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**Modulation of the inflammatory response by taurine and its structural
analogue, HEPES.**

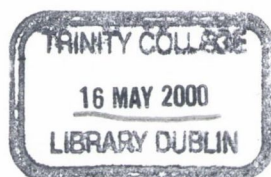
A dissertation submitted to the University of Dublin for the degree of
Doctor of Philosophy.

by

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1999

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TABLE OF CONTENTS

Page

Chapter I General Introduction

1.1	Inflammatory Bowel Disease (IBD)	2
1.1.1	Historical perspective	2
1.1.2	Etiology	3
1.1.3	The inflammatory response in IBD	4
1.1.4	Medical and Surgical treatment	6
1.2	Neutrophil involvement in the inflammatory response	7
1.2.1	Neutrophil adhesion	7
1.2.2	Neutrophil respiratory burst activity	9
1.2.3	Signal transduction in PMN oxidative metabolism	10
1.2.4	Second messengers in the neutrophil	11
1.3	Nitric oxide	13
1.3.1	Role of NO in the inflammatory response	13
1.3.2	Production of NO by PMNs	14
1.3.3	Production of NO by enterocytes	15
1.4	Apoptosis	16
1.4.1	Role in the inflammatory response	17
1.4.2	Neutrophil cell death in the inflammatory response	17
1.4.4	Enterocyte cell death in IBD	19
1.5	Taurine and HEPES	20
1.5.1	Biochemistry & Biosynthesis	21
1.5.2	Taurine and the Neutrophil inflammatory response	21
1.5.3	Taurine and apoptosis	23
1.6	Aims of project	24

Chapter II Materials & Methods

2.1	Preparation of solutions	26
2.2	Tissue Culture techniques	26
2.2.1	Preparation of PMNs	26
2.2.2	Enterocyte preparation	27
2.3	Cell surface receptor expression	
2.3.1	CD11b receptor expression	27

2.4	Intracellular Calcium, $[Ca^{2+}]_i$	
2.4.1	Measurement of $[Ca^{2+}]_i$	28
2.5	Cellular oxidative metabolites	
2.5.1	Overall oxidative stress	28
2.5.2	NADPH Oxidase activity	29
2.5.3	Myeloperoxidase activity	30
2.5.4	NO production	30
2.5.5	Peroxynitrite anion formation	31
2.6	Degranulation	
2.6.1	β -Glucuronidase activity	31
2.7	Cyclooxygenase and lipoxygenase activity	
2.7.1	LTB_4 and PGE_2 production	32
2.8	Assessment of apoptosis	
2.8.1	Cell Morphology	32
2.8.2	Propidium iodide staining	32
2.8.3	DNA gel electrophoresis	33
2.8.4	Annexin V Binding	33
2.9	Western Blotting	
2.9.1	Protein determination	34
2.9.2	Measurement of nitric oxide synthase protein	34
2.10	Animal model of experimental colitis	
2.10.1	Animals	34
2.10.2	Experimental model of ulcerative colitis	35
2.10.3	Assessment of tissue injury	35
2.10.4	Organ culture	36
2.11	Statistical analysis	36
2.12	Reagents	37

Chapter III The role of taurine and HEPES on neutrophil function in the inflammatory response *in vitro*

3.1	Introduction	39
3.2	Aims	41
3.3	Results	42
3.4	Discussion	46

Chapter IV The role of taurine and its substituted analogue, HEPES on the modulation of NO-mediated apoptosis

A study to investigate the ability of taurine and HEPES to modulate oxidant-induced cell death in the PMN.

4.1	Introduction	53
4.2	Aims	54
4.3	Results	55
4.4	Discussion	59

The role of taurine and HEPES on NO-mediated cell death in the enterocyte.

4.5	Introduction	64
4.6	Aims	66
4.7	Results	67
4.8	Discussion	70

Chapter V The modulation of experimental colitis by taurine and its structural analogue, HEPES

5.1	Introduction	75
5.2	Aims	76
5.3	Results	77
5.4	Discussion	79

Chapter VI Concluding Remarks 84

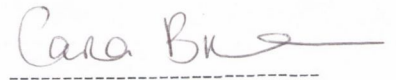
Bibliography 91

Declaration

I declare that none of the work described in this thesis has been submitted for any degree or diploma at this or any other University.

I declare that unless otherwise stated, the work described is entirely my own, and has been carried out in the Department of Surgical Research, Beaumont Hospital, Dublin.

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A handwritten signature in cursive script, reading "Cara Brennan", written in dark ink. The signature is positioned above a horizontal dashed line.

Cara Brennan

Abbreviations

AA	Arachidonic acid
ANOVA	Analysis of variance
5-ASA	5-aminosalicylic acid
Ca ²⁺	Calcium ion
[Ca ²⁺] _i	Intracellular calcium concentration
CAT	Catalase
CD(no.)	Cluster differentiation number
CD	Crohn's disease
CO ₂	Carbon dioxide
CMS	Colon macroscopic score
CMiS	Colon microscopic score
cNOS	Constitutive nitric oxide synthase
DAG	Diacylglycerol
DMEM	Dulbecco's Minimal Essential Medium
DMSO	Dimethyl sulfoxide
<i>E. coli</i>	<i>Escheria coli</i>
EtoH	Ethanol
GPX	Glutathione peroxidase
H & E	Haemotoxylin and Eosin
h	hour
H ₂ O ₂	Hydrogen peroxide
HBSS	Hank's balanced salt solution
HEPES	N-[2-hydroxyethyl] piperazine-N'-(2-ethanesulphonic acid)
HETES	Hydroxyeicosatetraenoic acids
HOCl	Hypochlorous acid
IBD	Inflammatory bowel disease

ICAM-1	Intracellular adhesion molecule-1
IFN- γ	Interferon-gamma
IL-6	Interleukin 6
iNOS	Inducible nitric oxide synthase
IP ₃	Inositol-1,4,5-triphosphate
ir	intrarectal
kDa	Kilodalton
Kg	Kilogram
L	Litre
L-NMA	NG-monomethyl-L-arginine
LPS	Lipopolysaccharide
LTB ₄	Leukotriene B ₄
M ϕ	Monocyte
MCF	Mean channel fluorescence
MEM	Minimal essential medium
MPO	Myeloperoxidase
n	number (sample size)
NF-kB	Nuclear transcription factor-kB
NP-40	Nonidet P-40
NO	Nitric oxide
O ₂ ⁻	Superoxide anion
OH ⁻	Hydroxyl radical
ONOO ⁻	Peroxynitrite
PBS	Phosphate buffered saline
PCD	Programmed cell death
PGE ₂	Prostaglandin E ₂
PI	Propidium iodide
PIP ₂	Polyphosphoinositol 4,5-bisphosphate

PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase c
PMA	Phorbol myristate acetate
PMN	Polymorphonuclear cell/neutrophil
ROS	Reactive oxygen species
rpm	revolutions per minute
RT-PCR	Reverse transcriptase polymerase chain reaction
SEM	standard error of the mean
SNP	Sodium nitroprusside
SOD	Superoxide dismutase
Taurine	2-aminoethanesulphonic acid
TNBS	Trinitrobenzenesulphonic acid
TNF- α	Tumour necrosis factor -alpha
UC	Ulcerative colitis

Dedication

This thesis is dedicated to my parents, Canice and Noel Brennan.

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I would like to acknowledge the help and guidance of Prof. David Bouchier Hayes and Dr. Philip Stapleton who gave me the opportunity to carry out research in the Dept. Of Surgery, Beaumont Hospital. A special thanks goes to Prof. Keith Tipton for his insightful comments and help during the write up of this thesis. I would also like to thank the friends I made during the time spent in Beaumont Hospital for friendship during the inevitable ups and downs of doing research.

Summary

Activated neutrophils (PMNs) have been implicated in the production of tissue injury in inflammatory bowel disease (IBD) through release of proinflammatory mediators during respiratory burst activity. Taurine, the most abundant free β -amino acid in human PMNs possesses anti-inflammatory properties. HEPES, a structural analogue of taurine is a potent anti-inflammatory agent *in vivo*. Experiments on the effects of taurine and HEPES on PMN function *in vitro* revealed that these compounds prevent receptor-mediated influx of Ca^{2+} , decrease NADPH oxidase and respiratory burst activity and modulate adhesion. In contrast, Upon stimulation by soluble stimuli, taurine and HEPES increase NADPH oxidase activity, MPO activity and respiratory burst activity. The differential effects of taurine and HEPES on respiratory burst activity are most likely due to the biphasic effects of taurine and HEPES on Ca^{2+} .

IBD is characterised by increased NO with the potential to modulate apoptosis within the colon. It was demonstrated that exogenously-added NO induced PMN apoptosis. Taurine decreased nitrite generation in PMNs cultured with an exogenous source of NO without affecting apoptosis. HEPES increased endogenous NO production in fMLP-activated PMNs without affecting PMN apoptosis. Activated colonic epithelial cells, Caco-2 and HT-29 released large amounts of NO in response to LPS/IFN- γ *in vitro*. Endogenously produced NO induced apoptosis in the HT-29 colonic epithelial cell line which retains a p53 allele. In contrast, LPS/IFN- γ increased apoptosis in the Caco-2 cell line through a p53/NO-independent mechanism. Supplementation with taurine or HEPES did not significantly protect against LPS/IFN- γ -activated apoptosis in colonic epithelial cells.

Experimental colitis resulted in an influx of PMNs into the colon with enhanced ROS activity and increased colonic nitrite generation. HEPES caused a significant reduction in tissue injury as assessed by a colon macroscopic score. The administration of taurine decreased

expression of the PMN adhesion receptor, CD11b/CD18 but did not decrease tissue injury *in vivo*. HEPES modulates the inflammatory response through a direct interaction with the respiratory burst pathway. The effectiveness of HEPES but not taurine on tissue injury in this rat experimental model of colitis may be explained by the ability of HEPES to be retained in the systemic circulation for longer periods of time than taurine. The efficacy of HEPES as a pharmacological agent demonstrated by this study suggest the future use of HEPES as an anti-inflammatory agent.

CHAPTER I

GENERAL INTRODUCTION

1.1 Inflammatory Bowel Disease (IBD)

The Inflammatory Bowel Diseases (IBD), ulcerative colitis (UC) and Crohn's disease (CD) are chronic inflammatory diseases in which periods of inactive disease are interspersed with spontaneous relapse and active disease. While in UC, the inflammation is essentially mucosal and limited to the colon, inflammation in CD can affect the entire gastrointestinal tract and affects both mucosal and muscle layers. UC and CD have clinical features in common which include diarrhoea, lower abdominal pain, urgency of defaecation and passage of blood or mucus from the rectum. These disorders afflict over 1 million people in the United States (U.S.), 70,000 to 100,000 of whom are seen for the first time in hospitals every year in the U.S. (Lichtenstein, 1994).

The incidences of UC and CD are approximately equal with between 3-6/100,000. However, there is a disparity of information in the literature on the incidence of IBD. Over a ten year period, the incidence of UC in Granada, Spain was 2 per 100,000 (Martinez-Salmeron *et al.*, 1993) whilst in Bologna, Italy, the incidence rate for UC was 7.7 per 100,000 (Tragnone *et al.*, 1993). Although conclusive data on this point is still lacking recent reports suggest that the incidence and the prevalence of IBD in Europe and Northern America has steadily increased over the past thirty years (Russel and Stockbrugger, 1996). The rising incidences of IBD since the second world war coincides with profound changes in dietary pattern and "modern living" has been implicated. The incidence of IBD shows marked geographical variations with UC and CD more frequent in Western countries and areas of high socioeconomic development but relatively infrequent in Southern Europe (Shivananda *et al.*, 1996).

1.1.1 Historical Perspective

Although diarrheal illnesses have been described since the early medical writings of Hippocrates (400 B.C.), there is little evidence that they were distinguished from the all too common infectious enteritides (Adams, 1939). A study of 200 cases that presented in the Union Army during the civil war in 1875 afforded the first formal documented pathologic

anatomy of IBD (Crohn, 1962). By the turn of the century, the disease was fully characterised as to its nonspecific nature and was distinguishable by clinical as well as pathologic criteria.

1.1.2 Etiology

While genetic predisposition, infectious agents, dietary factors and immunologic disorders have been implicated, the precise etiology of IBD remains unknown. A genetic origin has been suggested as the incidence of ulcerative colitis is significantly higher in relatives of patients with IBD than spouses who share many environmental exposures. UC is two to four times more common in Jewish than in non-Jewish white populations (Shapira *et al.*, 1998). In addition, the disease occurs with greater frequency in twins. Patients with IBD share genetic markers (HLA DR2, HLA DR4) in common with patients with other inflammatory diseases such as rheumatoid arthritis. The HLA phenotype, AW24 and BW35 is also increased in frequency in patients with early onset UC. The presence of antineutrophil cytoplasmic antibodies (ANCA) has been identified in 72% of UC patients (Hardarson *et al.*, 1993).

Environmental factors may play an important role in the development of the disease with more evidence pointing to an environmental etiology with UC than CD. A remarkably decreased incidence of UC was reported among Jews in Israel (3.7 per 100,000) compared to Jews living in Europe (7.3 per 100,000 in London, Copenhagen) and North America (7.2 per 100,000 in Rochester, USA) (Gilat *et al.*, 1974). There is some evidence of causative infectious agents in CD including *Mycobacteria paratuberculosis*, mycobacteria of unknown origin, paramyxovirus, measles virus, *Angiostrongylus costaricensis* (reviewed by Lashner and Brzezinski, 1994). The examination of bacterial and viral agents in UC suggest that *Chlamydia*, cytomegaviruses, *Yersinia*, *Clostridium difficile* may be involved in the pathogenesis of ulcerative colitis (reviewed by Becker and Moody, 1989). There is some evidence for *E. coli* being a causative agent in both UC and CD (Hartley *et al.*, 1993). Other environmental agents implicated in the etiology of IBD include cigarette smoking and oral contraceptive use. Cigarette smoking continues to be the single most important environmental agent associated with the development of IBD.

1.1.3 The inflammatory response in IBD

Active episodes of IBD are characterized by infiltration of a large number of phagocytic leukocytes into the mucosal interstitium accompanied by extensive mucosal injury (Grisham and Granger, 1998). Chronic gut inflammation is characterized by enhanced production of reactive oxygen species (ROS) and NO (Grisham, 1994). Because it is well established that activated inflammatory phagocytes release large amounts of potentially cytotoxic oxidants upon stimulation by several proinflammatory mediators, it is reasonable to postulate that infiltrated phagocytes may mediate tissue injury within the chronically inflamed gut. Furthermore, activated colonic epithelial cells exert bactericidal activity through an oxidant-dependant pathway *in vitro* (Deitch *et al.*, 1995). In this study, it was demonstrated that intestinal epithelial cells can kill certain strains of bacteria and may function as "nonprofessional" phagocytes.

Enhanced production of NO has been observed in chronically inflamed colon from patients with IBD and in experimental animal models of UC (Rachmilewitz *et al.*, 1995, Miller *et al.*, 1994). The sources of NO generation within the gut include phagocytic leukocytes influxing the colon and activated tissue cells including colonic epithelial and vascular endothelial cells. There is increasing evidence that colonic epithelial cells produce large amounts of NO within the inflamed gut (Singer *et al.*, 1996) and upon stimulation by proinflammatory cytokines *in vitro* (Salzman *et al.*, 1996, Dignass *et al.*, 1995, Kolios *et al.*, 1995). Phagocytic leukocytes may also represent an important source. Because several cytokines such as tumour necrosis factor- α (TNF- α), interferon- γ (IFN- γ) and interleukin 1 β (IL-1 β) are increased in the inflamed colon, it is reasonable to postulate that they may also induce nitric oxide synthase (NOS) in PMNs, macrophages and epithelial cells.

Tissue associated antioxidants and free radical scavengers can be overwhelmed during active colonic inflammation, resulting in oxidation of cellular components. The human colon contains much smaller amounts of antioxidants than other organs such as the liver and the heart (Grisham, 1990, Mulder *et al.*, 1991). Most of the antioxidant mucosal enzyme activity is associated with colonic epithelial cells (Grisham, 1990, Mulder *et al.*, 1991). Mucosal

antioxidant defenses are depleted in inflamed gut from biopsy samples of IBD patients (Buffington and Doe, 1995), suggesting that IBD may render the inflamed mucosa susceptible to oxidative tissue damage. Several lines of evidence demonstrate a beneficial effect of antioxidant supplementation in inflamed gut. In animal models of colitis, some therapeutic benefits have been observed with superoxide dismutase (SOD), catalase, α -tocopherol, and novel or nonspecific antioxidant compounds (Keshavarzian *et al.*, 1992, Blumerstein *et al.*, 1994, Emerit *et al.*, 1989). The active component of sulphasalazine, 5-aminosalicylate (5-ASA) possesses potent anti-oxidant and free radical scavenging properties. ROS have also been implicated as mediators of gut inflammation because of the beneficial effects of 5-ASA in the treatment of IBD. A role for NO-derived metabolites in experimental models of IBD has been demonstrated by several laboratories. In one study, oral administration of iNOS inhibitors attenuates tissue injury in experimental colitis (Neilly *et al.*, 1995). However, there has been much focus on the development of highly specific inhibitors of iNOS for use as potent anti-inflammatory agents but these drugs have proved to be of limited success (reviewed by Evans, 1995).

Certain oxidants are known to promote apoptosis, induce cell death and modulate electrolyte and fluid secretion in the normal gut (Conner *et al.*, 1996). Some of these oxidants are known to modulate the expression of a variety of genes that are involved in the immune and inflammatory responses (reviewed by Jourdain *et al.*, 1997). For example, micromolar concentrations of H_2O_2 can activate the nuclear transcription factor κB (NF- κB), which regulates the expression of a variety of different adhesion molecules, cytokines and enzymes (reviewed by Jourdain *et al.*, 1997). Oxidants are believed to promote intestinal epithelial cell apoptosis. The proinflammatory cytokine, TNF- α induces enterocyte apoptosis in mice (Piguet *et al.*, 1998). The reaction product of O_2^- and NO, $ONOO^-$ can induce apoptosis which is attenuated by mesalamine (Sandoval *et al.*, 1997). It has also been demonstrated that sulphasalazine induces PMN apoptosis (Akahoshi *et al.*, 1997).

Taken together, these observations suggest that ROS and NO produced in IBD may play an important role in regulating the inflammatory response and in modulating apoptosis.

1.1.4 Medical and Surgical treatment of IBD

Despite the progress made in understanding the disease process, modern treatment has not altered the natural history of chronic IBD. Medical treatment in IBD is complicated by the fact that their etiology is unknown. Treatment is based on a current understanding of the pathophysiology of the intestinal inflammatory process. The drugs most commonly used are the aminosalicylates and corticosteroids. The use of aminosalicylates has long been established in the treatment of patients with inflammatory diseases. The active component of the aminosalicylates is 5-aminosalicylic acid (5-ASA) which has numerous effects on the inflammatory process. 5-ASA possesses strong antioxidant properties, inhibits the synthesis of leukotrienes and various cytokines. Corticosteroids are efficacious for the treatment of active UC and CD regardless of the disease distribution. However, corticosteroids have been shown to be ineffective in the maintenance of remission and have numerous side effects. The use of immunosuppressants, antibiotics, antimycobacterial agents have been used with some success in the treatment of IBD.

If medical treatment in IBD fails, surgical intervention may be used as a form of treatment. Total removal of affected organs provides a complete cure, but at a sacrifice since patients must live with an external abdominal stoma (an ileostomy) for the remainder of their lives. In the case of ulcerative colitis, restorative proctocolectomy has become the standard technique worldwide. A total resection is necessary in the vast majority of patients with ulcerative colitis. The role of surgical intervention in CD is based on the premise that surgery be considered only if conservative treatment has failed or if serious complications develop.

1.2 PMN involvement in the inflammatory response

Neutrophils or polymorphonuclear leukocytes (PMNs) play an important role in host defence against invading microorganisms but paradoxically are also implicated in the pathology of various inflammatory diseases. PMNs are rich in cytoplasmic granules and contain a lobulated nucleus. Azurophilic or primary granules within the PMN contain bactericidal enzymes such as myeloperoxidase (MPO) and proteinases. Specific or

secondary granules contain collagenases, lysozymes and metalloproteinases. PMNs have been implicated as the major source of tissue damaging agents produced by the inflammatory response (reviewed by Smith *et al.*, 1994).

PMNs represent 50 to 60% of the total circulating leukocytes. Upon initiation of an inflammatory response, PMNs are summoned from the bone marrow and are the first cells to be recruited to sites of infection or injury. Activation of a family of PMN cell surface receptors, the β 2-integrins bind adhesion molecules on the surface of activated endothelium. This facilitates PMN penetration through the endothelial layer and migration through connective tissue to sites of infection in a process called diapedesis. Upon entering tissue pools, PMNs survive for 1 to 2 days. PMNs are terminally differentiated cells and undergo programmed cell death or apoptosis prior to removal by macrophages (Savill *et al.*, 1993). This prevents the expelling of cytotoxic contents into the extracellular milieu and plays a role in the termination of PMN inflammatory responses (Savill *et al.*, 1993).

PMNs function to phagocytose their targets which include bacteria, fungi, viruses and tumour cells. The PMN microbicidal armory consists of both oxidative and non-oxidative processes which are activated simultaneously upon initiation of phagocytosis. Oxidative processes result in the formation of ROS through respiratory burst activity and NO. PMNs contain proteinases and anti-microbial polypeptides which are involved in killing through non-oxidative (enzymatic) processes. Release of the contents of azurophilic and specific granules into the phagosome during degranulation create a highly microbicidal environment.

1.2.1 PMN Adhesion Receptors and modulation of the inflammatory response

PMNs undergo a series of morphological changes associated with adhesion to the endothelium. Upon recognition of endothelium by phagocytes at an inflamed site, selectin-carbohydrate interactions lead to PMN adhesion to the endothelium. This results in the rolling of PMNs along blood vessel walls at a much reduced velocity compared to normal blood flow. Activation of PMN integrins leads to an interaction with endothelial ICAMs resulting in a stronger adhesion to the endothelium that stops PMN movement (Figure 1.1).

The PMN can then migrate through endothelial cell junctions and travel to the inflammatory site using amoeboid movements under the influence of chemoattractants. Resting post-capillary venule endothelium cannot recognise circulating leukocytes. However, in the presence of proinflammatory cytokines and interleukins, endothelial cells express cell surface molecules which interact with circulating leukocytes.

There are three groups involved in leukocyte-endothelial interactions - the IgG family, the integrins and the selectins. The integrin family mediate cell to cell and cell to extracellular matrix interactions. Integrins are plasma membrane receptors composed of two gene products, termed α and β chains which are linked non-covalently. Both chains are required for normal receptor expression and receptor-ligand binding. Integrins have been classified into three groups according to the gene product used. β_1 integrins are known as the very late activation antigen family. Integrins which use β_2 are known as leukocyte cell adhesion molecules because their expression is limited to white blood cells.

There are three β_2 integrins, LFA-1 (CD11a/CD18), Mac-1 (CD11b/CD18) and glycoprotein 150, 95 (CD11c/CD18). Each has a unique α chain in combination with β_2 (CD18). CD11b/CD18 binds ICAM-1 on activated endothelial cells and the iC3b product of activated complement. The importance of the CD11b/CD18 integrin is observed in patients with congenital leukocyte adhesion deficiency (LAD) which have mutations in the common β_2 subunit. Patients with LAD have recurring infections, often fatal in childhood unless they are corrected by bone marrow transplantation.

The PMN β_2 integrin, CD11b/CD18 exist in presynthesized intracytoplasmic reserves and is recruited from intracellular stores upon cell activation (Sengelov *et al.*, 1993). Upon PMN stimulation by agonists, the CD11b/CD18 contents of the intracellular organelles are summoned to the plasma membrane (Buyon *et al.*, 1997). Translocated CD11b/CD18 must be modified at the plasma membrane before it comes functional. There are two distinct pathways of activation of CD11b dependent upon activation by PKC activators or chemoattractants (Merrill *et al.*, 1990). PMA-activated CD11b receptor expression is dependent upon phosphorylation of the CD18 β -chain through a PKC-dependent mechanism (Merrill *et al.*, 1990). Treatment of PMNs with the calcium based stimulus, fMLP results in

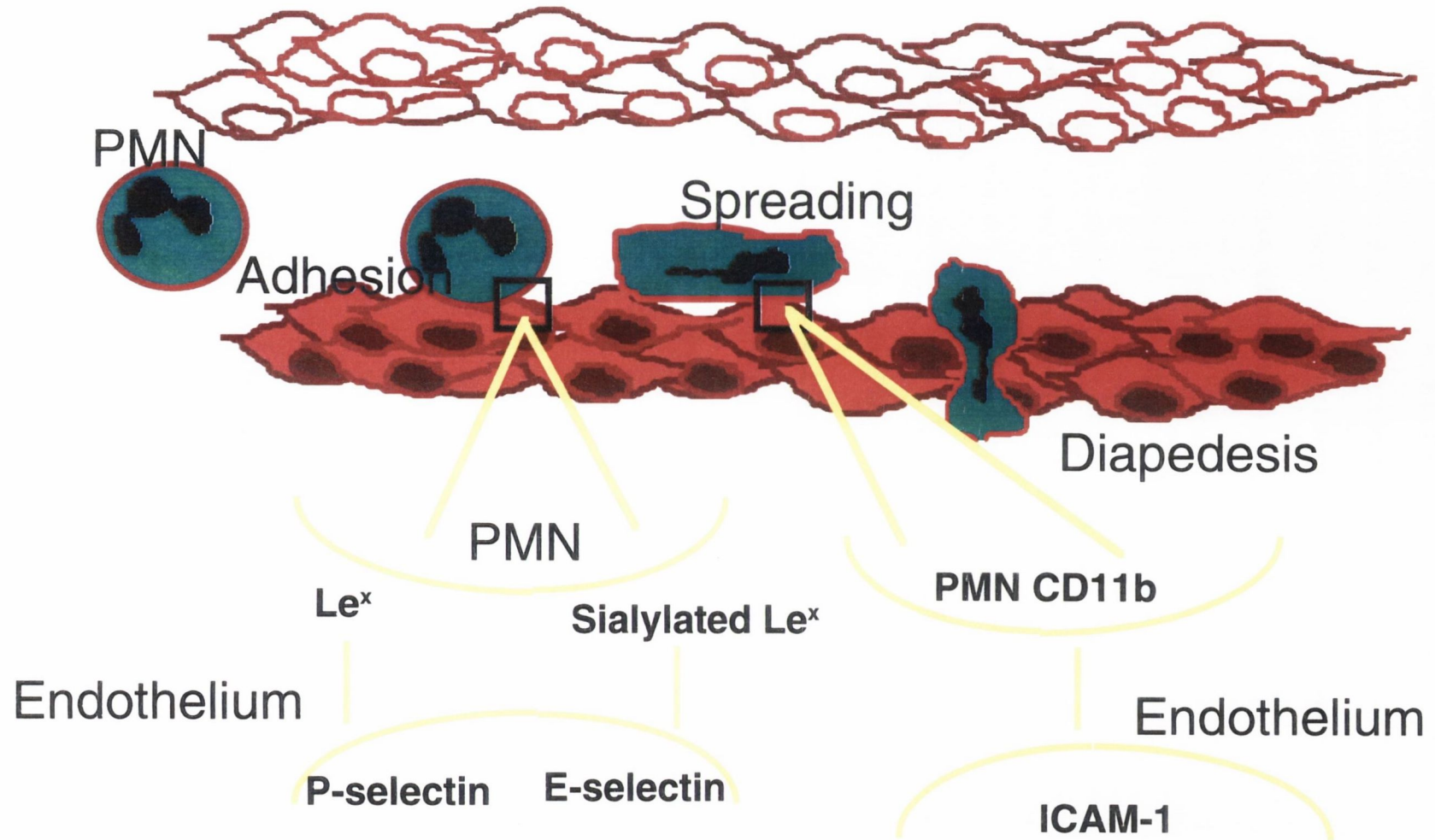


Figure 1.1 Molecular interactions during PMN adhesion to endothelium at sites of inflammation including exploded view of PMN-endothelial cell interactions

no detectable phosphorylation of CD18 (Merrill *et al.*, 1990). Recent evidence confirms that regulation of leukocyte cell adhesion via $\beta 2$ integrins is modulated directly by calcium levels (reviewed by Sjaastad *et al.*, 1997). In some cases phosphorylation events occur concomitantly with calcium signalling and/or the activation of PKC (reviewed by Sjaastad *et al.*, 1997). It is clear however that there are different pathways of integrin-mediated signalling upon activation by differing stimuli.

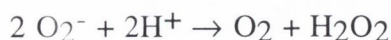
1.2.2 PMN respiratory burst activity

PMN respiratory burst activity is triggered upon phagocytosis or activation by an appropriate synthetic stimulus *in vitro*. The respiratory or oxidative burst results in the production of a number of microbicidal reactive oxygen species (ROS) (Figure 1.2). Initially, superoxide (O_2^-) is formed by the reduction of molecular oxygen by NADPH oxidase:



This process is catalysed by the combined action of a number of components: cytochrome b558 (a heterodimer composed of gp91-phox and p22-phox), cytosolic proteins (p47-phox, p67-phox, p40-phox), and two Rac-related GTP-binding proteins (Rap1A, rac) (Leusen *et al.*, 1996). The NADPH oxidase system is dissociated and thus inactive in the plasma membrane of resting PMNs. Upon PMN activation, the cytosolic components translocate to the plasma membrane and form an active complex. There are multiple signal transduction leading to activation of the NADPH oxidase. This most likely reflects the toxicity of ROS produced by an active NADPH oxidase. Once an infection is cleared, a rapid mechanism to deactivate NADPH oxidase is essential.

Hydrogen peroxide (H_2O_2) is formed rapidly from O_2^- by the catalytic action of superoxide dismutase (SOD) or spontaneous dismutation:



Hypochlorous acid (HOCl), a very powerful oxidising antimicrobial agent between 100 to 1000 times more antimicrobial than H_2O_2 is then rapidly formed (Eaton, 1993). HOCl is

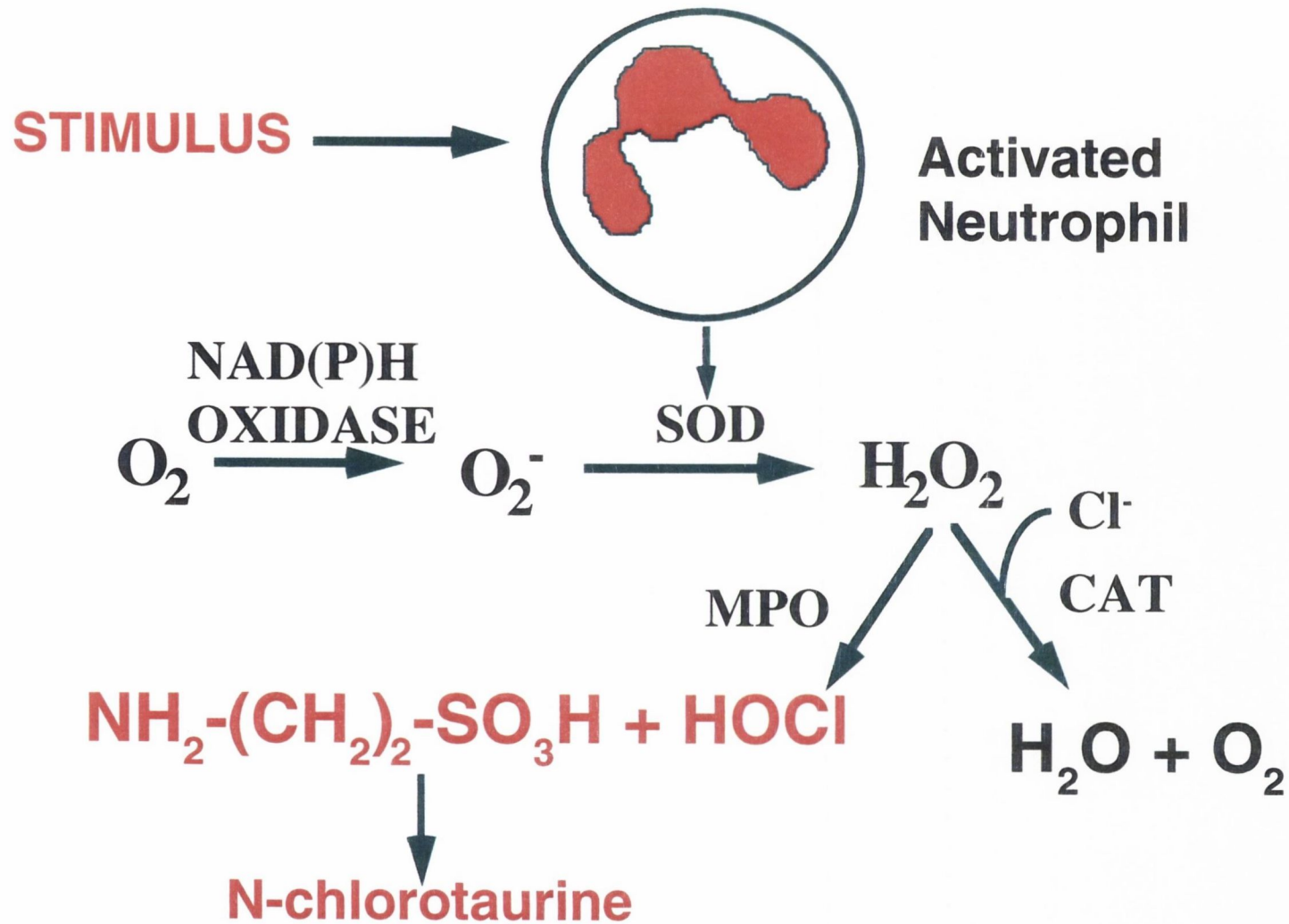
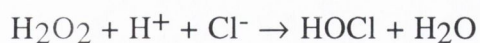
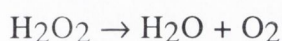


Figure 1.2 The respiratory burst pathway in activated PMNs

generated by the reaction of H_2O_2 with chloride ions taken up from the extracellular environment which is catalysed in the following reaction by myeloperoxidase (MPO):



The percentage of H_2O_2 converted to HOCl varies from 30 to 70% depending on the experimental system used (Klebanoff *et al.*, 1992). The removal of H_2O_2 can also occur by catalase (CAT) or glutathione peroxidase (GPX):



The GPX-catalysed reaction serves two purposes, the removal of H_2O_2 and the oxidation of NADPH to NADP which is utilised by the hexose monophosphate shunt (HMPS). CAT detoxifies H_2O_2 serving to protect the PMN from oxidative damage. PMNs also produce NO through an oxidative process which may contribute to the microbicidal activity of PMNs by reacting with O_2^- forming peroxynitrite, ONOO^- .

1.2.3 Signal transduction in PMN oxidative metabolism

Synthetic stimuli used to activate PMN functions (respiratory burst activity, chemotaxis and lysosomal enzyme secretion) *in vitro* comprise of two separate groups, particulate stimuli including chemoattractants and soluble stimuli such as phorbol esters and calcium ionophores. Binding of chemoattractants to specific cell surface receptors on human PMNs initiates a variety of biological responses (reviewed by Dillon *et al.*, 1988). Phorbol esters such as PMA bypass receptor-mediated events at the plasma membrane and directly activate protein kinase C (PKC) with subsequent activation of the PMN. Calcium ionophores increase intracellular Ca^{2+} by increasing the influx of Ca^{2+} into the cell from the extracellular environment. There are common and divergent pathways utilized by particulate and soluble stimuli in the signalling mechanisms that trigger respiratory burst activity.

Chemoattractant receptors are coupled to G proteins within the PMN plasma membrane. Receptor occupancy leads to phospholipase C (PLC)-mediated hydrolysis of polyphosphoinositol 4,5-bisphosphate (PIP_2) yielding inositol 1,4,5-triphosphate (IP_3) and 1,2 sn-diacylglycerol (DAG) (Figure 1.3). These products synergise to initiate cell activation

via calcium mobilization (IP₃) and protein kinase C activation (DAG). Metabolism of 1,4,5-IP₃ to inositol proceeds via two distinct pathways in PMNs: degradation to 1,4-IP₂ and 4-IP₁ or conversion to 1,3,4,5-IP₄, 1,3,4-IP₃, 3,4-IP₂ and 3-IP₁. Initial formation (0-30 s) of 1,4,5-IP₃ and DAG occurs at ambient intracellular Ca²⁺ levels, whereas formation of 1,3,4-IP₃ and a second sustained phase of DAG production (30 s-10 min) require elevated cytosolic Ca²⁺ influx. Receptor agonists empty intracellular Ca²⁺ stores which leads to Ca²⁺ influx across the plasma membrane. Products formed during activation can feed back to attenuate chemoattractant receptor-mediated stimulation of PLC by uncoupling receptor/G protein/PLC interaction.

A variety of studies indicate that protein kinases and protein phosphorylation events are important for activation of the respiratory burst (reviewed by Farago *et al.*, 1990). PKC is a multifunctional serine/threonine protein kinase requiring Ca²⁺ and phospholipid. Under physiological conditions, DAG greatly increases the affinity of PKC for Ca²⁺ and phospholipid, thereby activating the enzyme. However, a study by Sharma demonstrated that certain PKC-isotypes can activate NADPH oxidase at resting Ca²⁺ levels (Sharma *et al.*, 1995). PKC is linked to the phosphorylation of NADPH oxidase in PMNs. Several phosphoproteins that are either NADPH oxidase components or may regulate the activation mechanism have been identified including p47-phox, cytochrome b558 and rap1A. PMNs stimulated with PMA, an activator of PKC exhibit rapid phosphorylation of p47-phox on multiple serine residues (reviewed by Leusen *et al.*, 1996) and p67-phox (Benna *et al.*, 1997), a process that accompanies translocation of this protein to assemble an active NADPH oxidase complex at the plasma membrane.

1.2.2 Second messengers in the PMN

The levels of several second messengers increase dramatically in PMNs in response to chemoattractant-receptor binding and/or synthetic stimuli. These include Ca²⁺, IP₃, DAG, arachidonic acid (AA), phosphatidic acid (PA) and cAMP. These second messengers are

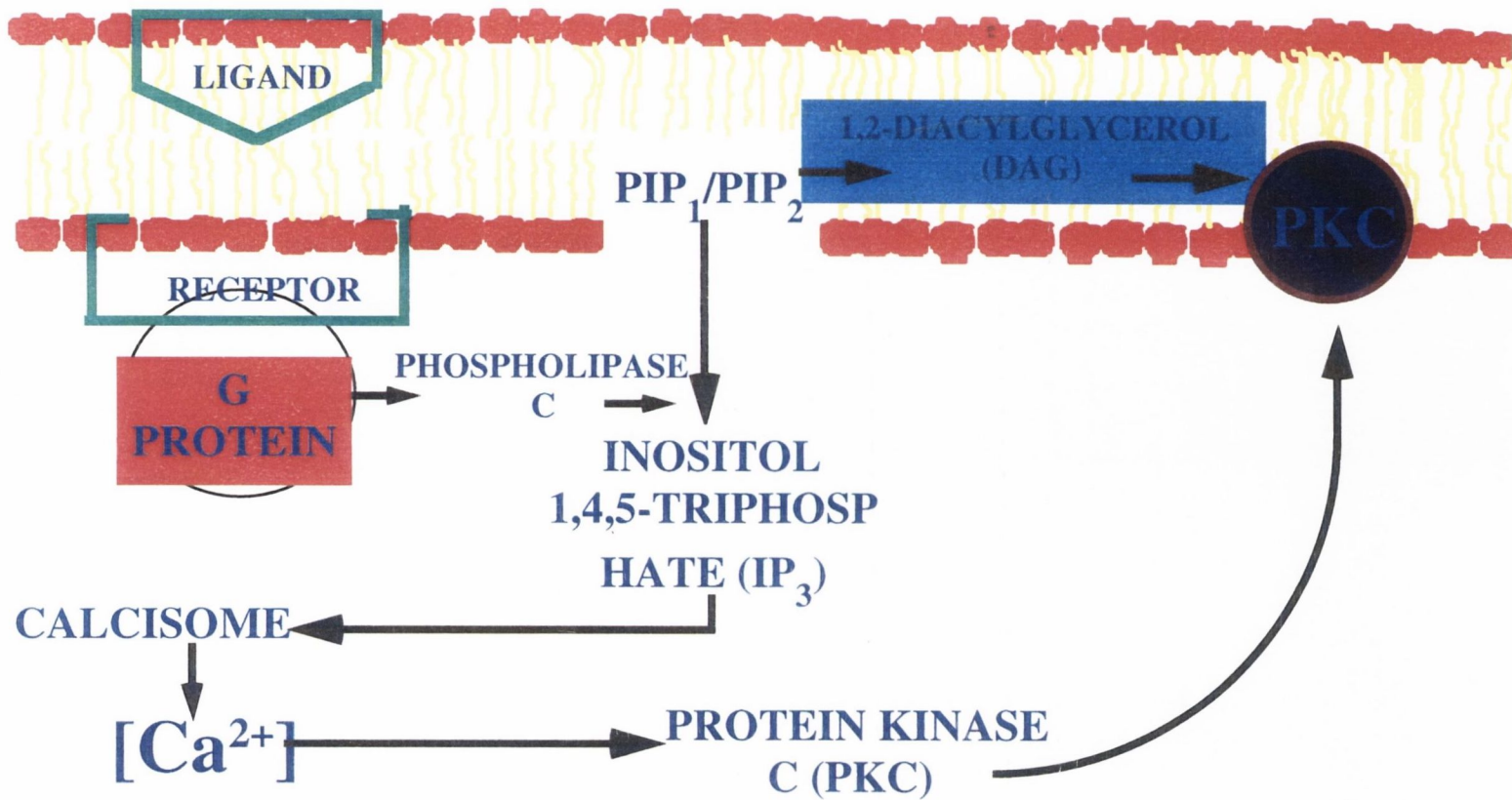


Figure 1.3 The signal transduction pathway in PMNs upon binding of a ligand or chemoattractant to its receptor.

generated as a consequence of chemoattractant-mediated activation of phospholipases. The phospholipases that are activated include: phospholipase A₂ (PLA₂) which hydrolyses phosphatidylcholine (PC) and/or phosphatidylethanolamine (PE) to produce AA and lyso PC and/or lyso PE; phospholipase C (PLC) which hydrolyses PC to produce DAG and IP₃; and phospholipase D (PLD) which hydrolyses PC to produce PA and choline.

An increase in the intracellular free calcium, $[Ca^{2+}]_i$ induced by chemoattractants begins rapidly following agonist binding (within 0.2-0.3 s) and involves at least two (possibly more) processes. Release from an intracellular storage compartment, the calciosome within the PMN is mediated by the binding of IP₃ to its receptor on the calciosome (Pittett *et al.*, 1998). Upon emptying of the internal stores, an influx of Ca^{2+} from the extracellular environment occurs. Increases in $[Ca^{2+}]_i$ induced by chemoattractants gradually returns to near baseline levels over 5-15 minutes by mechanisms involving PKC and/or PKA (reviewed by Di Virigilio *et al.*, 1990). PKC can induce activation of the plasma membrane Ca^{2+} -ATPase, which pumps calcium out of the cell, and both PKC and PKA can inhibit the generation of IP₃ (reviewed by Traynor-Kaplan, 1990). Increases in $[Ca^{2+}]_i$ can be achieved rapidly with calcium ionophores which bypass signal transduction mechanisms at the plasma membrane.

Arachidonic acid (AA) is formed via activation of PLA₂ which results in the release of free AA from membrane phospholipids. AA functions both as a second messenger and as a precursor for other lipid mediators. As a second messenger, AA is an activator of PKC (McPhail *et al.*, 1984). AA can be converted by the lipoxygenase pathway into leukotrienes and hydroxyeicosatetraenoic acids (HETEs) or through the cyclooxygenase pathway into prostaglandins (such as PGE₂) and thromboxanes. Prostaglandins are involved in pain and swelling in the inflammatory response. In contrast to leukotrienes, prostaglandins suppress most PMN functions, possibly through their ability to elevate intracellular cAMP (Sedgewick *et al.*, 1985). There is evidence to suggest that prostaglandins have an anti-inflammatory role in the inflammatory response. In contrast, leukotriene B₄ (LTB₄) is a potent chemoattractant

and may play a role in the pathogenesis of inflammatory diseases (Ford-Hutchinson *et al.*, 1980).

1.3 Nitric oxide

NO is synthesized from the conversion of L-arginine to L-citrulline by the enzyme, nitric oxide synthase (NOS). There are two types of this enzyme, the constitutive NOS (cNOS) and the inducible NOS (iNOS). cNOS releases NO for short periods of time in response to receptor or physical stimulation. This enzyme is cytosolic, Ca^{2+} /calmodulin dependent and acts as a transduction mechanism for the activation of soluble guanylate cyclase underlying several physiological responses (reviewed by Moncada *et al.*, 1995). In contrast, iNOS is cytosolic, Ca^{2+} -independent and requires a number of co-factors. iNOS synthesizes NO for longer periods of time in response to activation by various cytokines (reviewed by Griffith *et al.*, 1995).

1.3.1 The role of nitric oxide (NO) in inflammation

Inflammatory disorders are characterized by an upregulation of NO through activation of the inducible NOS (Rachmilewitz *et al.*, 1995). Within the inflamed area, NO is released from activated tissue cells, the vascular endothelium and cells migrating to the area such as neutrophils (PMNs) and monocytes/macrophages (Mφs) which release NO possibly upon phagocytosis. It remains controversial whether migrating phagocytes contribute significantly to the production of NO in inflammatory conditions. Low levels of NO produced by PMNs and Mφs may play a role in the mobilisation of more inflammatory cells to the area. Because of its cytotoxic properties, NO has been implicated in causing tissue injury in a number of inflammatory situations (Wiseman *et al.*, 1996). The extent of tissue injury is likely to be due to the fluxes between NO and oxygen radicals with maximal tissue insult where the balance between NO and superoxide (O_2^-) is exactly equal (Grisham *et al.*, 1995). Recent research has suggested that NO may be anti-inflammatory by inhibiting phagocyte function,

downregulating proinflammatory cytokine production and modulating apoptosis (Moilanen *et al.*, 1993, Meyer *et al.*, 1995, Albina *et al.*, 1993). There has been much focus on the development of highly specific inhibitors of iNOS for use as potent anti-inflammatory agents but these drugs have proved to be of limited success (Evans *et al.*, 1995). However, the literature to date would suggest that it may be worthwhile maintaining levels of NO high to avail of its protective properties whilst downregulating reactive oxygen species (ROS) to reduce its cytotoxicity.

1.3.2 Neutrophil production of NO

It is well established that rodent PMNs produce NO as part of their non-specific immunity. Rodent PMNs express iNOS when activated (McCall *et al.*, 1991, McCall *et al.*, 1989). There has been much controversy as to whether human PMNs synthesize NO and contribute to increased production of NO in inflamed areas. Numerous studies have been unable to detect NO in response to a wide variety of proinflammatory mediators and stimuli known to stimulate many PMN functions (Keller *et al.*, 1990, Yan *et al.*, 1994). It was demonstrated that any nitrite production by human, rat or mouse neutrophils *in vitro* was as a result of contamination by monocytes or macrophages in the preparation (Padgett *et al.*, 1995).

However, it is now generally accepted that human PMNs produce NO. NO has been detected in response to the chemoattractant, fMLP at low concentrations over short time points (Riesco *et al.*, 1993, Wright *et al.*, 1989). The formyl peptide, fMLP is a calcium linked stimulus which may activate cNOS due to its requirements for calcium. In addition, it has also been demonstrated that the proinflammatory cytokines, LPS, IFN- γ , IL-6 and PMA can upregulate NO release from human PMNs which was inhibitable by L-NMMA (Larfars and Gyllenhammer, 1998). Another study found that incubation of PMNs with LPS, IFN- γ and TNF- α in combination increased nitrite generation (Carreras *et al.*, 1993). The majority of studies showing increases in nitrite were found over short periods of time (1-2 hours) suggesting the involvement of cNOS rather than iNOS. Although cNOS has been purified in human PMNs (Bryant *et al.*, 1992), there is no evidence of an active iNOS in human PMNs nor any evidence of the presence of iNOS. A recent publication by Grisham has partially

resolved this controversy. He found that neither circulating rat or human PMNs contained iNOS mRNA, protein, enzymatic activity or nitrite generation when cultured *in vitro* (Grisham *et al.*, 1995). In contrast, inflammation-induced extravasation of rat but not human PMNs upregulated transcription and translation of iNOS in a time-dependent manner (Grisham *et al.*, 1995). More sensitive methods such as RT-PCR and Northern blot analysis show "constitutively expressed" iNOS mRNA in PMNs (Amin *et al.*, 1997). A study by Zhang investigated NO production from activated macrophages at the level of the iNOS gene. He found that human macrophages do not possess an LPS-inducible NF- κ B/Rel complex and have multiple inactivating nucleotide substitutions in the human enhancer elements that controls LPS/IFN- γ induced expression within the mouse iNOS gene (Zhang *et al.*, 1996). The genetic differences between human and rodent iNOS may account for the hyporesponsiveness of the human iNOS gene upon stimulation by LPS/IFN- γ .

Collectively, these studies indicate that PMNs produce low levels of NO following stimulation with a number of proinflammatory cytokines and chemoattractants at short time points most likely due to cNOS. PMNs may express very low levels of iNOS protein but may not have the functional or genetic capacity to produce NO through iNOS upon activation by proinflammatory cytokines.

1.3.3 Enterocyte production of NO

Activated tissue cells produce large amounts of NO at the site of inflammation. Enterocytes within the inflamed intestine of patients with IBD possess inducible NOS (iNOS) (Rachmilewitz *et al.*, 1995). Induction of iNOS usually requires stimulation with LPS and IFN- γ whereby nanogrammes of NO is produced within hours and remain for several days. This form of NOS has been implicated in causing tissue destruction as resident tissue cells do not express iNOS under normal homeostatic conditions.

The cellular distribution of iNOS in IBD was examined using immunoperoxidase microscopy with a monospecific human iNOS antibody (Singer *et al.*, 1996). This study found intense focal iNOS labelling localized to the inflamed human colonic epithelium. Because several cytokines such as tumour necrosis factor- α (TNF- α), interferon- γ (IFN- γ)

and interleukin 1 β (IL-1 β) are increased in the inflamed colon, it is reasonable to speculate that they may also induce nitric oxide synthase (NOS) in colonic epithelial cells. *In vitro* studies indicate that colonic epithelial cell lines are capable of releasing large amounts of nitrite upon stimulation by proinflammatory cytokines and endotoxin (Salzman *et al.*, 1996, Dignass *et al.*, 1995, Kolios *et al.*, 1995). A variety of agents including bacterial products and cytokines alone or in combination enhance the production of NO by rat intestinal epithelial cells *in vitro* (Dignass *et al.*, 1995, Grisham *et al.*, 1995). Stimulation with IL-1 and IFN- γ upregulates iNOS mRNA and nitrite production in HT-29 cells (Kolios *et al.*, 1995).

There is little information on the regulation of NOS activity within the gut. However, activated colonic epithelial cells within the inflamed gut may upregulate their production of iNOS upon stimulation by proinflammatory cytokines. Over a period of days, these activated resident tissue cells may release large amounts of NO into the inflamed site. iNOS in the inflamed colonic epithelium is associated with the formation of peroxynitrite anion and nitration of cellular proteins (Singer *et al.*, 1996, Miller *et al.*, 1995). Induction of iNOS in enterocytes may be responsible for tissue injury in IBD through the formation of peroxynitrite anion or perhaps through its own toxicity.

1.4 Apoptosis

Apoptosis or programmed cell death is a distinct mode of eukaryotic cell death. It is responsible for the deletion of cells in normal tissues and complements cell proliferation in normal tissue homeostasis. Dysregulated apoptosis is associated with disease states which include cancer, autoimmunity, AIDS, allergic disorders, neurodegenerative and inflammatory diseases (reviewed by Westin and Bergh, 1998, Signore *et al.*, 1998, Schwartz and Klotman, 1998, Grayson and Bochner, 1998, Jacobson, 1998, Fiocchi *et al.*, 1997).

Apoptosis is fundamentally different from degenerative cell death or necrosis. It characteristically affects single cells, not groups of contiguous cells as in necrosis. The apoptotic cell undergoes a relatively ordered form of cell death characterised by cell shrinkage, cellular crenation, cytoplasmic and chromatin condensation and internucleosomal DNA fragmentation. Changes in membrane glycosylation and lipid profiles, and alteration in

expression of surface receptors have been observed (Martin *et al.*, 1995). The apoptotic cells are rapidly phagocytosed and degraded by tissue macrophages without eliciting an inflammatory response. This mechanism prevents the release of cytotoxic agents into the extracellular milieu with potential provocation of an inflammatory response (Savill *et al.*, 1993). This process differs significantly to cell death by necrosis or lysis. During necrosis, the release of the contents of the cell into the surrounding tissue occurs thereby perpetuating the inflammatory response. As cells undergo apoptosis their DNA is cleaved in an orderly fashion by endonucleases resulting in the generation of 180-200 base pairs. DNA fragmentation is characteristic of apoptosis and produces a ladder pattern on DNA gel electrophoresis. During necrosis, there is a random cleavage of DNA which appears as a banding effect on DNA gel electrophoresis.

1.4.2 PMN apoptosis and its role in the inflammatory response

During the normal resolution of inflammation, PMN apoptosis and their subsequent ingestion by macrophages plays an important role in limiting autotoxic potential of the PMN (Savill *et al.*, 1997). Failure of the activated PMN to undergo apoptosis and thereby terminate the inflammatory response has been proposed as a precipitant of inflammatory diseases. Apoptotic PMNs are functionally effete with a number of downregulated PMN functions including depressed chemotaxis, phagocytosis, granule release and respiratory burst activity (Narayanan *et al.*, 1997). In addition, uptake of apoptotic PMNs failed to stimulate macrophage release of proinflammatory mediators. This may serve to limit tissue injury and promote resolution of inflammation.

The PMN remains the key proinflammatory cell in the initial response to injury or an inflammatory insult. PMNs are responsible for mediating the first line of host immune function resulting in a rapid systemic neutrophilia and rapid PMN influx into the inflammatory site. Although PMNs contain powerful oxidative and non-oxidative mechanisms critical in host defence, uncontrolled release of these toxic agents during necrosis may exacerbate local tissue injury. The induction of PMN apoptosis over necrosis limits the potential for injury. Phagocytic recognition mechanisms of apoptotic PMNs are

beginning to be understood. Recognition by macrophages of apoptotic cells occur by multiple overlapping mechanisms. Savill proposed that macrophage recognition of apoptotic PMNs occurs by at least two distinct mechanisms including recognition of exposed phosphatidylserine on the surface of apoptotic cells and recognition of a putative thrombospondin binding motif recognised by the $\alpha_v\beta_3$ (vitronectin) integrin and CD36 (thrombospondin) receptor complex on the macrophage (Savill *et al.*, 1993).

Although the metabolic cellular events involved in the apoptotic process have been investigated in a wide variety of cells, a common pathway for induction of apoptosis is unclear. One common underlying mechanism is through an oxidative process. There is a large body of evidence that implicates ROS in the induction of apoptosis based on the fact that many of the chemical and physical treatments that induce apoptosis are themselves inducers of oxidative stress (Buttke and Sandstrom, 1994). In addition, NO is released upon activation of NADPH oxidase during the oxidative burst. One recent biological effect attributed to NO is its induction of apoptosis in the macrophage, the effector cell for NO-mediated cytotoxicity. Murine peritoneal macrophages when activated to express nitric oxide synthase (NOS) die prematurely in culture (Albina *et al.*, 1993). Since this discovery, NO has been found to regulate apoptosis in a number of cell types. NO induces apoptosis in chondrocytes (Blanco *et al.*, 1995). However, there are also reports of inhibition of apoptosis by NO in B-lymphocytes (Mannick *et al.*, 1994) and eosinophils (Beauvais *et al.*, 1995).

A number of proinflammatory cytokines are released at the inflammatory site with the potential to modulate apoptosis. The process of apoptosis is regulated by signals generated when cytokines bind to their receptors. There are two types of cytokine-induced signals. Cytokines inducing apoptosis include TNF- α and Fas ligand (CD95). The proapoptotic cytokine, TNF- α activates Cytosolic Aspartate-Specific cysteine Proteases (Caspases). These cleave cellular substrates leading to membrane changes and eventual nuclear damage. The second group of cytokines are inhibitory signals that suppresses apoptosis. LPS and IFN- γ have been shown to be anti-apoptotic to PMNs (Colotta *et al.*, 1992).

1.4.3 Enterocyte apoptosis and its role in the inflammatory response

The epithelium of the gastrointestinal tract has a highly stereotyped organisation with a continuous high level of cell proliferation (Wright and Alison, 1984). Epithelial cells of the gut are characterized by rapid, constant cell renewal. The lining of the gastrointestinal tract is replaced every 2 to 3 days in the rodent and 6 to 7 days in the human. In the adult, mammalian gastrointestinal tract apoptosis has been found in the proliferative and non-proliferative compartments (Hall *et al.*, 1994) and this increases in inflammatory conditions (Fiocchi, 1997). In order to balance the enormous cell production that is reported to occur, cell loss occurs by shedding into the gut lumen (Wright and Alison, 1984). The mechanism of cell loss is believed to be a form of exfoliation from the small intestinal villus, the top of the colonic crypt and inter-crypt epithelium into the lumen of the gut. However, the most recent evidence points to the involvement of intraepithelial lymphocytes in killing and engulfment of apoptotic enterocytes by macrophages (reviewed by Iwanaga, 1995). More recently, the involvement of intraepithelial lymphocytes has been suggested in enterocyte cell death under physiological conditions (Shiner *et al.*, 1998).

At present, there is little information as to the mechanisms that regulate cell loss within the gut. However, two mechanisms of cell loss in the gut have been proposed by Mayhew (Mayhew *et al.*, 1999). In the first (type 1), complete cells are extruded into the lumen. Apoptosis by loss of adherence is a recently described phenomenon termed "anoikis". Survival of colonic epithelia crucially depends on matrix adhesion and is likely to be mediated through a β -1 integrin/matrix interaction. An *in vitro* study demonstrated rapid onset of apoptosis following disruption of extracellular matrix in human colonic crypt cells (Strater *et al.*, 1996). In the second, only anucleate apical cell fragments pass into the lumen. Type 2a refers to cell loss through the creation of large intercellular spaces extending from the preserved apical cap to the basal lamina containing enterocyte debris for phagocytosis. Type 2b involves the gradual shrinkage of individual cells and in situ degeneration of nucleated subapical portions in narrow intercellular spaces between adjacent enterocytes.

Enterocyte apoptosis can occur through p53-dependent or p53-independent pathways. Many of the cell lines used in the study of intestinal biology are p53 mutants as

primary culture has proved difficult. The p53 tumour suppressor gene plays a central role in the regulation of cell proliferation, primarily at the G1 phase of the cell cycle. Under physiological conditions p53 acts to limit proliferation and induce apoptosis following damage to genomic DNA. Inactivation or loss of p53 function is associated with the deregulation of a number of growth related processes, including events underlying cell cycle progression and apoptosis. Although the mechanism whereby p53 induces apoptosis is controversial, several studies have suggested that p53 regulates apoptosis by transcriptional suppression of bcl-2 and induction of bak (Manne *et al.*, 1997).

Apoptosis within the gastrointestinal tract is regulated by a number of cytokines including TNF- α and TGF- β (Piguet *et al.*, 1998). NO is a diffusible messenger involved in pathophysiological processes including cell killing and cytotoxicity. Nitric oxide induces apoptosis in a number of cell types (Blanco *et al.*, 1995, Mannick *et al.*, 1994, Beauvais *et al.*, 1995). NO is produced in large quantities by enterocytes in IBD (Singer *et al.*, 1996) and by colonic epithelial cell lines upon stimulation by a combination of LPS and proinflammatory cytokines *in vitro* (Dignass *et al.*, 1995). The reaction product of NO and O₂⁻, ONOO⁻ has been shown to induce apoptosis in human intestinal epithelial cells, an effect which is attenuated by mesalamine, a standard therapy for IBD (Sandoval *et al.*, 1997). The effect of NO on enterocyte apoptosis is currently unknown. A proposed mechanism for NO-induced apoptosis within the gut is illustrated in Figure 1.4.

1.5 Taurine and its structural analogue, HEPES

Taurine (2-aminoethane sulphonic acid) is a naturally occurring β -amino acid having the molecular structure, NH₂-(CH₂)₂-SO₃H. Taurine differs from α -amino acids in possessing a sulphonic rather than carboxylic acid group. It was first discovered in ox bile in 1827 (Tiedman *et al.*, 1827) and since then has become a physiologically significant compound. Taurine has effects on a number of physiological processes including osmoregulation, calcium modulation and membrane stabilising effects through its interaction with phospholipids within the plasma membrane. In addition, taurine possesses anti-oxidant

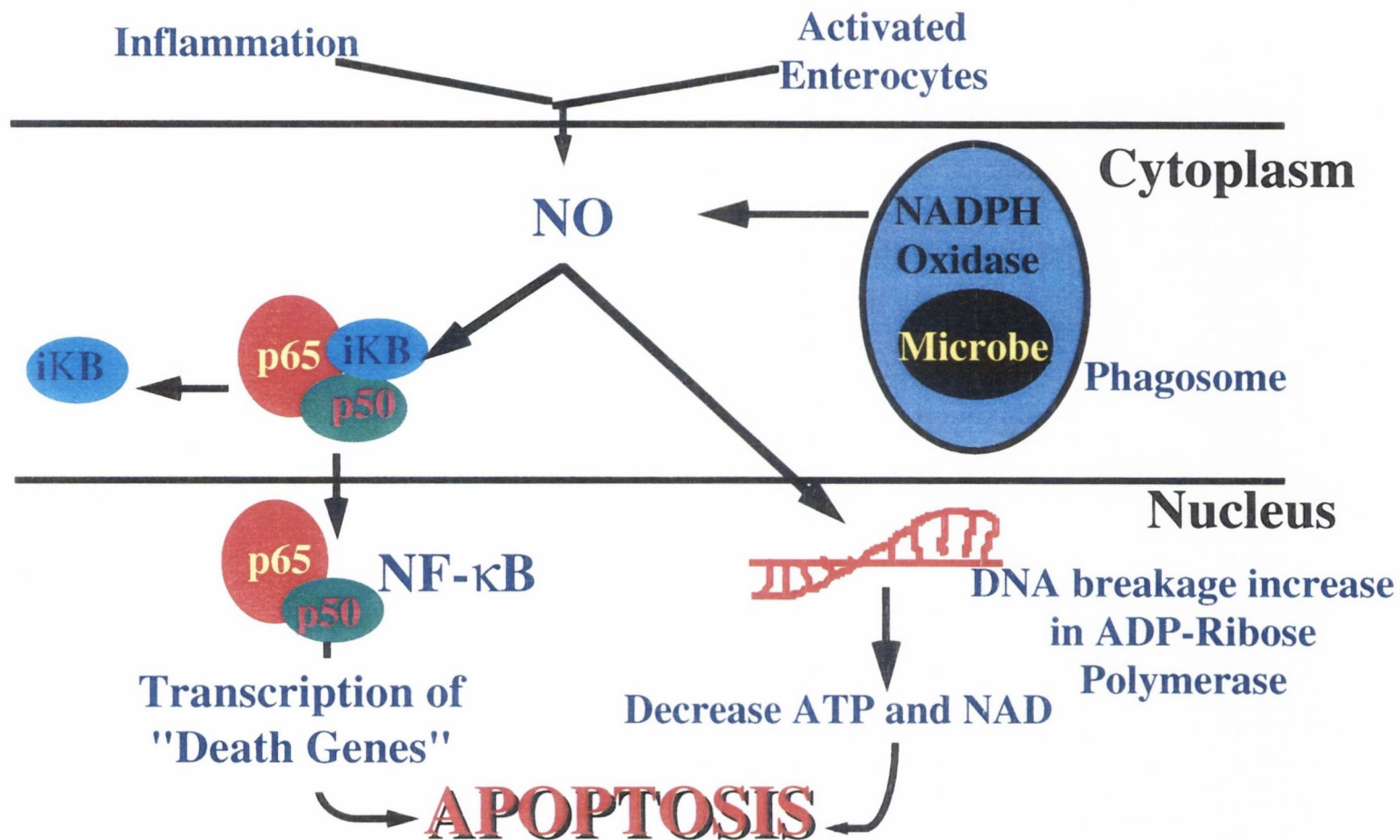


Figure 1.4 Proposed role for NO as a mediator of PMN and enterocyte apoptosis.

properties through the formation of taurine-chloramine by sequestering HOCl. HEPES (N-2-Hydroxyethylpiperazine-N'2-ethanesulphonic acid), a structural analogue of taurine possesses potent anti-inflammatory properties *in vivo* (Stapleton *et al.*, 1993).

1.5.1 Biochemistry and Biosynthesis of taurine

Taurine is an inert compound which does not enter into the composition of proteins and exists as a free form or in some simple peptides (Huxtable, 1979). Taurine is taken in via the diet but can be synthesized from methionine and cysteine primarily in the liver. There are a number of possible routes of synthesis which include (1) the oxidation of cysteine to cysteinesulphinic acid, subsequent conversion to cysteic acid and decarboxylation to taurine and (2) oxidation of cysteine to cysteine sulphinic acid, subsequent decarboxylation to hypotaurine and spontaneous oxidation of hypotaurine to taurine. The capacity to synthesize taurine depends on the availability of methionine and cysteine and varies between species. Its biosynthesis can be limited in humans due to low activity of cysteinesulphinic acid decarboxylase (CSAD), the enzyme that catalyses the conversion of cysteinesulphinic acid to hypotaurine. Humans and mammals are dependent on a dietary source of taurine with cats being incapable of synthesising taurine (Huxtable, 1992).

1.5.2 Taurine and the PMN inflammatory response

High levels of taurine have been reported in tissues that generate reactive oxidants. Taurine makes up to 76% of the total free amino acid of human neutrophils (Fukuda *et al.*, 1982) with the intracellular concentrations ranging from 20-50 mM (Soupart, 1962). There is a 1000 fold gradient between cellular taurine levels and plasma concentrations as concentrations of taurine in the plasma range from 50 to 100 μ M (Soupart, 1962). Stringent control of PMN taurine levels indicate an important role for this amino acid during inflammation. Huxtable suggested such stringent control of PMN cellular taurine may augment physiological competence (Huxtable, 1992).

Taurine possesses anti-inflammatory activity in a diverse range of animal models of inflammation including arthritis in which neutrophil recruitment is believed to play a role (Schuller-Levis *et al.*, 1994, Witso-Sarvat *et al.*, 1995, Wang *et al.*, 1992, Son *et al.*, 1997). Research from our lab has demonstrated that taurine has the capacity to decrease PMN influx across the pulmonary vasculature in an animal inflammatory model (Barry *et al.*, 1995). CD11b/CD18 belongs to the β_2 -integrin family of cell surface adhesive receptors that play a role in PMN adherence to endothelium. Taurine attenuates MPO-upregulated CD11b receptor expression (Stapleton *et al.*, 1998). LTB₄, the most potent chemoattractant produced by activated PMNs is decreased by taurine which was postulated to be mediated through upregulated MPO activity in human PMNs (McLoughlin *et al.*, 1991).

Taurine has a number of anti-inflammatory effects on the PMN respiratory burst pathway (Stapleton *et al.*, 1993, Stapleton *et al.*, 1994). HOCl is a powerful oxidising agent produced during respiratory burst activity. It has been implicated as the most destructive oxidant synthesized by activated PMNs in the inflammatory response (Eaton, 1993). It can cause tissue damage by oxidising carbohydrates, nucleic acids and amino acids. Taurine scavenges HOCl to form taurinechloramines (Lampert *et al.*, 1983) which is markedly less toxic to host tissue (Thomas *et al.*, 1979). The reaction of taurine or its analogues with HOCl leads to the formation of either a monotaurochloramine or a ditaurochloramine (Wright *et al.*, 1985). The monochloramine is the predominant species produced by intact human PMNs *in vivo* which is illustrated as follows:



The production of chloramines was proposed by Thomas (1979) to be an anti-inflammatory control mechanism.

The conversion of molecular oxygen to O_2^- via NADPH oxidase is a Ca^{2+} -dependent process. The effect of taurine and HEPES on NADPH oxidase activity and NADPH oxidase-dependent activation of the respiratory burst pathway is unknown. It has been well established that taurine possesses calcium-modulatory effects (Huxtable *et al.*, 1992). Taurine has been shown to stimulate ATP-dependent calcium ion uptake on photoreceptor cells (Kuo *et al.*, 1980) and affect calcium uptake in excitable tissues (Kramer

et al.,1981). As an increase in $[Ca^{2+}]_i$ is one of the triggers for activation of NADPH oxidase, taurine and HEPES may affect respiratory burst activity through effects on Ca^{2+} thereby modulating NADPH oxidase activity.

1.5.3 Taurine and apoptosis

Apoptosis plays a major role in promoting resolution of the acute inflammatory response (Savill *et al.*, 1997). Reactive oxygen and nitrogen species induce apoptosis in PMNs and macrophages. There is a large body of evidence that implicates reactive oxygen species in the induction of apoptosis based on the fact that many of the chemical and physical treatments that induce apoptosis are themselves inducers of oxidative stress (reviewed by Buttke and Sandstrom, 1994). Conversely, many inhibitors of apoptosis have antioxidant activities or enhance cellular antioxidant defenses (reviewed by Buttke and Sandstrom, 1994).

Apoptosis in any cell type is in part related to the ability of the cell to maintain an appropriate oxidant-antioxidant balance. There is evidence to indicate that onset of apoptosis is mediated by oxidative stress resulting from the downregulation of key antioxidant defence systems of the cell namely SOD and glutathione (Narayanan *et al.*, 1997). Research from our laboratory has demonstrated that the antioxidant, taurine can decrease apoptosis in a number of cell types including PMNs, endothelial cells and hepatocytes. Taurine significantly protects against sodium arsenite-induced PMN apoptosis through its anti-oxidant properties (Watson *et al.*, 1996). Taurine attenuates hepatocyte apoptosis and necrosis through an inhibition of both NO and ROS (Redmond *et al.*, 1996). Taurine prevents endothelial cell death through its antioxidant activity and regulation of intracellular calcium flux (Wang *et al.*, 1996). In other studies, taurine did not prevent apoptosis in NK cells by monocyte-derived reactive oxygen species (Hansson *et al.*, 1996).

1.6 Aims of the project

The aims of this project were to study the role which taurine and its structural analogue, HEPES play in the inflammatory process and apoptosis and evaluate a possible mode of action for these compounds in the modulation of inflammatory bowel disease. This work comprises of three major aspects:

(1) an *in vitro* investigation to clarify the mechanism through which taurine and its analogue, HEPES modulate inflammatory cell activity and establish the biochemical pathways through which taurine and its structural analogue, HEPES regulate neutrophil function.

(2) an *in vitro* study to determine the effects of taurine and HEPES on the regulation of cell death within IBD. This involved an assessment of apoptosis within the polymorphonuclear leukocyte, the major inflammatory cell influxing the colon and activated colonic epithelial cells.

(3) an *in vivo* investigation to study the effects of taurine or taurine analogues in an animal model of colitis. The effects of pharmacological doses of these compounds on the induction of colitis was assessed to establish the efficacy of taurine and its structural analogues as prophylactic and pharmacological agents in the management of IBD.

CHAPTER II

MATERIALS AND METHODS

2.2.1 Preparation of solutions

Reagents are listed in Section 2.12 with the address of the reagent supplier. Reagents were stored according to manufacturer's instructions. Powder form reagents were weighed using a Sartorius AC1215 electronic balance and solutions were prepared in distilled deionised water unless otherwise stated. pH was measured using a Radiometer A/S meter calibrated with buffers at pH 4.0, 7.0 and 10.0 (Sigma Chemicals). For transferring volumes ranging from 0.5 µl to 10 ml, Gilson pipettes were used.

2.2 Tissue culture techniques

All cell culture work was carried out in a Laminar Air Flow cabinet (Holten Lamin Air) using an aseptic technique. The laminar flow cabinet was sterilised with 70% ethanol prior to use.

2.2.1 Isolation of human PMNs.

Human peripheral blood anticoagulated with heparin (10 U/ml) was collected from healthy volunteers. PMNs were isolated from human peripheral blood and subjected to dextran (6%, saline) sedimentation for 35-40 min at room temperature. The leukocyte-rich layer was layered onto a Ficoll-Paque gradient and centrifuged at 300g for 20 min in an Eppendorf bench centrifuge (Model 5403). Contaminating erythrocytes were removed from the PMN fraction by hypotonic lysis. The remaining pellet was washed and resuspended in Dulbecco's Modified Eagle Medium containing 10% foetal calf serum (FCS) and penicillin/streptomycin (1 U/ml). Cells were incubated in polypropylene tubes to prevent adherence in a temperature controlled (37°C) humidified 5% CO₂ incubator. PMN purity was >95% as assessed by both flow cytometry and light microscopy. Cell numbers were calculated using a Neubauer haemocytometer. Briefly, an equal volume of cells were mixed with an equal volume of trypan blue (Sigma Chemicals) prior to counting under 400X magnification.

2.2.2 Enterocyte tissue culture and subculture

The Caco-2 cell line was grown as a monolayer on plastic sterile 25 cm² tissue culture flasks in Minimal essential medium (MEM) supplemented with heat inactivated FBS (10% v/v), 100U penicillin/streptomycin, non-essential amino acids and L-glutamine (2mM) in a temperature controlled (37°C) humidified 5% CO₂ incubator. The HT-29 cell line was grown as a monolayer on plastic in McCoy's medium supplemented with 10% heat inactivated FBS (10% v/v) in a temperature controlled (37°C) humidified 5% CO₂ incubator. Cell lines were subcultured 2-3 times weekly in the case of the HT-29 cell line and 1-2 times weekly in the case of the Caco-2 cell line. Subculture was achieved by incubating confluent flasks (approximately 80%) in 0.05% trypsin/0.02% EDTA until cells detached. The activity of trypsin was terminated by addition of fresh medium containing FCS. The resulting cell suspension was spun at 300g at room temperature in an Eppendorf 5403 refrigerated benchtop centrifuge. The pellet was resuspended and cells were seeded in a ratio of 3:1 in fresh flasks. Cells were routinely tested for mycoplasma using the Mycoplasma PCR ELISA kit and were found to be free of mycoplasma.

If cells were not immediately required, Cells were stored frozen in a 10% stock solution of DMSO/FCS at a final concentration of 5×10^6 cells/ml. 1-2 mls of the cell suspension were pipetted into sterile 1 ml cryotubes, labelled and gradually frozen to a temperature of -80°C over a period of 12 hours before final storage in liquid nitrogen. Cells were revived by removing from liquid nitrogen and quickly transferring to a 37°C waterbath until thawed. The ampoule was pipetted into a sterile tube containing 10 mls of prewarmed medium. The cells were centrifuged to remove any DMSO and cultured as previously described.

2.3.1 CD11b Receptor Expression

Human CD11b Receptor expression was assessed in resting and stimulated PMNs. 50 µl of PMNs (5×10^6 cells) was incubated with 10 µl of Mac-1 monoclonal antibody for 10 minutes at room temperature and then 3 mls of lysing solution (Becton Dickinson, Mountain

View, CA) was added, vortexed and spun at 250g for 5 minutes after which it was washed with cold PBS and spun again. The samples were analysed by flow cytometry within an hour.

Rat PMN CD11b expression was determined using a Mac-1 anti-rat antibody (Serotec, Oxford, U.K.). 100µl of blood was incubated with 10µl of antibody for 15 minutes at room temperature. FACS lysing solution was added to lyse the red blood cells prior to washing with PBS. The samples were then assessed immediately flow cytometrically). Forward and right angle scatter were used to selectively gate the PMN population.

2.4.1 Measurement of PMN intracellular calcium, $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ activity was assessed in stimulated PMNs using a calcium probe, Fluo-3/AM. The synthesis and spectral properties of Fluo-3 have been well described (Rijkers *et al.*, 1990). A 2mM stock solution of fluo-3/AM was prepared in anhydrous dimethylsulfoxide, containing 37.5g/l Pluronic F-127. PMNs (5×10^6 cells/ml) were removed from culture, washed twice in Kreb's Ringer Phosphate Dextrose buffer (KRPD) and incubated at 37°C for 30 minutes in KRPD (120mM NaCl, 5mM KCl, 0.5mM $CaCl_2$, 1.3mM $MgCl_2$, 11mM glucose in a sodium phosphate buffer (10mM)) containing fluo-3/AM (final concentration of 1µM). Cells were then washed once in KRPD to remove any free Fluo-3 and resuspended in KRPD and allowed 10 minutes incubation at 37°C for optimal deesterification. Cells were subsequently stimulated with fMLP (0.1 µM) and analyzed on a FACSCAN flow cytometer every 30 seconds out to 5 minutes or every minute out to 10 mins with A23187 (1 µM). Forward and right angle scatter were used to selectively gate the PMN population. Excitation was from an argon laser at 488 nm. Emission at 525 nm was measured on a linear scale. A discontinuous strategy was performed with FACSCAN software, acquiring 2000 events for each sample.

2.5.1 PMN Respiratory burst activity

PMNs were assessed for respiratory burst activity flow cytometrically as described previously (Rothe *et al.*, 1988, Smith and Weidemann, 1993) using the fluorogenic substrate

dihydrorhodamine 123 (DHR 123). Briefly, 50µl of isolated human PMNs (1×10^6 /ml) were incubated with 10 µl of opsonised *E. coli* (1×10^9 bacteria/ml) or PMA (1.62 mM) for 10 minutes at 37°C followed by 10 µl of DHR 123 which fluoresces on interaction with oxygen radicals. The samples were centrifuged in a refrigerated centrifuge at 250g for 5 minutes, washed in cold PBS and maintained on ice until acquired by flow cytometry (Becton Dickinson, Mountain View, CA).

Rat PMN respiratory burst activity was assessed in whole blood using a modified version of the above assay. Briefly, 50 µl of blood was incubated with 10 µL of rat opsonized *E. coli* (1×10^9 bacteria/ml) for 10 minutes at 37°C followed by 10 µL of DHR 123. FACS lysing solution (1 mL) was added to lyse the red blood cells. The samples were centrifuged at 250g for 5 minutes, washed in cold PBS and analysed immediately. Rat PMN respiratory burst activity was assessed by gating the PMN region.

2.5.2 NADPH oxidase activity

NADPH oxidase was assessed indirectly by superoxide dismutase (SOD)-inhibitable reduction of ferricytochrome C, in response to the following stimuli, PMA (1 mM), the calcium ionophore, A23187 (1mM) or the chemotactic peptide, fMLP (1 µM) using the discontinuous method as described (Jones and Hancock, 1986). Briefly, PMNs (1×10^6 /ml) were washed and cultured in prewarmed ferricytochrome C (100 µM in KRPD) with or without Superoxide Dismutase (SOD, 250 U/ml). Cells were stimulated by incubation with the stimulus in a shaking water bath at 37°C with PMA (30 mins), A23187 (60 mins) and fMLP (45 mins). The progress of cytochrome c reduction was followed using a microtitre plate reader equipped with a 550nm filter. The reduction of ferricytochrome C was calculated and the results represent the difference between stimulated and unstimulated cultures with or without SOD.

2.5.3 Myeloperoxidase (MPO) Activity

MPO activity was assayed according to the o-dianisidine method (Williams *et al.*, 1983). PMNs (5×10^6 cells/ml) were lysed with the detergent, Triton X-100 (0.05%), washed and centrifuged at 300g for 5 mins. 10 μ l of the supernatant containing MPO activity was added to 100 μ l of assay solution (0.1M sodium phosphate buffer, pH 7.3 (3 mls), 0.01M H₂O₂ (3 mls), H₂O (22 mls) and 0.2 M o-dianisidine (1:1 in H₂O/MeOH) (2 mls)). This assay solution was prepared on ice directly before use and protected from light. Samples were left for 10 minutes at room temperature before addition of 100 μ l of HCl (1M) to stop the reaction. Samples were then read spectrophotometrically at 450 nm. Units are calculated according to the following formula, $\text{MPO units/ml} = (\Delta\text{O.D.} \times V_t \times 4) / (E \times D_t \times V)$ where V_t = total volume (mls), V_s = sample volume (mls), $\Delta\text{O.D.}$ is the change in optical density and D_t = time of measurement (min).

For the determination of tissue MPO in animal model of colitis, an adaptation of the above method was used. After weighing colonic tissue (inflamed and minimally inflamed areas), it was homogenised in 10 mls of 0.5% hexadecyltrimethyl ammonium bromide (HTAB) in 50 mM potassium phosphate buffer (pH 6.0). This was then subjected to 3 freeze/thaw cycles, and centrifuged (30 minutes, 2000g). MPO was assayed in the resultant supernatant spectrophotometrically using the o-dianisidine method. The change in absorbance with time, at 460 nm (CPU 8720, UV/VIS Scanning spectrophotometer, Philips, Eindhoven, Netherlands), was continuously recorded over 10 minutes. One unit of MPO was defined as that which degrades 1 μ mole of peroxide/minute at 25°C which was calculated per gram of tissue using the following formula: $\text{Absorbance}/10/\text{weight of tissue}/0.0113 = \text{MPO/gram of tissue}$ where 0.0113 is a constant (Williams *et al.*, 1983).

2.5.4 Determination of NO release

NO was measured indirectly by measuring the stable end product, nitrite (NO₂⁻) by the Griess assay (Green *et al.*, 1982). 100 μ l aliquots of PMN culture supernatants were

incubated with an equal volume of Greiss reagent (1% sulphanilamide in 5% H_3PO_4 / 0.1% N-(1-Naphthyl)ethylenediamine) for 10 min at room temperature. Concentrations were determined using sodium nitrite for standard curves. Absorbance was read at 550 nm .

2.5.5 PMN Peroxynitrite anion formation

Circulating blood PMNs (in PBS without $\text{Ca}^{2+}/\text{Mg}^{2+}$) were assessed for peroxynitrite formation using the fluorogenic substrate dihydrorhodamine as described (Koody *et al.*, 1994). Briefly, activated PMNs (100 μl) at $5 \times 10^6/\text{ml}$ were incubated with 10 μl of DHR 123 (final concentration of $1\mu\text{M}$) for 10 minutes at room temperature. Samples were washed, resuspended in PBS and analysed immediately using a FACS flow cytometer. Analysis was carried out on a FACScan equipped with an argon laser and a standard fluorescein isothiocyanate-phycoerythrin filter pack providing an excitation wavelength of $\lambda_{\text{ex}} = 488\text{nm}$ and the collection of fluorescence signals at $\lambda_{\text{em}} = 530 \pm 15\text{nm}$ (green fluorescence).

2.6.1 β -Glucuronidase Assay

Degranulation was assessed as the release of granule contents into the extracellular medium (exocytic degranulation). PMNs were stimulated with both a soluble and particulate stimulus plus an inhibitor of ingestion (cytochalasin B). PMNs (5×10^6 cells/ml) were mixed with stimulus plus cytochalasin B (5mg) in KRPD. The cells are incubated for 30 mins at 37°C and the reaction stopped by placing the mixture on ice and centrifuging at 300g for 10 mins. As a control, cells without stimuli plus cytochalasin B were carried out. The supernatant was removed and assessed for enzyme contents. The reaction is initiated by adding 5×10^6 cells (0.3 mls) from the degranulation supernatant preparation to sodium acetate buffer, 0.01M (0.5 mls), pH 5.0, Triton X-100 (0.1 mls), 0.01 M p-nitrophenyl-B-D-glucuronide (0.1 mls). The assay mixture was incubated in a shaking water bath at 37°C for 2 hours. The reaction was stopped by the addition of 0.2M sodium hydroxide and the change in absorbance at 405 nm was recorded. Reagent blanks, containing sodium acetate buffer instead of supernatant

was subtracted. The β -glucuronidase activity was calculated as follows, β -glucuronidase activity [nmoles/min/5 x 10⁶] = 0.45 [DA405 (units/2 hr)]

2.7.1 LTB₄ and PGE₂ production

LTB₄ and PGE₂ production were assayed in the supernatants of stimulated cells as determined by competitive enzyme immunoassay kits following manufacturers instructions. The prostaglandin synthetase inhibitor, indomethacin was added to all samples at 10 mg/ml. Total activity, non-specific binding, maximum binding and substrate blank wells were run as a means of quality control for each assay along with appropriate standard curves.

2.8.1 Cell Morphology

Wright Giemsa staining was used to assess morphological changes in PMNs. PMNs were cytopun and viewed under a light microscope to assess for apoptotic morphology or necrosis as described (Martin *et al.*, 1990). Cell shrinkage, nuclear condensation (typical PMN characteristic), increased vacuole formation are characteristic of apoptotic cells.

2.8.2 Propidium iodide staining

PMN apoptosis was assessed according to the percent of cells with hypodiploid DNA as described (Nicoletti *et al.*, 1991). Following co-culture with the apoptotic inducing agent, PMNs were pelleted by centrifuging at 300g for 5 min. Cells were gently resuspended in 1 ml of hypotonic propidium iodide (PI) fluorochrome solution (50 mg/ml PI, 3.4 mM sodium citrate, 1 mM Tris, 0.1 mM EDTA, 0.1% Triton X-100), incubated in the dark at 4°C for 3 hours before they were analysed by a FACScan flow cytometer. Apoptotic PMN nuclei were distinguished by their hypodiploid DNA content from normal PMN nuclei. Forward scatter and side scatter of PMN particles were simultaneously measured. The PI fluorescence of individual nuclei with an acquisition of FL2 was plotted against forward scatter, and the data

plotted on a logarithmic scale. A minimum number of 5,000 events was collected and analysed using software Lysis II. PMN debris was excluded from analysis by raising the forward threshold. All measurements were performed under the same instrument settings.

2.8.3 DNA Gel Electrophoresis

PMN DNA fragmentation was assessed using gel electrophoresis by a modification of the method described (Martin *et al.*, 1990). Briefly, 4×10^6 PMNs were washed twice with HBSS and pelleted by centrifugation at 250g for 5 min. Cells were resuspended in 30 μ l of lysis buffer (20 mM EDTA, 100 mM Tris, 0.8% N-lauroylsarcosine, pH 8.0) and 20 μ l of RNase A (1 mg/ml in 100 mM sodium acetate (pH 4.8) and 0.3 mM EDTA) for 6 hours at 37°C in the water bath. 15 μ l of proteinase K (20 mg/ml) was added overnight at 50°C. Loading buffer (10 mM EDTA, 0.25% bromophenol blue, and 50% glycerol, 5mg) was added to the DNA preparations and electrophoresed on 1.5% agarose gel containing 0.3 mg/ml of ethidium bromide in TBE buffer (2 mM EDTA, 89 mM Tris, and 89 mM boric acid, pH 8.0) for 4 hrs. A HAE III digest of fX174-DNA was used as molecular weight size markers of 1.4, 1.1, 0.9, 0.6 and 0.3 kb respectively. Gels were photographed using UV transillumination.

2.8.4 Annexin V binding

Apoptosis was assessed by virtue of apoptotic cells ability to bind annexin V and exclude propidium iodide. Enterocytes were lifted with trypsin (0.05%). Cells were washed in cold PBS containing 10% FCS and resuspended in binding buffer (HEPES (10 mM)-buffered saline solution (150 mM NaCl, 5 mM KCl) supplemented with 1 mM $MgCl_2$ and 1.8 mM $CaCl_2$, pH 7.4) 10 μ l of fluorescein-conjugated annexin V (10 mg/ml) and 10 μ l of propidium iodide (50 mg/ml) reagent were added to 10^5 cells. This mixture was vortexed and incubated for 15 minutes in the dark at room temperature. 400 μ l of binding buffer is then added and analysed on a flow cytometer. A positive control for apoptosis was included in the

assay by incubating enterocytes for approximately 30 minutes before trypsinization with *E. coli* (10^7 bacteria). Likewise, a positive control for necrosis was included by similarly incubating enterocytes in a 10% ethanol solution.

2.9.1 Protein determination

Protein was measured using a Coomassie Protein Assay Reagent (Pierce) suitable for determining protein concentrations in the range from 1-25 mg/ml (Bradford, 1976) against a bovine serum albumin standard. Samples were read at an absorbance of 595 nm.

2.9.2 Measurement of NOS protein

Following incubation, PMNs were lysed in Lysis buffer (50 mM Tris-HCl, pH7.5, 150 mM NaCl, 5mM EDTA, 1% NP40, 0.5% SDS, 1 mM PMSF) for detection of iNOS protein. Samples of 10 µg/lane were electrophoresed and transferred to nitrocellulose overnight. Non-specific binding was blocked by incubation in TBST (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% (v/v) Tween 20) containing 5% (w/v) non-fat milk and washed in TBST. Blots were incubated for 1h with a murine immunoglobulin G (IgG) monoclonal anti-macrophage iNOS antibody at a 1:2500 dilution in TBST, washed in TBST and further incubated with an anti-murine IgG conjugated to alkaline phosphatase for 1h. Blots were developed using a chemiluminescence substrate and photographed.

2.10.1 Animals

Male sprague-dawley rats (Charler River, U.K.) weighing 280-320g were studied. They were housed in wire mesh cages in controlled conditions and given free access to standard lab chow. The experimental group was supplemented with taurine (4%) or HEPES (4%) (Sigma, Dorset, U.K.) in their drinking water for eight days prior to TNBS or saline administration and for a further three days following administration. All of the animal studies described in this report adhere to the standards established by the "Guide for the Care and Use of

2.10.2 Experimental model of ulcerative colitis

Experimental colitis was induced in rats by intrarectal administration of trinitrobenzenesulphonic acid (TNBS, 30 mg) in 50% ethanol (EtOH, 1 ml) as described (Kankuri et al., 1999). This was delivered through a Foley catheter inserted 7-8 cm into the anus. Control animals received 0.9% saline. Animals were anaesthetised by inhalational halothane (May & Parker Ltd., Dagenham, U.K.). Male Sprague-Dawley rats were pretreated with 4% of either taurine or HEPES in their drinking water for 8 days prior to induction of colitis or saline and for 3 days following administration. 3 days after administration of TNBS/EtOH, portal blood was cannulated from the inferior mesenteric vein (blood coming from the colon) and systemic blood taken from the superior vena cava to assess ROS activity and CD11b receptor expression. Tissue explants were taken from minimally inflamed and chronically inflamed areas of the colon to determine tissue injury, tissue NO production and MPO activity.

2.11.3 Tissue injury

Colonic inflammation was assessed by a blinded pathologist using both a colon macroscopic score (CMS) and colon microscopic score (CMiS). Formalin-fixed, 5 µm H&E stained sections of the colon were evaluated in a blinded fashion by a pathologist (5 or more longitudinal sections per colon).

<u>CMS</u>	<u>Gross morphology</u>
0	No damage
1	Localized hyperaemia
2	Ulceration without hyperaemia or bowel wall thickening
3	Ulceration with inflammation at one site

- | | |
|------|---|
| 4 | Two or more sites of ulceration/inflammation |
| 5 | Major sites of inflammation extending more than 1 cm along length of colon |
| 6-10 | If damage extends more than 2 cm along length of colon, score is increased by one for each additional 1 cm. |

CMiS

- | | |
|----|---|
| 0. | intact epithelium, no leucocytes or haemorrhage |
| 1. | < 25% disrupted epithelium, focal leucocytes infiltrates, and focal haemorrhage |
| 2. | 25% disrupted epithelium, focal leucocytes infiltrates and focal haemorrhage |
| 3. | ≤ 50% disrupted epithelium, wide-spread leucocytes, and haemorrhage; |
| 4. | >50% disrupted epithelium, extensive leucocyte infiltration, and haemorrhage. |

2.10.4 Organ culture

Colonic explants were placed in NaCl (0.9%) at 4⁰C and within 30 minutes were organ-cultured for 24 hours at 37⁰C, 95% O₂, 5% CO₂ in DMEM phenol red medium containing penicillin, streptomycin and augmentin.

2.11 Statistical analysis

Data is expressed as the mean value ± standard error of the mean (SEM) of at least 3 experiments (n values are indicated in figures). Statistical analysis was carried out using Analysis of Variance (ANOVA) with LSD post hoc tests for analysing three or more groups. Repeated Measures Analysis of Variance was utilized where repeated measurements were made between control and treated groups on the same subject's cells or cells from cell lines passaged at a specified passage number. A Mann-Whitney statistical test was carried out to

assess differences in tissue injury scores between groups. A p value of <0.05 was considered statistically significant.

2.12 Reagents

GIBCO Life Technologists Ltd., Longbridge, U.K.

Dulbecco's Modified Eagle Medium (DMEM), Minimal Essential Medium (MEM), penicillin and streptomycin, L-glutamine, non-essential amino acids, foetal calf serum (FCS)

SIGMA Chemical Co., Dorset, U.K.

Taurine, HEPES, Phorbol Myristate Acetate (PMA), fMLP, A23187, Superoxide dismutase (SOD), Ferricytochrome C, Sulphanilamide, N-(1-Naphthyl)ethylenediamine, Fluorescein isothiocyanate (FITC), NaCl, KCL, Na₂HPO₄, Glucose, CaCl₂, MgCl₂, NaHCO₃, Sodium deoxycholate, Dextran, Ficoll, Sulphosalicylic acid, Ethanol, Propidium iodide (PI), Sodium citrate, Tris, EDTA, Triton X-100, Agarose, N-laurylsarcosine, Proteinase K, RNase A, Bromophenol blue, Glycerol, Ethidium bromide, Boric acid, Actinomycin D, H₃PO₄, Wright Stain, Rat serum, Trypan blue

SEROTEC, Oxford, U.K.

Murine Mac-1 (CD11b) monoclonal antibody

BECTON DICKINSON, Mountain View, California, U.S.A.

FACScan flow cytometer, Lysing solution (Bursttest reagent), Polypropylene snap cap tubes, Human Mac-1 monoclonal antibody

AMERICAN TYPE CULTURE COLLECTION, Maryland Drive, U.S.A.

Caco-2 and HT-29 cell lines

GENZYME, Massachusetts, U.S.A.

Human recombinant Tumor Necrosis Factor- α and Interferon- γ

BOEHRINGER MANNHEIM, East Sussex, U.K.

HAE III

MOLECULAR PROBES, Oregon, U.S.A.

Flou-3, Dihydrorhodamine 123

CHAPTER III

THE ROLE OF TAURINE AND HEPES ON NEUTROPHIL FUNCTION *IN VITRO*

3.1 Introduction

Neutrophils (PMNs) play an important role in host defence against infectious agents but paradoxically, are also involved in the pathology of various inflammatory diseases. PMNs are the first phagocytes to be recruited to the inflammatory site. Activation of the β_2 -integrin, CD11b/CD18 on the surface of stimulated PMNs binds ICAM-1 on endothelial cells in postcapillary venules. Adhesion to endothelium primes PMNs for physiological activation in response to proinflammatory cytokines and other stimuli. This facilitates PMN migration through endothelial cell junctions which occurs under the influence of chemoattractants in a process called diapedesis. PMNs can then exert their microbicidal effects through a combination of both oxidative and enzymatic (non-oxidative) processes.

The oxidative or respiratory burst pathway is activated upon phagocytosis or when the pathway is triggered by an appropriate stimulus *in vitro*. This leads to the production of reactive oxygen species (ROS) and nitric oxide (NO) with the release of granule contents into the phagosome during degranulation. During the respiratory burst, superoxide (O_2^-) is formed initially by the reduction of molecular oxygen by NADPH oxidase. Upon PMN activation, two cytosolic proteins (p47-phox and p67-phox), a Rac-related GTP-binding protein translocate to the plasma membrane to form an active complex with the membrane bound-oxidase and cytochrome b558. More potent ROS are rapidly formed with O_2^- forming hydrogen peroxide (H_2O_2) by the action of superoxide dismutase (SOD). Hypochlorous acid (HOCl), a potentially cytotoxic agent (Eaton, 1993) is generated by myeloperoxidase (MPO). Inflammatory diseases are characterised by enhanced production of nitric oxide (NO). PMNs produce NO which is derived from the guanido nitrogen in the conversion of L-arginine to L-citrulline (Wright *et al.*, 1989). Recent evidence suggests that the balance between levels of ROS and NO is likely to be critical in determining the extent of inflammation-induced tissue injury (Beckman and Koppenol, 1996).

Distinct pathways of activation of a common NADPH oxidase exist upon activation by differing stimuli. The classical signal transduction pathway is activated upon binding of a chemoattractant to its receptor, coupling to G proteins/phospholipase C (PLC) and generation

of two second messengers, inositol 1,4,5-triphosphate (IP₃) and diacylglycerol (DAG). IP₃ leads to a transient rise in cytosolic free calcium, [Ca²⁺]_i (Pittet *et al.*, 1992) whereas DAG remains associated with the plasma membrane and participates in the activation of protein kinase C (PKC) (Farago *et al.*, 1990). Stimulation of the inflammatory response also activates phospholipase A₂ (PLA₂) resulting in release of free arachidonic acid from membrane phospholipids generating prostaglandins and leukotrienes. Leukotriene B₄ (LTB₄) is a potent proinflammatory mediator inducing leukocyte migration and activation (Ford-Hutchinson *et al.*, 1980). In contrast, soluble stimuli such as calcium ionophores and phorbol esters bypass signal transduction events at the plasma membrane and directly increase [Ca²⁺]_i or activate PKC.

Taurine, the most abundant free amino acid in human inflammatory cells (Fukuda *et al.*, 1982) scavenges the most destructive agent synthesized by activated PMNs, HOCl forming taurinechloramine (Lambert and Weiss, 1983). This compound maintains bactericidal activity but is far less toxic to surrounding tissues (Thomas, 1979). Structural analogues of taurine such as HEPES also have the capacity to form chloramines (Stapleton *et al.*, 1993) with HEPES being retained in the circulation for longer periods of time than taurine (Stapleton *et al.*, 1994). Taurine and HEPES possess anti-inflammatory properties both *in vitro* and *in vivo* (Stapleton *et al.*, 1993, Stapleton *et al.*, 1994). Taurine attenuates oxidative cellular damage in chemically-induced models of inflammation which were attributed to an inhibition of ROS and NO (Redmond *et al.*, 1996) and modulation of arachadonic acid metabolites (Son *et al.*, 1998). It was demonstrated that taurine reduced the production of ROS and lowered LTB₄ levels in stimulated PMNs *in vitro* through its effects on the MPO-H₂O₂-halide system (Mc Loughlin *et al.*, 1991). However, there are few reports of the direct effects of taurine and HEPES on the respiratory burst pathway *in vitro* and particularly, the signalling pathways triggering activation of NADPH oxidase.

3.2 Aims

It has been well established that taurine possesses calcium-modulatory effects (Huxtable *et al.*, 1992). As an increase in $[Ca^{2+}]_i$ is one of the triggers for activation of NADPH oxidase, it was hypothesized that taurine and its structural analogue, HEPES may function to modulate ROS by affecting activation of NADPH oxidase through effects on Ca^{2+} . The effect of taurine and HEPES on the subsequent release of ROS, NO and the production of cytotoxic mediators by the PMN was assessed.

3.3 Results

3.3.1 Setting up experimental protocol

The effects of taurine and HEPES on PMN function were investigated in PMNs activated with either the particulate stimuli, human opsonised *E. coli* or fMLP or soluble stimuli such as a calcium ionophore, A23187 or a phorbol ester, PMA. Initially, PMNs were stimulated with the membrane bound stimulus, *E. coli* or the soluble stimulus, PMA. However, neither of these stimuli significantly upregulated intracellular calcium, $[Ca^{2+}]_i$. For the remainder of the experiments, fMLP was used as a particulate stimulus and A23187 as a soluble stimulus, both of which significantly upregulated $[Ca^{2+}]_i$. PMNs were preincubated with taurine (0.25, 0.5, 1 mg/ml equivalent to 2, 4, 8 mM) and HEPES (0.25, 0.5, 1 mg/ml equivalent to 0.9375, 1.875, 3.75 mM) for 1h as maximal effects on respiratory burst activity (as described in section 3.3.3) were seen at this time point.

3.3.2 Effect of Taurine and HEPES on PMN adhesion

The PMN receptor responsible for the recognition of opsonised particles is CD11b. PMN CD11b receptor expression was assessed after stimulation with both a particulate stimulus, heat killed human opsonized *E. Coli* and a soluble stimulus, phorbol 12-myristate acetate (PMA). PMNs (1×10^6 cells/ml) were preincubated with taurine and HEPES (0.25, 0.5 and 1 mg/ml) for 1 hour prior to stimulation. Both stimuli significantly upregulated PMN CD11b receptor expression compared to unstimulated PMNs (109.61 ± 14.0 mean channel fluorescence (MCF)) with receptor expression higher in PMA-stimulated PMNs (342.03 ± 24.1 MCF) than upon stimulation with opsonised *E. coli* (208.74 ± 42.0 MCF) (Table 3.1). Taurine and HEPES had no effect on CD11b receptor expression in resting PMNs or upon stimulation with the internal stimulus, PMA (Table 3.1). Stimulation with opsonised *E. Coli* resulted in a significant ($p < 0.001$) increase in CD11b receptor expression in the presence of taurine and HEPES at all concentrations (Table 3.1).

PMN CD11b Receptor Expression (MCF)			
Inducing agents	NONE	PMA	Opsonised <i>E. Coli</i>
Spontaneous	109.61 ± 14.0	342.03 ± 24.1	208.74 ± 42.0
+ Taurine (0.25 mg/ml)	112.56 ± 11.1	357.72 ± 19.6	263.92 ± 11.0*
+ Taurine (0.5 mg/ml)	107.94 ± 16.2	323.25 ± 30.5	281.83 ± 16.9*
+ Taurine (1 mg/ml)	105.58 ± 15.4	325.77 ± 31.2	256.92 ± 33.3*
+ HEPES (0.25 mg/ml)	110.64 ± 11.2	317.79 ± 24.2	279.95 ± 31.1*
+ HEPES (0.5 mg/ml)	102.70 ± 12.1	337.57 ± 33.4	245.90 ± 22.7*
+ HEPES (1 mg/ml)	108.52 ± 16.5	321.75 ± 18.6	237.02 ± 19.8*

Table 3.1 The effect of Taurine and HEPES on CD11b Receptor Expression in resting and activated PMNs. PMNs (1×10^6 cells/ml) were preincubated with varying concentrations (as shown) of taurine or HEPES for 1 h before activation with a soluble stimulus, PMA or a receptor agonist, opsonised *E. Coli*. CD11b Receptor expression was assessed flow cytometrically using a MAC 1 anti-human antibody. Data is expressed as mean channel fluorescence (MCF) ± SEM of 3 experiments, * $p < 0.001$ vs spontaneous (Repeated Measures Analysis of Variance)

3.3.3 Taurine and HEPES modulate overall oxidative stress

Respiratory burst activity was assessed using the fluorogenic substrate, Dihydrorhodamine 123 (DHR 123) which is a measure of overall oxidative stress. PMN respiratory burst activity was assessed after stimulation with the membrane-bound stimulus, *E. coli* or the soluble stimulus, PMA. PMNs (1×10^6 cells/ml) were preincubated with taurine and HEPES (0.25, 0.5 and 1 mg/ml) for 1 hour prior to stimulation. Both stimuli significantly increased respiratory burst activity compared to unstimulated cells (<10 MCF) with PMA (791.22 ± 62.3 MCF) inducing a larger burst than opsonised *E. coli* (229.62 ± 85.8 MCF). Taurine (1 mg/ml) and HEPES (0.5 and 1 mg/ml) significantly ($p < 0.05$) decreased *E. coli*-activated respiratory burst activity (Figure 3.1a). However, HEPES (0.5 and 1 mg/ml) significantly ($p < 0.01$) increased PMA-stimulated respiratory burst activity (Figure 3.1b).

3.3.4 Taurine and HEPES have differential effects on NADPH oxidase

In order to assess if taurine and HEPES modulate respiratory burst activity at the initial step in the respiratory burst pathway, the production of O_2^- by NADPH oxidase was measured by the SOD-inhibitable reduction of ferricytochrome c. PMNs (1×10^6 cells/ml) were preincubated with taurine and HEPES (0.25, 0.5 and 1 mg/ml) for 1 hour prior to stimulation with both a particulate stimulus, n-formyl-methionyl-leucyl-phenylalanine (fMLP) and two soluble stimuli, a phorbol ester, PMA or a calcium ionophore, A23187. Figure 3.2a illustrates the effect of taurine and HEPES on fMLP-stimulated NADPH oxidase activity. HEPES (1 mg/ml) significantly ($p < 0.05$) decreased fMLP-activated NADPH oxidase activity with taurine having no significant effect. Upon stimulation with soluble stimuli, taurine and HEPES significantly ($p < 0.05$) increased PMA-induced (Figure 3.2b) and A23187-stimulated NADPH oxidase activity (Figure 3.2c).

3.3.5 Effect of taurine and HEPES on intracellular Calcium, $[Ca^{2+}]_i$

The mechanism through which taurine and HEPES modulates NADPH oxidase may be through its calcium modulatory effects. Intracellular calcium, $[Ca^{2+}]_i$ was assessed flow cytometrically in suspensions of human PMNs loaded with the fluorescent Ca^{2+} indicator

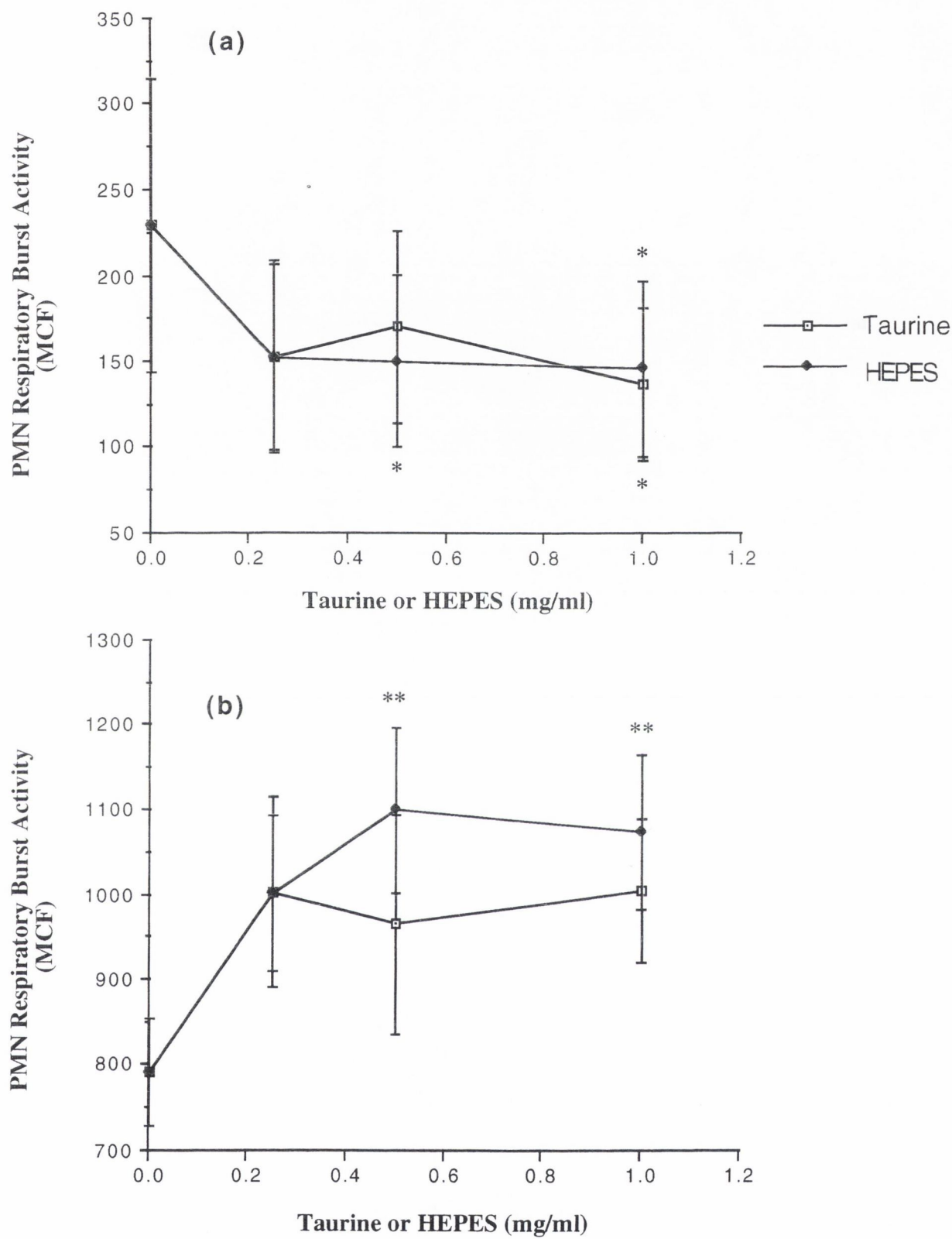


Figure 3.1 The effect of taurine and HEPES on PMN respiratory burst activity. PMNs (1×10^6 cells/ml) were preincubated with varying concentrations of taurine and HEPES for 1h before activation with (a) a particulate stimulus, human opsonised *E. Coli.* or (b) a soluble stimulus, PMA. Respiratory burst activity was assessed flow cytometrically by the oxidation of dihydrorhodamine 123. Data are expressed as the mean $\pm$ SEM of 6 experiments, * $p < 0.05$, ** $p < 0.01$ vs stimulus alone (Repeated Measures Analysis of Variance).

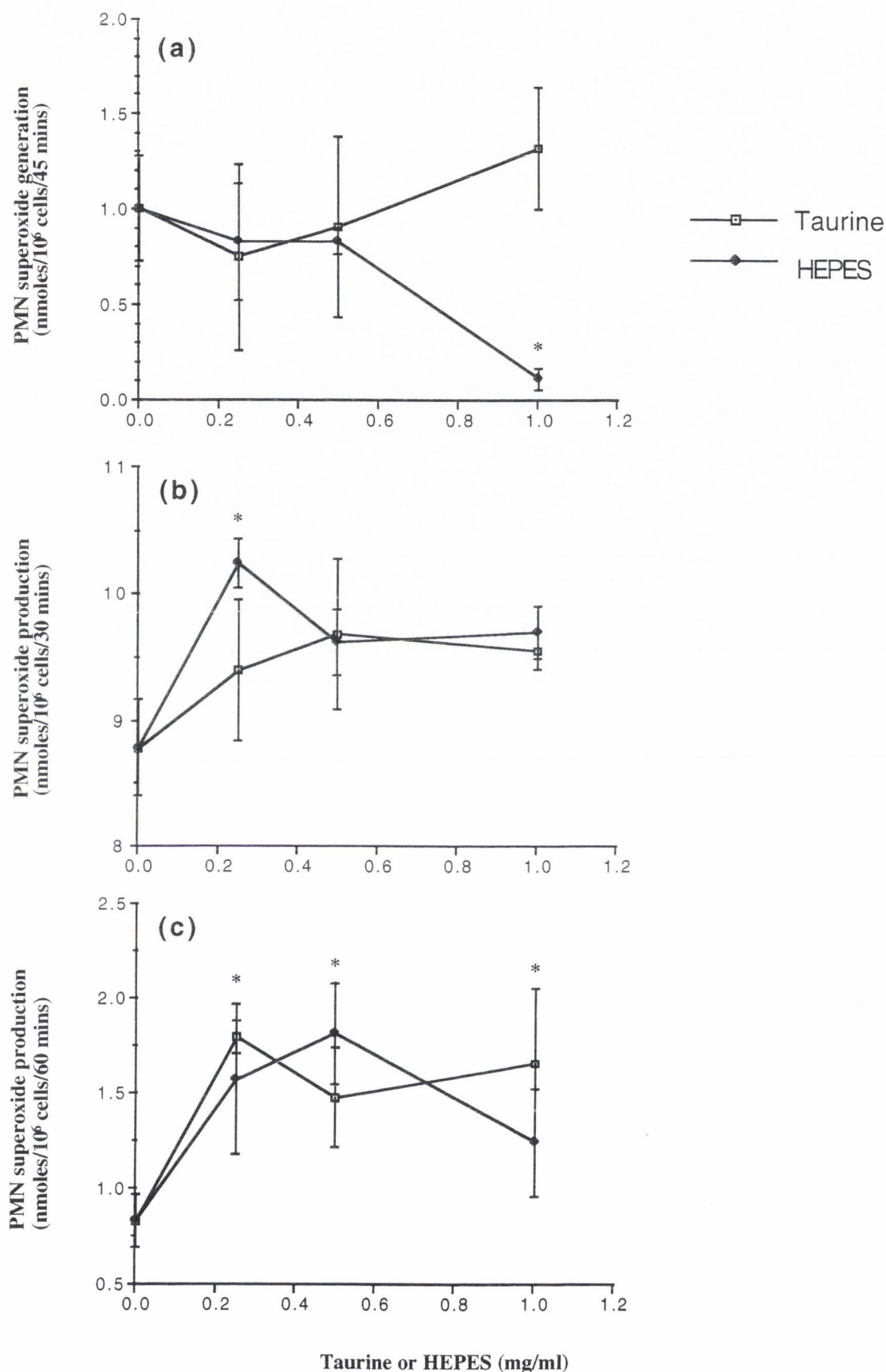


Figure 3.2 The effect of taurine and HEPES on PMN NADPH oxidase activity. NADPH oxidase was assessed indirectly through the SOD-inhibitable reduction of ferricytochrome c in PMNs (1×10^6 cells/ml) preincubated with varying concentrations of taurine and HEPES upon activation with (a) a particulate stimulus, fMLP or the soluble stimuli, (b) the phorbol ester, PMA or (c) the calcium ionophore, A23187. Data are expressed as the mean $\pm$ SEM of 3 experiments in triplicate, * $p < 0.05$ vs stimulus alone (Repeated Measures Analysis of Variance)

dye, Fluo-3. The fMLP-induced increase in $[Ca^{2+}]_i$ consists of two phases which is illustrated in Figure 3.3. Addition of fMLP causes an immediate rapid increase in $[Ca^{2+}]_i$ which is released from intracellular stores (within the first 30 seconds) and a slower influx of Ca^{2+} lasting up to several minutes. $[Ca^{2+}]_i$ declined significantly ($p < 0.05$) faster in fMLP-stimulated PMNs pretreated with taurine (0.25 mg/ml) (Figure 3.3a). The calcium ionophore, A23187 was used to assess the effects of taurine and HEPES on $[Ca^{2+}]_i$ independent of receptor stimulation. A23187 induces a steady increase in $[Ca^{2+}]_i$ which lasts for up to 10 minutes (Figure 3.3) due to an influx of extracellular Ca^{2+} . Taurine and HEPES had no significant effect on A23187-upregulated $[Ca^{2+}]_i$ (Figure 3.3b).

3.3.6 Taurine increases PMN myeloperoxidase (MPO) activity.

It has been previously demonstrated *in vitro* that ROS may be reduced by manipulation of the MPO- H_2O_2 -halide system upon addition of taurine and HEPES (Mc Loughlin *et al.*, 1991). A significant increase in PMN A23187-stimulated MPO activity was observed at all concentrations of HEPES and taurine (0.25, 0.5 mg/ml) (Figure 3.4).

3.3.7 Taurine and HEPES increase fMLP-stimulated PMN nitrite production

The constitutive form of nitric oxide synthase (cNOS) has been purified in human PMNs (Bryant *et al.*, 1992). Taurine (1 mg/ml) increased NO production significantly ($p < 0.05$) in resting PMNs (Table 4.1, Chapter 4). PMNs were stimulated with fMLP (1 nM) in the presence of taurine and HEPES for 1 hour to examine expression of NO through cNOS. HEPES (0.25 mg/ml) significantly ($p < 0.01$) increased PMN nitrite production from 92.59 ± 13.35 pmoles/1h/5 x 10^6 to 266.67 ± 65.1 pmoles/1h/5 x 10^6 cells in fMLP-activated PMNs (Figure 3.5). Taurine increased fMLP-stimulated nitrite production non-significantly (Figure 3.5).

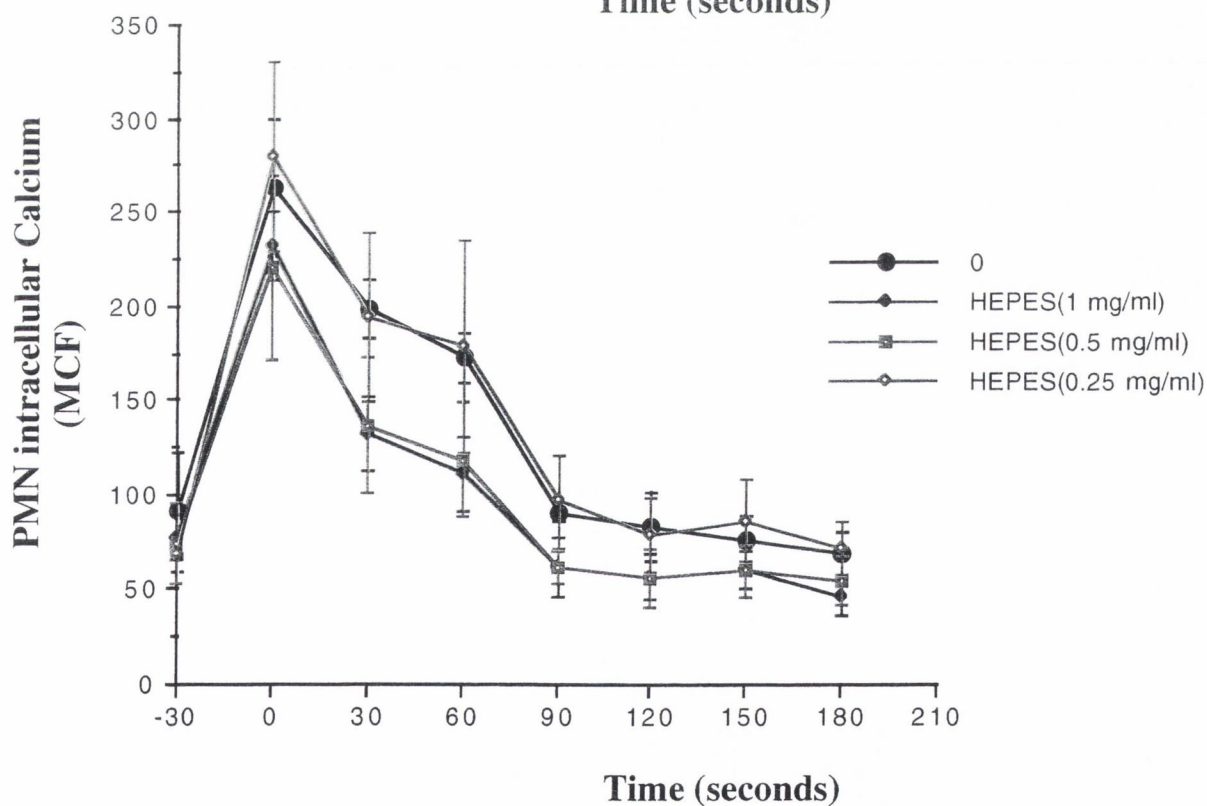
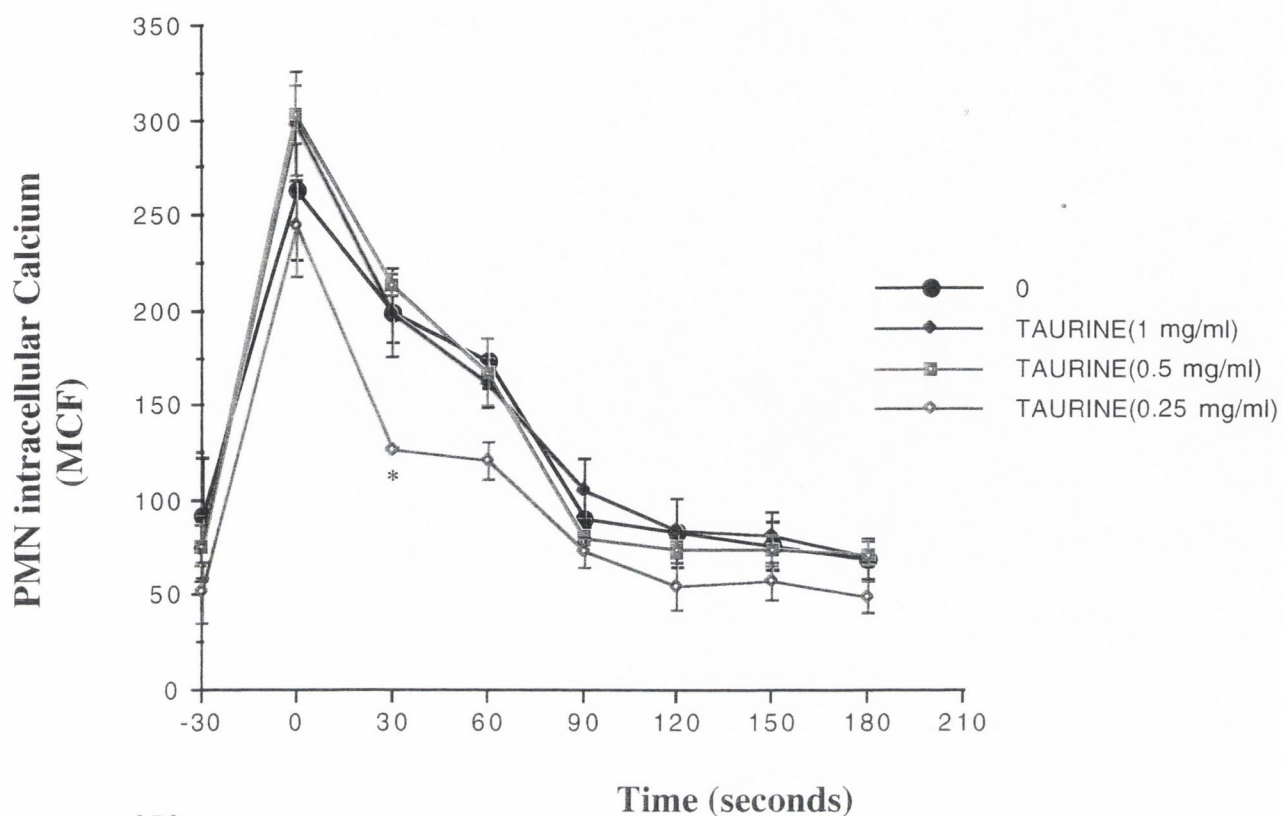


Figure 3.3a The effect of taurine and HEPES on intracellular calcium, $[Ca^{2+}]_i$ upon stimulation by the receptor agonist, fMLP. PMNs preincubated with taurine or HEPES (1 hour) were loaded with Fluo-3 before addition of fMLP and $[Ca^{2+}]_i$ assessed flow cytometrically over time. Data are expressed as the mean channel fluorescence $\pm$ SEM of 3 individual experiments, * $p < 0.05$ vs stimulus alone (MANOVA).

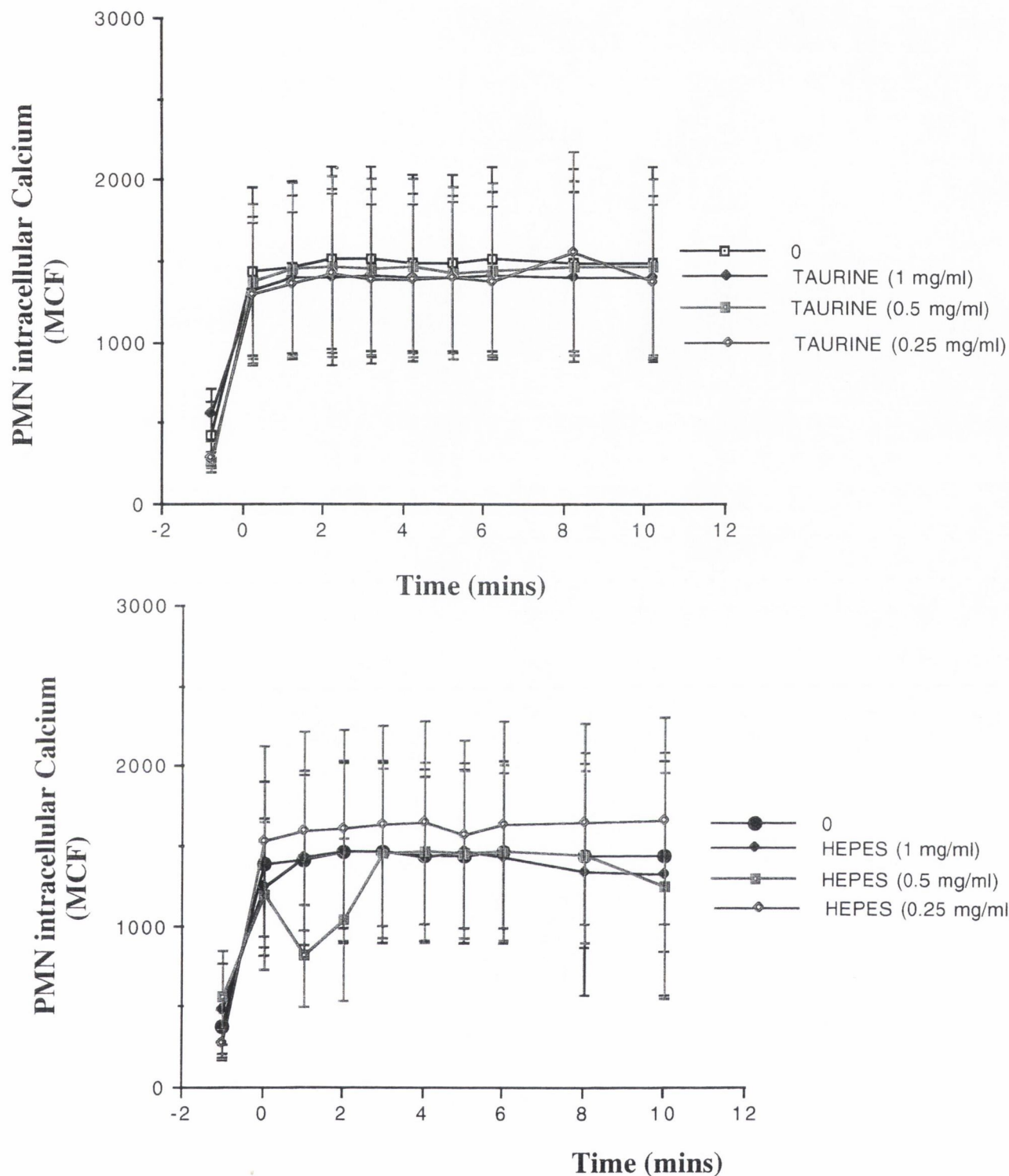


Figure 3.3b The effect of taurine and HEPES on intracellular calcium, $[Ca^{2+}]_i$ upon stimulation by the calcium ionophore, A23187. PMNs preincubated with taurine or HEPES (1 hour) were loaded with Fluo-3 before addition of A23187 and $[Ca^{2+}]_i$ assessed flow cytometrically over time. Data are expressed as the mean channel fluorescence $\pm$ SEM of 4 individual experiments.

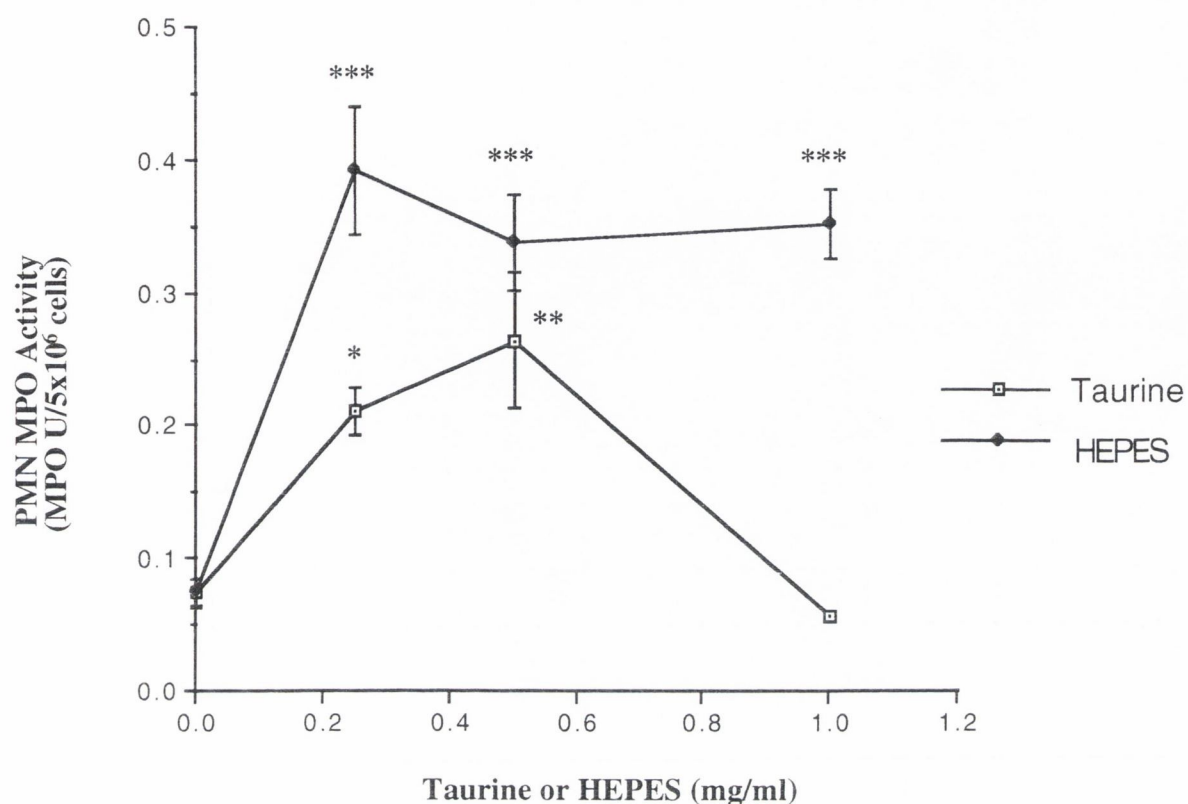


Figure 3.4 The effect of taurine and HEPES on MPO activity in A23187-stimulated PMNs. PMNs (5×10^6 cells/ml) were preincubated with varying concentrations of taurine and HEPES for 1h before activation with A23187. MPO activity was measured in the supernatant of lysed PMNs by the o-dianisidine method. Data are expressed as the mean $\pm$ SEM of 3 experiments in triplicate, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs stimulus alone (Repeated Measures Analysis of Variance).

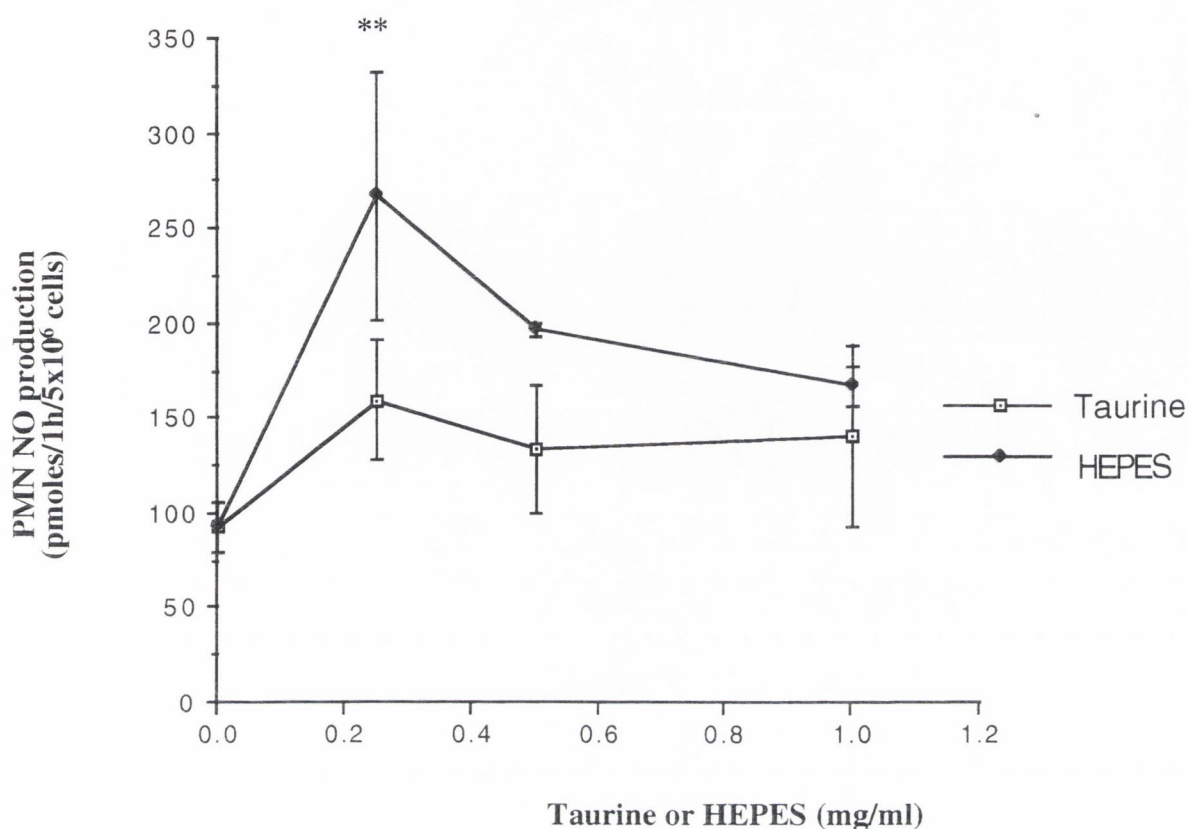


Figure 3.5 The effect of taurine and HEPES on NO production in fMLP-stimulated PMNs. PMNs (5×10^6 cells/ml) were incubated with varying concentrations of taurine and HEPES in the presence of fMLP (1 nM) for 1 hour. NO production was measured as nitrite accumulation in the cell supernatant using the griess assay. Data are expressed as the mean $\pm$ SEM of 3 experiments in triplicate, ** $p < 0.01$ vs stimulus alone (Repeated Measures Analysis of Variance).

3.3.8 The effects of taurine and HEPES on arachidonic acid metabolism

Prostaglandin E₂ (PGE₂) and leukotriene B₄ (LTB₄) are primary products of arachidonic acid metabolism which are released upon PMN degranulation. PGE₂ and LTB₄ were assessed in supernatants of fMLP and A23187-stimulated PMNs cultured with taurine or HEPES using enzyme immunoassay kits. Taurine or HEPES did not significantly affect fMLP- or A23187-stimulated PGE₂ production (Table 3.3). Taurine decreased non-significantly LTB₄ production from 1591.5 ± 1313 pg/10⁶ cells in cells stimulated with fMLP alone to 262.5 ± 68 pg/10⁶ cells in PMNs cultured with taurine (1 mg/ml) (Table 3.4).

3.3.9 Taurine and HEPES have no effect on PMN degranulation

Extracellular degranulation of the lysosomal enzyme, β -glucuronidase was measured in the supernatants of A23187- and fMLP-stimulated PMNs. Both fMLP and A23187 increased β -glucuronidase release with A23187 being more effective than fMLP. Taurine and HEPES had no significant effect on PMN release of β -glucuronidase upon stimulation with either fMLP or A23187 (Table 3.4).

PMN PGE ₂ PRODUCTION (pg/10 ⁶ cells)		
Inducing agents	fMLP	A23187
Spontaneous	474.27 ± 404.5	272.44 ± 65.0
+ Taurine (0.25 mg/ml)	355.83 ± 280.5	437.42 ± 176.3
+ Taurine (0.5 mg/ml)	207.75 ± 144.8	147.38 ± 84.0
+ Taurine (1 mg/ml)	223.28 ± 147.8	132.31 ± 45.0
+ HEPES (0.25 mg/ml)	130.35 ± 43.71	202.20 ± 24.4
+ HEPES (0.5 mg/ml)	413.68 ± 288.2	229.45 ± 18.5
+ HEPES (1 mg/ml)	259.96 ± 121.53	162.27 ± 25.5

Table 3.2 The effect of Taurine and HEPES on PGE₂ production in activated PMNs. PMNs (1 x 10⁶ cells/ml) were preincubated with varying concentrations (as shown) of taurine or HEPES for 1 h before activation with a calcium ionophore, A23187 or a receptor agonist, fMLP. PGE₂ production was measured using a standard commercially available kit. Data is expressed as mean ± SEM of 3 experiments.

PMN LTB ₄ PRODUCTION (pg/10 ⁶ cells)		
Inducing agents	fMLP	A23187
Spontaneous	1591.5 ± 1313	16362 ± 2074
+ Taurine (0.25 mg/ml)	757.2 ± 408	23830 ± 4090
+ Taurine (0.5 mg/ml)	362.0 ± 45	24899 ± 4145
+ Taurine (1 mg/ml)	262.5 ± 68	11915 ± 6648
+ HEPES (0.25 mg/ml)	883.7 ± 265	24236 ± 19877
+ HEPES (0.5 mg/ml)	881.7 ± 292	37127 ± 29047
+ HEPES (1 mg/ml)	1266.2 ± 604	33255 ± 10497

Table 3.3 The effect of Taurine and HEPES on LTB₄ production in activated PMNs. PMNs (1 x 10⁶ cells/ml) were preincubated with varying concentrations (as shown) of taurine or HEPES for 1 h before activation with a calcium ionophore, A23187 or a receptor agonist, fMLP. LTB₄ production was measured using a standard commercially available kit. Data is expressed as mean ± SEM of 3 experiments.

PMN β -Glucuronidase release (pmoles/ 10^6 cells/2h)		
Inducing agents	fMLP	A23187
NONE	2.07 $\pm$ 1.7	
Stimulus alone	19.30 $\pm$ 3.5	40.00 $\pm$ 6.5
+ Taurine (0.25 mg/ml)	18.05 $\pm$ 2.3	37.93 $\pm$ 8.1
+ Taurine (0.5 mg/ml)	18.56 $\pm$ 2.5	41.87 $\pm$ 1.4
+ Taurine (1 mg/ml)	17.48 $\pm$ 1.4	36.58 $\pm$ 1.8
+ HEPES (0.25 mg/ml)	16.65 $\pm$ 4.8	40.03 $\pm$ 9.8
+ HEPES (0.5 mg/ml)	19.58 $\pm$ 2.0	34.12 $\pm$ 14.9
+ HEPES (1 mg/ml)	17.44 $\pm$ 16.5	37.57 $\pm$ 3.8

Table 3.4 Taurine and HEPES has no effect on PMN degranulation. PMNs (1×10^6 cells/ml) were preincubated with varying concentrations (as shown) of taurine or HEPES for 1 h before activation with a calcium ionophore, A23187 or a receptor agonist, fMLP. PMN degranulation was measured by the release of β -glucuronidase into the supernatant. Data is expressed as mean $\pm$ SEM of 3 experiments in triplicate.

3.4 Discussion

Influx of activated PMNs across the endothelium is central to initiation and perpetuation of the inflammatory response. PMN migration occurs by attachment and rolling of PMNs along blood vessels, subsequent activation and transendothelial migration which is mediated by the $\beta 2$ integrin, CD11b/CD18. PMN CD11b/CD18 binds ICAM-1 on activated endothelial cells and is activated *in vitro* upon ligation of PMN receptors by opsonised particles. Stimulation of PMNs with both PMA or opsonised *E. Coli* induced a significant upregulation of CD11b with receptor expression higher in PMNs stimulated with PMA than opsonised *E. Coli*, suggesting different activation pathways. There are two different pathways of activation of CD11b dependent upon activation with PKC activators or chemoattractants (Merrill *et al.*, 1990). PMA-activated CD11b receptor expression is dependent upon phosphorylation of the CD18 β -chain by a PKC-dependent pathway (Merrill *et al.*, 1990). Taurine and HEPES had no effect on resting or PMA-induced CD11b expression indicating that taurine and its analogues do not affect CD11b/CD18 receptor expression through PKC. However, taurine and HEPES served to increase opsonised *E. Coli*-induced CD11b receptor expression. This was an unexpected finding as research from our laboratory had previously demonstrated that taurine has the capacity to decrease PMN influx across the pulmonary vasculature in animal inflammatory models (Barry *et al.*, 1995). *E. Coli* opsonised with serum components (IgG, C3b) interact with three kinds of transmembrane receptors in human PMNs: the IgG receptors, Fc γ R11 and Fc γ R111 and the receptor for C3b (CR1). In addition, PMNs undergoing CD11b/CD18 integrin-dependent adhesion show repeated elevations in $[Ca^{2+}]_i$ (Pettitt *et al.*, 1997). Stimulation by serum-opsonised bacteria may induce changes in $[Ca^{2+}]_i$ dependent on the strain of bacteria used (Ruotsalainen *et al.*, 1995). Experiments from our lab indicate that human opsonised *E. Coli* could not significantly upregulate intracellular Ca^{2+} . Taurine has the ability to increase Ca^{2+} availability under conditions of low Ca^{2+} availability (Huxtable, 1992). Taurine and its analogue may increase the release from intracellular stores upon stimulation by opsonins accounting for increased surface expression of CD11b/CD18. Thus, these results suggest that taurine and HEPES modulate PMN adhesion through a

calcium dependent mechanism without affecting PKC-dependent phosphorylation of the CD11b/CD18 receptor.

In this study, Taurine and HEPES exhibited differential effects on PMN respiratory burst activity dependent upon stimulation by receptor-dependent or receptor-independent stimuli. It was observed that there was a significant reduction in overall PMN oxidative stress by taurine and its substituted analogue, HEPES upon stimulation by human opsonised *E.Coli.*. Taurine and HEPES inhibited the production of O_2^- in response to the receptor agonist, fMLP and attenuated fMLP-stimulated rises in cytosolic free Ca^{2+} concentration. These results suggest that taurine and HEPES decrease fmlp-induced NADPH oxidase activity through a modulation of calcium fluxes. Receptor-dependent stimuli such as the chemoattractant, fMLP bind to specific cell surface receptors on human PMNs initiating a variety of biological responses including NADPH oxidase activation. G proteins, or heterotrimeric GTP-binding proteins are coupled to chemoattractant receptors and phospholipase C (PLC) within the plasma membrane. fMLP receptor occupancy leads to PLC-mediated hydrolysis of polyphosphoinositol 4,5-bisphosphate (PIP_2) yielding 1,4,5-triphosphate (IP_3) and 1,2 sn-diacylglycerol (DAG). IP_3 stimulates the discharge of Ca^{2+} from intracellular stores resulting in a rapid rise in $[Ca^{2+}]_i$. The emptying of intracellular Ca^{2+} stores leads to the influx of Ca^{2+} across the plasma membrane. The first peak of fMLP-induced increases in $[Ca^{2+}]_i$ is a sum of Ca^{2+} release from intracellular stores and the early influx of Ca^{2+} from the extracellular milieu (Hallett *et al.*, 1990, Davies *et al.*, 1991). The second rise in $[Ca^{2+}]_i$ induced by fMLP is due to an influx of extracellular Ca^{2+} (Hallett *et al.*, 1990, Davies *et al.*, 1991). The first phase of this rapid, intracellular calcium response in human PMNs induced by fMLP was unchanged by taurine indicating that calcium mobilization from intracellular stores may be unaffected by taurine. It is worth noting however, that the release of Ca^{2+} from internal stores occurs within seconds (Sage *et al.*, 1990). Therefore, the effects of taurine or HEPES on calcium mobilization from intracellular stores may have been masked by the time dependence of this measurement. However, the fmlp-evoked calcium response declined significantly faster in PMNs pre-incubated with

taurine and HEPES. This indicates that a second phase of the calcium response in PMNs was inhibited by taurine and HEPES. Thus, taurine and HEPES affect receptor-mediated influx of Ca^{2+} from the extracellular environment and decrease ROS.

In contrast, responses to receptor-independent agonists, PMA and calcium ionophore, A23187 were enhanced by taurine and HEPES. PMA-stimulated respiratory burst activity was enhanced by PMNs cultured with taurine or HEPES. In addition, treatment with taurine or HEPES increased both PMA- and A23187-activated PMN O_2^- production. In the presence of taurine or HEPES, there was enhanced metabolism through the MPO- H_2O_2 -halide system in response to the pertussis toxin resistant stimulus, A23187. The mechanism through which taurine and its substituted analogue, HEPES may increase A23187- and PMA-activated NADPH oxidase activity are unclear. Receptor-independent stimuli such as phorbol esters (PMA) and ionophores (A23187) bypass G protein/PLC coupling at the plasma membrane to directly activate PKC (PMA) or increase $[\text{Ca}^{2+}]_i$ influx from the extracellular environment (A23187). Taurine may modulate respiratory burst activity through effects on PKC and/or effects on calcium processes. Alternatively, taurine may affect kinases such as a Ca^{2+} -PLC-independent kinase identified in cytosolic fractions from PMA-stimulated cells (Melloni *et al.*, 1985). This, however is unlikely as most effects of taurine occur through calcium mobilization. In addition, taurine is unlikely to directly affect PKC but there is evidence that taurine inhibits phosphorylation of some proteins by PKC (Li and Lombardini, 1991, Lombardini, 1985). Experiments on the effects of taurine and HEPES on $[\text{Ca}^{2+}]_i$ by PMA were not carried out as PMA either does not affect $[\text{Ca}^{2+}]_i$ at resting Ca^{2+} levels (Sharma *et al.*, 1991) or is known to stimulate an efflux of Ca^{2+} from PMNs where there are elevated intracellular Ca^{2+} levels (McCarthy *et al.*, 1989). Research by Kramer has demonstrated that taurine affects cell metabolism through a calcium biphasic effect that depends on calcium concentration (Kramer *et al.*, 1981). It is possible that taurine may increase $[\text{Ca}^{2+}]_i$ in the PMN upon stimulation by ionophores which raise $[\text{Ca}^{2+}]_i$ or perhaps, prevent PMA-induced Ca^{2+} efflux from the PMN. Results found in this study suggest that the effects of taurine and HEPES on A23187-stimulated respiratory burst

activity are independent of changes in intracellular calcium levels. Taurine and HEPES did not significantly affect A23187-stimulated increases in $[Ca^{2+}]_i$ but HEPES (0.25 mg/ml) non-significantly increased $[Ca^{2+}]_i$ (Fig. 3.3b). However, there are a number of reports indicating that taurine stimulates ATP-dependent calcium uptake in photoreceptor cells and excitable tissues (Kuo *et al.*, 1980, Kramer *et al.*, 1981). Therefore, taurine and HEPES may increase respiratory burst activity through increases in $[Ca^{2+}]_i$ thereby further activating NADPH oxidase. The biphasic effects of taurine and HEPES on Ca^{2+} may explain the stimulatory effects of these compounds on the respiratory burst pathway upon activation by non-receptor mediated agonists.

Unpublished reports from our laboratory demonstrated that taurine increase cNOS and NO production in human umbilical vein endothelial cells. PMNs produce NO which is derived from the guanido nitrogen in the conversion of L-arginine to L-citrulline catalysed by nitric oxide synthase (NOS). The constitutive form of NOS (cNOS) has been purified and cloned from human PMNs (Bryant *et al.*, 1992). This enzyme is regulated by Ca^{2+} but unlike the constitutive form found in the brain is independent of calmodulin (Yui *et al.*, 1991). We confirmed reports that NO production in human PMNs could be stimulated by low concentrations of fMLP (Schmidt *et al.*, 1989, Mc Call *et al.*, 1989). Taurine and HEPES increased NO production in resting cells and upon stimulation for one hour by fMLP. This production of NO is most likely due to the activity of cNOS as induction of cNOS does not require new protein synthesis and produces NO continually. Thus, these results suggest that taurine and its analogue, HEPES increase NO production through cNOS in human PMNs upon stimulation by the receptor agonist, fMLP. It has been demonstrated recently that the Ca^{2+} content of the internal stores regulate cNOS activity (Xu *et al.*, 1994). Although taurine and HEPES inhibit extracellular Ca^{2+} influx upon activation by fMLP, these compounds may increase receptor-agonist evoked Ca^{2+} from intracellular stores with subsequent increases in NO production through cNOS. Increased production of NO may downregulate the inflammatory response as constitutively-derived NO has been shown to inhibit leukocyte adhesion (Kubes *et al.*, 1991) and NO donors inhibit PMN function (Moilanen *et al.*, 1993).

Synthesis of eicosanoids is initiated by activation of phospholipase A₂ (PLA₂) stimulating lipoxygenase and cyclooxygenase pathways. Leukotriene B₄ (LTB₄) is a potent chemotactic agent synthesized by activated PMNs, elevated levels of which have been implicated in the pathogenesis of inflammatory diseases (Wallace *et al.*, 1989, Brain *et al.*, 1984). Taurine decreased non-significantly LTB₄ and PGE₂ production in response to the receptor-mediated peptide, fMLP. However, findings by Mc Loughlin *et al.* found a significant reduction in LTB₄ levels in PMN supernatants preincubated with taurine or HEPES (Mc Loughlin *et al.*, 1991). It has been demonstrated that LTB₄ can conjugate with taurine forming tauro-18-carboxy-19,20-dinor-LTB₄ (Shirley *et al.*, 1990) and a prostaglandin analogue, trimoprostil can be metabolized into taurine conjugates (Kolios *et al.*, 1986). The formation of these metabolites may provide an important pathway for inactivation and elimination of leukotrienes and prostaglandins by taurine and HEPES.

PMNs release their granule contents into phagolysosomes in an oxygen-independent fashion. Pharmacologic inhibition of secretion of toxic oxidants and proteases may limit PMN-mediated tissue damage. Taurine and HEPES had no effect on PMN degranulation suggesting that taurine and HEPES modulate the inflammatory response through direct effects on PMN adhesion and respiratory burst activity.

Most of the affects of taurine and HEPES closely relate to the modulation of calcium ions through cell membranes or the binding of calcium ions to membranes. Two distinct Ca²⁺ storage and release sites have been identified in PMNs, one below the plasma membrane and the other at the centre of the PMN possibly in calciosomes (Pettitt *et al.*, 1998). Increases in PMN [Ca²⁺]_i consist of two components, an immediate increase in [Ca²⁺]_i from intracellular stores and a more delayed response which is dependent on extracellular Ca²⁺. The function of the intracellular Ca²⁺ store depends on a Ca²⁺ pump with similarities to the cardiac reticulum Ca²⁺-ATPase, a Ca²⁺-storage protein and a Ca²⁺ release channel which is sensitive to IP₃ (reviewed by Krause *et al.*, 1990). The Ca²⁺ regulatory function of the plasma membrane depends on a Ca²⁺ pump similar to the erythrocyte-type Ca²⁺-ATPase and a Ca²⁺ channel, the activity of which is closely coupled

to PI turnover (reviewed by Krause *et al.*, 1990). The exact mechanism through which taurine directly affects any of these processes in modulating PMN $[Ca^{2+}]_i$ is unclear. Our results indicate that upon stimulation by receptor mediated stimuli, taurine and HEPES prevent the extracellular influx of $[Ca^{2+}]_i$ through calcium channels and although not demonstrated in this study, these compounds may increase the release of Ca^{2+} from IP_3 -sensitive intracellular calcium stores. Additionally, taurine and HEPES may increase the influx of extracellular calcium upon stimulation by non-receptor agonists. Research by Huxtable shows that taurine may not directly affect the transport of Ca^{2+} through Ca^{2+} channels but rather may modify the binding of Ca^{2+} to biological membranes (Huxtable, 1990). Ca^{2+} is released into the cytosol from extracellular fluid through phospholipid binding sites within the PMN plasma membrane. Taurine interacts with the polar head group of a neutral phospholipid possibly affecting Ca^{2+} binding sites within the phospholipid portion of the membrane (Huxtable, 1990). A study by Nakashima demonstrated that taurine decreases membrane mobility but not fluidity in erythrocytes (Nakashima *et al.*, 1996). Since calcium channels are surrounded by phospholipids, this would suggest that taurine and its structural analogue, HEPES may affect the binding of calcium fluxes to cell membranes by changing the dynamics of membrane phospholipids.

These results demonstrate that taurine and its structural analogue, HEPES differentially modulate the inflammatory response. Upon stimulation by receptor-dependent stimuli, taurine and HEPES prevent receptor-mediated influx of Ca^{2+} from the extracellular environment and subsequently have protective effects on PMN respiratory burst activity, NO production and PMN adhesion. Taurine and HEPES have the capacity however, to enhance respiratory burst activity upon stimulation by non-receptor agonists which is most likely due to the differential effects of taurine on Ca^{2+} . The anti-inflammatory effects of taurine and HEPES in responses to receptor agonists may have more physiological relevance to the inflammatory response where PMN activation occurs upon receptor binding. This is supported by studies demonstrating that one of the mechanisms through which taurine exerts its protective effects is through membrane-stabilisation (Huxtable *et al.*, 1992).

CHAPTER IV

THE ROLE OF TAURINE AND HEPES ON THE MODULATION OF NITRIC OXIDE-MEDIATED APOPTOSIS.

4.1 Introduction (PMN studies)

Inflammatory diseases are characterized by upregulation of nitric oxide (NO). Enhanced production of nitric oxide (NO) found at inflammatory sites is associated with upregulation of the inducible nitric oxide synthase (iNOS) (Rachmilewitz *et al.*, 1994, Wong *et al.*, 1995). NO possesses protective, anti-inflammatory effects but is also associated with the capacity to cause tissue injury during the inflammatory response (reviewed by Smith *et al.*, 1994). The most recent biological effect attributed to NO is its induction of apoptosis in the macrophage, the effector cell for NO-mediated cytotoxicity. Murine peritoneal macrophages when activated to express nitric oxide synthase (NOS) die prematurely in culture (Albina *et al.*, 1993). Since this discovery, NO has been found to regulate apoptosis in a number of cell types. NO induces apoptosis in chondrocytes (Blanco *et al.*, 1995). However, there are also reports of inhibition of apoptosis by NO in b-lymphocytes (Mannick *et al.*, 1994) and eosinophils (Beauvais *et al.*, 1995).

Polymorphonuclear leukocytes (PMNs) constitute the majority of influxing leucocytes into an inflamed area and play an important role in the pathophysiology of various inflammatory diseases. There has been much controversy as to whether human PMNs synthesize NO. Although several laboratories have reported that human PMNs produce NO in response to a number of stimuli (Wright *et al.*, 1989, Riesco *et al.*, 1993), there has been no biochemical nor molecular confirmation of the presence of an active iNOS in human PMNs (Miles *et al.*, 1995). Simultaneous production of NO and superoxide (O_2^-) by the PMN may form peroxynitrite ($ONOO^-$) which has been implicated as a mediator of NO-induced cellular injury in many inflammatory states (Radi *et al.*, 1991, Wize mann *et al.*, 1994). Maximal production of NO occurs where NADPH oxidase and iNOS are not simultaneously induced (Bastian *et al.*, 1994).

Clearance of PMNs is essential for resolution of the inflammatory response which occurs by apoptosis and ingestion by macrophages (Savill *et al.*, 1989, Savill *et al.*, 1997). Two distinct mechanisms of cell death have been identified, that of apoptosis and necrosis. During apoptosis, the cells undergo shrinkage and zeiosis, the nucleus collapses and the chromatin is cleaved into nucleosomal fragments (Cohen, 1991). These apoptotic bodies are subsequently

engulfed without eliciting an inflammatory response. In contrast, necrosis is always the outcome of catastrophic injury to the cell with the release of its contents into the surrounding tissue space with potential provocation of an exuberant inflammatory response (Buttke *et al.*, 1994). The proinflammatory cytokines, LPS/IFN- γ act synergistically to increase NO in a number of cell types (Kilbourn and Belloni, 1990, Billiar *et al.*, 1990, Dignass *et al.*, 1995). Endogenous upregulation of NO by proinflammatory cytokines may have the potential to modulate apoptosis at the site of inflammation. The effect of endogenous upregulation of NO on PMN apoptosis is currently unknown although there is one report that demonstrated exogenous NO enhanced PMN cell death (Fortenberry *et al.*, 1998).

Taurine, a semi-essential amino acid is the most abundant free β -amino acid in PMNs constituting up to 76% of the total free amino acid pool (Fukuda *et al.*, 1982). Research from our laboratory has demonstrated that taurine can decrease PMN apoptosis which were attributed to its antioxidant properties (Watson *et al.*, 1996a) In addition, taurine can decrease endothelial cell apoptosis (Wang *et al.*, 1995). Taurine reduces NO production in LPS/IFN- γ -activated murine peritoneal macrophages (Watson *et al.*, 1996b) and hepatocytes (Redmond *et al.*, 1996) suggesting that taurine may have the ability to decrease iNOS. On the other hand, taurine can increase NO production in human umbilical vein endothelial cells through cNOS. However, the effect of taurine and its structural analogue, HEPES on NO-mediated PMN apoptosis are unknown.

4.2 Aims

This aims of this study were to determine if exogenously added NO or endogenously generated NO could induce PMN apoptosis and secondly, to assess the effects of taurine and HEPES on NO-modulated apoptosis. This involved a study to determine if the PMN could produce NO and generate NOS protein and also, the relationship between NO and ROS was investigated.

4.3 Results

4.3.1 Spontaneous PMN apoptosis

A time course was performed to determine rates of spontaneous apoptosis. PMNs (1×10^6 cells/ml) were cultured in DMEM containing FCS (10% v/v) and penicillin/streptomycin (5% v/v). PMN apoptosis was assessed at 0, 3, 6, 12, 18 and 24 hours which gave values of 10.29 ± 3.93 , 14.24 ± 6.33 , 15.89 ± 4.68 , 21.24 ± 5.0 , 28.84 ± 2.23 and $45.6 \pm 7.9\%$ apoptosis respectively (Figure 4.1). PMN apoptosis was assessed using propidium iodide (PI) which binds hypodiploid DNA allowing the percentage of apoptotic cells to be quantified flow cytometrically. An optimum time of 12 hours was taken as the culture time for the remainder of the experiments.

4.3.2 Effects of sodium nitroprusside (SNP) on PMN apoptosis

To evaluate the effect of an exogenous source of NO on PMN apoptosis, PMNs (1×10^6 cells/ml) were cultured with the NO donor, SNP at the following concentrations: 0.5, 1, 2, 4, 8, 16 and 32 mM. After 12 hours incubation, PMN apoptosis was assessed using PI DNA staining. There was a significant increase in apoptosis above spontaneous levels at 8mM ($p < 0.05$), 16 and 32mM ($P < 0.01$) SNP (Figures 4.2a and 4.2b). Apoptosis was confirmed by morphology and DNA gel electrophoresis. DNA gel electrophoresis revealed the typical DNA laddering effect with 200 kb pair fragments (Figure 4.2c). At the highest concentration (32 mM), a smearing effect was seen on the gel indicative of necrosis. Morphology showed typical nuclear condensation, vacuole formation and some cell shrinkage in PMNs cultured with 1, 2, 4, 8, 16 and 32 mM concentrations of SNP (Figure 4.2d). However, at the higher concentration of SNP (32 mM), necrosis was also visible. Necrotic cells can be distinguished from apoptotic cells as clusters of cells with damage to the cell membrane. Collectively, these results indicate that NO, when generated from exogenous donors, can induce morphological changes and DNA fragmentation characteristic of apoptosis but at high doses can induce both apoptosis and necrosis.

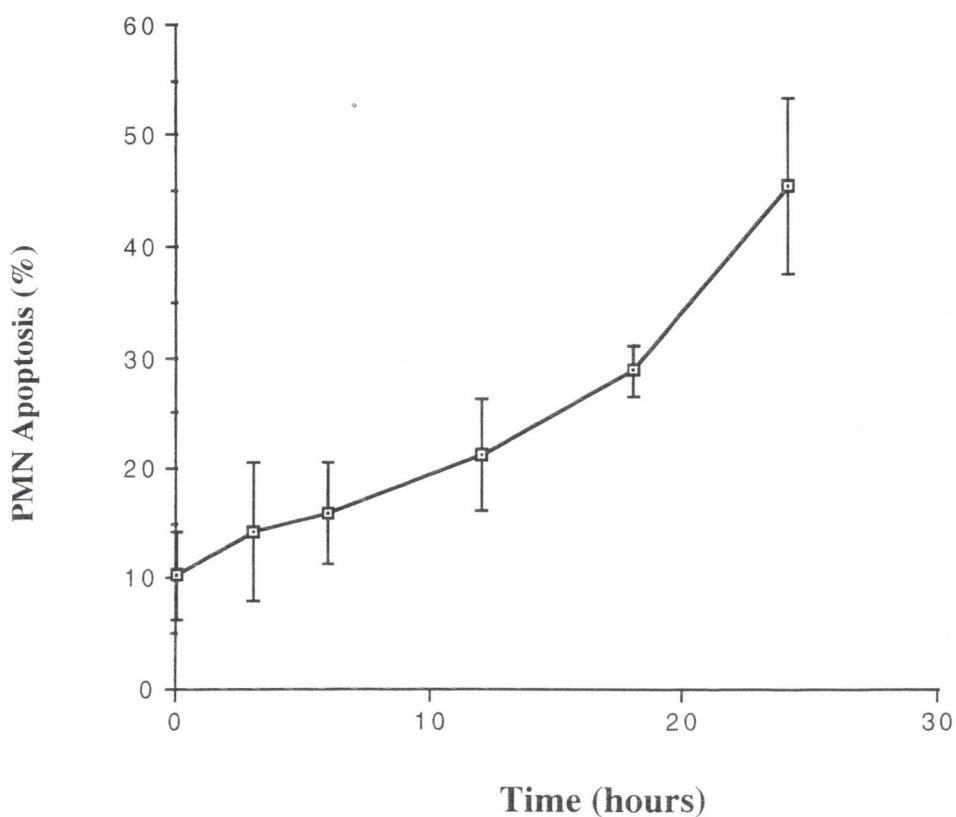


Figure 4.1 Time course of spontaneous rates of PMN apoptosis. PMNs (1×10^6 cells/ml) were cultured in DMEM culture medium in polypropylene tubes (to prevent adherence) and apoptosis was assessed flow cytometrically using propidium iodide at various times as shown. Data are expressed as the mean $\pm$ SEM of 3 individual experiments carried out in duplicate.

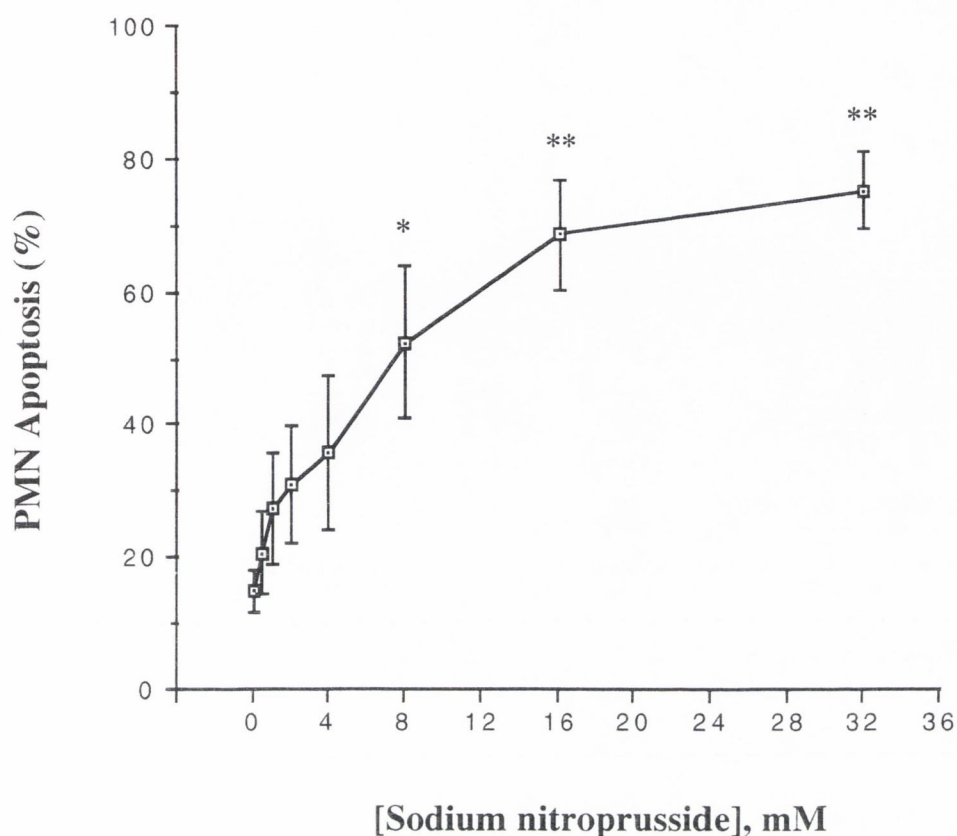
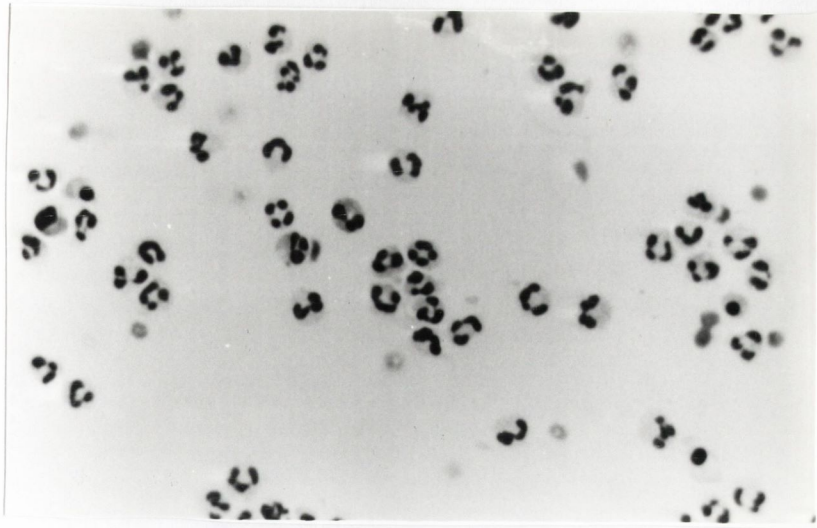
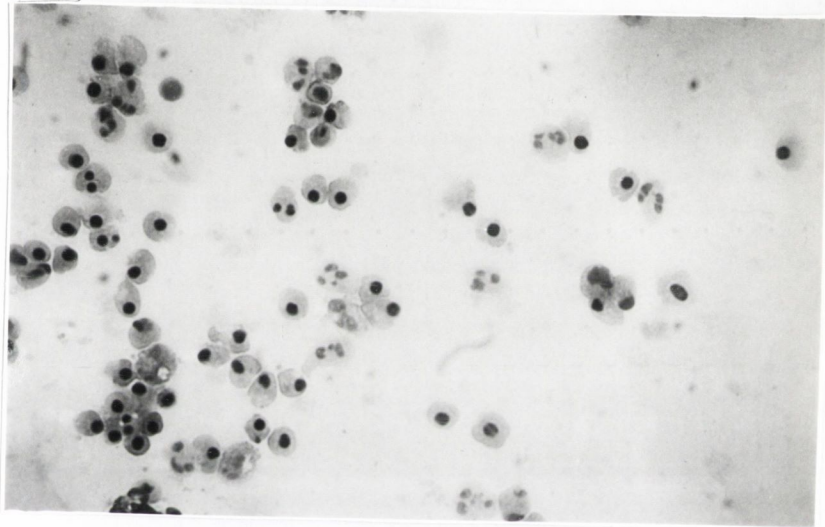


Figure 4.2 The effect of the nitric oxide donor, sodium nitroprusside (SNP) on PMN apoptosis. PMNs (1×10^6 cells/ml) were cultured with varying concentrations of SNP and apoptosis was assessed flow cytometrically using propidium iodide at a 12 hour time point. Data are expressed as the mean $\pm$ SEM of 4 individual experiments carried out in duplicate. * $p < 0.05$, ** $p < 0.01$ vs stimulus alone (Repeated Measures Analysis of Variance).

A



B



C

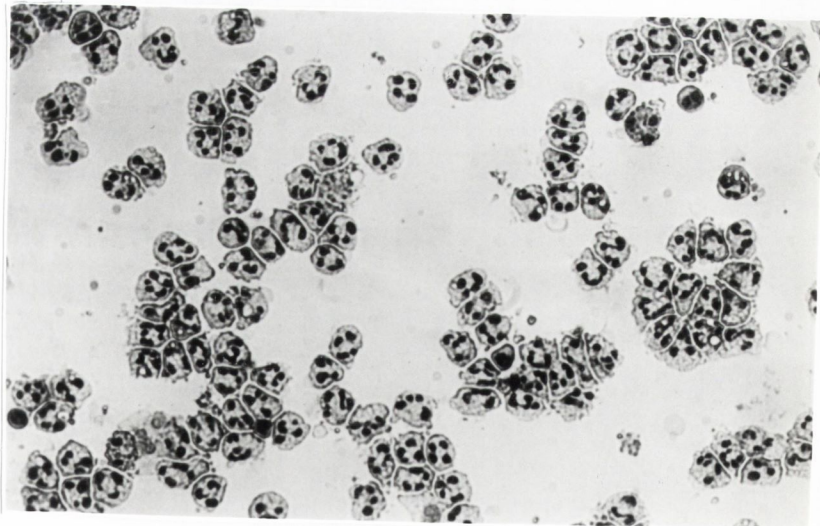


Figure 4.2b Morphological appearance of PMNs cultured in (A) medium alone, (B) sodium nitroprusside (8mM) or (C) sodium nitroprusside (32mM) for 12 hours. PMNs ($1 \times 10^6/\text{ml}$) were cultured in polypropylene tubes (to prevent adherence) in DMEM with or without sodium nitroprusside for 12 hours. Cytocentrifuge preparations were fixed and stained with Wrights stain. Sodium nitroprusside culture resulted in PMN apoptosis as shown in (B) and necrosis (C).

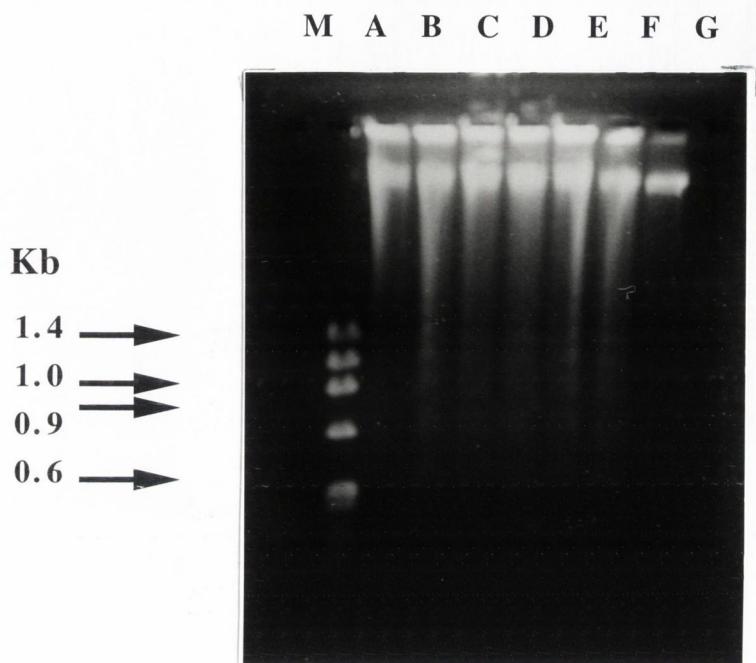


Figure 4.2c Gel electrophoresis of DNA extracted from PMN that had been cultured in the presence of 1 mM (lane B), 2 mM (lane C), 4mM (lane D), 8mM (lane E), 16mM (lane F) or 32mM (lane G) sodium nitroprusside or DMEM only (lane A) for 12 hours. Sodium nitroprusside-cultured PMNs show increased chromatin fragmentation compared to control. However, treatment with SNP (32 mM) induced non-specific banding (lane G) indicative of necrosis.

4.3.3 Endogenous upregulation of PMN nitric oxide production

As the NO donor induced PMN apoptosis, it was of interest to test whether endogenously generated NO was capable of inducing similar effects on PMN apoptosis. Previous studies have demonstrated that dual stimulation with LPS and IFN- γ increase NO production in many cell types through inducible nitric oxide synthase (iNOS) (Kilbourn and Belloni, 1990, Billiar *et al.*, 1990, Dignass *et al.*, 1995). However, there are conflicting reports as to whether human PMNs produce NO (Wright *et al.*, 1989, Riesco *et al.*, 1993, Miles *et al.*, 1995). PMNs were stimulated with LPS (2 μ g/ml), IFN- γ (200 U/ml) and TNF- α (500 U/ml) alone or in combination for 12 hours at 37°C in 5% CO₂ to examine formation of NO through iNOS. PMNs incubated for 12 hours produced an average of 1.677 ± 0.93 nmoles nitrite/1 x 10⁶ cells/12h. All of the cytokines alone or in combination reduced PMN nitrite production below unstimulated levels at this time point (Figure 4.3a). Western blotting did not detect the presence of iNOS protein in either resting or cytokine-stimulated PMNs. Figure 4.3b shows an immunoblot of iNOS protein in these PMNs. The LPS/IFN- γ -activated murine macrophage cell line RAW 264.7 was used as a positive control (lane 1). Neither resting (lane 2) nor cytokine-activated human PMNs (lanes 3-7) expressed detectable protein for iNOS (Fig 4.3b).

PMNs were activated with fMLP (10⁻⁶, 10⁻⁷, 10⁻⁸, 10⁻⁹, 10⁻¹⁰ M) for 1h to determine production of NO through constitutive form of NOS (cNOS). Figure 4.3a illustrates PMN NO₂⁻ production stimulated with fMLP. At 1 hour, resting PMNs released 2.27 ± 1.461 pmol NO/1 x 10⁶/1h into the medium whereas PMNs stimulated with 1nM fMLP significantly increased PMN NO production to 83.91 ± 28.09 pmol NO/10⁶/1h. The production of pmoles is consistent with the production of NO through cNOS. However, cNOS protein could not be detected in these samples using the available antibody for endothelial cNOS (Figure 4.3b). Cell lysates from human endothelial cells line were used as a positive control (lane 1). Neither resting nor fMLP-stimulated PMNs contained cNOS protein (lanes 2-8).

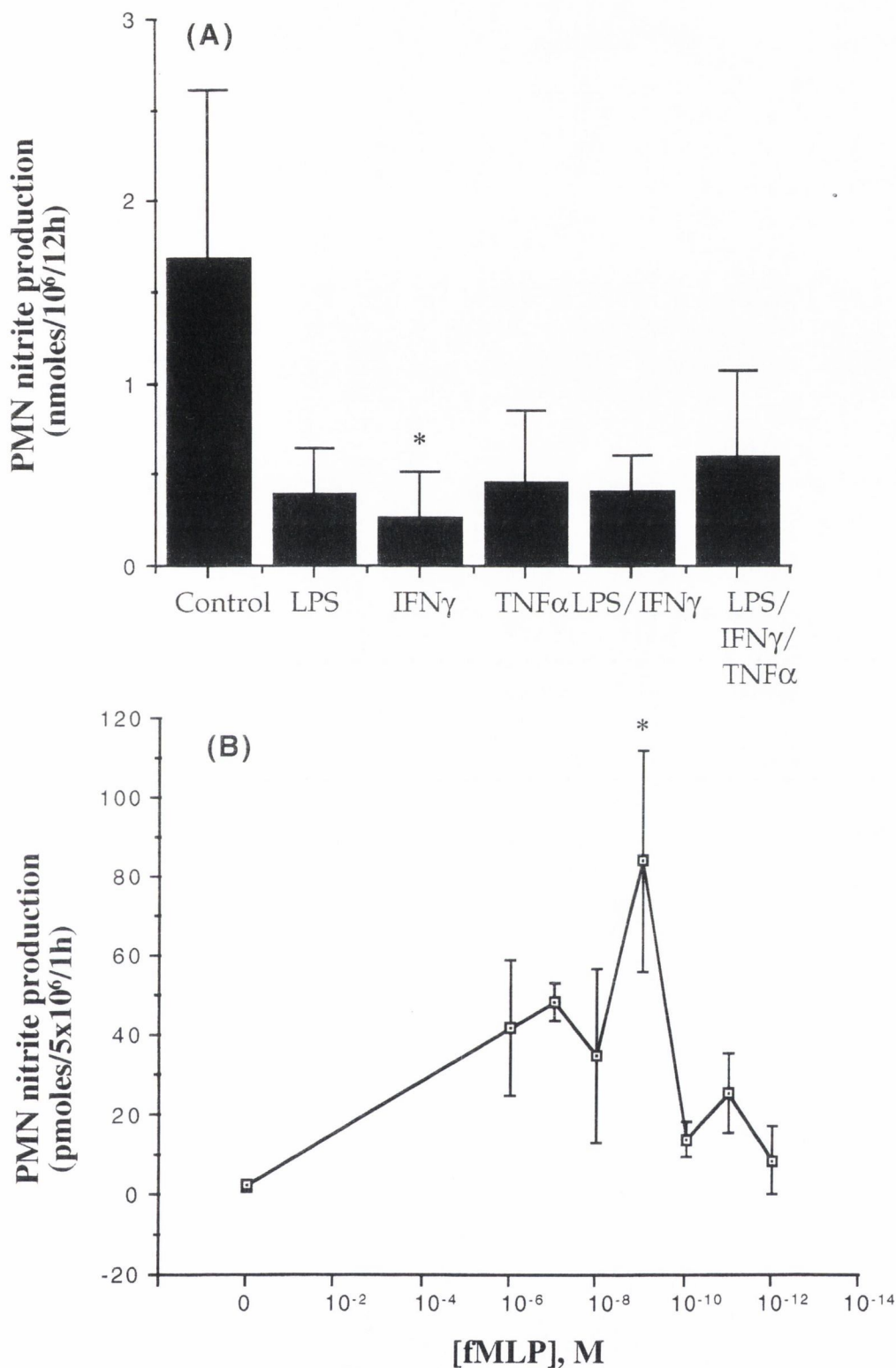
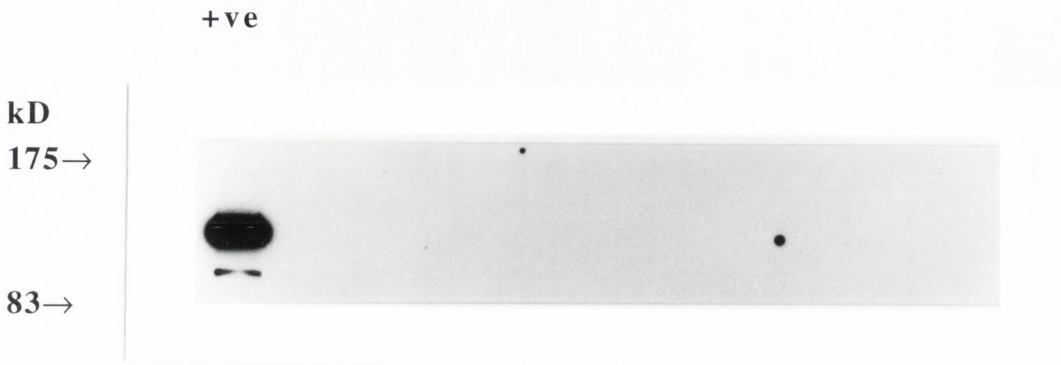


Figure 4.3a The effect of proinflammatory cytokines and fMLP on endogenous PMN NO production. (A) PMNs were stimulated with LPS (2 μ g/ml), IFN- γ (200 U/ml) and TNF- α (500 U/ml) alone or in combination for 12 hours at 37°C in 5% CO₂ to examine formation of NO through iNOS. (B) PMNs were stimulated with various concentrations of the chemoattractant, fMLP for 1 hour at 37°C in 5% CO₂ to examine expression of NO through cNOS. Nitrite production was assessed using griess assay. Data are expressed as the mean $\pm$ SEM of 5 individual experiments carried out in triplicate. *p<0.05 vs stimulus alone (Analysis of Variance with LSD post hoc test).

A



B



Figure 4.3b The effect of proinflammatory cytokines and fMLP on PMN iNOS and cNOS production. (A) PMNs were stimulated with LPS (2 μ g/ml), IFN- γ (200 U/ml) and TNF- α (500 U/ml) alone or in combination for 12 hours at 37°C in 5% CO₂ to examine formation of NO through iNOS. (B) PMNs were stimulated with various concentrations of the chemoattractant, fMLP for 1 hour at 37°C in 5% CO₂ to examine expression of NO through cNOS. NOS protein was assessed by western blotting using a chemiluminescent probe to detect cNOS or iNOS protein with positive controls included (+ve). Data is representative of a single experiment.

4.3.4 PMN NO production is dependent on its interaction with ROS

As we had demonstrated a reduction in the levels of biologically active NO in cytokine-stimulated PMNs, it was proposed that this may be due to its reaction with oxygen radicals forming peroxynitrite (ONOO^-). The effect of these cytokines on PMN reactive oxygen species (ROS) and ONOO^- was assessed. PMNs (1×10^6 cells/ml) were incubated with LPS (2 $\mu\text{g/ml}$), IFN- γ (200 U/ml) and TNF- α (500 U/ml) alone or in combination. PMNs were assessed for intracellular ROS activity after 2 hours. All of the cytokines, alone or in combination, significantly increased ROS generation except incubation with IFN- γ alone (Figure 4.4a). Thus, incubation with proinflammatory cytokines was associated with significant increases in PMN ROS generation which preceded the observed decreases in NO production at 12 hours.

It was proposed this was due to the scavenging of NO by superoxide to form ONOO^- . Thus, the levels of peroxynitrite anion were assessed at the 12 hour time point. The oxidation of dihydrorhodamine 123 by peroxynitrite has been successfully used for the measurement of peroxynitrite formation *in vitro* and *in vivo* (Wizemann *et al.*, 1994, Szabo *et al.*, 1995). The method is sensitive and specific because neither NO nor superoxide causes dihydrorhodamine 123 oxidation (Koody *et al.*, 1994). It was found that all cytokines except TNF- α increased the formation of peroxynitrite and this became significant upon stimulation by LPS/IFN- γ or LPS/IFN- γ /TNF- α (Figure 4.4b). TNF- α did not have any effect on ONOO^- anion formation and there was no difference in mean channel fluorescence (MCF) upon stimulation with either LPS/IFN- γ or LPS/IFN- γ /TNF- α . In conclusion, cytokine-activated PMNs produce ROS which precedes reductions in the levels of nitrite resulting in increased peroxynitrite formation.

4.3.5 Effects of endogenous NO production on PMN apoptosis

PMNs (1×10^6 /ml) were stimulated with LPS (2 $\mu\text{g/ml}$), IFN- γ (200 U/ml) and TNF- α (500 U/ml) alone or in combination for 12 hours at 37°C in 5% CO_2 . LPS and IFN- γ either alone or in combination did not affect rates of PMN apoptosis. The addition of TNF- α to LPS/IFN- γ promoted a synergistic and significant increase in PMN apoptosis (Figure 4.5a).

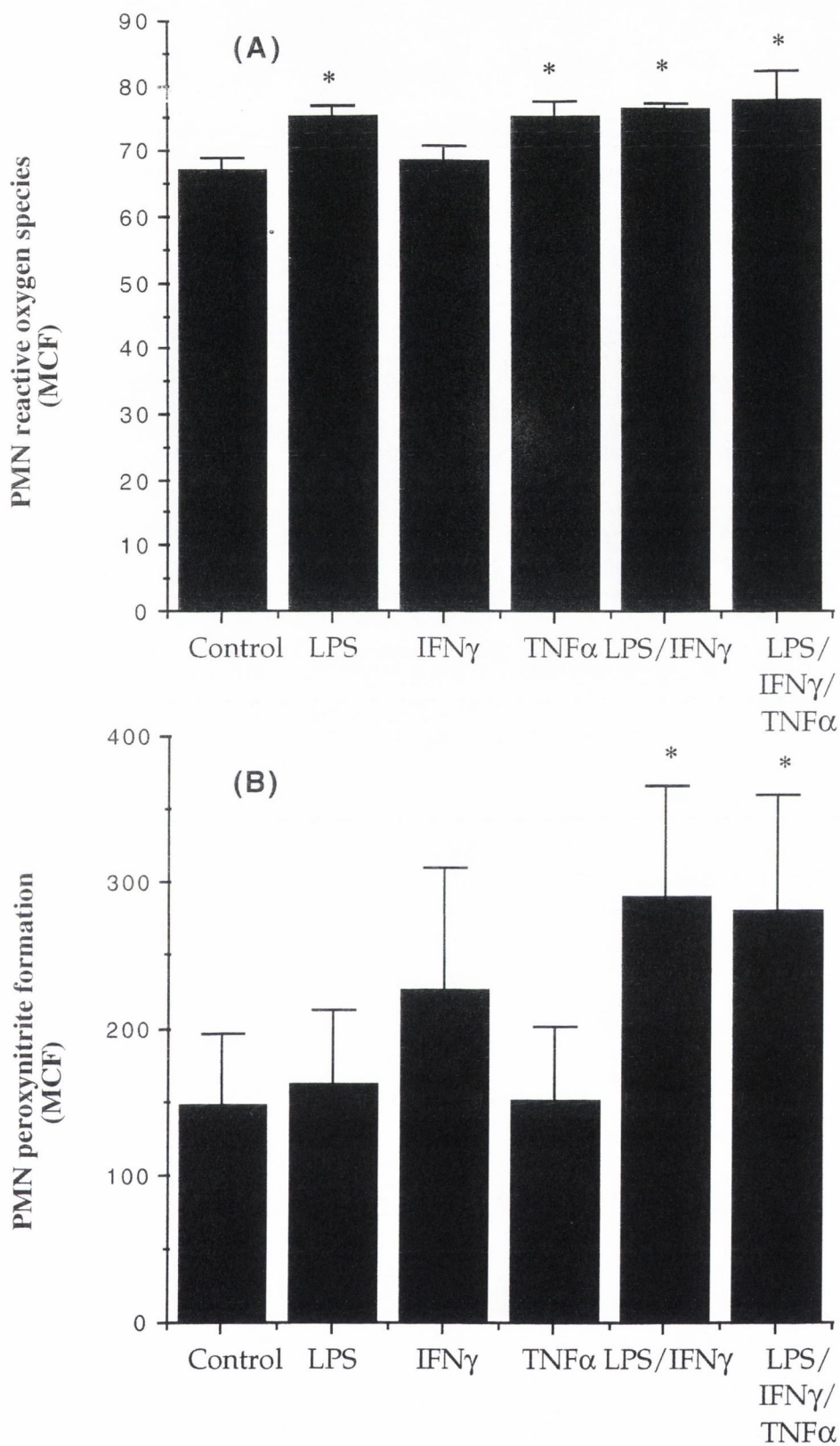


Figure 4.4 The effect of proinflammatory cytokines on reactive oxygen species (ROS) and peroxynitrite formation (ONOO⁻) in the PMN. (A) PMNs were stimulated with LPS (2 μ g/ml), IFN- γ (200 U/ml) and TNF- α (500 U/ml) alone or in combination for 2 hours at 37°C in 5% CO₂ and ROS measured flow cytometrically by the oxidation of DHACR. (B) ONOO⁻ formation was assessed flow cytometrically by the oxidation of dihydrorhodamine 123 in these PMNs upon culture for 12 hours. Data are expressed as the mean $\pm$ SEM of 3 experiments carried out in duplicate. * p <0.05 vs stimulus alone (Repeated Measures Analysis of Variance).

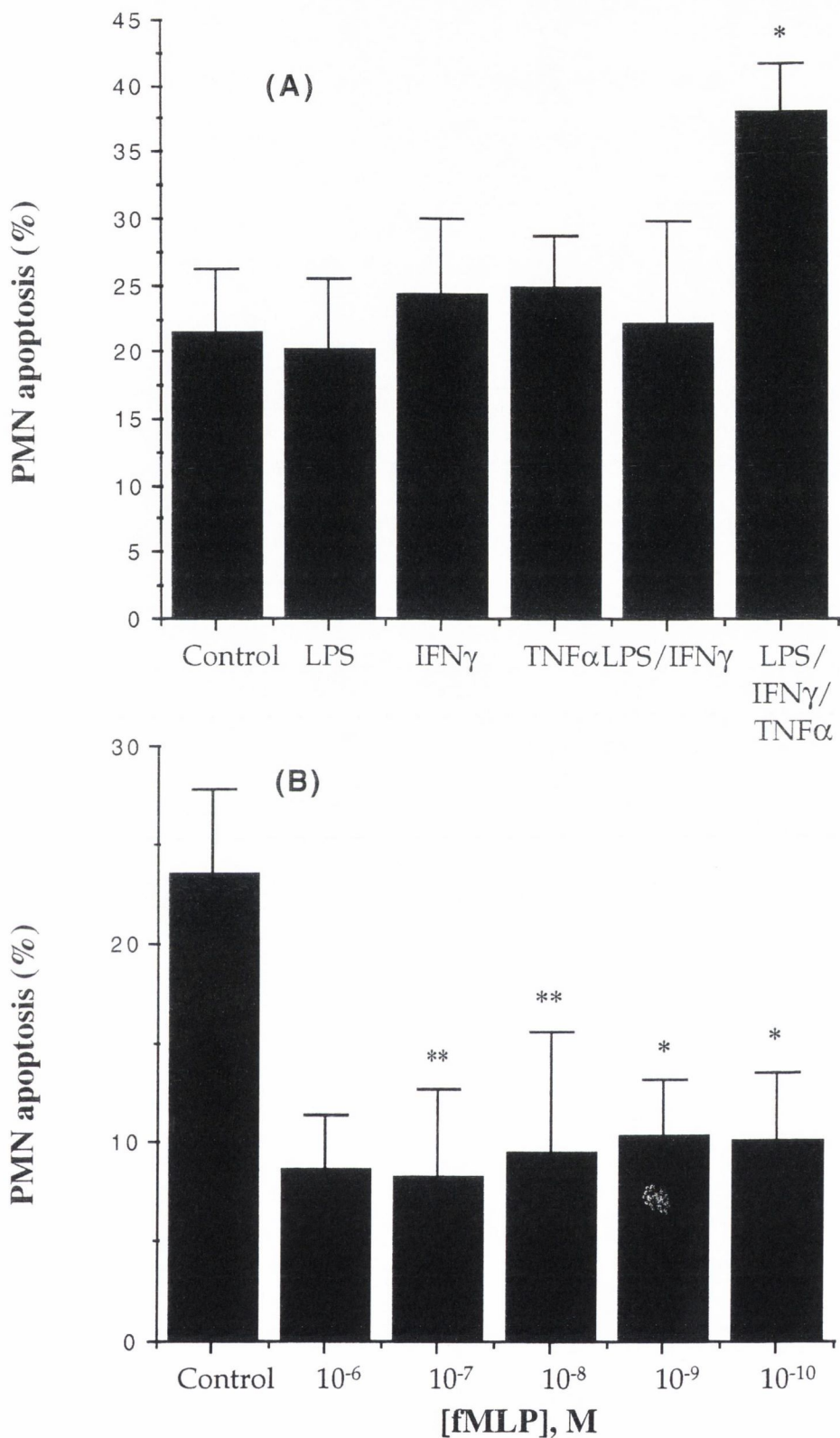


Figure 4.5 The effect of proinflammatory cytokines and fMLP on PMN apoptosis. (A) Apoptosis was measured flow cytometrically using propidium iodide staining in PMNs stimulated with LPS (2 μ g/ml), IFN- γ (200 U/ml) and TNF- α (500 U/ml) alone or in combination for 12 hours at 37°C in 5% CO₂. (B) PMNs were stimulated with various concentrations of the chemoattractant, fMLP for 1 hour at 37°C in 5% CO₂ and PMN apoptosis assessed flow cytometrically by annexin V staining. Data is expressed as the mean $\pm$ SEM of 3 experiments carried out in duplicate. Data was analysed using Repeated Measures Analysis of Variance in the case of (A) or Analysis of Variance with LSD post-hoc test in the case of (B), * p <0.05, ** p <0.01 vs stimulus alone.

To confirm the role of NO in PMN apoptosis, PMNs (1×10^6 cells/ml) were cultured with varying concentrations of formylmethionyl-leucyl-phenylalanine (fMLP) for 1 hour and PMN apoptosis was assessed. Early events in apoptosis can be assessed using Annexin V/PI DNA staining which assays phosphatidylserine flipping to the outer membrane. fMLP significantly decreased PMN apoptosis at all concentrations in the range from $1\mu\text{M}$ to 0.1nM fMLP (Figure 4.5b).

4.3.6 Effects of taurine and HEPES on NO-mediated apoptosis

The effects of the anti-oxidant, taurine and its structural analogue, HEPES were assessed on NO-modulated PMN apoptosis. To evaluate a direct effect of taurine and HEPES on NO, PMNs were cultured with an exogenous source of NO, SNP (8 mM) in the presence of 0.25 , 0.5 and 1 mg/ml taurine or HEPES. SNP at the concentration used in this experiment released 1.389 ± 0.013 nmoles nitrite/12 hours into the culture medium and induced $60.76 \pm 3.5\%$ apoptosis at this 12 hour time point. Addition of taurine or HEPES at any of these concentrations failed to prevent PMN apoptosis but did abrogate SNP-induced nitrite generation (Figure 4.6b).

Upon incubation with taurine or HEPES for 12 hours, it was discovered that these compounds had no effect on resting PMN apoptosis but taurine (1 mg/ml) did significantly ($p < 0.01$) increase PMN nitrite production in resting cells (Table 4.1). In order to assess if taurine and HEPES had the capacity to affect endogenous upregulation of NO-modulated PMN apoptosis, PMNs were cultured with fMLP (1nM) in the presence of 0.25 , 0.5 and 1 mg/ml taurine or HEPES. Taurine or HEPES failed to significantly attenuate PMN apoptosis, necrosis or NO production (Table 4.2). However, HEPES (0.25 mg/ml) increased PMN nitrite generation from 60.55 ± 17.3 pmoles/ 5×10^6 cells/1h in fMLP-treated cells to 147.95 ± 61.27 pmoles/ 5×10^6 cells/1h whilst decreasing PMN apoptosis to $13.22 \pm 2.5\%$ from $18.16 \pm 1.4\%$ in fMLP-stimulated cells.

In conclusion, taurine and HEPES have the capacity to directly decrease exogenously added NO without having any significant effects on PMN apoptosis. Taurine increases NO production in resting PMNs and in fMLP-stimulated PMNs at short time points where the production of NO is most likely due to the constitutive form of NOS.

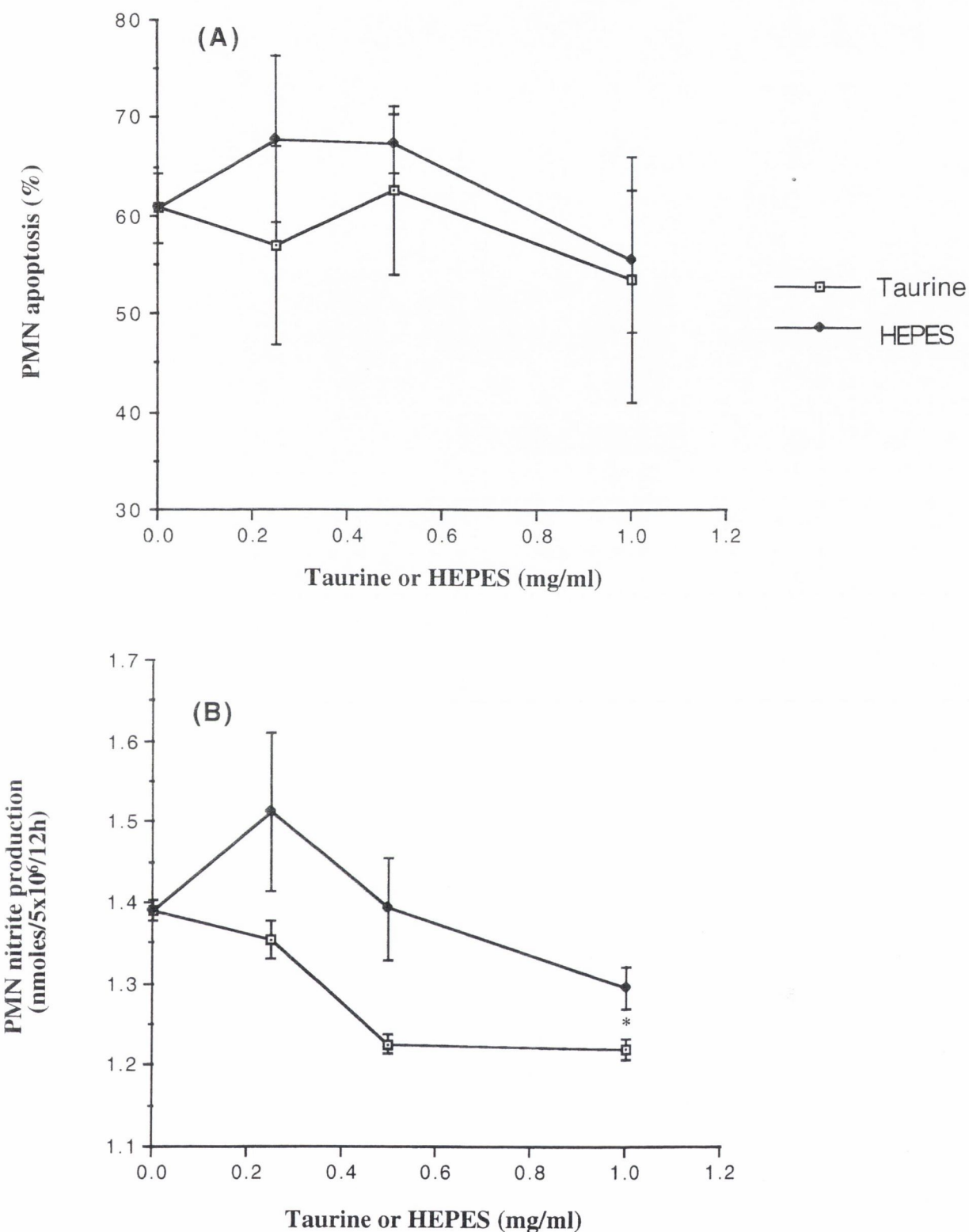


Figure 4.6 The effect of taurine and HEPES on PMN apoptosis and NO production in sodium nitroprusside (SNP)-treated PMNs. PMNs were incubated at 37°C in 5% CO₂ with SNP (8 mM) and various concentrations of taurine or HEPES for 12 hours. PMN apoptosis was assessed flow cytometrically using propidium iodide staining. PMN NO production was assessed as the stable end product of NO metabolism, nitrite by the griess assay. Data is expressed as the mean $\pm$ SEM of 3 experiments carried out in duplicate, * $p < 0.05$ vs stimulus alone (Analysis of Variance with LSD post hoc test).

	PMN apoptosis (%)	PMN nitrite production (pmoles/10 ⁶ cells/12h)
Spontaneous	22.75 ± 4.4	66.51 ± 7.7
+ Taurine (0.25 mg/ml)	23.59 ± 0.4	54.15 ± 9.5
+ Taurine (0.5 mg/ml)	27.17 ± 3.9	73.96 ± 8.8
+ Taurine (1 mg/ml)	21.75 ± 0.3	105.95 ± 7.8*
+ HEPES (0.25 mg/ml)	18.91 ± 2.5	64.55 ± 6.9
+ HEPES (0.5 mg/ml)	23.87 ± 1.5	45.32 ± 4.3
+ HEPES (1 mg/ml)	18.54 ± 1.3	50.62 ± 2.8

Table 4.1 The effect of Taurine and HEPES on PMN apoptosis and NO production in resting PMNs. PMNs (1 x 10⁶ cells/ml) were preincubated with varying concentrations (as shown) of taurine or HEPES for 12 hours. PMN apoptosis was assessed flow cytometrically using propidium iodide staining and nitrite generation was assessed in supernatants using the griess assay. Data is expressed as mean ± SEM of 3 experiments, * p<0.01 vs spontaneous (Analysis of Variance with LSD post-hoc test)

	PMN apoptosis (%)	PMN nitrite production (pmoles/10 ⁶ cells/12h)	PMN necrosis (%)
fMLP (1 nM)	18.16 ± 1.4	60.55 ± 17.3	1.50 ± 0.6
fMLP + Taurine (0.25 mg/ml)	23.38 ± 5.0	97.17 ± 31.5	1.16 ± 0.5
fMLP + Taurine (0.5 mg/ml)	17.96 ± 2.8	75.44 ± 30.4	1.18 ± 0.5
fMLP + Taurine (1 mg/ml)	21.16 ± 2.8	76.95 ± 35.9	1.12 ± 0.6
fMLP + HEPES (0.25 mg/ml)	13.22 ± 2.5	147.59 ± 61.3	1.63 ± 0.7
fMLP + HEPES (0.5 mg/ml)	20.28 ± 2.2	133.24 ± 30.3	1.16 ± 0.4
fMLP + HEPES (1 mg/ml)	22.36 ± 3.7	96.49 ± 32.4	1.66 ± 0.9

Table 4.2 The effect of Taurine and HEPES on PMN apoptosis, necrosis and NO production in fMLP-stimulated PMNs. PMNs (1 x 10⁶ cells/ml) were incubated with fMLP and varying concentrations (as shown) of taurine or HEPES for 1 hour. PMN apoptosis and necrosis was assessed flow cytometrically using annexin V binding and propidium iodide exclusion. PMN nitrite generation was assessed in supernatants using the griess assay. Data is expressed as mean ± SEM of 6 experiments.

4.4 Discussion

PMN's are the first cells to be recruited to the inflamed area whereupon they exert their microbicidal properties. PMNs have great potential to injure tissues during the inflammatory response through the production and release of oxidants such as NO and ROS, proteolytic enzymes and products of arachidonic acid metabolites into the extracellular milieu (reviewed by Smith *et al.*, 1994). Thus, the clearance of activated PMNs from the site of inflammation is central to the resolution of the inflammatory response. This process of apoptotic cell death is distinct from necrosis in that apoptosis occurs through a controlled manner minimizing PMN proinflammatory potential allowing tissues to return to homeostasis (Savill *et al.*, 1997). PMNs are constitutively programmed to undergo apoptosis within 5-6 h in the circulation (Savill *et al.*, 1989). This study found that ageing PMNs in culture undergo a spontaneous time-dependent apoptosis which is consistent with findings by Savill.

It was demonstrated in this study that exogenous NO as applied through sodium nitroprusside (SNP) induced a significant increase in PMN apoptosis. PMN apoptosis was significantly increased in SNP-treated PMNs as determined by staining with the DNA-intercalating dye, propidium iodide (PI). SNP-treated PMNs exhibited classical morphological features of apoptosis of cell shrinkage, cytoplasmic condensation and dramatic condensation of nuclear chromatin (Darzynkiewicz *et al.*, 1992). Additional information sought from DNA gel electrophoresis indicated the presence of a distinctive ladder pattern of cleaved DNA in SNP-treated PMNs. DNA isolated from apoptotic cells is cleaved into 200 bp integer multiples as a result of activation of endogenous endonucleases (McGahon *et al.*, 1995). However, the higher doses of SNP (16 and 32 mM) were associated with PMN cell death through necrosis as visualised through PMN morphology. In addition, the presence of non-specific banding on DNA gel electrophoresis in SNP (32mM)-treated PMNs was indicative of necrosis. There has been one study to date assessing the effects of exogenous NO on PMN apoptosis. This study found that exogenous NO gas, at clinically relevant concentrations (20, 50 ppm of NO) induces cell death in PMNs (Fortenberry *et al.*, 1998). Our results indicate that NO derived from exogenous sources induces PMN apoptosis but at higher doses can induce cell death through necrosis.

In a number of cell types, the combination of lipopolysaccharide (LPS) and interferon- γ (IFN- γ) induce high levels of NO production through iNOS (Kilbourn and Belloni, 1990, Billiar *et al.*, 1990, Dignass *et al.*, 1995). Stimulation of PMNs with a combination of LPS, IFN- γ , TNF- α alone or in combination failed to upregulate PMN nitrite production as originally anticipated but rather, cytokine-activated PMNs had lower nitrite production than resting circulating PMNs at 12 hours culture. In addition, this study demonstrates that neither human resting PMNs nor those activated with LPS, IFN- γ , TNF- α alone or in combination contained detectable levels of iNOS protein. In this study, enhanced chemiluminescence was used to detect iNOS protein which can detect as low as 10pg of protein when using horse radish peroxidase-labelled antibodies. This is in agreement with findings by Amin who reported that iNOS could not be detected in human neutrophils under conditions sufficient to induce iNOS in the rodent system (Amin *et al.*, 1995/6). It was also demonstrated that neither circulating nor extravasated human PMNs contain iNOS mRNA, iNOS protein or enzymatic activity (Miles *et al.*, 1995). No study to date biochemically confirms the presence of iNOS in human PMNs although one study showed "constitutively expressed" iNOS mRNA in human activated neutrophils using more sensitive methods such as RT-PCR and northern blot analysis (Amin *et al.*, 1995/6). In another study, the hyporesponsiveness of the human iNOS gene was related to an inability to initiate transcription of the iNOS gene upon stimulation with LPS/IFN- γ (Zhang *et al.*, 1996).

It has been demonstrated that the addition of TNF- α to LPS/IFN- γ -activated cells to resting PMNs promoted maximal release of nitrite in human PMNs over three hours (Carreras *et al.*, 1994). Our data suggest that NO and O₂⁻ produced by LPS/IFN- γ -activated PMNs react over short time points (2 hours) producing ONOO⁻ anion observed in this study at 12 hours culture. We confirmed that cytokine-activated PMNs had increased ROS activity which preceded significant reductions in nitrite and increased ONOO⁻ formation. This study suggests that NO is inactivated by O₂⁻ in cytokine-activated PMNs resulting in the formation of ONOO⁻. Indeed, It has been demonstrated by a number of investigators that the levels of biologically active NO decrease as the degree of stimulation and O₂⁻ production increase (McCall *et al.*, 1989). Thus, these results suggest that NO production in activated PMNs is

dictated by the fluxes between ROS and NO with maximal NO production perhaps where ROS and NO are not simultaneously induced.

Incubation with the combination of LPS, IFN- γ and TNF- α significantly increased PMN apoptosis (Carreras *et al.*, 1993). In this study, the addition of TNF- α to LPS/IFN- γ -activated PMNs was critical for the induction of PMN apoptosis. PMN ONOO⁻ formation was found to be significantly elevated in LPS/IFN- γ -activated PMNs but not TNF- α stimulated PMN. In addition, there was no difference in ONOO⁻ formation between LPS/IFN- γ - and LPS/IFN- γ /TNF- α -activated PMNs. This would indicate that endogenously-generated NO produced by LPS/IFN- γ /TNF- α -stimulated PMNs may not be shunted into the formation of ONOO⁻ anion and may be free to increase PMN apoptosis observed in this study. The rate of apoptosis in PMN populations *in vitro* can be accelerated or inhibited by inflammatory mediators. The inflammatory mediator, TNF- α increases PMN apoptosis (Takeda *et al.*, 1993). In summary, stimulation with LPS/IFN- γ /TNF- α may increase "constitutively expressed" iNOS activity with the ability to increase PMN apoptosis.

We have shown that stimulation of PMNs with fMLP enhances the synthesis of NO. These results confirm other studies that have demonstrated PMNs produce NO in response to fMLP over short time points (1-2 h) (Schmidt *et al.*, 1989, McCall *et al.*, 1989). This production of NO is undoubtedly due to the activity of the constitutive form of NOS (cNOS) as induction of iNOS requires new protein synthesis and requires a lag time of a couple of hours. Indeed, cNOS has been purified and cloned from resting human PMNs (Bryant *et al.*, 1992). However, cNOS protein was undetected using an endothelial cNOS antibody in this study. It was reported that human PMNs did not have any detectable amounts of the endothelial type NOS protein (Amin *et al.*, 1995/6). This may be due to differences between a PMN NOS and the endothelial isoform. A recent study demonstrated cNOS protein in PMNs using an antibody to the neuronal NOS (nNOS) (Akahoshi *et al.*, 1997) suggesting nNOS is closer in homology to neutrophil cNOS. However, fMLP-stimulated PMNs did not have an nNOS signal as assessed by RT-PCR in another study (Amin *et al.*, 1995/6). fMLP is a formyl peptide that leads to increases in Ca²⁺ and activates NADPH oxidase. Elevation of cytosolic Ca²⁺ in a number of cell types is involved in the early induction of apoptosis

(Wyllie *et al.*, 1984, Takahashi *et al.*, 1989). However, Research by Haslett's group have shown that elevating intracellular calcium concentration by calcium ionophores inhibits the rate of PMN apoptosis (Whyte *et al.*, 1993). Additionally, PMN lifespan is prolonged via the inhibition of apoptosis by fMLP (Lee *et al.*, 1989). Thus, although endogenously generated NO through cNOS is associated with decreased PMN apoptosis in this study, a wide range of fMLP concentrations (1 μ M to 0.1 nM) used in this experiment inhibited PMN apoptosis with only one concentration significantly increasing PMN nitrite production (1 nM).

Oxidative stress has been implicated in the induction of PMN apoptosis with anti-oxidants inhibiting PMN apoptosis (Oishi and Machida *et al.*, 1997, Buttke *et al.*, 1994). Taurine acts as an anti-oxidant in human inflammatory cells. Research from our laboratory demonstrated that taurine was capable of decreasing NO production in peritoneal macrophages (Watson *et al.*, 1996) and hepatocytes (Redmond *et al.*, 1996). In this study, taurine significantly decreased NO production in SNP-treated PMNs but did not significantly reduce PMN apoptosis. Thus, taurine and HEPES have the capacity to reduce NO production in SNP-treated PMNs which appears to be through a direct interaction with NO itself. Taurine and HEPES significantly increased NO production in resting and fMLP-stimulated PMNs most likely through increasing cNOS activity. Similarly, taurine and HEPES failed to significantly decrease PMN cell death either through apoptosis or necrosis in resting or fMLP-activated PMNs. Therefore, it appears that endogenous production of NO may be necessary but is not sufficient for the induction of apoptosis in PMNs. It has been demonstrated recently that the product of NO and superoxide, peroxynitrite can increase PMN apoptosis (Blaylock *et al.*, 1998).

These findings suggest that exogenous NO is a major trigger for apoptosis in human PMNs. The observation that high levels of NO derived from exogenous sources induce PMN apoptosis may be critical in the resolution of the inflammatory response. Activated tissue cells in inflammatory sites release large amounts of NO which may contribute to cell death in PMNs through apoptosis. The terminally differentiated PMN is short-lived and destined to die rapidly by apoptosis (Fliender *et al.*, 1964). Upon stimulation, PMNs are capable of producing large quantities of ROS and smaller amounts of NO (Mc Call *et al.*, 1993). Endogenously produced NO may induce PMN apoptosis depending on the relative balance between oxygen radicals

and NO. The anti-oxidant, taurine significantly decreased PMN nitrite production without affecting PMN apoptosis indicating that endogenously generated NO may not be critical in the induction of PMN apoptosis. These results taken together suggest that exogenous NO is capable of inducing PMN apoptosis whereas endogenously produced NO may alter the balance of oxygen radicals thereby determining PMN survival. Elucidation of factors regulating cell death should provide valuable information towards our understanding of tissue injury.

4.5 Introduction (Enterocyte studies)

Inflammatory bowel disease is characterized by increased synthesis of nitric oxide through inducible nitric oxide synthase (iNOS) (Rachmilewitz *et al.*, 1994). iNOS is generated in several cell types by treatment with lipopolysaccharides and/or cytokines (Kilbourn and Belloni, 1990, Billiar *et al.*, 1990, Dignass *et al.*, 1995). The cellular sources of NO synthesis in the gut are the intrinsic intestinal tissue include colonic epithelial cells and cells influxing the colon including neutrophils and mononuclear cells. The intestinal epithelium is exposed to a hostile environment within the lumen of the gut including proinflammatory cytokines and endotoxin in active colitis. As human leukocytes are limited in their ability to produce NO upon activation, it is possible that colonic epithelial cells are primarily responsible for enhanced NO in IBD. Human HT-29 and DLD-1 colonic epithelial cells express iNOS mRNA and produce large quantities of nitrite in response to specific combinations of cytokines (Kolios *et al.*, 1995, Salzman *et al.*, 1996). However, little is known about the regulation of NO synthase activity in intestinal epithelial cells.

Nitric oxide (NO) is synthesized from the conversion of L-arginine to L-citrulline by nitric oxide synthase (NOS). NO is a reactive free radical gas with a variety of functions including vasodilator and neurotransmitter. Within the gut, NO has specific roles including regulation of the intestinal mucosal barrier integrity and salt and water secretion (Salzman *et al.*, 1995, Stark *et al.*, 1992). NO may protect the gastrointestinal mucosa from a variety of stimuli by maintaining blood flow, mucosal perfusion, prevention of mast cell activation (reviewed by Salzman *et al.*, 1995), inhibit PMN and platelet adhesion to the mesenteric endothelium (Kubes *et al.*, 1991, Miller *et al.*, 1994) and through its antioxidant properies (Kanner *et al.*, 1991). Endogenous NO and NO donors appear to reduce the sequelae of inflammation in the gut (Boughton-Smith *et al.*, 1990, Johnson *et al.*, 1991). However, Excessive NO or prolonged production of NO as in IBD may directly injure the mucosa through its interactions with other biological molecules enhancing its cytotoxicity (Wiseman *et al.*, 1996).

Apoptosis or programmed cell death is an active physiological process characterized by defined morphological features including membrane blebbing, cell shrinkage, nuclear

pyknosis and DNA fragmentation (Martin *et al.*, 1994). Within the gastrointestinal tract, there are defined zones of proliferation and migration of epithelial cells (Hall *et al.*, 1994). In order to maintain homeostasis, apoptosis must occur and has been noted in both proliferative and non-proliferative areas of the gut. It is believed that cell loss occurs through shedding into the gut lumen (Iwanaga *et al.*, 1995, Hall *et al.*, 1994). It has been proposed that the presence of the constitutive NOS in gastric mucosa cells plays a role in the modulation of epithelial cell integrity (Tepperman *et al.*, 1993). NO can act as a messenger molecule in apoptotic cell death since it has previously shown to induce apoptosis in activated macrophages (Albina *et al.*, 1993), chondrocytes (Blanco *et al.*, 1995), pancreatic b-cells (Kaneto *et al.*, 1996) and Swiss 3T3 fibroblasts (Khan *et al.*, 1996). Therefore, NO may play a role in the regulation of cell number in the mammalian gastrointestinal tract in IBD.

Taurine (2-aminoethanesulphonic acid) is one of the most abundant intracellular free amino acids present in various mammalian tissues particularly those tissues exposed to high concentrations of oxidants such as the intestine (Fukuda *et al.*, 1982, Green *et al.*, 1991, Wright *et al.*, 1985). Taurine and its substituted analogue, HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid) possess protective properties in models of inflammation which were attributed to their ability to attenuate oxidative cellular damage through neutrophil modulation and scavenging of reactive oxygen species (ROS) (Stapleton *et al.*, 1996, Son *et al.*, 1996, Son *et al.*, 1998). There is direct evidence of *in vivo* oxidant-induced injury in colonic epithelial cells isolated from inflamed mucosa of IBD patients (McKenzie *et al.*, 1996). The human colon contains much smaller amounts of anti-oxidants than many other tissues including the liver and heart (Grisham *et al.*, 1990, Mulder *et al.*, 1991, Buffington *et al.*, 1995). Thus, tissue-associated antioxidants and free radical scavengers may be overwhelmed during active episodes of IBD. Recent work in our laboratory has shown that supplementation with taurine attenuates NO production by the activated macrophage (Watson *et al.*, 1996). In addition, taurine attenuates hepatocyte cell death through an inhibition of both NO and ROS (Redmond *et al.*, 1996).

4.6 Aims

We hypothesized that the anti-oxidant taurine and its structural analogue, HEPES may be protective against NO-induced cell death within the gut. Thus, the aims of this study were to determine if endogenously produced NO may induce cell death in two colonic epithelial cell lines, Caco-2 and HT-29 and evaluate the effects of taurine and its analogue, HEPES on NO-mediated enterocyte apoptosis.

4.7 Results

4.7.1 Upregulation of endogenous NO production induces HT-29 apoptosis

Previous studies have demonstrated that in human intestinal epithelial cells, stimulation with various cytokines results in NO production (Kolios *et al.*, 1995, Salzman *et al.*, 1996). HT-29s were stimulated with LPS (2 µg/ml) and IFN-γ (200 U/ml) alone or in combination for 48 hours at 37°C in 5% CO₂ to examine expression of NO through iNOS. NO was significantly ($p < 0.05$) increased in LPS/IFN-γ-stimulated cells compared with monolayers that were treated with LPS alone (Figure 4.7a). The addition of the L-arginine analogues, L-NMMA (100 µM) or L-NAME (100 µM) to LPS/IFN-γ-containing media decreased NO generation by 24% and 32% respectively but this did not reach statistical significance. HT-29 apoptosis was assessed in the same cultures using an annexin V flow cytometric assay. Both LPS and IFN-γ increased HT-29 apoptosis and this became significant ($p < 0.01$) in cultures with the dual stimuli, LPS/IFN-γ ($22.06 \pm 5.06\%$) compared to media alone ($9.8 \pm 2.79\%$) (Figure 4.7b). However, Addition of the NOS inhibitors, L-NMMA and L-NAME did not significantly decrease apoptosis in LPS/IFN-γ-activated HT-29 cells. The addition of any of the cytokines and nitric oxide synthase inhibitors did not affect cell death by necrosis (Table 4.3). These results indicate that endogenously produced NO induces cell death by apoptosis rather than necrosis in the HT-29 cell line.

4.7.2 LPS and IFN-γ induce apoptosis in Caco-2 cell monolayers without significantly affecting NO production

Subconfluent Caco-2 cell monolayers were stimulated with LPS (2 µg/ml) and IFN-γ (200 U/ml) alone or in combination for 48 hours. NO production was unchanged in cultures containing LPS and IFN-γ (1.022 ± 0.14 nmoles/well/48h) compared to spontaneous levels (0.942 ± 0.15 nmoles/well/48h). However, LPS/IFN-γ-activated Caco-2 monolayers produced significantly more nitrite generation than culture with LPS alone (Figure 4.8a). NO production by Caco-2 cell monolayers was significantly ($p < 0.001$) decreased by the NOS inhibitors, L-NAME and L-NMMA compared to LPS/IFN-γ-activated Caco-2 cells at 48h of culture. Stimulation with both LPS and IFN-γ induced significant ($p < 0.01$) levels of

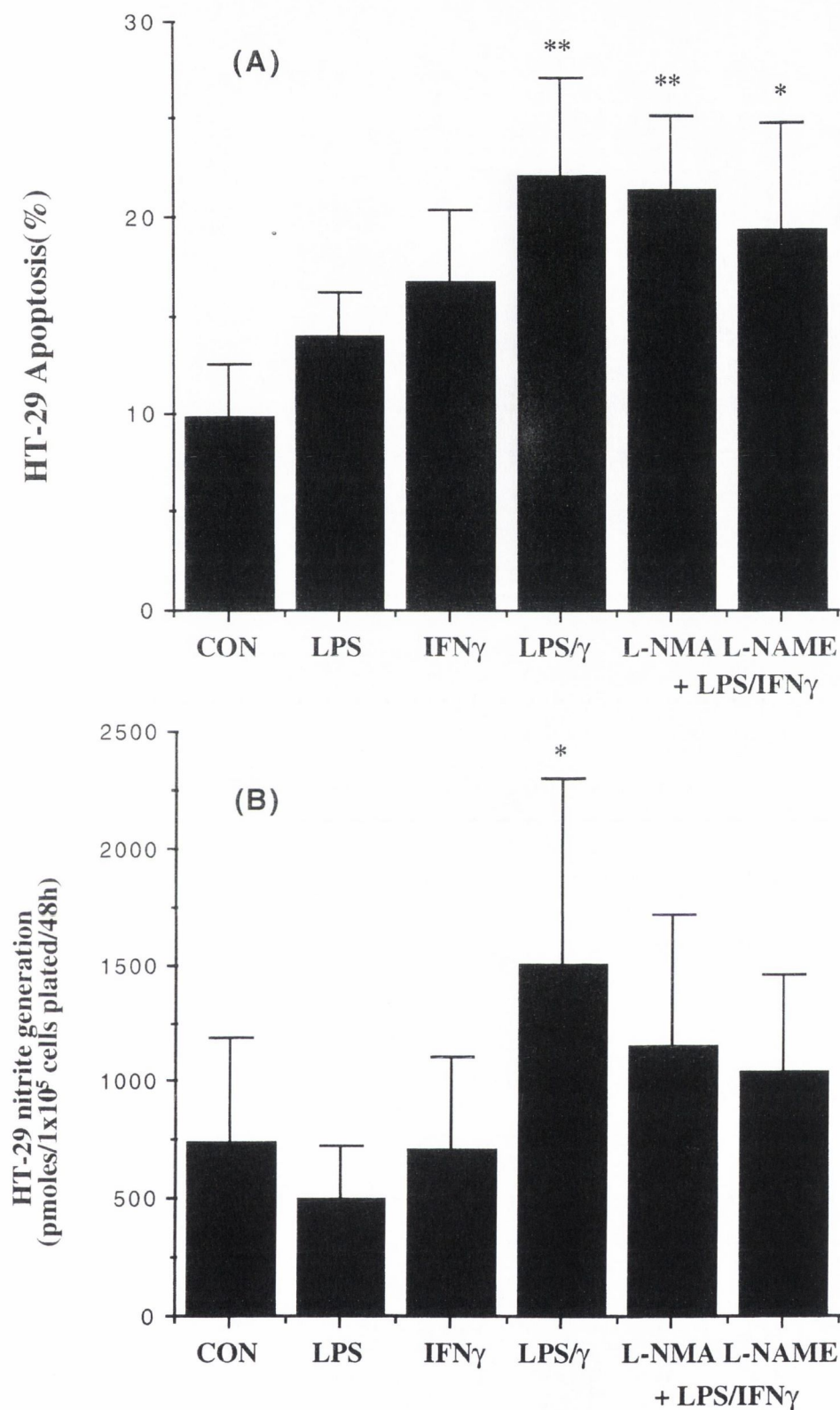


Figure 4.7 The effect of cytokines and NOS inhibitors on (A) enterocyte apoptosis and (B) nitrite generation in cultured HT-29 cells. Subconfluent cells were plated (1×10^5 cells) with LPS ($2 \mu\text{g/ml}$), IFN- γ (200 U/ml) alone or in combination with L-NMMA ($100 \mu\text{M}$) or L-NAME ($100 \mu\text{M}$) in McCoy's medium for 48 hr. Nitrite generation in the culture medium was determined using the Griess assay. Apoptosis was determined flow cytometrically using annexin V/PI staining. Results are expressed as mean value $\pm$ SEM of 5 separate experiments in the case of (A), ** $p < 0.01$, * $p < 0.05$ vs no stimulus (Repeated Measures Analysis of Variance) or the mean triplicate value of 4 separate experiments in (B), * $p < 0.05$ vs LPS alone (Repeated Measures Analysis of Variance).

Inducing agents	Enterocyte necrosis (%)	
	HT-29	Caco-2
	(n=5)	(n=3)
NONE	12.61 ± 3.2	3.64 ± 0.9
LPS (2 µg/ml)	12.02 ± 2.1	4.53 ± 0.3
IFN-γ (200 U/ml)	10.43 ± 1.2	5.58 ± 0.9
LPS/IFN-γ (2 µg/ml, 200 U/ml)	13.29 ± 2.1	3.35 ± 0.2
LPS/IFN-γ + L-NMMA (100 µM)	12.09 ± 1.0	5.79 ± 0.5
LPS/IFN-γ + L-NAME (100 µM)	13.81 ± 1.8	4.11 ± 0.7

Table 4.3 Enterocyte necrosis in resting and activated colonic epithelial cell lines, Caco-2 and HT-29. A variety of cytokines and NOS inhibitor (as shown) were added to subconfluent monolayers of Caco-2 and HT-29 cells for 48 hours. Necrosis was measured flow cytometrically using annexin V/PI staining. Data is expressed as mean ± SEM of n experiments (as shown).

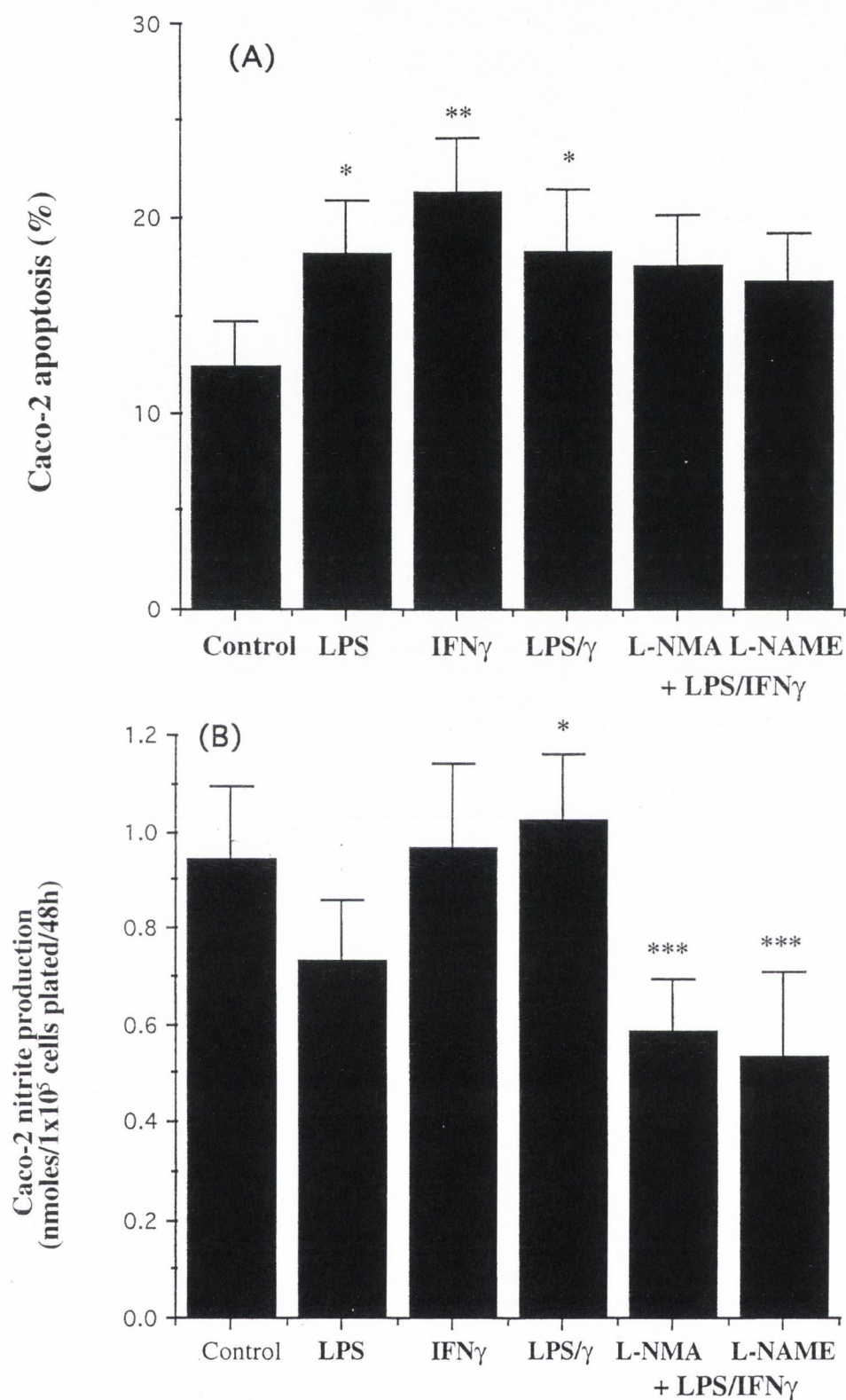


Figure 4.8 The effect of cytokines and NOS inhibitors on (A) enterocyte apoptosis and (B) nitrite generation in Caco-2 cell monolayers. Subconfluent cells (1×10^5 cells) were stimulated with LPS ($2 \mu\text{g/ml}$), IFN- γ (200 U/ml) alone or in combination with L-NMA ($100\mu\text{M}$) or L-NAME ($100\mu\text{M}$) in MEM medium for 48 hr. Nitrite generation released into the culture medium was determined using the griess assay. Apoptosis was determined flow cytometrically using annexin V/PI staining. Results are expressed as mean value $\pm$ SEM of 3 separate experiments in the case of (A), ** $p < 0.01$, * $p < 0.05$ vs no stimulus (Repeated Measures Analysis of Variance) or the mean triplicate value of 4 separate experiments in (B), ** $p < 0.01$ vs LPS alone, *** $p < 0.001$ vs LPS/IFN- γ culture (Repeated Measures Analysis of Variance)

apoptosis in the Caco-2 cell monolayers ($18.25 \pm 6.0\%$) compared to spontaneous levels ($12.23 \pm 3.6\%$). Incubation with IFN- γ alone was sufficient to cause significant increases in apoptosis over media alone (Figure 4.8b). Addition of $100\mu\text{M}$ L-NAME or L-NMMA to LPS/IFN- γ -activated Caco-2 cells was not significantly different from control rates of apoptosis. The addition of any of the cytokines and nitric oxide synthase inhibitors did not induce cell injury by necrosis (Table 4.4). These results suggest that the proinflammatory cytokines induce apoptosis without significantly affecting NO production in Caco-2 cell monolayers.

4.7.3 LPS and IFN- γ increase NO production in undifferentiated Caco-2 cells without significantly affecting Caco-2 apoptosis

Previous studies have demonstrated decreased activity of iNOS during the differentiation of Caco-2 cells (Vecchini *et al.*, 1997, Hague *et al.*, 1997). In order to prevent Caco-2 cell anchorage and thus enterocyte differentiation, Caco-2 cells were maintained in a polypropylene container at 37°C . To confirm if enterocyte differentiation may affect endogenous NO production by Caco-2 cells, Caco-2's (1×10^6 cells/ml) were stimulated with LPS ($2 \mu\text{g/ml}$) and IFN- γ (200 U/ml) in a single cell suspension for 48 hours. The combination of LPS and IFN- γ significantly ($p < 0.01$) increased NO production from 0.206 ± 0.15 in unstimulated cells to $0.505 \pm 0.13 \text{ nmoles}/10^6/48 \text{ h}$ (Figure 4.9a). The presence of L-NAME in LPS/IFN- γ cultures decreased NO levels back to that of control ($0.156 \pm 0.08 \text{ nmoles}/10^6/48 \text{ h}$). Loss of anchorage in these cell suspensions leads to increased apoptosis over cells maintained in monolayers as spontaneous levels of apoptosis in cell suspensions were $27.72 \pm 5.6\%$ in Caco-2 cell suspensions compared to $9.81 \pm 3.5\%$ in monolayers at 48 hours culture. LPS and IFN- γ in combination non-significantly increased Caco-2 apoptosis in cells maintained in a cell suspension (Figure 4.9b). Treatment of Caco-2 cells with LPS/IFN- γ resulted in $34.97 \pm 4.4\%$ apoptosis compared to $27.72 \pm 5.6\%$ in media alone. The NOS inhibitor, L-NAME decreased rates of apoptosis to that of control Caco-2 cells maintained in a cell suspension.

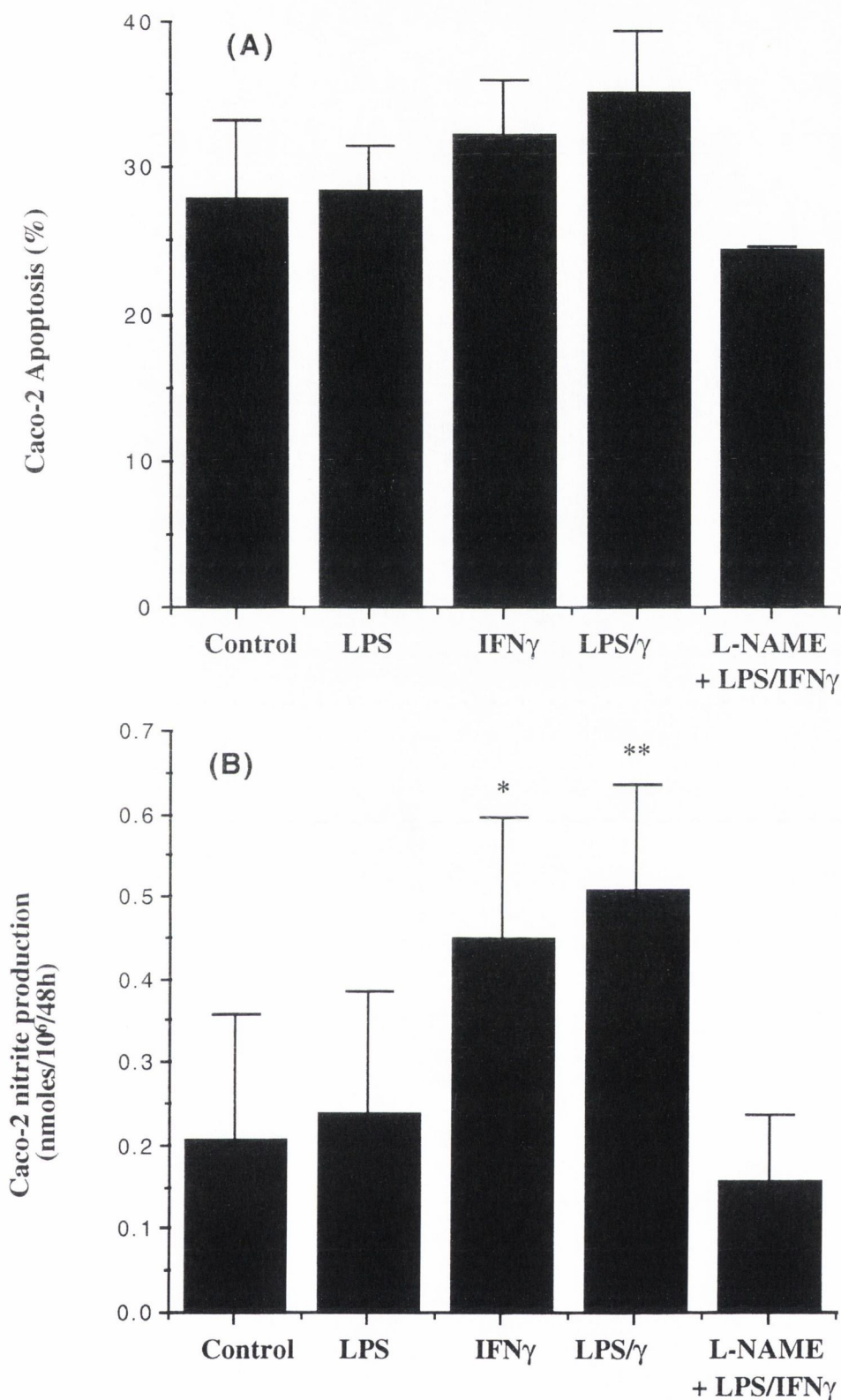


Figure 4.9 The effect of cytokines and NOS inhibitors on (A) enterocyte apoptosis and (B) nitrite generation in Caco-2 cells maintained in a cell suspension. Caco-2 (1×10^6 cells/ml) were stimulated with LPS ($2 \mu\text{g/ml}$), IFN- γ (200 U/ml) alone or in combination with L-NAME ($100 \mu\text{M}$) in MEM for 48 hr. Nitrite generation in the culture medium was determined using the griess assay. Apoptosis was determined flow cytometrically using PI staining. Results are expressed as the mean value $\pm$ SEM of 3 separate experiments, * $p < 0.05$, ** $p < 0.01$ vs no stimulus (Repeated Measures Analysis of Variance).

4.7.4 Taurine and HEPES have no effect on LPS/IFN- γ -activated colonic epithelial cell apoptosis or NO production

In order to assess the effects of the anti-oxidant, taurine and its structural analogue, HEPES on apoptosis in LPS/IFN- γ -activated enterocytes, subconfluent HT-29 and Caco-2 cell monolayers were cultured with LPS and IFN- γ in the presence of 0.25, 0.5 and 1 mg/ml taurine or HEPES. Addition of taurine or HEPES at any of these concentrations failed to significantly decrease HT-29 and Caco-2 NO production (Table 4.4) or apoptosis (Table 4.5). However, addition of taurine (1 mg/ml) to LPS/IFN- γ -activated HT-29 cell monolayers or taurine (0.25 mg/ml) to LPS/IFN- γ -activated Caco-2 monolayers decreased NO production and apoptosis which approached statistical significance. The presence of taurine and HEPES in the culture media did not affect necrosis in the HT-29 cell line but both taurine and HEPES increased necrosis in the Caco-2 cell line at all concentrations (Table 4.6).

Inducing agents	NO production (nmoles/well/48h)	
	HT-29	Caco-2
LPS/IFN- γ (2 μ g/ml, 200 U/ml)	1.50 $\pm$ 0.8	1.022 $\pm$ 0.14
LPS/ γ + Taurine (0.25 mg/ml)	1.48 $\pm$ 0.4	1.125 $\pm$ 0.23
LPS/ γ + Taurine (0.5 mg/ml)	1.30 $\pm$ 0.6	0.845 $\pm$ 0.26
LPS/ γ + Taurine (1 mg/ml)	1.09 $\pm$ 0.4	0.810 $\pm$ 0.23
LPS/ γ + HEPES (0.25 mg/ml)	1.50 $\pm$ 0.7	1.173 $\pm$ 0.14
LPS/ γ + HEPES (0.5 mg/ml)	1.12 $\pm$ 0.5	0.924 $\pm$ 0.27
LPS/ γ + HEPES (1 mg/ml)	0.91 $\pm$ 0.7	1.092 $\pm$ 0.36

Table 4.4 Taurine and HEPES have no significant effects on NO production in LPS/IFN- γ -activated HT-29 and Caco-2 cells. Taurine or HEPES were added to LPS/IFN- γ -cultures of Caco-2 and HT-29 cell monolayers for 48 hours. NO production was assessed as the stable end product of nitrogen metabolism, nitrite by the griess assay. Data is expressed as mean $\pm$ SEM of 4 seperate experiments.

Inducing agents	Enterocyte Apoptosis (%)	
	HT-29	Caco-2
	(n=5)	(n=3)
LPS/IFN- γ	22.06 $\pm$ 5.1	18.25 $\pm$ 6.0
LPS/ γ + Taurine (0.25 mg/ml)	17.86 $\pm$ 4.5	17.81 $\pm$ 4.5
LPS/ γ + Taurine (0.5 mg/ml)	17.68 $\pm$ 1.6	13.09 $\pm$ 2.1
LPS/ γ + Taurine (1 mg/ml)	18.43 $\pm$ 4.0	17.83 $\pm$ 7.0
LPS/ γ + HEPES (0.25 mg/ml)	20.01 $\pm$ 4.3	21.19 $\pm$ 3.7
LPS/ γ + HEPES (0.5 mg/ml)	19.09 $\pm$ 5.6	19.26 $\pm$ 5.6
LPS/ γ + HEPES (1 mg/ml)	18.23 $\pm$ 6.1	22.74 $\pm$ 8.4

Table 4.5 Taurine and HEPES have no significant effects on apoptosis in LPS/IFN- γ -activated HT-29 and Caco-2 cells. Taurine or HEPES were added to LPS/IFN- γ -cultures of Caco-2 and HT-29 cell monolayers for 48 hours. Apoptosis was measured flow cytometrically by annexin V/PI staining. Data is expressed as mean $\pm$ SEM of n experiments (as shown).

Inducing agents	Enterocyte Necrosis (%)	
	HT-29	Caco-2
	(n=5)	(n=3)
LPS/IFN- γ	13.29 $\pm$ 2.1	3.35 $\pm$ 0.2
LPS/ γ + Taurine (0.25 mg/ml)	13.81 $\pm$ 2.5	4.60 $\pm$ 0.9*
LPS/ γ + Taurine (0.5 mg/ml)	11.69 $\pm$ 1.0	7.29 $\pm$ 4.9*
LPS/ γ + Taurine (1 mg/ml)	14.33 $\pm$ 3.5	4.61 $\pm$ 0.5*
LPS/ γ + HEPES (0.25 mg/ml)	11.51 $\pm$ 2.0	5.77 $\pm$ 1.0*
LPS/ γ + HEPES (0.5 mg/ml)	11.07 $\pm$ 1.1	6.06 $\pm$ 0.7*
LPS/ γ + HEPES (1 mg/ml)	12.03 $\pm$ 2.1	5.82 $\pm$ 0.8*

Table 4.6 The effect of taurine and HEPES on necrosis in LPS/IFN- γ -activated enterocytes. Taurine or HEPES were added to LPS/IFN- γ -cultures of Caco-2 and HT-29 cell monolayers for 48 hours. Necrosis was measured flow cytometrically by annexin V/PI staining. Data is expressed as mean $\pm$ SEM of n experiments (as shown), * p<0.001 vs LPS/IFN- γ alone (Repeated Measures Analysis of Variance)

4.8 Discussion

Under resting conditions, minute amounts of NO (pmoles) are synthesized in the bowel wall by cNOS regulating motility, salt and water absorption and blood flow. Under pathophysiological conditions, however, an inducible isoform of NOS is produced *de novo*, involving transcription of new mRNA and biosynthesis of new protein producing large amounts of NO (nmoles) for up to several days. Enhanced production of NO through increased iNOS activity has been demonstrated in patients with ulcerative colitis (Rachmilewitz *et al.*, 1994, Singer *et al.*, 1996). Although the sources of this NO generation have not been identified, there is some evidence that gastrointestinal epithelial cells represent an important source (Salzman *et al.*, 1996, Dignass *et al.*, 1995, Kolios *et al.*, 1995). The intestinal mucosa is composed of intrinsic resident tissue cells including epithelial cells and immune cell populations which rapidly turnover. Epithelial cells of the gut are characterized by rapid constant cell renewal. Enterocytes have a life span of 6-7 days in the human and 2 to 3 days in the mouse and rat (Iwanaga *et al.*, 1995). Enterocyte cell number in the gastrointestinal tract is highly regulated with cell loss occurring through a highly controlled process called programmed cell death or apoptosis.

In this study, it was demonstrated that endogenously generated NO induces apoptosis in the HT-29 cell line. HT-29 colonic epithelial cells produce large quantities of nitrite in response to dual stimulation with LPS and IFN- γ . This is in agreement with *in vitro* studies that have shown iNOS activity in the human colon epithelial cell lines, HT-29 and DLD-1 cells upon stimulation by a combination of cytokines (Kolios *et al.*, 1995, Salzman *et al.*, 1996). Increases in NO production by LPS/IFN- γ -activated HT-29 cells correlated with increased expression of annexin V. Annexin V is an early marker of apoptosis as it binds phosphatidylserine exposed on the surface of apoptotic cells, one of the earliest events in the apoptotic process (Martin *et al.*, 1995). Neither cytokine alone significantly affected NO production or apoptosis suggesting LPS and IFN- γ act synergistically to induce NO-mediated apoptosis in the HT-29 cell line. In addition, cytokines and NOS inhibitors alone or in combination did not affect cell death by necrosis. These results are similar to studies in murine macrophages where NOS is induced by cytokines (IFN- γ) and bacterial derived

molecules (LPS) acting synergistically. The NOS inhibitors, L-NAME and L-NMMA reduced nitrite production and apoptotic rates non-significantly in LPS/IFN- γ cultures with L-NAME being more potent than L-NMMA. The lack of significance may be due to the concentration of inhibitor used (100 μ M) as some studies required concentrations of 1 mM to inhibit nitrite formation (Dignass *et al.*, 1995, Grisham, 199x). In conclusion, increased nitrite generation by LPS/IFN- γ -activated HT-29 cells is associated with increased HT-29 apoptosis.

Stimulation with LPS and IFN- γ increased Caco-2 apoptosis without upregulating nitrite production in Caco-2 monolayers. However, IFN- γ alone was sufficient to induce Caco-2 cell apoptosis. There are no reports of upregulation of Caco-2 NO production of cytokines by LPS or proinflammatory cytokines although enteroinvasive bacteria have been shown to directly induce expression of iNOS in Caco-2 cell monolayers (Witthoft *et al.*, 1998). Addition of the NOS inhibitors, L-NMMA and L-NAME to LPS/IFN- γ cultures decreased NO production without affecting Caco-2 apoptosis. Stimulation with LPS/IFN- γ may not therefore be a sufficient stimulus to upregulate epithelial iNOS expression within these well-differentiated epithelial cell monolayers. A recent study demonstrated that nitrite generation was greater in subconfluent cultures than fully confluent cultures suggesting contact inhibition (Hague *et al.*, 1997). Another study demonstrated that the expression of iNOS decreases during the differentiation of Caco-2 cells (Vecchini *et al.*, 1997). In order to prevent effects of cell differentiation on NO production, Caco-2 cells were maintained in a cell suspension. In these experiments, an upregulation of nitrite was seen upon stimulation with LPS and IFN- γ in Caco-2 cells which was inhibitable by L-NAME. Caco-2 cells maintained in a cell suspension had greater rates of apoptosis than monolayers confirming reports by Hague that cell-cell contacts may be an important survival factor for colonic epithelial cells (Hague *et al.*, 1997). However, LPS and IFN- γ failed to significantly increase Caco-2 cell apoptosis where there was a loss of anchorage. These results suggest that the ability of Caco-2 monolayers to produce NO is impaired or that NO is not a sufficient signal to induce apoptosis in the Caco-2 cell line.

As epithelial cells are difficult to culture, the majority of studies on colonic epithelial cells have been carried out using adenocarcinoma cell lines. The HT-29 and Caco-2 cell lines

have served as useful tools to study intestinal epithelial cell biology. Studies have demonstrated the similarity between Caco-2 cells ("type 1" enterocyte) and normal human intestinal epithelial cells possessing epithelial polarity, presence of an actin-binding protein, villin and the occurrence of enterocytic differentiation (Chantret *et al.*, 1988). The HT-29 cell line ("type 2" enterocyte) is an undifferentiated colon carcinoma cell line (Chantret *et al.*, 1988). Alterations in p53 gene expression are common in intestinal epithelial cell lines which are associated with colon cancer in humans.

Apoptosis can occur through p53-dependent or p53-independent pathways (Liebermann *et al.*, 1995). A study by Baker showed that expression of wild type p53 gene can suppress the growth of human colorectal carcinoma cells (Baker *et al.*, 1990) presumably by inducing apoptosis. NO induces nuclear accumulation of p53 protein in a time and dose-dependent manner which precedes apoptotic cell death in a number of cell types including RAW 264.7 macrophages (Messmer *et al.*, 1994, Messmer *et al.*, 1995). Apoptosis is more easily induced in response to endogenously generated NO in cells containing wild type 53 (Elledge and Lee, 1995). The differences between NO signalling in the two colonic epithelial cell lines used in this study may reflect the finding that the HT-29 cell line retains one wild type p53 allele and has high levels of immunoreactive p53 protein whereas the Caco-2 cell line contains deleted and mutant p53 alleles and has no detectable p53 protein (Djelloul *et al.*, 1997). These findings suggest that apoptosis within the Caco-2 cell line in comparison to the HT-29 cell line is not under the control of the tumour suppressor gene, p53. As p53 is so closely interlinked with NO in the induction of apoptosis, It appears that LPS and IFN- γ induce apoptosis through a p53/NO-independent pathway in Caco-2 cells.

As the Caco-2 cell line has no detectable p53 protein (due to deleted and mutated alleles), results obtained with the HT-29 cell line which retains a wild-type allele may be more relevant to actual apoptotic events within the gut. The demonstration that endogenously produced NO induces cell death through apoptosis in HT-29 cells is potentially important in understanding the effects of NO on homeostasis within the colonic epithelium. NO-induced apoptosis within the gut has important implications in IBD. Increased NO production from enterocytes in IBD may enhance cell death through apoptosis rather than necrosis thereby selectively removing enterocytes within the gut.

The anti-oxidant, taurine and HEPES have protective effects against TNBS-induced IBD in rats (Son *et al.*, 1996). In addition, taurine has previously been shown to attenuate apoptosis through an inhibition of both NO and ROS in hepatocytes (Redmond *et al.*, 1996). It was of interest if one of the cytoprotective effects of taurine within the gut would include an attenuation of NO-mediated enterocyte cell death. In this study, taurine and HEPES failed to significantly reduce LPS/IFN- γ -induced apoptosis or NO production in both epithelial cell lines, HT-29 and Caco-2. Taurine and HEPES increased Caco-2 necrosis but had no effects on necrosis in the HT-29 cell line. Levels of necrosis rose to a maximum of 7% in LPS/IFN- γ cultures in Caco-2 cells cultured with 0.5 mg/ml of taurine compared to 3% in control. Although these effects were statistically significant, the biological effects are likely to be insignificant. Taurine is a naturally occurring amino acid which has been used in numerous clinical situations and supplemented in food/drinks with minimal or no adverse effects.

In conclusion, endogenously produced NO induces enterocyte apoptosis in HT-29 cells which retain a p53 wild type allele. Results from this study are based on colon cancer cell lines which are generally resistant to apoptosis (O'Connell *et al.*, 1997) and may not be representative of actual effects of NO on enterocyte apoptosis within the gut. NO-induced apoptosis may have important consequences in causing tissue injury in IBD. The antioxidant, taurine and HEPES did not significantly protect against LPS/IFN- γ -induced apoptosis.

CHAPTER V

THE MODULATION OF EXPERIMENTAL COLITIS BY TAURINE AND ITS STRUCTURAL ANALOGUE, HEPES

5.1. Introduction

Ulcerative colitis (UC) is a chronic recurrent inflammatory disorder of unknown etiology characterized by neutrophil (PMN) influx into the colonic mucosa with enhanced production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Keshavarzian *et al.*, 1992, Rachmilewitz *et al.*, 1995). In active intestinal inflammation, PMNs transmigrate across the intestinal epithelium which is mediated by the cell surface adhesion receptor, CD11b/CD18. Both ROS and RNS have been implicated in extensive cell and tissue damage in colitis (Evans, 1995). Hypochlorous acid (HOCl) produced by the H_2O_2 -myeloperoxidase- Cl^- (H_2O_2 -MPO- Cl^-) system is the most potent ROS produced by activated PMNs (Eaton, 1993, Weiss, 1989). Also upregulated in ulcerative colitis, nitric oxide (NO) possesses beneficial vasodilatory and anti-inflammatory properties (Moilanen *et al.*, 1993, Stadler *et al.*, 1993) although paradoxically is associated with tissue injury in colitis (Brune *et al.*, 1995). However, recent evidence suggests that tissue injury is primarily mediated through the reaction between NO and various redox forms of oxygen (Grisham *et al.*, 1995). Simultaneous production of O_2^- and NO form peroxynitrite (ONOO^-), a powerful oxidant and nitrating agent.

Current therapies used in the treatment of inflammatory bowel disease have met with little success. The most effective prevailing treatments are the glucocorticosteroids and sulphasalazines administering limited beneficial effects (Podolski *et al.*, 1991a, Podolski *et al.*, 1991b). HEPES (N-2-hydroxyethylpiperazine-N'-2-ethane sulphonic acid) is a structural analogue of the β -amino acid, taurine, the most abundant free amino acid in inflammatory cells (Fukuda *et al.*, 1982). Taurine and its structural analogues function as anti-oxidants through their ability to sequester hypochlorous acid producing their respective chloramines (McLoughlin *et al.*, 1991, Lambert and Weiss, 1983). Taurine reduces NADP(H)-dependent respiratory burst activity *in vitro*. In addition, taurine reduces LTB_4 levels which were postulated to be mediated through upregulated MPO activity (Stapleton *et al.*, 1993).

Taurine possesses anti-inflammatory activity in a diverse range of animal models of inflammation including arthritis and autoimmune diseases in which neutrophil recruitment is believed to play a role (Schuller-Levis *et al.*, 1994, Witso-Sarvat *et al.*, 1995, Wang *et al.*,

1992). Research from our laboratory has demonstrated that taurine attenuates PMN influx across the pulmonary vasculature in an animal inflammatory model (Barry *et al.*, 1995). Carrageenan-induced paw edema was attenuated following intraperitoneal injection of HEPES which was not found with taurine (Stapleton *et al.*, 1994). The greater efficacy of HEPES as an anti-inflammatory agent compared to taurine was attributed to its slower systemic clearance (Stapleton *et al.*, 1994). Within the gut, taurine protects against nonsteroidal antiinflammatory drug-induced gastric mucosal injury which was attributed to its anti-oxidant properties which inhibit lipid peroxidation and PMN activation. Although taurine through its chlorinated derivative attenuates proinflammatory mediator generation including NO by macrophages (Park *et al.*, 1995, Marcinkiewitz *et al.*, 1995), the efficacy of taurine or its analogues to influence NO *in vivo* remains to be elucidated. In particular, the ability of taurine or HEPES to modulate the balance between reactive oxygen and nitrogen species in an experimental model of inflammation is unknown. In particular, HEPES coupled with its relative slow clearance from the systemic circulation may be beneficial in an experimental model of colitis.

5.2 Aims

In the present study, the efficacy of the natural compound, taurine and its structural analogue, HEPES was assessed in a rat model of TNBS/ethanol-induced colitis. We hypothesized that taurine and HEPES through regulation of PMN adhesion and modulation of leukocyte derived oxygen and nitrogen species may downregulate the inflammatory response *in vivo*.

5.3. Results

5.3.1 Tissue injury

Administration of TNBS/EtOH produced an acute inflammation of the colon characterized by infiltration of neutrophils primarily to the mucosa and submucosa. Extensive thickening of the bowel wall (<2 cm along length of colon), ulceration, fecal impaction, haemorrhage and oedema were prominent features observed in this inflammatory model (Figure 5.1a and 5.1b). A non-significant reduction in TNBS/EtOH-induced colitis was observed using a colon macroscopic score (CMS) following oral supplementation with taurine. However, HEPES significantly ($p<0.05$) reduced tissue injury by reducing CMS from an average score of 6.5 ± 0.8 to 4.5 ± 0.7 in animals treated with TNBS/EtOH (Table 5.1). A colon microscopic score was used to assess leukocyte infiltration and mucosal disruption. The HEPES-treated group had reduced leukocyte accumulation in the tissue with less mucosal disruption (Table 5.1). Reduction in tissue injury assessed by CMS was found to correlate with the extent of leukocyte infiltration in untreated colitis ($R^2=0.835$) (Figure 5.1a). PMN counts indicated that in colitis, there was a greater influx of PMNs into the mucosa than the submucosa. HEPES served to reduce PMN counts in both the submucosa and the mucosa (Table 5.1).

5.3.2 Taurine and HEPES decrease PMN adhesion in experimental colitis

PMN CD11b receptor expression was elevated in experimental colitis (Figure 5.2). CD11b, the receptor responsible for neutrophil adhesion to ICAM-1 on the vascular endothelium was increased in both portal and systemic blood in experimental colitis. There was a 2.5 fold increase in portal blood and 1.4 fold increase in systemic blood CD11b receptor expression over group treated with saline (Figure 5.2). The administration of taurine significantly ($p<0.05$) decreased CD11b receptor expression in portal blood but did not significantly affect receptor expression in systemic blood. HEPES did not significantly decrease PMN CD11b receptor expression in either portal or systemic bloods sampled (Figure 5.3).

	Colon Macroscopic score (CMS)	Colon Microscopic score (CMiS)	PMN counts	
			Submucosa	Mucosa
COLITIS	6.45 ± 0.8 (n=11)	3.1 ± 0.3 (n=11)	140 ± 20 (n=9)	113 ± 18 (n=9)
+ Taurine (4%)	6.00 ± 1.0 (n=8)	2.8 ± 0.6 (n=8)	ND	ND
+ HEPES (4%)	4.47 ± 0.7*(n=15)	2.3 ± 0.4 (n=15)	113 ± 28 (n=4)	70 ± 20 (n=4)

Table 5.1 Tissue injury scores in Sprague-Dawley rats pretreated with taurine or HEPES before induction of experimental colitis. Male Sprague-Dawley rats were administered with a 4% solution of taurine or HEPES in their drinking water for 11 days. On Day 8, experimental colitis was induced with a single intra-rectal administration of trinitrobenzenesulphonic acid (30 mg) in 50% ethanol (1 ml). Tissue colonic samples were retrieved 3 days later, preserved in formalin and tissue injury scores were assessed by a blinded pathologist in the Histopathology Department, Beaumont Hospital. Data is expressed as mean ± SEM of n experiments (as shown), *p<0.05 Mann Whitney. (ND = not determined)

A



B



C



Figure 5.1a Macroscopic appearance of colons in (A) saline-treated animals, (B) animals with experimental colitis or (C) animals pretreated with HEPES prior to induction of experimental colitis. Male Sprague-Dawley rats were administered trinitrobenzenesulphonic acid (30 mg) in 50% ethanol to induce colitis. Animals were pretreated with HEPES for eight days prior to induction of colitis in group C.

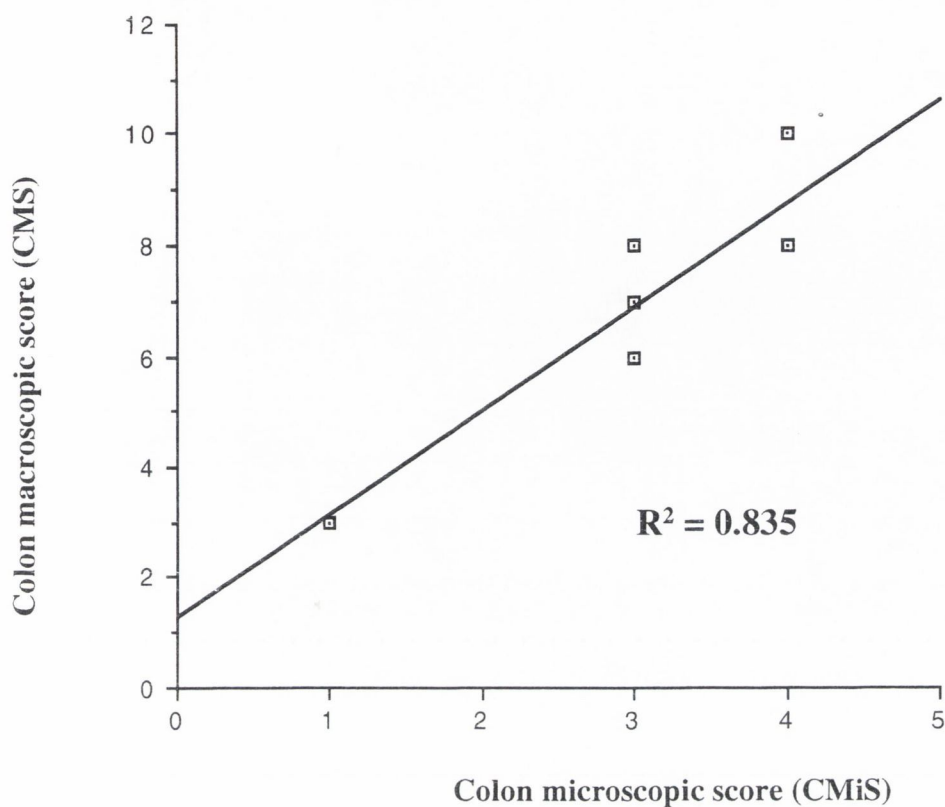


Figure 5.1b A graph comparing the two scoring system used to assess tissue injury in TNBS/EtOH-induced colitis in untreated colitis (n=11). A colon macroscopic score (CMS) is an assessment of gross morphological changes whereas the colon microscopic score (CMiS) assesses the extent of leukocyte influx and epithelial destruction within the colonic tissue.

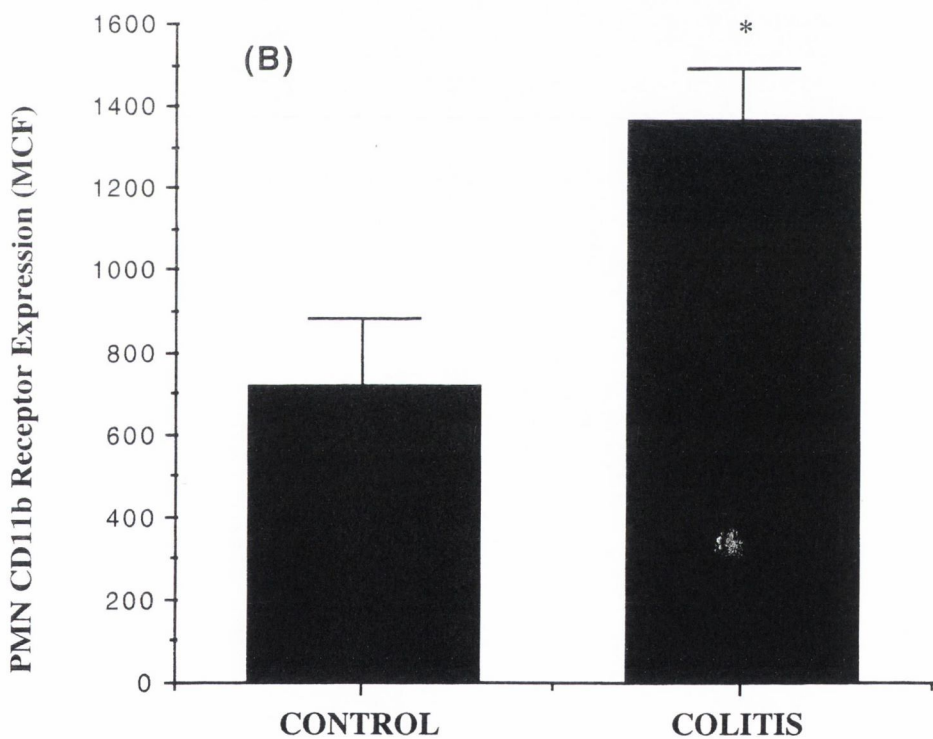
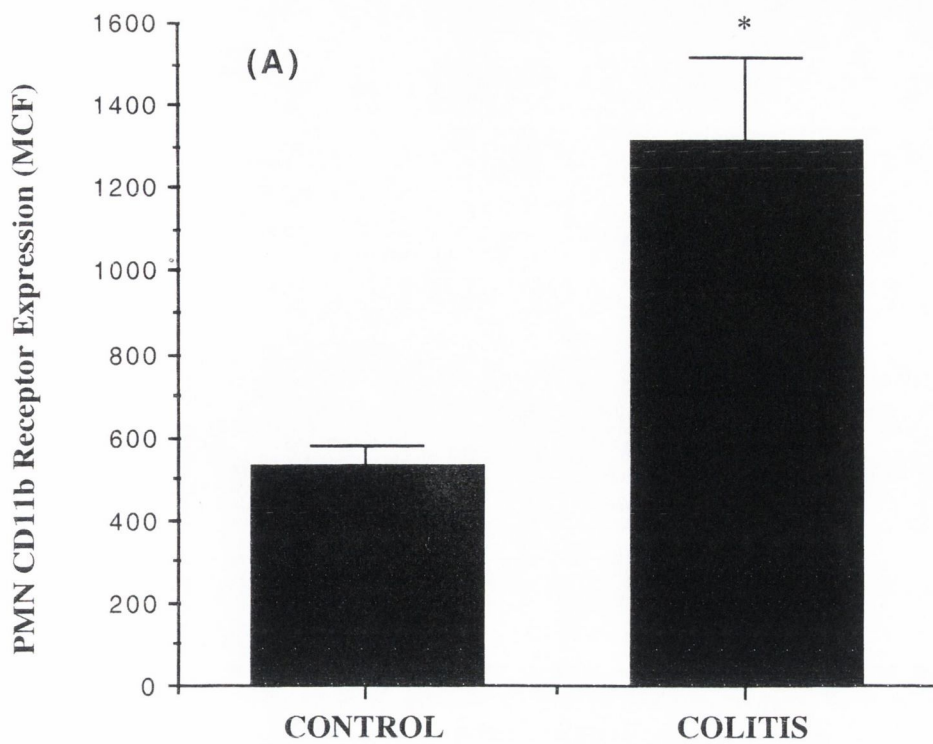


Figure 5.2 PMN CD11b receptor expression in (a) portal and (b) systemic bloods taken from male Sprague-Dawley rats with experimental colitis. Experimental colitis was induced with a single intrarectal administration of trinitrobenzenesulphonic acid (30 mg) in 50% ethanol (1 ml). 3 days later, portal blood was cannulated from the inferior mesenteric vein and systemic blood taken from the superior vena cava. PMN CD11b receptor expression was assessed flow cytometrically using a rat anti-MAC-1 antibody in portal and systemic bloods. Results are expressed as the mean value $\pm$ SEM of 5 animals, * $p < 0.05$ vs saline treated (Analysis of Variance with LSD post-hoc test).

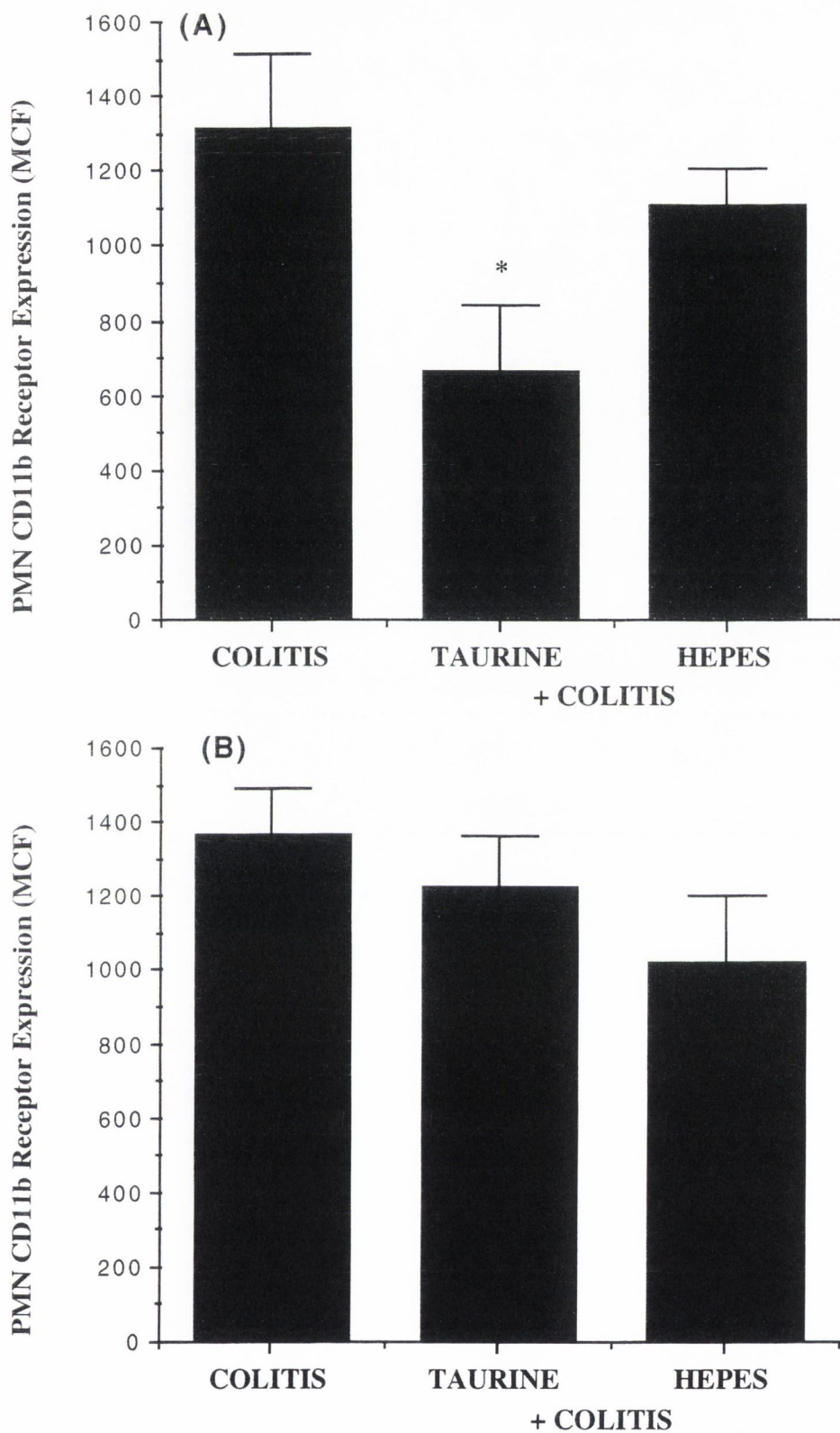


Figure 5.3 The effect of administration of taurine and HEPES on PMN CD11b receptor expression in (a) portal and (b) systemic bloods taken from Sprague-Dawley rats with experimental colitis. Male Sprague-Dawley rats were administered a 4% solution of taurine or HEPES prior to administration of trinitrobenzenesulphonic acid (30 mg) in 50% ethanol (1 ml). CD11b receptor expression was assessed flow cytometrically using an anti-rat MAC-1 antibody in portal and systemic bloods. Results are expressed as the mean value $\pm$ SEM of 5 animals, * $p < 0.05$ vs saline treated (Analysis of Variance with LSD post-hoc test).

5.3.3 PMN respiratory burst activity in experimental colitis

Stimulating respiratory burst activity with rat opsonised *E. coli* resulted in a significant reduction in PMN respiratory burst activity in animals with experimental colitis compared to the saline-treated group (Figure 5.4). No differences were found between portal or systemic blood sampled. Administration of taurine led to a reduction in PMN respiratory burst activity but this was significant ($p<0.05$) in HEPES-treated animals in portal and systemic blood sampled (Figure 5.5)

5.3.5 Tissue MPO activity in experimental colitis

Tissue MPO activity was significantly increased ($p<0.05$) in TNBS/EtOH-induced colitis in both minimally inflamed and chronically inflamed areas of the colon (Figure 5.6). HEPES served to significantly ($p<0.05$) increase tissue MPO activity from 13.09 ± 2.3 to 24.44 ± 3.0 MPO U/g tissue in inflamed areas with taurine having no significant effect (Figure 5.7a). In minimally inflamed areas of the colon, taurine or HEPES had no significant effect on tissue MPO activity (Figure 5.7b).

5.3.5 Taurine and HEPES increase tissue NO generation in experimental colitis

Nitric oxide generation by cultured mucosal explants obtained from inflamed and minimally inflamed areas of the colon from animals with colitis was 2.2 and 1.8-fold higher than by mucosal explants obtained from saline-treated subjects (Figure 5.8). Mean nitric oxide in the saline-treated group was 6.12 ± 1.0 compared to 14.8 ± 1.7 nmoles/g tissue/24 hrs in inflamed areas of the colon in experimental colitis animals. Nitric oxide generation by explants obtained from healthy, uninflamed mucosa was significantly lower than by inflamed ($p<0.01$) or minimally mucosa ($p<0.05$) obtained from the same animals. Taurine and HEPES increased NO generation non-significantly in TNBS/EtOH-treated groups with HEPES approaching statistical significance (Figure 5.9).

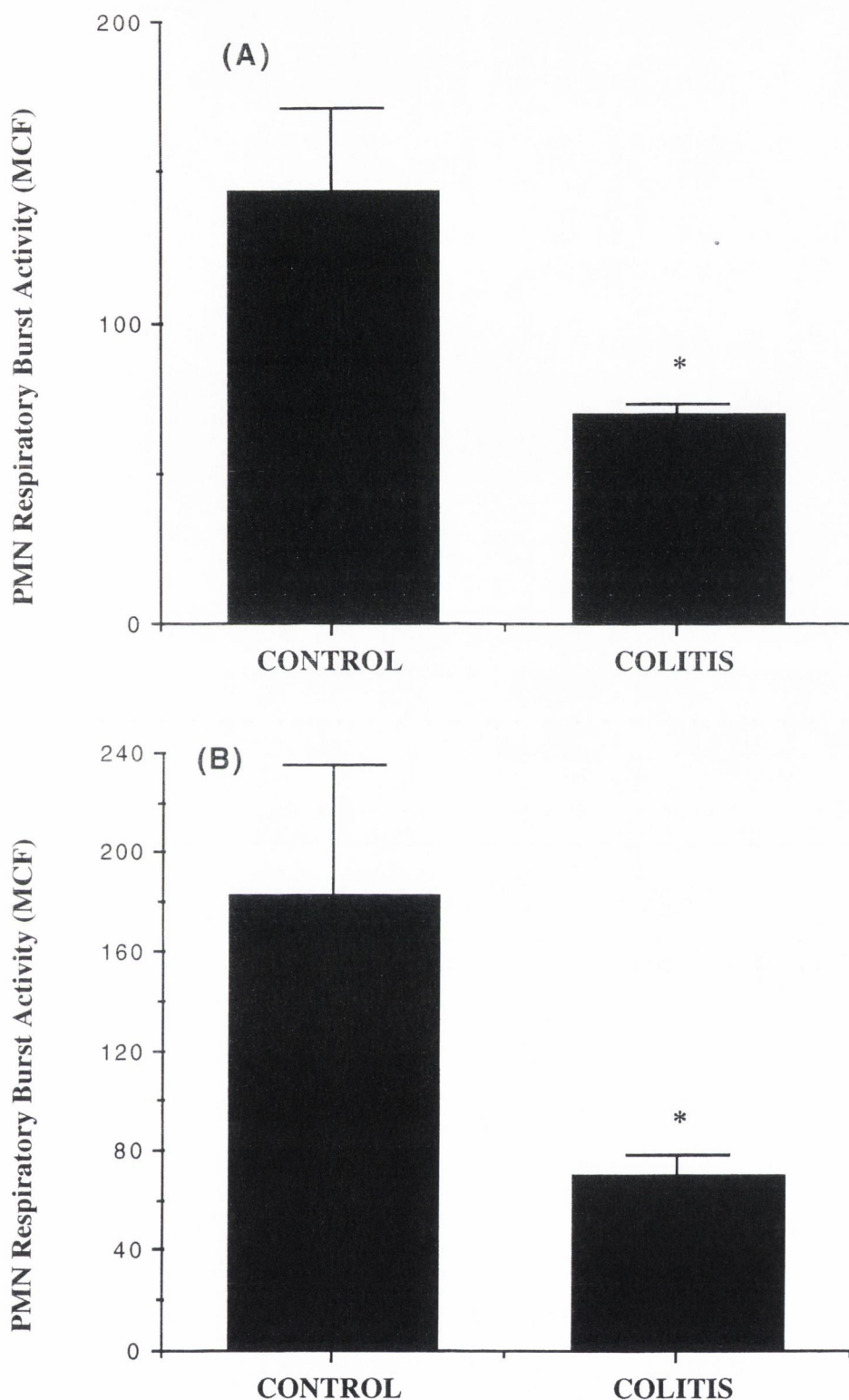


Figure 5.4 PMN respiratory burst activity in (a) portal and (b) systemic bloods taken from male Sprague-Dawley rats with experimental colitis. Experimental colitis was induced with a single intrarectal administration of trinitrobenzenesulphonic acid (30mg) in 50% ethanol (1 ml). 3 days later, portal blood was cannulated from the inferior mesenteric vein and systemic blood taken from the superior vena cava. Respiratory burst activity was assessed flow cytometrically in PMNs stimulated with rat opsonised *E. Coli* using the intracellular probe, Dihydrorhodamine 123 in portal and systemic bloods. Results are expressed as the mean channel fluorescence $\pm$ SEM of 11 animals, * $p < 0.05$ vs saline treated (Analysis of Variance with LSD post-hoc test).

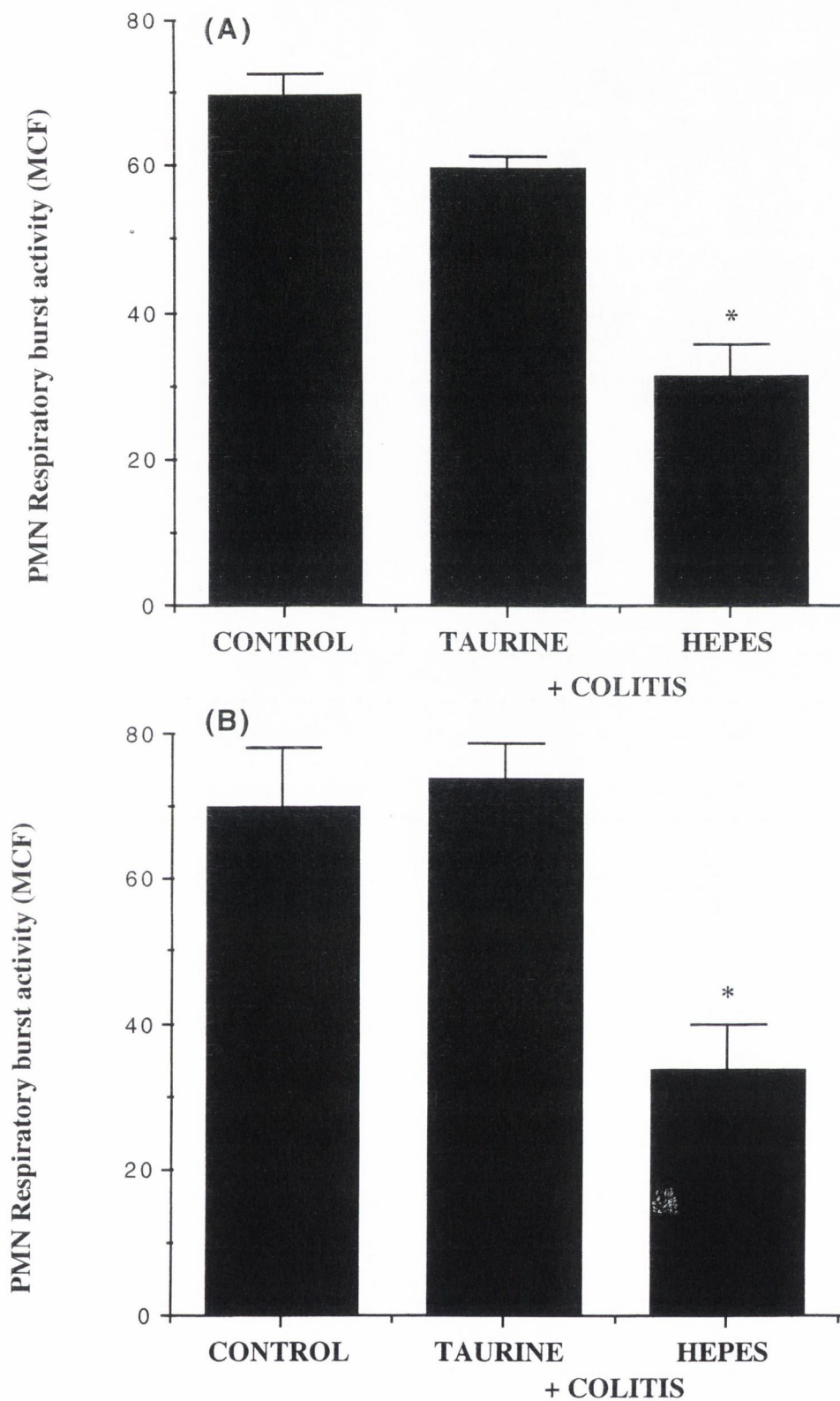


Figure 5.5 The effect of administration of taurine and HEPES on PMN respiratory burst activity in (a) portal and (b) systemic bloods taken from Sprague-Dawley rats with experimental colitis. Male Sprague-Dawley rats were administered a 4% solution of taurine or HEPES prior to administration of trinitrobenzenesulphonic acid (30 mg) in 50% ethanol (1 ml). Respiratory burst activity was assessed flow cytometrically in PMNs stimulated with rat opsonised *E. Coli* using the fluorogenic probe, Dihydrorhodamine 123 in portal and systemic bloods. Results are expressed as the mean channel fluorescence $\pm$ SEM of 11 animals in saline-treated, 8 animals in taurine-treated group and 15 animals in HEPES-treated groups, * $p < 0.05$ vs saline treated (Analysis of Variance with LSD post-hoc test).

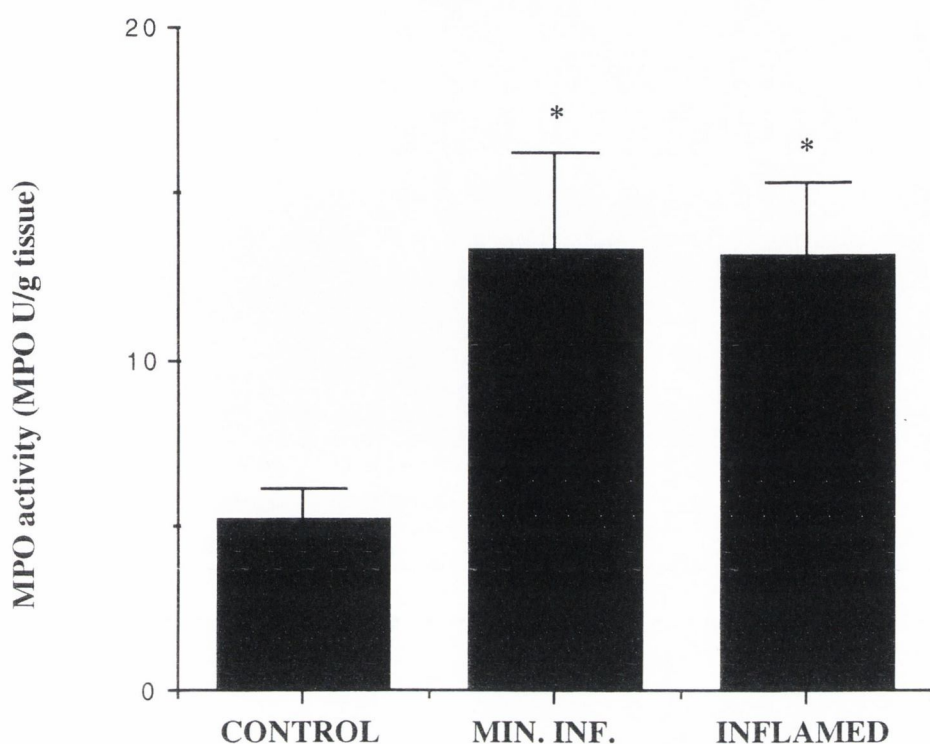


Figure 5.6 Tissue MPO activity in minimally inflamed and inflamed areas of the colon of male Sprague-Dawley rats with experimental colitis. Experimental colitis was induced with a single intrarectal administration of trinitrobenzenesulphonic acid (30 mg) in 50% ethanol (1 ml). 3 days later, colonic tissue (approximately 0.1g) was excised from minimally inflamed and inflamed areas of the colon, homogenised and frozen in solution A. Tissue MPO activity was assessed in the supernatant of centrifuged tissue samples subjected to 3 freeze-thaw cycles using the o-dianisidine method. Results are expressed as the mean value $\pm$ SEM of 5 animals, * $p < 0.05$ vs saline treated (Analysis of Variance with LSD post-hoc test).

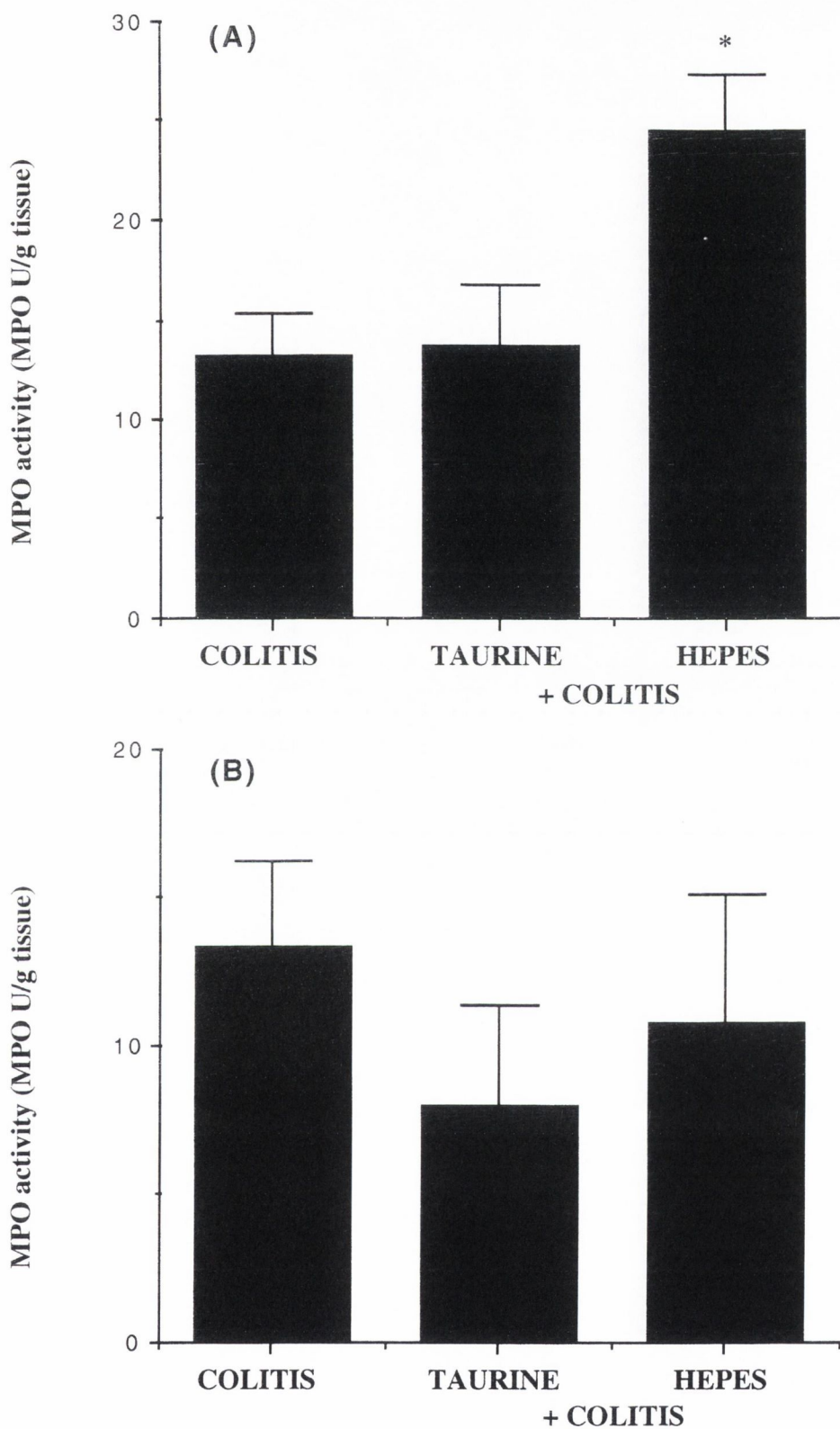


Figure 5.7 The effect of administration of taurine and HEPES on tissue MPO activity in (a) inflamed and (b) minimally inflamed areas of the colon of sprague-dawley rats with experimental colitis. Male Sprague-Dawley rats were administered a 4% solution of taurine or HEPES prior to administration of trinitrobenzenesulphonic acid (30 mg) in 50% ethanol (1 ml). Tissue MPO activity was assessed in the supernatant of centrifuged tissue samples subjected to 3 freeze-thaw cycles using the o-dianisidine method. Results are expressed as the mean $\pm$ SEM of 5 animals, * $p < 0.05$ vs saline treated (Analysis of Variance with LSD post-hoc test).

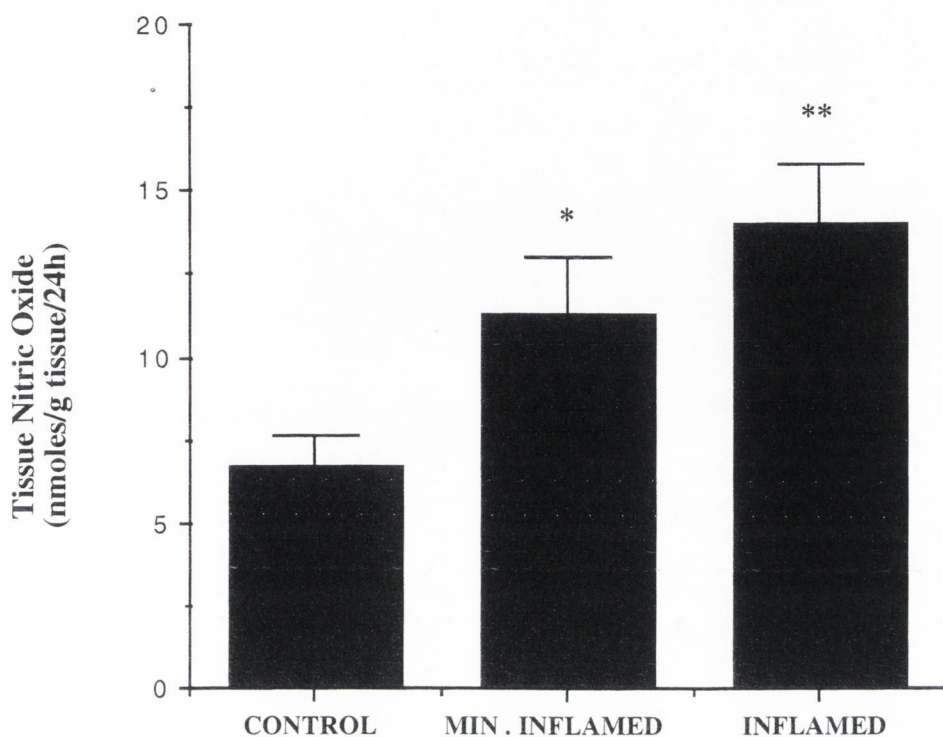


Figure 5.8 Tissue NO production in minimally inflamed and inflamed areas of the colon of male Sprague-Dawley rats with experimental colitis. Experimental colitis was induced with a single intrarectal administration of trinitrobenzenesulphonic acid (30 mg) in 50% ethanol (1 ml). 3 days later, colonic explants were taken from minimally inflamed and inflamed areas of the colon and cultured for 24 hours. Tissue NO production was assessed as nitrite accumulation in culture media using the Griess assay. Results are expressed as the mean value $\pm$ SEM of 11 animals, * $p < 0.05$, $p < 0.01$ vs saline treated (Analysis of Variance with LSD post-hoc test).

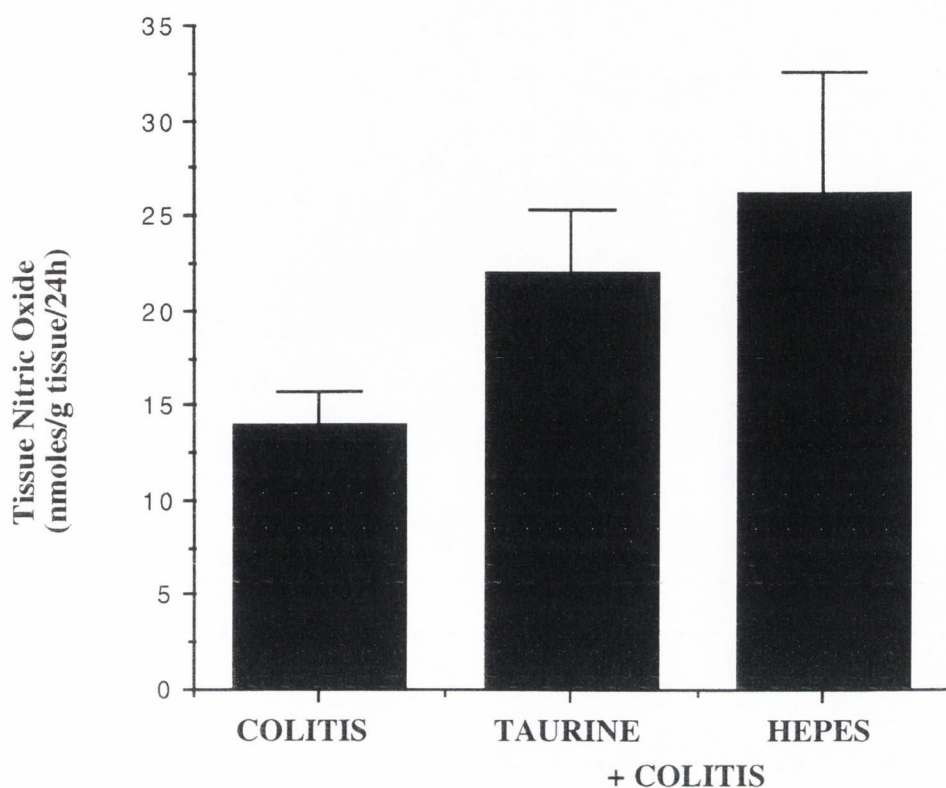


Figure 5.9 The effect of administration of taurine and HEPES on tissue NO production in inflamed areas of the colon of Sprague-Dawley rats with experimental colitis. Male Sprague-Dawley rats were administered a 4% solution of taurine or HEPES prior to administration of trinitrobenzenesulphonic acid (30 mg) in 50% ethanol (1 ml). Tissue NO production was assessed as nitrite accumulation in culture media of colonic explants cultured for 24 hours using the Griess assay. Results are expressed as the mean value $\pm$ SEM of 11 animals in control, 8 animals in taurine-treated group and 15 animals in HEPES-treated group.

5.4 Discussion

Intrarectal administration of TNBS/EtOH results in colonic inflammation with biological and pathological similarities to human ulcerative colitis (Morris *et al.*, 1989). Intrarectal administration of TNBS/EtOH induces a profound colonic inflammation which is accompanied by widespread haemorrhage, oedema and pronounced neutrophil infiltration. A greater number of PMNs were recruited into the colonic mucosa than the submucosa in this TNBS/EtOH experimental model of colitis. This study demonstrated a significant reduction in macroscopic tissue injury with administration of HEPES. In addition, HEPES-treated animals had fewer inflammatory cells as assessed by a colon microscopic score and fewer PMN counts in both the colonic mucosa and submucosa. Taurine treatment resulted in non-significant reductions in tissue injury scores in experimental colitis.

The significance of HEPES but not taurine on tissue injury in this rat experimental model of colitis may be explained by the ability of HEPES to be retained in the systemic circulation for longer periods of time than taurine (Stapleton *et al.*, 1993). Pharmacokinetic studies using a rat model of carrageenan-induced hind paw inflammation have shown that HEPES is retained in the circulation for longer periods of time. Intravenous injection of [^{14}C]HEPES or [^3H]taurine showed the $t_{1/2}$ value for HEPES (44.48 mins) to be greater than taurine (18.08 mins). The K_e value calculated by JANA was $0.0492 \mu\text{mol min}^{-1}$ for taurine compared with $0.0168 \mu\text{mol min}^{-1}$ for HEPES. This indicates a significantly slower clearance for the synthetic analogue in comparison with the natural compound.

PMNs influxing the colon have enhanced ability to adhere to the vascular endothelium as seen through an upregulation of PMN CD11b levels in this model of colitis. CD11b is an adhesion receptor present on both PMNs and monocytes that ligates endothelial ICAM-1. PMNs derived from rats treated with TNBS/EtOH had increased CD11b levels in both portal and systemic bloods sampled indicating that this experimental model of colitis affects PMN adhesion in the systemic circulation as well as the portal blood supply. This demonstrates that colonic inflammation profoundly influences systemic blood PMN function which may account for some of the systemic complications associated with inflammatory

bowel disease (IBD) such as distal arthropathy. Taurine decreased PMN CD11b receptor expression in portal blood in experimental colitis with HEPES causing non-significant reductions. Downregulation of CD11b receptor expression is a potent anti-inflammatory strategy. Reduced PMN infiltration into inflamed areas in the presence of taurine or HEPES may decrease the influx of proinflammatory mediators into the colon with subsequent attenuation in colonic tissue damage observed in this study. The mechanism of attenuation of this adhesion receptor by taurine and HEPES are likely to be through its membrane stabilising properties.

The respiratory burst pathway generates highly reactive metabolites of oxygen such as hypochlorous acid (HOCl) through the MPO-H₂O₂-halide system as well as superoxide (O₂⁻) and hydroxyl radicals (OH⁻). Although these reactive oxygen species are involved in bacterial killing, their production in IBD in the absence of any known stimuli may promote host tissue injury. In this experimental model, PMN respiratory burst activity in response to rat opsonised *E. Coli* was reduced although tissue myeloperoxidase (MPO) activity was augmented. Increased MPO activity in UC has been well established suggesting enhanced tissue PMN activity in colitis. Enhanced respiratory burst activity has been demonstrated in inflamed tissue from both patients with ulcerative colitis (Sedghie *et al.*, 1993, Williams, 1990) and in animal models of experimental colitis (Keshavarzian *et al.*, 1992). Sources of ROS within the gut include phagocytic leukocytes infiltrating the inflammatory site or activated tissue cells such as colonic epithelium or vascular endothelium but may also reflect production of O₂⁻ from xanthine oxidase.

Although activated leukocytes are well known sources of reactive oxygen species (ROS), reports on PMN respiratory burst activity in colitis have been conflicting. Many authors have reported a reduction in PMN superoxide (O₂⁻) formation and chemiluminescence responses from patients with ulcerative colitis (UC) and Crohn's disease (Gionchetti *et al.*, 1994, Verspaget *et al.*, 1986, Williams *et al.*, 1990, Worsaae *et al.*, 1982) whilst there are fewer reports demonstrating increased PMN O₂⁻ generation or enhanced chemiluminescence (Faden and Rossi, 1985). These conflicting results are difficult to explain although they may be due to methodological differences or different stimuli used to activate

PMNs. In contrast, there is overwhelming evidence that there is enhanced respiratory burst activity in monocytes and macrophages (Williams *et al.*, 1989, Mahida *et al.*, 1989). Increased chemiluminescent responses were found in monocytes from patients with UC and Crohn's disease, however, PMNs from these same patients produced significantly lower chemiluminescent responses (Williams *et al.*, 1990). Recently, it has been demonstrated that colonic epithelial cells are capable of producing large amounts of ROS (Deitch *et al.*, 1995). In this study, colonic epithelial cells were found to kill a greater percentage of bacteria than PMNs and it was concluded that these cells may function as "nonprofessional" phagocytes (Deitch *et al.*, 1995). Therefore, this present study suggests that there is enhanced tissue respiratory burst activity in colitis but the capacity of PMNs influxing the colon to produce ROS is impaired. This may be due to the existence of recently synthesized or immature and thus less responding PMNs in the circulation. It has been suggested that this inability of the PMN to mount an effective inflammatory response may contribute to the disease process. However, it should be remembered that these cells are not present at the site of inflammation and their relevance to events within the bowel wall is questionable. Enhanced ROS production by mucosal phagocytes in IBD may play an important role in the pathogenesis of tissue damage in colonic inflammation (reviewed by Williams, 1990)

HEPES significantly downregulated PMN respiratory burst activity in experimental colitis with taurine reducing PMN respiratory burst activity non-significantly. The significance of this finding may not be important, however, as there is already reduced PMN ROS production in portal and systemic blood sampled in this experimental model of colitis. More importantly, this finding does not give any information on the effects of taurine or HEPES on ROS production by mucosal phagocytes. It has previously been reported that HEPES like taurine conjugates form its chlorinated derivative with HOCl (Stapleton *et al.*, 1994). HEPES may exert part of its anti-inflammatory effects through sequestration of HOCl to form HEPESchloramine. Taurine and HEPES have been shown to reduce ROS production both *in vitro* and in animal models of inflammation and injury. Taurine and its structural analogue, HEPES may downregulate ROS production in mucosal phagocytes and/or activated tissue cells within the bowel wall.

Tissue MPO activity was found to be elevated in our HEPES-treated group in this model of experimental colitis. Taurine and its analogues are known activators of MPO activity *in vitro* and *in vivo* (Stapleton *et al.*, 1994, Stapleton *et al.*, 1995, Finnegan *et al.*, 1998). HOCl is the most destructive agent synthesized by activated PMNs approximately 100 to 1000 times more toxic than H₂O₂ (Eaton, 1993). HOCl is scavenged by taurine or HEPES to form taurinechloramine and HEPESchloramine respectively (Lambert *et al.*, 1983, Stapleton *et al.*, 1993). Chloramines maintain bactericidal activity but are markedly less toxic to host tissue (Thomas *et al.*, 1979). Enhanced MPO levels in this study with taurine and its analogues may reflect enhanced activity through the MPO-H₂O₂-halide system thereby increasing the formation of chloramines from HOCl. HOCl is indiscriminate and will oxidize host cell membranes (Weissmann *et al.*, 1978). An essential methionine residue at position 358 of human plasma α_1 -proteinase inhibitor is susceptible to oxidation by HOCl. Therefore, the ability of taurine and its analogues to scavenge HOCl may result in less tissue damage through inactivation of α_1 -proteinase inhibitor. Although tissue MPO activity is generally regarded as a marker of PMN infiltration (Williams *et al.*, 1983, Barone *et al.*, 1991), We have demonstrated enhanced tissue MPO activity with fewer PMNs within the colonic mucosa and submucosa in a model of colitis. PMNs may be at various states of activation within inflamed tissue in colitis. MPO activity is a measure of activation of the MPO enzyme and not necessarily an indication of the degree of PMN presence within an inflammatory site, as generally accepted. Thus, the administration of taurine and HEPES is associated with increased MPO activity most likely reflecting increased activity through the MPO-H₂O₂-halide system forming chloramines.

Enhanced colonic nitric oxide (NO) generation was demonstrated in inflamed and minimally inflamed areas of the colon of animals administered TNBS/EtOH. This confirms reports which demonstrated increases in colonic nitric oxide generation and inducible NOS (iNOS) compared to controls (Rachmilewitz *et al.*, 1995). NO was non-significantly increased in the HEPES-treated groups in this chemical model of colitis. Nitrite accumulation was determined in mucosa cultured at 24 hours as it has been reported that mucosal morphology of surface epithelial cell structure is preserved in culture for up to 24 hours (Eastwood *et al.*, 1973). Taurine and HEPES increase nitrite accumulation non-significantly

by cultured mucosa reflecting increased NO production from surface epithelial cells. In this study, taurine and HEPES most likely increase NO through constitutive NOS (cNOS) as inducible NOS requires new protein synthesis and is unlikely to be induced at the 24 hour culture time point chosen in this study. Additionally, NO release is likely to be due to the epithelial cells within the colonic explant as it would be more difficult for NO to diffuse from cells underlying colonic epithelium. Unpublished research from our laboratory has demonstrated that taurine increases NO production and cNOS within the vascular endothelium. NO possesses many gastroprotective and anti-inflammatory properties. NO is a vasodilator which inhibits PMN adhesion to the vascular endothelium (Kubes *et al.*, 1991). It mediates reduced LTB₄ levels (Ney *et al.*, 1990), one of the most potent chemotactic agents synthesized by activated neutrophils (Ford-Hutchinson, 1993). NO reduces the production of IL-6 by Kupffer cells and chondrocytes, and the production of IFN- γ and TNF- α by splenocytes (Stadler *et al.*, 1993, Evans, 1995). Tissue injury associated with NO is believed to be mediated through the formation of the ONOO⁻ anion (Grisham, 1995).

This study indicates that taurine and HEPES have beneficial effects in a chemically induced model of colitis. TNBS/EtOH-mediated tissue damage occurs through enhanced PMN influx across the endothelial barrier, production of ROS and NO and is pathologically similar to tissue damage observed in human colitis. HEPES but not taurine caused significant reductions in tissue injury in this experimental model of colitis. The efficacy of any pharmacological agent is dependent in part on its retention within the circulation. The half-life of intravenously administered HEPES as previously demonstrated in the rat (Stapleton *et al.*, 1994) indicated that it is sufficiently long to exert its anti-inflammatory effects. Enhanced levels of NO with a lowering of O₂⁻ within the inflammatory site by taurine and its analogues may limit the formation of ONOO⁻, a potent oxidant and nitrating agent. This may be one mechanism through which HEPES decreases tissue injury in this model of colitis. These findings suggest that HEPES warrants further investigation as a novel therapeutic agent in the management of inflammatory bowel disease.

CHAPTER VI

CONCLUDING REMARKS

Taurine is a conditionally essential amino acid but unlike α -amino acids is not incorporated into proteins but rather, is the most abundant free β -amino acid in inflammatory cells and many tissues. Since its discovery as a component of ox bile acid in 1827, the commonly established function of taurine up until the 1970's was bile salt formation, yet this accounts for only a tiny proportion of the functions of taurine. Synthesized from methionine and cysteine metabolism, taurine has been shown to play an important role in numerous physiological functions including detoxification, membrane stabilization, osmoregulation and the modulation of cellular calcium levels. *In vivo* studies have demonstrated that taurine deficiency is associated with different pathologies including cardiomyopathy, retinal degeneration and growth retardation especially if this deficiency occurs during development (Sturman, 1993). Clinically, taurine has been shown to reduce cellular damage associated with ischaemic-reperfusion injuries (Kramer *et al.*, 1981), to protect against lung injury mediated by various toxins and augment anti-microbial function in normal human PMNs and monocytes.

Anti-inflammatory properties of taurine *in vitro* have been previously reported. Stringent control of cellular taurine is maintained during periods of oxidative stress indicating an important role for this amino acid during inflammation (Huxtable, 1992). The structural analogue of taurine, HEPES possesses potent anti-inflammatory properties *in vivo* (Stapleton *et al.*, 1994). The greater anti-inflammatory effects of HEPES compared with taurine were attributed to its slower clearance *in vivo* (Stapleton *et al.*, 1994). This present study further assessed the ability of taurine and HEPES to act as anti-inflammatory agents in *in vivo* and *in vitro* models. The results obtained in this body of research suggest that a naturally occurring amino acid, taurine and its structurally related analogue, HEPES may offer a novel therapeutic strategy in the treatment of inflammatory diseases.

Modern treatment has not altered the natural history of chronic inflammatory bowel diseases (CIBD). The drugs currently used, mainly aminosalicyclates and corticosteroids intervene at numerous steps in the inflammatory process. 5-ASA is the active component of sulphasalazine, the most commonly used medication to treat IBD. Sulphasalazine will induce remission in 35% to 80% of patients with CIBD (reviewed by Lichtenstein, 1994). Side effects are commonly seen in patients (approximately 15%) taking sulphasalazine

which are dose-dependent (Bachrach, 1988). Sulphapyridine, the "carrier" molecule of sulphasalazine is responsible for side effects and intolerance to sulphasalazine. Corticosteroids are the most effective drugs in the treatment of CIBD since they induce remission in 3/4 of cases but have numerous side effects.

Activated PMNs have been implicated in the production of tissue injury in IBD through the release of proinflammatory mediators. Taurine acts as an antioxidant through the formation of taurinechloramine by sequestering HOCl, the most destructive agent synthesized by activated PMNs. Taurine possesses anti-inflammatory activity in a wide range of animal models of inflammation (Son *et al.*, 1996, Son *et al.*, 1998, Stapleton *et al.*, 1994). The *in vitro* effects of taurine or HEPES on PMN function have not been widely investigated. Results obtained from this study demonstrate that taurine and its structural analogue, HEPES differentially modulate the inflammatory response *in vitro*. Upon activation by receptor-dependent stimuli, taurine and HEPES prevent receptor-mediated influx of Ca^{2+} from the extracellular environment and subsequently have protective effects on Ca^{2+} -dependent PMN functions such as PMN respiratory burst activity, NO production and PMN adhesion. Taurine and HEPES have the capacity however, to enhance respiratory burst activity upon stimulation by non-receptor agonists which is most likely due to the biphasic effects of taurine on Ca^{2+} . The effects of taurine on Ca^{2+} have been referred to as the "calcium paradox" (Huxtable, 1992, Kramer, 1981).

Research on the effects of taurine and its analogues on PMN function *in vitro* have concentrated on the latter part of the respiratory chain, the MPO- H_2O_2 -halide system (Mc Loughlin *et al.*, 1991). The anti-inflammatory properties are dependent on the formation of taurinechloramines during respiratory burst activity. The conversion of taurine to taurinechloramine occurs in response to oxidative stress (Cunningham *et al.*, 1998). The formation of taurinechloramine is an anti-inflammatory mechanism as this compound modulates NO and other proinflammatory mediators (Park *et al.*, 1999). Taurinechloramine maintains bactericidal activity but is markedly less toxic to surrounding tissues (Thomas *et al.*, 1979). Analogues of taurine form stable chloramines *in vitro* (Stapleton *et al.*, 1993). Research from this study demonstrated that taurine and its analogue, HEPES affect the

initial steps in the respiratory burst pathway i.e. NADPH oxidase activation. Receptor-mediated activation of NADPH oxidase is Ca^{2+} -dependent. Using a Ca^{2+} -specific fluorescent probe, it was demonstrated that taurine inhibited Ca^{2+} influx into the PMN. Inhibition of Ca^{2+} influx is well documented as an anti-inflammatory strategy. For example, tolafenamic acid, a non-steroidal anti-inflammatory drug (NSAID) inhibits PMN function through an inhibition of Ca^{2+} influx (Kankaanranta *et al.*, 1995). Thus, a decrease in the production of ROS through NADPH oxidase by taurine and its analogues due to their Ca^{2+} -regulatory abilities may be a potent anti-inflammatory mechanism.

IBD is characterised by enhanced colonic production of NO. Within the inflamed gut, NO is released from activated tissue colonic epithelial cells, the vascular endothelium and cells migrating to the area such as PMNs and monocytes which release NO possibly upon phagocytosis. Cell death either can occur by apoptosis or necrosis in response to NO. Activated macrophages release NO as part of their own cell suicide (Albina *et al.*, 1993). This study assessed the role of NO in the modulation of apoptosis in the major inflammatory cell influxing the colon, the PMN and activated resident colonic epithelial cells, enterocytes.

This study demonstrated exogenously-added NO induced PMN apoptosis. There is one other study to date indicating that clinically relevant doses of NO induce apoptosis (Fortenberry *et al.*, 1998). Increased NO production by activated tissue cells such as in IBD may play a role in the termination of the PMN inflammatory response by inducing apoptosis. Results obtained from this study also demonstrate that at higher concentrations, NO can induce necrosis over apoptosis. This may support a number of studies implicating NO as a mediator of cell/tissue injury. Taurine supplementation (1 mg/ml) decreased nitrite generation in SNP-treated PMNs without significantly affecting PMN apoptosis. This study indicates that taurine directly interacts with NO in the PMN. Taurine supplementation in our laboratory has been shown to attenuate NO production in hepatocytes and endothelial cells (Redmond *et al.*, 1996, Wang *et al.*, 1996).

Endogenously produced NO was associated with decreased PMN apoptosis in this study. PMNs generated small amounts of NO in response to fMLP at 1h where expression

of NO is most likely through cNOS. However, fMLP-stimulated PMNs did not produce detectable amounts of cNOS protein using a commercially available antibody to the endothelial cNOS. Although cNOS has been purified in the PMN, there is only one report of the presence of cNOS protein which was detected using an antibody to the neuronal NOS. Future studies using more sensitive methods such as RT-PCR or the development of an antibody with closer homology to "neutrophil" cNOS may facilitate the measurement of cNOS in the PMN. Taurine increased NO production in resting and fMLP-stimulated PMNs without affecting PMN apoptosis in this study. Unpublished reports from our laboratory have demonstrated that taurine increased NO production through cNOS in HUVEC cells. Increased production of NO may downregulate the inflammatory response as constitutively-derived NO has been shown to inhibit leukocyte adhesion (Kubes et al., 1991) and NO donors inhibit PMN function (Moilanen *et al.*, 1993)

Stimulation of PMNs with a combination of proinflammatory cytokines, LPS, IFN- γ , TNF- α for 12h to examine expression of NO through iNOS failed to upregulate PMN nitrite production but increased PMN apoptosis. In addition, iNOS protein was undetected in these samples. No study to date has biochemically confirmed the presence of iNOS within the PMN. Increased superoxide production preceded increased peroxynitrite formation in LPS/IFN- γ /TNF- α -activated PMNs at 12h. These study suggests that NO production in activated PMNs is dictated by the fluxes between ROS and NO with maximal NO production where ROS and NO are not simultaneously induced. Endogenous production of NO may alter the balance of oxygen radicals thereby determining PMN survival.

The HT-29 and Caco-2 colonic epithelial cell lines are commonly used in the study of intestinal biology due to their similarity to enterocytes in culture. This study demonstrated that LPS/IFN- γ -HT-29 and Caco-2 cells are capable of producing large amounts of NO (nmoles) *in vitro*. Recent studies indicate that activated colonic epithelial cells are primarily responsible for increased NO production within the gut. iNOS has been detected in the inflamed colonic epithelium (Singer *et al.*, 1996). Results from this study would suggest that colonic epithelial cells release large amounts of NO through iNOS within the inflamed colon and may be primarily responsible for increased NO production in

IBD. Endogenously produced NO induced apoptosis in the HT-29 cell line which retains a p53 wild type allele. In contrast, LPS/IFN- γ activated Caco-2 cells may have important consequences in causing tissue injury in IBD. Tissue destruction caused by NO is likely to be mediated through the reaction of NO with various redox forms of oxygen (Wiseman *et al.*, 1995). ONOO⁻ possesses enhanced toxicity over O₂⁻ or NO alone. Nitrotyrosine labelling was observed in the inflamed colonic epithelium indicative of nitration of cellular proteins by ONOO⁻. Taurine did not significantly protect against LPS/IFN- γ -modulated apoptosis.

Induction of colitis by 2,4,6-trinitrobenzene sulphonic acid (TNBS) is a widely used experimental model of colitis. TNBS induces colitis involving infiltration of colonic mucosa by PMNs and increased production of inflammatory mediators (Kankuri *et al.*, 1999, Neilly *et al.*, 1995). HEPES caused a significant reduction in tissue injury in this model of experimental colitis. Taurine, having been shown to be anti-inflammatory *in vitro* did not administer significant reductions in tissue injury *in vivo*. However, taurine decreased the expression of the β 2 integrin, CD11b/CD18. The administration of HEPES did not alter PMN CD11b expression suggesting that HEPES acts through a non receptor-mediated pathway. HEPES modulates the inflammatory response through a direct interaction with the respiratory burst pathway. HEPES reduced PMN respiratory burst activity whilst enhancing tissue MPO activity ($p < 0.05$). This would indicate that HEPES increases the flux through the H₂O₂-MPO-halide system forming chloramines. In addition, HEPES may alter the balance between the levels of NO and ROS thereby reducing the formation of ONOO⁻. Pharmacokinetic studies using a rat model of carageenan-induced hind paw inflammation have shown that HEPES is retained for significantly longer periods than taurine in the circulation (Stapleton *et al.*, 1993). This longer retention period may account for the greater efficacy of HEPES *in vivo*. The efficacy of HEPES as a pharmacological agent demonstrated by this study suggest the future use of HEPES as an anti-inflammatory agent.

Decreased PMN taurine levels have been previously demonstrated in the inflammatory disease, psoriasis (Stapleton *et al.*, 1996). Correction of tissue homeostasis may limit tissue destruction in inflammatory diseases. Taurine and HEPES supplementation

offers the possibility of a fresh therapeutic approach in the management of inflammation. The administration of HEPES is a potent anti-inflammatory agent *in vivo*. As taurine has been used in a number of clinical situations without any adverse side effects, the administration of taurine and HEPES may eliminate side effects commonly observed in patients on medication for inflammatory diseases. Taurine and HEPES are potent inhibitors of specific mediators of inflammation which clinically should translate as anti-inflammatory agents with improved tolerability compared to current treatments in IBD. The administration of HEPES is a potent anti-inflammatory agent *in vivo* and may offer an improved approach to current treatment of inflammatory bowel disease.

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