the division of ΔHR by ΔBP for each animal. In order to determine the variability of the relationship a Pearson's correlation test was employed. Statistically significant differences between means were calculated using a 1-way ANOVA followed by a Student Newmann Keuls post-hoc test and a Student's unpaired *t*-test. $P<0.05$ was deemed significant. All data are expressed as mean ± S.E.M.

6.3 Results

6.3.1 Resting animal characteristics

Table 6.1. Resting characteristics of normotensive female (N F), normotensive male (N M), genetically hypertensive female (GH F) and genetically hypertensive male (GH M) animals. n = 12 in all groups. All values are expressed as mean ± S.E.M.

	N F	*GH F*	*N M*	*GH M*
Age (weeks)	14-18 weeks	14-18 weeks	14-18 weeks	14-18 weeks
Body Weight (g)	238±6.9	190±6.7§§§	346±5.6***	298±12.2§§***
SBP tail-cuff (mmHg)	129±2.9	199±4.8§§§	132±2.9	196±4.7§§§
MAP Intra-arterial (mmHg)	114±5.9	150±2.8§§§	106±5.1	146±4.6§§§
Heart rate (bpm)	390±7.8	357±6.8§§	404±12.9	356±8.3§§

* shows a significant difference between male and female animals within the one strain, § shows a significant difference between animals of the same gender between strains. */§ $P<0.05$, **/§§ $P<0.01$, ***/§§§ $P<0.001$.

Table 6.2 Resting characteristics of normotensive female control (con F), normotensive ovariectomised female (OV F), normotensive male control (con M) and normotensive castrated male (Cast M) animals. All values are expressed as mean ± S.E.M.

	con F	*OV F*	*Con M*	*Cast M*
Age (weeks)	14 - 18 weeks	14 - 18 weeks	14 -18 weeks	14 - 18 weeks
Body Weight (g)	264±14.4	259±14.4	377±8.1***	315±7.1§§§
SBP tail-cuff (mmHg)	118±4.4	116±8.8	129±7.0	136±5.9
MAP intra-arterial (mmHg)	109±7.4	101±3.4	116±7.8	107±6.6
Heart rate (bpm)	376±15.5	387±9.6	394±13.2	383±11.9

* shows a significant difference between male and female animals within the one strain, § shows a significant difference between intact and gonadectomised animals. ***/§§§ $P<0.001$.

Table 6.3 Resting characteristics of genetically hypertensive female control (con F), genetically hypertensive ovariectomised female (OV F), genetically hypertensive male control (con M) and genetically hypertensive castrated male (Cast M) animals. n = 12 in all groups. All values are expressed as mean ± S.E.M.

	con F	*OV F*	*Con M*	*Cast M*
Age (weeks)	14 - 18 weeks	14 - 18 weeks	14 -18 weeks	14 - 18 weeks
Body Weight (g)	202±6.4	263±9.2§§§	298±6.9***	312±6.1
SBP tail-cuff (mmHg)	192±4.7	200±3.5	204±3.5	206±6.7
MAP intra-arterial (mmHg)	153±4.3	158±7.8	138±4.5*	146±5.8
Heart rate (bpm)	403±11.3	399±15.0	383±6.2	370±10.6

* shows a significant difference between male and female animals within the one strain, § shows a significant difference between intact and gonadectomised animals. */§ $P<0.05$, **/§§ $P<0.01$, ***/§§§ $P<0.001$.

6.3.2 Resting blood pressure and heart rate

GH male and female animals had significantly higher tail-cuff and intra-arterial blood pressure than their normotensive counterparts (see Table 6.1). There was no difference in MAP between normotensive male and female animals or between GH male and female animals (see Table 6.1). Normotensive animals had significantly greater heart rates than GH animals however there was no difference between the genders (see Table 6.1). No differences in resting blood pressure or heart rate were evident between gonadectomised and intact animals in either the normotensive or hypertensive strain (see Table 6.2 and Table 6.3).

6.3.3 Responses to vasoactive drugs in normotensive and genetically hypertensive animals

Both strains and genders responded to the same concentrations of sodium nitroprusside and phenylephrine. There was no difference in the maximal (high-dose) or minimum (low-dose) pressor or depressor response (corresponding to the two doses of substance infused) between control male and female animals of the same strain (see Table 6.4). However, GH animals exhibited significantly greater maximal responses than their normotensive counterparts (see Table 6.4). When

looking at the minimum pressor and depressor responses there was no difference between the magnitude of blood pressure change between normotensive and GH rats or between the genders (see Table 6.5).

Table 6.4 Maximum depressor and pressor responses (mmHg) to sodium nitroprusside (SNP) and phenylephrine (PE) infusion. n = 12 in all groups. All values are expressed as mean ± S.E.M.

	N Female	N male	GH Female	GH Male
SNP	55±5.0	53±5.5	83±5.5**	76±5.5*
PE	71±8.0	74±7.9	90±6.6	98±5.4*

* shows a significant difference between normotensive and GH animals. * $P<0.05$, ** $P<0.01$.

Table 6.5 Minimum depressor and pressor responses (mmHg) to sodium nitroprusside and phenylephrine infusion. n = 12 in all groups. All values are expressed as mean ± S.E.M.

	N Female	N male	GH Female	GH Male
SNP	36±4.2	29±4.1	35±5.4	43±7.1
PE	28±8.2	39±6.9	51±8.6	43±5.3

6.3.4 Responses to vasoactive drugs in intact and gonadectomised normotensive and genetically hypertensive animals

Both intact and gonadectomised rats responded to the same concentrations of sodium nitroprusside and phenylephrine. Gonadectomy had no effect on maximal pressor and depressor responses (see Table 6.6 and Table 6.7) or minimum pressor and depressor responses (see Table 6.8 and Table 6.9) to vasoactive substances in normotensive and GH rats.

Table 6.6 Maximum depressor and pressor responses (mmHg) to sodium nitroprusside and phenylephrine infusion in N rats. n = 8 in all groups. All values are expressed as mean ± S.E.M.

	con Female	con male	OV Female	Cast Male
SNP	44±4.6	36±3.0	36±1.3	35±5.3
PE	87±10.2	70±10.5	73±10.6	81±11.3

Table 6.7 Maximum depressor and pressor responses (mmHg) to sodium nitroprusside and phenylephrine infusion in GH rats. n = 12 in all groups. All values are expressed as mean ± S.E.M.

	con Female	con male	OV Female	Cast Male
SNP	67±5.7	57±4.0	76±6.4	63±4.2
PE	106±4.5	112±6.7	90±7.2	111±5.3

Table 6.8 Minimum depressor and pressor responses (mmHg) to sodium nitroprusside and phenylephrine infusion in N rats. n = 8 in all groups. All values are expressed as mean ± S.E.M.

	con Female	con male	OV Female	Cast Male
SNP	25±4.4	23±3.0	22±2.8	23±4.5
PE	48±7.3	45±8.7	52±8.5	50±7.8

Table 6.9 Minimum depressor and pressor responses (mmHg) to sodium nitroprusside and phenylephrine infusion in GH rats. n = 12 in all groups. All values are expressed as mean ± S.E.M.

	con Female	con male	OV Female	Cast Male
SNP	40±5.1	34±3.2	47±5.9	43±5.5
PE	66±6.9	64±7.7	59±9.2	69±8.3

6.3.5 Baroreflex gain in normotensive and genetically hypertensive control animals

Regression lines relating maximum changes in heart rate evoked by each vasoactive substance and reciprocal changes in blood pressure were constructed, and the slopes of the regression lines were taken as an indication of BRS. The line was

put through zero to obtain the slope and then without placing the line through zero the r^2 value was obtained (see Figure 6.1 and 6.2 for control sodium nitroprusside and Figure 6.3 and 6.4 for control phenylephrine graphs, respectively).

There was no difference between reflex tachycardia in either the normotensive or GH animals or between the sexes (see Figure 6.5).

When examining the effect of gender within the one strain a significantly greater bradycardic response in normotensive males than in normotensive females was observed and male GH animals had a tendency towards greater reflex bradycardia than their female counterparts (see Figure 6.6). The baroreflex gain evident during phenylephrine infusion was over three-fold greater in normotensive than GH animals (see Table 6.7 and Figure 6.6). To investigate whether the difference in reflex bradycardia was in any way related to resting blood pressure levels regression analysis was carried out (see Figure 6.7). No significant relationship was observed in any of the animal groups.

In normotensive animals absolute gain for bradycardic responses to pressure elevation were significantly greater than the absolute gain for tachycardic responses to blood pressure depression (see Table 6.10). For GH males a similar situation was seen. No difference in gains between either limb of the baroreflex was found in GH females (see Table 6.10).

Table 6.10 Reflex tachycardic and bradycardic responses to sodium nitroprusside and phenylephrine infusion respectively, in N and GH control animals. n = 12 in all groups. All values are expressed as mean ± S.E.M.

	N Female	N male	GH Female	GH Male
Tachycardic (bpm.mmHg^{-1})	0.16±0.036	0.21±0.032	0.20±0.044	0.20±0.036
Bradycardic (bpm.mmHg^{-1})	0.68±0.113#	0.98±0.157§#	0.21±0.031**	0.31±0.041***#

* shows a significant difference between normotensive and GH animals. § shows a difference between male and female animals, # shows a difference between the two limbs of the baroreflex within the one group. */§/# $P<0.05$, ** $P<0.01$, *** $P<0.001$

6.3.6 Baroreflex gain in intact and gonadectomised normotensive animals

For regression lines relating maximum changes in heart rate evoked by each vasoactive substance and changes in blood pressure see Figures 6.8 and 6.9 for sodium nitroprusside and Figure 6.10 and 6.11 for phenylephrine.

There was no difference in the tachycardic response to blood pressure depression between male and female normotensive or intact and gonadectomised animals (see Figure 6.12). Bradycardic responses to phenylephrine infusion were greater in N males than females (see Figure 6.13) while ovariectomised and castrated animals had similar responses to their intact counterparts.

The bradycardic response to pressure elevation was significantly greater than the tachycardic response to pressure depression in all groups (see Table 6.11).

Table 6.11 Reflex tachycardic and bradycardic responses to sodium nitropruside and phenylephrine infusion, respectively in normotensive animals

n = 8 in all groups. All values are expressed as mean ± S.E.M.

	con Female	OV Female	con Male	Cast Male
Tachycardic (bpm.mmHg^{-1})	0.04±0.019	0.07±0.030	0.20±0.090	0.12±0.050
Bradycardic (bpm.mmHg^{-1})	0.52±0.094###	0.64±0.110###	1.06±0.225##	0.75±0.139##

* shows a significant difference between male and female animals. # shows a difference between the two limbs of the baroreflex within the one group */# $P<0.05$, **/## P<0.01, ***/### P<0.001.

6.3.7 Baroreflex gain in intact and gonadectomised genetically hypertensive animals

For regression lines relating maximum changes in heart rate evoked by each vasoactive substance and changes in blood pressure see Figure 6.14 and 6.15 for sodium nitroprusside and Figure 6.16 and 6.17 for phenylephrine.

Intact females were found to have increased tachycardic responses to sodium nitroprusside infusion when compared with control males (see Figure 6.18). There was no difference in bradycardic responses to phenylephrine infusion between the

genders (see Figure 6.19). Ovariectomised females did not exhibit any differences in tachycardic responses when compared with controls (see Figure 6.18) and the same was evident when comparing bradycardic responses (see Figure 6.19). When comparing castrated and intact male animals a similar result was found (see Figures 6.18 and 6.19, respectively).

The bradycardic response to pressure elevation was significantly greater than the tachycardic response to pressure depression in control males, castrated males and ovariectomised females (see Table 6.12). However no difference in gains between either limb of the baroreflex was observed in female controls (see Table 6.12).

Table 6.12 Reflex tachycardic and bradycardic responses to sodium nitroprusside and phenylephrine infusion, respectively in genetically hypertensive animals. n = 12 in all groups. All values are expressed as mean ± S.E.M.

	con Female	OV Female	con Male	Cast Male
Tachycardic (bpm.mmHg^{-1})	0.26±0.031*	0.29±0.041	0.12±0.028	0.11±±0.021
Bradycardic (bpm.mmHg^{-1})	0.33±0.025	0.38±0.069#	0.28±0.027#	0.28±0.039#

* shows a significant difference between male and female animals. # shows a difference between the two limbs of the baroreflex within the one group */# $P<0.05$.

Figure 6.1 Regression graphs relating maximum changes in heart rate (Δ HR) to reciprocal changes in blood pressure (Δ BP) in response to sodium nitroprusside infusion. A. Normotensive females. B. Normotensive males. C. Genetically hypertensive females. D. Genetically hypertensive males. n=12. y=slope of line through zero.

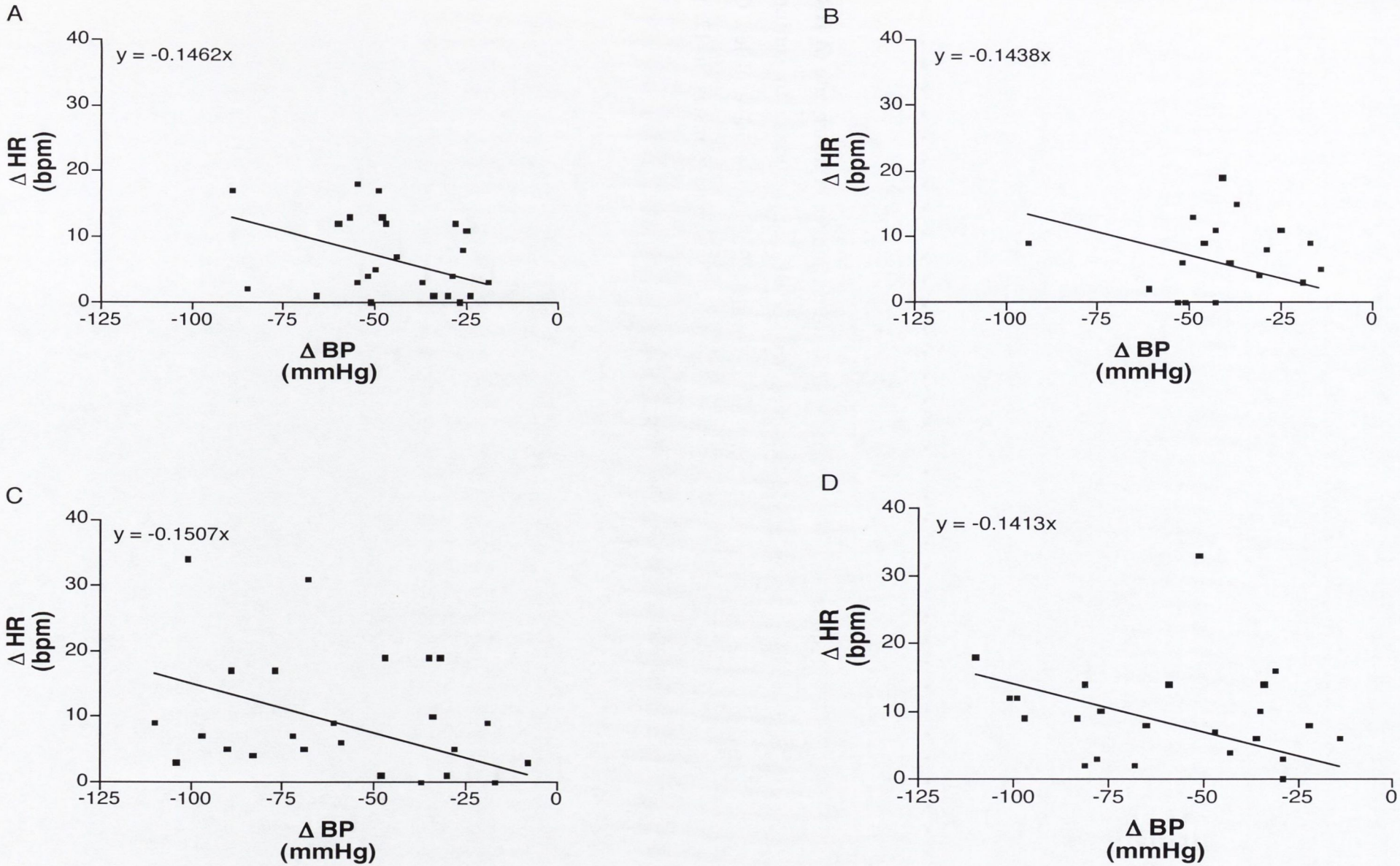

A
y = -0.1462x
Δ HR (bpm)
Δ BP (mmHg)
B
y = -0.1438x
Δ HR (bpm)
Δ BP (mmHg)
C
y = -0.1507x
Δ HR (bpm)
Δ BP (mmHg)
D
y = -0.1413x
Δ HR (bpm)
Δ BP (mmHg)

Figure 6.2 Regression graphs relating maximum changes in heart rate (Δ HR) to reciprocal changes in blood pressure (Δ BP) in response to sodium nitroprusside infusion. A. Normotensive females. B. Normotensive males. C. Genetically hypertensive females. D. Genetically hypertensive males. n=12. r^2 represents the correlation coefficient.

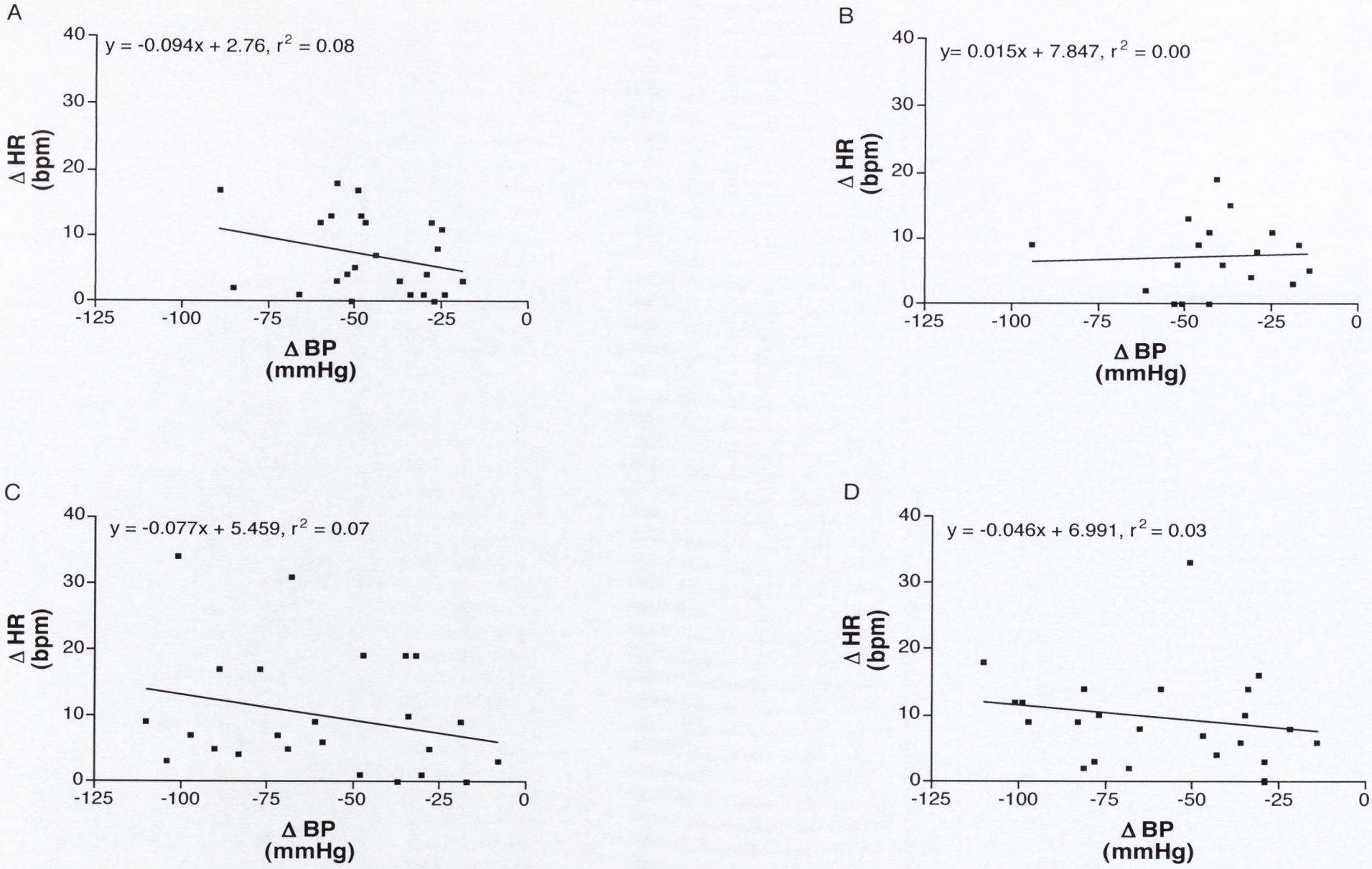

A
y = -0.094x + 2.76, r2 = 0.08
Δ HR (bpm)
Δ BP (mmHg)
B
y= 0.015x + 7.847, r2 = 0.00
Δ HR (bpm)
Δ BP (mmHg)
C
y = -0.077x + 5.459, r2 = 0.07
Δ HR (bpm)
Δ BP (mmHg)
D
y = -0.046x + 6.991, r2 = 0.03
Δ HR (bpm)
Δ BP (mmHg)

Figure 6.3 Regression graphs relating maximum changes in heart rate (Δ HR) to reciprocal changes in blood pressure (Δ BP) in response to phenylephrine infusion. A. Normotensive females. B. Normotensive males. C. Genetically hypertensive females. D. Genetically hypertensive males. n=12. y=slope of line through zero.

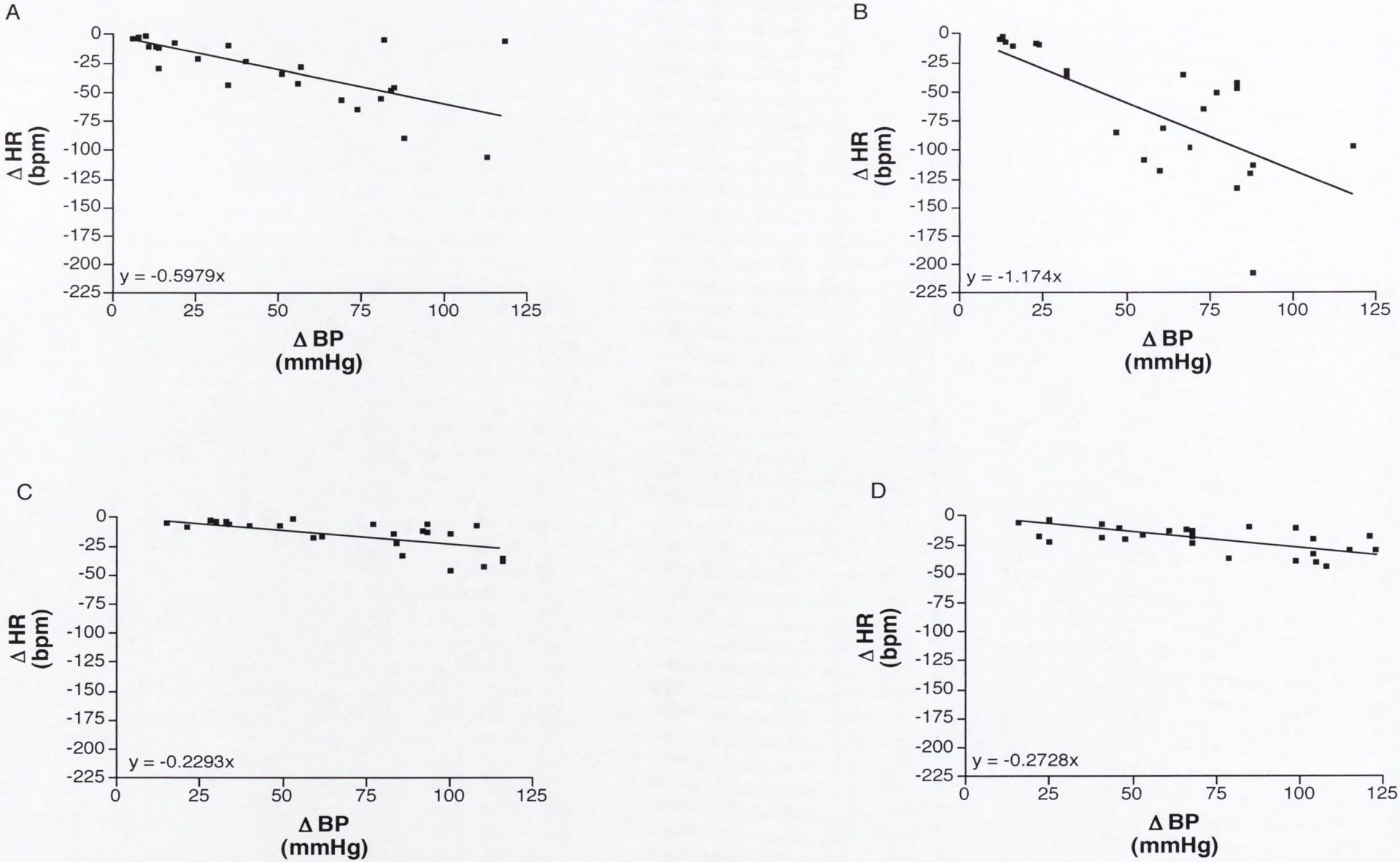

A
0
-25
-50
-75
-100
-125
-150
-175
-200
-225
Δ HR (bpm)
y = -0.5979x
0
25
50
75
100
125
Δ BP (mmHg)
B
0
-25
-50
-75
-100
-125
-150
-175
-200
-225
Δ HR (bpm)
y = -1.174x
0
25
50
75
100
125
Δ BP (mmHg)
C
0
-25
-50
-75
-100
-125
-150
-175
-200
-225
Δ HR (bpm)
y = -0.2293x
0
25
50
75
100
125
Δ BP (mmHg)
D
0
-25
-50
-75
-100
-125
-150
-175
-200
-225
Δ HR (bpm)
y = -0.2728x
0
25
50
75
100
125
Δ BP (mmHg)

Figure 6.4 Regression graphs relating maximum changes in heart rate (Δ HR) to reciprocal changes in blood pressure (Δ BP) in response to phenylephrine infusion. A. Normotensive females. B. Normotensive males. C. Genetically hypertensive females. D. Genetically hypertensive males. n=12. r^2 represents the correlation coefficient.

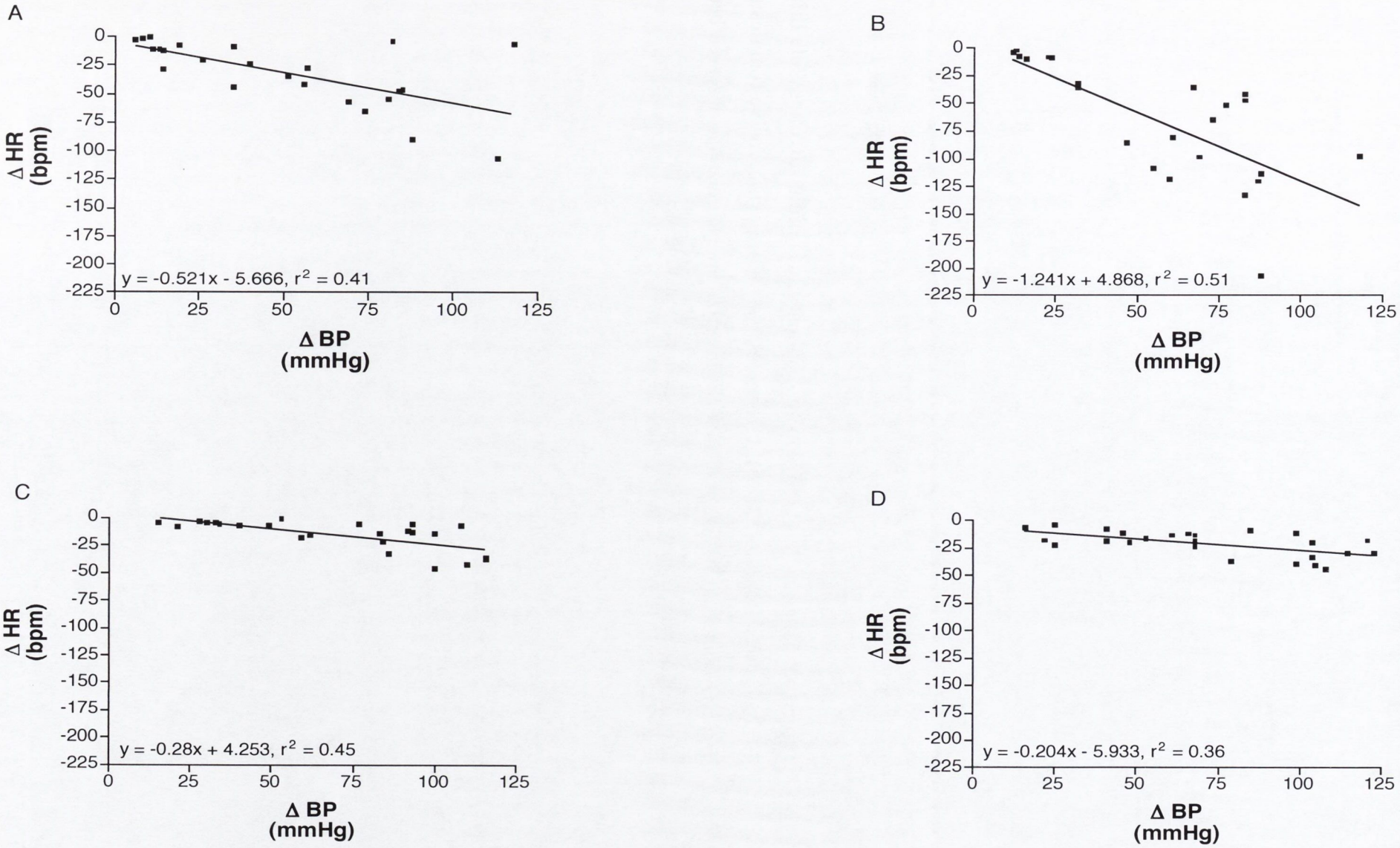

A
0
-25
-50
-75
-100
-125
-150
-175
-200
-225
Δ HR (bpm)
y = -0.521x - 5.666, r2 = 0.41
0
25
50
75
100
125
Δ BP (mmHg)
B
0
-25
-50
-75
-100
-125
-150
-175
-200
-225
Δ HR (bpm)
y = -1.241x + 4.868, r2 = 0.51
0
25
50
75
100
125
Δ BP (mmHg)
C
0
-25
-50
-75
-100
-125
-150
-175
-200
-225
Δ HR (bpm)
y = -0.28x + 4.253, r2 = 0.45
0
25
50
75
100
125
Δ BP (mmHg)
D
0
-25
-50
-75
-100
-125
-150
-175
-200
-225
Δ HR (bpm)
y = -0.204x - 5.933, r2 = 0.36
0
25
50
75
100
125
Δ BP (mmHg)

Figure 6.5 Mean reflex tachycardic responses to sodium nitroprusside infusion in normotensive males (N M), GH males (GH M), normotensive females (N F) and GH females (GH F). No difference in reflex bradycardia was observed between the groups (n=12, 1-way ANOVA). Data represents mean ± S.E.M.

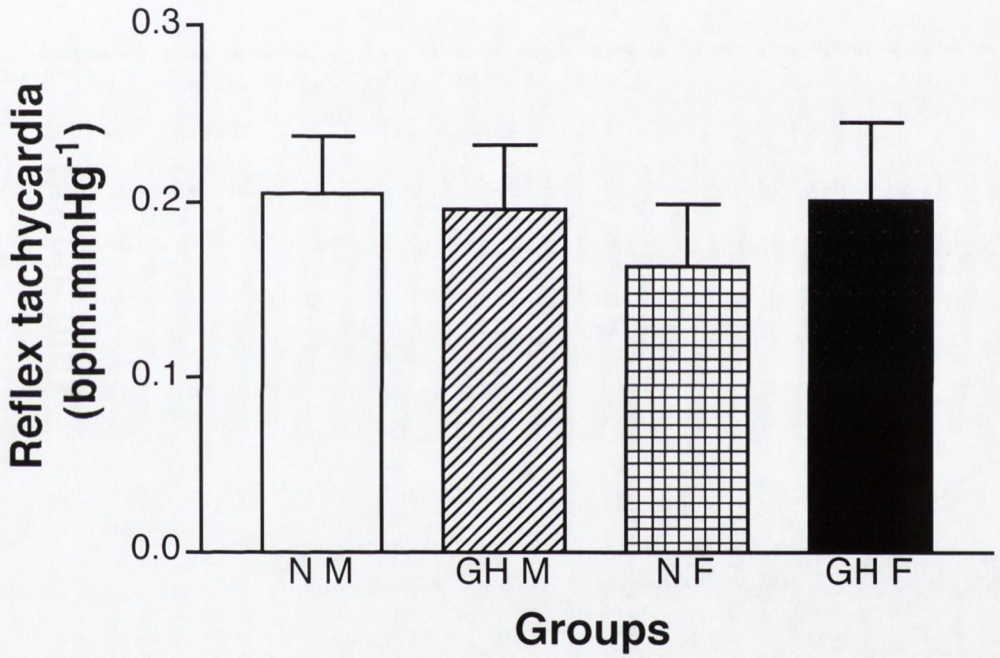

Reflex tachycardia
(bpm.mmHg-1)
0.3
0.2
0.1
0.0
N M
GH M
N F
GH F
Groups

Figure 6.6 Mean reflex bradycardic responses to phenylephrine infusion in normotensive males (N M), GH males (GH M), normotensive females (N F) and GH females (GH F). Male and female GH animals had significantly lower reflex bradycardia than normotensive animals. Female normotensive animals had attenuated bradycardia when compared to males. (n=12; * $P<0.05$, ** $P<0.01$; *** $P<0.001$; 1-way ANOVA). Data represents mean ± S.E.M.

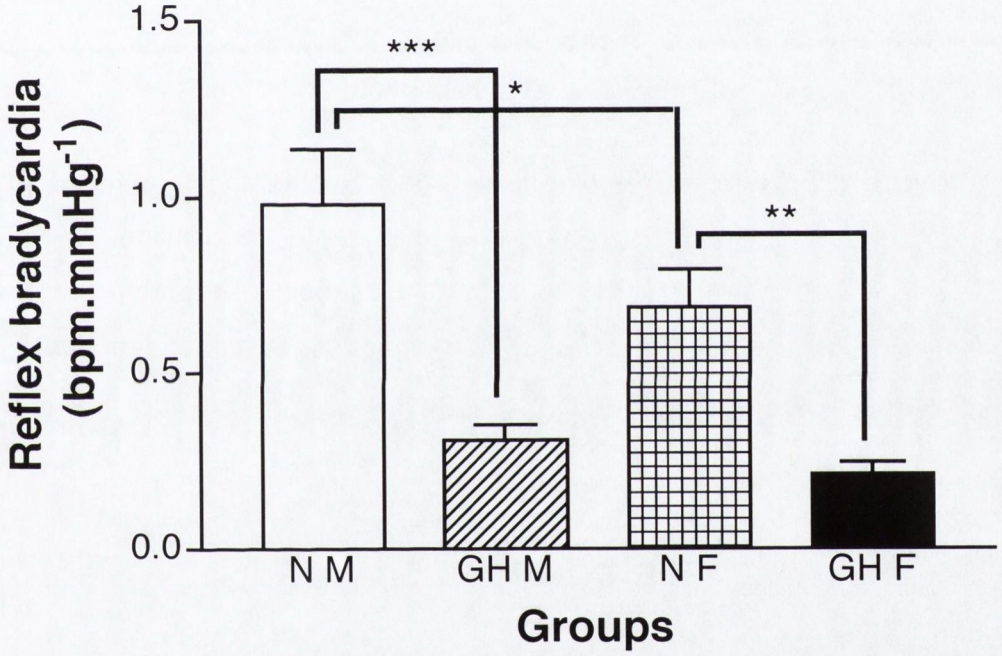

Reflex bradycardia
(bpm.mmHg-1)
1.5
1.0
0.5
0.0
***
*
**
N M
GH M
N F
GH F
Groups

Figure 6.7 Regressions graphs relating resting mean arterial pressure to baroreflex sensitivity in response to phenylephrine infusion. A. Normotensive females. B. Normotensive males. C. Genetically hypertensive females. D. Genetically hypertensive males. n=12. r^2 represents the correlation coefficient.

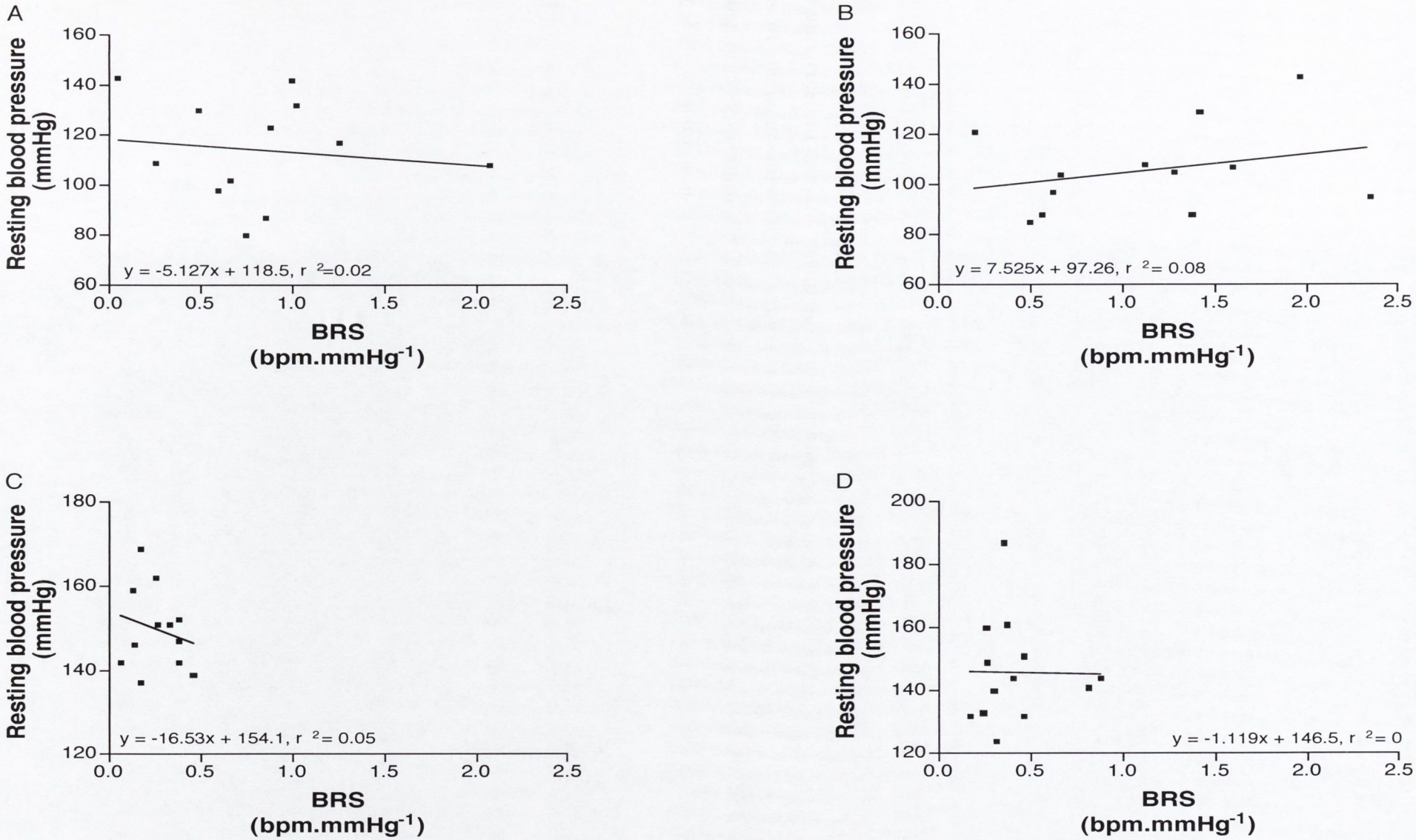

A
Resting blood pressure (mmHg)
160
140
120
100
80
60
y = -5.127x + 118.5, r 2=0.02
0.0
0.5
1.0
1.5
2.0
2.5
BRS
(bpm.mmHg-1)
B
Resting blood pressure (mmHg)
160
140
120
100
80
60
y = 7.525x + 97.26, r 2= 0.08
0.0
0.5
1.0
1.5
2.0
2.5
BRS
(bpm.mmHg-1)
C
Resting blood pressure (mmHg)
180
160
140
120
y = -16.53x + 154.1, r 2= 0.05
0.0
0.5
1.0
1.5
2.0
2.5
BRS
(bpm.mmHg-1)
D
Resting blood pressure (mmHg)
200
180
160
140
120
y = -1.119x + 146.5, r 2= 0
0.0
0.5
1.0
1.5
2.0
2.5
BRS
(bpm.mmHg-1)

Figure 6.8 Regression graphs relating maximum changes in heart rate (Δ HR) to reciprocal changes in blood pressure (Δ BP) in response to sodium nitroprusside infusion. A. Normotensive males. B. Normotensive castrated males. C. Normotensive females. D. Normotensive ovariectomised females. n=8. y=slope of line through zero.

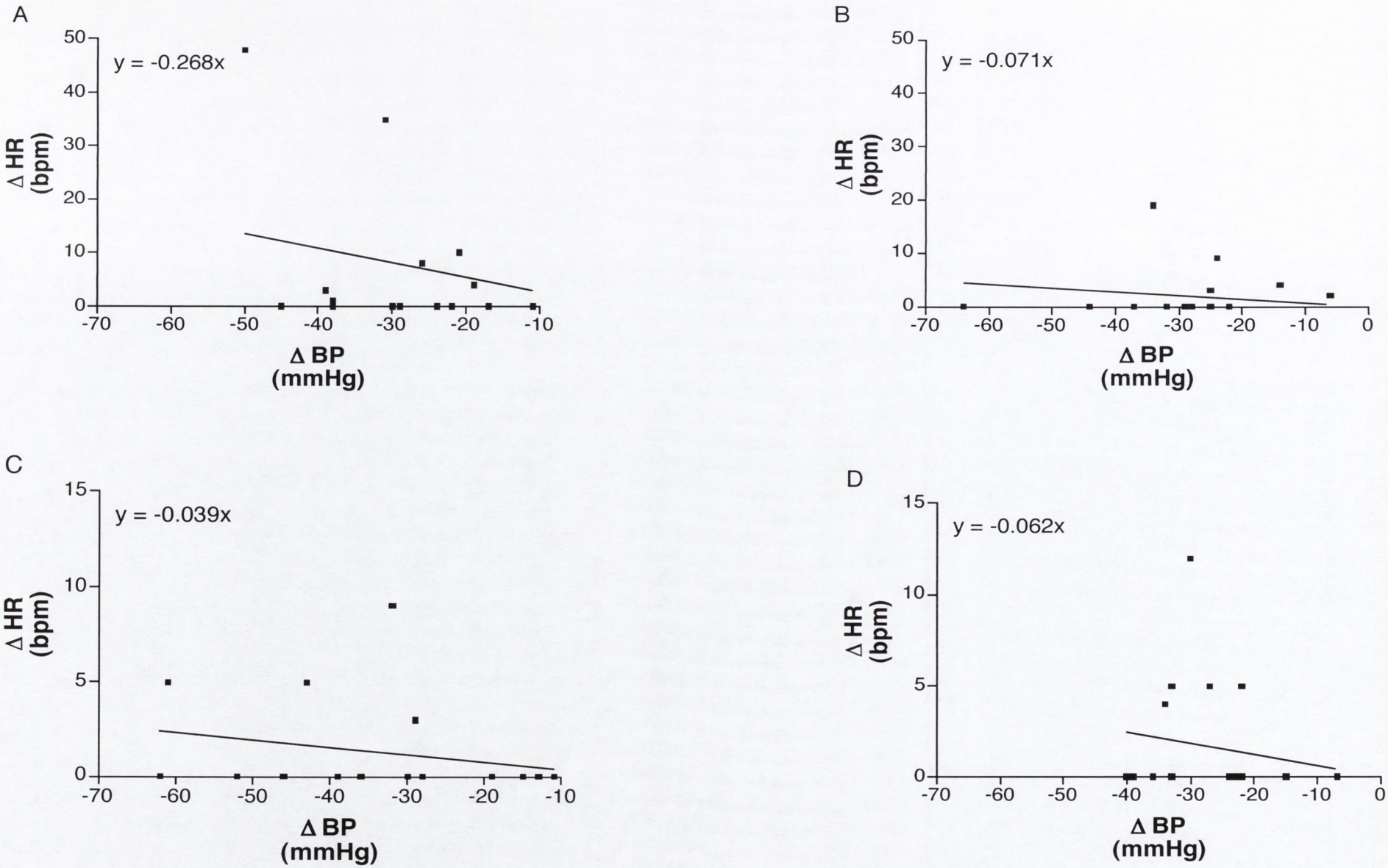

A
y = -0.268x
Δ HR (bpm)
Δ BP (mmHg)
B
y = -0.071x
Δ HR (bpm)
Δ BP (mmHg)
C
y = -0.039x
Δ HR (bpm)
Δ BP (mmHg)
D
y = -0.062x
Δ HR (bpm)
Δ BP (mmHg)

Figure 6.9 Regression graphs relating maximum changes in heart rate (Δ HR) to reciprocal changes in blood pressure (Δ BP) in response to sodium nitroprusside infusion. A. Normotensive males. B. Normotensive castrated males. C. Normotensive females. D. Normotensive ovariectomised females. n=8. r^2 represents the correlation coefficient.

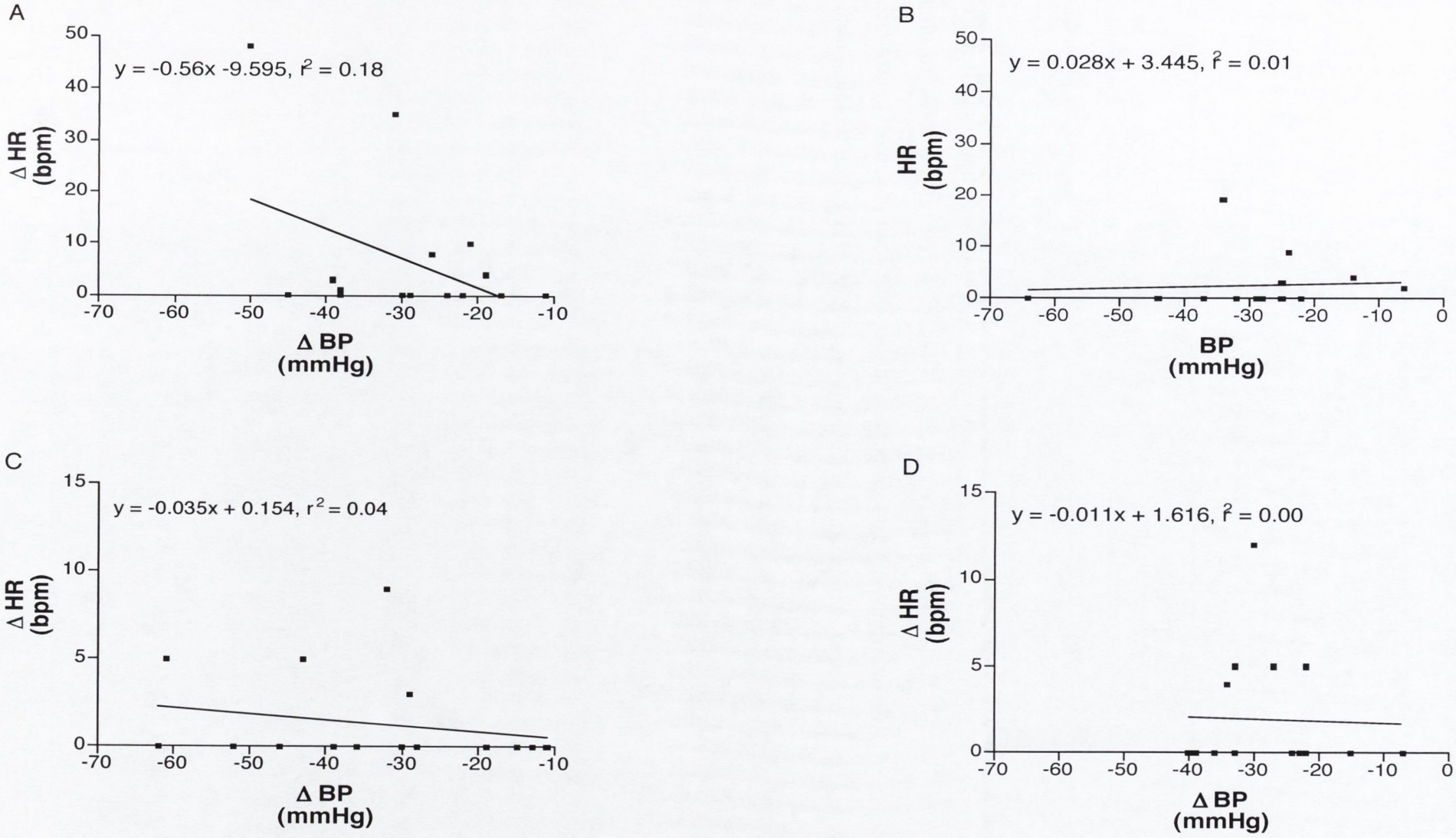

A
y = -0.56x -9.595, r² = 0.18
Δ HR (bpm)
Δ BP (mmHg)
B
y = 0.028x + 3.445, r² = 0.01
HR (bpm)
BP (mmHg)
C
y = -0.035x + 0.154, r² = 0.04
Δ HR (bpm)
Δ BP (mmHg)
D
y = -0.011x + 1.616, r² = 0.00
Δ HR (bpm)
Δ BP (mmHg)

Figure 6.10 Regression graphs relating maximum changes in heart rate (Δ HR) to reciprocal changes in blood pressure (Δ BP) in response to phenylephrine infusion. A. Normotensive males. B. Normotensive castrated males. C. Normotensive females. D. Normotensive ovariectomised females. n=8. y=slope of line through zero.

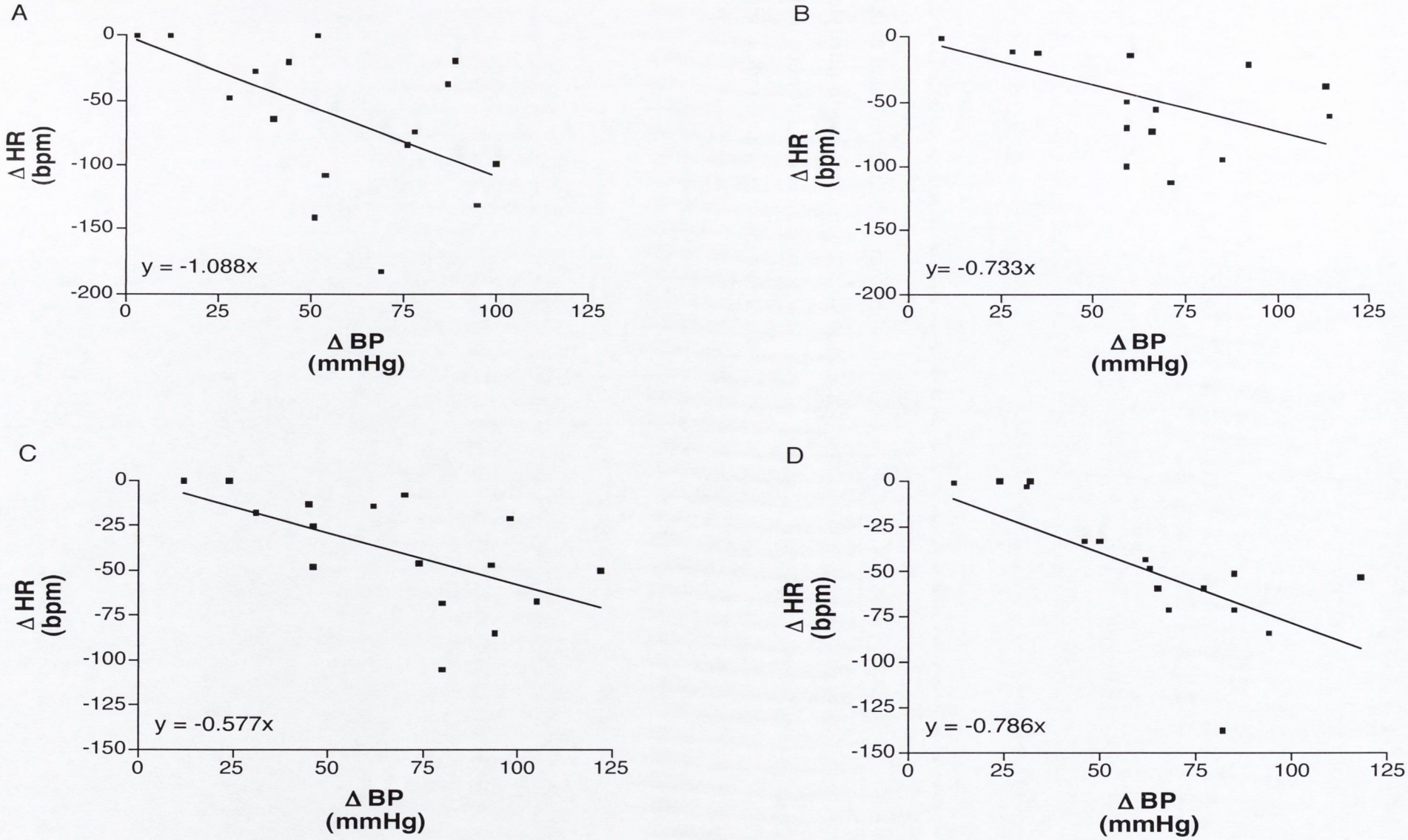

A
0
-50
-100
-150
-200
Δ HR (bpm)
y = -1.088x
0
25
50
75
100
125
Δ BP (mmHg)
B
0
-50
-100
-150
-200
Δ HR (bpm)
y= -0.733x
0
25
50
75
100
125
Δ BP (mmHg)
C
0
-25
-50
-75
-100
-125
-150
Δ HR (bpm)
y = -0.577x
0
25
50
75
100
125
Δ BP (mmHg)
D
0
-25
-50
-75
-100
-125
-150
Δ HR (bpm)
y = -0.786x
0
25
50
75
100
125
Δ BP (mmHg)

Figure 6.11 Regression graphs relating maximum changes in heart rate (Δ HR) to reciprocal changes in blood pressure (Δ BP) in response to phenylephrine infusion. A. Normotensive males. B. Normotensive castrated males. C. Normotensive females. D. Normotensive ovariectomised females. n=8. r^2 represents the correlation coefficient.

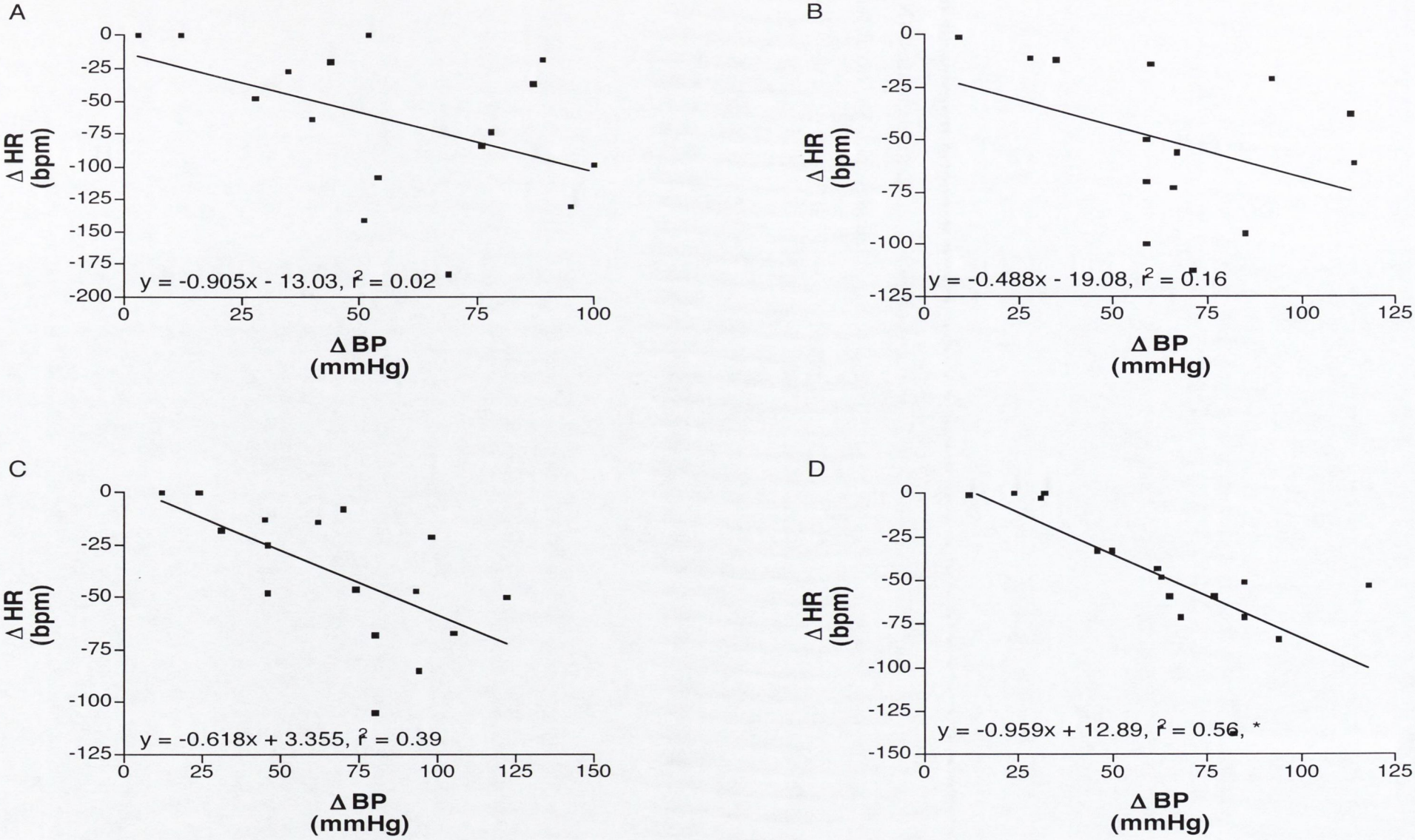

A
0
-25
-50
-75
-100
-125
-150
-175
-200
Δ HR (bpm)
y = -0.905x - 13.03, r² = 0.02
0
25
50
75
100
Δ BP (mmHg)
B
0
-25
-50
-75
-100
-125
Δ HR (bpm)
y = -0.488x - 19.08, r² = 0.16
0
25
50
75
100
125
Δ BP (mmHg)
C
0
-25
-50
-75
-100
-125
Δ HR (bpm)
y = -0.618x + 3.355, r² = 0.39
0
25
50
75
100
125
150
Δ BP (mmHg)
D
0
-25
-50
-75
-100
-125
-150
Δ HR (bpm)
y = -0.959x + 12.89, r² = 0.56, *
0
25
50
75
100
125
Δ BP (mmHg)

Figure 6.12 Mean reflex tachycardic responses to sodium nitroprusside infusion in normotensive males (N M), normotensive castrated males (N cast), normotensive females (N F) and ovariectomised females (N OV). No difference in reflex tachycardic was observed between the groups (n=8, 1-way ANOVA). Data represents mean ± S.E.M.

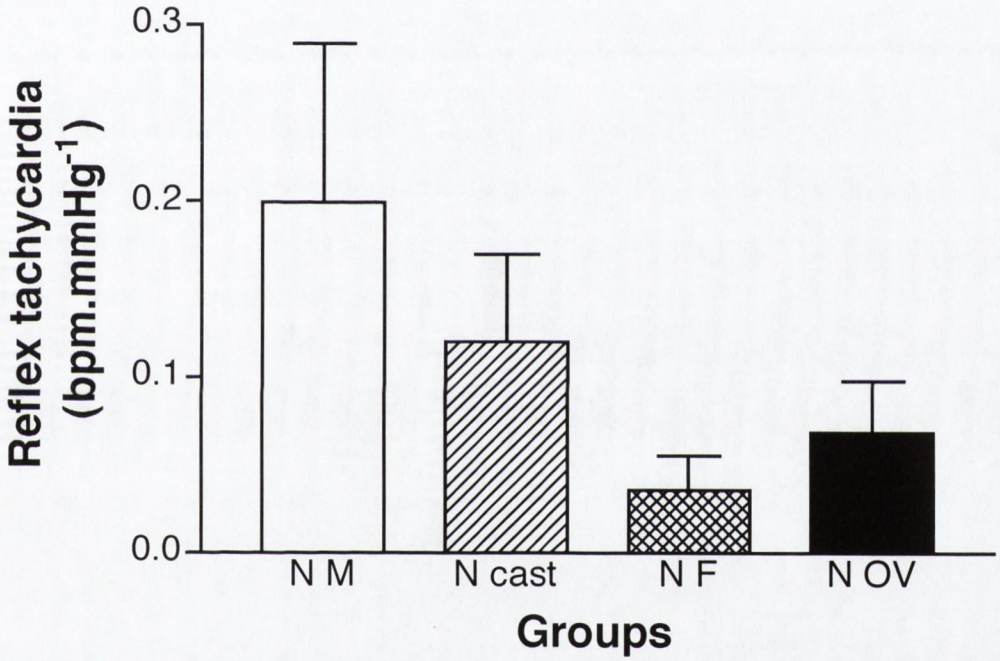

Reflex tachycardia
(bpm.mmHg-1)
0.3
0.2
0.1
0.0
N M
N cast
N F
N OV
Groups

Figure 6.13 Mean reflex bradycardic responses to phenylephrine infusion in normotensive males (N M), normotensive castrated males (N cast), normotensive females (N F) and ovariectomised females (N OV). Female normotensive animals had attenuated bradycardia when compared to males. (n=8; * $P<0.05$, 1-way ANOVA). Data represents mean ± S.E.M.

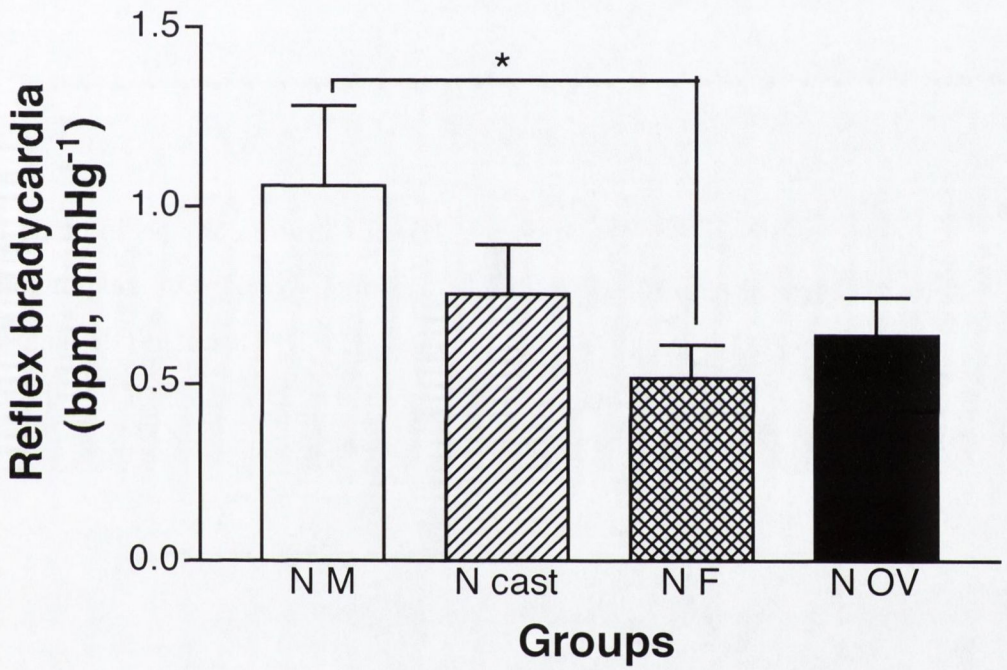

1.5
1.0
0.5
0.0
*
Reflex bradycardia
(bpm, mmHg-1)
N M
N cast
N F
N OV
Groups

Figure 6.14 Regression graphs relating maximum changes in heart rate (Δ HR) to reciprocal changes in blood pressure (Δ BP) in response to sodium nitroprusside infusion. A. Control GH males. B. Castrated GH males. C. Control GH females. D. Ovariectomised GH females. n=12. y=slope of line through zero.

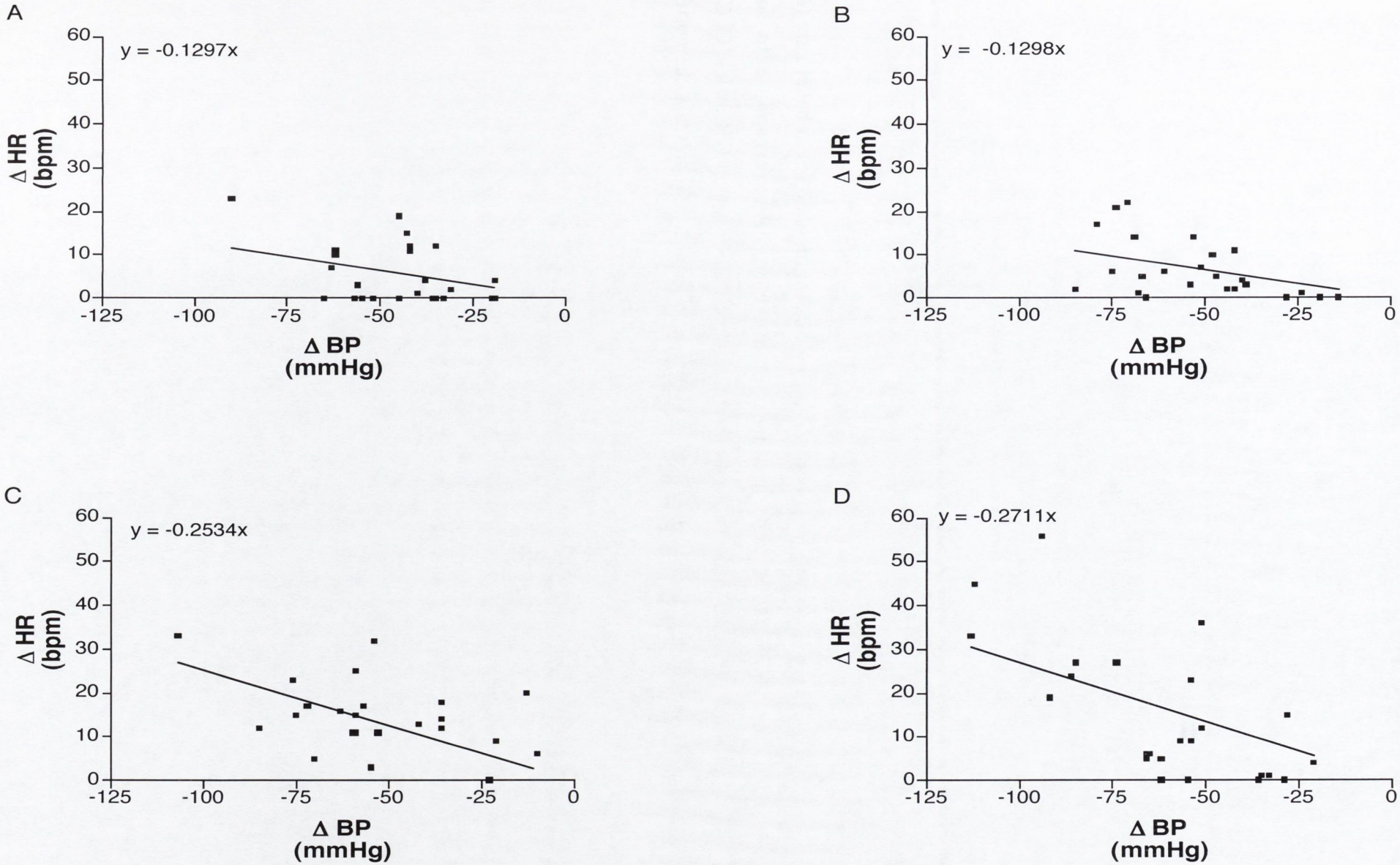

A
y = -0.1297x
Δ HR (bpm)
Δ BP (mmHg)
B
y = -0.1298x
Δ HR (bpm)
Δ BP (mmHg)
C
y = -0.2534x
Δ HR (bpm)
Δ BP (mmHg)
D
y = -0.2711x
Δ HR (bpm)
Δ BP (mmHg)

Figure 6.15 Regression graphs relating maximum changes in heart rate (Δ HR) to reciprocal changes in blood pressure (Δ BP) in response to sodium nitroprusside infusion. A. Control GH males. B. Castrated GH males. C. Control GH females. D. Ovariectomised GH females. n=12. r^2 represents the correlation coefficient.

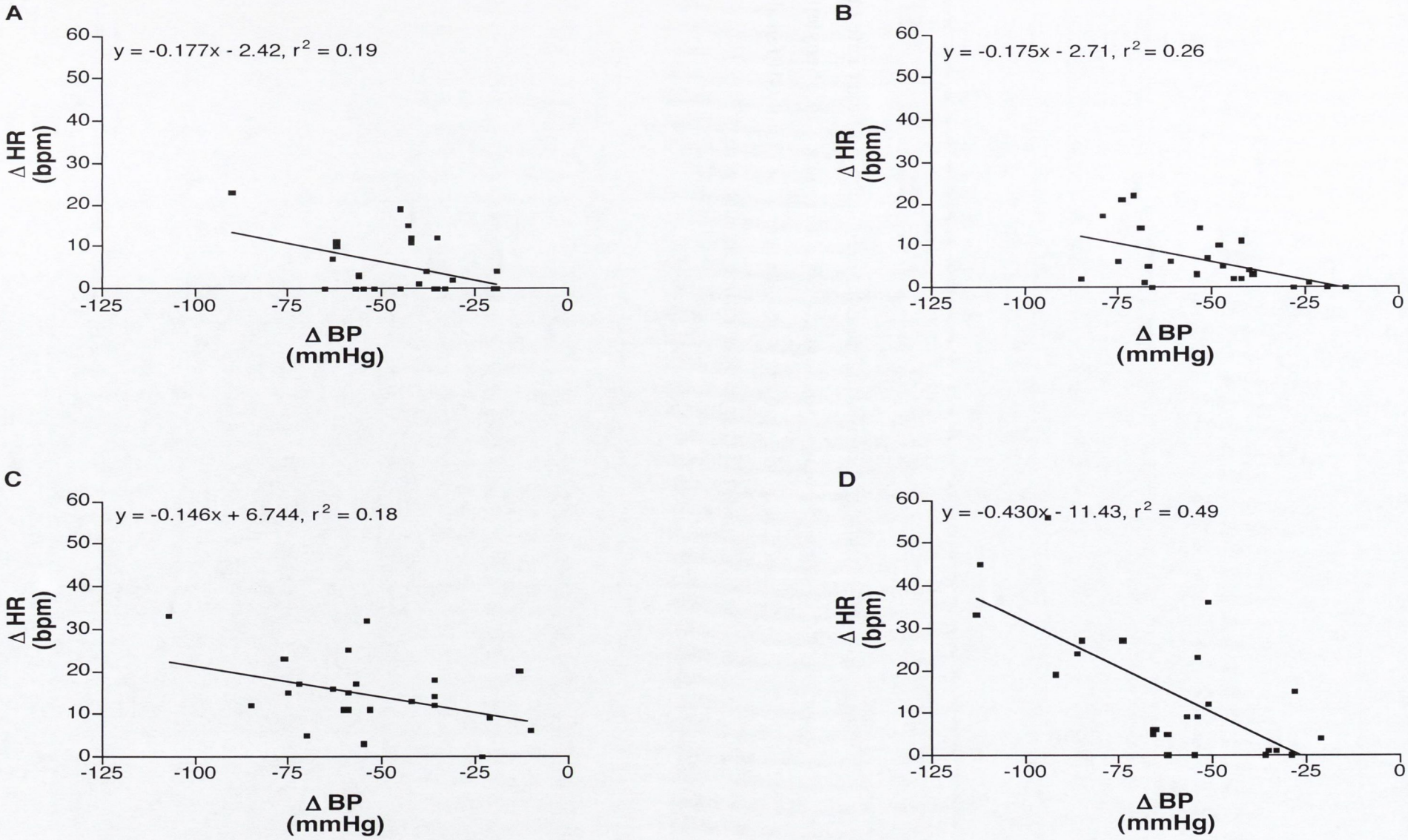

A
y = -0.177x - 2.42, r2 = 0.19
Δ HR (bpm)
Δ BP (mmHg)
B
y = -0.175x - 2.71, r2 = 0.26
Δ HR (bpm)
Δ BP (mmHg)
C
y = -0.146x + 6.744, r2 = 0.18
Δ HR (bpm)
Δ BP (mmHg)
D
y = -0.430x - 11.43, r2 = 0.49
Δ HR (bpm)
Δ BP (mmHg)

Figure 6.16 Regression graphs relating maximum changes in heart rate (Δ HR) to reciprocal changes in blood pressure (Δ BP) in response to phenylephrine infusion. A. Control GH males. B. Castrated GH males. C. Control GH females. D. Ovariectomised GH females. n=12. y=slope of line through zero.

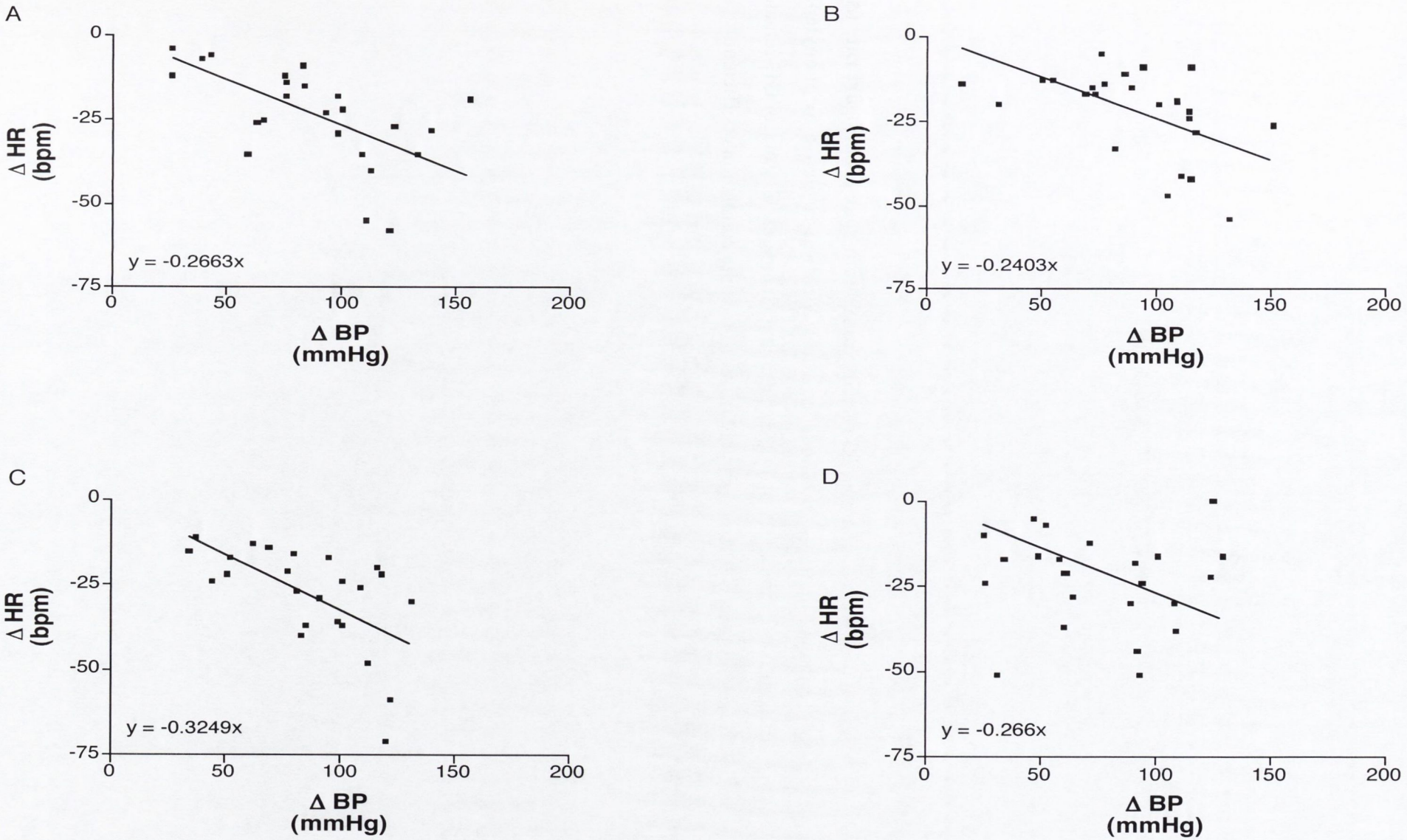

A
0
-25
-50
-75
Δ HR (bpm)
y = -0.2663x
0
50
100
150
200
Δ BP (mmHg)
B
0
-25
-50
-75
Δ HR (bpm)
y = -0.2403x
0
50
100
150
200
Δ BP (mmHg)
C
0
-25
-50
-75
Δ HR (bpm)
y = -0.3249x
0
50
100
150
200
Δ BP (mmHg)
D
0
-25
-50
-75
Δ HR (bpm)
y = -0.266x
0
50
100
150
200
Δ BP (mmHg)

Figure 6.17 Regression graphs relating maximum changes in heart rate (Δ HR) to reciprocal changes in blood pressure (Δ BP) in response to phenylephrine infusion. A. Control GH males. B. Castrated GH males. C. Control GH females. D. Ovariectomised GH females. n=12. r^2 represents the correlation coefficient.

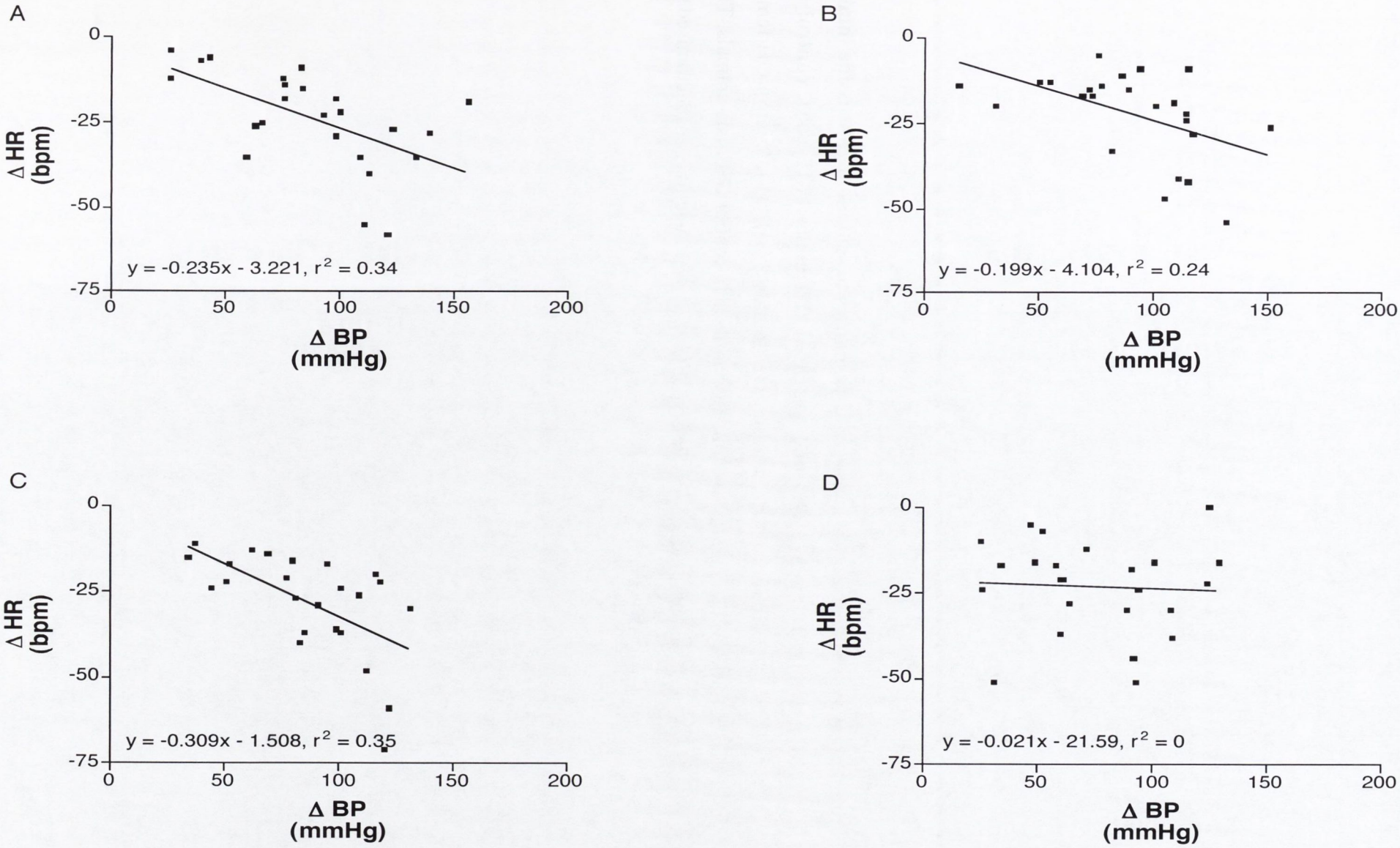

A
0
-25
-50
-75
Δ HR (bpm)
y = -0.235x - 3.221, r2 = 0.34
0
50
100
150
200
Δ BP (mmHg)
B
0
-25
-50
-75
Δ HR (bpm)
y = -0.199x - 4.104, r2 = 0.24
0
50
100
150
200
Δ BP (mmHg)
C
0
-25
-50
-75
Δ HR (bpm)
y = -0.309x - 1.508, r2 = 0.35
0
50
100
150
200
Δ BP (mmHg)
D
0
-25
-50
-75
Δ HR (bpm)
y = -0.021x - 21.59, r2 = 0
0
50
100
150
200
Δ BP (mmHg)

Figure 6.18 Mean reflex tachycardic responses to sodium nitroprusside infusion in control GH males. (GH M), castrated GH males (GH cast), control GH females (GH F) and ovariectomised GH females (GH OV). Control GH females had significantly greater tachycardic responses than control GH male animals. There were no differences in tachycardic responses between intact or gonadectomised rats. (n=12; 1-way ANOVA). Data represents mean ± S.E.M.

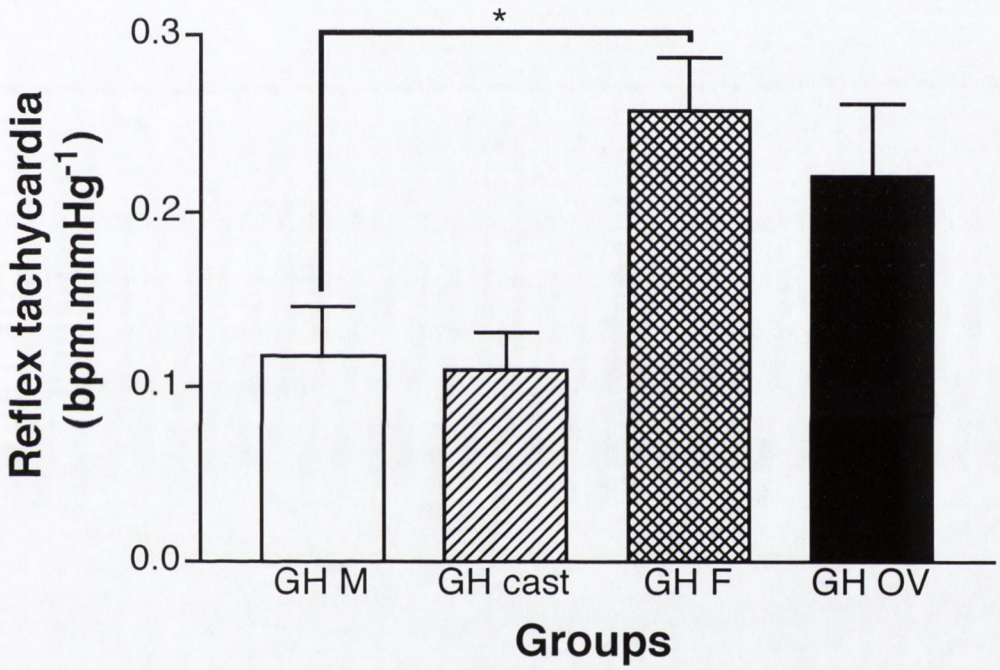
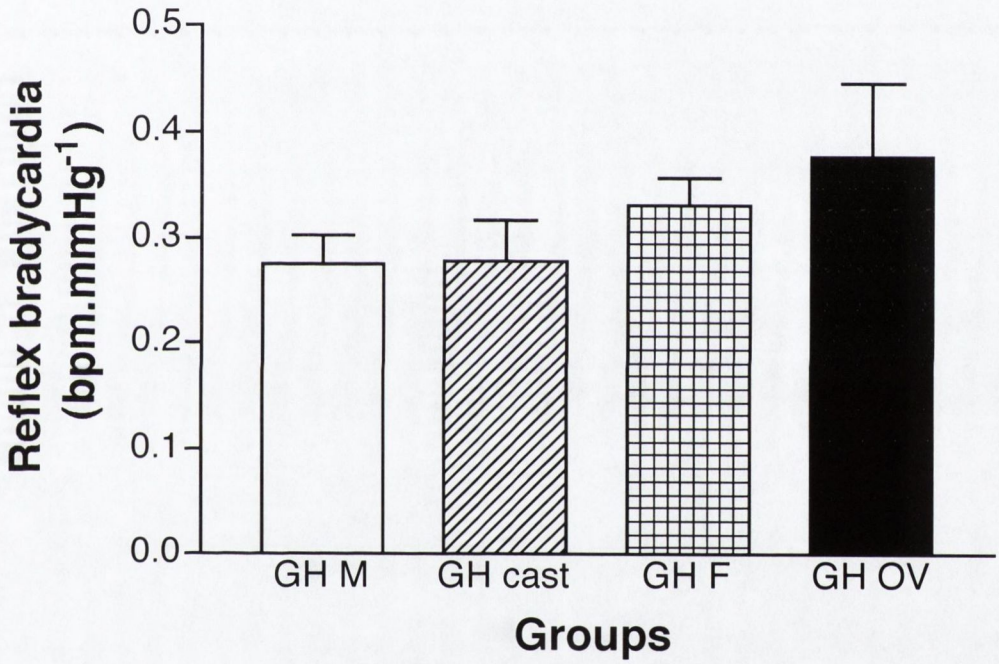

Reflex tachycardia
(bpm.mmHg-1)
0.3
0.2
0.1
0.0
*
GH M
GH cast
GH F
GH OV
Groups

Figure 6.19 Mean reflex bradycardic responses to phenylephrine infusion in control GH males. (GH M), castrated GH males (GH cast), control GH females (GH F) and ovariectomised GH females (GH OV). There were no differences in bradycardic responses between intact or gonadectomised rats. (n=12; 1-way ANOVA). Data represents mean ± S.E.M.


Reflex bradycardia
(bpm.mmHg-1)
0.5
0.4
0.3
0.2
0.1
0.0
GH M
GH cast
GH F
GH OV
Groups

6.4 Discussion

This study demonstrates lower bradycardic baroreflex gain in GH compared with normotensive controls. Tachycardic responses were not affected by hypertension. A significantly lower reflex bradycardia was present in normotensive female animals compared with males and a similar although non-significant trend was evident in hypertensive animals. There was no difference between the genders when examining tachycardic responses to blood pressure depression. The bradycardic response appears to be the dominant limb of the baroreflex.

There is no clear consensus on the influence of gender on resting blood pressure in GH animals. In the present study there was no difference in resting blood pressure values between male or female animals in either strain of rat. This result is comparable with others where there was no influence of gender on MAP in hypertensive or normotensive rats (Chen and Dicarlo, 1996; Chandler and Dicarlo, 1998). However Ashton and Balment (1991) found that male New Zealand GH rats had greater blood pressures than their female counterparts while Pallot and Barer (1985) found the contrary.

No difference in blood pressure levels in intact and gonadectomised animals were observed in this study. Some studies using ovariectomised female SHR have found that ovarian hormones do not provide protection against the elevated blood pressure in this strain of rat when compared with males (Reckelhoff *et al.*, 1998; 2000). Moreover, previous studies (Alper and Schmitz, 1996; El-Mas and Abdel-Rahman, 1998) reported no changes in blood pressure after ovariectomy or short- and long-term oestrogen treatment. However other studies in deoxycorticosterone-salt hypertension and in SHR have found that ovariectomy causes a dramatic increase in blood pressure when compared with either males or intact females (Crofton and Share, 1997; Dantas *et al.*, 1999) and that oestrogen therapy corrects this (Dantas *et al.*, 1999). In male animals no difference in blood pressure has been found between castrated and testosterone-replaced rats (El-Mas *et al.*, 2001).

Pressor responses to the vasoactive substances used to elicit blood pressure changes in this study were higher in GH than in normotensive animals. This result confirmed that of Phelan and colleagues (Phelan, 1966). After ganglion blockade when the blood pressures of both GH and N rats are similar the reactivity to noradrenaline was still increased in GH animals (Phelan *et al.*, 1962).

The present findings confirm reduced cardiovagal baroreflex gain in hypertension (Grassi *et al.*, 1998; Pikkujamsa *et al.*, 1998). In experiments on Dahl-hypertensive and normotensive rats similar results have been found (Murphy and McCarty, 1995). These rats, comparable to New Zealand GH rats, also have increased vasopressin levels. Impaired BRS in hypertension could be caused by a number of factors: attenuated vessel compliance (Tomiyama *et al.*, 1996), cardiac hypertrophy (Head and Minami, 1992; Minami and Head, 1993), high blood pressure itself (Lantelme *et al.*, 1994) even in the absence of cardiac hypertrophy and a direct effect of humoral factors, e.g. angiotensin II, which can depress baroreflex function by a pressure-independent mechanism (Brooks, 1995). All these factors are evident in the New Zealand GH rat strain.

This study demonstrates defective bradycardic baroreflex regulation in female normotensive rats when compared with males. This difference exists independently of the presence of phyto-oestrogens in the diet. Several lines of evidence suggest that baroreceptor control of heart rate is sexually dimorphic. An attenuated baroreflex function has been demonstrated in female rats compared with males (El-Mas and Abdel-Rahman, 1998; Abdel-Rahman, 1999) as well as in human subjects (Abdel-Rahman *et al.*, 1994; Huikuri *et al.*, 1996; Laitinen *et al.*, 1998; Beske *et al.*, 2001). Interestingly, in a study looking at the effects of gender on BRS in normotensive and hypertensive humans, Peckerman *et al.*, (2001) found a lower BRS in normotensive women than in men and lower sensitivity in hypertensive men than normotensive men. However hypertensive females had comparable sensitivity to their normotensive counterparts.

It is very difficult to account for the decreased baroreflex function in females. There is a large body of evidence to suggest that ovarian hormones are cardioprotective and women develop CVDs at a much later stage than men (Simon and Jaillon, 2000). There therefore seems to be a paradox between defective baroreflex gain in females and premenopausal women having lower levels of CVD. However, females appear to have the benefit of more favourable cardiovascular risk profiles than do men; for example, oestrogen positively effects lipid metabolism, thrombogenic factors, endothelial function and vascular reactivity (Oparil *et al.*, 1996). Perhaps a lower baroreflex gain in females is compensated for by oestrogen's cardioprotective qualities. It must be remembered however that it has not been clearly shown whether changes in BRS can lead to a sustained alteration in blood

pressure and so a diminished baroreflex gain may not necessarily mean a higher blood pressure level.

The sex-related differences in baroreflex function observed in this study could partly be due to the opposing effects of the ovarian hormones (oestrogen and progesterone) on baroreflexes (Masilamani and Heesch, 1997; El-Mas and Abdel-Rahman, 1998). Unlike oestrogen, progesterone has been shown to attenuate baroreflex function (Masilamani and Heesch, 1997). Unfortunately in this study we did not get an opportunity to assess oestrus cycle phase. The role of oestrogen in the brain centres responsible for baroreflex function is also unclear. Oestrogen has been shown to have a modulatory effect on central glutamatergic neurotransmission (Wong and Moss, 1992) while it has inhibitory effects on GABAergic (Kelly *et al.* 1992) neurotransmission, both of which are essential for the modulation of the baroreflex at a central level (Lawrence and Jarrott, 1996). Moreover studies have found varied effects of oestrogen on the binding activity of α_1- and α_2-adrenoceptors in the brain (Shackelford *et al.*, 1988), which are known to facilitate baroreflex functioning (Sved *et al.*, 1992).

The difference between the genders in reflex bradycardia in this present study may not be due to either oestrogen or progesterone but may be a direct result of the male sex hormone, testosterone. El-Mas *et al.* (2001) found that testosterone enhances vagally-mediated reflex bradycardia and castration decreased the baroreflex while testosterone replacement returned the gain to control levels; reflex tachycardic responses were unaffected. The mechanism by which testosterone modulates baroreflex responsiveness is not clear. However testosterone has been shown to interact with at least two central mechanisms involved in baroreflex regulation. Testosterone facilitates glutamatergic neurotransmission in several central areas including the hypothalamus (Pouliot *et al.*, 1996) and hippocampus (Diano *et al.*, 1997). Glutamate is an essential neurotransmitter in the central processing of baroreceptor information (Van Giersbergen *et al.*, 1992). Few studies have investigated the direct relaxing effects of testosterone on the vasculature. However some that have, show that testosterone induces endothelium-dependent and -independent relaxations in experimental animals (Yue *et al*, 1995; Chou *et al*, 1996; Costarella *et al*, 1996). Therefore testosterone may also have a favourable effect on vascular function and hence BRS.

An important difference to note between studies on BRS is the time-course of the administration of the pressor/depressor agent. Abdel-Rahman *et al.* (1994) found that control of bradycardic responses to pressure elevation were indeed gender related with young, normotensive women having a lower gain than matched males. This effect was independent of resting heart rate but was dependent on the method of administration of the pressor agent. Some studies use bolus injections of the vasoactive substance to elicit changes in blood pressure while others use an infusion protocol, which differs from study to study depending on the infusion rate. Using these different protocols the bolus method generally takes 30-45 seconds to rise to its peak while the infusion method would usually reach a maximum by 2 minutes depending on the rate. Abdel-Rahman *et al.* (1994) found that the BRS was 54% lower in females than in males using the bolus procedure but that there was no difference in BRS between the sexes after infusion. This difference is probably due to differences in the rate of change of blood pressure, a fact which is known to influence baroreflex function (Sullebarger *et al.*, 1990). There may be a difference therefore in gender reactivity of the baroreflex to immediate or prolonged blood pressure elevation. This view is supported by two studies by He and colleagues. The first (He *et al.*, 1998) investigated the effects of phenylephrine on sympathetic activity in ovariectomised rats treated with 17ß-oestradiol. They found that in rats chronically treated with 17ß-oestradiol, the pressor response to a 30-minute infusion of phenylephrine was smaller than in control animals and they also determined that BRS was greater in 17ß-oestradiol-treated rats. However in 1999 this same group using a modified protocol found BRS was unchanged by 17ß-oestradiol treatment in ovariectomised rats when phenylephrine was used to elevate blood pressure within a 100-s interval (He *et al.*, 1999). It appears therefore, that the more prolonged the blood pressure elevation, the more beneficial the effect of oestrogen (He *et al.*, 1998), whereas intermediate time intervals such as that used by He *et al.* (1999) do not evince an oestrogen-dependent effect, while my own study with an even shorter time to peak effect (approx. 30-s) demonstrated lower reflex bradycardia in females than males. Other studies that have found increased baroreflex gain in female and male rats treated with oestrogen do not specify the infusion rate or the length of time allowed for infusion (Saleh and Connell, 2000; 1998, respectively) and many use blood pressure changes of very different ranges.

The result of the present experiment could therefore be partly due to differences in the time-course of parasympathetic and sympathetic activation after stimulation and activation of the baroreceptors. The vagal arm of the baroreceptor increases its activation more rapidly than the sympathetic withdrawal component (Coleman, 1980). The rapid decreases in heart rate in my experiment are more likely due to an increase in vagal activity rather than sympathetic withdrawal since the time-course of blood pressure responses was similar to that of a bolus injection. Perhaps the CNS adapts so as to acutely reset the baroreceptors (Dorward *et al.*, 1982) in response to slow elevations of blood pressure. In fact it appears that there are two types of carotid sinus baroreceptor: Type 1 primarily respond to rapid changes in blood pressure whereas Type 2 react to prolonged elevations in blood pressure (Seagard *et al.*, 1993). The terminal sites of the Type 1 receptor appear to be located in the dorsomedial subnucleus of the rostral nucleus tractus solitarius while that of the Type 2 baroreceptors are linked to neurons in the commissural, medial and dorsolateral subnuclei of the nucleus tractus solitarius (Dean and Seagard, 1997). The responses of these subtypes of baroreceptors to blood pressure change may be responsible for the disparity within the literature.

According to the present study's results female animals appear to exhibit less cardiac responsiveness to increases in blood pressure compared to males. This suggests that there may be a less pronounced activation of vagal activity in females than in males. However this is mere speculation because we did not block the interaction of the vagal or sympathetic nerves with the baroreflex arc although previously, in conscious rats, bradycardic responses to phenylephrine infusion were found to be primarily controlled by the parasympathetic nervous system (Stornetta *et al.*, 1987). These findings would support the view that the cardiac vagal component of the baroreflex plays a substantially smaller role in the baroreflex-mediated bradycardia in females and that males have a heightened parasympathetic tone and therefore an enhanced ability to resist increases in sympathetic output. However it is possible that various pressor agents may elicit different response in males and females for example, Girdler *et al.* (1990) found that females reacted to stress with increased cardiac output and heart rate while males responded with more vascular reactivity.

No differences in tachycardic responses to sodium nitroprusside were evident between the sexes in this study. The absence of a gender effect on reflex

tachycardic responses confirms findings by Mohamed *et al.* (1999) and El-Mas *et al.* (2001) who found that neither ovariectomy nor oestrogen administration or castration nor testosterone administration, respectively, caused any change in baroreflex-mediated tachycardia. The absence of an effect of sex hormones on reflex tachycardia but an enhanced reflex bradycardia in males suggests a gender-specific modulation by the autonomic nervous system of baroreflex control of heart rate. However, when repeating the experiment with control male and female GH animals there was a significantly greater tachycardic response in control GH females compared with males. This elevation could partially be due to the fact that the absolute baroreflex gain was lower in GH males when the study was repeated. Also at rest the hearts of rats are predominantly under sympathetic control and the resting heart rates were higher in the second study than in the first. Perhaps there is a gender difference in heart rate control at the upper limits of the heart rate range.

Although reflex tachycardia is generally accepted as representing sympathetic nervous control a study by Stornetta *et al.* (1987) demonstrated that reflex tachycardia is attenuated to an equal extent by the sympathetic blocking agent atenolol and the parasympathetic blocking agent methyl-atropine This indicates that, at least in conscious rats, the sympathetic and parasympathetic systems may contribute equally to reflex tachycardia. Given the fact that there was a gender difference in reflex bradycardia, it seems likely that it is the sympathetic component of the reflex that is responsible for the lack of a gender effect in this present study and when specifying reflex tachycardia in this and the following chapters it is the sympathetic component of the response that is referred to.

It must be noted that the reflex tachycardic responses observed in the present study were small despite the substantial depressor response to sodium nitroprusside. The general anaesthetics: pentobardital and urethane, are known to have effects on autonomic nervous system control. Both are commonly used in experiments on BRS and both are known to attenuated the tachycardic response to blood pressure depression (Stornetta *et al.*, 1987). However when looking at the bradycardic response pentobarbital results in a greater attenuation of reflex bradycardia than urethane (Stornetta *et al.*, 1987). Perhaps therefore the urethane anaesthetic acts so as to depress reflex tachycardia and preserve, as much as possible, reflex bradycardia. In general however urethane is the best anaesthetic to use when investigating autonomic nervous system responses. In a study by Shimokawa *et al.*

(1998) it was found that although pentobarbital sodium and chloralose anaesthesia caused no change in tonic renal SNA they produced an inhibitory effect on baroreceptor renal SNA. Urethane on the other hand increased tonic renal SNA but had no effect on the baroreceptor renal SNA reflex. It therefore appears that pentobarbital sodium and chloralose anaesthesia may exert inhibitory effects while urethane does not. However it must still be taken into account that urethane increases renal SNA and a mixture of chloralase and urethane may be a better choice. In fact Faber *et al.* (1989) found that urethane-chlorase anasethesia did not significantly alter BRS.

In this study neither reflex tachycardic nor bradycardic responses were influenced by ovariectomy or castration. However while El-Mas *et al.* (2001) found that castration and subsequent testosterone administration had no effect on reflex tachycardia in response to sodium nitroprusside, which is similar to the present study, they found that castration attenuated reflex bradycardia while testosterone replacement restored the baroreflex to pre-castration values. Furthermore this same group, through blockade of the vagal and sympathetic components of the baroreflex found that testosterone causes its effects at least partly by an enhancement of vagal efferent activity. Ovariectomy has also been previously shown to significantly attenuate baroreflex heart rate responses and oestrogen administration reverses this (El-Mas and Abdel-Rahman, 1998). Similar findings have been demonstrated in male rats administered with oestrogen (Saleh and Connell, 1998). Given the evidence to suggest gonadectomy influences reflex bradycardia the results of this present study are suprising although studies conducted in adult rats of different strains and ages may not be comparable and the vast majority of studies on BRS use normotensive animals.

The absolute values obtained for both tachycardic and bradycardic responses in this study are similar from those obtained in other laboratories, although tachycardic responses in this present study are quite small. Other studies have found that reflex bradycardiac responses in oestrogen-replaced rats are significantly higher (0.65 ± 0.05 bpm.mmHg^{-1}) than tachycardic responses (0.51 ± 0.05 bpm.mmHg^{-1}) (Saleh and Connell, 2000). This agrees with the result of normotensive females in the present study: bradycardic -0.68 ± 0.11 bpm.mmHg^{-1} cf tachycardic 0.16 ± 0.04 bpm.mmHg^{-1}. In another study this same group found no difference between tachycardic or bradycardic responses in male rats (0.55 bpm.mmHg^{-1}) (Saleh and

Connell, 1999) while males had a BRS of 0.62 ± 0.05 bpm.mmHg^{-1} in a previous study (Saleh and Connell, 1998). Mohamed *et al.* (1999) found female rats had a BRS of 0.91 ± 0.12 bpm.mmHg^{-1} and ovariectomised animals had a BRS of 0.54 ± 0.05 bpm.mmHg^{-1}. Differences in values between studies could be due to the animal strain used or the absolute blood pressure level. In the present studies only the GH females showed no difference between the sympathetic and parasympathetic components of the baroreflex. The tachycardic effect was similar for all groups so it was the small bradycardic response in this group that caused the difference. This indicates that heart rate in female GH animals appears to be equally controlled by the sympathetic and parasympathetic nervous system. This would suggest that females with hypertension are less influenced by vagal control than matched males.

Conclusion

In summary, the gain of baroreflex tachycardic responses is substantially less than that of bradycardic responses, in both male and female normotensive rats. GH animals exhibit reduced bradycardic BRS but no alteration in tachycardic BRS. This is compatible with selective reduction in the efficiency of cardiovagal drive in the hypertensive strain although an influence of the anaesthetic, urethane, on parasympathetic and sympathetic cardiac drive cannot be ruled out. Tachycardic BRS is of similar magnitude in male and in female animals, but bradycardic BRS is lower in normotensive females than in males. This suggests a differential effect of gender on cardiovascular autonomic control in normotensive animals. However gonadectomy at an early age does not appear to affect the baroreflex.

Chapter 7 'Effects of hypertension and gender on markers of endothelial cell function and turnover in the rat'

7.1 Introduction

In view of the fact that CEC number was increased with smoking in humans and that hypertension is also associated with increased CEC number, this chapter was designed to address whether elevations in CEC number are related to increases in cell turnover rates and/or cell death.

The functional integrity of the endothelium is paramount to the prevention of vascular disorders such as thrombosis, atherosclerosis and vascular inflammatory diseases. The endothelium also has an important modulating effect on peripheral resistance and hence blood pressure through its effect on small resistance arteries (Mulvany *et al.*, 1978). Endothelial dysfunction plays a critical part in the pathophysiology of hypertension (Lüscher, 1992). In some forms of genetic and experimental hypertension, a decreased responsiveness to endothelium-dependent vasodilator substances such as ACh has been found (Wu *et al.*, 1996).

Endothelial cell dysfunction, injury and regeneration seem to be associated processes. The presence of dying and regenerating endothelial cells may be a basis for endothelial dysfunction. Acceleration of the turnover rate of endothelial cells could potentially lead to their dysfunction. The aortic endothelium normally has a fixed cell population number with a low rate of replication (Schwartz and Benditt, 1976). Hansson *et al.* (1985) demonstrated that lipopolysaccaride, a substance that is cytotoxic to endothelial cells in culture, causes a massive cell death in the endothelium, which is paralleled by increased cell replication. When atherosclerotic lesions develop and grow, an increase in both endothelial turnover (Hansson and Bondjers, 1980; Walker *et al.*, 1986) and injury (Bondjers and Bjorkerud, 1977) is observed. Sympathetic activation appears to accelerate the development of atherosclerosis. Pettersson *et al.* (1990) found that SNS activation via chloralose anaesthesia, caused a 5-fold increase in endothelial cell death. This was exclusively caused by the $ß_1$ adrenoceptor. Increased endothelial cell turnover and death may therefore influence the progression of CVD.

Endothelial cells of the aorta are constantly exposed to haemodynamically generated mechanical forces. Shear stress regulates gene expression of various proteins, including vasoactive substances (e.g. NOS and endothelin-1), growth factors, adhesion and chemoattractant molecules, coagulation factors and antioxidant enzymes (Chien *et al.*, 1998). Furthermore, shear stress completely inhibits apoptosis of endothelial cells in response to various stimuli (Dimmeler *et al.*, 1996) thus preventing the development of CVD.

During apoptosis there is an increase in the adhesiveness of endothelial cells for platelets and increased thrombus formation, reflecting a loss of membrane anticoagulant properties and an increased expression of adhesion molecules (Greeno *et al.*, 1996; Bombeli *et al.*, 1997; 1999). Transiently leaky junctions occurring during endothelial cell turnover result in the increased transport or leakage of macromolecules, including atherogenic LDL (Lin, 1996) across the vascular endothelium. Many risk factors for vascular disease promote apoptotic death of endothelial cells. These include oxLDL (Dimmeler *et al.*, 1997; Li *et al.*, 1998) high glucose and insulin (Du *et al.*, 1999; 1998, respectively), angiotensin II (Dimmeler *et al.*, 1997; Li *et al.*, 1999) and oxidative stress (Buttke and Sandstrom, 1994).

As well as the effect of hypertension on the endothelium gender differences in endothelial function and the effects of hormone therapy on vascular function have been the focus of considerable research interest. Oestrogen, progesterone and androgen receptors have all been identified in human vascular endothelium (Venkov *et al.*, 1996; Ba *et al.*, 2001; Welter *et al.*, 2003). Oestrogens inhibit monocyte adhesion (Mikkola and St Clair, 2002), the expression of adhesion molecules (Simoncini *et al.*, 2000) and protect endothelial cells from apoptotic damage (Alvarez *et al.*, 1997; Spyridopoulos *et al.*, 1997). Razandi *et al.* (2000) found that oestradiol prevents hypoxia-induced apoptosis and induces both the migration of endothelial cells and the formation of primitive capillary tubes. However the cellular actions of oestrogens, which lead to the preservation of the structure and function of endothelial cells are not well understood.

Little is known about the effects of androgens on vascular cell apoptosis in animals. Testosterone may aggravate vascular endothelial cell dysfunction in men. In a recent study testosterone was found to reduce DNA synthesis and enhance apoptosis (Ling *et al.*, 2002). Animal studies have shown that testosterone administration increases plaque formation in chicks (Toda *et al.*, 1984) and monkeys

(Adams *et al.*, 1995). Androgens may directly stimulate the proliferation of vascular SMCs (Fujimoto *et al.*, 1994) and increase human monocyte adhesion to endothelial cells via the expression of adhesion molecules (McCrohon *et al.*, 1999) thereby accelerating atherosclerosis. However, in castrated male animals an anti-atherosclerotic role of androgen supplementation has been proposed (Alexandersen *et al.*, 1999). It has been suggested that testosterone may propagate the effects of NO and act as an antioxidant (Guetta and Cannon, 1996). Testosterone has also been shown to elicit a direct vasodilatory effect in rabbit coronary arteries and aorta (Yue *et al.*, 1995).

The aim of this study therefore was to investigate whether hypertension and/or gender influences endothelial cell function, death and proliferation in rats.

7.2 Methods

7.2.1 Animals used

Age-matched adult male and female (14-18 weeks) normotensive (N) and New Zealand genetically hypertensive (GH) Wistar rats were used in the study. For apoptosis, proliferation, CECs and vWF n=8 for all groups, for inhibition of NOS n=12 for all groups.

7.2.2 Experimental protocol

Blood studies

Animals were anaesthetised with urethane (1.25 g/kg i.p.). A 5 ml blood sample was collected by cardiac puncture. Blood samples were separated into 2 ml and 3 ml aliquots. The 2ml sample was centrifuged to obtain plasma for analysis of vWF (via ELISA as previously described in Chapter 2). The 3ml blood sample was required for the isolation of CECs under light microscopy (see Chapter 2).

7.2.3 Isolation of the aortic endothelial cell layer via the *en face* method

The *en face* method is described in detail in Chapter 2. Briefly the thoracic aorta was removed, opened longitudinally and cut into 10 mm sections. The sections were placed inward side down on polylysine-coated slides and left on a metal block at -20°C to cool. At the first sign of freezing, one corner of the tissue was raised and rolled back carefully leaving an intact layer of endothelial cells on the slide. These slides were then stored at -80°C for further analysis.

7.2.4 Cell proliferation

All animals were injected with BrdU (1 ml/kg body weight, i.p.) 1 hour before sacrifice. Newly formed DNA incorporating BrdU was identified by immunostaining using a commercially available kit (5-Bromo-2′-deoxy-uridine Labeling and Detection Kit II, Roche Diagnostics, Germany) by a method already described in Chapter 2. Briefly the cells were incubated with anti-BrdU monoclonal antibody, washed and then immersed in anti-mouse-Ig-alkaline phosphatase. The colour reaction was then visualised by the addition of the chromogen NBT and a phosphate solution. BrdU staining was performed on *en face* preparations. Cells displaying positive BrdU immunoreactivity (stained blue) were counted and

expressed as a percentage of the total number of cells examined, which was a minimum of 500 cells.

7.2.5 Cell apoptosis

Apoptotic cell death was detected using a commercially available kit - DeadEnd Colorimetric Apoptosis Detection System (Promega Corporation, Madison, USA). TUNEL was performed on *en face* preparations. The TUNEL procedure was described in detail in Chapter 2. Briefly a biotinylated nucleotide was incorporated at the 3'-OH DNA ends using the enzyme TdT. Streptavidin HRP was then bound to the nucleotides. These biotinylated nucleotides were then detected using the chromogen, DAB and hydrogen peroxidase. Apoptotic cells (TUNEL-positive - stained brown) were counted and expressed as a percentage of the total number of cells examined, which was a minimum of 500 cells.

7.2.6 Endothelial nitric oxide production

NOS was inhibited using N^G-nitro-L-arginine methyl ester (L-NAME, 30 mg/kg i.v., Sigma). The magnitude of the increase in MAP to L-NAME infusion was expressed as a percentage of the basal MAP. The absolute increase in MAP was also noted.

7.2.7 Statistical analysis

Statistically significant differences between means were calculated for multiple comparisons using a 1-way ANOVA followed by a Student Newmann Keuls post-hoc test and for single comparisons using a Student's *t*-test. $P<0.05$ was deemed significant. All data are expressed as mean ± S.E.M.

7.3 Results

7.3.1 Von Willebrand Factor

No difference in vWF concentration was evident between male and female N (17 ± 8.3 cf 13 ± 4.5 Units) or GH (12 ± 1.1 cf 11 ± 4.6 Units) rats. No differences in levels between N or GH animals were observed. See Figure 7.1.

7.2.2 Circulating endothelial cells

Rats of both strains and gender presented with CECs in their blood samples. The shape and size of these cells were similar to those previously observed in humans and a sample can be seen in Figure 7.2 A. CEC numbers were elevated in the male and female GH animals when compared to N (109 ± 23.6 and 96 ± 21.7 cf 47 ± 12.2 and 19 ± 8.4 cells.ml^{-1}blood, $P<0.05$), respectively (see Figure 7.2 B).

7.3.3 Cell proliferation

An example of an *en face* BrdU preparation counter-stained with methyl green is shown in Figure 7.3 A. An example of a proliferating cell i.e. a BrdU-positive endothelial cell is shown in Figure 7.3 B.

GH female animals demonstrated a significantly greater percentage of mitotic cells than their normotensive counterparts (1.5 ± 0.20 cf 0.5 ± 0.16 %, $P<$ 0.01). Male GH and N animals had similar numbers of proliferating cells (0.5 ± 0.13 cf 0.8 ± 0.19 %). GH females had significantly greater numbers of mitotic cells than did male GH animals while no difference was observed between female and male N animals (see Figure 7.3 C).

7.3.4 Apoptosis

An example of an *en face* TUNEL preparation counter-stained with methyl green is shown in Figure 7.4 A. An example of a TUNEL-positive cell i.e. an apoptotic endothelial cell is shown in Figure 7.4 B.

Male and female N animals demonstrated similar percentages of apoptotic cells (0.57 ± 0.002 cf 0.32 ± 0.001 %) as did male and female GH animals (0.39 ± 0.002 cf 0.51 ± 0.003 %). No difference was observed in apoptotic cell levels between N and GH animals (see Figure 7.4 C).

7.3.5 Nitric oxide synthase inhibition

L-NAME caused substantial elevation of resting blood pressure in both N and GH male and female rats. Resting blood pressure was: 106 ± 5.1 cf 114 ± 5.9 mmHg in N male and females and 146 ± 4.6 cf 150 ± 2.8 mmHg in GH male and females. When looking at the absolute increase in MAP male and female N animals displayed a 72 ± 5.0 cf 65 ± 5.2 mmHg increase while male and female GH animals displayed a 81 ± 7.0 cf 88 ± 3.8 mmHg increase, respectively. Except for a significantly higher elevation in GH females than N females ($P<0.05$) no other differences were noted. The elevation observed was an increase of 67 ± 6.5 cf 56 ± 4.9 % in male and female N and 59 ± 4.6 cf 59 ± 2.3 % in GH rats, respectively. There was no difference in the percentage increase of blood pressure from rest between either male and female or N and GH animals (see Figure 7.5).

In order to compare normalised vasoconstrictory reactivity to endothelium-dependent reactivity, absolute blood pressure increases to infusions of phenylephrine (giving a maximal pressor response) and L-NAME were compared. Absolute increases for phenylephrine in male and female N animals were 74 ± 7.9 cf 71 ± 8.0 mmHg and in male and female GH animals were 98 ± 5.4 cf 90 ± 6.6 mmHg, respectively. There were no differences in the absolute blood pressure increases observed with either phenylephrine or L-NAME within the one group.

Figure 7.1 Effects of gender and hypertension on plasma von Willebrand factor.

Plasma vWF concentration was not different between male and female N and GH animals. No gender differences in vWF levels were evident. (n=8 in all groups). Data represents mean ± S.E.M.

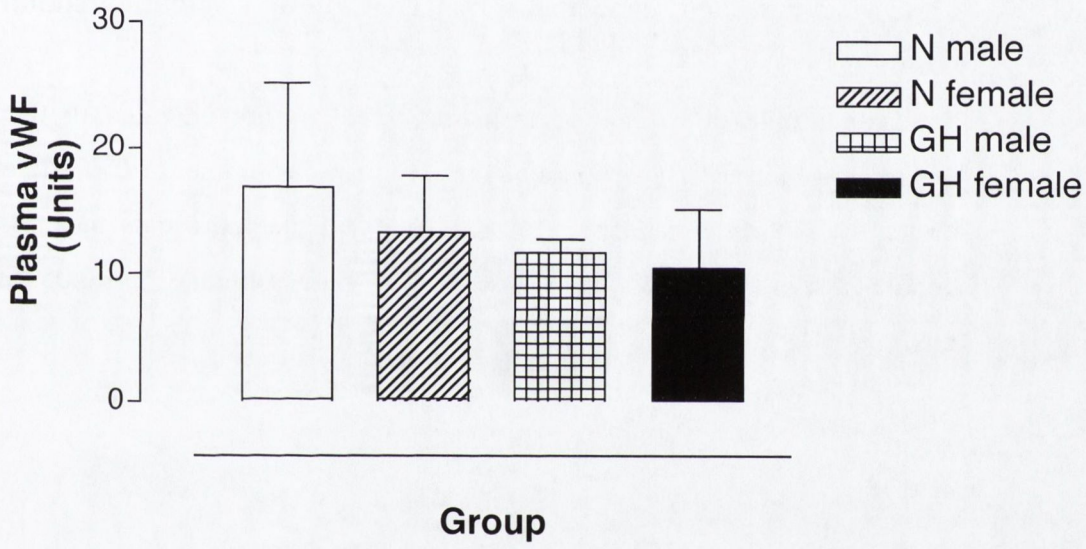
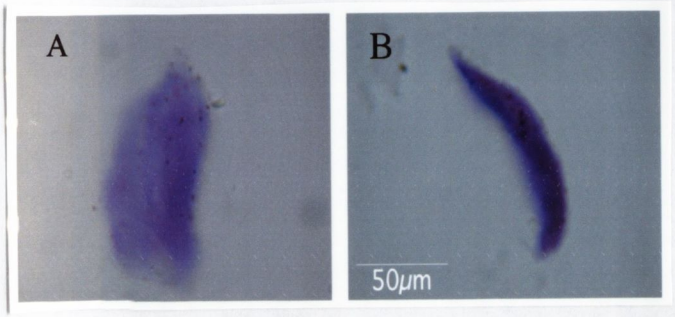

30
20
10
0
Plasma vWF
(Units)
N male
N female
GH male
GH female
Group

Figure 7.2 Effects of gender and hypertension on circulating endothelial cell number

A. An example of a CEC from a rat. B. CECs are elevated in the blood of hypertensive animals. There was no difference in the number of CECs in either male or female N or GH rats. (n=8 in all groups). Data represents mean ± S.E.M. * denotes a significant difference between GH and N animals * $P<0.05$, Student's *t*-test.

A


A
B
50μm

B

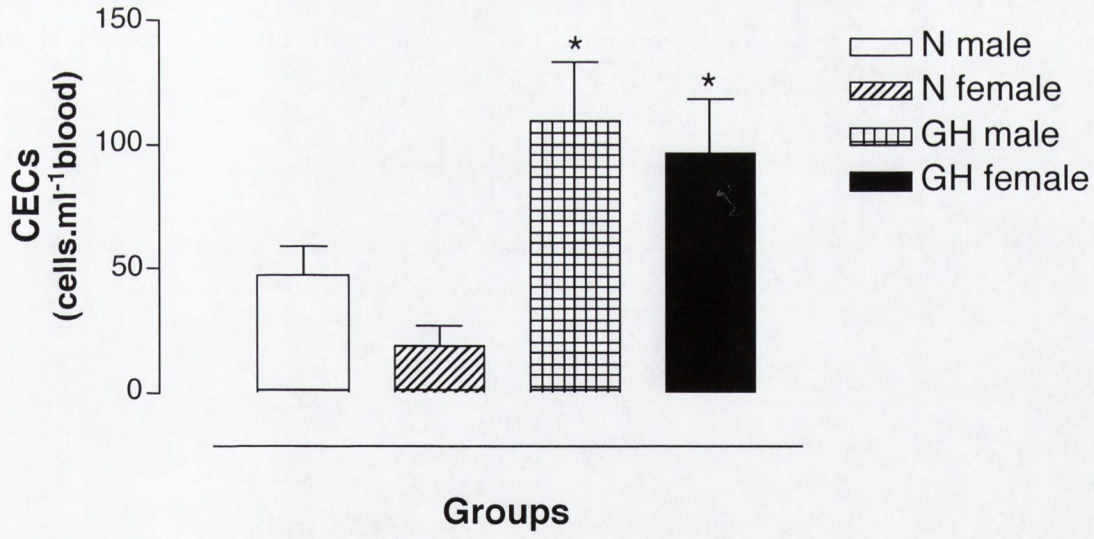
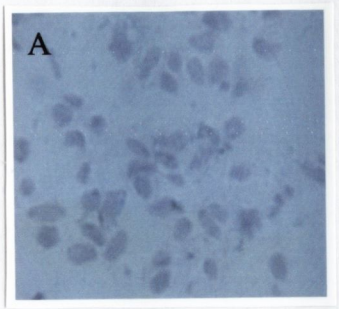

150
100
50
0
CECs
(cells.ml-1blood)
*
*
N male
N female
GH male
GH female
Groups

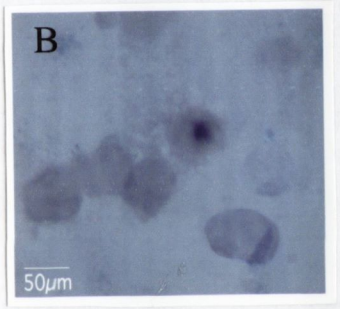
Figure 7.3 Effects of gender and hypertension on proliferating cell number in the rat

A. An example of a BrdU preparation counter-stained with methyl green. B. An example of a BrdU-positive cell. C. The percentage of proliferating cells was not different between N and GH male animals however female GH animals had a greater number of proliferating cells than female N and male GH animals. (n=8 in all groups). Data represents mean ± S.E.M. * denotes a significant difference between female GH and male GH animals and between female GH animals and female N animals ** $P<0.01$, 1-way ANOVA).

A


A

B


B
50μm

C

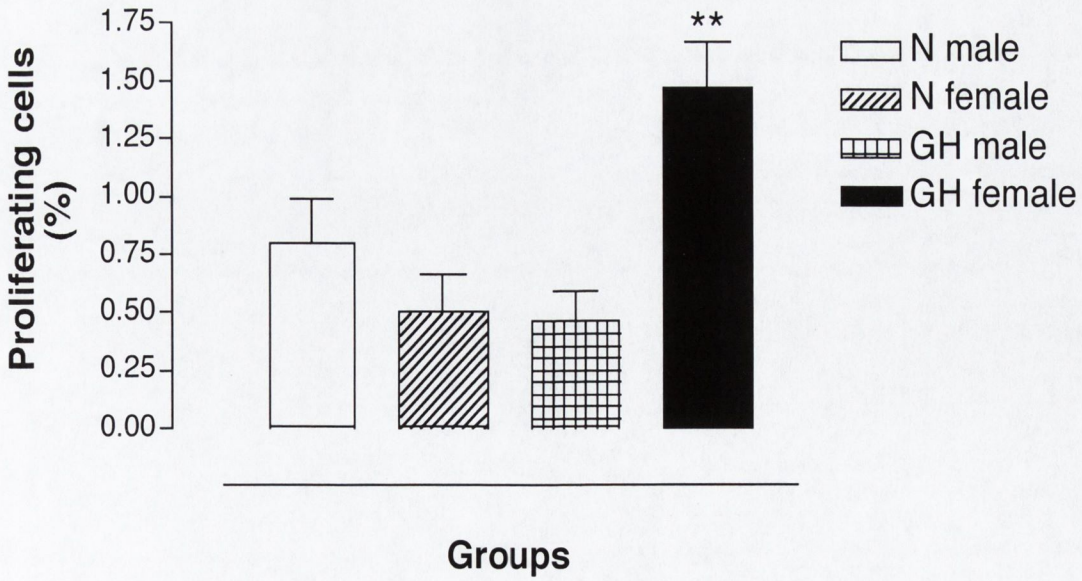
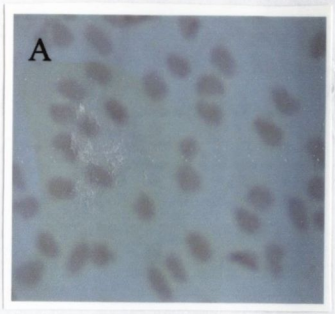

Proliferating cells (%)
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
**
N male
N female
GH male
GH female
Groups

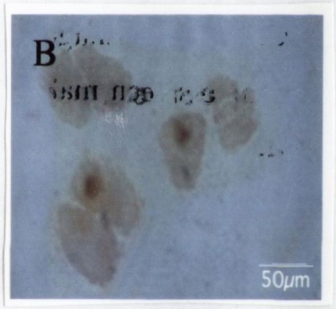
Figure 7.4 Effects of gender and hypertension on apoptotic cell number in the rat

A. An example of a TUNEL preparation counter-stained with methyl green. B. An example of a TUNEL-positive cell. C. The percentage of apoptotic cells was not different between N and GH animals or between males and females. (n=8 in all groups). Data represents mean ± S.E.M.

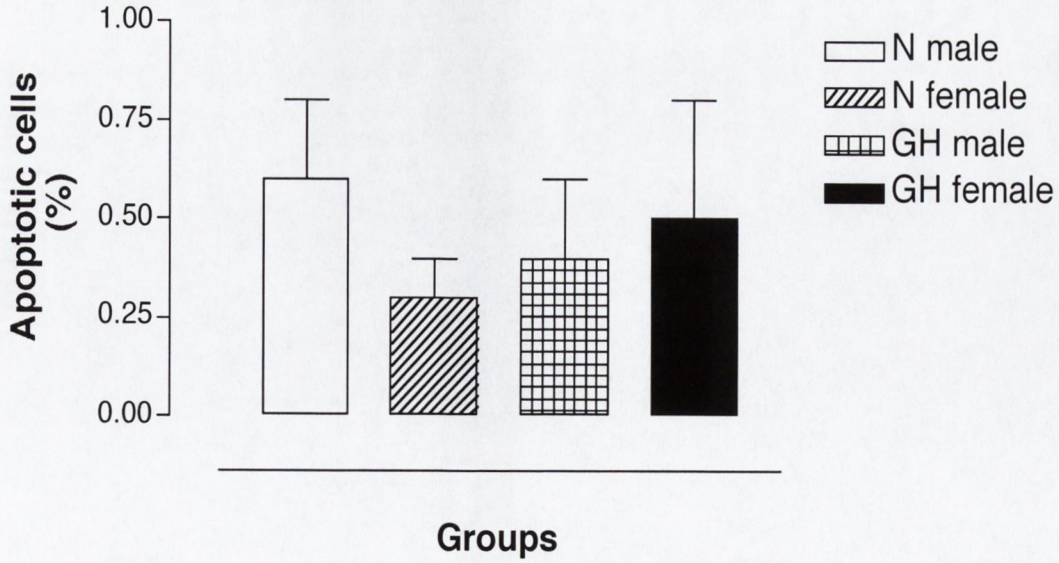
A


A

B


B
50μm

C


1.00
0.75
0.50
0.25
0.00
Apoptotic cells (%)
N male
N female
GH male
GH female
Groups

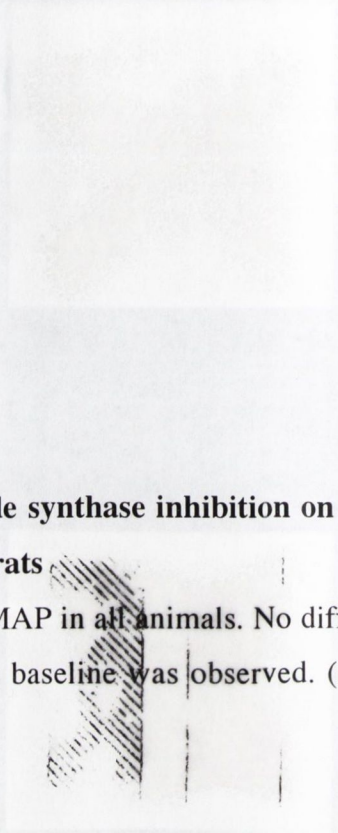
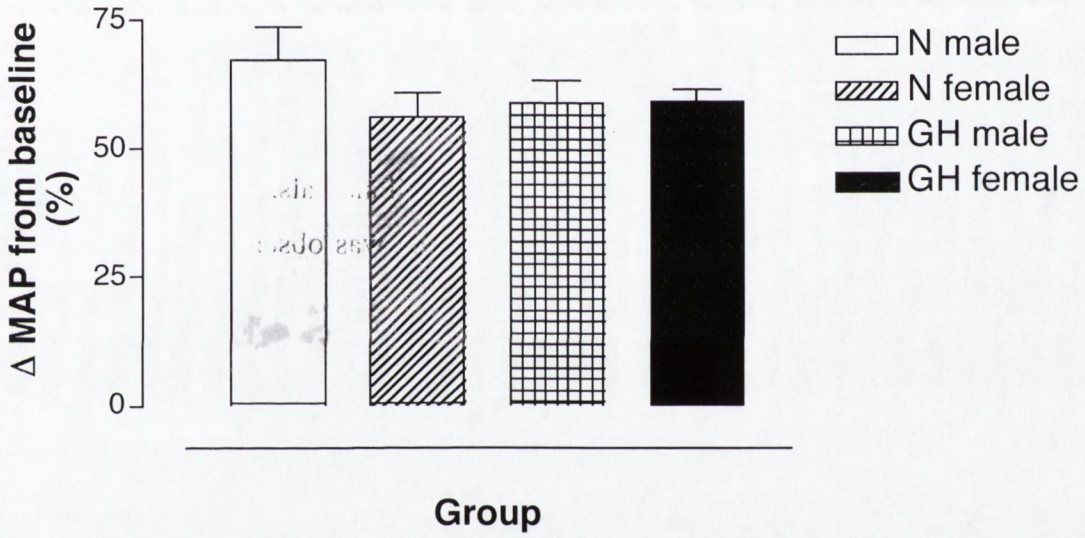


Figure 7.5 Effect of nitric oxide synthase inhibition on mean arterial pressure in male and female N and GH rats

LNAME caused an increase in MAP in all animals. No difference in the percentage increase in blood pressure from baseline was observed. (n=8 in all groups). Data represents mean ± S.E.M.


Δ MAP from baseline (%)
75
50
25
0
N male
N female
GH male
GH female
Group

7.4 Discussion

Endothelial cell growth, death and function are important determinants of vascular homeostasis. The GH rat strain has been extensively studied as a model for human essential hypertension (Simpson *et al.*, 1973). In many GH rat strains, endothelial injury and replication occur simultaneously during the hypertensive process (Schwartz and Lombardi, 1982; Mulvany, 1987). When Schwartz and Lombardi (1982) compared the replicative activity of the endothelium in SHR receiving anti-hypertensive agents, the decreased cell replication in the endothelium of treated animals suggested that cell replication was increased during chronic hypertension. Wu *et al.* (1990) found that endothelial injury and replication were significantly increased in the aortas of adult SHR compared to WKY. Endothelial cell turnover and the number of leaky junctions are increased in the aorta of SHRs, hyperlipidaemic rats, nicotine-treated rats and diabetic rats (Lin, 1996; Lin and Ding, 1996).

The results of the present study show that rates of endothelial cell death and proliferation are not different between the GH and N male rats. The most likely reason for no difference having been found is that NO levels were similar between the strains and NO is antiproliferative (Garg and Hassid, 1990). In essential hypertension the majority of evidence suggest there is an abnormality in the NO pathway (Cannon, 1998), which has also been documented in some rat models of hypertension (Dominiczak and Bohr, 1995). A deficiency has previously been shown in the basal production of NO by the endothelium of arterioles of some strains of GH rat (Boegehold, 1992) and in some forms of genetic hypertension, the flow/shear stress-induced dilation in skeletal and mesenteric muscle arterioles is significantly reduced, because of the absence of the NO-mediated portion of the response (Koller *et al.*, 1994; Koller and Huang, 1994; Matrougui *et al.*, 1997). Perhaps the comparable levels of NO in the N and GH rats in the present study acted to preserve endothelial function.

A strain difference between the GH rats used in the present study and those used in others cannot be ruled out. Apart from a previous study that suggested an impairment of endothelium-dependent relaxation in conduit arteries of these animals (Winquist *et al.*, 1984) more recent evidence concurs with the results of the present study (Orange *et al.*, 2000; Tucker *et al.*, 2000; Brady *et al.*, 2002). Moreover

inhibition of NOS in SHR has been found to elevate blood pressure to a greater extent in SHR than in WKY (Akiba *et al.*, 1995). This is consistent with greater endothelium-dependent vasodilator function at rest in SHR perhaps leading to reduced shear stress-dependent NO release. In the present study the magnitude of blood pressure increase was similar between GH animals and their N controls except for GH females, which had a higher level of MAP increase. Although a positive relationship between oestrogen and basal or stimulated releases of NO has been established in both large and small vessels, *in vivo* and *in vitro* (Rosselli *et al.*, 1995; Huang *et al.*, 1998) this was not observed in the present study.

In the present study L-NAME infusion caused a significant increase in MAP in all groups of animals and there was no gender difference in the percentage blood pressure increase. It is notable that within all groups NOS inhibition caused a similar increase in blood pressure as phenylephrine infusion, which was taken as an example of normalised reactivity. This would suggest that the structural properties of the vessel wall in GH animals, such as an increased wall-lumen ratio, did not affect the response. The significant increase in blood pressure elicited by systemic inhibition of NOS (Rees *et al*, 1989), in part, is likely to be caused by the lack of shear stress-induced release of NO in the peripheral circulation. In hypertension, impairment of the regulation of shear stress in arterioles, because of a lesser release of endothelial NO, may contribute to the elevated peripheral resistance (Koller and Huang, 1994; Cockell and Poston, 1997). Pulsatile stretch of cultured SMCs and endothelial cells stimulates their proliferation (Chien *et al.*, 1998) and growth factor production (Hu *et al.*, 1998). Because blood flow can also change in hypertension (van Boom and Saxena, 1980), alterations in shear stress and pulsatility may also contribute to vascular structural changes (Ibrahim *et al.*, 2003). Low shear stress, or changing shear stress direction, promotes endothelial proliferation and apoptosis, shape change, and secretion of substances that promote vasoconstriction, coagulation, and platelet aggregation (Paszkowiak and Dardik, 2003).

Hansson *et al.* (1985) found that dead and replicating endothelial cells in the aorta are clustered and that cell death probably leads to stimulation of cell replication. It is true that in some preparations from the present study the apoptotic cells were clustered together in areas. Unfortunately it was not possible to treat the same cell preparation for both apoptotic and mitotic cells, a technique that could identify co-localisation of both processes. The finding of an apoptotic index between

0.3 and 0.6% and a proliferative index of between 0.46 and 1.47% is slightly larger than reported in previous studies. Using IgG immunocytochemistry for identifying dying endothelial cells an average of 0.1% (Chen *et al.*, 1997), 0.19% (Hansson and Schwartz, 1983) and 0.75% (Lin and Ding, 1996) have been found in various studies. Mitotic cells in the range of 0.01% (Chen *et al.*, 1997) to 0.013% (Lin and Ding, 1996) have been observed using a haematoxylin technique. Differences in results could be due to differences in the rat strains and/or methodologies used.

Concentrations of CECs were similar in the plasma of male and female N and GH animals but GH animals had significantly elevated numbers when compared to N. Higher levels of CECs in hypertensive animals concurs with the result of a previous study in humans (Zhu, 1991). Although CECs are elevated in GH animals this was not accompanied by a concurrent loss of NO vasodilator capacity. Also plasma vWF levels were similar in both male and female and N and GH animals even though elevated levels of vWF have previously been associated with essential hypertension in humans (Blann *et al.*, 1993). This suggests that the increase in CEC in GH animals observed in this study does not reflect endothelial cell damage. Nor is it indicative of an increase in endothelial death since the percentage of apoptotic cells in these animals is not different. Some prior apoptotic assays in humans on CECs have been negative (Mutin *et al.*, 1999; Woywodt *et al.*, 2003). In fact Reidy and Schwartz (1981) found that injury causing a 2-cell wide denuded area is healed by endothelial migration without cell replication within 8 hours. Larger wounds may therefore require replication in order to re-endothelialise while cell loss below a threshold level may be compensated by a reorganisation of the endothelium without the requirement for DNA synthesis and cell division. The elevation in CEC number observed in this study could be due to defective adhesive properties of endothelial cells (Dignat-Gorge and Sampol, 2000) with hypertension or perhaps it is not apoptosis but rather necrosis that is responsible for endothelial cell detachment. The involvement of necrosis has been proposed recently (Woywodt *et al.*, 2003). It is also possible that the greater shear stress in the vessels of hypertensive animals caused the mechanical dislodgement of endothelial cells, which cannot be compensated for by an increase in endothelial cell turnover.

Finally the elevated proliferation rate in GH female animals in this study is difficult to explain. Vessel stretch, which is often present in hypertensive conditions, is accompanied by activation of JNK and p38 that cause vascular SMC apoptosis

(Sotoudeh *et al.*, 2002). Oestradiol has been shown to inhibit p38α (Razandi *et al.*, 2000). This isoform participates in the induction of apoptosis (Whitmarsh and Davis, 1996). Oestradiol helps maintain cytoskeletal integrity and may thereby prevent apoptosis in response to stresses in various cell types (Huot *et al.*, 1997; Landry and Huot, 1995). Oestradiol however can stimulate local vascular endothelial growth factor (VEGF) production (Dabrosin *et al.*, 2003). VEGF can increase vascular permeability and endothelial cell proliferation and migration (Ferrara and Davis-Smyth, 1997). Therefore although oestrogen inhibits apoptosis it has a positive effect on proliferation, which is reflected in the results of the present study.

Conclusion

CEC numbers are elevated in GH animals. This is not accompanied by an elevation in vWF and so does not appear to be due to endothelial dysfunction. The detachment of these endothelial cells does not involve an elevation in apoptosis or proliferation rates, although an interaction between oestrogens and growth factors cannot be completely ruled out. However this study does not confirm changes in cell proliferation rates with hypertension. NOS activity is similar in normotensive and GH animals indicating no impairment in the NO cycle in genetic hypertension.

The primary objective of this thesis was to investigate the interactive effects of hormonal status, smoking and ageing on SNS activation and endothelial function in women so as to further understand the mechanisms underlying the cardioprotective effects of female sex hormones. Sympathetic activation was accomplished through three well-known physiological stressors and endothelial integrity was assessed via endothelium-dependent vasodilatation and the quantification of plasma markers of endothelial function. In light of the data obtained in the human studies and in order to elucidate further the role of hypertension and gonadal steroid hormones on autonomic nervous system control of blood pressure and heart rate and on endothelial cell function, BRS and endothelial cell turnover rates were investigated in the rat.

8.1 Effects of hormonal status and ageing on resting cardiovascular parameters in young and middle-aged females

In Chapter 3 it was shown that menstrual cycle phase did not have any effect on resting cardiovascular parameters in young women. No difference in blood pressure was observed between menses and ovulation. This result is consistent with a previous finding from this laboratory using the Omron HEM-705CP automated oscillometric brachial artery monitor (Tansey and Bell, 2001). In that previous study brachial artery blood pressure was compared with that recorded at the digital artery, where wave reflection from peripheral sites leading to increased pressure pulse amplitude is likely (Taylor, 1965). At ovulation mean finger diastolic pressure was found to be significantly lower than mean arm diastolic pressure. However this was not the case at menses, perhaps due to lower peripheral vascular resistance at ovulation, which is only apparent in the extremities. Other groups have also found no difference in brachial MAP, systolic pressure or diastolic pressure during the menstrual cycle (Hashimoto *et al.*, 1995; van Beek *et al.*, 1996; Lewandowski *et al.*, 1998). The fact that combined oestrogen and progesterone oral contraceptive use, the presence of these hormones is evident in the luteal phase of the cycle, had no

influence on resting blood pressure also supports this conclusion. Cardiac parameters such as heart rate, stroke index, cardiac index and rate pressure product were similar between the two phases of the menstrual cycle suggesting physiological levels of oestrogen do not effect the heart directly via changes in cardiac contractility or work. Further evidence that oestrogen and progesterone do not affect resting blood pressure was found in Chapter 4 where postmenopausal HRT users had similar levels relative to non-users. This result is in agreement with those of others (Akkad, *et al.*, 1997; Pripp *et al.*, 1999). However the fact that the subjects in the present study were using a variety of HRT regimens may have been a confounding factor. The presence of progesterone, the route of administration, the dose of treatment or the duration of HRT use could potentially have significant effects. Nevertheless Mills *et al.* (2003) found no difference in daytime or nighttime blood pressure between women given combined HRT, oestrogen replacement therapy or placebo. Both HRT and oestrogen replacement therapy have been found to have beneficial effects on aortic distensibility and elastic properties (Kawecka-Jaszcz *et al.*, 2002; Gorgulu *et al.*, 2003) but this improvement seems to be independent of blood pressure changes (Kawecka-Jaszcz *et al.*, 2002). These studies suggest the presence of progesterone may not have played a major role in the result. Also a recent study has demonstrated no differences between oral and transdermal HRT in office blood pressure levels (Zacharieva *et al.*, 2002). In the context of this thesis therefore it is also unlikely that route of administration played a role.

Previous comparisons of young with elderly subjects have indicated that ageing is associated with increased plasma NA, increased SNA and elevated MAP in both men and women (Ziegler *et al.*, 1976; Ng *et al.*, 1993; Burt *et al.*, 1995). Men have elevated blood pressure relative to women until the age of 40 when thereafter the rate of rise increases past that of men (Burt *et al.*, 1995). This suggests that hormonal status may contribute to blood pressure stability. In the present study postmenopausal women had significantly elevated resting MAP, diastolic pressure and systolic pressure relative to younger controls. The elevation of MAP seemed to be due primarily to a rise in peripheral vascular resistance without cardiac involvement, since resting TPR in the postmenopausal cohort was considerably greater than that found in young subjects and heart rate was constant while stroke index was reduced. There was also significantly greater wave reflection in the older group suggesting reduced arterial distensibility. The changes observed in the present

study are similar to the situation observed recently in sedentary middle-aged men (O' Sullivan, 2002), suggesting that they are related to the ageing process *per se* rather than to postmenopausal withdrawal of hormonal influence. In support of this, the data showed that age-matched postmenopausal women on HRT exhibited identical changes in MAP and TPR to those in the untreated postmenopausal group.

8.2 Effects of hormonal status and ageing on sympathetic activity in young and middle-aged females

Many studies have demonstrated an association between cardiovascular reactivity and CVD development (Barnett *et al.*, 1997; Kamarck, 1997; Matthews *et al.*, 1998). Groups that differ in reproductive hormone status differ in the magnitude of their cardiovascular responsiveness to various forms of stress as well as risk of CVD such as atherosclerosis. Men are at higher risk of coronary heart disease than premenopausal women and have elevated blood pressure and catecholamine responses to laboratory stressors (Allen *et al.*, 1993; Stoney *et al.*, 1997). Postmenopausal and öophorectomised women have an increased coronary heart disease risk relative to premenopausal women (Colditz *et al.*, 1987; Matthews, 1989) and pregnant women exhibit decreases in stress-induced diastolic pressure responses compared with pre-pregnant levels or matched controls (Matthews and Rodin, 1992). These instances would support a role for reproductive hormones influencing cardiovascular responses to stress.

As discussed in Chapter 3 menstrual cycle phase did not influence responses to physiological stress except during the cold pressor test where a greater increase in MAP was observed at ovulation than at menses, most likely due to the much elevated systolic pressure and the substantially greater cardiac work. Female sex hormones are also known to effect thermoregulation (Brooks *et al.*, 1997). In fact during the menstrual cycle fluctuations in core temperature occur depending on the concentration of oestrogen and progesterone. Core temperature increases with the presence of progesterone during the luteal phase and is at its lowest during the late follicular phase, which is accompanied by a surge of oestrogen (Stephenson and Kolka, 1999). Sex steroids most likely operate to impact thermoregulation through the hypothalamus. In animals oestrogen and progesterone can act directly on specific neurons in the preoptic and anterior hypothalamus (Silva and Boulant,

1986). Perhaps oestrogen lowered the set-point at which responses to the cold pressor test take place and hence a elevated pressor response at ovulation was observed. However whether blood pressure increases in response to the cold pressor test rely on hormone-dependent thermoregulation remains to be determined. Other plausible alternatives to a thermoregulatory basis for this increase in MAP are a change in α-adrenergic receptor density or affinity for oestrogen (Colucci *et al.*, 1982) during the menstrual cycle. Oral contraceptive users on the other hand displayed blunted blood pressure responses to mental stress possibly due to the presence of progesterone. This is supported by the fact that Kirschbaum *et al.* (1995) found reduced cortisol production in response to psychological stress in women using oral contraceptives. In a recent animal study oestrogen and progesterone administration together were found to significantly attenuate blood pressure and heart rate responses to stress (Eikelis and van den Buuse, 2000).

In addition to having increased resting blood pressure, the postmenopausal group responded to a sustained handgrip and mental stress with larger increments in systolic pressure and greater rate pressure products for a sustained handgrip than their younger counterparts. In young subjects while TPR rose during handgrip and diastolic pressure rose during mental arithmetic, no effect on either of these parameters was seen in the postmenopausal group. Collectively, these observations are not supportive of enhanced sympathetic reactivity in the older women. A previous study using similar stress protocols found that hyperreactivity of the SNS is not present with ageing. This group found similar plasma NA, muscle SNA and blood pressure stress responses between young and older subjects (Ng *et al.*, 1994). The increase in systolic pressor responsiveness in the present study could have been partly due to the greater arterial stiffness at rest in the older group. Previous studies have documented that reduced arterial distensibility is associated with menopause (McGrath *et al.*, 1998; Westendorp *et al.*, 1999) as well as with the normal ageing process (Cheitlin, 2003). However, since similar elevations of systolic pressure were found in untreated postmenopausal women and in the cohort undertaking HRT, the effect that was observed seems likely to have been age-related rather than secondary to hormonal changes.

The cold pressor test produced somewhat conflicting results in that younger women tended to have greater heart rate and blood pressure responses than older women. This effect could be due to age-related deficits in thermoregulation (Young

and Lee, 1997). Thermoregulatory efficiency decreases with age, predisposing older individuals to hypothermia in cold environments (Inoue *et al.*, 1992; Young and Lee, 1997). Older persons have a reduced cutaneous thermal sensitivity, and a reduced subjective thermal perception during cooling. Both vasoconstriction and shivering, the two primary responses that maintain body temperature during cold challenge, are less effective in older people (Khan *et al.*, 1992). Central and peripheral components seem to be involved in the age-related changes in thermoregulatory functions (Smolander, 2002). Cold-induced changes in vasomotor tone depend primarily on NA release and adrenoceptor-mediated vasoconstriction in the cutaneous vasculature (Frank *et al.*, 1995; 1997). Older persons' skin vasoconstrictive response to cold is diminished compared to younger persons. Reasons for impaired thermoregulatory vasoconstriction with ageing may be decreased NA release (Frank et a. 2000) or a decreased vasomotor response for a given amount of NA at its receptors (Scott and Reid, 1982; Nielsen *et al.*, 1992; Frank *et al.*, 2000) or downregulation of adrenoceptor numbers (Dinenno *et al.*, 2002). In fact Nagashima *et al* (2002) found that in a cool environment finger temperature was inversely correlated with NA levels in young women. Furthermore, the contribution of sympathetic α-adrenergic vasoconstriction to basal forearm vascular tone is reduced with age in healthy men (Dinenno *et al.*, 2002).

Hormonal status does not seem to be involved since HRT users and non-users had comparable responses. This result is supported by a recent study where short-term oestradiol administration did not have any effect on blood pressure or heart rate responses to the cold pressor test (Weitz *et al.*, 2001).

8.3 Effects of smoking on resting cardiovascular parameters in young and middle-aged females

Young smokers had elevated MAP, diastolic pressure and systolic pressure at ovulation when compared with non-smokers and smokers had elevated MAP at ovulation when compared with menses, as shown in Chapter 3. The elevated resting blood pressure in smokers at ovulation and the absence of this effect at menses is somewhat suprising. However it may indicate that there is some interaction between oestrogen concentration and smoking's pressor effects. Cigarette smoke is a known anti-oestrogenic compound that works so as to increase inactive catechol oestrogens

and lower effective plasma oestrogen levels (Baron *et al.*, 1990; Bolego *et al.*, 2002). Another possible explanation is that increased oestrogen levels during the menstrual cycle leads to an elevation in aldosterone levels (Chidambaram *et al.*, 2002) as does smoking (Garcia Calzado *et al.*, 1990) and taken together these factors could perhaps have substantial blood pressure elevating effects. Changes in adrenergic receptor number during the menstrual cycle or as a result of exposure to cigarette smoke or a reduction in basal NO production due to smoking (Barua *et al.*, 2001) may also play a part. The elevation of blood pressure at ovulation is somewhat supported by results from Girdler *et al.* (2000) who found that postmenopausal non-smokers using HRT had lower MAP with treatment relative to control values while this difference was not seen in smokers, suggesting smokers may experience less benefit from HRT use.

No differences in resting cardiovascular parameters were seen between middle-aged smokers and non-smokers except for a lower cardiac index in the smoking group perhaps due to an elevated TPR. Interestingly no difference in resting blood pressure was evident between middle-aged and young smokers (at menses) however resting TPR was elevated and cardiac index was lower in the middle-aged smoking group. The absence of an age-effect on the blood pressure of middle-aged smokers is peculiar. However several previous studies have reported similar or lower blood pressure levels in smokers when compared to non-smokers (Berglund and Wilhelmsen, 1975; Green *et al.*, 1986; Imamura *et al.*, 2002). In the present study the middle-aged smokers had lower blood pressure than middle-aged non-smokers although not significantly so. It is possible that smokers, having smoked on average 4.7 ± 1.4 h before testing, were more at ease than non-smokers in the laboratory setting. In a recent study craving intensity in abstaining smokers was less with nicotine patch treatment than with placebo (Teneggi *et al.*, 2002) indicating the stress-relieving effects of nicotine. It is also known that feelings of stress and anxiety are attenuated immediately after smoking (Parrott *et al.*, 1995). However whether these feeling were present in smokers in the present study is unclear since the half-life of nicotine is thought to be 2.6 h (Benowitz and Jacob, 1993). Cotinine, the principal proximate metabolite of nicotine, however has a half-life of about 17 h (Benowitz and Jacob, 1993). Its involvement is unlikely, however since it does not appear to have any cardiovascular effects (Keenan *et al.*, 1994) and

antagonises the beneficial effects of nicotine on withdrawal symptoms (Hatsukami *et al.*, 1998).

8.4 Effects of smoking on sympathetic activity in young and middle-aged females

When looking at cardiovascular responses to stress at menses young smokers and non-smokers did not differ markedly. In the middle-aged group no difference in cardiovascular reactivity was observed between non-smokers and smokers during a sustained handgrip or the cold pressor test but smokers displayed elevated blood pressure responses to mental stress compared with non-smokers. A similar albeit insignificant trend was evident when looking at middle-aged and young smokers.

This increased blood pressure reactivity in middle-aged smokers during mental stress was perhaps due to the additive effects of ageing, stress and smoking. It has been previously suggested that smokers may get greater effects from cigarettes consumed temporally far apart than from cigarettes smoked closer together. Perkins *et al.* (1995) found that widely spaced cigarettes produced greater arousal responses to nicotine. A study using psychological stress found that after 12 hours of smoking abstinence smokers reacted with greater blood pressure responses (Tsuda *et al.*, 1996) and Tersman *et al.* (1991) found that smokers are more reactive to stress than non-smokers. However this fact is disputed (Adan and Sánchez-Turet, 2001). Another study found that prior cigarette smoking in women produced blood pressure and heart rate responses to mental stress that were larger than the additive effects of smoking and stress taken separately (Dembroski *et al.*, 1985). It therefore does not seem likely that length of time since last cigarette played a significant role in the present study since middle-aged smokers abstained for greater than 2 h and less than 12 h. Also there was no difference between length of time since last cigarette in young and middle-aged smokers and no such increase in blood pressure in response to mental stress was observed in the younger group. The fact that this form of stress produced greater reactivity in middle-aged smokers may suggest a link between smoking and CVD risk in these women. Middle-aged smokers had been smoking for over 30 years whereas the young smokers were relatively short-term smokers. It is likely the results of the present study could be due to changes caused by longer tobacco exposure. It has also recently been suggested that nicotine

administration to young females enhanced feelings of calmness while the same dose in males was associated with feelings of anxiety and discontent (File *et al.*, 2001). Since postmenopausal women are oestrogen-deprived, as are males, perhaps there is a hormonal basis to this reaction. It is however important to investigate whether length of time smoking and what duration of abstinence have effects on cardiovascular reactivity to mental stress.

There is also compelling evidence that excessive cardiovascular reactivity associated with stress can promote damage to the endothelium and favour the development of atherosclerosis (Manuck *et al.*, 1988). The normal functioning of the endothelium may therefore be both a cause and a consequence of cardiovascular responses elicited by stress. As discussed later there was an impairment of endothelium-dependent vasodilatation in postmenopausal smokers when compared with younger smokers and middle-aged non-smokers perhaps mirroring the excessive reactivity observed. In fact anger, anxiety and depression are associated with impaired brachial artery dilation among postmenopausal women (Harris *et al.*, 2003) demonstrating that psychological factors can influence endothelial function. In a study in young adolescents systolic pressure reactivity to postural change, forehead cold stimulation and dynamic exercise significantly predicted flow-mediated dilation (Treiber *et al.*, 1997).

8.5 Effects of hormonal status and ageing on endothelial function in young and middle-aged females

As discussed in Chapter 5 there were no phase differences in hyperaemic responses to arterial occlusion during the menstrual cycle. This would suggest that in this study physiological concentrations of oestrogen at ovulation did not have any influence on endothelium-dependent vasodilatation. Measurements of brachial artery diameter, another means of assessing endothelium-dependent function, during the menstrual cycle have previously demonstrated greater diameters in the follicular and luteal phases of the cycle, where oestrogens are present (Hashimoto *et al.*, 1995; Kawano *et al.*, 1996; Arora *et al.*, 1998; Chan *et al.*, 2001). The differences between these studies and the present are probably due to differences in methodology. Recently Malik *et al.* (2001) found when using duplex Doppler ultrasonography for the simultaneous assessment of both flow-mediated dilation and reactive hyperaemia

that peak blood flow response was more reproducible than FMD because of considerably lower intra-subject variability. Therefore the results of the present study may be more accurate using the 3 minutes standard reactive hyperaemia protocol. Further study in this area is warranted however. No differences between endothelium-dependent vasodilatory responses at withdrawal or during oral contraceptive use or between responses in oral contraceptive users are non-users were evident in this study. This results agrees with the available literature (John *et al.*, 2000) and goes somewhat to validate the menstrual cycle result since oral contraceptives contain oestrogen and progesterone i.e. are comparable to the luteal phase of the menstrual cycle.

Endothelial dilator capacity did not seem to be substantially altered with either ageing or hormonal withdrawal in this study. Percentage increase in blood flow after release of the occlusive cuff was not significantly correlated with age. The magnitudes of flow in response to temporary arterial occlusion were similar in postmenopausal controls and in postmenopausal HRT users to that seen in young premenopausal women. Previous studies have shown that oestrogen therapy enhances flow-mediated brachial artery dilation (Williams *et al.*, 1992; Gilligan *et al.*, 1994; Lieberman *et al.*, 1994). However progesterone seems to negate this effect (Miller and Vanhoutte, 1991). The fact that the majority of subjects in the present study were on combined HRT may have played a significant role. In agreement with this combined HRT has produced no difference in endothelium-dependent responses (Sorensen *et al.*, 1998; Van Baal *et al.*, 1999; Teede *et al.*, 2001). In addition there were no differences between postmenopausal women and young women in CEC number or in plasma levels of vWF, TM or CRP all of which are widely used as markers of endothelial damage (Califano *et al.*, 2000; Dignat-George and Sampol, 2000; Conway *et al.*, 2002; Brevetti *et al.*, 2003).

It is not known to what extent postmenopausal women benefit from the long-term effects of premenopausal endogenous oestrogen exposure. In several studies age at menopause was found to be related to risk of CVD (van der Schouw *et al.*, 1996; Cooper and Sandler, 1998). Furthermore, DeKleijn *et al.* (2002) found that age at menopause was positively correlated with duration of exposure to oestrogens ($r = 0.725$) and that cardiovascular mortality risk was lower for women with a late menopause. All subjects in the present study were comparable in time since menopause and HRT users started the treatment immediately at menopause onset

except for one who started 4 years later. However, in light of the literature it cannot be excluded that impaired endothelial dilation and changes in circulating levels of these markers may be associated with more extreme ageing or longer periods of oestrogenic deprivation at menopause (Sarabi *et al.*, 1999). Also the possibility that longer periods of HRT use could have altered the fact that HRT had no effect on endothelium-dependent vasodilatation in the present study cannot be ruled out.

8.6 Effects of smoking on endothelial function in young and middle-aged females

In contrast to the lack of a smoking effect on endothelial function in young women, the results of Chapter 5 demonstrate that middle-aged smokers have a significantly lower endothelium-dependent vasodilator capacity than middle-aged non-smokers and young smokers. The loss of the normal vasodilatory response in older smokers suggests that the increased incidence of CVD in smokers could be due to loss of endothelial function. The fact that ageing or menopause did not have any detrimental effects on endothelium-dependent vasodilatation in the non-smoking cohort suggests that although ageing was perhaps a contributory factor, smoking itself seems to be the primary cause. This is supported by the fact that there was a significant relationship between numbers of cigarettes smoked and percentage increase in blood flow from rest. The restriction of the decline in vasodilatory capacity to middle-aged smokers alone may reflect their greater exposure to tobacco smoke products. In fact in a recent study both ACh- and sodium nitroprusside-induced skin blood flow increases in the microvasculature were significantly attenuated in older smokers (mean 30 pack years) compared with non-smokers, whereas no such effect was evident in young smokers (mean 7.2 pack years) (Pellaton *et al.*, 2002). A similar situation now seems likely in forearm resistance vessels.

The mechanisms by which cigarette smoking leads to endothelial dysfunction are unclear. However, a number of scenarios are likely. Oxidative stress may be a mediator since cigarette smoke contains large amounts of free radicals such as superoxide anion and hydroquinone (Freed *et al.*, 2001) that degrade NO released from the endothelium. Long-term smoking could potentially lead to greater prooxidant production and consequently prolonged oxidative stress. Indeed, anti-

oxidants such as vitamin E and C have been shown to attenuate the impairment of endothelium-dependent vasodilatation after heavy cigarette smoking, due to an improvement of the antioxidant status (Heitzer *et al.*, 1996; Neunteufl *et al.*, 2000). Nicotine could potentially be the tobacco component involved. A recent study in humans has shown that flow-mediated dilation is directly impaired by nicotine (Neunteufl *et al.*, 2002). This could be due to a loss of the vasodilatory action of NO due to oxidants and ensuing endothelial cell damage possibly via membrane lipid peroxidation (Radi *et al.*, 1991) or the production of prostaglandin H_2, which causes vasospasm (Zou and Bachschmid, 1999). However as regards the present study it is unlikely that acute nicotine exposure was the primary cause as its half-life is approximately 2.6 h, although years of chronic exposure probably have longer lasting effects. This is also supported by the fact that young smokers displayed no dysfunction whatsoever. Nevertheless it has also previously been shown that the antioxidant capacity of smokers does not return to non-smoking levels until between 6 and 12 months after smoking cessation and lower antioxidant levels in middle-aged men are correlated with longer smoking duration and greater daily smoking quantity (Zhou *et al.*, 2000).

In agreement with published evidence for damaging effects of smoke constituents on the endothelium smokers in the present study had a two-fold increase in CEC number when compared with non-smokers. It was however noticeable that middle-aged smokers did not display greater CEC number than young smokers despite blunted vasodilator responses in the middle-aged group. Therefore women who had been smoking for over thirty years exhibited similar numbers as young women who had only been smoking for several years. This evidence suggests that the rise in CEC number with smoking may not be reflective of endothelial cell damage. This is supported by the fact that plasma vWF, TM and CRP levels were not different between middle-aged smokers and non-smokers. The reason for this increase in CECs is not known however a number of mechanisms could be involved. Apoptosis of endothelial cells because of an increase in oxidative stress (Vayssier-Taussat *et al.*, 2001) in combination with a decrease in NO (Dimmeler and Zeiher, 1999), a known apoptosis inhibitor, may be involved. Increased stimulation of the endothelium by the products of cigarette smoke may not actually lead to cell death but rather could increase the rate of cell proliferation. In fact both nicotine and cotinine have previously been shown to up-regulate

endothelial growth factor expression (Conklin *et al.*, 2002) and rats given nicotine in their water have been shown to have increased levels of endothelial cell death (Lin, 1996). Alternatively endothelial detachment could involve ß-adrenergic receptor stimulation (Pettersson *et al.*, 1990) although it must be noted that resting blood pressure and heart rates were similar among smokers and non-smokers. However the fact that middle-aged smokers display elevated responses to mental stress may be indicative of increased ß-adrenergic activation in this group. Previously a ß-adrenergic antagonist has been shown to inhibit cell death caused by sympathetic activation (Pettersson *et al.*, 1990).

8.7 Effects of hypertension and gender on baroreflex sensitivity in female and male intact and gonadectomised rats

Having investigated the effects of female sex hormones on SNS activation, a role for oestrogens and progesterones on blood pressure regulation via the baroreflex was considered. Since BRS is not easily assessed in humans a rat model allowing for hormonal manipulation was used. Long-term hormonal changes were then assessed by removing the influence of pubertal sex hormones. Using a GH rat model the role of hypertension on BRS was also assessed.

Initially the lack of a hormone effect on blood pressure and heart rate when comparing postmenopausal HRT users and non-users was somewhat validated by the fact that no difference was observed between blood pressure or heart rate levels in normotensive female intact and gonadectomised rats in this study. Although it must be remembered that while postmenopausal females have been exposed to oestrogens for at least 30 years the rats were ovariectomised before their first oestrus cycle. No differences in blood pressure and heart rate were also seen between male and female rats again demonstrating no effect of sex homones on these parameters.

BRS is impaired in hypertension and sustained elevation of blood pressure is associated with the resetting of baroreflex function towards a higher value (Mussalo *et al.*, 2002). There is an important hereditary component in the development of hypertension and defective baroreflex control has been previously demonstrated in both genetically hypertensive animals (Andresen *et al.*, 1980; Lantelme *et al.*, 1998; Murphy and McCarty, 1995) and humans (Parmer *et al.*, 1992; Grassi *et al.*, 1998; Pikkujamsa *et al.*, 1998). In agreement with the previously published data the results

of Chapter 6 confirm that New Zealand GH rats from the inbred GH strain exhibit considerably lower baroreflex gain than age-matched inbred normotensive controls. This attenuated baroreflex gain amounted to approximately one-third that of normotensive controls.

Maximum pressor and depressor responses to sodium nitroprusside and phenylephrine in the GH strain were larger than normotensive responses. This increased cardiovascular reactivity is probably due to increased vasoconstrictor responses brought about by an elevated wall-lumen ratio in these animals (Ledingham and Laverty, 1996). This in turn may help to maintain the elevated blood pressure in GH rats due to deficits in autonomic control of vascular resistance. Also in most animals the vagal limb elicited a greater baroreflex response when compared with the sympathetic limb suggesting it is the bradycardic response that is dominant and therefore plays a greater part in blood pressure control. Interestingly the sympathetic limb of the baroreflex arc was unaffected by hypertension in these animals suggesting that it is vagally-mediated baroreflex control that is defective.

Oestrogen receptors have been found in many areas of the baroreflex arc and evidence suggests it may modulate baroreflex function (Saleh and Connell, 2000). Results from this thesis show that female normotensive rats display defective bradycardic baroreflex regulation when compared with male animals. This result agrees with those of prior studies undertaken in rats (El-Mas and Abdel-Rahman 1998; Abdel-Rahman, 1999). However there is a large body of evidence to suggest that oestrogen has beneficial effects on baroreflex gain (He *et al.*, 1998; Saleh *et al.*, 2000; Sahel and Connell, 2000). These studies generally use either oestrogen replacement or oestrogen administration and the evidence to suggest that intact female animals have greater baroreflex gain than males is limited. It may be that physiological concentrations of oestrogen may not produce similar results. In support of this consensus Cooke *et al.* (2002) found no change in baroreflex gain over the duration of the menstrual cycle. Studies in pregnant women suggest BRS can decrease by as much as 50% in normotensive pregnant women (Blake *et al.*, 2000). Even though females are thought to have a higher level of parasympathetic tone than males the vagal limb of the baroreflex seems to be attenuated. This is supported by the fact that the majority of human studies show defective cardiovagal BRS in women when compared to men (Huikuri *et al.*, 1996; El-Mas and Abdel-Rahman, 1998; Laitinen *et al.*, 1998; Abdel-Rahman, 1999; Beske *et al.*, 2001;

Peckerman *et al.*, 2001). Indeed Sevre *et al.* (2001) found that hypertensive females had comparatively lower BRS than hypertensive men. This trend was seen in the first experiment using GH rats but could not be repeated. Also the New Zealand GH rat may produce more comparable results to studies performed in humans since they are often used as a model for human essential hypertension.

The reasons for lower BRS in females are unknown. However when considering gender control of blood pressure more closely it is a well-established fact that women have reduced orthostatic tolerance compared with men (Gotshall *et al.*, 1991; Gotshall, 2000) and this has been associated with a number of factors, including reduced cardiovagal baroreflex gain (Convertino, 1998). Nevertheless given that decreased BRS is a risk factor for CVD and that oestrogens are cardioprotective, the situation seems highly contradictory. It is known however that these cardioprotective effects include favourable changes in lipid metabolism, thrombogenic factors, endothelial function and distensibility perhaps compensating for defective BRS. In the present study oestrus cycle phase was not taken into account and hence the possibility of unfavourable effects of progesterone cannot be excluded. Progesterone has previously been shown to attenuate BRS (Masilamani and Heesch, 1997). Some progestins prevent the decrease in pulsatility index observed on oestrogen administration (Mercuro *et al.*, 2000) a factor that could have a detrimental impact on baroreceptor function. Circulating NA is increased during the luteal phase of the menstrual cycle (Goldstein *et al.*, 1983) when progesterone levels are high and progesterone administration alone has been reported to increase the concentration of NA in the blood (Mercuro *et al.*, 1999). Plasma NA concentration has been shown to correlate inversely with BRS (Laitinen *et al.*, 1998). This may indicate that progesterones act in opposition to oestrogen via effects on the SNS. Moreover testosterone has been shown to enhance and castration to attenuate reflex bradycardia (El-Mas *et al.*, 2001) and androgen receptors have also been found in brainstem areas that are involved in baroreflex integration: the nucleus tractus solitarius (Simerly *et al.*, 1990), the nucleus ambiguus and the dorsal motor nucleus of the vagus (Sheridan and Weaker, 1982; Freeman *et al.*, 1995). However the fact that gonadectomy did not have any influence on BRS in either normotensive or GH animals would suggest that ovarian hormone deprivation via ovariectomy or testosterone hormone deprivation via castration may not be the primary regulators involved.

Also the age of the animal at gonadectomy could play an important role in the outcome of experiments on gender. Surgery was performed in animals of 3-4 weeks. This age was chosen as it is known female rats begin their first oestrous cycle at 35 days old and GH animals have elevated systolic blood pressure when compared with normotensive animals as young as at 2 days old (Jones and Dowd, 1970). It was therefore known that sex hormones would be totally absent from these animals and hypertension would be well established at this young age. Studies in the literature have performed the surgery in animals between the age of 9-16 weeks (He *et al.*, 1998, 1999; Mohamed *et al.*, 1999) or a weight of 230-280 g (El-Mas *et al.*, 2001, 2002; Saleh and Connell, 1998, 2000). This differs from the case in the present experiment as these animals have previously been exposed to sex hormones whereas those in my study were not. Perhaps in animals gonadectomised at an early age other factors compensate for the lack of sex hormone production.

8.8 Effects of hypertension and gender on endothelial function in rat

The endothelium has an important barrier function and is thought to be a relatively static single layer of cells with slow turnover rates. Changes in numbers of dying and regenerating endothelial cells could be indicative of endothelial cell dysfunction. A variety of situations have been reported to increase endothelial turnover. These include lipopolysaccharide injection (Hansson *et al.*, 1985), fluid shear stress, which stimulates DNA synthesis and is suggestive of mitosis (Davies *et al.*, 1986), sympathetic activation (Pettersson *et al.*, 1990), the development and growth of atherosclerotic lesions (Hansson and Bondjers, 1980), and a variety of risk factors for CVD – hypertension, nicotine and diabetes (Lin, 1996). Previously in Chapter 5 it was found that smoking causes an elevation in CEC number. It is also know that hypertension increases the number of these cells. However it is unknown what causes this rise in CECs in the peripheral blood. One plausible explanation is an elevation in the rate of endothelial cell death and turnover.

The results from Chapter 7 indicate that hypertension in the GH rat is not associated with increased levels of cell death by apoptosis nor increased endothelial cell regeneration via DNA synthesis. However an elevated number of CECs was found in the blood of hypertensive animals. The reason for the detachment of endothelial cells from the basal lamina is not known. However results from Chapter

7 suggest it is not apoptosis nor increased cell turnover that is responsible. New evidence proposes that endothelial cells may experience greater levels of death via necrosis (Woywodt *et al.*, 2003). Another alternative is that the higher pressures in the arteries of hypertensive animals caused mechanical dislodgement of the cells. Hypertension could alternatively have influences on the metabolic milieu. It has previously been shown that increases in shear stress as might be seen in hypertension itself is often associated with increases in reactive oxygen species (Rodrigo *et al.*, 2003), which may exert potentially damaging effects. Although CECs were elevated in the hypertensive condition no corresponding increase in vWF concentration was observed and therefore this increase is probably not associated with a loss of endothelial function. This is further supported by the fact that NOS activity was similar in hypertensive and normotensive animals. It is known that endothelial cells are more resistant to apoptosis possibly due to the fact that this process is inhibited by NO (Dimmeler and Zeiher, 1999; Haendeler *et al.*, 1999).

8.9 Conclusions

In summary, resting blood pressure in young women is elevated by moderate cigarette consumption, which varies with hormonal profile. Postmenopausal women exhibit increased resting blood pressure and TPR and decreased cardiac index when compared with young women. This change in cardiovascular profile appears to be due solely to ageing since postmenopausal women on HRT demonstrate identical changes to those in the untreated postmenopausal group.

Cardiovascular reactivity to stress is minimally affected, except for the cold pressor test, by menstrual cycle phase or smoking status. Cigarette smoking in postmenopausal women is associated with elevated cardiovascular reactivity to mental stress when compared to age-matched controls. Cardiovascular reactivity to stress in middle-aged women is on the whole independent of hormonal status since HRT use does not affect the response.

Endothelium-dependent vasodilatation is not significantly affected by ageing. Hormonal status is not a determining factor since postmenopausal women had similar responses to those on HRT and there is no difference between responses seen at menses and at ovulation. Long-term smoking however blunts vasodilatory capacity in the forearm possibly due to prolonged exposure to tobacco products.

CEC numbers are consistently elevated in smokers however this is not associated with increased vWF levels, or in young smokers with blunted vasodilatory capacity and so is perhaps due to some effect on endothelial cell turnover rates, as opposed to endothelial dysfunction.

The results of Chapter 6 showed that in normotensive animals the gain of baroreflex tachycardic responses is substantially less than that of bradycardic responses, suggesting a dominating effect of the parasympathetic limb of the baroreflex. GH animals exhibit attenuated bradycardic responses but tachycardic responses are unaltered. This suggests a selective reduction in the efficiency of cardiovagal drive in the hypertensive strain. Normotensive female animals have reduced bradycardic responses to pressure elevation when compared with male animals suggesting some effect of gender on autonomic control of blood pressure. However sex hormone deprivation via gonadectomy does not seem to have any role.

Hypertension is not associated with increased endothelial cell death and turnover at least in animals with similar NOS activity to controls. Although CEC number increases with the condition this is not indicative of endothelial cell injury since vWF levels were similar to controls.

8.10 Further studies

In light of the results presented in this thesis a number of questions arise and further studies could be undertaken to address these. One of the primary limitations of the study was that group numbers were quite small. A larger sample size, particularly in the smoking and HRT groups would help to better understand the roles of these interventions in cardiovascular health. In addition to a larger number of smokers in the middle-aged and young groups a set time of smoking abstention so as to investigate the separate influences of acute and chronic effects of smoking on cardiovascular and endothelial function should be enforced. In view of the fact that the literature suggests that route of administration, dose and the presence of progesterone all might have an effect on the functioning of HRT in relation to sympathetic activation and endothelial function, postmenopausal women could be given a standardised HRT regimen.

A comparison of the effects of different anaesthetics on BRS would be useful to distinguish between parasympathetic and sympathetic influences. Finally

further investigation needs to be undertaken to elucidate how endothelial cells are lost from the basal lamina with smoking and hypertension and once they are detected in the peripheral blood what is their half-life and how are they degraded.

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APPENDIX I

Material	*Source*
Accutrend cholesterol strips	Roche Diagnostics
Adenosine di-phosphate (ADP)	Boehringer Mannheim
Bovine serum albumin (BSA)	Sigma
BrdU Labelling and Detection kit	Roche Diagnostics
Citric acid	May and Baker Ltd.
Cresyl violet	DIFCO Laboratories
DPX	BDH Laboratories
EDTA and Heparin vacutainers	BD vacutainers systems
Gelatine	BDH Laboratories
H_2SO_4	BDH Laboratories
Haemostasis Reference Plasma	Biopool International
HEPES	Sigma
Hydrogen peroxide (H_2O_2)	Sigma
KCl	BDH Laboratories
KH_2PO_4	Sigma
L-NAME	Sigma
Methyl green	DIFCO Laboratories
Na_2CO_3	Sigma
Na_2HPO_4	Sigma
NaCl	Sigma
$NaHCO_3$	BDH Laboratories
Ortho-phenylenediamine (OPD)	Sigma
Paraformaldehyde	Sigma
Phenylephrine	Sigma
phytoestrogens free diet	GEFCO UK
Red dot monitoring electrodes	3M Health Care
sodium nitroprusside	M & B Laboratory
Thrombomodulin (CD141) ELISA kit	Diaclone Research
Triton X-100	Sigma
TUNEL apoptosis detection kit	Promega
Tween-20	Sigma
von Willebrand Factor ELISA kit	Kordia

Suppliers	*Address*
BDH Laboratory Supplies	BDH Laboratory Poole, BH15 1TD, U.K.
BD Vacutainer Systems	BD Vacutainer Systems Preanalytical solutions Belliver Industrial Estate Plymouth PL6 7BP, U.K.
Biopool International	Biopool International 6025 Nicolle Street Ventura, CA 93003 U.S.A
Boehringer Mannheim	D-68298 Mannheim Germany
Diaclone Research	Diaclone Research 1, Bd A Fleming BP 1985 25020 Besancon Cedex France
DIFCO Laboratories	West Molesey Surrey, U.K.
GEFCO UK	Lillico Ltd., Surrey U.K.
Kordia Laboratory Supplies	Kordia bv Oude Singel 158 2312 RG Leiden the Netherlands
May and Baker Ltd.	Dagenham, U.K.
Promega Corporation	Madison U.S.A.
Roche Diagnostics Ltd.	Bell Lane BN7 1LG, Lewes East Sussex, U.K.
Sigma Chemical Ltd.	Fancy Road Poole, Dorset, U.K.

APPENDIX II

UNIVERSITY OF DUBLIN

Department of Physiology

MEDICAL EXAMINATION FORM

PERSONAL DETAILS

Name	***e-mail***
Date of Birth	***Telephone***
Address	***Do you smoke?*** **If you do, how many do you smoke per day and for how long have you been smoking?**
Do you perform regular exercise? **eg. of weekly training schedule…**	

MEDICAL HISTORY

Have you ever had any of the following?

	Yes	*No*		*Yes*	*No*
Angina			***Heart Attack***		
Rheumatic Fever			***Irregular heart beat (Arrhythmia)***		
Heart Murmur			***Diabetes***		
Stroke			***Mini-Stroke (TIA)***		
Epilepsy			***Asthma***		
Tuberculosis (TB)			***Cancer***		
Jaundice			***Arthritis***		
Anaemia			***Sporting Injury***		
Are you on any medicines?			***Are you allergic to anything?***		

SURGICAL HISTORY

Have you ever had any operations?
If yes, what did you have done and when?

Operation	*Year*

Were there any complications? _____

SYSTEMS REVIEW

Do you suffer from any of the following?

	Yes	*No*		*Yes*	*No*
Headaches			*Sore Throat*		
Loss of Vision			*Abdominal Pain*		
Loss of power to the legs or arms			*Constipation*		
Numbness or unusual feeling in legs or arms			*Diarrhoea*		
Chest Pain			*Nausea*		
Shortness of Breath			*Vomiting*		
Palpitations			*Unusual Bleeding or bruising*		
Cough			*Kidney or Bladder problems*		

EXAMINATION FINDINGS

Blood Pressure	*Pulse*		
Respiratory	*Cardiovascular*		
Other findings	*Chol*	*Height*	*Weight*
ECG	*Signed*		*Date*

APPENDIX III

TRINITY COLLEGE DUBLIN
HUMAN EXPERIMENTATION SUBJECT INFORMATION & INFORMED CONSENT FORM

PROJECT:

INFLUENCES OF HORMONAL STATUS AND LIFESTYLE ON CARDIOVASCULAR FUNCTION IN YOUNG PREMEOPAUSALWOMEN

Principal Investigators:

Ms E Tansey; Professor C Bell

BACKGROUND:

High blood pressure and heart attacks are far less common in premenopausal women than in men but this difference disappears after the menopause. In order to be able to optimise prevention and treatment of these cardiovascular diseases, it is therefore important to understand what processes are involved in the cardiovascular protective effect of female sex hormones. Since there is evidence that smoking can impair cardiovascular regulation, we wish also to determine whether these effects can be modulated by hormonal status. At this stage, we are requesting participation by young women who are either non-smokers or usually smoke at least 10 cigarettes daily.

Participation in this study will involve the following:

- a routine medical examination to exclude subjects who would be recommended not to perform maximal exercise.
- a maximal fitness test (VO_{2max}) test to establish your level of fitness. This involves cycling on a stationary cycle ergometer at a constant pace, while the peddling resistance is increased every three minutes until you are unable to continue. During the test, you will breath into a mouthpiece for analysis of your oxygen consumption and will have several self-adhesive electrodes attached to your skin to monitor your ECG. A maximal fitness test usually lasts 20 minutes. A 9 ml sample of blood for analysis will be taken from your arm, by a registered medical practitioner or an approved phlebotomist for detection of circulating endothelial cells. At this time, those with normal, regular menstrual cycles will also be issued with a dipstick testing kit to use at home, so the precise timing of your cycle can be established.
- up to four subsequent visits to the laboratory for testing –
  On each day, you will be seated quietly in a chair for about 45 min. Flexible rubber bands will be wrapped around one forearm and one finger and inflatable cloth-covered cuffs will be placed on the same arm, in order to monitor local blood volumes. Small adhesive pads will be placed in each shoulder to record heart beat and blood pressure will be measured periodically using a conventional inflatable cuff and a stethoscope. Four stimuli will be used to evoke standard cardiovascular reflexes. These are (i) maintained handgrip for as long as possible at 50% of your maximal voluntary force, (ii) simple mental arithmetic for 2 min, (iii) inflation of a cuff around one arm to prevent blood flow, for 3 min and (iv) placing one foot in iced water for 2 min. For those with

normal regular menstrual cycles the first two sessions will be arranged soon after your initial assessment visit, to coincide with menstrual and mid-cycle times or withdrawal and oral contraceptive use; the second two would be about 6 weeks later, after a period of physical training, if you agree to participate.

- A 5-6 week period of gentle physical training. These sessions will consist of 30 min cycling on a stationary cycle ergometer, at a moderate pace, three times per week for 5-6 weeks.

The testing sessions will take place in the department of physiology on days and at times to suit you. Training sessions will be arranged at any time, in the department, to fit in with your daily schedules.
If you are willing, further small blood samples will be taken during each test session, so as to monitor circulating markers. All blood samples taken will be used exclusively for this study and held in storage anonymously for 5 years.

IF YOU HAVE ANY QUESTIONS OR CONCERNS AT ANY TIME, PLEASE CONTACT THE CHIEF INVESTIGATOR, ETAIN TANSEY (608 1131) OR PROFESSOR C BELL (608 1076).

DECLARATION:
This study and this consent form have been explained to me. The investigators have answered all my questions to my satisfaction. I believe I understand what will happen if I agree to be part of this study.
I have read, or had read to me, this consent form. I have had the opportunity to ask questions and all my questions have been answered to my satisfaction. I freely and voluntarily agree to be part of this research study, though without prejudice to my legal and ethical rights and understand that I am entitled to leave the study at any time, without penalty. I have received a copy of this agreement.

PARTICIPANT'S NAME:.....
CONTACT DETAILS:.....
PARTICIPANT'S SIGNATURE: .....
Date:.....

Statement of investigator's responsibility: I have explained the nature, purpose, procedures, benefits, risks of, or alternatives to, this research study. I have offered to answer any questions and fully answered such questions. I believe that the participant understands my explanation and has freely given informed consent.

INVESTIGATOR'S SIGNATURE:.....
Date:.....

APPENDIX IV

TRINITY COLLEGE DUBLIN
HUMAN EXPERIMENTATION SUBJECT INFORMATION & INFORMED CONSENT FORM

PROJECT:

INFLUENCES OF HORMONAL STATUS AND LIFESTYLE ON CARDIOVASCULAR FUNCTION IN POSTMENOPAUSAL WOMEN

Principal Investigators:

Ms E Tansey; Professor C Bell

BACKGROUND:

High blood pressure and heart attacks are far less common in premenopausal women than in men but this difference disappears after the menopause. In order to be able to optimise prevention and treatment of these cardiovascular diseases, it is therefore important to understand what processes are involved in the cardiovascular protective effect of female sex hormones. Since there is evidence that smoking can impair cardiovascular regulation, we wish also to determine whether these effects can be reduced by exercise. At this stage, we are requesting participation by postmenopausal women who are either non-smokers or usually smoke at least 10 cigarettes daily or are taking Hormone Replacement Therapy.

Participation in this study will involve the following:

- a routine medical examination to exclude subjects who would be recommended not to perform maximal exercise.
- a maximal fitness test (VO_{2max}) test to establish your level of fitness. This involves cycling on a stationary cycle ergometer at a constant pace, while the peddling resistance is increased every three minutes until you are unable to continue. During the test, you will breath into a mouthpiece for analysis of your oxygen consumption and will have several self-adhesive electrodes attached to your skin to monitor your ECG. A maximal fitness test usually lasts 20 minutes. A 9 ml sample of blood for analysis will be taken from your arm, by a registered medical practitioner or an approved phlebotomist for detection of circulating endothelial cells. At this time, those with normal, regular menstrual cycles will also be issued with a dipstick testing kit to use at home, so the precise timing of your cycle can be established.
- up to four subsequent visits to the laboratory for testing –
  On each day, you will be seated quietly in a chair for about 45 min. Flexible rubber bands will be wrapped around one forearm and one finger and inflatable cloth-covered cuffs will be placed on the same arm, in order to monitor local blood volumes. Small adhesive pads will be placed in each shoulder to record heart beat and blood pressure will be measured periodically using a conventional inflatable cuff and a stethoscope. Four stimuli will be used to evoke standard cardiovascular reflexes. These are (i) maintained handgrip for as long as possible at 50% of your maximal voluntary force, (ii) simple mental arithmetic for 2 min, (iii) inflation of a cuff around one arm to prevent blood flow, for 3 min and (iv) placing one foot in iced water for 2 min. The first

session will be arranged soon after your initial assessment visit; the second two would be about 6 weeks later, after a period of physical training, if you agree to participate.

- A 5-6 week period of gentle physical training. These sessions will consist of 30 min cycling on a stationary cycle ergometer, at a moderate pace, three times per week for 5-6 weeks.

The testing sessions will take place in the department of physiology on days and at times to suit you. Training sessions will be arranged at any time, in the department, to fit in with your daily schedules.
If you are willing, further small blood samples will be taken during each test session, so as to monitor circulating markers. All blood samples taken will be used exclusively for this study and held in storage anonymously for 5 years.

IF YOU HAVE ANY QUESTIONS OR CONCERNS AT ANY TIME, PLEASE CONTACT THE CHIEF INVESTIGATOR, ETAIN TANSEY (608 1131) OR PROFESSOR C BELL (608 1076).

DECLARATION:
This study and this consent form have been explained to me. The investigators have answered all my questions to my satisfaction. I believe I understand what will happen if I agree to be part of this study.
I have read, or had read to me, this consent form. I have had the opportunity to ask questions and all my questions have been answered to my satisfaction. I freely and voluntarily agree to be part of this research study, though without prejudice to my legal and ethical rights and understand that I am entitled to leave the study at any time, without penalty. I have received a copy of this agreement.

PARTICIPANT'S NAME:.....
CONTACT DETAILS:.....
PARTICIPANT'S SIGNATURE: .....
Date:.....

Statement of investigator's responsibility: I have explained the nature, purpose, procedures, benefits, risks of, or alternatives to, this research study. I have offered to answer any questions and fully answered such questions. I believe that the participant understands my explanation and has freely given informed consent.

INVESTIGATOR'S SIGNATURE:.....
Date:.....

APPENDIX V

Brand of cigarettes smoked by subjects:

Brand	Young smokers	Middle-aged smokers
Silk Cut Purple	1	2
Silk Cut Blue	4	4
Silk Cut Ultra		1
Marlboro Lights	2	

APPENDIX VI

Combined oral contraceptives used by subjects:

Brand	Oestrogen	Progesterone
Cilest	Ethinyloestradiol (35mg)	Norgestimate (0.25mg)
Marviol	Ethinyloestradiol (30mg)	Desogestrel (150mg)
Mercilon	Ethinyloestradiol (20mg)	Desogestrel (150mg)
Microgynon 30	Ethinyloestradiol (30mcg)	Levonorgestrel (150mcg)

Cilest, n=2
Marviol, n=1
Mercilon, n=4
Microgynon 30, n=3

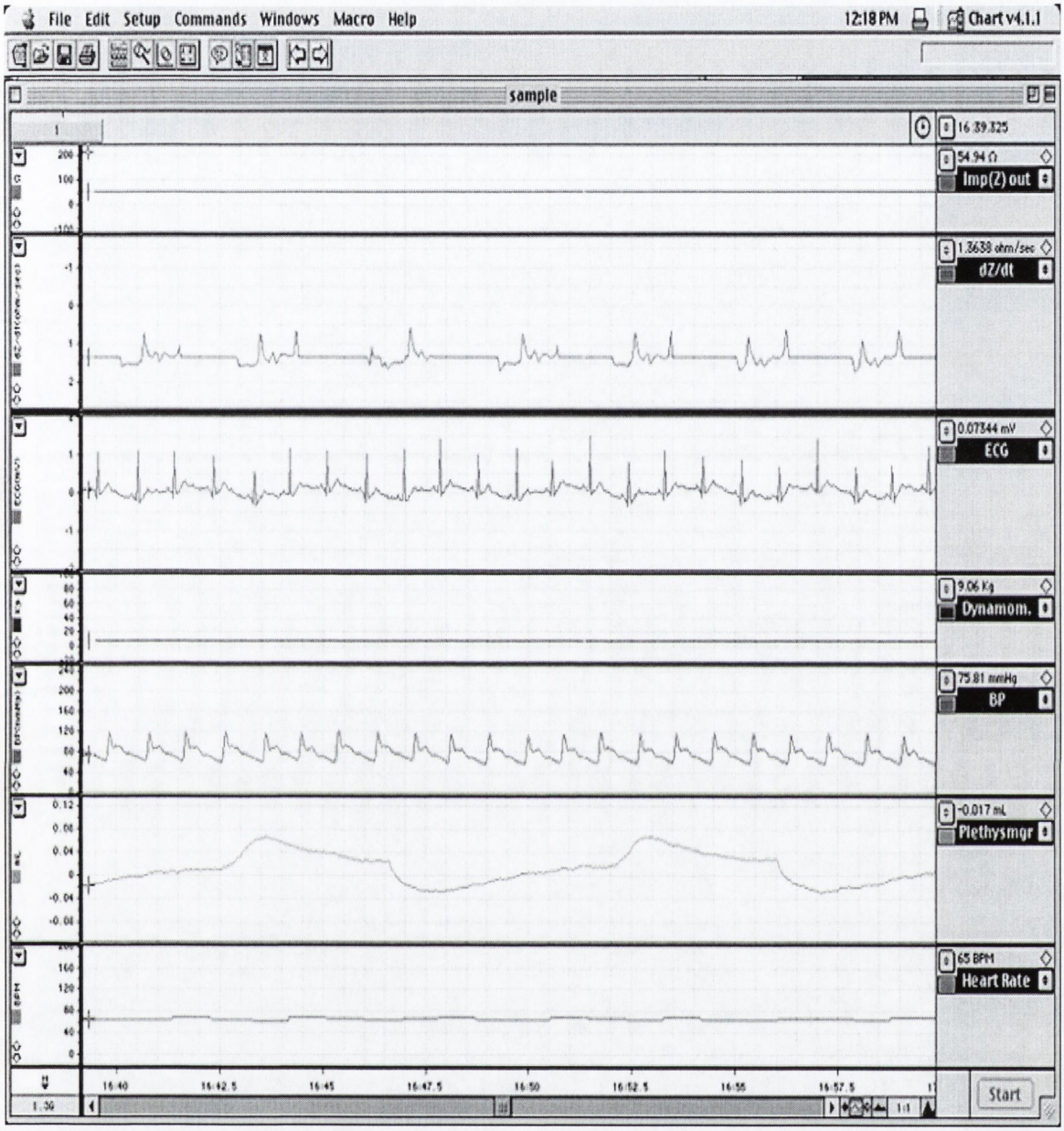
APPENDIX VII

Hormone Replacement Therapy regimens used by subjects:

Brand	Oestrogen	Progesterone	Formulation
Estraderm TTS	oestradiol (100mcg)		Patches
Premique 5	oestrogens (0.625mg)	Medroxyprogesterone (5mg)	Tablets
Prempak-C	oestrogens (0.625mg)	Norgestrel (0.15mg)	Tablets
Premarin	oestrogens (1.25mg)		Tablets

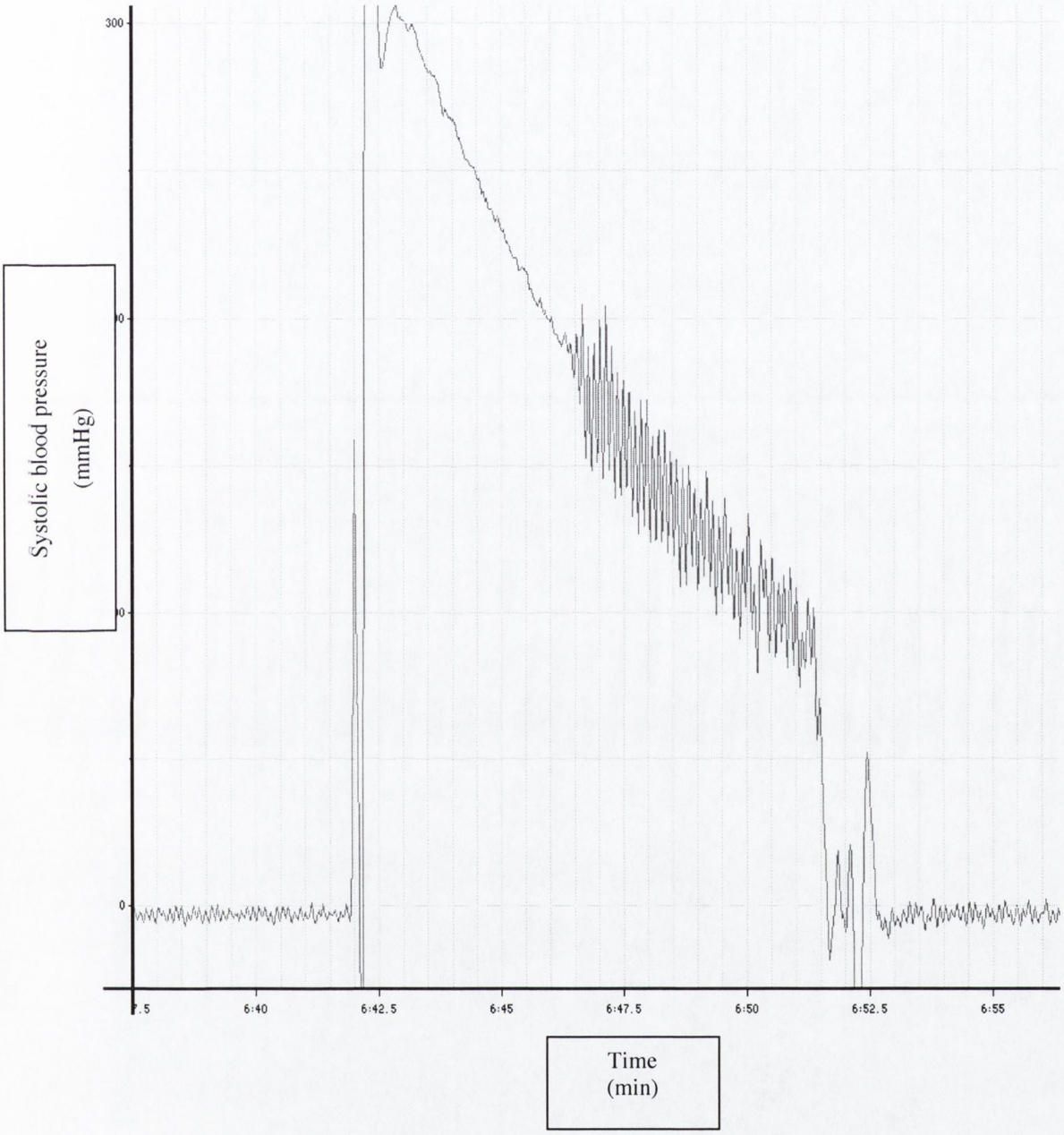
Estraderm TTS, n=2
Premique 5, n=2
Prempak-C, n=2
Premarin, n=1

APPENDIX VIII



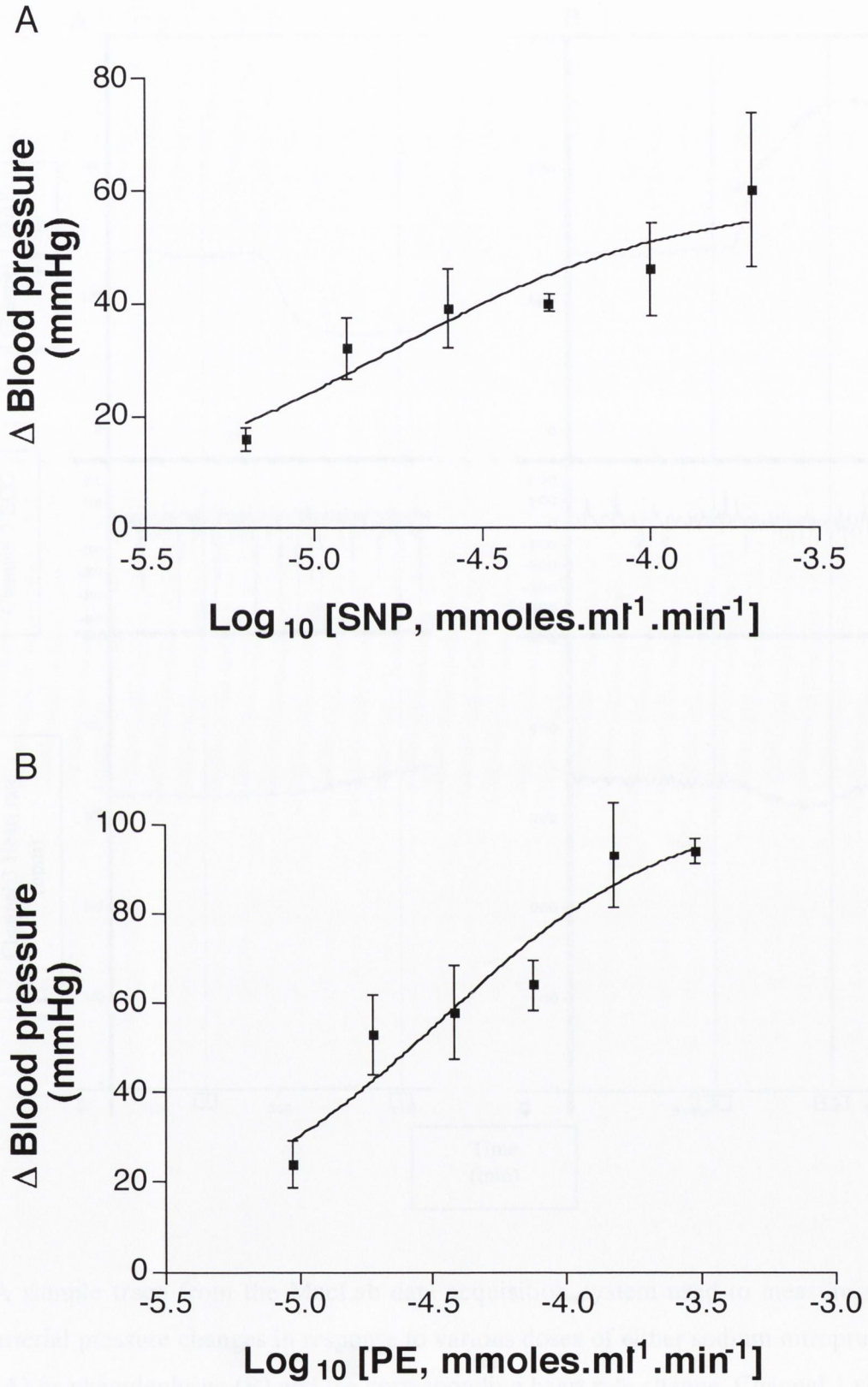
A screen-shot of the MacLab data acquisition system. Channel 1 recorded basal impedance levels while channel 2 recorded the changes in impedance. Channel 3 displayed a continuous ECG, which triggered a ratemeter to give heart rate displayed on 7. Channel 5 was used to help subjects maintain 50% MVC during sustained handgrip and channel 6 was used to monitor blood pressure continuously. Blood flow was measured using the changes in circumference of the forearm, which was recorded on channel 6.

APPENDIX IX



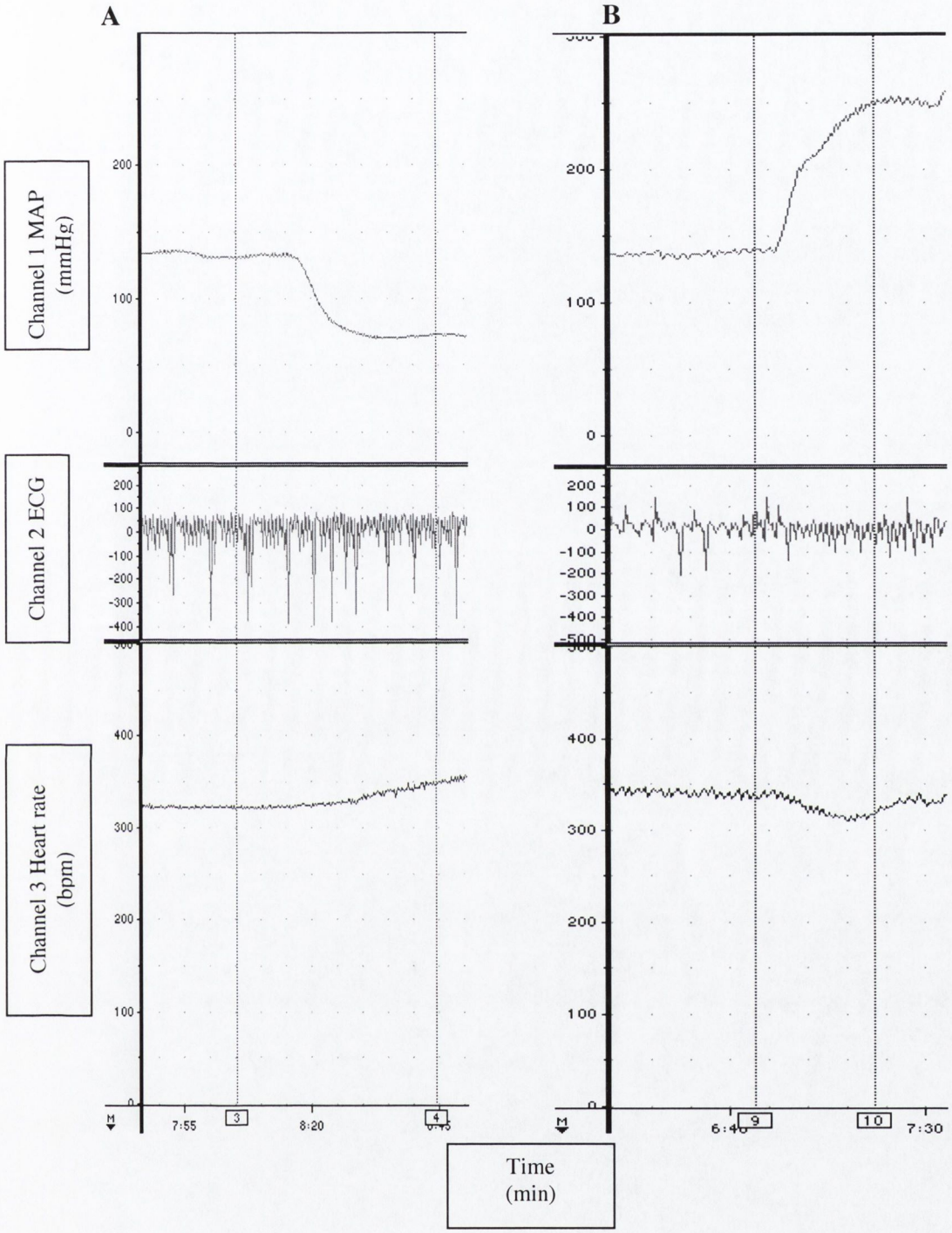
A sample trace from the MacLab data acquisition system used to measure systolic blood pressure via tail-cuff plethysmography. The cuff was inflated to 300 mmHg for GH rats. The tail pulses were not visible above the occlusion pressure. The cuff was then deflated slowly and the reappearance of the tail pulses was noted. The pressure at the onset of the pulses was the systolic blood pressure point.

APPENDIX X



Dose response curves. A. SNP – sodium nitroprusside, B. PE - phenylephrine

APPENDIX XI



A sample trace from the MacLab data acquisition system used to measure mean arterial pressure changes in response to various doses of either sodium nitroprusside (A) or phenylephrine (B) and the corresponding heart rate change. Channel 1 shows mean arterial pressure, channel 2 shows a 3-lead ECG, which in turn triggered a ratemeter to give heart rate, displayed on channel 3. Dotted lines indicate beginning and end of infusion period, respectively.