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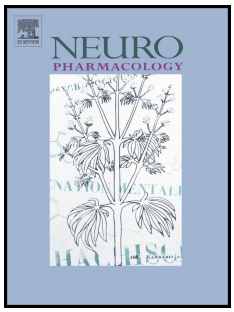

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Noradrenaline acting at β-adrenoceptors induces expression of IL-1β and its negative regulators IL-1ra and IL-1RII, and drives an overall anti-inflammatory phenotype in rat cortex

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Running Title: β_2-adrenoceptors activate the IL-1 system yet drive an anti-inflammatory phenotype in rat brain

Abstract

Evidence indicates that noradrenaline elicits anti-inflammatory actions in the central nervous system (CNS), and plays a neuroprotective role where inflammatory events contribute to pathology. Here we examined the ability of pharmacological enhancement of central noradrenergic tone to impact upon activation of the IL-1 system in rat brain. Treatment with the noradrenaline reuptake inhibitor reboxetine combined with the α_2-adrenoceptor antagonist idazoxan induced expression of IL-1β as well as its negative regulators, IL-1 receptor antagonist (IL-1ra) and IL-1type II receptor (IL-1RII) in rat cortex. The ability of reboxetine/idazoxan treatment to activate the IL-1 system was mediated by β-adrenoceptors, as the aforementioned effects were blocked by the β-adrenoceptor antagonist propranolol. Moreover, administration of the brain penetrant β_2-adrenoceptor agonist clenbuterol induced expression of IL-1β, IL-1ra and IL-1RII in rat brain. This action was selective to the IL-1 system, as other inflammatory cytokines including TNF-α, IL-6 or IFN-γ were not induced by clenbuterol. Induction of IL-1β was accompanied by activation of NFκB and of the MAP kinase ERK, and clenbuterol also induced expression of the IL-1β-inducible gene CINC-1. The ability of clenbuterol to activate the IL-1 system was blocked by propranolol, and was mimicked by the highly selective β_2-adrenoceptor agonist formoterol. Despite the ability of clenbuterol to activate the central IL-1 system, it largely combated the neuroinflammatory response induced by systemic inflammatory stimulus (bacterial lipopolysaccharide; LPS). Specifically, whilst the ability of clenbuterol to induce expression of IL-1RII and IL-1Ra was maintained following the inflammatory challenge, its ability to induce IL-1β was reduced. In addition, clenbuterol suppressed LPS-induced expression of the inflammatory cytokines TNF-α and IL-6, the inflammatory chemokines RANTES and IP-10, the co-stimulatory molecules CD40 and ICAM-1. Thus overall, clenbuterol suppresses the innate inflammatory response in rat brain.

Keywords: Noradrenaline, β-adrenoceptor, IL-1, IL-1ra, IL-1RII, neuroinflammation, LPS

1. Introduction

Neuroinflammation, characterised by inappropriate microglial activation and inflammatory mediator production, contributes to the pathophysiology of neurodegenerative states such as Alzheimer's disease, Parkinson's disease and ischaemic stroke (Allen et al., 2005; Block et al., 2007; Heneka and O'Banion, 2007). Evidence indicates that the neurotransmitter noradrenaline has anti-inflammatory properties, and plays an important role in maintaining the immunosuppressive environment of the brain (Feinstein et al., 2002; Heneka et al., 2002; Marien et al., 2004). Noradrenergic innervation is widespread throughout the brain, and following its release noradrenaline can reach proximal glial cells (Aoki, 1992) and modulate glial function by activating β_2-adrenoceptors (Mori et al., 2002).

Interleukin-1β (IL-1β) has been implicated as a key mediator of neuroinflammation (see Allan et al., 2005). Whilst IL-1β plays a number of physiological roles in the CNS (see Goshen et al., 2007; Pinteaux et al., 2009; Spulber et al., 2009), excessive production of this cytokine, particularly in the context of microglial activation, has been implicated in the neurodegenerative process (see Allan *et al*., 2005; Basu *et al*., 2004 for review). IL-1β signals by binding to the IL-1 type I receptor (IL-1RI) and recruitment of IL-1 receptor accessory protein (IL-1RAcp) (Sims *et al.,* 1988, Korherr *et al*., 1997), and this signalling system is present in the CNS (Ericsson *et al.,* 1995; Lynch and Lynch, 2002). The actions of IL-1β can be regulated by interleukin-1 receptor antagonist (IL-1ra), which prevents IL-1β from acting on the IL-1 type I receptor (IL-1RI) (Carter et al., 1990), and by the IL-1 type II receptor (IL-1RII); a decoy receptor that serves to sequester IL-1 (Colotta et al., 1994). Both IL-1ra and IL-1RII are expressed in brain (Pinteaux et al. 2002, 2006), and these molecules are known to prevent excessive or prolonged activation of the IL-1 system (Arend, 2002; Bessis et al., 2000), thus increased expression of these two molecules may serve to combat the deleterious effects of IL-1β on brain function. We recently demonstrated that noradrenaline induces expression of IL-1ra and IL-1RII in primary glial cells without altering expression of IL-1β, and the ability of noradrenaline to increase IL-1ra and IL-1RII was mediated via β-adrenoceptor activation (McNamee et al., 2010a).

Despite the established anti-inflammatory actions of noradrenaline and β-adrenoceptor agonists on glial cell activation *in vitro* (Dello Russo et al., 2004; Mori et al., 2002; McNamee

et al., 2010a), and the role of endogenous noradrenaline in limiting CNS inflammation *in vivo* (Feinstein et al., 2002; Heneka et al., 2002; Kalinin et al., 2007), the anti-inflammatory potential of agents that enhance central noradrenergic tone, or directly activate central β_2-adrenoceptors have been little examined to date. Recent data from our laboratory demonstrate that noradrenaline reuptake inhibitors (NRIs) reduce neuroinflammation following a systemic inflammatory challenge (O'Sullivan et al., 2009, 2010). In the present study we assessed the ability of the NRI reboxetine, alone and in combination with the α_2-adrenoceptor antagonist idazoxan, to impact upon expression of IL-1β, its receptors (IL-1RI, IL-1Racp and IL-1RII) and IL-1ra in rat cortex. Reboxetine is an antidepressant drug that increases noradrenaline availability by blocking the its reuptake via the noradrenaline transporter (Burrows et al., 1998; Wong et al., 2000). Idazoxan is an antagonist of inhibitory pre-synaptic α_2-adrenoceptors and greatly augments the ability of reboxetine to increase the synaptic availability of noradrenaline in rat brain (Sacchetti et al., 1999). Due to the key role of β_2-adrenoceptors in meditating the actions of noradrenaline on inflammatory events in the brain (Mori et al., 2002), we assessed the role of β-adrenoceptor activation in the ability of reboxetine/idazoxan to activate the IL-1 system. Furthermore, we assessed the ability of the β_2-adrenoceptor agonists clenbuterol and formoterol to induce expression of IL-1 system components in the brain. Clenbuterol and formotorol are bronchodilators used to treat respiratory disorders including asthma and chronic obstructive pulmonary disease (Baronti et al., 1980; Boner et al., 1988; Humbert et al., 2008; Papiris et al., 1986; Steiropoulos et al., 2008), and clenbuterol has been shown to have neuroprotective properties both *in vitro* and *in vivo* (Culmsee et al., 1999a,b; Gleeson et al., 2010).

As neurodegenerative diseases are associated with a neuroinflammatory state (see Block and Hong, 2005; Heneka and O'Banion, 2007) we determined if β_2-adrenoceptor activation could induce central IL-1ra and IL-1RII expression in the presence of an inflammatory stimulus (systemic challenge with bacterial lipopolysaccharide; LPS). We also determined if β_2-adrenoceptor stimulation could combat the more generalised neuroinflammatory response induced by LPS administration. Specifically, we examined the ability of clenbuterol to suppress LPS-induced expression of inflammatory cytokines IL-1β, TNF-α and IL-6, chemokines RANTES and IP-10 and co-stimulatory molecules CD40 and ICAM-1.

2. Materials and methods

2.1 Animals

Male Sprague-Dawley rats (200-250g) were obtained from Harlan, UK. Rats were maintained on a 12 h light: 12 h dark cycle (lights on at 08:00 h) in a temperature controlled room (22 ± 2˚C) and food and water were available *ad libitum.* The experimental protocols were in compliance with the European Communities Council directive (86/609/EEC).

2.2 Drugs

Reboxetine, idazoxan, clenbuterol and formoterol were obtained from Tocris Bioscience, UK. Propranolol, dexamethasone and lipopolysaccharide (E.coli: serotype 0111:B4) were obtained from Sigma-Aldrich, Ireland. All drugs were dissolved in 0.9 % NaCl and administered via the intraperitoneal (i.p.) route in an injection volume of 1ml/kg and 0.9 % NaCl was administered alone as a vehicle to control animals.

2.3 Experimental design

2.3.1 Experiment I: Effect of combined treatment with reboxetine and idazoxan on IL-1β, IL-1ra, IL-1RI, IL-1Racp and IL-1RII expression in rat cortex: This experiment was conducted to test the hypothesis that increasing central noradrenergic tone using combined treatment with the NRI reboxetine and the α_2-adrenoceptor antagonist idazoxan could induce expression of the anti-inflammatory components of the IL-1 system, IL-1ra and IL-1RII in rat brain. **We chose the cortex for analysis in this study as an in vivo comparator to our in vitro study where we show that noradrenaline and β-adrenoceptor agonists induces expression of IL-1ra and IL-1RII in glial cells prepared from rat cortex (McNamee et al., 2010a). In addition, the cortex receives rich noradrenergic input from the dorsal bundle arising from the locus coeruleus (Dahlstrom and Fuxe 1964), and we have shown that noradrenaline reuptake inhibitors (NRIs) elicit anti-inflammatory actions in rat cortex (O'Sullivan et al., 2009). Moreover the cortex is involved in higher cognitive processing and is adversely affected in many neurodegenerative diseases (Burton et al., 2004; Whitwell et al., 2007).**

The drug treatment regimen employed here was chosen based on its ability to increase extracellular noradrenaline concentrations in the CNS following acute administration.

Specifically, combined treatment with reboxetine (15mg/kg; i.p.) and idazoxan (1 mg/kg; i.p.) has been shown to elicit a robust increase in extracellular noradrenaline in rat cortex (Sacchetti *et al*., 1999).

Reboxetine (15mg/kg), Idazoxan (1mg/kg), or both drugs in combination were administered intraperitoneally (i.p.), and rats (*n*=6 per group) were killed 4 h later. 0.9% NaCl was administered to control animals as a vehicle treatment. The brain was dissected on an ice-cold plate and a portion of cortical tissue was harvested and placed into microtubes containing RNA later® (Ambion, UK) to ensure integrity of RNA for gene expression analysis. Another portion of cortex was rapidly frozen on dry ice in order to facilitate protein analysis. Samples were stored at -80˚C until mRNA expression or protein analysis was performed.

2.3.2 Experiment II: Can pre-treatment with the β-adrenoceptor antagonist propranolol block the ability of reboxetine/idazoxan treatment to activate the central IL-1 system?

Rats were pre-treated with either vehicle or the β-adrenoceptor antagonist propranolol (10 mg/kg; i.p.) 30 min prior to administration of reboxetine (15 mg/kg;ip)/idazoxan (1 mg/kg; i.p.) combination treatment. Rats were killed 4 h later and brain tissue was prepared and stored for analysis of IL-1β, IL-1ra and IL-1RII expression as outlined in Experiment I above.

2.3.3 Experiment III: Can treatment with the brain penetrant β_2-adrenoceptor agonist clenbuterol activate the central IL-1 system?

(a) A time-course analysis: Vehicle or clenbuterol (0.5 mg/kg; i.p.) was administered to rats and separate groups of rats were killed 1, 4 or 8 h following clenbuterol treatment. Brain tissue was prepared and stored as outlined in Experiment I above for measuring expression of IL-1 ligands (IL-1β and IL-1ra), IL-1 receptors (IL-1RI, IL-1RAcp, IL-1RII), IL-1 signalling (IκBα expression, MAP Kinase activation and CINC-1 expression), expression of other pro-inflammatory cytokines including IL-6, TNF-α and IFN-γ, and of apoptotic markers including caspase-3, bax and bcl-2 . A dose of clenbuterol (0.5 mg/kg) was used based on studies demonstrating that this dose has neuroprotective effects in a rat model of stroke (Zhu et al., 1999) and against kainic acid-induced neurotoxicity (Gleeson et al., 2010).

(b) A dose-response analysis: Vehicle or clenbuterol (0.03 – 0.3 mg/kg; i.p.) was administered to rats which were killed 4 h later, and brain tissue was prepared and stored as outlined in Experiment I above for analysis of IL-1β, IL-1ra and IL-1RII expression.

2.3.4 Experiment IV: Can pre-treatment with the β-adrenoceptor antagonist propranolol block the ability of clenbuterol to activate the central IL-1 system?

Rats were pre-treated with either vehicle or the brain penetrant β-adrenoceptor antagonist propranolol (10 mg/kg; i.p.) 30 min prior to clenbuterol (0.5 mg/kg; ip) treatment. Rats were killed 4 h later and brain tissue was prepared and stored as outlined in Experiment I above for analysis of expression of IL-1β, IL-1ra, IL-1RII and IκBα expression.

2.3.5 Experiment V: Can treatment with the highly selective β_2-adrenoceptor agonist formoterol mimic the ability of clenbuterol to activate the central IL-1 system? Vehicle or formoterol (0.5 mg/kg; i.p.) was administered to rats which were killed 4 h later. Brain tissue was prepared and stored as outlined in Experiment I above for analysis of IL-1β, IL-1ra, IL-1RII and IκBα expression.

2.3.6 Experiment VI: Effect of clenbuterol treatment on neuroinflammation induced by a systemic LPS challenge: Rats received either vehicle or clenbuterol (0.03 mg/kg; i.p.) co-administered with LPS (250 μg/kg; i.p.) and were killed 4 h later. Brain tissue was prepared and stored as outlined in Experiment I above for analysis of a range of markers of neuroinflammation.

We have previously found that this dose and route of administration of LPS produces quantifiable increases in cytokine, chemokine and co-stimulatory molecule expression in the CNS (O'Sullivan et al., 2009, 2010). This is a sub-septic dose of LPS that is approximately 10-fold lower than the LD_{50} (Hawes et al., 1992) and provokes modest stimulation of the immune response in rats, characterised by a transient increase in inflammatory mediator production.

2.4 Analysis of markers of neuroinflammation and neurodegeneration using Real-Time PCR

RNA was extracted from brain tissue using the NucleoSpin® RNA II total RNA isolation kit (Macherey-Nagel, Germany). Any genomic DNA contamination was removed with the addition of DNase to the samples according to the manufacturer's instructions. RNA was reverse transcribed into cDNA using a High Capacity cDNA Archive Kit (Applied Biosystems, Darmstadt, Germany). Real-time PCR was performed using an ABI Prism 7300 instrument (Applied Biosystems) as previously described (Boyle & Connor 2007). Taqman Gene Expression Assays (Applied Biosystems) containing forward and reverse primers and a FAM-labeled MGB Taqman probe were used to quantify each gene of interest. Assay ID's for the genes examined were as follows: : IL-1β (Rn00580432_m1), IL-1ra (Rn00573488_m1), IL-1RI (Rn00565482_m1), IL-1RII (Rn00588589_m1), IL-1RAcp (Rn00492642_m1), IκBα (Rn01473658_g1), TNF-α (Rn99999017_m1), IFN-γ (Rn00594078_m1), IL-6 (Rn00561420_m1), CD40 (Rn01423583_m1), CINC-1 (Rn00578225_m1), IP-10 (Rn00594648_m1), RANTES (Rn00579590_m1), ICAM-1 (Rn00564227_m1), bax (Rn02532082_g1), bcl-2 (Rn99999125_m1), caspase-3 (Rn00563902_m1). PCR was performed using Taqman® Universal PCR Master Mix and samples were run in duplicate. The cycling conditions consisted of 90˚C for 10 min and 40 cycles of 90˚C for 15 seconds followed by 60˚C for 1 min. β-actin was used as an endogenous control to normalize gene expression data. Relative gene expression was calculated using the ΔΔCT method with Applied BioSystems RQ software (Applied BioSystems, UK).

2.5 Analysis of MAP kinase phosphorylation using Western Immunoblotting

Brain tissue was lysed in ice-cold Hepes lysis buffer (Hepes (25mM), $MgCl_2$ (5mM), EDTA (5mM) in d.d.H_2O.) with the addition of DTT (5mM) and 1% (v/v) of both protease / phosphatase inhibitors (Sigma, UK). The resultant lysate was assayed for protein content using a BCA protein assay (Pierce) and diluted to give equal protein concentrations. Samples were diluted 1:1 in sample buffer (0.5 mM Tris-HCl, pH 6.8; 10% glycerol v/v; 10% SDS w/v, 5% 2 β-mercaptoethanol v/v, 0.05% bromophenol blue w/v) and boiled for 5 min prior to loading onto 10 % SDS-polyacrylamide gels. Proteins were separated by application of a constant voltage of 30mA for 25-35 minutes and transferred onto PVDF membranes (225 mA for 85mins). To assess the expression of specific proteins, membranes were incubated overnight at 4˚C in 10 ml TBS/Tween (0.1 % v/v) containing 1% BSA with one of the following

antibodies: pERK (Santa Cruz Biotechnology; Diluted 1:1000), ERK (Cell Signalling Technologies; Diluted 1:1000), pP38 (Cell Signalling Technologies; Diluted 1:1000), P38 (Cell Signalling Technologies; Diluted 1:1000), JNK (Cell Signalling Technologies; Diluted 1:1000); pJNK (Cell Signalling Technologies; Diluted 1:750). Following washing, immunoreactive bands were detected by incubating blots with either peroxidase-linked donkey anti-rabbit IgG (Amersham Biosciences; 1:10000) or peroxidase-linked goat anti-mouse IgG (Sigma, UK; 1:10000), where appropriate, in 10ml TBS/Tween (0.1% Tween-20 v/v) containing 1% BSA for 1h at room temperature.

Blots were exposed to Immobilon Western HRP substrate (Millipore) for 5 min and developed using an automated developer (Agfa CP1000). Bands were quantified by densitometric analysis using Gelworks ID, Version 2.51. Gelworks provides a single value (in arbitrary units) representing the density of each band. Blots were stripped using Re-Store Western Blot Stripping Buffer (Pierce) prior to re-probing.

2.6 Analysis of IL-1β concentrations by ELISA

IL-1β concentrations were measured in cortical tissue lysates using ELISA with cytokine specific antibodies and standards (R&D systems, UK). Assays were performed according to the manufacturer's instructions, and absorbance read at 450 nm using a microplate reader (Biotek instruments). Absorbance was then recalculated as a concentration (pg/ml) using a standard curve derived using GraphPad Prism Software Version 4.00 (GraphPad software, Inc).

2.7 Statistical analysis of data

All values are expressed as mean ± standard error of the mean (S.E.M). Data was analysed using a Student's *t*-test or a one- or two-way analysis of variance (ANOVA) followed, where appropriate, by a Newman-Keuls *post hoc* test (GB-Stat). A value of $P<0.05$ was considered statistically significant.

3. Results

3.1 Enhancement of the central noradrenergic tone induces expression of IL-1β and its negative regulators IL-1ra and IL-1RII in rat cortex: A role for β-adrenoceptor activation

Combined treatment with reboxetine and idazoxan induces IL-1β, IL-1ra and IL-1RII expression: Despite our recent finding that *in vitro* exposure of primary mixed glial cells to noradrenaline increases expression of IL-1ra and IL-1RII via activation of β-adrenoceptors (McNamee et al., 2010a), there is a paucity of research regarding the potential anti-inflammatory effects of pharmacological agents that enhance central noradrenergic tone *in vivo*. Consequently, in this study we assessed the impact of treatment with the NRI reboxetine (15 mg/kg; i.p.) and the α_2-adrenoceptor antagonist idazoxan (1mg/kg; i.p.), alone or in combination, on expression of ligands and receptors of the IL-1 cytokine system in rat cortex. Specifically, we examined expression of the pro-inflammatory cytokine IL-1β, the anti-inflammatory cytokine IL-1ra, IL-1RI and IL-1RAcp through which IL-1β signals, and IL-1RII; a decoy receptor with anti-inflammatory properties.

Combined treatment with reboxetine (15 mg/kg; i.p.) and idazoxan (1 mg/kg; i.p.) induced a robust increase in IL-1β (P<0.001), IL-1ra (P<0.05) and IL-1RII (P<0.001) expression in rat cortex (fig 1 a, b & e), without altering IL-1RI or IL-1RAcp expression (Fig 1c & d). Consistent with the fact that acute treatment with idazoxan alone does not increase noradrenaline release (Sacchetti et al., 1999), idazoxan treatment failed to induce the expression of IL-1ra, IL-1RI, IL-1II or IL-1RAcp. However, consistent with the fact that treatment with reboxetine alone provokes a modest increase in extracellular noradrenaline concentrations (Sacchetti et al., 1999), reboxetine induced a modest increase in cortical IL-1β expression (Fig 1a; P<0.05) but failed to alter IL-1ra, IL-1RII, IL-1RI or IL-1RAcp expression (Fig 1b-e).

β-adrenoceptors mediate the increase in IL-1β, IL-1ra and IL-1RII induced by combined treatment with reboxetine and idazoxan: Pre-treatment with the β-adrenoceptor antagonist propranolol (10 mg/kg; i.p.), blocked the increase of IL-1β (P<0.01), IL-1ra (P<0.05) and IL-1RII (P<0.01) induced by combined treatment with reboxetine and idazoxan (Fig 2).

3.2 Direct stimulation of β_2-adrenoceptors activates the central IL-1 system

Clenbuterol induces expression of IL-1β, IL-1ra and IL-1RII in rat cortex: Treatment with the brain penetrant β_2-adrenoceptor agonist clenbuterol (0.5 mg/kg; i.p.), induced a time-dependent increase in cortical IL-1β (*P*<0.01), IL-1ra (*P*<0.001) and IL-1RII (*P*<0.001) expression (Fig 3 a, b & e), without altering the expression levels of IL-1RI or IL-1RAcp (Fig 3 c & d). The induction of IL-1β mRNA is maximal at 1 h post-treatment, while its negative regulators, IL-1ra and IL-1RII, show a delayed expression profile peaking 4 h post-treatment (fig. 3). The results of our dose-response study show that IL-1β, IL-1ra and IL-1RII expression can be induced by doses of clenbuterol as low as 0.03mg/kg (Fig 5).

Clenbuterol induces IL-1β protein expression and signalling in rat cortex: Induction of IL-1β mRNA was accompanied by increased IL-1β protein production (*P*<0.01; Fig 4a) and increased expression of IκBα mRNA expression (*P*<0.01; Fig 4b), which is indirect readout of NFκB activation. The clenbuterol-induced increase in IL-1β was also accompanied by increased activation (phosphorylation) of the MAP kinase ERK (P<0.05; Fig 4e). In contrast, clenbuterol failed to activate JNK and P38 MAP kinase pathways. This clenbuterol-induced increase in IL-1β protein expression and signalling was accompanied by induction of the IL-1β inducible gene, CINC1 (*P*<0.01; Fig 4f).

Clenbuterol fails to induce expression of other pro-inflammatory cytokines or induce evidence of apoptotic signalling in rat cortex: In contrast to its ability to activate the IL-1 system, clenbuterol failed to induce expression of other inflammatory cytokines including TNF-α, IL-6 or IFN-γ (Table 1). In fact, clenbuterol induced a transient suppression of TNF-α and IL-6 expression (Table 1). In addition, clenbuterol failed to alter expression of the apoptotic markers, bcl-2, bax or caspase-3 in rat cortex, at any of the time points assessed (Data not shown).

The β-adrenoceptor antagonist propranolol blocks, and the highly selective β_2-adrenoceptor agonist mimics the ability of clenbuterol to activate the central IL-1 system: The ability of clenbuterol to activate the central IL-1 system is clearly mediated by central β-adrenoceptor activation, as the ability of clenbuterol to induce IL-1β (*P*<0.01), IL-1ra (*P*<0.01), IL-1RII (*P*<0.01) and IκBα (*P*<0.05) was blocked by pre-treatment with the brain

penetrant β-adrenoceptor antagonist, propranolol (Fig 6a-d). In addition, the highly selective β_2-adrenoceptor agonist formoterol (0.5 mg/kg; i.p.), mimicked the ability of clenbuterol to induce IL-1β (*P*<0.01), IL-1ra (*P*<0.01), IL-1RII (*P*<0.01) and IκBα (*P*<0.05) (Fig 6 e-h).

3.3 Clenbuterol attenuates neuroinflammation induced by a systemic challenge with bacterial LPS: As neurodegenerative diseases are associated with an inflammatory state within the CNS (see Allen et al., 2005; Block and Hong, 2005; Heneka and O'Banion, 2007) it was important for us to determine if β_2-adrenoceptor activation could induce central IL-1ra and IL-1RII expression in the presence of an inflammatory stimulus (a systemic challenge with bacterial lipopolysaccharide; LPS). In addition, we determined the ability of β_2-adrenoceptor stimulation to combat the more generalised neuroinflammatory response induced by LPS administration.

The ability of clenbuterol to induce cortical IL-1β expression was attenuated (P<0.05) in LPS-treated animals (Fig 7a). In contrast, the clenbuterol-induced induction of IL-1ra and IL-1RII was not altered following LPS administration (Fig. 7 b & c). Furthermore, a general anti-inflammatory effect was observed following β_2-adrenoceptor agonist administration as clenbuterol suppressed LPS-induced expression of the inflammatory cytokines IL-6 and TNF-α (*P*<0.05), the inflammatory chemokines RANTES and IP-10 (*P*<0.05) and the co-stimulatory molecules CD40 (*P*<0.01) and ICAM-1 (*P*<0.05) in the rat cortex (Fig 7 d-i).

4. Discussion

4.1 Enhancement of noradrenergic tone activates the central IL-1 system via β-adrenoceptor activation: Here we demonstrate that combined treatment with reboxetine and idazoxan, a drug combination that induces a robust increase in central noradrenaline availability (Sacchetti et al., 1999), induced expression of IL-1β in rat cortex, as well as its negative regulators IL-1ra and IL-1RII, both of which serve to inhibit IL-1β signalling and prevent excessive or prolonged activation of the IL-1 system. Consistent with the fact that idazoxan alone does not increase noradrenaline release (Sacchetti et al., 1999), it failed to induce IL-1β, IL-1ra or IL-1RII expression. Whilst treatment with reboxetine alone induced a slight increase in IL-1β, IL-1ra and IL-1RII expression this was greatly augmented when reboxetine was administered in combination with idazoxan. This observation is consistent with the profound synergistic increase in noradrenaline release in rat cortex that occurs when idazoxan and reboxetine are administered in combination (Sacchetti *et al*., 1999). Consistent with a role for β-adrenoceptors in mediating the immunomodulatory affects of noradrenaline, the effects of combined reboxetine/idazoxan treatment on the IL-1 system were blocked by pre-treatment with the β-adrenoceptor antagonist, propranolol.

In contrast to the profound increase in IL-1RII induced by the reboxetine/idazoxan combination, no significant change in IL-1RI or IL-1RAcp expression occurred. This is consistent with our *in vitro* study, in which noradrenaline failed to alter glial IL-1RI or IL-1RAcp expression (McNamee et al., 2010a), and with previous research indicating that the low constitutive levels of IL-1RI expression in the brain don't appear to be easily altered, at least in animal models (Parnet *et al*., 1994, Gayle *et al*., 1999).

4.2 Direct stimulation β_2-adrenoceptors selectively activates the central IL-1 system: It is clear from the present study that treatment with the brain penetrant β_2-adrenoceptor agonist clenbuterol mimicked the ability of reboxetine/idazoxan treatment to induce IL-1β, IL-1ra and IL-RII expression in rat cortex. However, the magnitude of increase was much greater than observed with reboxetine/idazoxan treatment, most likely due to the low agonist potency of noradrenaline at β_2-adrenoceptors relative to selective β_2-adrenoceptor agonists. Clenbuterol's actions were selective to the IL-1 system, in that expression of the

inflammatory cytokines TNF-α, IL-6 or IFN-γ were not induced by clenbuterol. Instead, clenbuterol treatment induced a transient suppression of TNF-α and IL-6 expression. This is an important finding as the neurotoxic actions of IL-1β are much more likely to emerge when it is expressed in concert with other inflammatory mediators.

Induction of IL-1β was accompanied by NFκB activation, indicated by increased expression of the proto-typical NFκB inducible gene IκBα (Read et al., 1994), and expression of the IL-1β-inducible gene, CINC-1 (Anthony et al., 1998). In addition to activating the NFκB pathway, IL-1 is known to activate MAP kinase pathways (Saklatlava et al., 1996). In this regard, the clenbuterol-induced increase in IL-1β was accompanied by increased activation (phosphorylation) of the MAP kinase ERK; a signalling pathway associated with pro-survival effects in neurons (Fukunaga and Miyamoto, 1998). In contrast, clenbuterol failed to activate JNK and P38 MAP kinase pathways, both of which have been implicated in mediating detrimental effects of IL-1 and glial cell activation in the brain (Curran et al., 2003; Kelly et al., 2002; Martin et al., 2003; Minogue et al., 2003; Xie et al., 2004). Consistent with this, clenbuterol failed to alter expression of the apoptotic markers, bcl-2, bax or caspase-3 in rat cortex, at any of the time points assessed (Data not shown). It is important to note that activation of IL-1 signalling, observed following β-adrenoceptor stimulation, occurred in a tightly controlled manner. Specifically, expression of IL-1ra and IL-1RII serve to inhibit prolonged IL-1 signalling which could be deleterious for brain function. In this regard, the increase in expression of the IL-1β-inducible gene, CINC-1, had completely dissipated 8h following clenbuterol treatment.

The ability of clenbuterol to activate the IL-1 system was blocked by propranolol, and was mimicked by the highly selective β_2-adrenoceptor agonist formoterol. These data are important in highlighting the specific role of β-adrenoceptor activation in the actions of clenbuterol on the central IL-1 system, as previous studies indicate that clenbuterol can elicit biological responses independent of β-adrenoceptors (Desaphy et al., 2003; Ngala et al., 2009). Our finding that systemic administration of clenbuterol induces IL-1β expression in brain tissue, is consistent with studies reporting that icv administration of the β-adrenoceptor agonists isoprenaline and procaterol induced IL-1β expression from glial cells

in rat brain parenchyma (Johnson et al., 2008; Maruta et al., 1997). However, to our knowledge this is the first study to demonstrate that β-adrenoceptors mediate induction of IL-1ra and IL-1RII expression in the brain. **Whilst the present investigation focuses specifically on the actions of β_2-adrenoceptors in rat cortex, we have recently demonstrated that clenbuterol also induces expression of IL-1β, IL-1ra and IL-1RII in rat hippocampus and hypothalamus (Griffin et al., 2010).** In contrast to these *in vivo* observations, noradrenaline and β-adrenoceptor agonists both fail to induce IL-1β expression in cultured glial cells (Kilroy, unpublished results; McNamee et al., 2010a). The reason for this discrepancy between *in vitro* and *in vivo* systems remains to be elucidated.

It is noteworthy that the increase in IL-1Ra and IL-1RII induced by clenbuterol do not occur simply as a downstream consequence of clenbuterol-induced IL-1β production. In this regard, we have shown that the anti-inflammatory agent dexamethasone completely blocks expression of IL-1β induced by clenbuterol, without altering its ability to induce the anti-inflammatory molecules IL-1ra and IL-1RII (Griffin et al., 2010). These data highlight are consistent with previous observations that IL-1ra and IL-1RII expression can occur independent of IL-1β both in cultured glial cells and in the intact brain (Docagne et al., 2005; McNamee et al., 2010a).

4.3 β-adrenoceptor-mediated activation of the central IL-1 system: Implications for brain function: There is now a general consensus that inflammatory mediators including IL-1β can exert both adaptive and maladaptive responses in the brain, depending on the concentration, the duration of exposure and the overall immune environment (Goshen et al., 2007; Li et al., 2007; Pinteaux et al., 2009; Spulber et al., 2009). For instance, whilst administration of exogenous IL-1β has been shown to inhibit memory and learning and block hippocampal long-term potentiation (LTP); an electrophysiological process thought to underline memory formation (Kelly et al., 2003; Pugh et al., 2001), constitutive expression of IL-1 is required for normal learning and memory and LTP (Avital et al., 2003; Goshen et al., 2007). Similarly, whilst there is a body of research outlining the contribution of IL-1β to the neurodegenerative process (Allan et al., 2005), evidence also indicates that IL-1β may elicit neuroprotective effects both *in vitro* and *in vivo*. For example, IL-1β protected cultured

cortical neurons in an NMDA-induced *in vitro* model of excitotoxicity (Strijbos and Rothwell, 1995), and enhanced survival of lesioned cultured dopaminergic neurons (Akaneya *et al*., 1995). In the *in vivo* context, IL-1β infusion into the substantia nigra 5 days prior to 6-hydroxydopamine administration elicits neuroprotective effects in this model of Parkinson's disease (Saura et al., 2003), and sustained overexpression of hippocampal IL-1β ameliorates amyloid-β (Aβ) plaque pathology in a mouse model of Alzheimer's disease (Shaftel et al., 2007). In line with this finding, IL-1β drives amyloid precursor protein processing in a non-amyloidogenic direction resulting in decreased Aβ production (Tachida et al., 2008). Finally, it has been shown that remyelination is dramatically reduced in the absence of IL-1β (Mason *et al*., 2001), and that IL-1β can induce oligodendrocyte proliferation and maturation (Vela *et al*., 2002). Taken together, these findings suggest that IL-1β can elicit protective or detrimental effects in the CNS depending on the context, and that constitutive expression of IL-1β is necessary for many aspects of normal brain function. In this regard, whilst both clenbuterol and noradrenaline reuptake inhibitors have neuroprotective (Culmsee et al., 1999a; Gleeson et al., 2010; Huang et al., 2007; Wang et al., 2007; Zhu et al., 1999), and memory enhancing properties (Clinton et al., 2006; Ramos et al., 2008; Seu et al., 2009), any role that IL-1 system components may have in such neuroprotective or memory enhancing actions of these drugs remains to be elucidated. **In addition, as combined treatment with reboxetine/idazoxan induced a very modest increase in IL-1β expression when compared to the large induction of IL-1β induced by β_2-adrenoceptor agonists or LPS, future studies are required to determine the functional significance of the different levels of IL-1β induced by these treatments for brain function.**

4.4 Clenbuterol attenuates neuroinflammation induced by a systemic LPS challenge : As neurodegenerative diseases are associated with an inflammatory state within the CNS (see Block et al., 2007; Heneka and O'Banion, 2007), it was important for us to determine if β-adrenoceptor activation could induce central IL-1ra and IL-1RII expression in the presence of an inflammatory stimulus. Consistent with our *in vitro* findings (McNamee et al., 2010a) clenbuterol was also effective at inducing IL-1ra and IL-1RII expression in the brains of animals that has been treated with a systemic inflammatory challenge with LPS. Moreover, whilst the ability of clenbuterol to induce expression of IL-1RII and IL-1Ra was maintained

following the inflammatory challenge, its ability to induce IL-1β was reduced. Thus in an inflammatory state clenbuterol has a multifaceted action on the IL-1 system resulting in its downregulation.

In addition to its actions on the central IL-1 system, clenbuterol induced a broad spectrum anti-inflammatory response in rat cortex following exposure to a systemic LPS challenge. Specifically, clenbuterol suppressed LPS-induced expression of the inflammatory cytokines TNF-α and IL-6, the inflammatory chemokines RANTES and IP-10, the co-stimulatory molecules CD40 and ICAM-1. These molecules mediate different aspects of the neuroinflammatory response, and have been implicated in the neurodegenerative process (see O'Sullivan et al, 2009, 2010). The ability of clenbuterol to suppress the central inflammatory response to LPS is consistent with studies demonstrating that noradrenaline reuptake inhibitors suppress expression of inflammatory mediators including TNF-α, IL-1β, CD40, ICAM-1, IP-10 and RANTES in rat brain following an LPS challenge (O'Sullivan et al, 2009, 2010). In addition, we have previously demonstrated that clenbuterol induces expression of the broad spectrum anti-inflammatory and neuroprotective cytokine IL-10 (McNamee et al., 2010b). Thus overall, both noradrenaline reuptake inhibitors and β_2-adrenoceptor agonists suppresses the innate inflammatory response in the CNS.

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Figure and table legends

Figure 1: Combined reboxetine and idazoxan treatment induces IL-1β, IL-1ra and IL-1RII mRNA expression in rat cortex

Co-treatment with reboxetine (15mg/kg) and idazoxan (1mg/kg) induced a significant increase in cortical IL-1b (a) IL-1ra (b) and IL-1RII (e) mRNA expression 4h later, but failed to alter expression of IL-1RI (c) or IL-1RAcp (d). Data is expressed as means + SEM (n=6). mRNA data is expressed as fold-change vs. saline vehicle. *P<0.05, **P<0.01 vs. saline vehicle (One-way ANOVA followed by *post-hoc* Newman-Keuls test).

Figure 2: β-adrenoceptors mediate the ability of combined reboxetine and idazoxan treatment to induce IL-1β, IL-1ra and IL-1RII expression in rat cortex

Pre-treatment with the β-adrenoceptor antagonist, propranolol (10mg/kg), attenuated the increase in cortical IL-1β (a), IL-1ra (b) and IL-1RII (c) expression induced by combined treatment with reboxetine (15mg/kg) and idazoxan (1mg/kg). Data is expressed as means + SEM as fold-change vs. saline vehicle (n=6). *P<0.05, **P<0.01 vs. saline vehicle, +P<0.05, ++P<0.01 vs. reboxetine/idazoxan (Two-way ANOVA followed by *post-hoc* Newman-Keuls test).

Figure 3: The β_2-adrenoceptor agonist, clenbuterol, induces IL-1β, IL-1ra and IL-1RII mRNA expression in rat cortex: A time-course analysis

Treatment with the selective β_2-adrenoceptor, clenbuterol (0.5 mg/kg), induces a time-dependent increase in cortical IL-1b (a) IL-1ra (b) and IL-1RII (e) mRNA expression, but failed to alter expression of IL-1RI (c) or IL-1RAcp (d). Data is expressed as means + SEM (n=6). mRNA data is expressed as fold-change vs. saline vehicle. *P<0.05, **P<0.01 vs. saline vehicle (One-way ANOVA followed by *post-hoc* Newman-Keuls test).

Figure 4: The β_2-adrenoceptor agonist, clenbuterol, induces IL-1β protein expression and signaling rat cortex: A time-course analysis

Treatment with the selective β_2-adrenoceptor, clenbuterol (0.5 mg/kg), induced a time-dependent increase in cortical IL-1β protein as measured by ELISA. This increase in IL-1b protein was accompanied by increased IκBα expression indicative of NFκB activation (b), activation of the MAP kinase ERK (e) and induction of the IL-1β-inducible genes CINC-1 and iNOS expression (f,g). Data is expressed as means + SEM (IL-1β protein expression and IκBα, CINC-1 and iNOS mRNA n=6; MAP kinase phosphorylation densitometry n=4). mRNA data is expressed as fold-change vs. saline vehicle. *P<0.05, **P<0.01 vs. saline vehicle (One-way ANOVA followed by *post-hoc* Newman-Keuls test).

Figure 5: The β_2-adrenoceptor agonist, clenbuterol, activates the IL-1 system in rat cortex: A dose-response analysis

Treatment with the selective β_2-adrenoceptor agonist, clenbuterol (0.03-0.3mg/kg), induced a significant increase cortical IL-1β (a), IL-1ra (b) and IL-1RII (c) expression 4h post-treatment. Data is expressed as means + SEM as fold-change vs. saline vehicle (n=6). *P<0.05, **P < 0.01 vs. saline vehicle (One-way ANOVA followed by *post-hoc* Newman-Keuls test).

Figure 6: The β-adrenoceptor antagonist propranolol blocks, and the highly selective β_2-adrenoceptor agonist formoterol mimics, the ability of clenbuterol to activate the IL-1 system in rat cortex

(a-d) Pre-treatment with the β-adrenoceptor antagonist, propranolol (10mg/kg), blocks the ability of clenbuterol (0.5mg/kg) to induce cortical IL-1β (a), IκBα (b), IL-1ra (c) and IL-1RII (d) expression 4h post-treatment. Data expressed as mean + SEM. mRNA data is expressed as fold-change vs. saline vehicle (n=6). **P<0.01 vs. saline vehicle, +P<0.05, ++P<0.01 vs. clenbuterol (Two-way ANOVA followed by *post-hoc* Newman-Keuls test).

(e-h) Treatment with the highly selective β_2-adrenoceptor agonist formoterol (0.5mg/kg) mimics the ability of clenbuterol to induce cortical IL-1β (e), Iκbα (f), IL-1ra (g) and IL-1RII (h) expression 4h post-treatment. Data expressed as mean + SEM. mRNA data is expressed as fold-change vs. saline vehicle (n=6). *P<0.05, **P<0.01 vs. saline vehicle (Student *t*-test).

Figure 7: Clenbuterol attenuates neuroinflammation induced by a systemic challenge with bacterial LPS

The β_2-adrenoceptor agonist clenbuterol (0.03mg/kg) induces cortical IL-1ra (b) and IL-1RII (c) mRNA expression when administered in the presence of the inflammagen bacterial LPS (250ug/kg). In contrast, the ability of clenbuterol to induce IL-1β expression was reduced in LPS-treated animals (a). In addition, clenbuterol blocked the LPS-induced induction of inflammatory cytokines IL-6 and TNF-α (d,e), the co-stimulatory molecules CD40 and ICAM-1 (f,g) and the inflammatory chemokines IP-10 and RANTES (h,i) in rat cortex. Data expressed as mean + SEM. mRNA data is expressed as fold-change vs. saline vehicle (n=6). *P<0.05, **P<0.01 vs. vehicle + vehicle, + P<0.05, ++P<0.01 vs. vehicle-treated counterparts (Two-way ANOVA followed by *post-hoc* Newman-Keuls test).

Table 1

Effect of treatment with the β_2-adrenoceptor agonist clenbuterol on expression of the pro-inflammatory cytokines TNF-α, IL-6 and IFN-γ in rat cortex

Figure 1

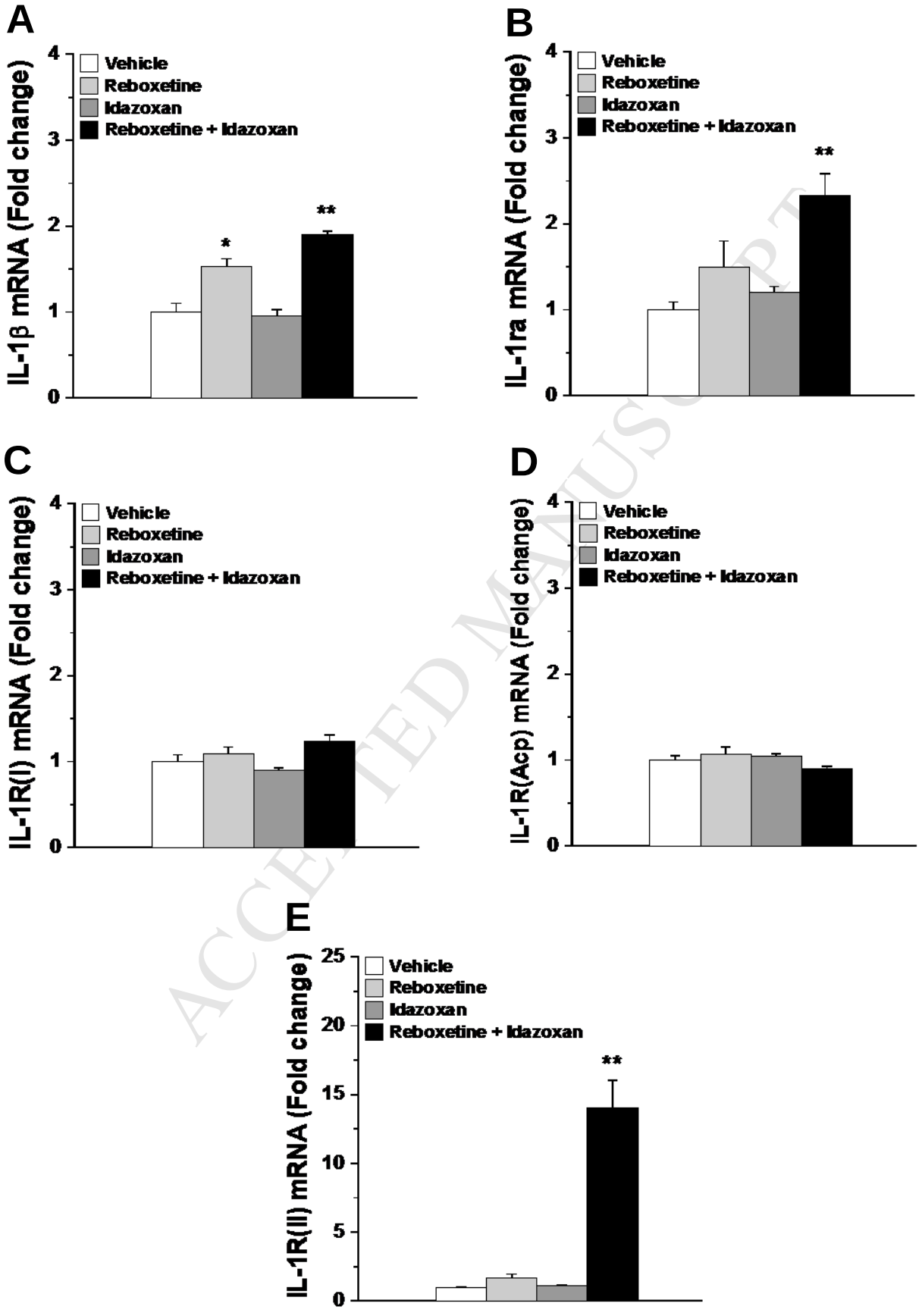
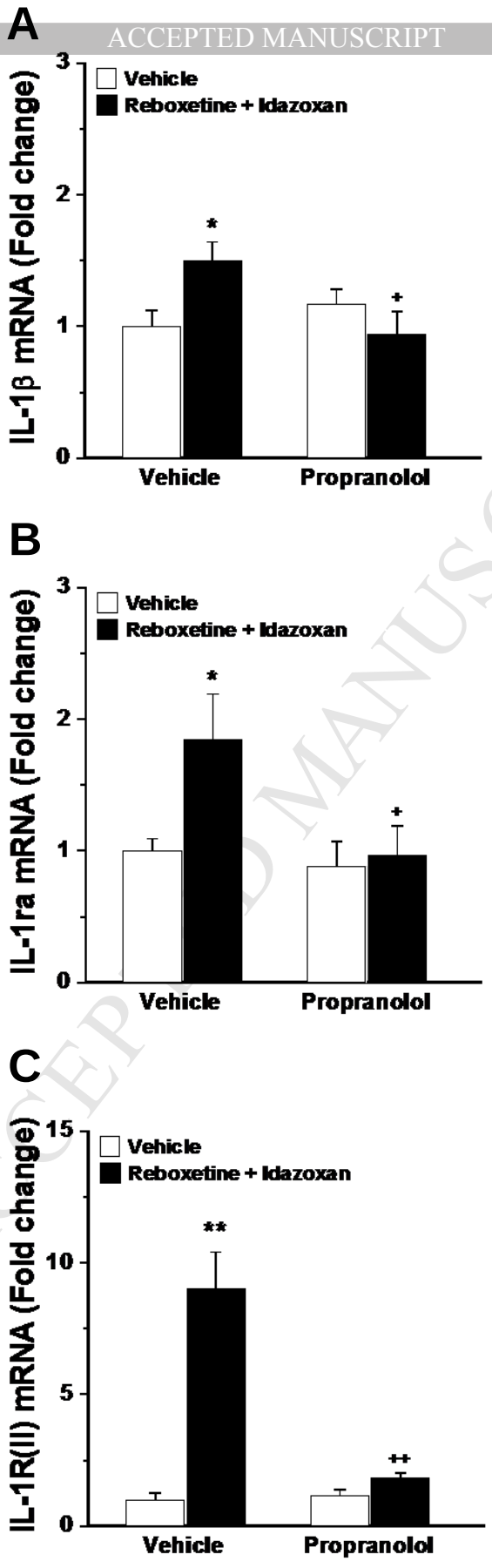


Figure 2



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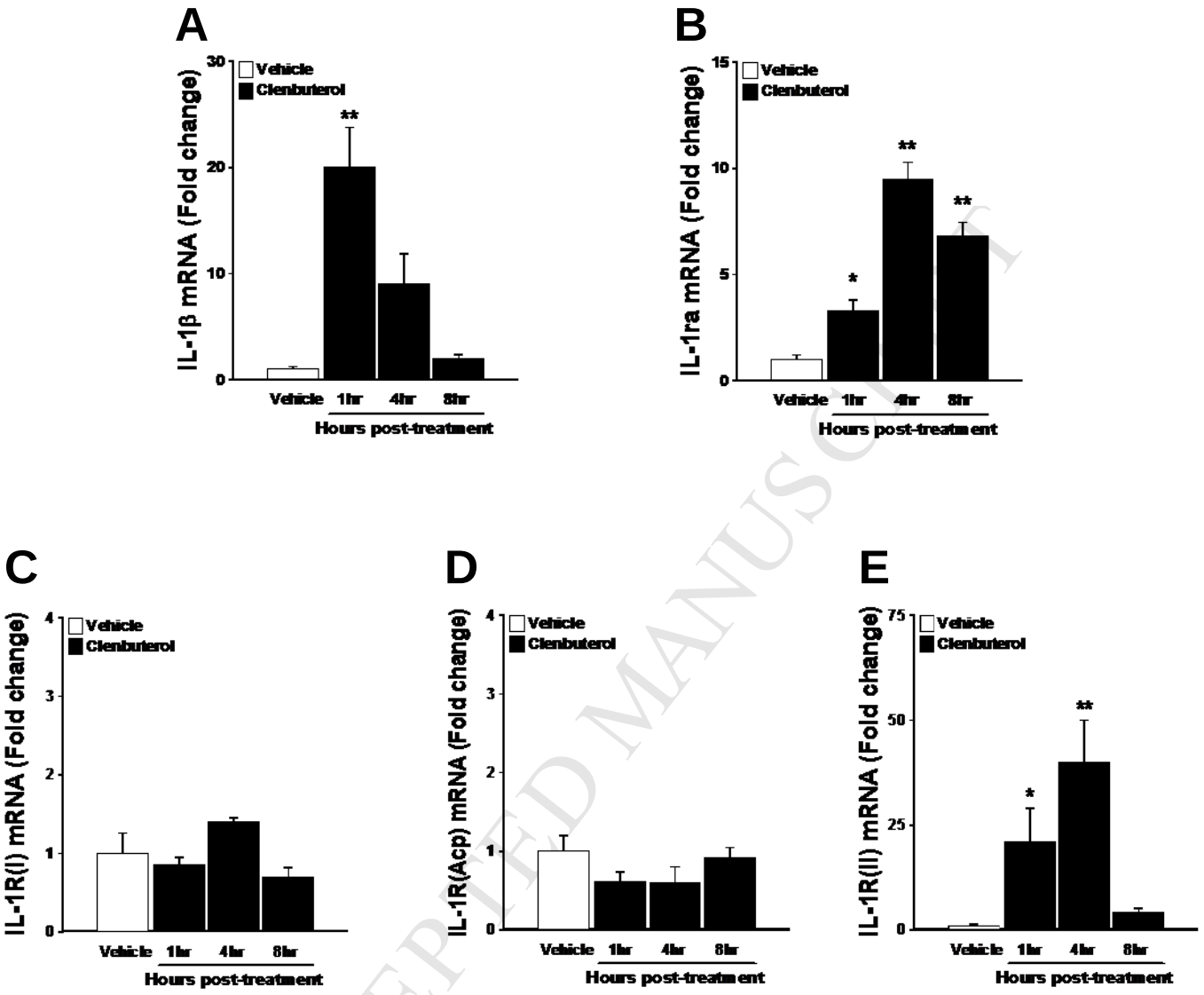

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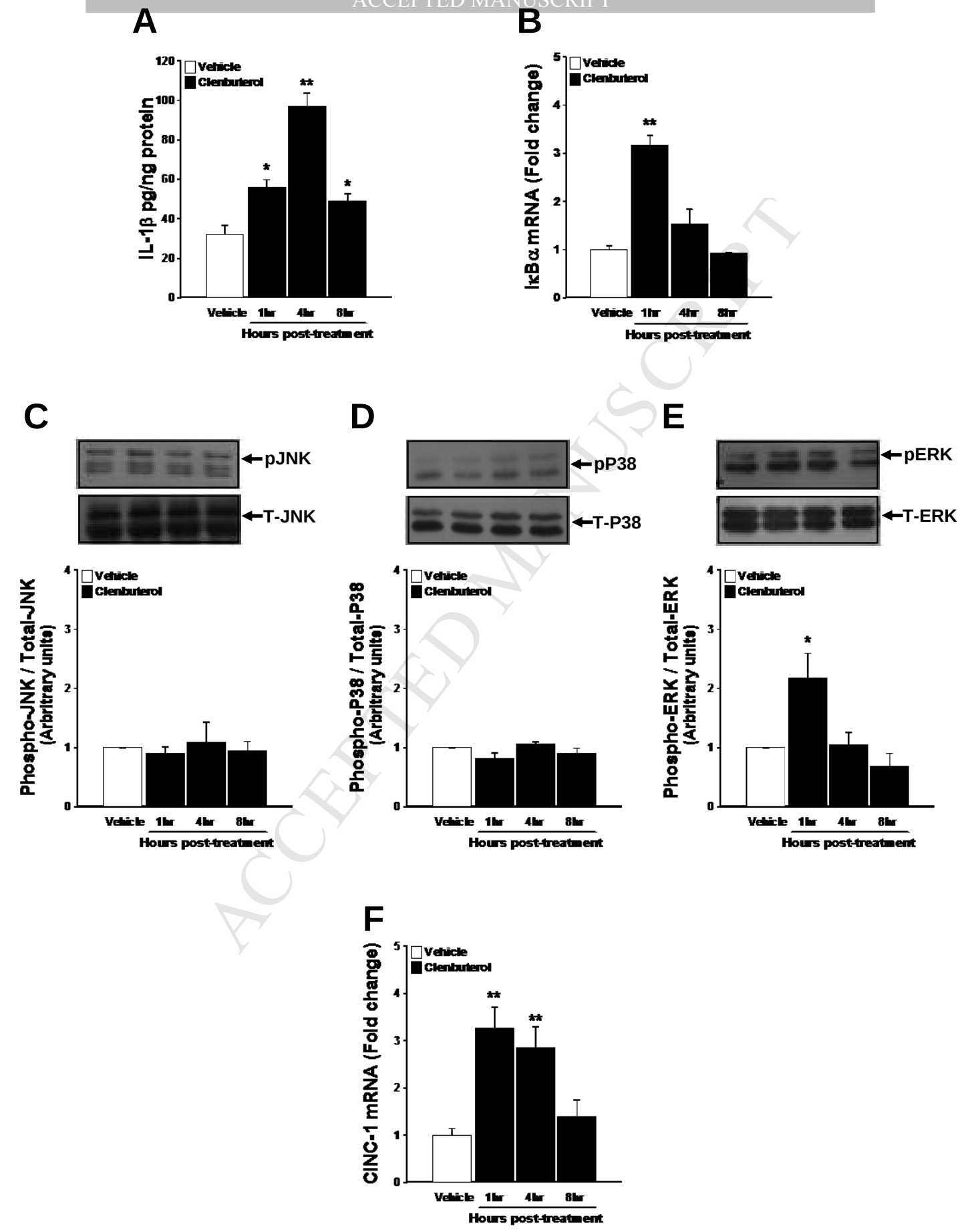
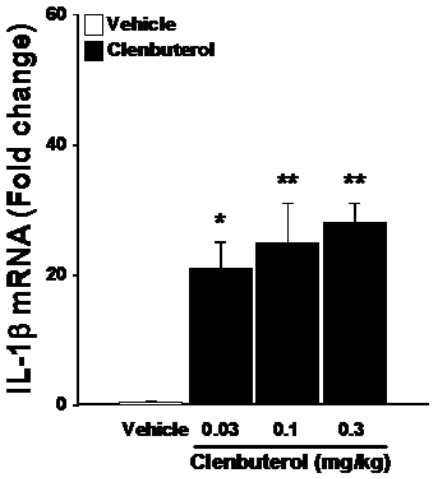


Figure 5

A



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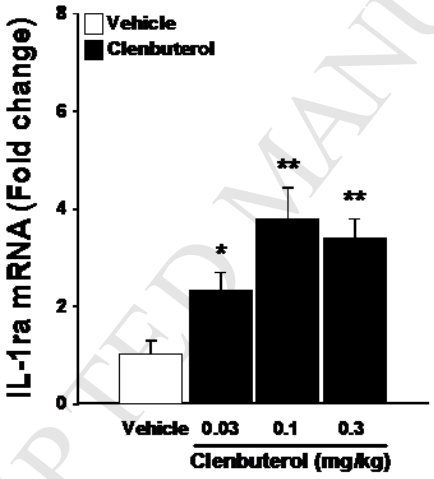

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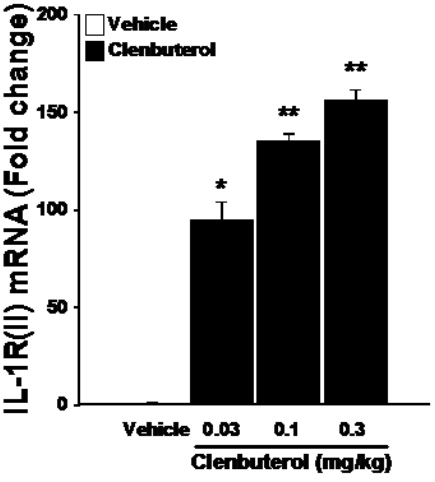

Figure 6

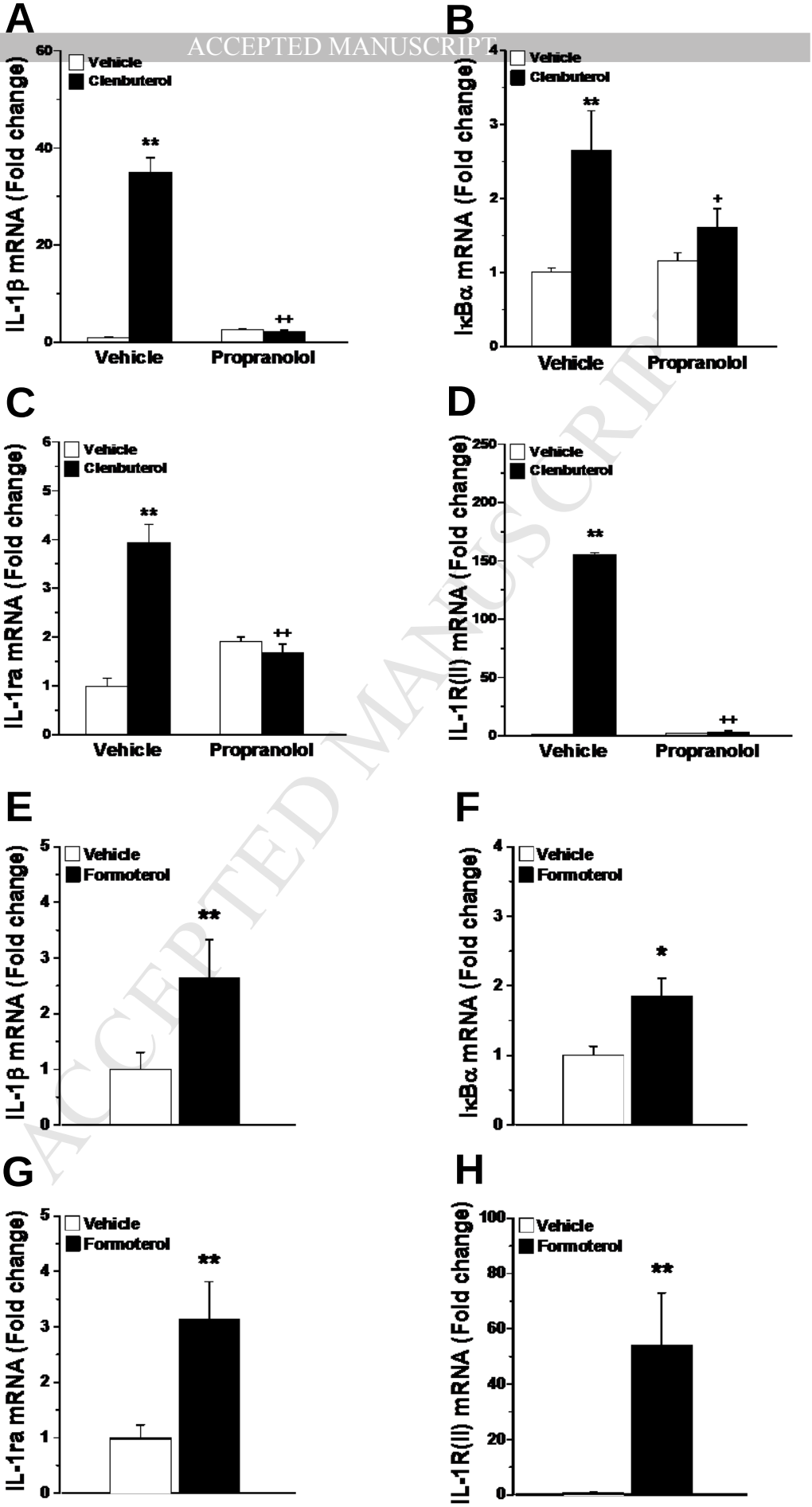

Figure 7

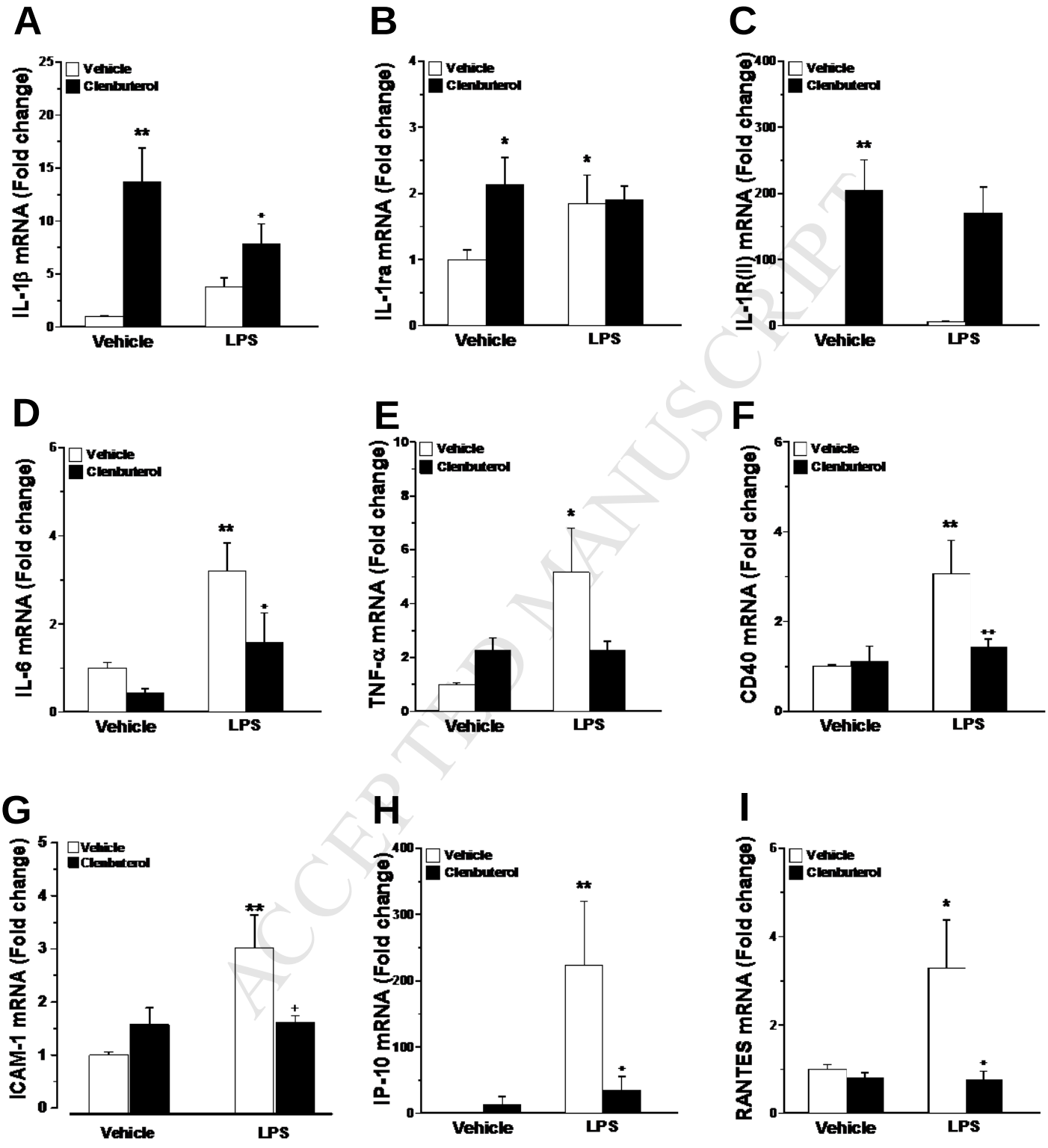

Table 1

		Clenbuterol (0.5 mg/kg) Hours post-treatment			
	Vehicle	**1**	**4**	**8**	***P* value**
TNF-α	1.0 ± 0.10	0.26 ± 0.05 **	0.88 ± 0.06	1.30 ± 0.16	*P* < 0.001
IL-6	1.0 ± 0.02	0.35 ± 0.07 *	0.99 ± 0.15	0.79 ± 0.14	*P* = 0.018
IFN-γ	1.0 ± 0.27	1.30 ± 0.31	1.14 ± 0.19	1.03 ± 0.15	*P* = 0.808

Data expressed as fold change of the mean mRNA expression ± S.E.M. vs. Saline vehicle (n=6). *P<0.05, **P<0.01 vs. saline vehicle (One-way ANOVA followed by *post-hoc* Newman-Keuls test).