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ORIGINAL ARTICLE

17β-Oestradiol Anti-Inflammatory Effects in Primary Astrocytes Require Oestrogen Receptor β-Mediated Neuroglobin Up-Regulation

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Neuroglobin (Ngb), so named after its initial discovery in brain neurones, has received great attention as a result of its neuroprotective effects both *in vitro* and *in vivo*. Recently, we demonstrated that, in neurones, Ngb is a 17β-oestradiol (E_2) inducible protein that is pivotal for hormone-induced anti-apoptotic effects against H_2O_2 toxicity. The involvement of Ngb in other brain cell populations, as well as in other neuroprotective effects of E_2, is completely unknown at present. We demonstrate Ngb immunoreactivity in reactive astrocytes located in the proximity of a penetrating cortical injury *in vivo* and the involvement of Ngb in the E_2-mediated anti-inflammatory effect in primary cortical astrocytes. Upon binding to oestrogen receptor (ER)β, E_2 enhances Ngb levels in a dose-dependent manner. Although with a lesser degree than E_2, the pro-inflammatory stimulation with lipopolysaccharide (LPS) also induces the increase of Ngb protein levels via nuclear factor-(NF)κB signal(s). Moreover, a negative cross-talk between ER subtypes and NFκB signal(s) has been demonstrated. In particular, ERα-activated signals prevent the NFκB-mediated Ngb increase, whereas LPS impairs the ERβ-induced up-regulation of Ngb. Therefore, the co-expression of both ERα and ERβ is pivotal for mediating E_2-induced Ngb expression in the presence of NFκB-activated signals. Interestingly, Ngb silencing prevents the effect of E_2 on the expression of inflammatory markers (i.e. interleukin 6 and interferon γ-inducible protein 10). Ngb can be regarded as a key mediator of the different protective effects of E_2 in the brain, including protection against oxidative stress and the control of inflammation, both of which are at the root of several neurodegenerative diseases.

Key words: astrocytes, oestrogen receptor, neuroinflammation, neuroglobin

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Neuroglobin (Ngb), so named as a result of its initial discovery in brain neurones (1), is a monomeric, approximately 150-amino acid-long haeme-protein displaying < 25% sequence identity to conventional vertebrate haemoglobin or myoglobin (1,2). Ngb has received great attention as a result of its neuroprotective effects *in vivo*. Indeed, Ngb exerts protection against middle cerebral artery occlusion-induced infarct, focal cerebral ischaemia, oxidative amyloid-β peptide toxicity, anoxia, and oxygen and glucose deprivation (3–6). Despite this wide range of effects, the molecular mechanisms underlying the neuroprotective function of Ngb remains poorly understood and openly debated.

Recently, it has been demonstrated that Ngb is a hormone-inducible protein. In particular, the female sex hormone 17β-oestradiol (E_2) up-regulates Ngb in human neuroblastoma cell line SK-N-BE and in mouse primary hippocampal neurones (7). At a physiological concentration (i.e. 1 nM), E_2 modulates Ngb expression by the synergy of genomic and membrane-initiated oestrogen receptor (ER)β signals. E_2-induced Ngb expression is pivotal for the neuroprotective effect of E_2 against H_2O_2-induced apoptosis (7).

Besides neurones, astrocytes represent one of the cellular targets of E_2 in the brain (8). The hormone regulates the morphology of astrocytes and the expression of several molecules that are relevant for the physiological and pathological responses of these cells, including the control of central nervous system (CNS) inflammation (8). Under pathological conditions, astrocytes release a number of pro-inflammatory cytokines and chemokines that attract macrophages/microglia and T cells to CNS inflammatory sites (9–11). In cultured astrocytes, E_2 decreases the expression of inflammatory

markers (12–14) such as interleukin (IL)-6 and interferon γ-inducible protein 10 (IP-10) induced by lipopolysaccharide (LPS) stimulation, a pro-inflammatory stimulus acting via nuclear factor-(NF)κB signalling (8,14,15). E_2 also decreases the activation of NFκB induced by amyloid-β peptide and LPS in cultured astrocytes (16). NFκB is a potent immediate-early transcriptional regulator of numerous proinflammatory genes and is involved in the regulation of the expression of IL-6 and IP-10 in astrocytes, as well as in other cell types (8). These anti-inflammatory effects of E_2 involve genomic and membrane initiated signals activated by both ER subtypes (17).

Recently, it has been demonstrated that Ngb is present in reactive astrocytes in neuropathological models of traumatic injury, infectious, autoimmune and excitotoxic diseases. Ngb positive astrocytes were found within regions associated with most severe pathology and in the astroglial scar (18). The distribution of Ngb positive astrocytes in these models suggests that Ngb is up-regulated in astrocytes that are exposed to significant stress from the necrotic core.

Whether Ngb is involved in the anti-inflammatory effects of E_2 is unknown. Therefore, the present study aimed to: (i) confirm the presence of Ngb in reactive astrocytes *in vivo* and (ii) investigate, in primary astrocytes, the possible involvement of Ngb in E_2-mediated effects against LPS-induced inflammation.

Materials and methods

Reagents

E_2, bacterial endotoxin LPS (*Escherichia coli* 026 : B6), Dulbecco's modified Eagle medium (DMEM) without phenol red, charcoal-stripped foetal calf serum, protease inhibitor cocktail, bovine serum albumin fraction V (BSA) and mouse monoclonal anti-β-actin (clone AC-74) antibody were purchased from Sigma-Aldrich (St Louis, MO, USA). Optimem was purchased from Gibco-BRL (Gaithersburg, MD, USA). NFκB inhibitor peptide SN50 was obtained from Calbiochem (San Diego, CA, USA). The E_2 antagonist fulvestrant (ICI 182,780; ICI), ERα-selective agonist 4,4′,4″–(4-propyl-[^{1}H]-pyrazole-1,3,5-triyl)trisphenol (PPT) and ERβ-selective agonist 2,3-bis(4-hydroxyphenyl)propionitrile (DPN) were obtained from Tocris (Ballwin, MO, USA). The Bradford protein assay was obtained from Bio-Rad Laboratories (Hercules, CA, USA). Human recombinant ERα and ERβ were obtained by Pan-Vera (Madison, WI, USA). Anti-ERα (MC20) and anti-ERβ (H150) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Monoclonal anti-human Ngb (13C8) antibody was purchased from Abcam (Cambridge, UK). Chemiluminescence reagent for Western blot ECL was obtained from GE Healthcare (Little Chalfont, UK). All the other products were obtained from Sigma-Aldrich. Analytical or reagent grade products were used without further purification.

Animals

CD1 mice were used in the present study. Mice were treated in accordance with the guidelines of the Council of Europe Convention ETS123, revised as indicated in the Directive 86/609/EEC. In addition, all protocols were approved by the Institutional Animal Care and Use Committee of CSIC-Cajal Institute (Madrid, Spain).

Cortical injury

Animals were anaesthetised with isofluorane and placed in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA; USA). To relieve postchirurgical pain, animals received one i.p. injection of buprenorphine (0.7 mg/kg; Buprex; Schering-Plough, Alcobendas, Spain) shortly after the induction of anaesthesia. An incision of the scalp was made and the cranium exposed. Then, a unilateral opening of the skull was made with a dental drill. Stab wound lesions were created with a needle blade by making a sagittal cut through the neocortex and corpus callosum, 1 mm lateral from the midline and extending from −1.3 mm to −2.5 mm posterior from the bregma. Coordinates were determined according to the stereotaxic atlas of Paxinos and Franklin (19).

Immunhistochemistry for glial fibrillary acidic protein (GFAP), vimentin and Ngb

Seven days after brain surgery, at the moment of maximal astrogliosis after brain injury (20), animals were deeply anaesthetised with pentobarbital (50 mg/kg body weight). Then, the animals were perfused through the left cardiac ventricle, first with pre-warmed (37 °C) 0.9% NaCl and then for 5 min with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Brains were postfixed for 5 h at 4 °C in the same fixative and washed three times with 0.1 M phosphate buffer, pH 7.4, at room temperature. Coronal sections of the brain, 30 μm thick, were obtained using a Vibratome (VT 1000 S; Leica Microsystems, Wetzlar, Germany).

Immunohistochemistry was carried out in free-floating sections under moderate shaking. For the double localisation of Ngb and GFAP (a marker of resting and reactive astrocytes) or vimentin (a marker of reactive astrocytes), sections were incubated overnight at 4 °C with a polyclonal rabbit GFAP antibody (Dako, Glostrup, Denmark), diluted 1 : 2000, or a polyclonal rabbit anti vimentin antibody (Abcam), diluted 1 : 2000 together with the Ngb antibody (1 : 1000). After three washes in buffer, sections were incubated for 2 h with an Alexa 488-labelled donkey anti-mouse IgG (diluted 1 : 1000; Jackson Immuno-Research Europe Ltd, Newmarket, UK.) together with an Alexa 568-labelled goat anti-rabbit IgG (diluted 1 : 1000; Jackson Immuno-Research Europe Ltd). Sections were observed under a Leica DMI6000 microscope equipped with a Leica DFC350 FX camera. Confocal images were obtained using a Leica TCS SP-5 microscope and are displayed as individual optical sections. For specificity controls, sections were processed in the same way but omitting the primary antibodies.

Mouse primary cortical astrocyte cultures

Astrocyte-enriched glial cultures were prepared by mechanical dissociation of the cerebral cortex from newborn (P0) CD1 mice (21). The cortex was isolated under a dissecting microscope and cleaned of choroid plexus and meninges. Cell suspensions were filtered through a 100-μm nylon cell strainer into phenol red free DMEM containing 10% foetal calf serum and penicillin-streptomycin. After centrifugation, cells were filtered through a 40-μm cell strainer and cultured in 75-cm^2 tissue culture flasks at 37 °C and 5% CO_2. The medium was changed after 4 days in culture and, subsequently, two times a week for the entire culture period. Cellular confluence was observed 10 days after plating, producing approximately 4×10^6 cells per flask, and showing a polygonal flat morphology. Enriched astrocyte cultures were obtained after overnight shaking at 37 °C at 250 r.p.m. in a table top shaker (Thermo Forma, Marietta, OH, USA) to minimise oligodendrocyte and microglia contamination. Astrocytes were removed from the flasks by incubation with 0.25% trypsin (type II-S; Sigma-Aldrich) and 0.04% ethylenediaminetetraacetic acid, plated onto poly-L-lysine-coated six-well plates at a density of 40 000 cells/cm^2 in serum-free medium and used within 24 h.

Determination of the proportion of microglia cells in the cultures

To determine the proportion of microglia cells in the cultures, cells were fixed for 20 min at room temperature in 4% paraformaldehyde and permeabilised

for 4 min with 0.12% Triton-X plus 0.12% gelatine in phosphate-buffered saline (PBS). Cells were then washed with PBS/gelatine and incubated for 1 h with anti-GFAP mouse monoclonal antibody (dilution 1 : 500 in PBS/gelatine; Sigma-Aldrich) and with a rabbit polyclonal antibody against Iba1, a marker of microglia (dilution 1 : 2000; Wako, Tokyo, Japan). After washing in the same buffer, cells were incubated for 45 min at room temperature with goat anti-mouse Alexa 594 (dilution 1 : 1000; Jackson Immuno-Research Europe Ltd) for the detection of GFAP and with goat anti rabbit Alexa 488 (dilution 1 : 1000; Jackson Immuno-Research Europe Ltd) for detection of Iba1. Cell nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). The proportion of microglia cells (cells Iba-1 immunoreactive) versus the total number of cells (DAPI stained cell nuclei) was determined in five cultures, under control conditions. The proportion of microglia cells in the cultures was 3.28 ± 0.50% (Fig. 1).

Cell stimulation

Cells were simultaneously treated with vehicle (ethanol/PBS 1 : 10, v/v) and/or E_2 (0.01–100 nM), PPT (0.01–100 nM), DPN (0.01–100 nM) and LPS (500 ng/ml). When indicated, the anti-oestrogen ICI (1 μM) and the NFκB inhibitor SN50 (10 μg/ml) were added 30 min before E_2 or LPS administration.

Transfection of small interfering (si)RNA

Mouse primary cortical astrocytes, reaching 40–60% confluence, were transfected in a serum-free condition with either Stealth RNAi™ Ngb siRNA (siNgb; Invitrogen, Carlsbad, CA, USA) or a mismatch sequence in accordance with the manufacturer's instructions, using oligofectamine (Invitrogen) as the transfection reagent. The sequence used for Ngb oligonucleotides was: 5′-CGUGAUUGAUGCUGCAGUGACCAAU-3′. The mismatch sequence used as a control for Ngb siRNA (siNgb) was: 5′-UGUGAUUUAUGGUGCAGUAACCAAC-3′.

Briefly, oligofectamine and oligonucleotides (400 pM) were mixed with Optimem. The mixture was incubated for 20 min at room temperature, diluted with Optimem, and added to the cell medium for 4 h at 37 °C. The medium was added to cells to reach the growing conditions [i.e. 10% (v/v) serum]. To evaluate the effective silencing of Ngb, total proteins from cells transfected with MOCK (control), with scramble (mismatch sequence, data not shown) and with siNgb oligonucleotides were extracted 48 h after transfection, and Ngb expression was tested by western blot analysis using anti-Ngb.

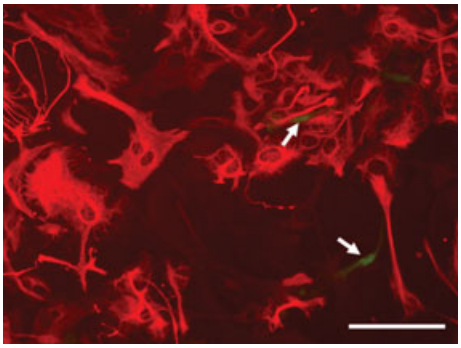

Fig. 1. Immunoreactivity for glial fibrillary acidic protein (GFAP) and Iba1 in astrocyte-enriched glial cultures. Representative culture incubated with a mouse monoclonal antibody against GFAP, a marker of astrocytes, and with a rabbit polyclonal antibody against Iba1, a marker of microglia. Secondary antibodies were an anti-mouse Alexa 594 (red) and anti rabbit Alexa 488 (green). Only a few cells were labelled in green (arrows), corresponding to microglia. Scale bar = 100 μm.

Western blot assays

After treatments, cells were lysed and solubilised in 0.125 M Tris, pH 6.8, containing 10% (w/v) sodium dodecyl sulphate (SDS) and protease inhibitor cocktail, and finally boiled for 2 min. Total proteins were quantified using the Bradford protein assay. Solubilised proteins (20 μg) were resolved by 7% or 15% SDS-polyacrylamide gel electrophoresis at 100 V for 1 h at 25 °C and then electrophoretically transferred to nitrocellulose for 45 min at 100 V and 4 °C. The nitrocellulose was treated with 3% (w/v) BSA in 138 mM NaCl, 25 mM Tris, pH 8.0 and 0.1% (w/v) Tween-20 at 25 °C for 1 h and then probed overnight at 4 °C with either anti-Ngb (final dilution 1 : 1000), or anti-ERα MC-20 (final dilution 1 : 500) or anti-ERβ H-150 (final dilution 1 : 3000). The nitrocellulose was stripped by Restore Western Blot Stripping Buffer (Pierce, Rockford, IL, USA) for 10 min at room temperature and then probed with anti-β-actin (final dilution 1 : 2500) to normalise total lysate. To determine ERα and ERβ levels, electrophoresis was performed in the presence of 5 ng of recombinant ERα and ERβ. The antibody reaction was visualised by electrochemiluminescence. Densitometric analyses were performed using IMAGEJ (NIH, Bethesda, MD, USA).

Quantitative real-time polymerase chain reaction (PCR)

After 5 h of treatment with LPS and/or E_2, culture plates were briefly centrifuged and supernatants removed. Cells were lysed and total RNA was extracted using the illustra RNAspin Mini RNA Isolation Kit (GE Healthcare) to measure the IP-10, IL-6, IL-1β, tumour necrosis factor (TNF)α and vimentin mRNA expression levels.

First-strand cDNA was prepared from 5 μg of RNA using the RevertAidTM H Minus First Strand cDNA Synthesis Kit (MBI Fermentas, Bath, UK) in accordance with the manufacturer's instructions. After reverse transcription (RT), the cDNA was diluted 1 : 10 and 5 μl were amplified by real-time PCR using SYBR Green master mix or TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA) in a ABI Prism 7000 Sequence Detector (Applied Biosystems), with conventional Applied Biosystems cycling parameters (40 cycles of 95 °C for 15 s and 60 °C for 1 min). Primer sequences were designed using Primer Express (Applied Biosystems) and, for IP-10, were: forward 5′-CAGTGAGAATGAGGGCCATAGG-3′ and reverse 5′-CGGATTCAGACATCTCTGCTCAT-3′; for IL-6: forward 5′-GAAACCGCTATGAAGTTCCTCTCTG-3′ and reverse 5′-TGTTGGGAGTGGTATCCTCTGTGA-3′; for IL1β: forward 5′-CGACAAAATACCTGTGGCCT-3′ and reverse 5′-TTCTTTGGGTATTGCTTGGG-3′; for TNFα: forward 5′-GAAAAGCAAGCAGGCAACCA-3′ and reverse 5′-CGGATCATGCTTTCTGTGCTC-3′; and, for Vimentin: forward 5′-GCTGCAGGCCCAGATTCA-3′ and reverse 5′-TTCATACTGCTGGCGCACAT-3′.

GADPH was selected as the control housekeeping gene. GADPH TaqMan probes and primers comprised the Assay-on-Demand gene expression products (Applied Biosystems). After amplification, a denaturing curve was performed to ensure the presence of unique amplification products. For visualising and sequencing of the PCR products, each mixture was electrophoresed in 2% (w/v) ethidium bromide-stained agarose gels. Then, bands were excised and cDNA was purified using the QIAquick PCR purification Kit (Qiagen GmbH, Hildenberg, Germany). One hundred nanograms of each sample was sequenced (Automatic Sequencing Centre, CSIC, Madrid, Spain) with the corresponding forward or reverse primer. The obtained sequence was aligned with the expected sequence of each transcript obtained from GenBank. All reactions were performed in triplicate and the quantity of target gene expression was normalised to the corresponding GAPDH expression in test samples and plotted.

Statistical analysis

Statistical analysis was performed using ANOVA followed by the Tukey–Kramer post-test with INSTAT3 (GraphPad software, La Jolla, CA, USA). $P < 0.05$ was considered statistically significant.

Results

Ngb immunoreactivity is detected in reactive astrocytes *in vivo* in the mouse brain

To confirm the presence of Ngb immunoreactivity in murine reactive astrocytes *in vivo* (18), we used a penetrating injury model of the cerebral cortex. Ngb immunoreactivity was detected in close proximity to the borders of the lesion (Fig. 2), where maximal GFAP immunoreactivity was also observed (Fig. 2). Ngb and GFAP immunoreactivity were colocalised in this region (Fig. 2). Confocal analysis of GFAP and Ngb colocalisation revealed that a subpopulation of GFAP immunoreactive astrocytes located in proximity of the lesion border were also Ngb immunoreactive (Fig. 2). Ngb immunoreactivity was also localised in cells immunoreactive for vimentin, a marker of reactive astrocytes, in the injured hemisphere (Fig. 2). By contrast, no Ngb immunoreactive astrocytes were detected in regions distant from the lesion site or in the contralateral hemisphere.

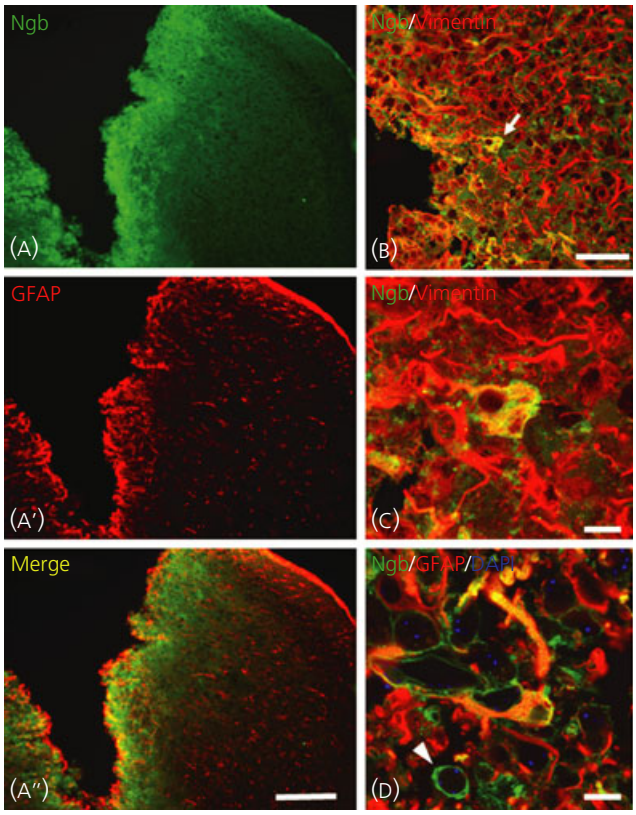

Fig. 2. Immunoreactivity for neuroglobin (Ngb), glial fibrillary acidic protein (GFAP) and vimentin in the cerebral cortex after a penetrating injury. Representative examples of Vibratome sections of the cerebral cortex of mice, 7 days after a penetrating brain injury. Brain sections were immunostained for Ngb (green) and GFAP (red) or vimentin (red). (A, A′, A″) Panoramic view of the injured cerebral cortex immunostained for Ngb (A) and GFAP (A′). The merged image is shown in (A″). Ngb and GFAP immunoreactivities are enriched in the proximity of the lesion. Scale bar = 300 μm. (B) Detail of the border of the lesion showing colocalisation (yellow) of vimentin (red) and Ngb (green). The cell pointed with an arrow is shown at high magnification in (C). Scale bar = 50 μm. (C) High magnification of the injured cerebral cortex showing colocalisation (yellow) of Ngb and vimentin. Scale bar = 10 μm. (D) High magnification of the injured cerebral cortex showing colocalisation (yellow) of Ngb and GFAP. The arrowhead points to a cell that is immunoreactive for Ngb (green) but not for GFAP. In this image, cell nuclei are stained with 4′,6-diamidino-2-phenylindole (DAPI). Scale bar = 10 μm.

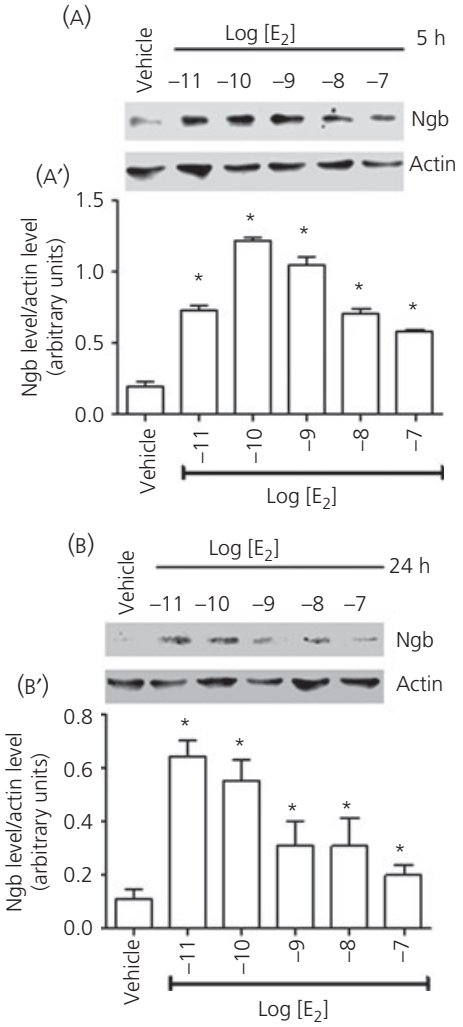

Fig. 3. Effect of 17β-oestradiol (E_2) on neuroglobin (Ngb) protein levels in mouse primary cortical astrocytes. Dose–dependent effect of E_2 treatment (0.01–100 nM) on Ngb levels at 5 h of stimulation (A) and at 24 h (B) of stimulation. The amount of protein was normalised to actin levels. Representative western blots of five independent experiments. The results of the densitometric analysis are represented in (A′) and (B′), respectively. Data are the mean ± SD of five different experiments. Significant differences ($P < 0.001$) were determined by ANOVA followed by the Tukey–Kramer post-test. *Significant difference versus vehicle.

Effect of E_2 on Ngb protein levels in mouse primary cortical astrocytes

Incubation of primary cortical astrocytes with E_2 for 5 h (Fig. 3a,a′) caused a significant increase in Ngb levels (an approximate 4.5-fold increase) with a maximum effect at an E_2 concentration of 10 and 100 pM, which decreased in the 1–100 nM concentration range. Twenty four hours after stimulation (Fig. 3b,b′), the effect of E_2 on Ngb protein level was still present, although with a lower intensity (an approximate 3.6-fold increase). In line with these results, 10 pM E_2 and 5 h of stimulation were used in subsequent experiments to stimulate primary cortical astrocytes.

Involvement of ERs and the effect of LPS on Ngb expression

Pretreatment of astrocytes with the E_2 antagonist, ICI (1 μM, 30 min before E_2 treatment), completely prevented the effect of E_2 on Ngb levels (Fig. 4a,a′), suggesting an ER-mediated mechanism. Because primary astrocytes contain both ERα and ERβ subtypes (Fig. 4b,b′), cells were stimulated with either the specific ERα agonist PPT or the specific ERβ agonist DPN to discriminate the role of each ER isoform with respect to the effect of E_2 on Ngb protein expression. Only DPN mimicked the effect of E_2 on Ngb levels (Fig. 4c,e), whereas PPT was unable to increase Ngb levels at any tested concentration (Fig. 4d,e). According to the literature (7), this

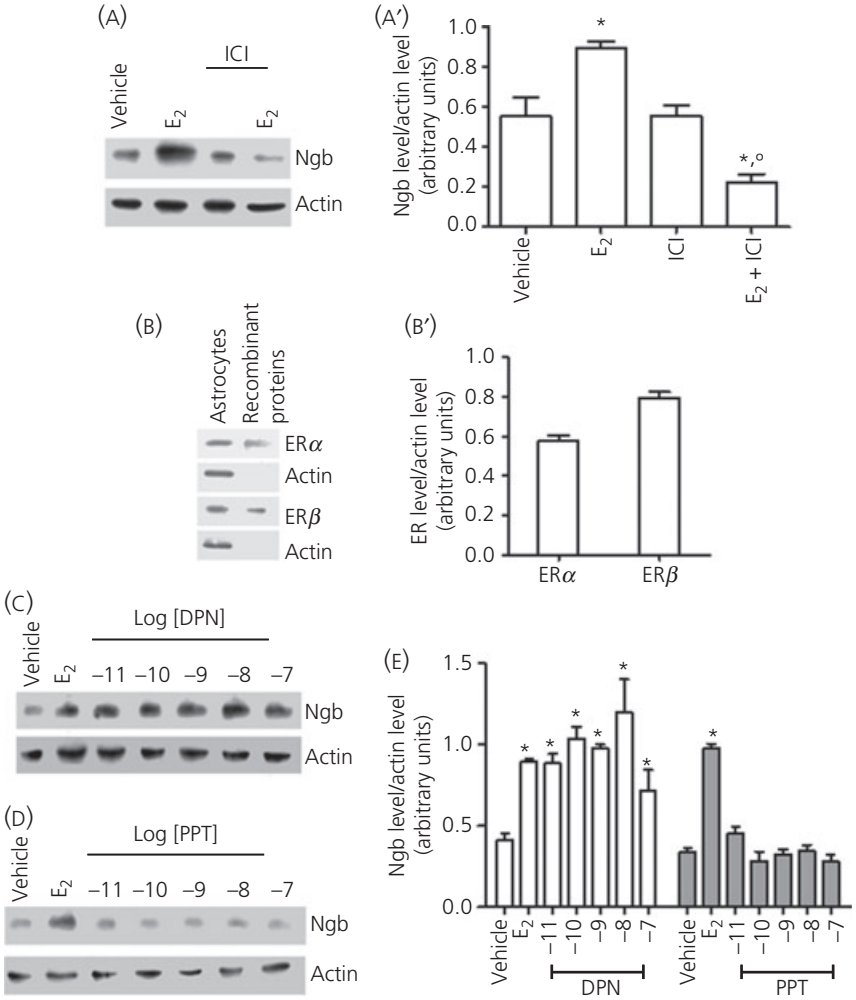

Fig. 4. Impact of oestrogen receptors (ERs) on neuroglobin (Ngb) protein levels in primary cortical astrocytes. (A) Western blot analysis of Ngb levels in cells stimulated for 5 h with either vehicle, or 17β-oestradiol (E_2) (10 pM) and/or the ER inhibitor fulvestrant (ICI 182,780; ICI) (1 μM). ICI was administrated 30 min before E_2. (B) Characterisation of ERs in mouse primary cortical astrocytes. ER subtype (ERα and ERβ) levels in nonstimulated cells compared to recombinant proteins (5 ng). (C) Analysis of Ngb levels in cells stimulated for 5 h with either vehicle, or E_2 (10 pM) or the ERβ agonist 2,3-bis(4-hydroxyphenyl)propionitrile (DPN) (0.01–100 nM). (D) Analysis of Ngb levels in cells stimulated for 5 h with either vehicle, E_2 (10 pM) or the ERα agonist 4,4′,4″–(4-propyl-[^{1}H]-pyrazole-1,3,5-triyl)trisphenol (PPT) (0.1–100 nM). The amount of protein was normalised to actin levels. The results of the densitometric analysis are represented in (A′), (B′) and (E), respectively. Data are the mean ± SD of five different experiments. Significant differences ($P < 0.001$) were determined by ANOVA followed by the Tukey–Kramer post-test. Significant difference versus vehicle (*) and E_2-treated (°) samples, respectively.

result strongly suggests the involvement of ERβ in the E_2-induced increase of Ngb protein levels.

The hypothesis that Ngb expression can be affected by inflammatory stimuli, such as LPS, has been evaluated. Compared to the control, LPS (i.e. 500 ng/ml for 5 h) significantly increased Ngb protein levels (an approximate 2.3-fold increase), even though the effect of LPS was less strong than that of E_2 (Fig. 5a,a′). E_2 and LPS co-treatment did not induce a significant difference in Ngb levels compared to treatment with E_2 alone, neither when E_2 was administrated before LPS (i.e. E_2 pretreatment 1 h before LPS), nor when LPS was administrated before E_2 (i.e. LPS pretreatment 1 h before E_2) (Fig. 5a,a′). After pretreatment of astrocytes with the NFκB cell-permeable inhibitor peptide SN50 (10 μg/ml, 30 min before E_2 and/or LPS), LPS was unable to increase Ngb levels, whereas the effect of E_2 was unchanged (Fig. 5b,b′).

Because E_2 interferes with LPS-activated signals (i.e. the NFκB pathway) (16,22), the cross-talk between ERs- and LPS-mediated signalling on Ngb protein levels has been investigated. Pretreatment with the ER antagonist ICI only prevented the ability of E_2 to increase Ngb protein levels, whereas it was ineffective on LPS-induced Ngb (Fig. 6a,a′). Co-administration of the ERβ agonist DPN (10 pM) with LPS indicated that LPS reduced the effect of DPN on Ngb induction, suggesting an antagonistic effect of LPS on ERβ signalling (Fig. 6b,b′). By contrast, in the presence of ERα agonist PPT (10 pM), the effect of LPS on the incresase in Ngb was barely detectable (Fig. 6c,c′).

Ngb involvement in E_2-mediated anti-inflammatory effects against LPS

The exposure of astrocyte cultures for 5 h to 500 ng/ml LPS resulted in a significant increase in mRNA levels of IL-6, IP-10 (Fig. 7), IL-1β and TNFα (data not shown). In addition, LPS increased mRNA levels of vimentin (Fig. 8), a marker of generalised astroglia activation. E_2 (10 pM) treatment prevented the LPS-mediated increase of IL-6, IP-10 and vimentin (Figs 7 and 8) but not of IL-1β and TNFα (data not shown). Notably, in cells transfected with siNgb, the reduced level of Ngb (Fig. 7c) is paralleled by the inability of E_2 to decrease the expression of IL-6, IP-10 (Fig. 7d, e) and vimentin in cultures treated with LPS (Fig. 8). On the other hand, siNgb transfection did not affect the ability of LPS to increase the mRNA levels of IL-6, IP-10 (Fig. 4c,c′), IL-1β and TNFα (data not shown). However, siNgb transfection decreased the effect of LPS on vimentin (Fig. 8).

Discussion

Beyond its reproductive function-related effects, E_2 is an important neurotrophic and neuroprotective hormone, affecting, amongst others, synaptic plasticity, neuronal excitability, neuronal survival, axonal outgrowth and neurogenesis (23,24). In addition, astrocytes have been identified as additional E_2 cellular targets (8). Although the role of astrocytes in the formation of immunological responses within the CNS is openly debated, there is evidence that these cells may actively take part in the initiation and modulation of immune reactions (25,26). The down-regulation exerted by E_2 on the production of cytokines and chemokines (e.g. IL-6 and IP-10) by reactive astrocytes has been included in the neuroprotective mechanisms of E_2, at least under chronic neurodegenerative conditions (8,12–14). The data reported in the present study strongly suggest that Ngb is part of the anti-inflammatory mechanism elicited by E_2 in reactive astrocytes.

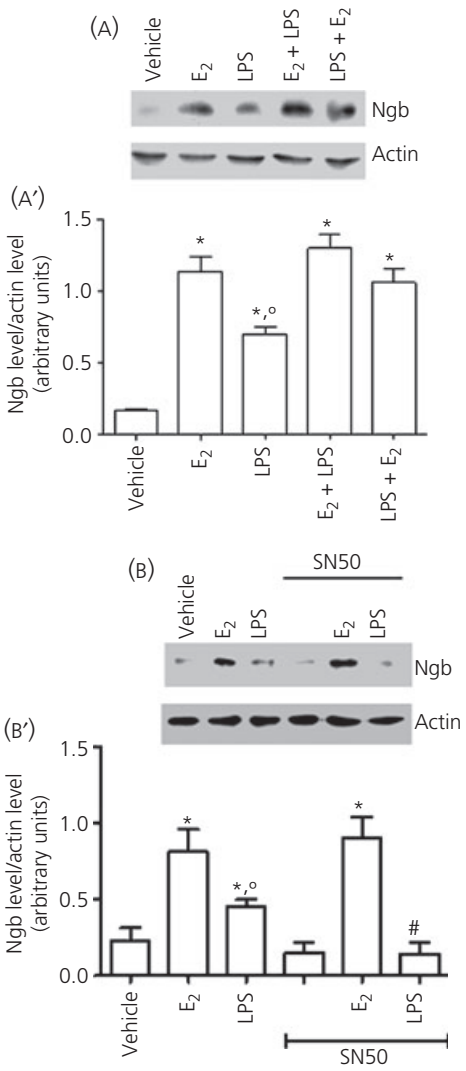

Fig. 5. Impact of lipopolysaccharide (LPS) and 17β-oestradiol (E_2) on neuroglobin (Ngb) protein levels in mouse primary cortical astrocytes. (A) Analysis of Ngb levels in cells stimulated for 5 h with either vehicle, or E_2 (10 pM), or LPS (500 ng/ml), or E_2 + LPS (E_2 1 h before LPS administration), or LPS + E_2 (LPS 1 h before E_2 administration). (B) Impact of nuclear factor (NF)κB inhibitor SN50 on Ngb protein levels in mouse primary cortical astrocytes. Analysis of Ngb levels in cells stimulated for 5 h with either vehicle, E_2 (10 pM), LPS (500 ng/ml) and/or NFκB inhibitor SN50 (10 μg/ml, administrated 30 min before E_2 or LPS). The amount of protein was normalised to actin levels. Representative western blots of five independent experiments. The results of the densitometric analysis are represented in (A′) and (B′), respectively. Data are the mean ± SD of five different experiments. Significant differences ($P < 0.001$) were determined by ANOVA followed by the Tukey–Kramer post-test. Significant difference versus vehicle (*), E_2 (°) and LPS (#) alone.

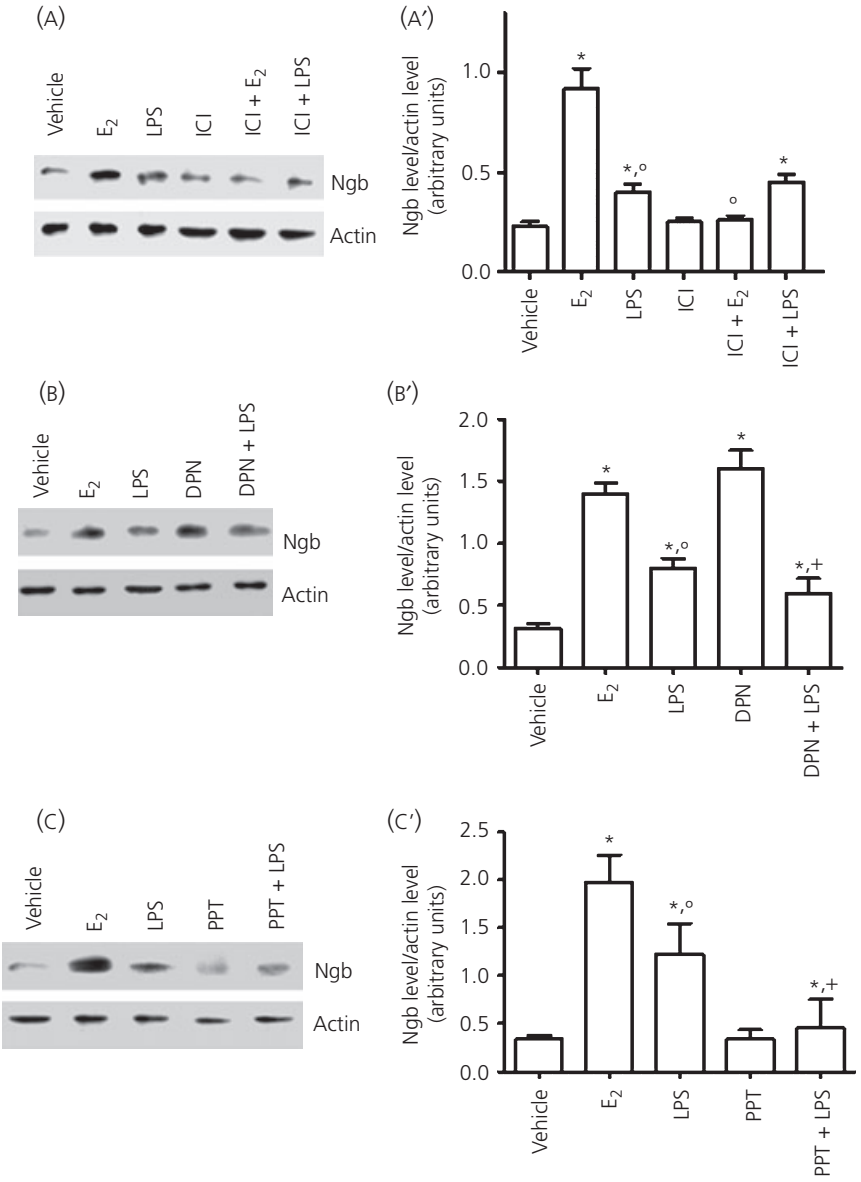

Fig. 6. Impact of lipopolysaccharide (LPS) and oestrogen receptors (ERs) on neuroglobin (Ngb) protein levels in mouse primary cortical astrocytes. (A) Analysis of Ngb levels in cells stimulated for 5 h with either vehicle, or 17β-oestradiol (E_2) (10 pM), or LPS (500 ng/ml), and/or fulvestrant (ICI 182,780; ICI) (1 μM, administrated 30 min before E_2 or LPS). (B) Analysis of Ngb levels in cells stimulated for 5 h with either vehicle, or E_2 (10 pM), or LPS (500 ng/ml), and/or 2,3-bis(4-hydroxyphenyl)propionitrile (DPN) (10 pM). (C) Analysis of Ngb levels in cells stimulated for 5 h with either vehicle, or E_2 (10 pM), or LPS (500 ng/ml), and/or 4,4′,4″–(4-propyl-[^{1}H]-pyrazole-1,3,5-triyl)trisphenol (PPT) (10 pM). Representative western blots of five independent experiments. The results of densitometric analysis are represented in (A′) (B′) and (C′), respectively. Data are the mean ± SD of five different experiments. Significant differences ($P < 0.001$) were determined by ANOVA followed by the Tukey–Kramer post-test. Significant difference versus vehicle (*), E_2(°), DPN(+) in (B′) and versus LPS(+) in (C′).

Despite the level of interest generated by the discovery of Ngb, the focus of research has primarily been on its roles in neurones. Indeed, the expression of functional Ngb in glial cells has been questioned previously (27). Subsequently, the presence of functional Ngb has been demonstrated in diving mammal astrocytes, which are the predominant glial cells in the brain (28–30). In these cells, Ngb antisense treatment enhanced apoptosis under ischemic conditions (28,30). In addition, Ngb expression has been detected in reactive astrocytes in murine models of traumatic injury, as well as infectious, autoimmune and excitotoxic diseases (18). Our present findings confirm the presence of Ngb immunoreactive astrocytes in close proximity to a penetrating injury in the mouse cerebral cortex *in vivo*. Ngb immunoreactivity is detected in astrocytes immunoreactive for GFAP (a marker of resting and reactive astrocytes) and vimentin (a marker of reactive astrocytes). By contrast, Ngb immunoreactive astrocytes are not detected in regions distant from the lesion site or in the non-injured hemisphere. This suggests that, under normal conditions, Ngb is barely expressed in resting

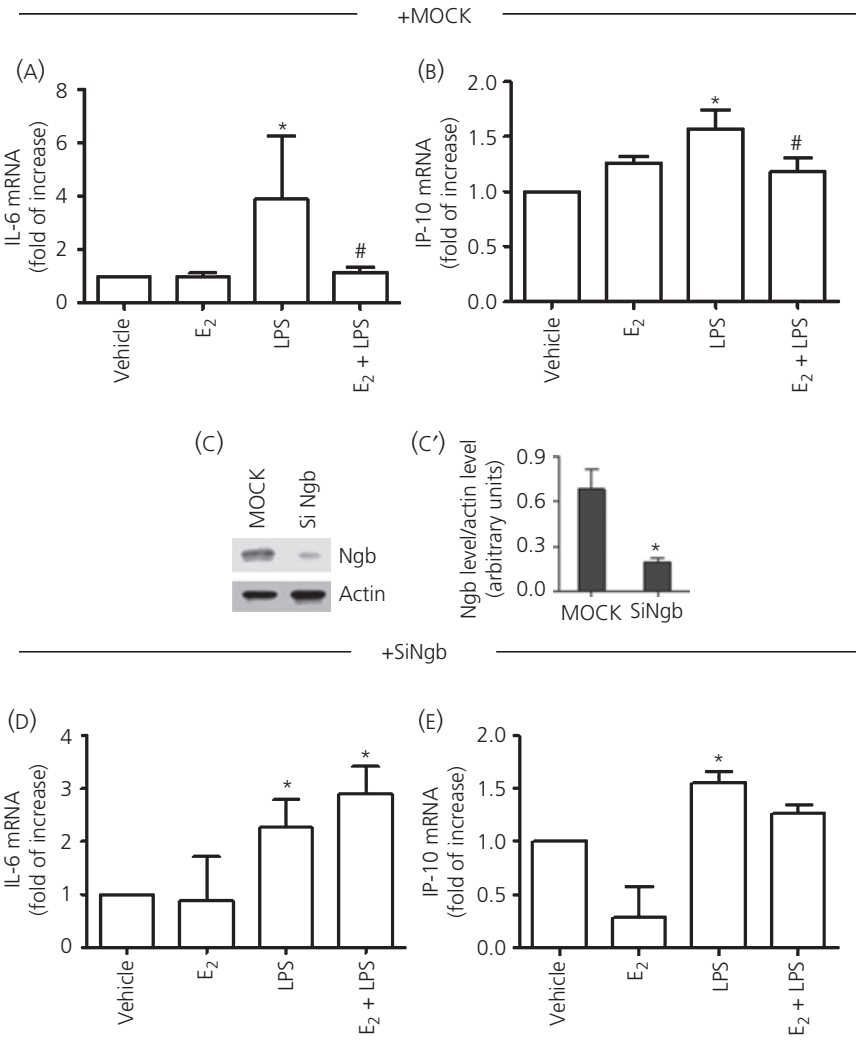

Fig. 7. Effect of 17β-oestradiol (E_2) and lipopolysaccharide (LPS) on interleukin (IL)-6 and interferon γ-inducible protein 10 (IP-10) mRNA levels in neuroglobin (Ngb)-silenced mouse primary cortical astrocytes. Cells transfected with either MOCK (control) (A, B) or small interfering RNA for Ngb (siNgb) (D,E) were stimulated for 5 h with either vehicle, or E_2 (10 pM) and/or LPS (500 ng/ml). The mRNA levels of IL-6 (A,D) and IP-10 (B,E) were measured on cell lysates by a real-time reverse transcriptase-polymerase chain reaction with primers for IL-6 or IP-10 using the amplification of GADPH as an internal control. The expression of IL-6 and IP-10 for each condition is expressed as the fold induction over the values with vehicle (set to 1). Data represent the mean $\pm$ SD of five different experiments. Significant differences ($P < 0.001$) were determined by ANOVA followed by the Tukey–Kramer post-test. Significant difference versus vehicle (*) and LPS (#). (C) Analysis of Ngb protein levels in cell transfected with either MOCK or siNgb. The amount of protein was normalised to actin levels. The results of the densitometric analysis are represented in (C′). Data are the mean $\pm$ sd of five different experiments. Significant differences ($P < 0.001$) were determined by ANOVA followed by the Tukey–Kramer post-test. *Significant difference versus MOCK.

astrocytes in the mouse brain. However, Ngb expression is induced in astrocytes *in vivo* after brain injury.

Having established that Ngb is expressed in mouse reactive astrocytes *in vivo*, we assessed the effect of E_2 on Ngb expression in primary mouse glial cultures enriched in astrocytes, which contain a small proportion (< 4%) of microglia cells. Our findings indicate that E_2 up-regulates Ngb levels in these cultures. The effect of E_2 on Ngb levels is already present after 5 h of hormone treatment and persists until 24 h of stimulation. The effect is dose-dependent, with the maximum effect at an E_2 concentration of 10 pM. Notably this concentration is two and three orders of magnitude lower than the effective dose in SK-N-BE cells (1 nM) and in primary hippocampal neurones (10 nM), respectively, where E_2 has been demonstrated to strongly up-regulate Ngb (7,31). Also in primary astrocytes, the E_2 dose–response curve is bell-shaped. The lack of an effect at higher concentrations could be considered as a receptor down-regulation phenomenon by which cells protect themselves against high hormone levels (32). Moreover, ERα and ERβ can regulate each other's expression, in this way affecting also the cell response to the hormone (32). Indeed, when cells are treated with the specific ERβ agonist DPN, the dose–response curve on Ngb level is hyperbolic, with ERα not being activated.

LPS stimulation also increases Ngb levels. These data suggest that Ngb could behave as a compensatory protein responding to

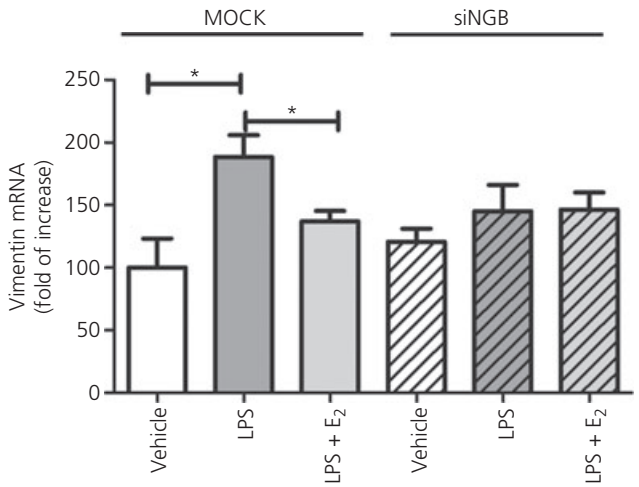

Fig. 8. Effect of 17β-oestradiol (E_2) and lipopolysaccharide (LPS) on vimentin mRNA levels in neuroglobin (Ngb)-silenced mouse primary cortical astrocytes. Cells transfected with either MOCK or small interfering RNA for Ngb (siNGB) were stimulated for 5 h with either vehicle, LPS (500 ng/ml) or E_2 (10 pM) and LPS. The mRNA levels of vimentin were measured on cell lysates by a real-time reverse transcriptase-polymerase chain reaction using amplification of GADPH as an internal control. The expression of vimentin for each condition is expressed as the fold induction over the values with vehicle and MOCK (set to 100). Data represent the mean ± SD of five different experiments. *Significant difference between the indicated groups.

injuring stimuli such as hypoxia (33,34), H_2O_2 toxicity (7) and LPS (present data). Although both E_2 and LPS are able to increase Ngb protein levels, siNgb does not affect the LPS-mediated over-expression of inflammatory markers, although it reduces the effect of LPS on vimentin expression. It is possible that only the high level of Ngb induced by E_2 (more than four-fold induction) could exceed the threshold of the Ngb concentration necessary for inhibition of the effect of LPS on the expression of inflammatory molecules. In line with this hypothesis, Ngb could only enhance cell survival under conditions of anoxia or hypoxia and glucose deprivation when it was over-expressed (4,6,35). However, the effect of LPS on Ngb levels may require a different timing than the effect of E_2.

Interestingly, E_2 and LPS induce the increase of Ngb levels via different pathways. ERα and ERβ are equally expressed in astrocytes and the ER pure antagonist ICI completely prevents the effect of E_2 on the increase of Ngb levels. In particular, the effect of E_2 on Ngb levels specifically requires ERβ, also confirming the direct involvement of ERβ in Ngb modulation in astrocytes, as observed in human neuroblastoma SK-N-BE and mouse hippocampal neurones (7,31). On the other hand, LPS-induced Ngb up-regulation requires NFκB signals. The present data indicate that a negative cross-talk between ERs and NFκB signals occurs, as already reported in macrophages and microglia (36,37). In addition, the stimulatory effect of LPS on Ngb levels was decreased by the ERα agonist PPT but not by the ERβ agonist DPN. The fact that E_2 did not imitate the effect of PPT may be a result of E_2 being an agonist of both ERs, and its effects on LPS-induced Ngb levels will result from a balance in the activation of ERα- and ERβ-mediated transcription. In addition, the lower affinity of E_2 for ERα, compared to that of PPT (38), may also contribute to the different outcome of the Ngb levels of these two ER ligands on LPS-induction. The effect of PPT suggests that ERα-activated signals, which are not involved in E_2-mediated Ngb up-regulation, prevent any NFκB-mediated Ngb increase. In parallel, LPS impairs the ERβ-mediated up-regulation of Ngb levels. Therefore, the synergic co-activation of ERα and ERβ is pivotal for regulating Ngb expression in the presence of NFκB-activated signals in astrocytes.

LPS induced the expression of IL-6, IP-10, IL-1β and TNFα in astrocyte cultures. In addition, LPS also induced the expression of vimentin, a generalised marker of astroglia activation. In agreement with previous studies (14,39,40), E_2 inhibited the effect of LPS on IL-6, IP-10 and vimentin. By contrast, E_2 was unable to reduce the mRNA levels of IL-1β and TNFα after LPS stimulation. Although E_2 has been reported to decrease TNFα expression after LPS stimulation in midbrain astrocytes (12), previous studies are in agreement with our present findings, showing that the hormone does not significantly affect TNFα and IL-1β expression after LPS stimulation in cortical astrocytes (12,41).

Our findings suggest that E_2-induced Ngb up-regulation is involved in the inhibitory effect of E_2 on IL-6, IP-10 and vimentin expression induced by LPS. Indeed, E_2 was no longer able to attenuate IL-6 and vimentin expression in cultures that were treated with siNgb and LPS. However, this may be the result, at least in part, of siNgb decreasing the effect of LPS on the expression of vimentin, suggesting that Ngb may be involved in the mechanism of activation of astrocytes in response to LPS. Furthermore, the effect of E_2 on LPS-induced IP-10 expression was only partially reversed by neuroglobin silencing. This suggests that Ngb only comprises one part of the anti-inflammatory mechanisms of E_2 on astrocytes. Being involved in the anti-inflammatory action of E_2, Ngb can now be regarded as a mediator of different E_2 protective effects in the brain; indeed, inflammatory events often may represent the cause of principal neurodegenerative diseases and determine the prognostic consequences of these pathologies (42–46). These findings, together with those of more recent studies (18,28), indicate that Ngb likely plays a central role in different CNS disorders such as neurotrauma, ischaemia, and infectious and autoimmune disease. Together with the well known effects observed in neurones, our findings suggest that glial cells may also participate in the neuroprotective function of Ngb.

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