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Stimulation of central β_2-adrenoceptors suppresses NFκB activity in rat brain: A role for IκB

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ABSTRACT

In this study we examined the impact of systemic treatment with the long-acting brain penetrant β_2-adrenoceptor agonist clenbuterol on NFκB activity and IκB expression in rat brain. Clenbuterol decreased NFκB activity (p65 DNA binding) in nuclear extracts prepared from rat cortex and hippocampus for up to 8 h following a single treatment. This was accompanied by increased expression of IκBα mRNA and protein. The temporal increase in IκB protein expression paralleled the suppression of NFκB activity, suggesting that IκBα mediates the suppression NFκB activity observed. These actions of clenbuterol were prevented by pre-treatment with the non-selective β-adrenoceptor antagonist propranolol, the β_2-adrenoceptor antagonist ICI-118,551, but not the β_1-adrenoceptor antagonist metoprolol, suggesting that the effects of clenbuterol on IκBα expression and NFκB activity are mediated specifically by the β_2-adrenoceptor. In addition, the actions of clenbuterol were mimicked by systemic administration of another highly selective long-acting β_2-adrenoceptor agonist formoterol. As neurodegenerative diseases are associated with inflammation we determined if clenbuterol could suppress NFκB activation that occurs in response to an inflammatory stimulus. In this regard we demonstrate that clenbuterol inhibited IκB phosphorylation and IκB degradation and inhibited NFκB activity in hippocampus and cortex of rats following a central injection of the inflammagen bacterial lipopolysaccharide (LPS). In tandem, clenbuterol blocked expression of the NFκB-inducible genes TNF-α and ICAM-1 following LPS administration. Our finding that clenbuterol and formoterol inhibit NFκB activity in the CNS further supports the idea that β_2-adrenoceptors may be an attractive target for treating neuroinflammation and combating inflammation-related neurodegeneration.

1. Introduction

Neuroinflammation, characterized 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 (Allan et al., 2005; Block et al., 2007; Heneka and O'Banion, 2007). Despite the evidence that the neurotransmitter noradrenaline elicits endogenous anti-inflammatory actions in the brain mediated by glial β_2-adrenoceptors (Feinstein et al., 2002; Heneka et al., 2002; Kalinin et al., 2007; McNamee et al., 2010a; Mori et al., 2002), the anti-inflammatory effects of agents that directly stimulate the β_2-adrenoceptor in the central nervous system (CNS) have not been extensively studied to date.

The brain-penetrant β_2-adrenoceptor agonist clenbuterol is used in the treatment of respiratory disorders including asthma and chronic obstructive pulmonary disease (COPD) (Baronti et al., 1980; Erichsen et al., 1994; Tondo et al., 1985; Papiris et al., 1986; Boner et al., 1988) and more recently it has been shown to have neuroprotective properties both *in vivo* and *in vitro* (Culmsee et al., 1999a,b). Specifically, clenbuterol has neuroprotective actions in rodent models of cerebral ischaemia (Semkova et al., 1996; Zhu et al., 1998; Culmsee et al., 1999b; Junker et al., 2002) and in both *in vitro* and *in vivo* models of excitotoxicity (Semkova et al., 1996; Gleeson et al., 2010). It has recently been demonstrated that clenbuterol induces expression of the pro-inflammatory cytokine IL-1β and its negative regulators IL-1ra and IL-1RII in rat brain and reduces expression of IL-6 and TNF-α (McNamee et al., 2010c). In addition, clenbuterol induces expression of the broad spectrum anti-inflammatory cytokine IL-10 and its

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downstream signalling molecule SOCS-3 in rat brain (McNamee et al., 2010b), and thus may be effective in the treatment of neuroinflammatory disease.

The transcription factor nuclear factor kappa-B (NFκB) is responsible for the regulation of genes encoding a variety of proteins involved in driving the immune response, such as cytokines, chemokines, and adhesion molecules (Subramaniam et al., 2004). It is composed of various dimeric complexes containing members of the Rel protein family, which include Rel (c-Rel), Rel A (p65), RelB, NF-κB1 (p50 and its precursor p105) and NF-κB2 (p52 and its precursor p100) (Ghosh et al., 1998). The most common dimeric combination is composed of the p50 and p65 subunits (Moynagh, 2005).

In the resting cell, NFκB resides in the cytoplasm where it is bound to the inhibitory molecule IκB, the most common forms being IκBα and to a lesser extent IκBβ (Ghosh et al., 1998). This interaction sequesters NFκB in the cytoplasm and inhibits its DNA-binding activity. Upon stimulation by a range of signals, such as bacterial lipopolysaccharide (LPS), pro-inflammatory cytokines and ultraviolet light, IκBα is phosphorylated and subsequently degraded by the 26S proteasome (Karin and Ben-Neriah, 2000). Degradation of IκBα releases NFκB allowing it to translocate to the nucleus where it regulates the expression of numerous genes involved in inflammation as well as IκBα (Karin and Ben-Neriah, 2000; Minagar et al., 2002; Simi et al., 2005). Notably, IκBα is rapidly re-synthesized following NFκB activation due to the presence of a transcriptional binding site for IκB on the NFκB promoter (Sun et al., 1993). Newly synthesized cytoplasmic IκBα translocates to the nucleus where it terminates the activity of NFκB by transporting it back to the cytoplasm (Arenzana-Seisdedos et al., 1995, 1997). This is a critical inhibitory step which results in transient NFκB activation and restoration of cytoplasmic pools of inactive NFκB.

In the present investigation we examined the effect of treatment with the β_2-adrenoceptor agonist clenbuterol on NFκB activity in the rat cortex and hippocampus. We chose the cortex and hippocampus for analysis in these studies as both regions receive rich noradrenergic innervation from the locus coeruleus (Dahlström and Fuxe, 1964), and are involved in higher cognitive processing such as learning, memory and reasoning that are adversely affected in many neurodegenerative diseases (Burton et al., 2004; Whitwell et al., 2007). Specifically, we investigated the effects of clenbuterol on NFκB activity (by measuring the DNA binding activity of the NFκB p65 subunit in nuclear extracts), on IκBα and IκBβ mRNA expression and on cytoplasmic IκBα protein levels. Additionally, we studied the involvement of the β_2-adrenoceptor in these processes, examining the ability of β-adrenoceptor antagonists (Non-selective: propranolol; β_2; ICI-118,551; β_1: metoprolol) to block, and the ability of a related β_2-adrenoceptor agonist, formoterol, to mimic the actions of clenbuterol on NFκB activity in rat brain. As neurodegenerative diseases are associated with a neuroinflammatory state (Heneka and O'Banion, 2007). We determined if β_2-adrenoceptor activation could inhibit NFκB activation and induction of NFκB-inducible genes that occurs in response to an inflammatory stimulus (intracerebroventricular challenge bacterial lipopolysaccharide; LPS).

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats (200–250 g) 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

Clenbuterol and LPS (*Escherichia coli*: serotype 0111:B4) were obtained from Sigma–Aldrich, Ireland. Clenbuterol was dissolved in 0.89% NaCl and administered via the intraperitoneal (i.p.) route in an injection volume of 1 ml/kg and 0.89% NaCl was administered alone as a vehicle to control animals. LPS was administered via the intracerebroventricular (i.c.v.) route in an injection volume of 5 μL.

2.3. Experimental design

2.3.1. Experiment I: effect of treatment with the β_2-adrenoceptor agonist clenbuterol on NFκB activity and IκBα expression in rat brain

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 post clenbuterol treatment. The brain was dissected on an ice-cold plate and cortical and hippocampal tissue was harvested and rapidly frozen on dry ice. Samples were stored at −80 °C until needed for analysis of NFκB p65 DNA binding (NFκB activity), and IκBα mRNA and protein expression. The dose of clenbuterol employed in this study was based on our studies demonstrating that administration of clenbuterol (0.5 mg/kg) to rats elicits anti-inflammatory effects in the CNS and has neuroprotective properties (Gleeson et al., 2010; McNamee et al., 2010a,b).

2.3.2. Experiment II: can pre-treatment with β_1- and β_2-adrenoceptor antagonists block the clenbuterol-induced effects on NFκB activity and IκBα expression in rat brain?

The aim of the present study was to determine a role for the β_2-adrencoceptor in the clenbuterol-mediated effects on NFκB activity and IκBα expression. Rats were pre-treated with either vehicle or one ofthe following drugs: the non-selective β-adrenoceptor antagonist propranolol (10 mg/kg; i.p.), the selective β_1-adrenoceptor antagonist metoprolol (10 mg/kg; i.p.) or the selective β_2-adrenoceptor antagonist ICI-118,551 (10 mg/kg; i.p.), 30 min prior to i.p. administration of clenbuterol (0.5 mg/kg; i.p.). Rats were killed 4 h post-clenbuterol injection and brain tissue was prepared and stored as outlined in Experiment I above for analysis of NFκB p65 DNA binding (NFκB activity) and IκBα mRNA expression.

2.3.3. Experiment III: does the β_2-adrenoceptor agonists formoterol mimic the effects of clenbuterol on NFκB activity and IκBα expression?

Rats received a vehicle or the β_2-adrenoceptor agonist formoterol (0.5 mg/kg; i.p.) and rats were killed 4 h later. Brain tissue was prepared and stored as outlined in Experiment I above for analysis of NFκB p65 DNA binding (NFκB activity), and IκBα mRNA and protein expression. The dose of formoterol was employed based on our studies demonstrating that administration of formoterol (0.5 mg/kg) mimicked the anti-inflammatory effects of clenbuterol in the rat brain (McNamee et al., 2010c).

2.3.4. Experiment IV: can pre-treatment with the β_2-adrenoceptor agonist clenbuterol suppress LPS-induced NFκB activity?

Rats were pre-treated with either vehicle or clenbuterol (0.5 mg/kg; i.p.). One hour later rats received a single intracerebroventricular (icv) injection of vehicle or LPS (1 μg/5 μL) into the left lateral ventricle. Rats were killed 2 h post-LPS treatment. Brain tissue was prepared and stored as outlined in Experiment I above for analysis of NFκB p65 DNA binding (NFκB activity), IκBα mRNA and protein, and TNF-α and ICAM-1 mRNA expression. The dose of LPS employed and time point that animals were killed was based on a pilot study which demonstrated that administration of LPS (1 μg/5 μL; icv) to rats suppressed NFκB activity at a 2 h time point post-treatment (data not shown). LPS was administered centrally (icv) in order to avoid any contribution that peripheral

anti-inflammatory actions of clenbuterol could have on its anti-inflammatory actions in the brain.

2.4. Intracerebroventricular surgery

Anaesthesia was induced using 5% gaseous isofluorane. Rats were placed in a stereotaxic frame and were maintained on 2.5–3% gaseous isofluorane. After shaving and sterilising the top of the head a midline sagittal incision of approximately 1.5 cm in length was made between the ears. A burr hole was drilled through the skull over the left lateral ventricle (stereotaxic coordinates: 0.09 cm posterior to bregma, 0.14 cm left lateral to midline, 0.36 cm ventral from skull surface) through which a glass micropipette delivered 5 μL of vehicle or LPS at a rate of 1 μL/min. The micropipette was withdrawn slowly and the incision was closed up using surgical staples. The rat was removed from the stereotaxic frame and singly housed while recovering from anaesthesia.

2.5. Measurement of NFκB activity

Nuclear fractions were prepared from homogenised cortex and hippocampus using an NE-PER Nuclear and Cytoplasmic Extraction kit (Thermo Scientific Pierce, UK) according to manufacturer's instructions. Protein concentrations were measured in the nuclear extracts using the Pierce BCA protein assay kit (Thermo Scientific Pierce, UK), and the protein content was equalised across all samples. NFκB p65 subunit DNA binding in nuclear fractions was measured using an NFκB p65 chemiluminescent transcription factor kit (Thermo Scientific Pierce, UK) as per manufacturer's instructions, and NFκB activity was expressed as Relative Light Units (RLU)/μg protein.

2.6. Analysis of gene expression of IκB and NFκB-inducible genes by 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 and Connor, 2007). Taqman Gene Expression Assays (Applied Biosystems) containing forward and reverse primers and a FAM-labeled MGB Taqman probe were used to quantify the gene of interest. The assay ID's for the gene expression assays employed were (IκBα: Rn01473658_g1; IκBβ: Rn00578384_m1; TNF-α: Rn99999017_m1; ICAM-1: Rn00564227_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 s followed by 60 °C for 1 min. β-actin was used as an endogenous control to normalise gene expression data. Relative gene expression was calculated using the ΔΔCT method with Applied BioSystems RQ software (Applied BioSystems, UK).

2.7. Analysis IκBα protein expression and IκBα phosphorylation using Western immunoblotting

Cytoplasmic fractions were prepared from homogenised cortex and hippocampus using an NE-PER Nuclear and Cytoplasmic Extraction kit (Thermo Scientific Pierce, UK) according to manufacturer's instructions. Protein concentrations were measured in the cytoplasmic extracts using the Pierce BCA protein assay kit (Thermo Scientific Pierce, UK), and the protein content was equalized across all samples. Each sample was combined with sample buffer [0.5 M Tris–HCl, pH 6.8; 10% (w/v) sodium dodecyl sulphate; 10% (v/v) glycerol; 5% (v/v) β-mercaptoethanol; 0.05% (w/v) bromophenol blue] in a 1:1 ratio. Samples were heated at 65 °C for 5 min prior to being loaded on the polyacrylamide gel.

Proteins were separated on a 10% SDS–polyacrylamide gel by application of a constant voltage of 40 mA and transferred onto a PVDF membrane via the semi-dry transfer method (225 mA for 50 min). To assess the expression of total-IκBα and phosphorylated-IκBα, PVDF membranes were incubated with primary antibody overnight at 4 °C. The primary antibodies used were: IκBα (L35A5) mouse mAb antibody, and Phospho-IκB-α (Ser32) (14D4) antibody (Cell Signaling) diluted 1:1000 in TBS/Tween (0.1% v/v) containing 5% BSA. Immunoreactive bands were detected using a peroxidase-linked donkey anti-rabbit IgG antibody (Amersham Biosciences) diluted 1:5000 in TBS/Tween (0.1% v/v) containing 1% BSA. PVDF membranes were stripped using ReBlot Plus (Chemicon) and probed with monoclonal mouse IgG antibody that targets actin (Sigma, UK) diluted 1:1000 in TBS/Tween (0.1% v/v) containing 1% BSA, and immunoreactive bands were detected using a peroxidase-linked goat anti-mouse IgG antibody (Sigma, UK) diluted 1:5000 in TBS/Tween (0.1% v/v) containing 1% BSA. Protein bands were visualised using Immobilon Western Chemiluminescent HRP Substrate (Millipore). Bands were quantified by densitometric analysis using ImageJ software (Wayne Rasband, NIH, USA).

2.8. Statistical analysis of data

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

3. Results

3.1. Clenbuterol suppresses NFκB activity and increases IκBα expression under normal resting conditions in the rat brain

3.1.1. The β_2-adrenoceptor agonist clenbuterol suppresses NFκB activity

Administration of the long-acting brain-penetrant β_2-adrenoceptor agonist clenbuterol (0.5 mg/kg; i.p.) induced a time-dependent suppression of NFκB p65 DNA binding activity (NFκB activity) in nuclear fractions of cortex and hippocampus (Fig. 1a and b). The suppression of NFκB activity was maximal at 4 h post-treatment ($p < 0.01$). Consistent with these results, a one-way ANOVA revealed a significant effect of clenbuterol on NFκB activity in both the cortex [$F_{(3,17)} = 5.25$, $p = 0.0095$] and hippocampus [$F_{(3,17)} = 15.72$, $p < 0.0001$].

3.1.2. The β_2-adrenoceptor agonist clenbuterol induces IκBα gene expression

Rat cortex and hippocampus were examined for inhibitory IκB expression 1, 4 and 8 h following clenbuterol (0.5 mg/kg; i.p.) administration. In keeping with findings by McNamee et al. (2010c) the present study found a robust and transient increase in IκBα mRNA ($p < 0.01$) 1 h post-clenbuterol administration in both cortex and hippocampus (Fig. 1c and d) and we also found a modest and transient increase in IκBβ mRNA to a lesser extent (data not shown). Consistent with these results, a one-way ANOVA revealed a significant effect of clenbuterol on IκBα mRNA in both the cortex [$F_{(3,14)} = 3.72$, $p = 0.0372$] and hippocampus [$F_{(3,16)} = 4.32$, $p = 0.0207$].

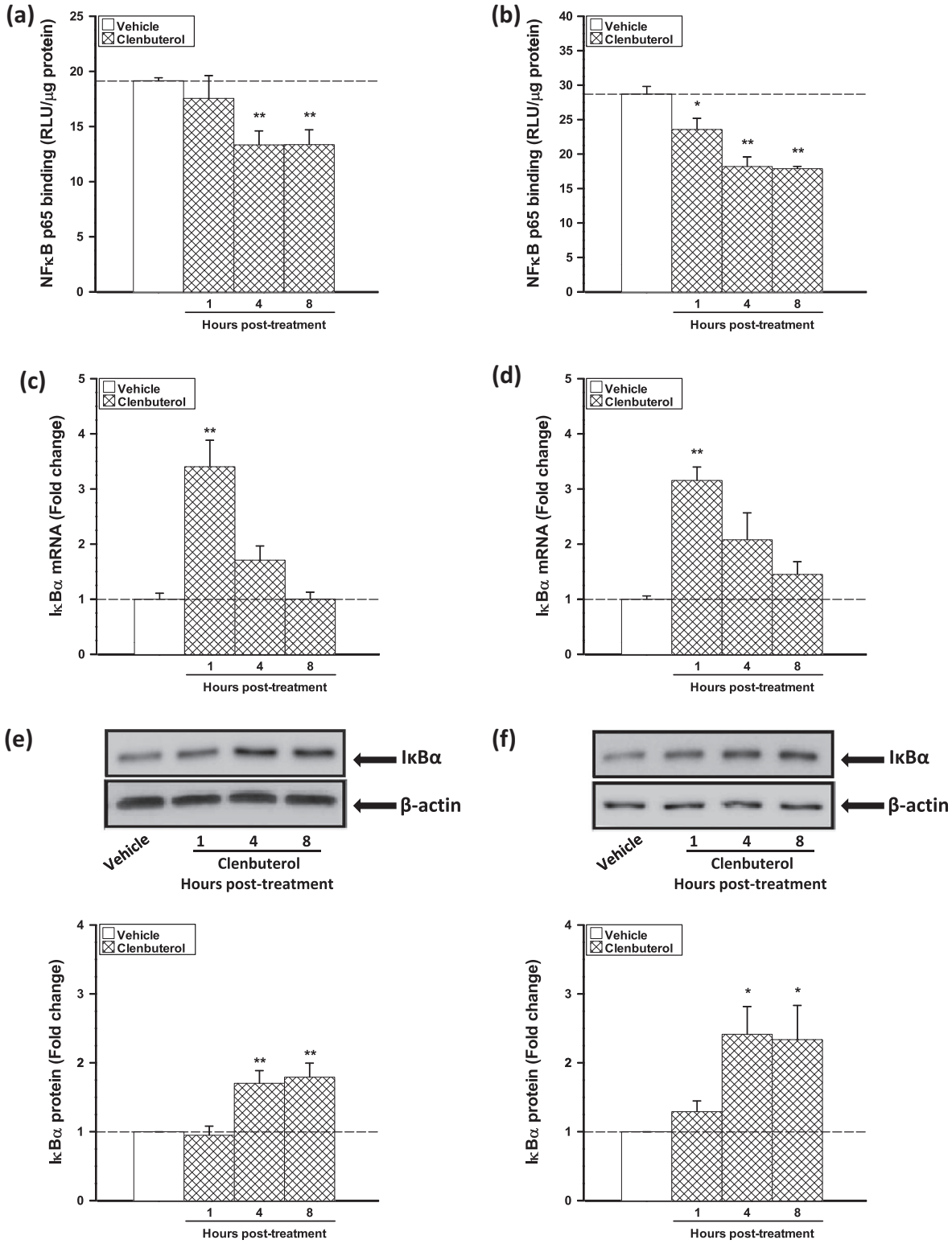

Fig. 1. The β_2-adrenoceptor agonist clenbuterol suppresses NFκB activity and increases IκBα expression in a time-dependent manner, in cortex and hippocampus. Clenbuterol (0.5 mg/kg; i.p.) or vehicle (0.89% w/v saline; i.p.) was administered to rats which were sacrificed at 1, 4 and 8 h time points post-injection. Clenbuterol induced a time-dependent suppression of NFκB p65 binding (NFκB activity) in rat cortex (a) and hippocampus (b). Clenbuterol induced a time-dependent increase in IκBα mRNA expression in cortex (c) and hippocampus (d) and an increase in IκBα protein expression in cortex (e) and hippocampus (f). Data expressed as mean + SEM ($n = 5–6$). mRNA data expressed as fold-change vs. saline vehicle. Results presented in upper panels (e and f) are representative images of Western immunoblots. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. saline vehicle (One-way ANOVA followed by *post hoc* Dunnett's procedure).

3.1.3. The β_2-adrenoceptor agonist clenbuterol induces IκBα protein expression

induction of IκBα mRNA was followed by a time-dependent increase in IκBα protein production 4 h post-clenbuterol treatment in cortex ($p < 0.01$) and hippocampus ($p < 0.05$) (Fig. 1e and f). Notably, this increase in IκBα protein mirrors the time-dependent decrease in NFκB activity seen in Fig. 1(a) and (b) 4 h after clenbuterol treatment. Thus, the increase in IκBα

protein expression coincides with the suppression of NFκB activity.

3.2. The effects of clenbuterol on NFκB activity and IκBα expression are mediated by the β_2-adrenoceptor

3.2.1. Pharmacological antagonism of β_2-adrenoceptors blocks the ability of clenbuterol to suppress NFκB activity and to induce IκBa expression

The clenbuterol-induced suppression of NFκB activity ($p < 0.05$) was blocked by pre-treatment with the non-selective β-adrenoceptor antagonist propranolol and the selective β_2-adrenoceptor antagonist ICI-118,551 in both cortex and hippocampus (Fig. 2a and b). Similarly, the clenbuterol-induced increase in IκBα expression ($p < 0.01$) was prevented by propranolol and ICI-118,551 pre-treatment cortex and hippocampus (Fig. 2c and d). In contrast, pre-treatment with the β_1-adrenoceptor antagonist metoprolol had no effect on the clenbuterol-induced changes in NFκB activity or IκBα expression (Fig. 2a–d). This clearly shows that the ability of clenbuterol to suppress NFκB activity and increase IκBα expression is mediated specifically by the central β_2-adrenoceptor subtype. Consistent with these findings a one-way ANOVA revealed a significant effect of drug treatment on NFκB activity in cortex [$F_{(4,21)} = 3.94$, $p = 0.0154$] and hippocampus [$F_{(4,21)} = 5.53$, $p = 0.0033$] and on IκBα mRNA expression in cortex [$F_{(4,23)} = 17.97$, $p < 0.0001$] and hippocampus [$F_{(4,23)} = 8.31$, $p = 0.0003$].

3.2.2. The selective β_2-adrenoceptor agonist formoterol mimics the ability of clenbuterol to suppress NFκB activity and to induce IκBα expression

A Student's *t*-test demonstrated that formoterol suppressed NFκB activity in both cortex and hippocampus 4 h post-administration ($p < 0.001$) (Fig. 3a and b), and induced an increase in IκBα mRNA ($p < 0.01$) and protein ($p < 0.05$) in both regions (Fig. 3c–f).

3.3. Pre-treatment with the β_2-adrenoceptor agonist clenbuterol suppresses LPS-induced NFκB activity, IκBα phosphorylation and degradation

The LPS-induced increase in NFκB activity ($p < 0.01$) was significantly attenuated ($p < 0.01$) by clenbuterol pre-treatment in cortex and hippocampus (Fig. 4a and b). Consistent with this finding, a two-way ANOVA revealed a significant clenbuterol × LPS interaction in both cortex [$F_{(1,18)} = 19.14$, $p = 0.0004$] and hippocampus [$F_{(1,18)} = 5.74$, $p = 0.0277$]. Notably, the LPS-induced phosphorylation of IκBα, which is responsible for NFκB activation, was also blocked by clenbuterol pre-treatment in cortex and hippocampus ($p < 0.05$) (Fig. 4c and d) where a two-way ANOVA revealed a significant clenbuterol × LPS interaction in cortex [$F_{(1,18)} = 3.87$, $p = 0.0069$] and a significant effect of clenbuterol in hippocampus [$F_{(1,15)} = 1.88$, $p = 0.0493$].

In addition, the LPS-induced suppression of IκBα protein ($p < 0.01$) was reversed by clenbuterol pre-treatment in cortex ($p < 0.01$) and hippocampus ($p < 0.05$) (Fig. 4e and f). Consistent

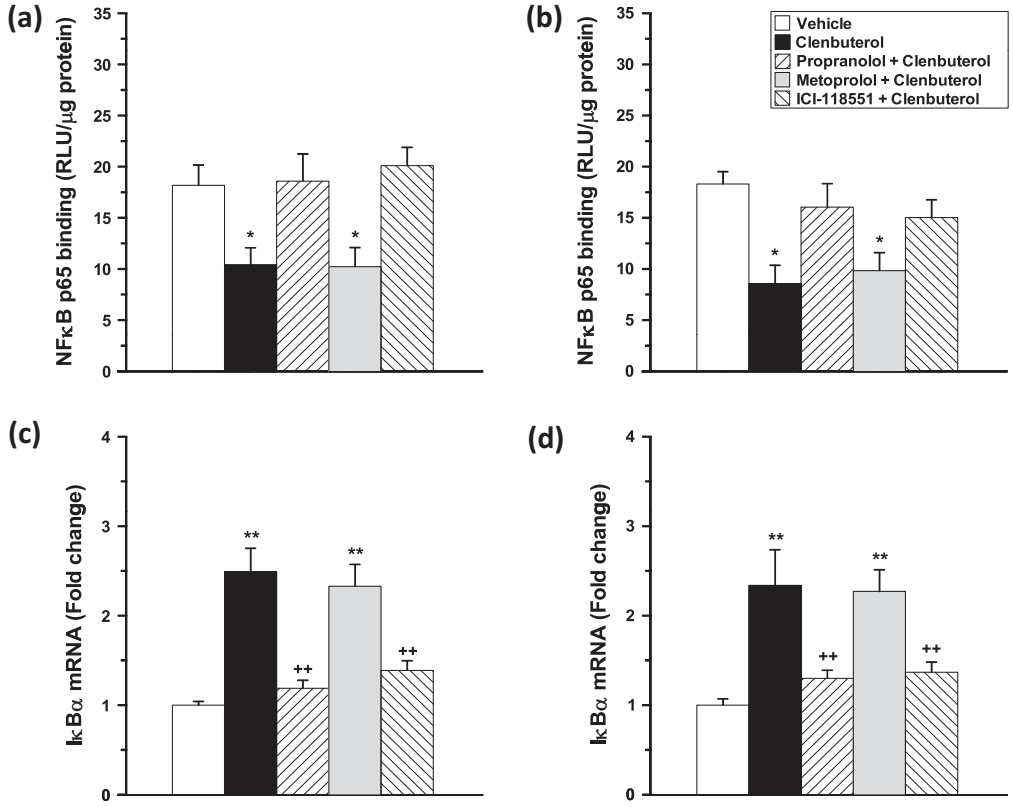

Fig. 2. Clenbuterol-induced suppression of NFκB activity and induction of IκBα mRNA expression is mediated specifically by the β_2-adrenoceptor in cortex and hippocampus Rats were pre-treated with the non-selective β-adrenoceptor antagonist propranolol (10 mg/kg; i.p.), the selective β_1-adrenoceptor antagonist metoprolol (10 mg/kg; i.p.) or the selective β_2-adrenoceptor antagonist ICI-118,551 (10 mg/kg; i.p.) 30 min prior to clenbuterol (0.5 mg/kg; i.p.). Rats were sacrificed 4 h post clenbuterol injection. The ability of clenbuterol to suppress NFκB activity and increase IκBα mRNA expression was prevented by pre-treatment with propranolol and ICI-118,551, but not metoprolol in cortex (a and c) and hippocampus (b and d). Data expressed as mean + SEM ($n = 5$–6). mRNA data expressed as fold-change vs. saline vehicle. $^{*}p < 0.05$, $^{**}p < 0.01$, vs. saline vehicle; $^{++}p < 0.01$, vs. clenbuterol vehicle (One-way ANOVA followed by *post hoc* Newman Keuls).

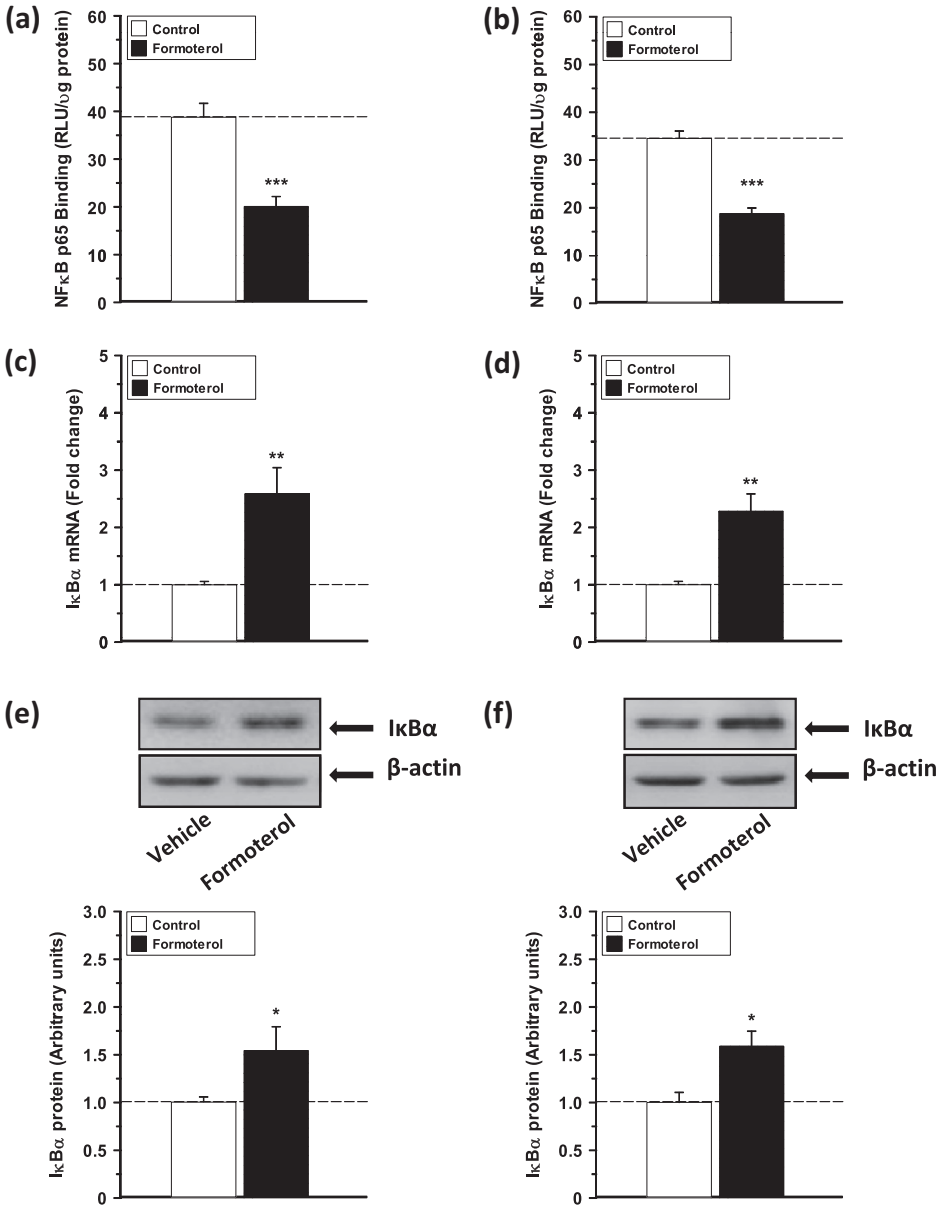

Fig. 3. The β_2-adrencoceptor agonist formoterol suppresses NFκB activity and increases IκBα expression in cortex and hippocampus. Formoterol (0.5 mg/kg; i.p) or vehicle (0.89% w/v saline; i.p) were administered and rats were sacrificed 4 h post-injection. Formoterol suppressed NFκB activity in cortex (a) and hippocampus (b). Formoterol also increased IκBα mRNA expression in cortex (c) and hippocampus (d), and IκBα protein expression in cortex (e) and hippocampus (f). Data expressed as mean + SEM ($n = 6$). mRNA data expressed as fold-change vs. saline vehicle. Results presented in upper panels (e and f) are representative images of Western immunoblots. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < .001$ vs. saline vehicle [Student's t-test].

with this finding, a two-way ANOVA revealed a significant effect of clenbuterol on IκBα protein expression in cortex [$F_{(1,18)} = 27.13$, $p = 0.0003$] and hippocampus [$F_{(1,18)} = 6.95$, $p = 0.0299$].

3.4. Pre-treatment with the β_2-adrenoceptor agonist clenbuterol suppresses LPS-induced expression of the NFκB-inducible genes TNF-α and ICAM-1

Consistent with the finding that clenbuterol suppresses LPS-induced NFκB activity, we also found that clenbuterol suppresses expression of the NFκB-inducible genes TNF-α and ICAM-1 induced by LPS in rat brain. The robust LPS-induced increase in TNF-α mRNA was significantly attenuated byclenbuterol pre-treatment in cortex (approximately 70% attenuation; $p < 0.01$) and in hippocampus (approximately 50% attenuation; $p < 0.01$) (Fig. 5a and b). Similarly, LPS-induced ICAM-1 mRNA was significantly attenuated by clenbuterol pre-treatment in both regions ($p < 0.01$) (Fig. 5c and d). Consistent with these findings, a two-way ANOVA revealed a significant clenbuterol × LPS interaction for both TNF-α [cortex: $F_{(1,18)} = 13.88$, $p = 0.0015$; hippocampus: $F_{(1,18)} = 17.97$, $p = 0.0005$] and ICAM-1 [cortex: $F_{(1,18)} = 17.88$, $p = 0.0005$; hippocampus: $F_{(1,18)} = 6.29$, $p = 0.022$].

4. Discussion

An increasing body of evidence indicates that noradrenaline via an action on glial β_2-adrenoceptors elicit endogenous anti-inflammatory actions in the brain (Feinstein et al., 2002; Heneka et al., 2002; Mori et al., 2002; Kalinin et al., 2007; McNamee et al., 2010a). Here we demonstrate that direct stimulation of central β_2-adrenoceptors following systemic treatment with the β_2-adre-

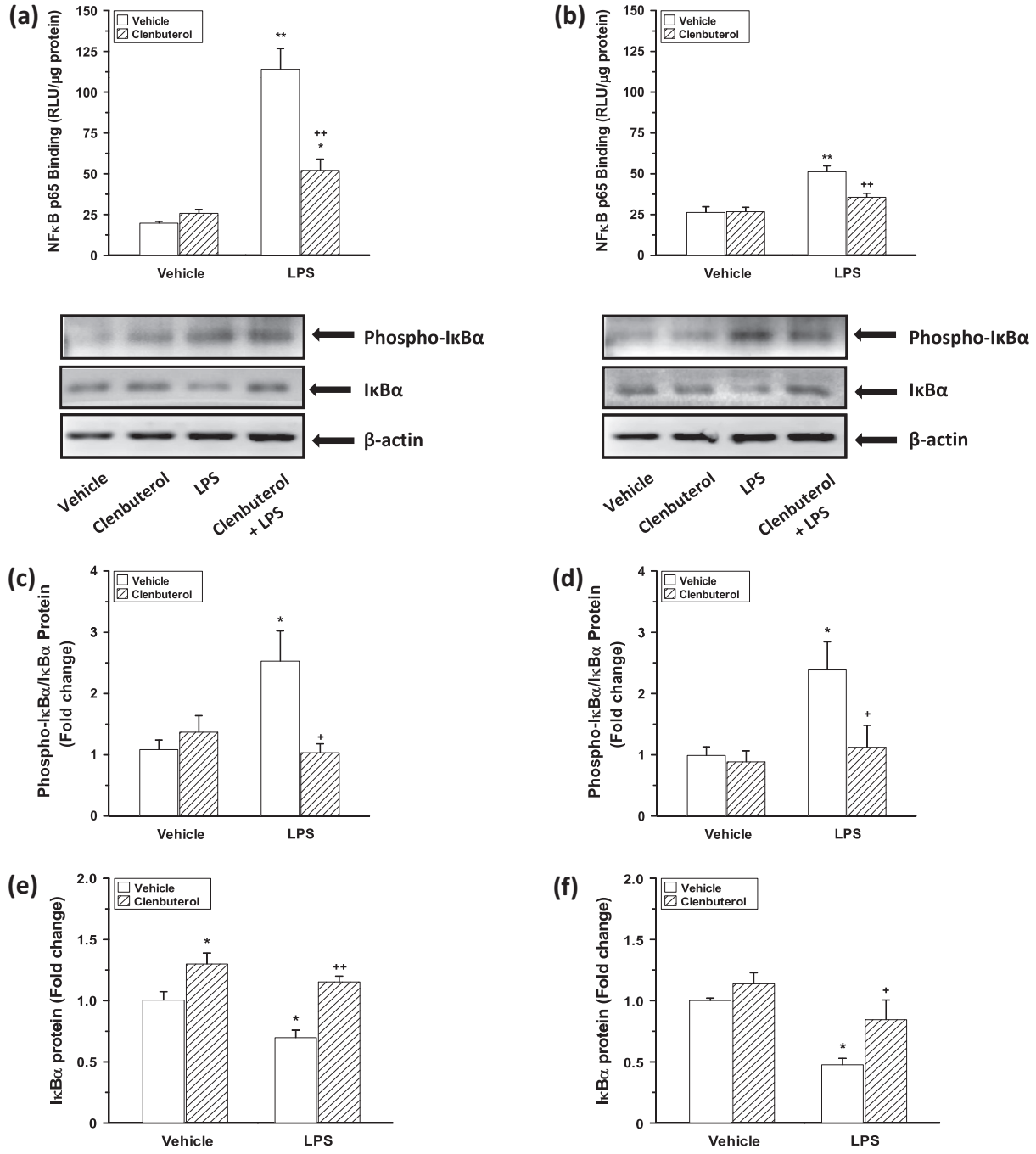

Fig. 4. Pre-treatment with the β_2-adrenoceptor agonist clenbuterol suppresses LPS-induced NFκB activation and blocks LPS-induced IκBα phosphorylation and degradation in cortex and hippocampus. Clenbuterol (0.5 mg/kg; i.p.) or vehicle (0.89% w/v saline; i.p.) were administered 1 h prior to LPS (1 μg/5 μL; i.c.v.) or vehicle (0.89% w/v saline; i.c.v.). Rats were sacrificed 2 h post LPS injection. Clenbuterol pre-treatment significantly attenuated LPS-induced NFκB activity in cortex (a) and hippocampus (b). Pre-treatment with clenbuterol attenuated the LPS-induced phosphorylation of IkBa in both cortex (c) and hippocampus (d). The LPS-induced decrease in IκBα protein was also blocked by clenbuterol pre-treatment in cortex (e) and hippocampus (f). Data expressed as mean + SEM ($n = 5$–6). Results presented in upper panels (above c and d) are representative images of Western immunoblots. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. saline vehicle; $^{+}p < 0.05$, $^{++}p < 0.01$ vs. LPS vehicle (Two-way ANOVA followed by *post hoc* Newman–Keuls test).

noceptor agonist clenbuterol suppresses NFκB activity in the rat brain under normal (non-inflammatory) conditions, as well as in response to an inflammatory stimulus (bacterial LPS). We also demonstrate that clenbuterol increases expression of the inhibitory IκBα protein, a result that supports our earlier finding that clenbuterol induced IκBα mRNA expression in rat brain (McNamee et al., 2010c). Time-course analysis of the actions of clenbuterol on IκB expression and NFκB activity demonstrates a robust early induction of IκBa mRNA expression peaking 1 h post-clenbuterol administration, followed by an increase in de novo synthesis of IκB protein expression and parallel suppression of NFκB activity at 4 and 8 h post-clenbuterol administration. Thus a single administration of clenbuterol induces a sustained suppression of NFκB activity that persists for at least 8 h post-administration which is paralleled by an increase in the inhibitory IκBα protein in the cytosol. In contrast to the robust increase in IκBα expression observed following clenbuterol treatment (3–3.3-fold), a very small and transient increase (approximately a 1.15 increase evident only at

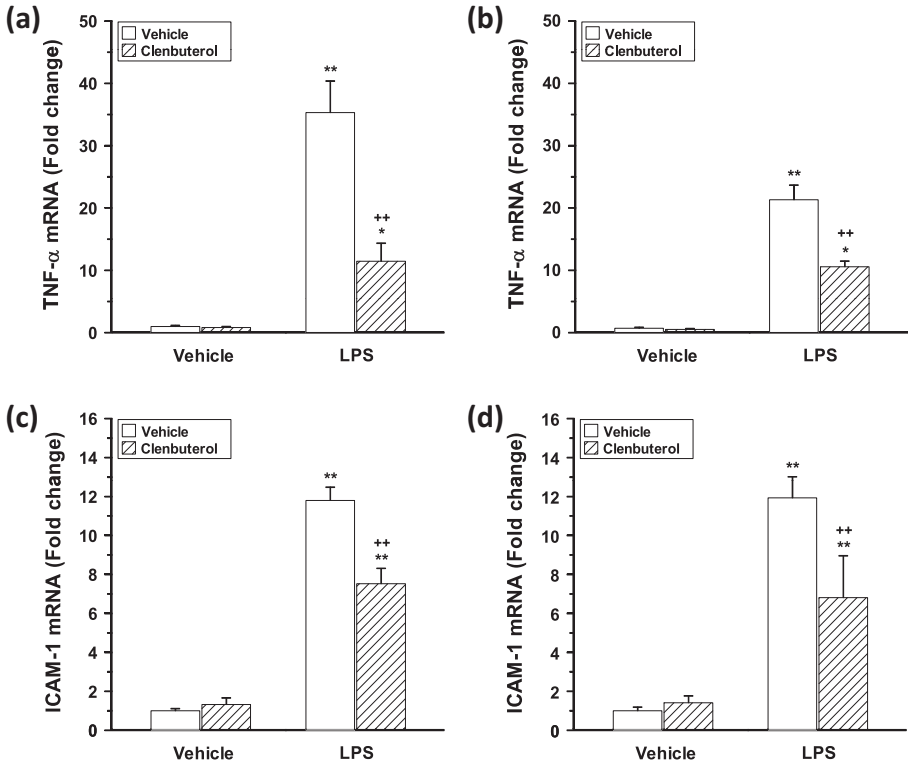

Fig. 5. Pre-treatment with the β_2-adrenoceptor agonist clenbuterol suppresses LPS-induced expression of the NFκB-inducible genes TNF-α and ICAM-1 in cortex and hippocampus. Clenbuterol (0.5 mg/kg; i.p.) or vehicle (0.89% w/v saline; i.p.) were administered 1 h prior to LPS (1 μg/5 μL; i.c.v.) or vehicle (0.89% w/v saline; i.c.v.). Rats were sacrificed 2 h post LPS injection. Pre-treatment with the β_2-adrenoceptor agonist clenbuterol significantly attenuated the LPS-induced increase in TNF-α mRNA expression in cortex (a) and hippocampus (b), and ICAM-1 mRNA expression in cortex (c) and hippocampus (d). Data expressed as mean + SEM (n = 5–6). mRNA data expressed as fold change compared to control. $^{**}p < 0.01$ vs. saline vehicle; $^{+}p < 0.05$, $^{++}p < 0.01$ vs. LPS vehicle (Two-way ANOVA followed by *post hoc* Newman–Keuls test).

1 h) in expression of the IκBβ isoform occurs following clenbuterol treatment (data not shown). Whilst the clenbuterol-induced increase in IκBβ expression was statistically significant in the cortex and approaching statistical significance in the hippocampus, the small magnitude of the increases observed suggest that a role for IκBβ in the suppressive effect of clenbuterol on NFκB expression is unlikely. Based on the magnitude of the increase of IκBα expression and the time-course of the response, we suggest that the increase in cytosolic IκBα protein observed at 4 and 8 h mediates the suppressive effect of clenbuterol on NFκB activity by sequestering NFκB p65 in the cytoplasm and preventing its translocation to the nucleus. In addition, IκBα has the ability to terminate NFκB activity by interacting with NFκB and transporting it back to the cytoplasm where is resides in an inactive form bound to IκBα (Arenzana-Seisdedos et al., 1995, 1997). This is a plausible mechanism behind the clenbuterol-induced suppression of NFκB activity as even a small (2-fold) increase in cytoplasmic IκBα (which is similar to the level induced by clenbuterol) is known to substantially inhibit NFκB activation (Miyamoto et al., 1994). While the exact mechanism by which clenbuterol induces de novo synthesis of IkBα remains to be clarified, it is likely to involve an increase in intracellular concentrations of cAMP which is long known to suppress immune responses (Rappaport and Dodge, 1982; Hasler et al., 1983; Gerlo et al., 2011). cAMP may suppress NFκB activation at the level of the differential binding of CREB and NFκB to the CREB-binding protein, which is necessary for efficient gene transcription (Parry and Mackman, 1997; Wen et al., 2010). Additionally, protein kinase A (PKA) which is activated by cAMP, has been reported to inhibit translocation of NFκB p65 to the nucleus by stabilisation of IκBα concentrations and blocking its phosphorylation in T-cells (Neumann et al., 1995). Given that clenbuterol and other β_2-adrenoceptor agonists signal via intracellular cAMP and activation of PKA (Ordway et al., 1987), it is possible that similar mechanisms are involved in the clenbuterol-induced suppression of NFκB.

Under normal circumstances, there is a natural increase in IκBα protein synthesis following NFκB activation, due to the presence of a transcriptional binding site for IκBα on the NFκB promoter (Sun et al., 1993), and this serves as a negative feedback mechanism to prevent over-activation of NFκB. Thus, the results presented here suggest that agents that are capable of increasing IκBα expression, such as the β_2-adrenoceptor agonist clenbuterol, may be useful in combating neuroinflammatory disorders where NFκB is dysregulated, such as in Alzheimer's disease (Kaltschmidt et al., 1999). In this regard, it is thought that activation of NFκB plays a role in the early phases of Alzheimer's disease when initiation of neuritic plaques and neuronal apoptosis occurs (Kaltschmidt et al., 1999).

Our group and others have shown that the effects of clenbuterol on cytokine expression in the CNS are mediated specifically by the β_2-adrenoceptor subtype (Junker et al., 2002; Culmsee et al., 2007; McNamee et al., 2010a). The findings from the present study demonstrate, through the use of selective and non-selective antagonists for β_1- and β_2-adrenoceptor subtypes, that the effect of clenbuterol on NFκB activity and IκBα expression is mediated by the β_2-adrenoceptor. These data are important in highlighting the specific role of β-adrenoceptor activation in the actions of clenbuterol on NFκB, as previous studies indicate that clenbuterol can elicit biological responses independent of β-adrenoceptors (Desaphy et al., 2003; Ngala et al., 2009). Furthermore we demonstrate that treatment with the related long-acting β_2-adrenoceptor agonist formoterol mimicked the ability of clenbuterol to suppress NFκB activity and upregulate IκBα. The fact that formoterol shares the ability of clenbuterol to suppress NFκB activity in the brain is of significance from a clinical perspective, as formoterol is currently

widely used for the treatment of asthma in humans (Welsh and Cates, 2010), whereas clenbuterol's use is confined to the veterinary setting (Erichsen et al., 1994). Combined with our previous finding that systemic formoterol treatment increases expression of the anti-inflammatory cytokine IL-10 in rat brain (McNamee et al., 2010a,b,c), these data further support the notion that formoterol can penetrate that brain following systemic administration. Taken together these data further reinforce the argument for the β_2-adrenoceptor as a neuroprotective target, and the ability of β_2-adrenoceptor activation within the CNS to suppress activity of NFκB independent of an immune stimulus adds to the hypothesis that increases in central noradrenergic tone and β_2-adrenoceptor activation provides an endogenous immunomodulatory mechanism within the brain to self-regulate inflammation.

As well as its ability to suppress NFκB activity under normal (non-inflamed) conditions in the rat brain, our data clearly shows for the first time, that clenbuterol suppresses NFκB activation that occurs in response to inflammation. It has been well documented that LPS treatment leads to phosphorylation and degradation of IκBα protein, thus freeing NFκB and allowing it to translocate to the nucleus where it induces transcription of pro-inflammatory genes (Karin and Ben-Neriah, 2000). Pre-treatment with clenbuterol significantly attenuated LPS-induced activation of NFκB in rat cortex and hippocampus. In tandem with this we found that clenbuterol blocked the LPS-induced phosphorylation of IκBα and its degradation. This lends support to our hypothesis that clenbuterol may inhibit nuclear translocation of NFκB through stabilisation of IκBα by blocking its phosphorylation (Fig. 6), in a similar manner to that seen in T-cells (Neumann et al., 1995). We hypothesise that by blocking IκBα phosphorylation, clenbuterol not only limits NFκB activity but also limits its downstream signalling as seen by the ability of clenbuterol to suppress LPS-induced TNF-α and ICAM-1, two NFκB-inducible genes.

Clenbuterol suppresses NFκB via de novo synthesis of IκBα through induction of gene expression under basal and LPS-stimulated conditions. Additionally under LPS-stimulated conditions, clenbuterol suppresses NFκB by stabilisation of IκBα by inhibition of its phosphorylation. It is however possible that pathways leading to stabilization of IkBα are also activated by clenbuterol under basal conditions but are not detectable given that basal expression is low (see faint representative bands in Fig. 4). An inflammatory stimulus (LPS) however initiates phosphorylation and changes are more readily detected.

From previous pilot investigations we determined that LPS-induced NFκB activity in the cortex peaks 2 h post-LPS and accordingly, this time was chosen as an appropriate point to assess the effects of clenbuterol on IκBα and NFκB under inflammatory conditions (Fig. 4). Clenbuterol is capable of suppressing NFκB activation over a time course from 1 to 4 h (Fig. 1) and based on this profile samples were taken 3 h post clenbuterol. However when tested under basal conditions the 3 h pre-treatment had no effect on NFκB binding. More robust reductions in NFκB binding are obtained at the 4 and 8 h time points (Fig. 1a and b) so it may be that the 3 h time point may have been too early to detect a reduction under basal conditions (as presented in Fig. 4). In any case pre-treatment with clenbuterol suppresses LPS-induced NFκB activation at this time.

Ubiquitination regulates at least three steps in the NFκB pathway. IκBα protein phosphorylation and degradation, the processing of NFκB precursors and activation of the IkB kinase (IKK) (reviewed by O'Donnell and Ting, 2012). Therefore, it is possible that clenbuterol may suppress NFκB activation not only via induc-

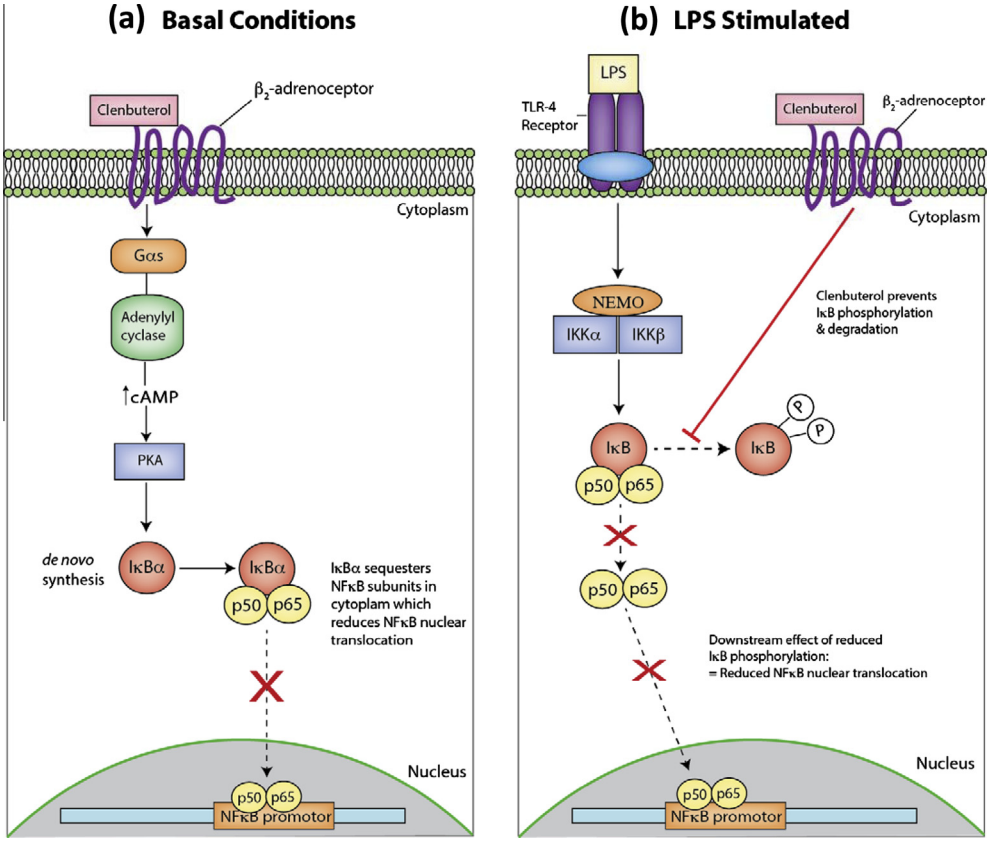

Fig. 6. Proposed mechanism by which β_2-adrenoceptor stimulation inhibits NFκB activity under (a) basal and (b) inflammatory conditions. Suppression of NFκB via de novo synthesis of IkBα under basal conditions while Fig. 6b shows the additional clenbuterol related suppression of NFkB by stabilisation of IkBα and prevention of IkBα phosphoylation.

tion of IκBα protein but also by acting upstream of IκBα protein phosphorylation. The delayed appearance of IκBα protein in the cytoplasm following mRNA expression indicates that inhibition of kinases upstream of NFκB may play a role in the prevention of IκBα degradation. In this regard, it has previously been reported that clenbuterol up-regulates the expression of the anti-apoptotic proteins BCL-2 and BCL-XL (Zhu et al., 1999) which are known in turn to prevent IκBα degradation to effect an anti-inflammatory action mediated by the inhibition of NFκB (Badrichani et al., 1999). In addition, several groups have reported that the broad-spectrum anti-inflammatory cytokine IL-10 is capable of reducing NFκB activity, not only through prevention of IκBα phosphorylation, but also by blocking IKK upstream of IκBα (Shames et al., 1998; Schottelius et al., 1999). Previous data from our group demonstrates that clenbuterol is an inducer of IL-10 and so it is possible that the suppressive effects of clenbuterol on NFκB activity may be mediated through the IKK complex (McNamee et al., 2010b).

The results of the present study support other work which implicates the regulation of NFκB activity in the anti-inflammatory effects of β_2-adrenoceptor agonists (Farmer and Pugin, 2000). Over-activation of the NFκB pathway is a pathogenic feature of neuroinflammatory disorders such as Alzheimer's disease (Kaltschmidt et al., 1999). In this regard, it is thought that activation of NFκB plays a role in the early phases of Alzheimer's disease when initiation of neuritic plaques and neuronal apoptosis occurs (Kaltschmidt et al., 1999). Thus, methods which inhibit NFκB may be a useful therapeutic tool in acute and chronic diseases where the inflammatory response becomes dysregulated. At present we are unable to confirm the specific cell type mediating these events. However, evidence suggests that β_2-adrenoceptors are found predominantly on astrocytes and microglia within the CNS (Zeinstra et al., 2002). of β_2-adrenoceptor we suspect that glial cells may be playing a role given the body of evidence which indicates that noradrenaline via an action on glial β_2-adrenoceptors elicit endogenous anti-inflammatory actions in the brain (Feinstein et al., 2002; Heneka et al., 2002; Mori et al., 2002; Kalinin et al., 2007; McNamee et al., 2010a). Future work, perhaps using single cell culture of glial cells, should be performed to show specifically where these events occur. The results of this study demonstrate that β_2-adrenoceptor activation inhibits NFκB activation and the induction of NFκB-inducible genes that occurs in response to an inflammatory stimulus. Thus, targeting the β_2-adrenoceptor is a potential mechanism to selectively target NFκB activity.

Acknowledgments

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