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β-Adrenergic receptor modulation of the LPS-mediated depression in CYP1A activity in astrocytes

Dalya Abdulla, Kenneth W. Renton*

Department of Pharmacology, Dalhousie University, Sir Charles Tupper Medical Building, Halifax, NS, Canada B3H 4H7

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Abstract

CYP1A1 and 1A2, two important P450 isoforms in the brain that metabolize many endogenous and exogenous substrates, are downregulated during central nervous system (CNS) inflammation. The stimulation of β-adrenergic receptors has been demonstrated to be anti-inflammatory in many cell types, leading us to hypothesize that stimulation of β-adrenergic receptors could prevent the down-regulation in CYP1A1 and 1A2 activity in an in vitro model of CNS inflammation. Isoproterenol, a general β_1/β_2 receptor agonist, and clenbuterol, a specific β_2 receptor agonist, were both able to prevent the LPS-induced downregulation in CYP1A1/2 activity in astrocytes. The involvement of β-adrenergic receptors was confirmed using the general β_1/β_2 receptor antagonist propranolol, which was able to abrogate the protection conferred by isoproterenol and clenbuterol in astrocytes treated with LPS. The isoproterenol and clenbuterol mediated protective effect on the LPS-induced downregulation in CYP1A activity was a cyclic AMP (cAMP) dependent process, since forskolin was able to mimic the protective effect. Isoproterenol and clenbuterol may also prevent the LPS-induced downregulation in CYP1A activity through changes in TNFα expression. Despite a slight reduction in the LPS-induced nuclear translocation of the p65 subunit of NF-κB, isoproterenol and clenbuterol had no effect on the DNA binding ability of this transcription factor, indicating that the β-adrenergic protective effects on CYP1A activity occurred independent of changes in NF-κB activity. The results presented in this paper reveal that β-adrenergic receptor stimulation can modulate cytochrome P450 activity in an in vitro model of CNS inflammation by a cAMP mediated pathway.

Keywords: Cytochrome P450; CNS inflammation; Astrocytes; β-Adrenergic receptors; EROD; NF-κB; IκBα; cAMP

1. Introduction

Cytochrome P450 (cyt P450) constitutes a superfamily of heme containing enzymes responsible for the metabolism and elimination of exogenous and endogenous substances [1,2]. The majority of the cyt P450 isoforms are located in the liver, however other extra-hepatic sites of cyt P450 localization include the central nervous system (CNS), gastrointestinal tract, kidney, lungs, and adrenal glands [3]. In the CNS, cyt P450 isoforms are thought to play a role in neurosteriod synthesis and drug metabolism [4]. In particular, members of the CYP1A family, whose roles in carcinogen detoxification and activation are well established, are thought to play a role in metabolism of eicosanoids and estrogen in the brain [5,6]. Additional substrates for the CYP1A family of cyt P450s include a diverse group of endogenous and exogenous compounds such as polyaromatic hydrocarbons, arylamines, nitrosamines, and heterocyclic amines [6,7].

Various immunostimulants cause an inflammatory response in the brain that is highly regulated and characterized by the production of cytokines, immune cell infiltration, and tissue damage [2,8–11]. Many inflammatory mediators, such as cytokines, can cause differential regulation of cyt P450 expression and activity in the brain [2,9,10,12–16]. Activity of CYP1A1 and CYP1A2

Abbreviations: LPS, lipopolysaccharide; CNS, central nervous system; cyt P450, cytochrome P450; DBA, dibenz[a,h]anthracene; NE, norepinephrine; βAR, β-adrenergic receptors; DMEM, Dulbecco's modified Eagle's medium; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol; TNFα, tumor necrosis factor alpha; EROD, 7-ethoxyresorufin *O*-dealkylase; IL-1β, interleukin-1β; IFNγ, interferon gamma; PDTC, pyrrolidine dithiocarbamate; iNOS, inducible nitric oxide synthase; NOS2, inducible nitric oxide synthase

* Corresponding author. Tel.: +1 902 494 2562; fax: +1 902 494 1388.
E-mail address: ken.renton@dal.ca (K.W. Renton).

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isoforms has been shown to be downregulated in response to LPS administered directly into the lateral cerebral ventricle in rat brain [2,4,9,10,15]. Similarly, in dibenz-[a,h]anthracene (DBA) treated astrocytes, LPS causes the downregulation of CYP1A1 and CYP1A2 activity in a dose-dependent manner [10]. Previously, our lab has shown that inflammatory conditions of the CNS lead to changes in the ability of the brain to metabolize endogenous and exogenous substrates and may exacerbate susceptibility to neurotoxic environmental agents, such as 1-methyl-4-phenylpyridinium; EROD, 7-ethoxyresorufin *O*-dealkylase MPP+ induced neurotoxicity [17].

The idea that norepinephrine (NE), one of the major catecholaminergic neurotransmitters, is neuroprotective and anti-inflammatory is not an old one. CNS diseases with an inflammatory component, such as Alzheimer's disease (AD) and multiple sclerosis (MS), involve NE depletion and/or perturbations in the NE signaling system [8]. Many studies support the notion that the increase in plasma levels of catecholamines seen during LPS-induced inflammation is an event that can be beneficial to the host response during endotoxemia [18]. β-Adrenergic receptor (βAR) ligands have been shown to modulate the release of pro-inflammatory mediators such as cytokines and cyclooxygenase-2 (COX2) from various immune cells when given concurrently with LPS in both in vivo and in vitro models of inflammation [8,19–24]. Szabo and coworkers have demonstrated that isoproterenol can protect endotoxemic mice from hypotension and causes a reduction in the amount of plasma tumor necrosis factor (TNFα) in these mice [21,24]. Clenbuterol was observed to be a potent inhibitor of the release of several LPS-induced cytokines both in vivo and in vitro [25]. Norepinephrine and isoproterenol were both shown to increase the protein and mRNA levels of IκBα, a protein that binds to the transcription factor nuclear factor κB (NF-κB) and prevents its translocation into the nucleus [8].

Given the anti-inflammatory role of norepinephrine in astrocytes, we chose to examine the effects of β-adrenergic receptor stimulation on CYP1A activity in an in vitro model of CNS inflammation. We hypothesize that β-adrenergic receptor stimulation would prevent the downregulation in CYP1A1/2 activity in astrocytes treated with LPS. Preventing the loss of P450 activity in the brain during conditions of CNS inflammation would protect the capacity of the brain to metabolize endogenous and exogenous agents.

2. Materials and methods

2.1. Materials

All reagents were purchased from Sigma–Aldrich Chemical Co. with the exception of fetal bovine serum (FBS), which was obtained from CanSera. Other exceptions are noted below. *E. coli* lipopolysaccharide (LPS) of sereotype 0127:B8 was utilized in experiments as outlined below. Several sereotypes of LPS exist, with each sereotype possessing a certain potency. The dose of LPS was chosen based on previously observed reductions in CYP1A1/2 activity seen in our laboratory [9].

2.2. Isolation and treatment of astrocytes

Astrocyte cultures were obtained using 1-day old Sprague-Dawley rats (Charles River Labs) as previously described [9]. Cell cultures were grown for 10–14 days in Dulbecco's Modified Essential Medium (DMEM) containing 10% FBS and 1% antibiotic/antimycotic (100 units penicillin, 0.1 mg streptomycin, and 0.25 μg amphotericin B) until cells reached a 90–95% confluency. We have previously shown that 14 days following the cell isolation, cell cultures consisted predominantly of astrocytes (>97% of the cells stained positive for the astrocyte marker glial fibrillary acid protein), with a minor contribution of oligodendrocytes and microglia [9,10].

On the day of drug treatment, fresh 10% FBS DMEM containing 1% antibiotic and antimycotic was added to the cells. Cell monolayers were treated with 50 nM DBA and either 10 or 50 μl drug of interest (10 μg mL^{-1} LPS, 50 μg mL^{-1} LPS, 10 nM isoproterenol, 10 nM clenbuterol, 10 nM propranolol, 1 μM forskolin, or 10 μM forskolin) administered concurrently. We have previously shown that EROD activity is not detectable in astrocytes that have not been induced with DBA [9]. Following the addition of drugs, cells were incubated for 24 h prior to measuring enzymatic activity.

2.3. 7-Ethoxyresorufin O-dealkylase (EROD) activity and protein determination

CYP1A1/2 activity in astrocyte cultures was determined by measuring the rate of formation of resorufin from the CYP1A1/2 substrate ethoxyresorufin using a modification of a previously described procedure [9]. CYP1A activity is expressed as the amount (pmoles) of resorufin formed per mg protein per min.

Following determination of EROD activity in astrocytes, the remaining cell medium was aspirated and 1 mL of fresh phosphate buffered saline (PBS) was added to each well. The astrocytes were scraped into the PBS then sonicated to lyse the cells. Protein concentrations in cell sonicates were determined using a modification of the method described by Lowry et al. [26]. For all experiments, EROD activity was normalized to the amount of protein present in the sample.

2.4. Nuclear extract preparations

Nuclear extracts from treated astrocytes were prepared according to a modified Dignam protocol [27]. Briefly, cell

monolayers were scraped in a total of 1 mL of phosphate buffered saline, and spun at 13 000 rpm for 10 min to collect the cellular pellet. The pellet was then lysed using Lysis Buffer (10 mM Hepes buffer pH 7.9, 10 mM KCl, 2 mM $MgCl_2$, 0.5 mM diothiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 5 μg mL^{-1} aprotonin, 5 μg mL^{-1} pepstatin A, and 5 μg mL^{-1} leupeptin) containing 0.1% Nonidet P-40 and incubated for a period of 15 min on ice to ensure complete lysis. Following 10 homogenization strokes with a Dounce homogenizer, the suspension was spun at 7500 rpm for 10 min to pellet the nuclei. The supernatants were stored at −80 °C until usage. The nuclear pellet was suspended in Nuclear Lysis Buffer (20 mM Hepes buffer pH 7.9, 0.25% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 5 μg mL^{-1} aprotonin, 5 μg mL^{-1} pepstatin A, and 5 μg mL^{-1} leupeptin) and incubated on ice for a period of 60 min to ensure lysis of the pellet. Nuclear proteins were collected following a spin at 10 000 rpm and stored at −80 °C until usage.

2.5. NF-κB p65 subunit immunoblotting

An amount of 4 μg of nuclear protein was electrophoresed on 7.5% gels and electrophoretically transferred onto an Immobilon-P membrane (Millipore Corporation) using a wet transfer apparatus (Bio-Rad, Ontario, Canada) running over night at 22 V. The p65 subunit of NF-κB was detected using a polyclonal antibody that detects non-phosphorylated murine p65 subunit (Santa Cruz) coupled with an anti-rabbit secondary antibody conjugated to peroxidase (1/4000 dilution). Band visualization was performed using enhanced chemiluminescence+ (ECL^+) substrate (Amersham Biosciences). Band densities were detected using a phosphor imager (Amersham) and quantified using ImageQuant™ 5.2 software.

2.6. Electrophoretic mobility shift assay (EMSA)

A double stranded oligonucleotide with high affinity for NF-κB binding (Santa Cruz, USA) with a κB site (5′-GGG GAC TTT CCC-3′) was utilized. A total of 2 μg of the NF-κB oligonucleotide was end labelled using γ-[^{32}P]-ATP (Perkin and Elmer) using PNK kinase (Fermentas). Binding reactions were performed in a 20 μL volume and were pre-incubated for a period of 15–20 min with the non-specific DNA competitor poly(dI-dC) prior to the addition of the specified oligonucleotides. Binding reactions contained 10 μg of nuclear extracts, 4 μL of 5× Binding Buffer (50 mM Tris, pH 7.6, containing 5 mM $MgCl_2$, 2.5 mM EDTA, 2.5 mM DTT, 250 mM NaCl, and 20% glycerol), 2 μg of poly(dI-dC), and 50 000 cpm of the [^{32}P] labelled NF-κB oligonucleotide. Following a 30 min incubation at room temperature, 2 μL of a 10× Loading Dye (250 mM Tris, pH 7.6, containing 0.2% bromophenol blue and 40% glycerol) was added to each reaction and the samples were then loaded onto a 5% – 0.4× TBE non-denaturing polyacrylamide gel (pre-run at 100 V for an hour) in 0.4× TBE, pH 8.0, for ∼2 h. In cases of competition, a 20× in excess non-radioactive oligonucleotide was utilized (NF-κB for the specific competitions and AP-1 for the non-specific competitions) and was included in the reaction mixture. Gels were dried using a Bio-Rad gel dryer (Bio-Rad) and exposed to a phosphor storage screen for a period of 16–24 h. Band densities were detected using a phosphor imager (Amersham) and quantified using ImageQuant™ 5.2 software.

2.7. RNA isolation and Northern blotting

Total RNA from confluent treated astrocytes was isolated using the TriZol® method, and quality was determined using 260/280 nm ratios. An amount of 2.5 μg of total RNA was electrophoresed on a 1.1% formaldehyde gel and transferred onto an immobolin–NY+ membrane (Millipore Corporation) overnight and fixed to the membrane by UV cross linking and heating for 1 h at 65 °C. Blots were prehybridized for 1 h in 10 mL of Sigma Perfecthyb™ Plus (Sigma–Aldrich Chemical Co.) after which the α-[^{32}P]-CTP (Perkin and Elmer) labelled probes (RmT Random Primer Labelling kit, Stratagene) were added to a specific activity of 1×10^7 cpm. Blots were exposed to a storage phosphor screen (Amersham Biosciences) for 16 h and scanned using a phospher imager (Amersham Biosciences). Bands were quantified using ImageQuant 5.2™ software (Amersham Biosciences). Probes specific for rat IκBα and TNFα were made using a TOPO TA Cloning® kit (Invitrogen) according to manufacturer's instructions and based on the following primers: IκBα FWD 5′-CAT GAA GAG AAG ACA CTG ACC ATG GAA-3′, IκBα REV 5′-TGG ATA GAG GCT AAG TGT AGA CAC G-3′ [8], TNFα FWD 5′-TAC TGA ACT TCG GGG TGA TTG GTC C-3′, and TNFα REV 5′-CAG CCT TGT CCC TTG AAG AGA ACC-3′ [28]. A human GAPDH probe (obtained from Dr. C.J. Sinal, Dalhousie University) that cross-reacts with rat GAPDH was utilized to determine equal loading.

2.8. MTT assay

Mitochondrial respiration, an indicator of cell viability, was assessed in astrocytes using an MTT assay. The basis of this assay is to determine the mitochondrial dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide MTT into formazan, which can then be quantified colorimetrically [29]. Astrocytes were grown in 96-well plates for 1 week in 10% FBS supplemented DMEM containing 1% antibiotic and antimycotic. On the day of the experiment, cells were incubated with 0.83 μg mL^{-1} of MTT for 2 h at 37 °C and 5% CO_2. Following the incubation period, the culture medium was removed by blotting the 96-well plate on blotting

paper and the cells were solubilized with 100 μL of dimethylsulfoxide (DMSO). The amount of formazan formed from MTT was quantified colorimetrically at a wavelength of 492 nm.

2.9. Statistical analysis

All data are reported as the mean ± the standard error of the mean. Multiple comparisons were made using the one-way or two-way analysis of variance (ANOVA) with statistical significance set at $P < 0.05$ and assessed by the Bonferroni post hoc analysis test.

3. Results

3.1. Isoproterenol and clenbuterol prevent the LPS-induced downregulation of EROD activity

The incubation of astrocytes with LPS for a period of 24 h caused a decrease in EROD activity (Fig. 1). To examine the effects of β adrenoceptor activation on EROD activity, astrocytes were incubated with 50 μg mL^{-1} LPS and either isoproterenol (a β_1/β_2 agonist) or clenbuterol (a specific β_2 agonist) (Fig. 1). A 70% decrease in EROD activity was observed in cells treated with LPS compared to those treated with media. When either isoproterenol or clenbuterol were co-incubated with 50 μg mL^{-1} LPS, the loss in EROD activity in response to LPS was prevented. Incubation of astrocytes with isoproterenol alone caused a 20% increase in EROD activity while incubation with clenbuterol alone had no effect on EROD activity. The protective effects of isoproterenol and clenbuterol occurred independent of cell viability, since astrocytes treated with 50 μg mL^{-1} of LPS experienced a 21.9% (± 3.0%) decrease in cell viability and the addition of isoproterenol or clenbuterol (10 nM) caused no further change in cell viability (18 ± 4.4 and 16 ± 8.0%, respectively). Both isoproterenol and clenbuterol alone had no effects on cell viability (4% increase (±10.8%) and 5% decrease (±10.6%), respectively). Similar effects were obtained with cells that were incubated with 10 μg mL^{-1} of LPS (data not shown).

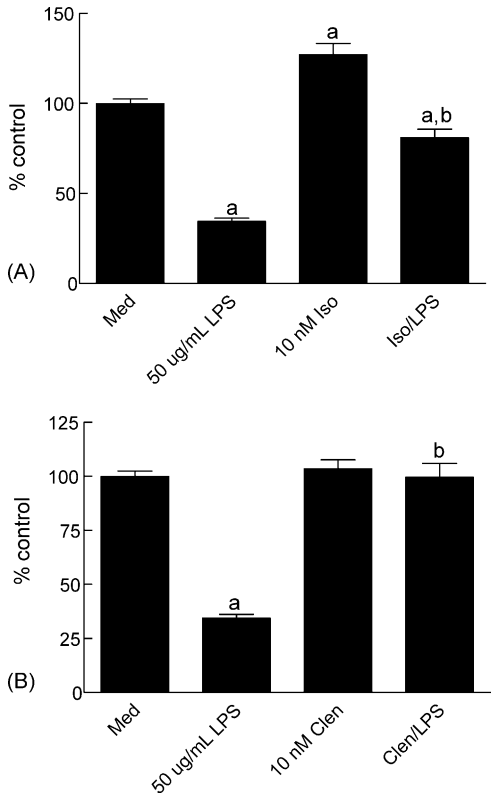

Fig. 1. Isoproterenol and clenbuterol protect against the LPS-induced downregulation in CYP1A1/2 activity in astrocytes. Astrocytes were incubated with 50 μg mL^{-1} of LPS with or without 10 nM isoproterenol (Iso) (A) or 10 nM clenbuterol (Clen) (B). Following incubation, the media was removed and the cells were incubated with 0.6 μM ethoxyresorufin and activity was measured 2 h later. Results are presented as % control of media only (med) treated samples and represent the averages of six separate experiments. Average EROD activity for media treated cells was 2.84 pmoles of resorufin per mg protein per min, which was normalized to 100%. "a" statistically different compared to media treated cells ($P < 0.05$). "b" statistically different compared to LPS treated cells ($P < 0.05$).

3.2. The isoproterenol and clenbuterol mediated protection of the LPS-induced downregulation in EROD activity is a β-adrenoceptor mediated process

The effect of 10 nM propranolol on the isoproterenol and clenbuterol mediated protection in EROD activity in the presence of either 10 or 50 μg mL^{-1} of LPS is shown in Fig. 2. LPS (10 or 50 μg mL^{-1}) reduced EROD activity by 50 and 70%, respectively, compared to media treated cells. Both isoproterenol and clenbuterol abrogated the loss in EROD activity in astrocytes treated with 10 or 50 μg mL^{-1} of LPS. Propranolol, a general β-adrenoceptor antagonist, was able to restore the ability of 10 and 50 μg mL^{-1} of LPS to depress CYP1A activity in isoproterenol and clenbuterol treated cells. Propranolol alone had no effect on EROD activity.

3.3. The isoproterenol and clenbuterol mediated protection of EROD activity is a cAMP dependent process

To examine the effects of cAMP elevation on EROD activity, cells were treated with 50 μg mL^{-1} LPS concurrently with either 1 or 10 μM of forskolin. These doses of forskolin was previously shown to cause an elevation in cAMP levels in astrocytes [30]. EROD activity was reduced by 80% with the 50 μg mL^{-1} dose of LPS when compared to media treated cells (Fig. 3). At a dose of 10 μM, forskolin caused a significant increase in EROD activity, however the 1 μM dose had no effect on EROD activity. In cells treated with either dose of forskolin, LPS was unable to depress EROD activity. Similar results were

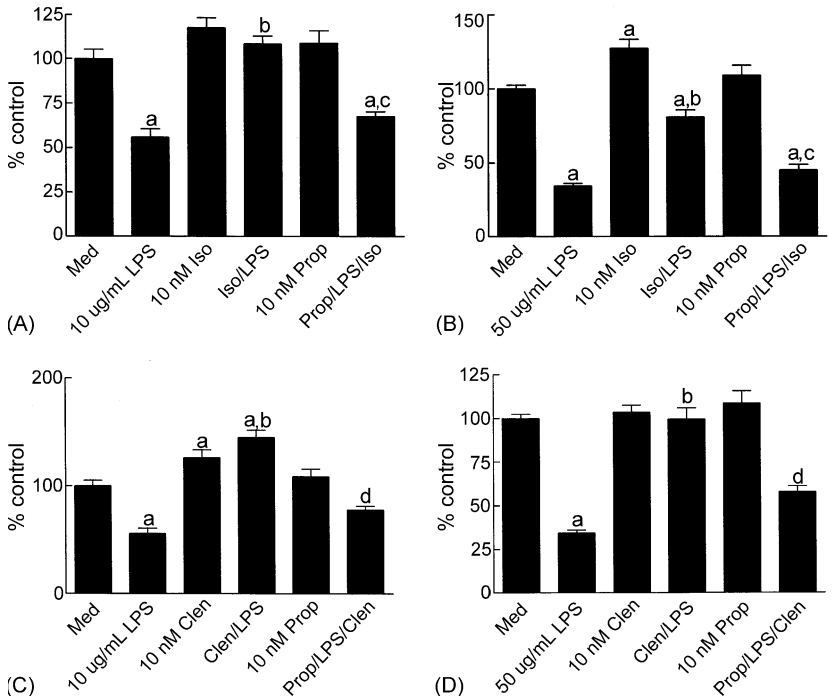

Fig. 2. The protection in the LPS-induced downregulation in CYP1A1/2 activity by isoproterenol and clenbuterol in astrocytes is a β-adrenergic receptor mediated effect. Astrocytes were incubated with 10 nM of propranolol (Prop) and treated with (A) 10 nM isoproterenol (Iso) and 10 μg mL^{-1} LPS, (B) 10 nM isoproterenol and 50 μg mL^{-1} LPS, (C) 10 nM clenbuterol (Clen) and 10 μg mL^{-1} LPS, and (D) 10 nM clenbuterol and 50 μg mL^{-1} LPS. CYP1A1/2 activity was measured 24 h later as described in Section 2 and results are presented as % control of media only (med) treated cells (average EROD activity was 6.109 pmoles of resorufin per mg protein per min). "a" is statistically different compared to media treated cells ($P < 0.05$). "b" is statistically different compared to LPS treated cells ($P < 0.05$). "c" is statistically different compared to isoproterenol and LPS treated cells ($P < 0.05$). "d" is statistically different when compared to clenbuterol and LPS treated cells ($P < 0.05$).

obtained when astrocytes were treated with 10 μg mL^{-1} of LPS and 10 μM of forskolin (data not shown).

3.4. Isoproterenol and clenbuterol prevent the LPS-induced upregulation in TNFα expression

The expression levels of TNFα were significantly increased in astrocytes 3 h following the treatment with 50 μg mL^{-1} of LPS (Fig. 4). When astrocytes were administered either isoproterenol or clenbuterol alone, TNFα expression levels were unchanged compared to media treated cells. The expression levels of TNFα in astrocytes treated with isoproterenol and LPS were significantly lower compared to LPS only treated cells (Fig. 4). Similarly, the expression levels of TNFα in astrocytes treated with clenbuterol and LPS were significantly lower compared to LPS only treated cells (Fig. 4).

3.5. Isoproterenol and clenbuterol do not exert their protective effect on the LPS-induced downregulation in EROD activity through direct changes in NF-κB activity

The effects of LPS on the translocation of the p65 NF-κB subunit was examined using a specific p65 NF-κB subunit antibody. The levels of nuclear p65 appeared to be increased by 74% and 51% at 1 and 2 h, respectively, following the addition of LPS to media (Fig. 5). Incubating cells with isoproterenol or clenbuterol appeared to reduce the LPS-mediated nuclear translocation of the p65 NF-κB subunit at 1 and 2 h. Isoproterenol (10 nM) and clenbuterol (10 nM) alone had a modest or no effect on nuclear p65 translocation.

The effect of LPS on the DNA binding ability of NF-κB was assessed using electromobility shift assays and changes in IκBα mRNA. Up to a six-fold increase in NF-κB binding was observed in astrocytes incubated with 50 μg mL^{-1} LPS for a period of 1 h compared to media treated cells. Isoproterenol and clenbuterol alone had no effects on NF-κB binding. When given in combination with LPS, neither isoproterenol nor clenbuterol were able to abrogate the LPS-induced upregulation in NF-κB binding (Fig. 6). IκBα mRNA levels were significantly increased (over five-fold) 1 h following the addition of 50 μg mL^{-1} LPS to astrocytes (Fig. 7A and C), but were not affected by treatment with either isoproterenol or clenbuterol. The levels of IκBα mRNA expression remained elevated 24 h following the administration of 50 μg mL^{-1} LPS, and were not affected by isoproterenol or clenbuterol (Fig. 7B and D).

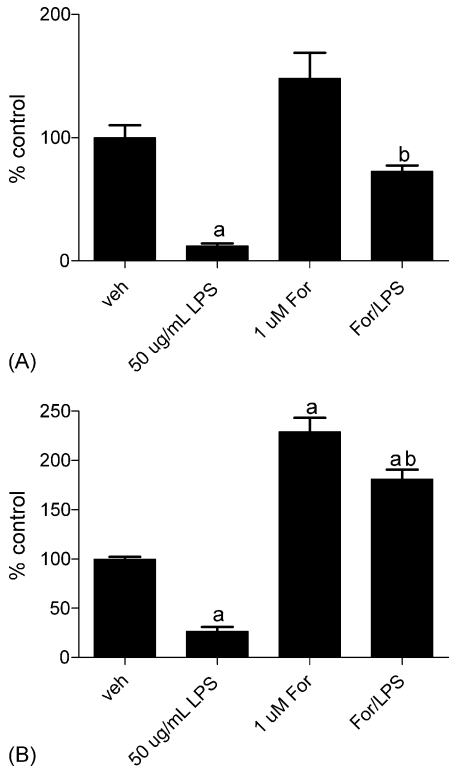

Fig. 3. The isoproterenol and clenbuterol mediated protection in CYP1A1/2 activity following LPS stimulation in astrocytes occurs through increases in cAMP. Astrocytes were incubated with 50 μg mL^{-1} of LPS and either 1 μM (A) or 10 μM (B) forskolin (For) for a period of 24 h. EROD activity was then measured as described in Section 2. The mean values from three separate experiments are shown for each treatment and are presented as % control of media (med) treated cells only. The average EROD activity for media treated cells was 8.38 pmoles of resorufin per mg protein per min. "a" is statistically different compared to media treated cells ($P < 0.05$). "b" is statistically different compared to LPS treated cells ($P < 0.05$).

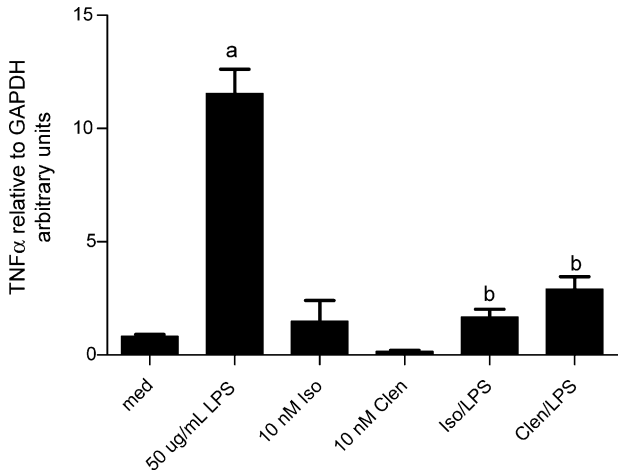

Fig. 4. Isoproterenol and clenbuterol abrogate the LPS-induced upregulation in TNFα expression. Astrocytes were incubated with 50 μg mL^{-1} of LPS and either 10 nM isoproterenol (Iso) or clenbuterol (Clen) for 3 h. Total RNA was isolated using TriZol Reagent as described in Section 2 and 2.5 μg of RNA was electrophoresed and probed using both a TNFα and GAPDH probe. Blot quantification was performed as described in the Section 2, and the results represent the pooled data of three separate experiments performed 3 h following drug addition. "a" is statistically different compared to media treated cells ($P < 0.05$). "b" is statistically different compared to LPS treated cells ($P < 0.05$).

4. Discussion

During CNS inflammation, a wide variety of inflammatory mediators such as cytokines, prostaglandins, nitric oxide, and reactive oxygen species are released from activated microglia, the resident macrophages of the CNS [11]. Astrocytes are the major glial cells in the CNS and have an important physiological role in integrating neuronal inputs, neurotransmitter release, and the protection and repair of nervous tissue after damage [31]. We have previously shown that stimulation of astrocytes with lipopolysaccharide (LPS) causes a loss in cyt P450 activity that is accompanied by the production of an inflammatory response characterized by increased levels of cytokines and NO production [9,10]. Pro-inflammatory cytokines such as TNFα, interleukin-1β (IL-1β), and interleukin-6 (IL-6), participate in the downregulation of several cyt P450 isoforms at the activity, protein, and mRNA levels in both peripheral and CNS models of inflammation [5,10,13,32]. Previous work in our laboratory has provided evidence that TNFα, IL-1β, and interferon-γ (IFNγ) are capable of downregulating CYP1A activity in astrocytes [10].

In the present study, we show that the administration of the β-adrenergic agonists isoproterenol and clenbuterol prevents the LPS-induced downregulation of CYP1A1/2

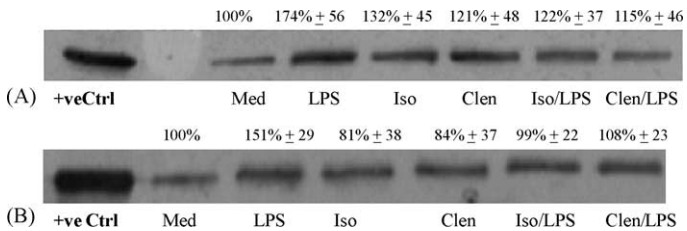

Fig. 5. The protective effects of isoproterenol and clenbuterol on the LPS-induced downregulation in CYP1A1/2 activity occurs independent of changes in p65 translocation. Astrocytes were incubated with 50 μg mL^{-1} of LPS and either 10 nM isoproterenol (Iso) or 10 nM clenbuterol (Clen) for either 1 or 2 h. Nuclear and cytoplasmic fractions were prepared as described in Section 2, electrophoresed and the p65 subunit determined using a specific antibody. The cytoplasmic fraction containing NF-κB was used as a positive control (+ve Ctrl). Representative blots for 1 h samples (A) and 2 h samples (B) are shown. The mean values from three separate experiments are shown for each treatment. Amido black staining was performed to ascertain equal protein loading. No treatments were statistically significant.

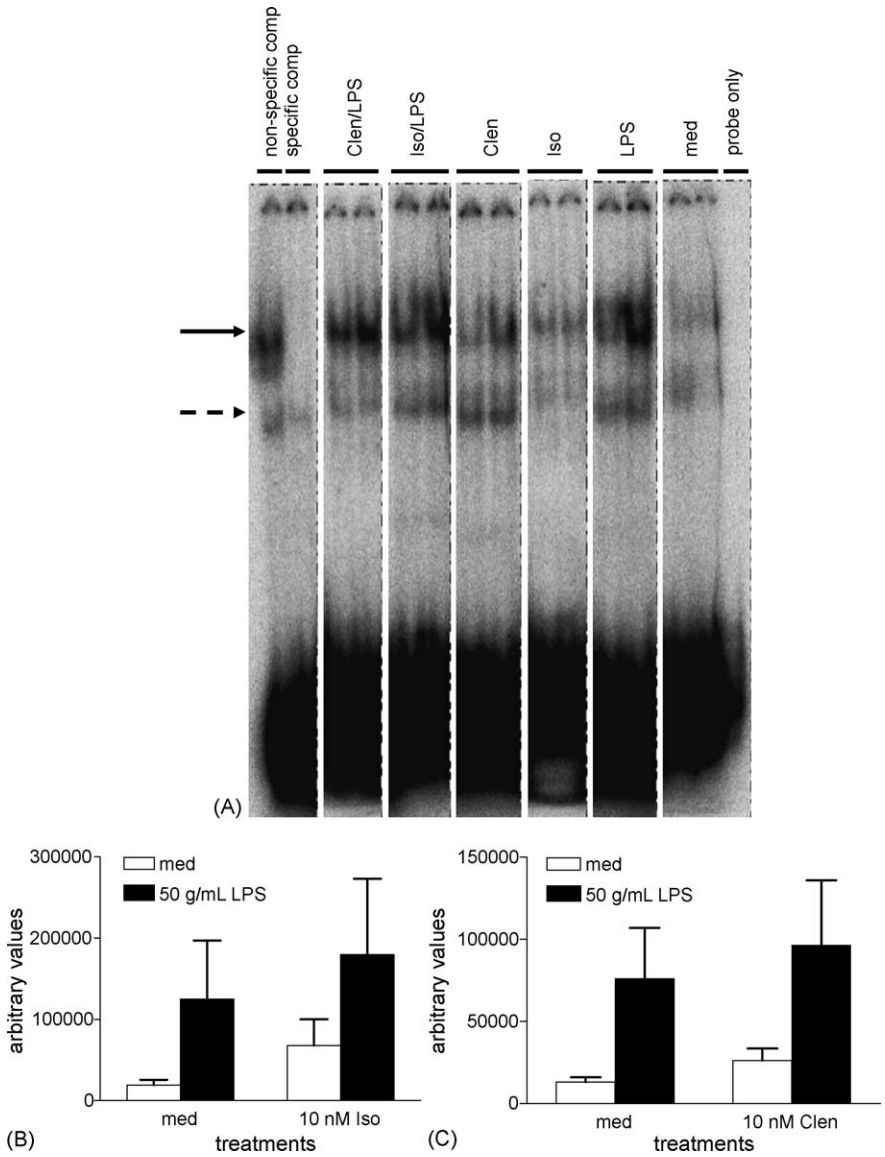

Fig. 6. Isoproterenol and clenbuterol have no effect on the LPS-induced upregulation in NF-κB binding in astrocytes. Astrocytes were incubated with 50 μg mL^{-1} of LPS and either (B) 10 nM isoproterenol (Iso) or (C) clenbuterol (Clen) for a period of 1 h, following which nuclear fractions were prepared and binding reactions were performed as described in Section 2. A representative blot is shown in (A), where solid line indicates specific binding and the dashed line indicates a non-specific binding. Specific comp and non-specific comp indicate competitions performed with excess non-radioactive NF-κB oligonucleotide and non-radioactive AP-1 oligonucleotide, respectively, as described in Section 2. The plotted results in (B) and (C) represent the pooled data from three separate experiments.

activity in astrocytes. Since the protective effect of these β-adrenergic agonists was blocked by propranolol (a general β_1/β_2 antagonist), we conclude that β-adrenergic receptor stimulation protects against the loss of CYP1A1/2 activity. To our knowledge, this is the first time an observation has been made linking CYP activity regulation through β-adrenergic receptor stimulation during inflammatory conditions. Norepinephrine has been reported to be neuroprotective in astrocytes (which express both β_1 and β_2 receptors) in various models of inflammation and neurotoxicity [23,33]. The protection provided by β-adrenergic receptor stimulation during the LPS-induced downregulation of CYP1A activity did not occur by the prevention of cell death, since MTT assays revealed a slight decrease in cell viability with 50 μg mL^{-1} LPS that was not altered by either isoproterenol or clenbuterol.

The β-adrenergic receptor is a seven-transmembrane protein coupled to a stimulatory G protein. Upon ligand binding, the Gα subunit dissociates and activates adenylyl cyclase (AC), eventually leading to increases in intracellular cyclic AMP (cAMP) concentrations. The second messenger cAMP activates protein kinase A (PKA), leading to a multitude of effects such as the release of intracellular Ca^{2+} stores and phosphorylation of cAMP response element binding (CREB) protein [34]. To examine the effects of cAMP elevation on the LPS-induced downregulation in CYP1A activity, we co-incubated astrocytes with forskolin and LPS. Forskolin was able to

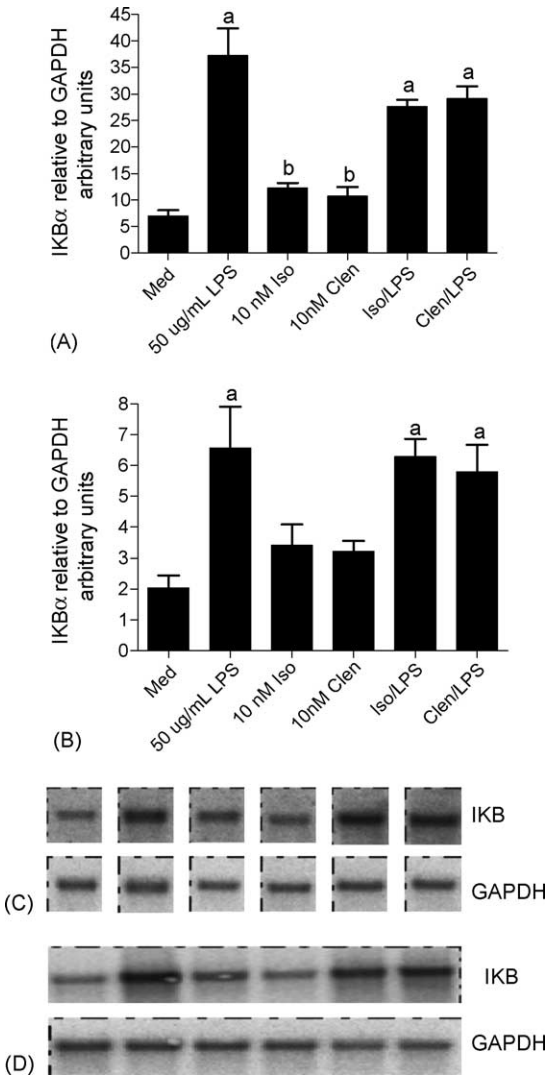

Fig. 7. The effects of isoproterenol and clenbuterol on the LPS-induced upregulation of IκBα expression in astrocytes. Astrocytes were incubated with 50 μg mL^{-1} of LPS and either 10 nM isoproterenol (Iso) or clenbuterol (Clen) for either 1 or 24 h. Total RNA was isolated using TriZol Reagent as described in Section 2 and 2.5 μg of RNA was electrophoresed and probed using both an IκBα and GAPDH probe. Representative blots for the 1 h samples (C) and the 24 h samples (D) are shown. Blot quantification was performed as described in Section 2, where (A) and (B) represent the pooled data of three separate experiments performed at 1 and 24 h, respectively, following drug addition. "a" is statistically different compared to media treated cells ($P < 0.05$). "b" is statistically different compared to LPS treated cells ($P < 0.05$).

completely prevent the LPS-induced downregulation in CYP1A activity. It has been shown that the phosphodiesterase (an enzyme involved in cAMP degradation) inhibitor, pentoxifylline, can protect against the LPS-induced downregulation in cyt P450 during models of systemic inflammation [35]. These observations, coupled with the fact that forskolin was able to abrogate the LPS-induced downregulation in CYP1A activity, indicate that the isoproterenol and clenbuterol mediated protection in CYP1A activity observed in this study is likely occurring through increases in intracellular cAMP.

The administration of LPS to astrocytes causes an increase in the release of pro-inflammatory cytokines such as TNFα and IL-1β [36]. We have previously shown that TNFα and IL-1β cause a downregulation in EROD activity in astrocytes [36]. β-Receptor agonists have been shown to modulate the LPS-induced release of these cytokines from various immune cells [24], and therefore to examine whether the isoproterenol and clenbuterol mediated effects observed in this study were mediated through changes in TNFα, we examined the effects of these agents on the LPS-induced upregulation in the expression of this cytokine. Our results reveal that both isoproterenol and clenbuterol were able to abrogate the LPS-induced increases in TNFα expression, indicating a possible mechanism for the protective role of these two agents on CYP1A activity in astrocytes. Norepinephrine has been shown to upregulate the expression of PPARγ (peroxisome proliferator-activated receptor gamma), a nuclear hormone receptor present in the CNS and adipose tissue [37]. Several PPARγ agonists have demonstrated anti-inflammatory effects in immune cells and diseases [38,39], and therefore it is possible that the β-adrenergic agonist mediated abrogation of the LPS-induced upregulation in TNFα expression in astrocytes is occurring through an upregulation in PPARγ.

We sought to examine the role of NF-κB in the β-adrenergic mediated protection observed in this study, since the anti-inflammatory effects of isoproterenol and clenbuterol have been attributed to changes in NF-κB signalling [8]. NF-κB is a major transcription factor responsible for the induction of pro-inflammatory genes and is present in the cytoplasm as a dimer of different components of the Rel family of proteins bound to and inhibited by IκBα [40]. There is a continuous shuttling of NF-κB bound to IκBα between the cytoplasm and nucleus, however upon stimulation by LPS and TNFα, proteosome-mediated degradation of IκBα occurs which allows the translocation of NF-κB into the nucleus to induce the transcription of numerous pro-inflammatory genes [41]. LPS was only able to cause a slight upregulation in p65 translocation compared to media treated cells, since the antibody utilized in these experiments identifies non-active and non-phosphorylated p65. The incubation of astrocytes with isoproterenol or clenbuterol was not able to completely prevent this slight upregulation in p65 translocation that occurs 1 h following the treatment of astrocytes with LPS. In order to ascertain the role of NF-κB on the β-adrenergic mediated protection in CYP1A activity, we performed electromobility shift assays (EMSAs) and Northern blot analyses to examine the DNA binding capacity of NF-κB and changes in IκBα mRNA, respectively. The EMSAs indicated that the LPS-mediated increase in NF-κB binding was not altered by isoproterenol or clenbuterol. We observed an increase in IκBα mRNA, and since it is known that the IκBα promoter contains several κB response elements [42], this result strengthens our observations of increased nuclear NF-κB translocation following LPS stimulation. Both β-adrenergic receptor

agonists were not able to abrogate the LPS-induced increase in IκBα mRNA levels. The LPS-induced upregulation in TNFα expression at 3 h observed in this study could still lead to an upregulation in NF-κB. Therefore the ability of isoproterenol and clenbuterol to abrogate the LPS-induced upregulation in TNFα expression could indicate that the β-adrenergic receptor mediated protection in CYP1A activity observed in this study could still be occurring through changes in NF-κB activity at later time points.

In conclusion, we have shown that the loss of CYP1A1/2 activity in astrocytes can be minimized by β-adrenergic receptor stimulation in an in vitro model of CNS inflammation. We also provide evidence that increases in intracellular levels of cAMP and changes in TNFα expression likely play a role in mediating this β-adrenergic effect. In addition, we provide evidence that this β-adrenergic effect occurs independent of direct changes in NF-κB activity induced by LPS. The results of this study support the idea that β-adrenoceptor stimulation during conditions of CNS inflammation may be protective against a reduction in the drug-metabolizing capacity of the brain.

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