Journal of Neuroinflammation

BioMed Central

Research

Inhibition of microglial inflammatory responses by norepinephrine: effects on nitric oxide and interleukin-1β production

Cinzia Dello Russo*[1,2], Anne I Boullerne[3], Vitaliy Gavrilyuk[1] and Douglas L Feinstein[1]

Address: [1]Department of Anesthesiology, University of Illinois, & West Side Veteran's Affairs Research Division, Chicago, Illinois, U.S.A, [2]Institute of Pharmacology, Catholic University Medical School, Rome, Italy and [3]Department of Neurology, University of Chicago, Chicago, Illinois, U.S.A

Email: Cinzia Dello Russo* - cdr@uic.edu; Anne I Boullerne - abouller@neurology.bsd.uchicago.edu; Vitaliy Gavrilyuk - picomol7@uic.edu; Douglas L Feinstein - dlfeins@uic.edu

* Corresponding author

Published: 30 June 2004

Journal of Neuroinflammation 2004, **1**:9 doi:10.1186/1742-2094-1-9

Received: 18 March 2004
Accepted: 30 June 2004

This article is available from: http://www.jneuroinflammation.com/content/1/1/9

Abstract

Background: Under pathological conditions, microglia produce proinflammatory mediators which contribute to neurologic damage, and whose levels can be modulated by endogenous factors including neurotransmitters such as norepinephrine (NE). We investigated the ability of NE to suppress microglial activation, in particular its effects on induction and activity of the inducible form of nitric oxide synthase (NOS2) and the possible role that IL-1β plays in that response.

Methods: Rat cortical microglia were stimulated with bacterial lipopolysaccharide (LPS) to induce NOS2 expression (assessed by nitrite and nitrate accumulation, NO production, and NOS2 mRNA levels) and IL-1β release (assessed by ELISA). Effects of NE were examined by co-incubating cells with different concentrations of NE, adrenergic receptor agonists and antagonists, cAMP analogs, and protein kinase (PK) A and adenylate cyclase (AC) inhibitors. Effects on the NFκB:IκB pathway were examined by using selective a NFκB inhibitor and measuring IκBα protein levels by western blots. A role for IL-1β in NOS2 induction was tested by examining effects of caspase-1 inhibitors and using caspase-1 deficient cells.

Results: LPS caused a time-dependent increase in NOS2 mRNA levels and NO production; which was blocked by a selective NFκB inhibitor. NE dose-dependently reduced NOS2 expression and NO generation, via activation of β2-adrenergic receptors (β2-ARs), and reduced loss of inhibitory IkBα protein. NE effects were replicated by dibutyryl-cyclic AMP. However, co-incubation with either PKA or AC inhibitors did not reverse suppressive effects of NE, but instead reduced nitrite production. A role for IL-1β was suggested since NE potently blocked microglial IL-1β production. However, incubation with a caspase-1 inhibitor, which reduced IL-1β levels, had no effect on NO production; incubation with IL-receptor antagonist had biphasic effects on nitrite production; and NE inhibited nitrite production in caspase-1 deficient microglia.

Conclusions: NE reduces microglial NOS2 expression and IL-1β production, however IL-1β does not play a critical role in NOS2 induction nor in mediating NE suppressive effects. Changes in magnitude or kinetics of cAMP may modulate NOS2 induction as well as suppression by NE. These results suggest that dysregulation of the central cathecolaminergic system may contribute to detrimental inflammatory responses and brain damage in neurological disease or trauma.

Introduction

Microglial activation including the production of pro-inflammatory cytokines and reactive oxygen species is now recognized as a key component of several neurological diseases including Multiple Sclerosis (MS) and Alzheimer's Disease (AD); as well as other conditions in which trauma, infection, or injury leads to inflammatory activation. Activated microglia produce the free radical NO synthesized by the inducible form of the enzyme nitric oxide synthase (iNOS or NOS2). NOS2 can be induced in enriched cultures of microglial cells upon treatment with proinflammatory cytokines or bacterial endotoxin [1-3], as well as in rodent brains following peripheral or intraparenchymal introduction of inflammatory inducers [4]. In some cases NOS2 expression was dependent upon IL1β production [5], and some anti-inflammatory treatments were shown to reduce both microglial IL-1β as well as NOS2 expression ([5] for review). However other studies reported distinct, and in some cases opposite effects of anti-inflammatory treatments upon IL-1β versus NOS2 expression [6]. Thus, the precise role for IL-1β in regulating NOS2 expression in microglia requires further study.

We demonstrated that the neurotransmitter norepinephrine (NE) prevents induction of NOS2 in rat cortical astrocytes [7,8], and more recently in vivo that depletion of NE exacerbates the cortical inflammatory response to amyloid beta (Aβ) [9]. Similarly, others have shown that NE reduces astroglial expression of pro-inflammatory cytokines including IL1β and TNFα [10-13] and of cell adhesion molecules [14]. The effects of NE appear to involve activation of β-adrenergic receptors (β-ARs) and elevation of intracellular cAMP, and in most cases lead to suppression of astrocytic inflammatory responses [15]. Perturbation in NE levels, or dysfunction in NE signaling might therefore exacerbate inflammatory responses and thus contribute to neurological damage, for example in AD and Parkinson's disease where noradrenergic locus coeruleus (LC) neurons are lost [16,17], or in MS where astrocytic β-AR expression is reduced [18,19].

Rat cortical microglia express all different types of ARs [20], and treatment with NE results in increased levels of cAMP within the cells which can be inhibited by the β-AR non selective antagonist propanol [21]. However the cellular effects of NE on microglial inflammatory responses are less well characterized. NE reduced NO production in N9 microglial cells [22] and in rat microglia [20], but increased IL-1β mRNA in rat microglia [21]. Other agents which increase microglial cAMP (analogs such as dibutyryl-cyclic AMP (dbcAMP), activators of adenylate cyclase (AC), or PGE2) also modulate inflammatory responses, however in contrast to astrocytes, both up as well as down regulation of NOS2 and IL-1β has been observed [23,24]. Since the regulation of microglial NOS2 differs from astroglial NOS2 [25] it is not surprising that anti-inflammatory treatments which attenuate astrocyte NOS2 or IL-1β may have distinct actions in microglial cells.

To better understand how NE reduces microglial inflammatory responses, we examined the effects of NE on NOS2 expression in rat cortical microglial cells. We observed that, as found for astrocytes, NE dose-dependently blocked microglial NOS2 expression, via β2-ARs activation. In the same cells, NE more potently reduced IL-1β production, reaching close to 100% attenuation at low concentrations of NE (1 to 10 μM). However, additional experiments suggest that while NE inhibits both these factors, the suppression of NOS2 expression is not directly due to the reduction of IL-1β levels. These findings indicate that, at least in vitro, microglial NOS2 expression is not dependent upon IL-1β production and therefore suggest that anti-inflammatory treatments designed to reduce IL-1β may be without effect on NOS2 levels.

Methods

Materials

Cell culture reagents (DMEM, and antibiotics) were from Cellgro Mediatech. Fetal calf serum (FCS) and DMEM-F12 were from GIBCO Life Technologies. Lipopolysaccharide (LPS, Salmonella typhimurium), NE, the NOS2 inhibitor (2-amino-dihydro-6-methyl-4H-1,3-thiazine, AMT) and the peptide aldehyde inhibitor benzyloxycarbonyl-Ile-Glu (Ot2butyl) 2Ala-leucinal (ZIE) were from Sigma. Adrenergic agonists and antagonists were from BIOMOL Research Laboratories. The protein kinase (PK) A inhibitors (KT-5270 and H-89) and activators (dbcAMP), the AC inhibitors (SQ 22536 and MDL-12,330A), the interleukin receptor antagonist (IL-1ra) and the irreversible cell permeable caspase-1 inhibitor (Ac-YVAD-CMK) were from Calbiochem (San Diego, CA). Taq polymerase and cDNA synthesis reagents were from Promega. Anti IkBa (SC-371) rabbit polyclonal antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). NOD.ICE-/- mice, which lack functional caspase-1, and therefore do not produce mature IL-1β or IL-18 [26] were obtained from Jackson Laboratories.

Cells

Rat cortical microglial cells were obtained as previously described [27]. Briefly, 1 day old Sprague Dawley rats (Charles River Laboratories) were used. The cortices were dissected under aseptic conditions, cut into small fragments, digested in 0.125% trypsin (Sigma) for 20 min at 37°C and a further 5 min in presence of 65 UI/ml of DNAse I. Cells were plated at a density of 4×10^4 cells/cm^2 in T75 flasks in 10 ml DMEM containing 10% FCS and antibiotics (100 IU/mL of penicillin and 100 μg/mL of streptomycin; Sigma), and incubated at 37°C in a humidified atmosphere containing 5% CO_2. The culture medium was changed within 24 h, and then after 5 days.

For studies shown in figure 8, microglia were prepared from caspase-1 deficient mice [26] using the same procedure except that the incubation time with trypsin was reduced to 5 minutes. Microglia were detached from the astrocyte monolayer by gentle shaking 11–13 days after the dissection and again after one week from the first shaking. The cells were plated in 96 well plates at a density of 3×10^5 cells/cm^2, using 100 μl/well of DMEM-F12 (10% FCS and antibiotics). Under these conditions, the cultures were 95–98% OX42-positive.

Experiments were carried out the day after the isolation from the astrocyte monolayer in DMEM-F12. In preliminary experiments, we assessed cell viability in presence of different concentrations of FCS, measuring lactic dehydrogenase (LDH) release in the incubation medium as an index of cell toxicity. We found a significant increase in LDH activity if the cells were incubated in serum free medium (see also [27]) or in medium containing 1% FCS; therefore all experiments were carried out in 10% FCS.

IL-1β Measurements

The levels of IL-1β in the incubation medium were detected by specific ELISA assays. For rat IL-1β we used an ELISA kit purchased by R&D System Inc and performed according to the manufacturer's instructions. For the assessment of IL-1β released by ICE-deficient microglial cells, we used an ELISA specific for mouse IL-1β (BD OptEIA™ Set, BD Bioscience).

NOS2 induction and activity measurements

NOS2 was induced in microglial cells by incubation with bacterial endotoxin LPS. NOS2 induction was assessed indirectly by nitrite production in the cell culture media [28]. An aliquot of the cell culture media (80 μl) was mixed with 40 μl of Griess reagent and the absorbance measured at 550 nm. In preliminary studies, we found that the LPS dependent nitrite production was greater when cells were incubated in DMEM-F12 medium, as compared to DMEM alone; and therefore all studies were carried out in DMEM-F12.

In some experiments, we assessed total levels of nitrites in the incubation media after enzymatic reduction of nitrates to nitrites [29]. Briefly, samples were incubated with nitrate reductase purified from Aspergillus (EC 1.6.6.2), reduced β–NADPH, and FAD for 2 hr at 37°C to convert nitrates into nitrites. Excess β–NADPH was removed by incubating the samples for 30 min at 37°C in presence of LDH from rabbit muscle (EC 1.1.1.27) and pyruvate (all reagents from Sigma). Samples were assayed before and after nitrate reduction by the Griess method, to obtain nitrite levels and calculate the ratio of nitrites/nitrates. The nitrite concentration was calculated from a NaNO2 standard curve, and complete conversion of nitrate into nitrite was confirmed by including a standard curve of NaNO3 in each test.

NOS2-derived NO production was measured by the oxidation of the cell-permeable fluorogenic probe, 2',7-dichlorodihydrofluorescein diacetate (H_2DCF-DA) [30]. Once inside the cells, H_2DCF-DA is deacetylated by cytosolic esterases to free H_2DCF, which can be oxidized to the fluorescent compound dichlorofluorescein (DCF). This reaction is catalyzed in vitro by the formation of the nitrogen radical peroxinitrite, while hydrogen peroxide and superoxide were found ineffective by themselves [31]. Since peroxinitrite is formed by reaction of NO with superoxide, we used oxidation of H_2DCF as a marker of NO production. Briefly, cells were activated by LPS for different periods of time. At the end of each experiment the incubation media was replaced by balanced salt solution (BSS, 124 mM NaCl, 5.8 mM KCl, 10 mM dextrose, 20 mM Hepes, 0.3 mM $CaCl_2(H_2O)_2$) [32]. Cells were incubated in plain BSS or in BSS containing 100 μM AMT, to selectively block NOS2 activity, for 20 min. At the end of this pre-incubation period 20 μM H_2DCF-DA was added to the cells, which were incubated for 45 min at 37°C in the incubator. The fluorescence signal due to H_2DCF oxidation within the cells was quantified using a plate fluorescence reader (GENios Multi-Detection Reader, TECAN) using 485 nm as excitation and 535 nm as emission wavelength.

mRNA analysis

Total cytoplasmic RNA was prepared from cells using TRIZOL reagent (Invitrogen); aliquots were converted to cDNA using random hexamer primers, and mRNA levels estimated by RT-PCR. The primers used for NOS2 detection were 1704F (5' CTG CAT GGA ACA GTA TAA GGC AAA C-3'), corresponding to bases 1704–1728; and 1933R (5' CAG ACA GTT TCT GGT CGA TGT CAT GA-3'), complementary to bases 1908–1933 of the rat NOS2 cDNA sequence which yield a 230 bp product. The primers used for glyceraldehyde 3- phosphate dehydrogenase (GDH) detection were 796F (5'-GCC AAG TAT GAT GAC ATC AAG AAG) and 1059R (5' TCC AGG GGT TTC TTA CTC CTT GGA) which yield a 264 bp product [33]. Quantitative changes in mRNA levels were estimated by real time PCR using the following cycling conditions: 35 cycles of denaturation at 94°C for 10 s; annealing at 61°C for 15 s; and extension at 72°C for 20 s; followed by 2 min at 72°C, in the presence of SYBR Green (1:10,000 dilution of stock solution from Molecular Probes) carried out in a 20 μL reaction in a Corbett Rotor-Gene (Corbett Research) [34]. Relative mRNA concentrations were calculated from the take-off point of reactions using the software included in the unit. At the end of real time PCR, the products were separated by electrophoresis through 2%

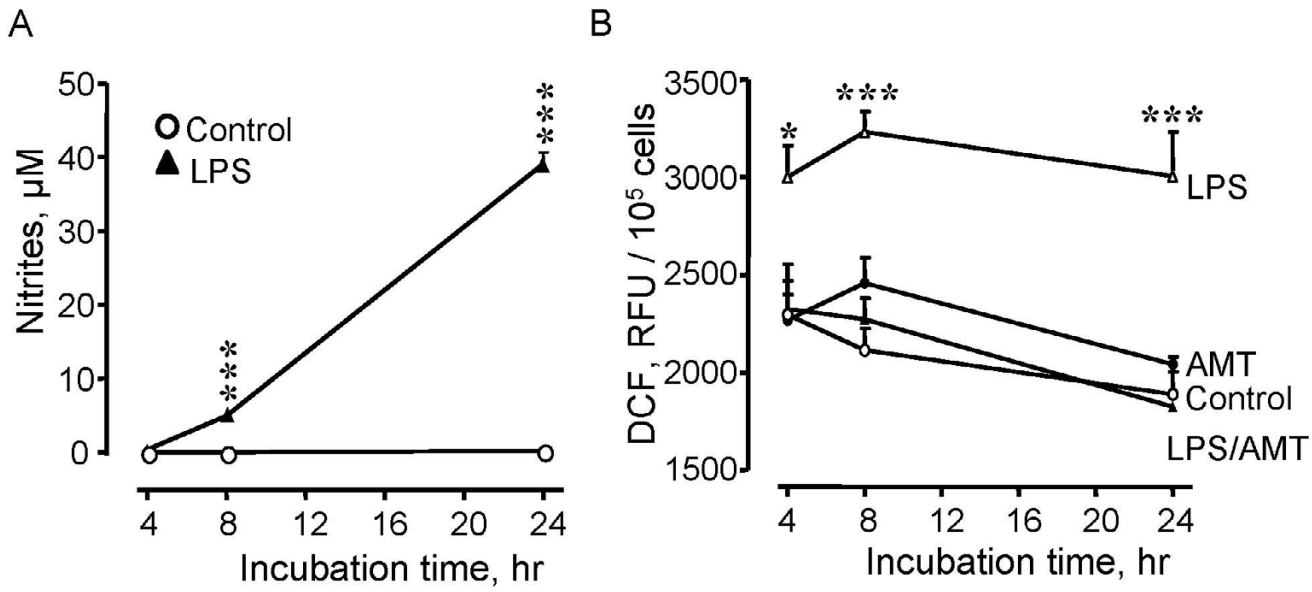

Figure 1

LPS increases microglial nitrite production. Rat microglia cells were incubated in the presence (▲) or absence (❍) of LPS (1 ng/ml) for indicated times. NO production was assessed (**A**) indirectly by measuring nitrite levels in the incubation medium; or (**B**) directly by the oxidation of H_2DCF added to the cells at the end of the experiment. For DCF studies, in each experimental group NOS2 activity was inhibited in parallel samples by preincubating cells 20 min with the selective NOS2 inhibitor, 2-amino-dihydro-6-methyl-4H-1,3-thiazine (AMT, 100 μM). Data are means ± s.e.m. of 3 different experiments. *** and *, P < 0.001 and 0.05 versus control; two-way ANOVA followed by Bonferroni's post hoc test.

agarose gels containing 0.1 μg/ml ethidium bromide to ensure production of correct sized product.

Western blotting

After desired incubations, cells were lysed using 8 M urea. The protein content in each sample was determined by Bradford's method using bovine serum albumin as standard. Ten μg of proteins were mixed 1: 3 with 3x gel sample buffer (150 mM Tris-HCl pH 6.8, 7.5% SDS, 45% glycerol, 7.5% of bromophenol blue, 15% β-mercaptoethanol), boiled for 5 min and separated through 10% polyacrylamide SDS gels. Apparent molecular weights were estimated by comparison to colored molecular weight markers (Sigma). After electrophoresis, proteins were transferred to polyvinylidene difluoride membranes by semi-dry electrophoretic transfer. The membranes were blocked with 10% (w/v) low-fat milk in TBST (10 mM Tris, 150 mM NaCl, 0.1% (w/v) Tween-20, pH 7.6) for 1 h, and incubated in the presence of anti-IκBα antibody (at 1:1,500 dilution) overnight with gentle shaking at 4°C. The primary antibody was removed, membranes washed 4 times in TBST, and further incubated for 1 h at room temperature in the presence of anti-rabbit IgG-HRP secondary antibody, diluted 1: 7,000. Following 4 washes in TBST, bands were visualized by incubation in enhanced chemiluminescence reagents for 1 min and exposure to X-ray film for 5 min.

Data analysis

All experiments were done at least in triplicate. Data are analyzed by one or two way ANOVA followed by Dunnett's multiple comparison or Bonferroni post hoc tests and P values < 0.05 were considered significant.

Results

LPS induces NOS2 expression in microglia

As shown by several groups, incubation of enriched cultures of rat cortical microglial cells with a low dose of LPS (1 ng/ml) led to a time-dependent increase in nitrite accumulation in the cell culture media. This concentration of LPS did not induce significant microglial cell death (assessed by LDH release); nor did higher concentrations of LPS result in significantly higher levels of nitrite production (data not shown). Nitrite levels were undetectable in control samples incubated for up to 24 hr, whereas LPS induced significant nitrite levels at 8 hr and 24 hr (0.49 and 3.9 nmole per 100,000 cells, respectively, Figure 1A); or approximately 2.1 μM nitrite accumulated per

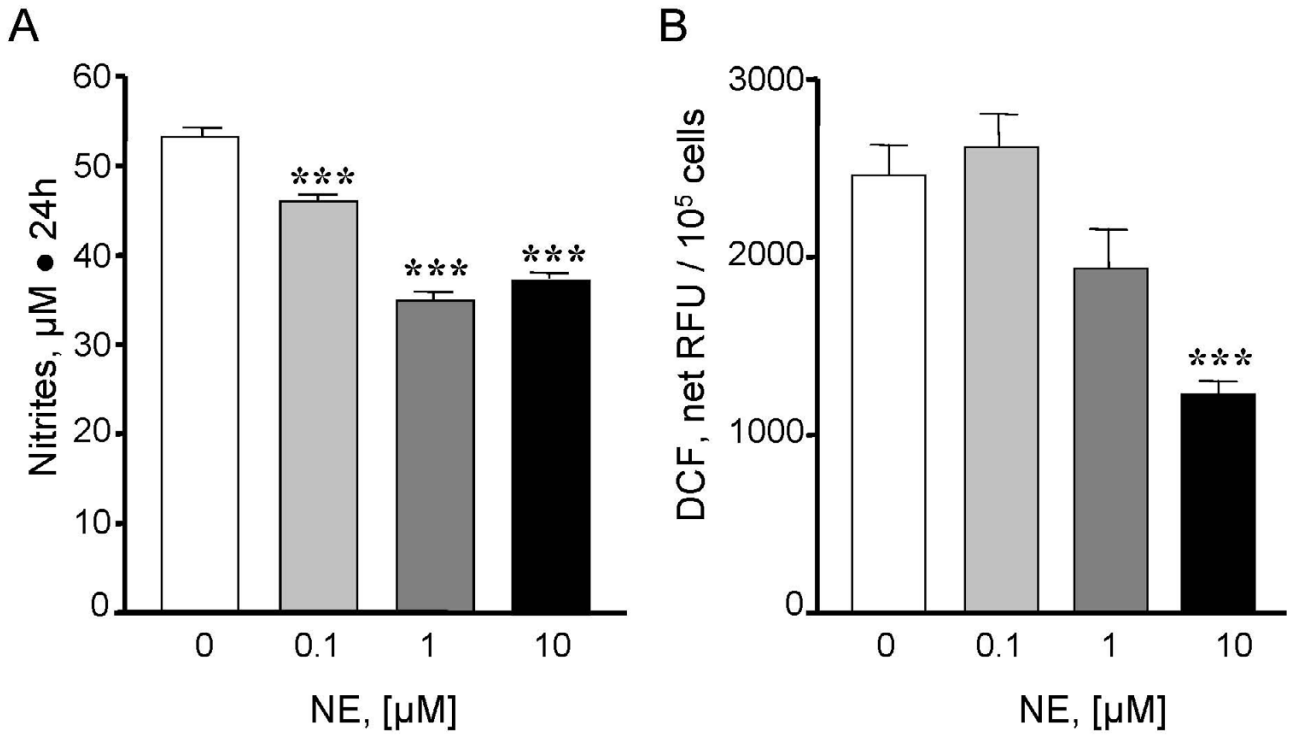

Figure 2

Microglial NOS2 activity is reduced by NE. Microglia were incubated with LPS and indicated concentrations of norepinephrine (NE). NO production was measured as in figure 1, (A) indirectly by nitrite levels measured after 24 hr; or directly by DCF fluorescence after 4 hr. In B, data are expressed as net relative fluorescence units (RFU) which is calculated as the difference between total RFU and the RFU values obtained by pre-blocking NOS2 activity in parallel samples with AMT (100 μM). Data are means ± s.e.m. of 3 experiments. ***, $P < 0.001$ versus LPS alone; one-way ANOVA.

hr per 100,000 cells (10 μg protein). Measurements using the fluorescent reporter H_2DCF-DA in the presence or absence of a selective NOS2 inhibitor (AMT, 100 μM) showed that NO production could be detected as soon as 4 hr after incubation with LPS and remained relatively unchanged for up to 24 hr incubation (Figure 1B).

NE inhibits nitrite accumulation and NO production

Microglia were incubated with LPS and varying concentrations of NE (Figure 2A). Co-incubation with NE dose-dependently reduced nitrite accumulation (measured after 24 hr incubation), with statistically significant inhibition occurring as low as 0.1 μM NE, and maximal inhibition reaching about 30% at 10 μM NE. Measurements of NO using H_2DCF-DA showed that NE reduced NOS2-derived NO after 4 hr of incubation, although at this time point significant inhibition was observed only at the higher (10 μM) NE concentration used (Figure 2B). Measurements of nitrite and nitrate levels (Figure 3) showed that the ratio of nitrite to nitrate (indicative of chemical breakdown) was unaffected by treatment with NE, ruling out that the reduction of nitrite accumulation due to NE was not due to increased conversion to nitrate.

NE effects are mediated by β2-ARs and may involve cAMP

The inhibition of nitrite accumulation by NE was mimicked by the β-AR agonist isoproterenol used between 0.1 and 10 μM (Figure 4A), and by the cAMP mimetic dbcAMP (Figure 4B). The inhibitory effects of NE were not reversed by the α-AR antagonist phenoxybenzamine (PB, Figure 5A) but were completely reverted using either a non-selective β-AR (propanolol, Figure 5A) or a selective β2-AR (ICI-118,551; Figure 5B) antagonist. Measurements of intracellular cAMP levels confirmed that NE (10 μM) significantly increased (approximately 10-fold versus control cells) cAMP levels between 15 and 60 minutes of incubation (not shown).

Since activation of PKA is mediated by cAMP, we tested a role for PKA in mediating NE inhibitory effects (Figure

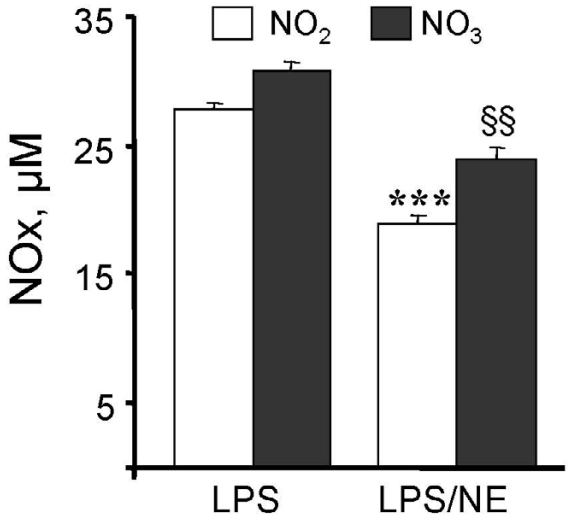

Figure 3
NE does not modify nitrite conversion to nitrate. Microglia cells were incubated with LPS (1 ng/ml) plus or minus 10 μM NE. After 24 hr, the levels of nitrite (open bars) and nitrate (filled bars) in the cell culture media were determined. ***, $P < 0.001$ versus LPS alone (NO_2); §§, $P < 0.05$ versus LPS alone (NO_3). The ratio of nitrite to nitrate in LPS treated cells was 0.91 ± 0.02, and in LPS/NE treated cells was 0.79 ± 0.03 (n = 3).

6A). However, co-incubation with the selective PKA inhibitor KT-5720 (Figure 6A) or compound H89 which inhibits both PKA and PKC (Figure 6B) did not reverse NE effects, suggesting that PKA activation does not play a major role in reducing NOS2 activity (or expression). Moreover, both inhibitors when used alone reduced nitrite accumulation due to LPS, suggesting that PKA and/or PKC activation may in fact play a role in potentiating microglial NOS2 induction.

To examine a role for cAMP in mediating NE actions, we treated microglia cells with two different AC inhibitors, SQ 22536 (IC_{50}= 200 μM) and MDL-12,330A (IC_{50}= 250 μM). Unexpectedly, in these cells inhibition of AC activity reduced LPS induced nitrite production (Figure 7A) and NOS2 expression (Figure 7B), and SQ 22536 further potentiated NE inhibitory effects (Figure 7A).

NE reduces NOS2 mRNA and increases IkBα levels

Quantitative RT-PCR analysis (Figure 8) showed that LPS increased NOS2 mRNA steady state levels approximately 15-fold versus control values after 4 hr of incubation, and further increased levels (to roughly 50-fold control levels) at 24 hr incubation. The presence of NE reduced the increase in NOS2 mRNA levels at both 4 and 24 hr, suggesting an effect of NE at the transcriptional and or post-transcriptional level.

In astrocytes, the suppression of NOS2 by NE involves modulation of the NFκB:IκB signaling system [33]. In microglia, nitrite production was also dependent upon NFκB activation, since treatment with the NFκB inhibitor ZIE dose-dependently reduced nitrite accumulation (Figure 9A). ZIE is a highly selective inhibitor of the 26S proteasome which blocks IκBα degradation and NFκB translocation into the nucleus [35]. In fact LPS induced a rapid loss of inhibitory IκBα protein (Figure 9B), which is affected by NE treatment. In the presence of NE, the reduction in IκBα protein levels occurring after 30 minutes incubation was less than that in control cells, while after 90 minutes NE caused an increase in IκBα levels. This suggests that, similar to what is observed in astrocytes [34], NE may increase IκBα re-synthesis.

NE reduces IL-1β release

As previously reported [12,36,37], LPS increased microglia IL-1β production (Figure 10A). As for nitrite production, co-incubation with NE (10 μM) reduced IL-1β release; however the magnitude of suppression was greater (approximately 80% inhibition) than the 30% suppression of nitrites observed. Incubation of cells with NE alone led to a small but non-significant IL-1β release after 7 hr. In contrast to nitrite reduction, maximal effects of NE on IL-1β levels were observed even at the lowest concentration (0.1 μM) tested (Figure 10B).

Effects of blocking IL-1β production on NOS2 expression

The above results suggested a link between microglial NOS2 expression and IL-1β production. However, incubation with a caspase-1 inhibitor (Figure 11A) reduced IL-1β production by 33% (Figure 11B) but had no effect on NO production (Figure 11B). Results using the IL-1ra were conflicting, since although we found a reduction (24%) of LPS-induced nitrite accumulation at the highest concentration tested (100 ng/mL), we found an increase (between 24–33%) at lower concentrations (10–30 ng/mL; data not shown). To further test an involvement of IL-1β in the induction of NOS2 by LPS, we used microglial cells derived from caspase-1 deficient mice which cannot produce the mature form of IL-1β. In these cells, LPS (10–1000 ng/ml) induced similar levels of nitrite production as did wild type cells; and the inhibitory effects of NE were maintained both at the highest concentration of LPS (Figure 11C) and at the lower ones (not shown). Together, these results suggest that the inhibition by NE is not primarily mediated via effects on IL-1β production.

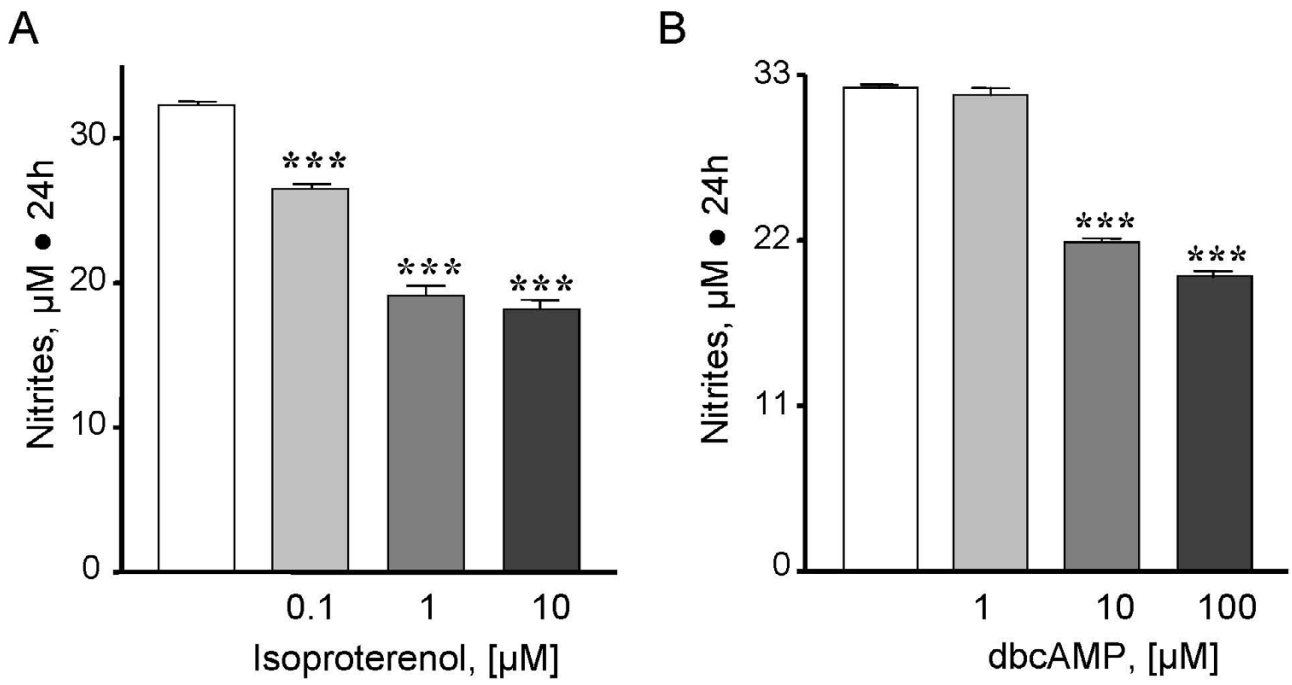

Figure 4
Inhibitory Effects of NE are mediated by β-ARs and replicated by cAMP. Microglia were incubated with LPS (1 ng/ml) and indicated concentrations of (A) isoproterenol or (B) dibutyryl-cyclic AMP (dbcAMP). Nitrite levels were measured after 24 hours. ***, $P < 0.001$ versus LPS alone.

Discussion

Consistent with previous reports, in the present study we show that rat cortical microglia can be activated in vitro by low doses of LPS leading to NOS2 expression, NO production and nitrite accumulation. Under our experimental conditions, co-incubation with NE (0.1–10 μM) inhibited LPS-dependent NOS2 expression and NO and nitrite production, via activation of β2-ARs most likely mediated by elevation of intracellular cAMP. Although NE has a high affinity for, and at low doses (100 nM to 1 μM) can increase cAMP via microglial β1 and β3 ARs, even greater increases in cAMP were found at higher (1–10 μM) NE concentrations which are needed to activate β2-ARs [21]. Thus, the amounts of NE needed to reduce NOS2 expression may reflect a requirement to activate β2-ARs in our studies, although other non-receptor mediated effects cannot be ruled out. Previous studies of adrenergic regulation of microglial NOS2 are limited: isoproterenol decreased NO release [38]; and in one study [20], NE, terbutaline (a β2-AR agonist), dobutamine (a β1-AR agonist) as well as phenylephrine (an α1-AR agonist) all reduced NO production despite different effects on cAMP elevation; suggesting that adrenergic stimulation can attenuate NOS2 irrespective of effects on cAMP.

Our results are consistent with several reports showing that intracellular levels of cAMP modulate microglial NOS2 expression. NOS2 expression was reduced by cAMP analogs in microglia [42]; by PGE2 (as well as FSK and dbcAMP) in enriched microglia [43,44]; and in mixed neuron:microglial co-cultures [36]. Microglial NOS2 was also reduced by treatment with phosphodiesterase (PDE) inhibitors [38,42-45]; as well as other agents which increase cAMP, including melanocortin peptides [46], and conditioned media from T. gondii infected astrocytes [48]. However, NOS2 is not always suppressed by elevated cAMP, and there are several studies showing that in contrast to being inhibitory, cAMP potentiates NOS2 expression [15]. For example, dbcAMP or IBMX treatment increased microglial NOS2 expression and activity due to Aβ [23]. The potentiating effects of cAMP appear to be mediated through activation of C/EBP family proteins which can be stimulatory [47], rather than through activation of CREB proteins which may be inhibitory [8]; and in macrophages may include activation of other kinases

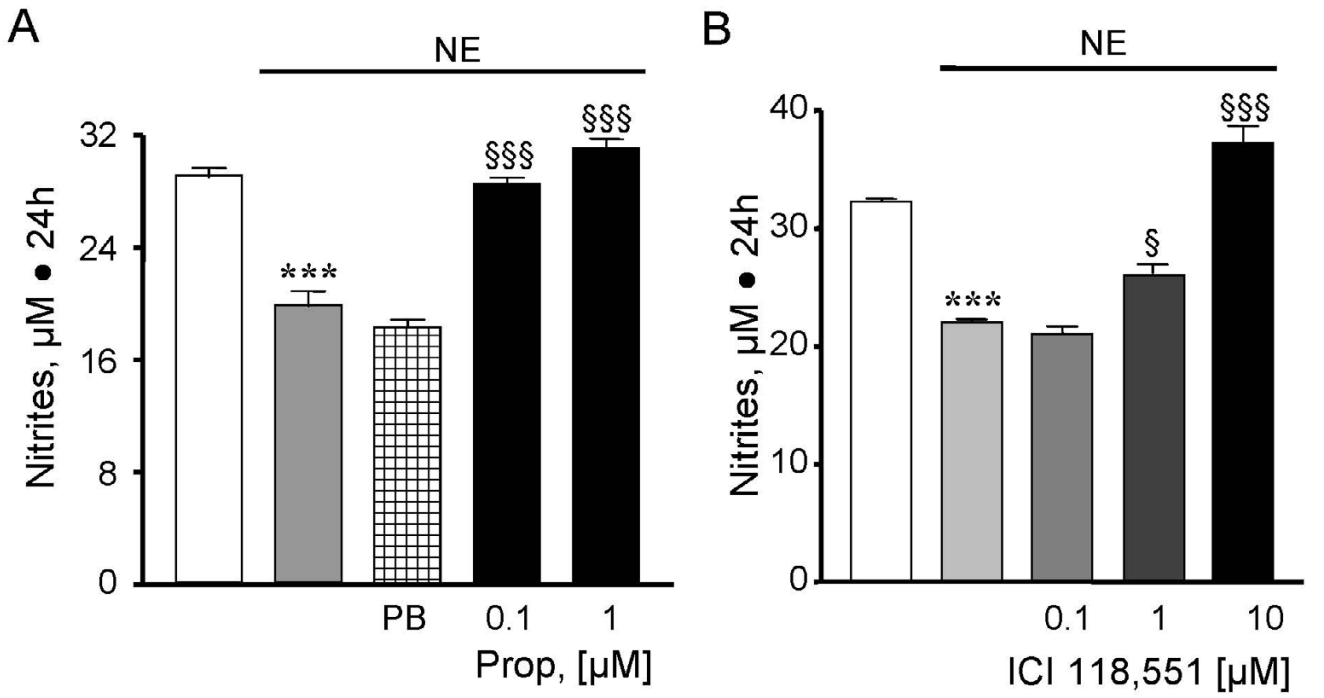

Figure 5

Inhibitory Effects of NE are mediated by β2-ARs. Microglia were incubated with LPS (1 ng/ml) alone or with NE (1 μM), and in the presence of (**A**) the α-AR antagonist phenoxybenzamine (PB, 10 μM) or the indicated amount of β-AR antagonist propanolol (Prop); or (**B**) indicated concentrations of the selective β2-AR antagonist ICI 118,551. Nitrite levels were measured after 24 hr. ***, P < 0.001 versus LPS alone; §§§ and §, P < 0.001 and 0.05 versus LPS plus NE.

including PKC isoforms and p38 MAPK [49]. Hence, activation of distinct cAMP-dependent transcription factors could account for observation of both activation as well as suppression by cAMP in microglial cells.

It should be pointed out that studies using dbcAMP should be interpreted cautiously since dbcAMP must first be metabolized to its active form, monobutyryl cAMP, a reaction catalyzed by intracellular esterases as well as extracellularly in the presence of serum, and that also releases the butyryl group from the 5'-position. The anti-inflammatory effects of dbcAMP on NO production could therefore be due, in part, to production of sodium butyrate which in rat primary microglial cells can reduce NO production and IL6 and TNFα release [39]. Nevertheless, findings that the effects of NE are mediated via β2-ARs which primarily increase intracellular cAMP, and also are mimicked using the βAR agonist isoproterenol are consistent with the idea that NE actions involve increases in cAMP.

Although PKA is a primary target for activation by cAMP, we found that selective PKA inhibitors did not reverse the inhibitory effects of NE, suggesting that other cAMP-dependent signaling pathways, such as the newly characterized EPAC/RAP system [40] may mediate NE inhibitory actions in microglia. However interpretation of results with PKA inhibitors are complicated by the fact that these inhibitors blocked LPS-dependent nitrite production (Figure 6B), suggesting a role for PKA activation in NOS2 induction.

Similarly, our results show that the AC inhibitor SQ22536 did not reverse NE actions, and by itself reduced LPS-induced NOS2 activity and expression (Figure 7). This finding is in contrast with previous studies showing no effects of this agent (or other AC inhibitors) on NOS2 induction, yet able to reverse NOS2 suppression due to activation of the prostaglandin EP2 receptors by PGE2 [41]. However, in the same study contradictory effects of AC activation on NOS2 were observed, since sulprostone, a potent agonist for the EP1 and EP3 receptors which inhibits AC activity, inhibited LPS-induced nitrite produc-

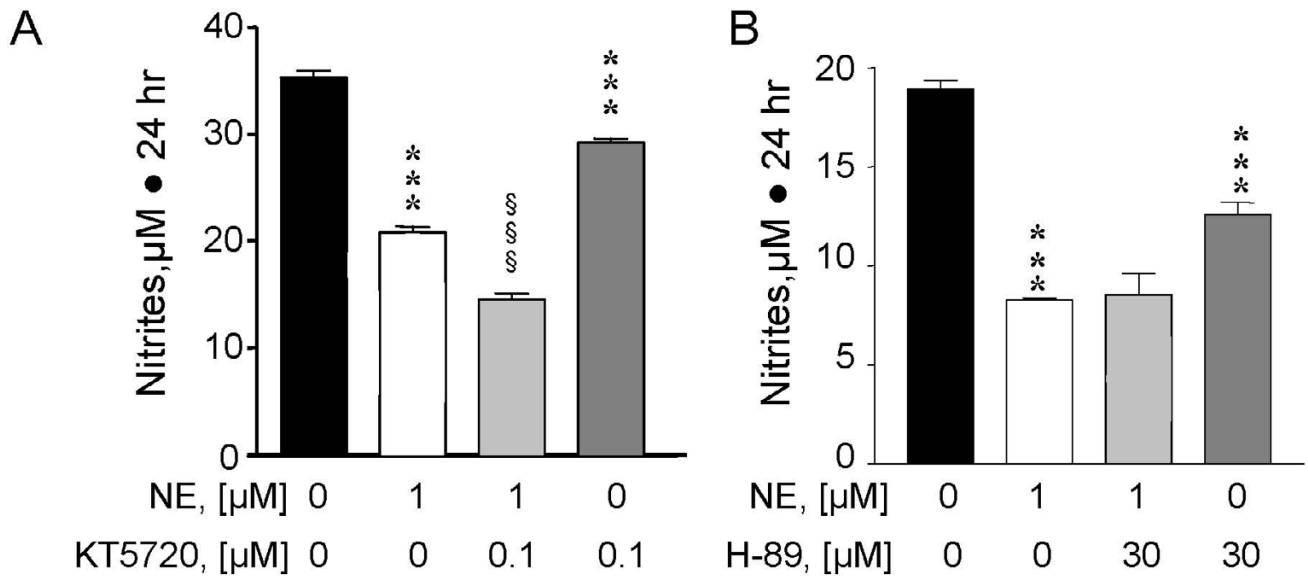

Figure 6
Protein Kinase A does not mediate Effects of NE. Microglia were incubated with LPS (1 ng/ml) alone or with NE (1 μM), and in the presence of (A) the selective PKA inhibitor KT5720; or (B) the PKA and PKC inhibitor H89. Nitrite levels were measured after 24 hr. ***, P < 0.001 versus LPS alone; §§§, P < 0.001 versus LPS plus NE.

tion; furthermore nitrite production was also increased at the higher concentrations of isoproterenol (>100 nM), the AC activator forskolin (FSK, > 100 μM) and dbcAMP (>10 μM). AC activators also had contrasting effects on Aβ induced nitrite production in microglia, where low doses of forskolin (10 to 50 μM) increased NO release, and a higher dose (100 μM) reduced NO release [23]. An understanding of the contrasting effects of PKA and AC inhibitors on nitrite production and NOS2 expression may therefore help to explain reported divergent effects of cAMP on microglial NOS2.

The effects of increasing cAMP levels on microglial IL-1β production and expression are also conflicting. Thus, Si et al. [50] showed that the PDE inhibitor propentofylline reduced LPS induced TNFα and IL-1β release; Caggiano and Kraig [12,51] showed that PGE2 acting via EP2 receptors (and increased cAMP) reduced IL-1β production; and Cho et al. [13] showed that the dopamine metabolite NAMDA which increases cAMP and CREB activation reduced IL-1β mRNA levels. In contrast, Hetier [24] found that the β-AR agonist isoproterenol reduced LPS induced IL-1β as well as TNFα production, however while TNFα mRNA was reduced, IL-1β mRNA was increased. Tomozawa et al. [52] similarly found that isoproterenol (and dbcAMP) increased IL-1β mRNA in microglial (although not in astrocytes); and Petrova et al. [11] reported that PGE2 also reduced IL-1β secretion, but increased IL-1β mRNA levels. More recently, Woo et al [53] showed that dbcAMP reduced TNFα expression, but increased IL-1β expression in BV2 cells; and Tanaka et al. [21] showed in that various β1- and β2-AR agonists alone could increase IL-1β mRNA levels in rat microglia. Our data is therefore the first to demonstrate the effects of an endogenous neurotransmitter on NOS2 expression and IL-1β levels in stimulated microglial cells.

The suppression by NE of IL-1β production was similar to that seen for NOS2, which suggested that the ability of NE to reduce NOS2 may be related to its ability to reduce IL-1β. However, several features suggest that these may be independent events. Thus, in contrast to suppression of NOS2, the effects of NE on IL-1β were observed at concentrations lower than that needed for maximal inhibition of NOS2 expression, and resulted in greater extent of inhibition (over 80% inhibition of IL-1β versus 30% of NO or nitrite production). Furthermore, NE was able to reduce nitrite production in caspase-1 deficient cells (Figure 10C) demonstrating that effects of NE on IL-1β are not necessary to observe effects on NOS2. Several previous reports suggest distinct regulation of microglial NOS2 and IL-1β. Thus, microglial cells cultured in the presence of

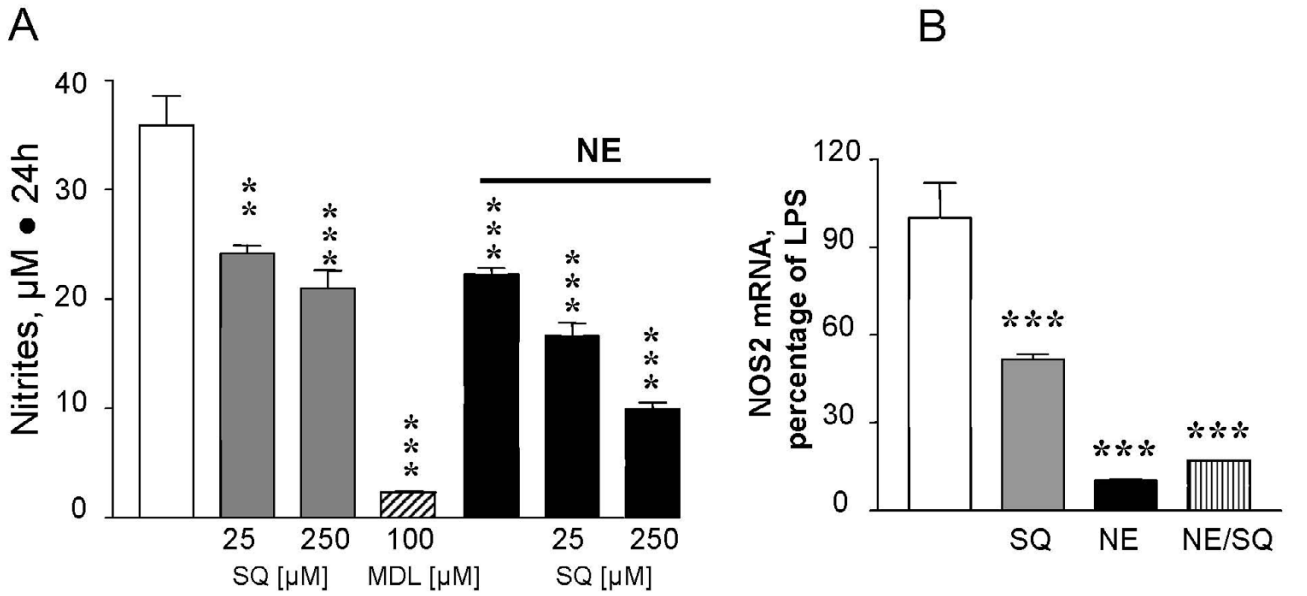

Figure 7

Adenylate cyclase activation mediates LPS induced NO production. (A) Microglia were incubated with LPS (1 ng/ml) alone or with NE (1 μM), and in the presence of the AC inhibitor SQ 22536 (SQ); or with LPS in presence of the irreversible cell permeable AC inhibitor MDL-12,330A (MDL). *** and **, P < 0.001 and 0.01 versus LPS alone. (B) Total cytosolic RNA was prepared from control microglia, or microglia incubated for 24 h with 1 ng/ml of LPS in presence of 200 μM SQ22536, or 10 μM NE or both SQ and NE and used for Q-PCR analysis of NOS2 mRNA. Data are expressed as percentage of LPS (100%). ***, P < 0.001 versus LPS alone; one-way ANOVA.

astrocytes lost their ability to produce NOS2 in response to LPS, although their IL-1β release was unaffected [54]. Petrova et al. [11] and Si et al. [50] showed cAMP dependent reductions in IL-1β production with no effect on NO production; and Woo et al [53] showed increased IL-1β expression due to dbcAMP with no effect on NO production. More recently, treatment of LPS activated microglia with malonic acid C60 derivatives reduced NOS2 mRNA expression, although these same reagents increased the release of IL-1β [55]. From these studies, it is clear that there is no necessary concordance between the regulation of IL-1β expression (or production) and that of NOS2 expression (or activity).

In general, the role that IL-1β plays in inducing glial (astrocytes or microglial) NOS2 is not clear. In astrocytes, IL-1β in combination with other cytokines (IFNγ and/or TNFα) can induce rodent NOS2 [25,56], and a few reports suggest that IL-1β alone may induce rodent astrocyte NOS2 [57]. In contrast, in human fetal and adult astrocytes, IL-1β alone can induce NOS2 [58,59] which is greatly increased by other cytokines [60]. Although astrocyte NOS2 induction can, in some cases, be reduced by treatment with IL-ra (hypoxia, [61]; using CM obtained from Gp41 activated of microglia, [62]; using Aβ stimulation, [63]) it is likely that other factor(s) are released which contribute to NOS2 induction. In contrast to astrocytes, there are no clear reports to indicate that IL-1β alone will induce microglial NOS2, and in fact human microglial appear more refractory to NOS2 inducers than do rodent cells [58]. Our results are consistent with the conclusion that the LPS induced IL-1β does not play an important role in mediating microglial NOS2 expression.

The molecular mechanism(s) by which NE reduces microglial NOS2 expression and IL-1β production and expression are not yet known. Work from several laboratories has shown in glial cells that LPS rapidly activates PK cascades which lead to phosphorylation of inhibitory IκB proteins, their degradation by the 26S proteasome, and subsequent activation of NFκB [64], necessary for the expression of pro-inflammatory genes [65]. We observed that LPS induced rapid loss of the microglial IκBα protein, while co-incubation with NE reduced that loss and moreover increased IκBα levels after longer times. Several reports suggest that increases in cAMP are associated with

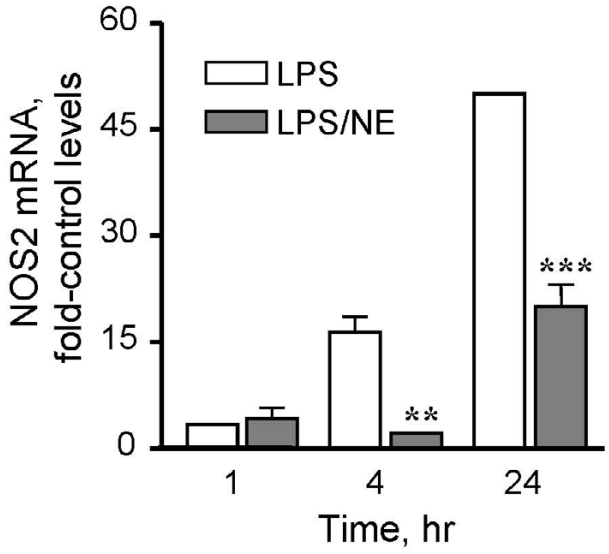

Figure 8
Microglial NOS2 mRNA is reduced by NE. Total cytosolic RNA was prepared from control microglia, or microglia incubated with LPS (open bars) or LPS plus 1 μM NE (filled bars), and used for Q-PCR analysis of NOS2 mRNA. Data are means ± s.e.m of 2 replicates per group, and are the values calculated for NOS2 mRNA levels relative to control (non-stimulated) cells. *** and **, P < 0.001 and 0.01 versus LPS alone; two-way ANOVA followed by Bonferroni's post hoc test.

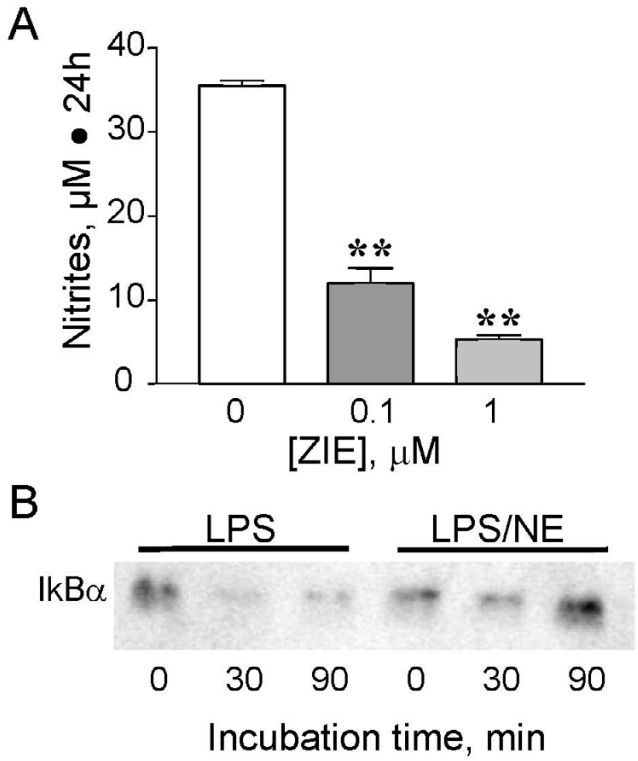

Figure 9
NE reduces LPS-induced IkBα loss. (A) NOS2 expression requires NFkB activation. Microglia were incubated with LPS (1 ng/ml) and the indicated concentration of the selective NFkB inhibitor ZIE. Nitrite levels were measured after 24 hr incubation. **, P < 0.01 versus LPS alone. (B) Microglial were incubated with LPS or LPS and NE (1 μM), and at the indicated time points, cytosolic lysates were examined for the presence of IkBα protein by western blot analysis. The gel shown is representative of experiments done on 3 separate microglial preparations.

increased levels of IκBα [66-68], and we demonstrated that NE directly increases transcription of the IκBα gene in astrocytes and reduces the overall magnitude of IkBa degradation [34]. Taken together these data suggest that the inhibitory effects of NE in microglia may also be mediated in part by interference with the NFkB:IkB signaling pathway.

The in vivo relevance of noradrenergic regulation of inflammatory gene expression, is suggested by observations that the noradrenergic neurons of the LC are damaged or lost in AD [69], leading to a loss (or at least a transient loss) in noradrenergic signaling within projection areas. A possible perturbation in noradrenergic signaling is also implicated in MS, since it has been shown that treatment with β-AR agonists [70,71] can provide protection in animal models of MS, and more recently that the levels of β2-ARs in astrocytes are decreased in MS patients as compared to healthy controls [72]. We recently demonstrated that LC loss increases both the magnitude as well as the duration of the inflammatory responses in vivo including the extent of microglia activation [73]. Furthermore, we showed that central NE depletion led to a dramatic decrease in cortical levels of the IkBa protein, consistent with the idea that NE normally keeps the IkBa gene transcriptionally active [34]. Together with the knowledge that IL-1β plays a prominent role in the pathogenesis of neurodegenerative diseases, such as AD [74], and in view of the fact that microglial NOS2 expression is implicated in the damage occurring in MS [75,76], AD [77] and cerebral ischemia [78,79], it may be of value to consider possible therapeutic strategies to increase microglial β-AR activation or intracellular cAMP levels.

List of abbreviations

inducible nitric oxide synthase (NOS2)

norepinephrine (NE)

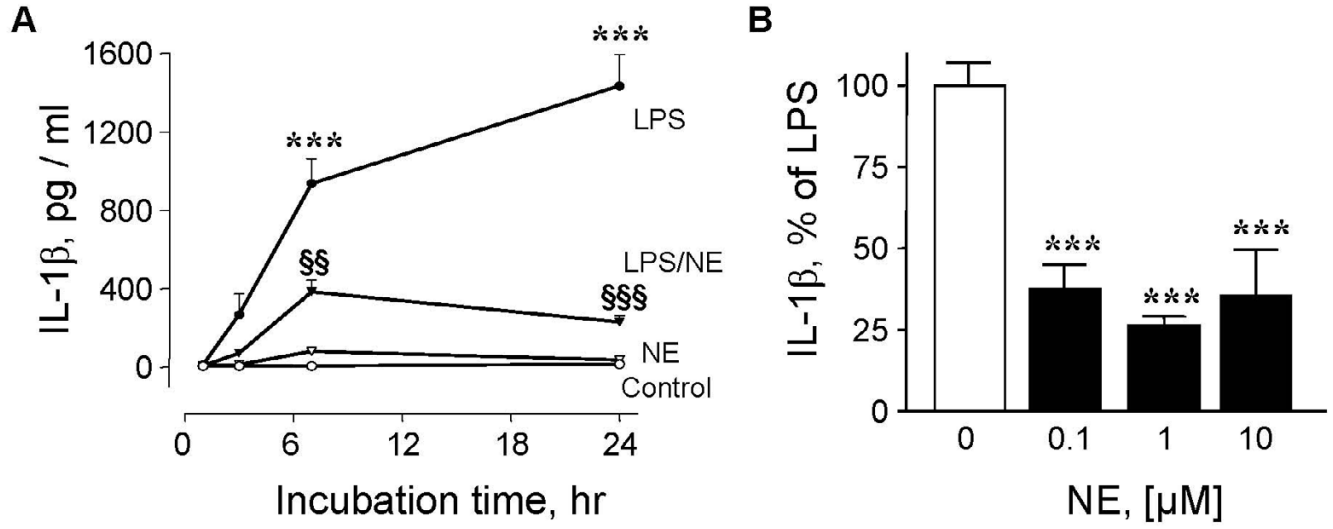

Figure 10

NE inhibits LPS dependent IL-1β production. (**A**) Microglia were incubated in medium alone (○), 1 ng/ml LPS (●), 10 μM NE (▽), or LPS plus NE (▼) and IL-1β levels were measured by ELISA at the indicated times. Data are means ± s.e.m. and are expressed as pg of IL-1β/ml. ***, P < 0.001 versus control; §§§ and §§, P < 0.001 and 0.01 versus LPS alone (n = 3); two-way ANOVA followed by Bonferroni's post hoc test. (**B**) Microglia were incubated in presence of 1 ng/ml LPS plus the indicated amounts of NE, and IL-1β levels measured by ELISA after 4 hours. Data are presented as % of the response to LPS alone (100% = 1160 ± 80 pg/ml). ***, P < 0.001 versus LPS alone (n = 3); one-way ANOVA followed by Dunnett's multiple comparison test.

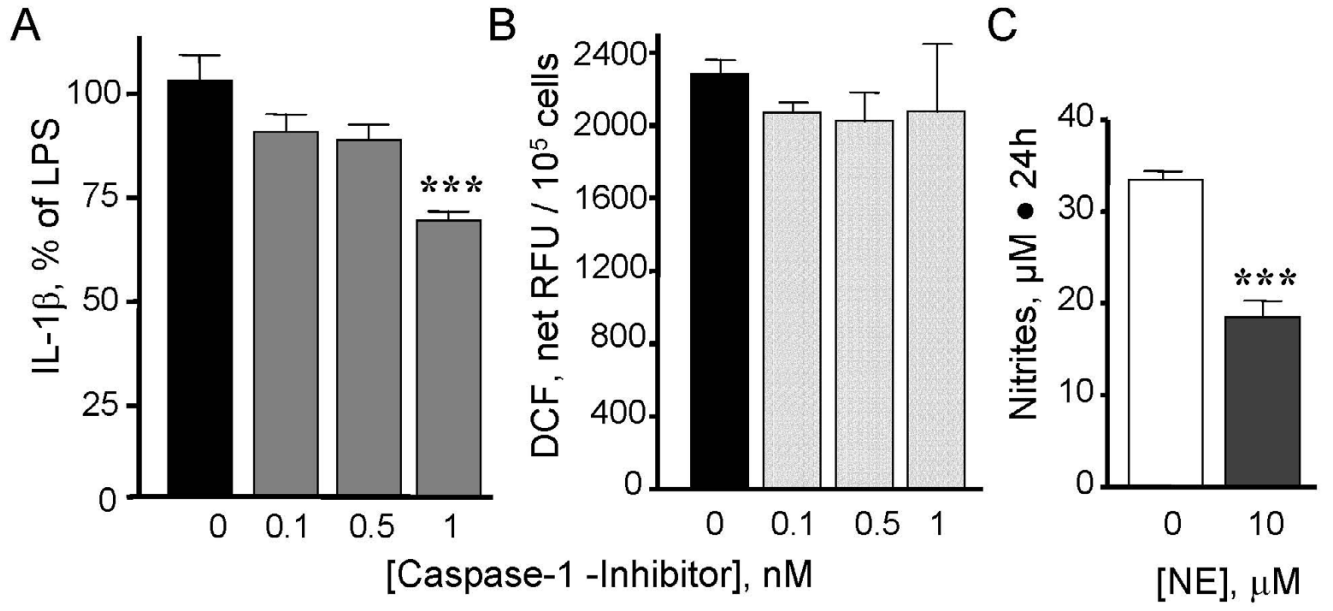

Figure 11

Effect of Caspase-1 inhibition on NOS2 activity. Microglia were incubated with 1 ng/ml LPS and the indicated concentrations of the caspase-1 cell-permeable irreversible inhibitor, Ac-YVAD-CMK. After 4 hr, (**A**) IL-1β levels in the incubation media were assessed by ELISA; and (**B**) NOS2 activity in the cells measured by increase in net DCF fluorescence (total RFU minus the RFU obtained by preincubating cells with 100 μM AMT NOS2 inhibitor). The data are means ± s.e.m of 3 experiments; the values for IL-1β are presented as % of the response to LPS alone (100% = 2520 ± 150 pg/ml). ***, P < 0.001 versus LPS alone. (**C**) Caspase-1 deficient microglia were incubated in 1 μg/ml LPS with 0 or 10 μM NE. Nitrite levels were measured after 24 hours. Data are means ± s.e.m. of 3 experiments. ***, P < 0.001 versus LPS alone.

interleukin 1-receptor antagonist (IL-1ra)

lipopolysaccharide (LPS)

protein kinase (PK)

adenylate cyclase (AC)

adrenergic receptor (AR)

multiple sclerosis (MS)

Alzheimer's disease (AD)

amyloid beta (Aβ)

locus coeruleus (LC)

dibutyryl-cyclic AMP (dbcAMP),

fetal calf serum (FCS)

benzyloxycarbonyl-Ile-Glu (Ot2butyl) 2Ala-leucinal (ZIE)

2',7-dichlorodihydrofluorescein diacetate (H_2DCF-DA)

dichlorofluorescein (DCF)

2-amino-dihydro-6-methyl-4H-1,3-thiazine (AMT)

phenoxybenzamine (PB)

forskolin (FSK)

phosphodiesterase (PDE)

Competing interests
None declared.

Authors' contributions
CDR carried out the majority of the experimental studies

AIB carried out nitrite and nitrate studies

VG carried out RTPCR and real time PCR analysis

DLF supervised the research, helped design experiments and analyze data, and helped write and edit the manuscript.

Acknowledgments
We thank Anthony Sharp for assistance with cell culture studies, and Dr. Pilar Mercado for help with ELISA measurements. This work was supported in part by NIH grants NS31556 and NS044945 to DLF and a postdoctoral award from the National Multiple Sclerosis Society (CDR).

References
1. Zielasek J, Tausch M, Toyka KV, Hartung HP: **Production of nitrite by neonatal rat microglial cells/brain macrophages.** *Cell Immunol* 1992, **141:**111-120.
2. Corradin SB, Mauel J, Donini SD, Quattrocchi E, Ricciardi-Castagnoli P: **Inducible nitric oxide synthase activity of cloned murine microglial cells.** *Glia* 1993, **7:**255-262.
3. Boje KM, Arora PK: **Microglial-produced nitric oxide and reactive nitrogen oxides mediate neuronal cell death.** *Brain Res* 1992, **587:**250-256.
4. Heneka MT, Dumitrescu L, Loschmann PA, Wullner U, Klockgether T: **Temporal, regional, and cell-specific changes of iNOS expression after intrastriatal microinjection of interferon gamma and bacterial lipopolysaccharide.** *J Chem Neuroanat* 2000, **18:**167-179.
5. Nakamura Y: **Regulating factors for microglial activation.** *Biol Pharm Bull* 2002, **25:**945-953.
6. Nakamura Y, Si QS, Kataoka K: **Lipopolysaccharide-induced microglial activation in culture: temporal profiles of morphological change and release of cytokines and nitric oxide.** *Neurosci Res* 1999, **35:**95-100.
7. Feinstein DL, Galea E, Reis DJ: **Norepinephrine suppresses inducible nitric oxide synthase activity in rat astroglial cultures.** *J Neurochem* 1993, **60:**1945-1948.
8. Feinstein DL: **Suppression of astroglial nitric oxide synthase expression by norepinephrine results from decreased NOS-2 promoter activity.** *J Neurochem* 1998, **70:**1484-1496.
9. Heneka MT, Galea E, Gavriluyk V, Dumitrescu-Ozimek L, Daeschner J, O'Banion MK, Weinberg G, Klockgether T, Feinstein DL: **Noradrenergic depletion potentiates beta-amyloid-induced cortical inflammation: implications for Alzheimer's disease.** *J Neurosci* 2002, **22:**2434-2442.
10. Willis SA, Nisen PD: **Inhibition of lipopolysaccharide-induced IL-1 beta transcription by cyclic adenosine monophosphate in human astrocytic cells.** *J Immunol* 1995, **154:**1399-1406.
11. Petrova TV, Akama KT, Van Eldik LJ: **Selective modulation of BV-2 microglial activation by prostaglandin E(2). Differential effects on endotoxin-stimulated cytokine induction.** *J Biol Chem* 1999, **274:**28823-28827.
12. Caggiano AO, Kraig RP: **Prostaglandin E receptor subtypes in cultured rat microglia and their role in reducing lipopolysaccharide-induced interleukin-1beta production.** *J Neurochem* 1999, **72:**565-575.
13. Cho S, Kim Y, Cruz MO, Park EM, Chu CK, Song GY, Joh TH: **Repression of proinflammatory cytokine and inducible nitric oxide synthase (NOS2) gene expression in activated microglia by N-acetyl-O-methyldopamine: protein kinase A-dependent mechanism.** *Glia* 2001, **33:**324-333.
14. Ballestas ME, Benveniste EN: **Elevation of cyclic AMP levels in astrocytes antagonizes cytokine-induced adhesion molecule expression.** *J Neurochem* 1997, **69:**1438-1448.
15. Galea E, Feinstein DL: **Regulation of the expression of the inflammatory nitric oxide synthase (NOS2) by cyclic AMP.** *FASEB J* 1999, **13:**2125-2137.
16. Zarow C, Lyness SA, Mortimer JA, Chui HC: **Neuronal loss is greater in the locus coeruleus than nucleus basalis and substantia nigra in Alzheimer and Parkinson diseases.** *Arch Neurol* 2003, **60:**337-341.
17. Bondareff W, Mountjoy CQ, Roth M, Rossor MN, Iversen LL, Reynolds GP, Hauser DL: **Neuronal degeneration in locus ceruleus and cortical correlates of Alzheimer disease.** *Alzheimer Dis Assoc Disord* 1987, **1:**256-262.
18. De Keyser J, Wilczak N, Leta R, Streetland C: **Astrocytes in multiple sclerosis lack beta-2 adrenergic receptors.** *Neurology* 1999, **53:**1628-1633.
19. De Keyser J, Zeinstra E, Frohman E: **Are astrocytes central players in the pathophysiology of multiple sclerosis?** *Arch Neurol* 2003, **60:**132-136.
20. Mori K, Ozaki E, Zhang B, Yang L, Yokoyama A, Takeda I, Maeda N, Sakanaka M, Tanaka J: **Effects of norepinephrine on rat cultured microglial cells that express alpha1, alpha2, beta1 and beta2 adrenergic receptors.** *Neuropharmacology* 2002, **43:**1026-1034.
21. Tanaka KF, Kashima H, Suzuki H, Ono K, Sawada M: **Existence of functional beta1- and beta2-adrenergic receptors on microglia.** *J Neurosci Res* 2002, **70:**232-237.

22. Chang JY, Liu LZ: **Catecholamines inhibit microglial nitric oxide production.** *Brain Res Bull* 2000, **52:**525-530.
23. Pyo H, Jou I, Jung S, Joe E: **cAMP potentiates beta-amyloid-induced nitric oxide release from microglia.** *Neuroreport* 1999, **10:**37-40.
24. Hetier E, Ayala J, Bousseau A, Prochiantz A: **Modulation of interleukin-1 and tumor necrosis factor expression by beta-adrenergic agonists in mouse ameboid microglial cells.** *Exp Brain Res* 1991, **86:**407-413.
25. Galea E, Feinstein DL, Reis DJ: **Induction of calcium-independent nitric oxide synthase activity in primary rat glial cultures.** *Proc Natl Acad Sci U S A* 1992, **89:**10945-10949.
26. Li P, Allen H, Banerjee S, Franklin S, Herzog L, Johnston C, McDowell J, Paskind M, Rodman L, Salfeld J, *et al.*: **Mice deficient in IL-1 beta-converting enzyme are defective in production of mature IL-1 beta and resistant to endotoxic shock.** *Cell* 1995, **80:**401-411.
27. Vairano M, Dello Russo C, Pozzoli G, Battaglia A, Scambia G, Tringali G, Aloe-Spiriti MA, Preziosi P, Navarra P: **Erythropoietin exerts anti-apoptotic effects on rat microglial cells in vitro.** *Eur J Neurosci* 2002, **16:**584-592.
28. Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR: **Analysis of nitrate, nitrite, and [15N]nitrate in biological fluids.** *Anal Biochem* 1982, **126:**131-138.
29. Boullerne AI, Rodriguez JJ, Touil T, Brochet B, Schmidt S, Abrous ND, Le Moal M, Pua JR, Jensen MA, Mayo W, Arnason BG, Petry KG: **Anti-S-nitrosocysteine antibodies are a predictive marker for demyelination in experimental autoimmune encephalomyelitis: implications for multiple sclerosis.** *J Neurosci* 2002, **22:**123-132.
30. Gunasekar PG, Kanthasamy AG, Borowitz JL, Isom GE: **Monitoring intracellular nitric oxide formation by dichlorofluorescin in neuronal cells.** *J Neurosci Methods* 1995, **61:**15-21.
31. Crow JP: **Dichlorodihydrofluorescein and dihydrorhodamine 123 are sensitive indicators of peroxynitrite in vitro: implications for intracellular measurement of reactive nitrogen and oxygen species.** *Nitric Oxide* 1997, **1:**145-157.
32. Imrich A, Kobzik L: **Fluorescence-based measurement of nitric oxide synthase activity in activated rat macrophages using dichlorofluorescin.** *Nitric Oxide* 1997, **1:**359-369.
33. Stasiolek M, Gavrilyuk V, Sharp A, Horvath P, Selmaj K, Feinstein DL: **Inhibitory and stimulatory effects of lactacystin on expression of nitric oxide synthase type 2 in brain glial cells. The role of Ikappa B-beta.** *J Biol Chem* 2000, **275:**24847-24856.
34. Gavrilyuk V, Dello Russo C, Heneka MT, Pelligrino D, Weinberg G, Feinstein DL: **Norepinephrine increases ikappa balpha expression in astrocytes.** *J Biol Chem* 2002, **277:**29662-29668.
35. Feinstein DL, Galea E, Reis DJ: **Suppression of glial nitric oxide synthase induction by heat shock: effects on proteolytic degradation of IkappaB-alpha.** *Nitric Oxide* 1997, **1:**167-176.
36. Kim EJ, Kwon KJ, Park JY, Lee SH, Moon CH, Baik EJ: **Neuroprotective effects of prostaglandin E2 or cAMP against microglial and neuronal free radical mediated toxicity associated with inflammation.** *J Neurosci Res* 2002, **70:**97-107.
37. Loughlin AJ, Woodroofe MN: **Inhibitory effect of interferon-gamma on LPS-induced interleukin 1 beta production by isolated adult rat brain microglia.** *Neurochem Int* 1996, **29:**77-82.
38. Zhang B, Yang L, Konishi Y, Maeda N, Sakanaka M, Tanaka J: **Suppressive effects of phosphodiesterase type IV inhibitors on rat cultured microglial cells: comparison with other types of cAMP-elevating agents.** *Neuropharmacology* 2002, **42:**262-269.
39. Huuskonen J, Suuronen T, Nuutinen T, Kyrylenko S, Salminen A: **Regulation of microglial inflammatory response by sodium butyrate and short-chain fatty acids.** *Br J Pharmacol* 2004, **141:**874-880.
40. Bos JL: **Epac: a new cAMP target and new avenues in cAMP research.** *Nat Rev Mol Cell Biol* 2003, **4:**733-738.
41. Minghetti L, Nicolini A, Polazzi E, Creminon C, Maclouf J, Levi G: **Inducible nitric oxide synthase expression in activated rat microglial cultures is downregulated by exogenous prostaglandin E2 and by cyclooxygenase inhibitors.** *Glia* 1997, **19:**152-160.
42. Fiebich BL, Butcher RD, Gebicke-Haerter PJ: **Protein kinase C-mediated regulation of inducible nitric oxide synthase expression in cultured microglial cells.** *J Neuroimmunol* 1998, **92:**170-178.
43. Levi G, Minghetti L, Aloisi F: **Regulation of prostanoid synthesis in microglial cells and effects of prostaglandin E2 on microglial functions.** *Biochimie* 1998, **80:**899-904.
44. Minghetti L, Nicolini A, Polazzi E, Creminon C, Maclouf J, Levi G: **Prostaglandin E2 downregulates inducible nitric oxide synthase expression in microglia by increasing cAMP levels.** *Adv Exp Med Biol* 1997, **433:**181-184.
45. Yoshikawa M, Suzumura A, Ito A, Tamaru T, Takayanagi T: **Effect of phosphodiesterase inhibitors on nitric oxide production by glial cells.** *Tohoku J Exp Med* 2002, **196:**167-177.
46. Delgado R, Carlin A, Airaghi L, Demitri MT, Meda L, Galimberti D, Baron P, Lipton JM, Catania A: **Melanocortin peptides inhibit production of proinflammatory cytokines and nitric oxide by activated microglia.** *J Leukoc Biol* 1998, **63:**740-745.
47. Eberhardt W, Pluss C, Hummel R, Pfeilschifter J: **Molecular mechanisms of inducible nitric oxide synthase gene expression by IL-1beta and cAMP in rat mesangial cells.** *J Immunol* 1998, **160:**4961-4969.
48. Rozenfeld C, Martinez R, Figueiredo RT, Bozza MT, Lima FR, Pires AL, Silva PM, Bonomo A, Lannes-Vieira J, De Souza W, Moura-Neto V: **Soluble factors released by Toxoplasma gondii-infected astrocytes down-modulate nitric oxide production by gamma interferon-activated microglia and prevent neuronal degeneration.** *Infect Immunol* 2003, **71:**2047-2057.
49. Chio CC, Chang YH, Hsu YW, Chi KH, Lin WW: **PKA-dependent activation of PKC, p38 MAPK and IKK in macrophage: implication in the induction of inducible nitric oxide synthase and interleukin-6 by dibutyryl cAMP.** *Cell Signal* 2004, **16:**565-575.
50. Si Q, Nakamura Y, Ogata T, Kataoka K, Schubert P: **Differential regulation of microglial activation by propentofylline via cAMP signaling.** *Brain Res* 1998, **812:**97-104.
51. Caggiano AO, Kraig RP: **Prostaglandin E2 and 4-aminopyridine prevent the lipopolysaccharide-induced outwardly rectifying potassium current and interleukin 1beta production in cultured rat microglia.** *J Neurochem* 1998, **70:**2357-2368.
52. Tomozawa Y, Yabuuchi K, Inoue T, Satoh M: **Participation of cAMP and cAMP dependent protein kinase in beta-adrenoceptor-mediated interleukin-1 beta mRNA induction in cultured microglia.** *Neurosci Res* 1995, **22:**399-409.
53. Woo MS, Jang PG, Park JS, Kim WK, Joh TH, Kim HS: **Selective modulation of lipopolysaccharide-stimulated cytokine expression and mitogen-activated protein kinase pathways by dibutyryl-cAMP in BV2 microglial cells.** *Brain Res Mol Brain Res* 2003, **113:**86-96.
54. Vincent VA, Van Dam AM, Persoons JH, Schotanus K, Steinbusch HW, Schoffelmeer AN, Berkenbosch F: **Gradual inhibition of inducible nitric oxide synthase but not of interleukin-1 beta production in rat microglial cells of endotoxin-treated mixed glial cell cultures.** *Glia* 1996, **17:**94-102.
55. Tzeng SF, Lee JL, Kuo JS, Yang CS, Murugan P, Ai Tai L, Chu Hwang K: **Effects of malonate C60 derivatives on activated microglia.** *Brain Res* 2002, **940:**61-68.
56. Hewett SJ, Corbett JA, McDaniel ML, Choi DW: **Interferon-gamma and interleukin-1 beta induce nitric oxide formation from primary mouse astrocytes.** *Neurosci Lett* 1993, **164:**229-232.
57. Petrova TV, Akama KT, Van Eldik LJ: **Cyclopentenone prostaglandins suppress activation of microglia: down-regulation of inducible nitric-oxide synthase by 15-deoxy-Delta12,14-prostaglandin J2.** *Proc Natl Acad Sci USA* 1999, **96:**4668-4673.
58. Lee SC, Dickson DW, Liu W, Brosnan CF: **Induction of nitric oxide synthase activity in human astrocytes by interleukin-1 beta and interferon-gamma.** *J Neuroimmunol* 1993, **46:**19-24.
59. Liu JS, John GR, Sikora A, Lee SC, Brosnan CF: **Modulation of interleukin-1beta and tumor necrosis factor alpha signaling by P2 purinergic receptors in human fetal astrocytes.** *J Neurosci* 2000, **20:**5292-5299.
60. Zhao ML, Liu JS, He D, Dickson DW, Lee SC: **Inducible nitric oxide synthase expression is selectively induced in astrocytes isolated from adult human brain.** *Brain Res* 1998, **813:**402-405.
61. Mollace V, Muscoli C, Rotiroti D, Nistico G: **Spontaneous induction of nitric oxide- and prostaglandin E2-release by hypoxic astroglial cells is modulated by interleukin 1 beta.** *Biochem Biophys Res Commun* 1997, **238:**916-919.
62. Hu S, Ali H, Sheng WS, Ehrlich LC, Peterson PK, Chao CC: **Gp-41-mediated astrocyte inducible nitric oxide synthase mRNA**

expression: involvement of interleukin-1beta production by microglia. *J Neurosci* 1999, **19:**6468-6474.
63. Akama KT, Van Eldik LJ: **Beta-amyloid stimulation of inducible nitric-oxide synthase in astrocytes is interleukin-1beta- and tumor necrosis factor-alpha (TNFalpha)-dependent, and involves a TNFalpha receptor-associated factor- and NFkappaB-inducing kinase-dependent signaling mechanism.** *J Biol Chem* 2000, **275:**7918-7924.
64. Zhang G, Ghosh S: **Molecular mechanisms of NF-kappaB activation induced by bacterial lipopolysaccharide through Toll-like receptors.** *J Endotoxin Res* 2000, **6:**453-457.
65. Lee SJ, Lee S: **Toll-like receptors and inflammation in the CNS.** *Curr Drug Targets Inflamm Allergy* 2002, **1:**181-191.
66. Farmer P, Pugin J: **beta-adrenergic agonists exert their "anti-inflammatory" effects in monocytic cells through the IkappaB/NF-kappaB pathway.** *Am J Physiol Lung Cell Mol Physiol* 2000, **279:**L675-L682.
67. Manna SK, Aggarwal BB: **Alpha-melanocyte-stimulating hormone inhibits the nuclear transcription factor NF-kappa B activation induced by various inflammatory agents.** *J Immunol* 1998, **161:**2873-2880.
68. Delgado M, Ganea D: **Vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide inhibit expression of Fas ligand in activated T lymphocytes by regulating c-Myc, NF-kappa B, NF-AT, and early growth factors 2/3.** *J Immunol* 2001, **166:**1028-1040.
69. Mann DM, Yates PO, Hawkes J: **The pathology of the human locus ceruleus.** *Clin Neuropathol* 1983, **2:**1-7.
70. Chelmicka-Schorr E, Kwasniewski MN, Thomas BE, Arnason BG: **The beta-adrenergic agonist isoproterenol suppresses experimental allergic encephalomyelitis in Lewis rats.** *J Neuroimmunol* 1989, **25:**203-207.
71. Wiegmann K, Muthyala S, Kim DH, Arnason BG, Chelmicka-Schorr E: **Beta-adrenergic agonists suppress chronic/relapsing experimental allergic encephalomyelitis (CREAE) in Lewis rats.** *J Neuroimmunol* 1995, **56:**201-206.
72. Zeinstra E, Wilczak N, De Keyser J: **[3H]dihydroalprenolol binding to beta adrenergic receptors in multiple sclerosis brain.** *Neurosci Lett* 2000, **289:**75-77.
73. Heneka MT, Gavrilyuk V, Landreth GE, O'Banion MK, Weinberg G, Feinstein DL: **Noradrenergic depletion increases inflammatory responses in brain: effects on IkappaB and HSP70 expression.** *J Neurochem* 2003, **85:**387-398.
74. Griffin WS, Mrak RE: **Interleukin-1 in the genesis and progression of and risk for development of neuronal degeneration in Alzheimer's disease.** *J Leukoc Biol* 2002, **72:**233-238.
75. Mitrovic B, Ignarro LJ, Vinters HV, Akers MA, Schmid I, Uittenbogaart C, Merrill JE: **Nitric oxide induces necrotic but not apoptotic cell death in oligodendrocytes.** *Neuroscience* 1995, **65:**531-539.
76. Merrill JE, Murphy SP, Mitrovic B, Mackenzie-Graham A, Dopp JC, Ding M, Griscavage J, Ignarro LJ, Lowenstein CJ: **Inducible nitric oxide synthase and nitric oxide production by oligodendrocytes.** *J Neurosci Res* 1997, **48:**372-384.
77. Wallace MN, Geddes JG, Farquhar DA, Masson MR: **Nitric oxide synthase in reactive astrocytes adjacent to beta-amyloid plaques.** *Exp Neurol* 1997, **144:**266-272.
78. Orihara Y, Ikematsu K, Tsuda R, Nakasono I: **Induction of nitric oxide synthase by traumatic brain injury.** *Forensic Sci Int* 2001, **123:**142-149.
79. Endoh M, Maiese K, Wagner J: **Expression of the inducible form of nitric oxide synthase by reactive astrocytes after transient global ischemia.** *Brain Res* 1994, **651:**92-100.