Journal of Cellular Biochemistry 96:428–438 (2005)

Roles of Ca^{2+} and the Ca^{2+}-Sensing Receptor (CASR) in the Expression of Inducible NOS (Nitric Oxide Synthase)-2 and Its BH_4 (Tetrahydrobiopterin)-Dependent Activation in Cytokine-Stimulated Adult Human Astrocytes

Ilaria Dal Pra,[1] Anna Chiarini,[1] Edward F. Nemeth,[2] Ubaldo Armato,[1] and James F. Whitfield[3]*

[1]Histology & Embryology Unit, Department of Biomedical & Surgical Sciences, University of Verona Medical School, Verona, Italy
[2]NPS Pharmaceuticals, Inc., Toronto, Ontario M5G1K2, Canada
[3]Institute for Biological Sciences, National Research Council of Canada, Ottawa, Ontario, Canada

Abstract Since NO production by NOS-2 made by astrocytes activated by proinflammatory cytokines contributes to the killing of neurons in variously damaged human brains, knowing the mechanisms responsible for NOS-2 expression should contribute to developing effective therapeutics. The expression and activation of NOS-2 in normal adult human cerebral cortical astrocytes treated with three proinflammatory cytokines, IL-1β, TNF-α, and IFN-γ, are driven by two separable mechanisms. NOS-2 expression requires a burst of p38 MAPK activity, while the activation of the resulting enzyme protein requires MEK/ERK-dependent BH_4 (tetrahydrobiopterin) synthesis between 24 and 24.5 h after adding the cytokines to the culture medium. Here we show that NOS-2 expression in the activated astrocytes requires that the culture medium contain 1.8 mM Ca^{2+}, but it is unaffected by inhibiting calcium-sensing receptors (CASRs) with NPS 89636. However, NOS-2 *activation* is inhibited by NPS 89626 during the MEK/ERK-dependent stage between 24 and 24.5 h after adding the cytokines, and this inhibition can be overridden by exogenous BH_4. Therefore, NOS-2 expression and the subsequent BH_4-dependent NOS-2-activation in human astrocytes need 1.8 mM Ca^{2+} to be in the culture medium, while NOS-2 activation also needs functional CASRs between 24 and 24.5 h after cytokine addition. These findings raise the possibility that calcilytic drugs prevent NO-induced damage and death of human neurons. J. Cell. Biochem. 96: 428–438, 2005.

Key words: astrocytes; calcium ion (Ca^{2+}); calcilytic NPS 89636; CASR (Ca^{2+}-sensing receptor); proinflammatory cytokines; nitric oxide synthase-2; tetrahydrobiopterin

When the brain is injured by stroke, head trauma or by the Alzheimer and Parkinson disease processes, astrocytes respond to the build-up of proinflammatory cytokines, such as IL-1β, TNF-α, and IFN-γ from activated microglia by making among other things the short-lived, weakly oxidizing NO, which is converted to the long-lived, cytotoxic, strongly oxidizing peroxynitrite radical ($ONOO^-$) that can harm and kill the neurons functionally and physically associated with the astrocytes [Ransom and Santheimer, 1992; Norenberg, 1994; McGeer and McGeer, 1995, 1999, 2003, 2004; Mrak et al., 1995; Dawson and Dawson, 1996; Hunot et al., 1996; Lincoln et al., 1997; Grzybicki et al., 1998; Wada et al., 1998; McGeer et al., 2000; Boczkowski et al., 2001; Rah et al., 2001; Estévez and Jordan, 2002; Khaldi et al., 2002; Leong et al., 2002; Liu et al., 2002; Hansson and Ronnback, 2003; Hirsch et al., 2003; Nedergaard et al., 2003; Ransom et al., 2003; Barbeito et al., 2004; Blasko et al., 2004; Dokmeci, 2004; Hertz and Zielke, 2004; Lin and

Grant sponsor: Italian Ministry for University and Research (MIUR; 60% allotment funds).

*Correspondence to: Dr. James F. Whitfield, PhD, Institute for Biological Sciences, National Research Council of Canada, Montreal Road 51, Ottawa K1A0N6, Ontario, Canada. E-mail: pthosteo@rogers.com

Received 18 February 2005; Accepted 18 March 2005
DOI 10.1002/jcb.20511

Bergles, 2004; Saez et al., 2004; Schipke and Kettenmann, 2004; Schultz and Falkenburger, 2004; Zhao, 2005]. This NO could be made by any one of the three main types of NOS (NO synthase)—neuronal NOS-1 (or nNOS), the inducible and the most productive NOS-2 (or iNOS), and endothelial cell NOS-3 (eNOS or ecNOS) [Lincoln et al., 1997; Baltrons and Garcia, 2001; Boczkowski et al., 2001]. But the large amount of NO made by proinflammatory cytokine-activated astrocytes is made by NOS-2 which, unlike the other two enzymes, is not directly controlled by Ca^{2+} or $Ca^{2+}\cdot$calmodulin because it carries its own "built-in" calmodulin [Lincoln et al., 1997; Baltrons and Garcia, 2001]. Also unlike the other two enzymes, NOS-2 is not expressed by the astrocytes in a normal brain or by proliferatively quiescent astrocytes in culture, but it is induced when the cells are activated by the signals from proinflammatory cytokine receptors [Suzuki et al., 1995; Grzybicki et al., 1998; Wada et al., 1998; Zamora et al., 2000; Baltrons and Garcia, 2001; Chiarini et al., 2005].

Since NOS-2 makes the NO that injures and kills neurons in variously damaged brains, finding out what induces it should help to design effective therapeutics. Indeed, for example, inhibiting the expression of the *NOS-2* gene with the p38 MAPK inhibitor SB203580 or inhibiting the activation of the NOS-2 protein by MEK1 with PD98059 significantly reduces damage from a head blow or focal ischemia in rat and mouse brains, respectively [Wada et al., 1998; Alessandrini et al., 1999; Piao et al., 2003]. Neufeld and Liu [2003b] have reported that NOS-2 inhibitors prevent the NO-mediated killing of retinal ganglion cells in glaucomatous neuropathy.

We have been explicating the mechanisms responsible for the build-up of NOS-2 in freshly isolated proliferatively quiescent, phenotypically stable, adult human cerebral astrocytes exposed to the three proinflammatory cytokines (IL-1β, IFN-γ, TNF-α) that are most often found in injured or diseased brains [Chiarini et al., 2005]. We have so far found that this cytokine triad turns on two mechanisms that together induce the appearance, accumulation, and activation of NOS-2. One mechanism stimulates the build-up of inactive NOS-2 protein and needs p38 MAPK activation to do so [Neufeld and Liu, 2003a,b; Chiarini et al., 2005]. The second mechanism, which operates between 24 and 24.5 h after adding the cytokines to the culture medium, stimulates the production of BH_4 (tetrahydrobiopterin) needed to activate the accumulating NOS-2 proteins and requires MEK1/MEK2 activities to do so [Chiarini et al., 2005].

In this communication, we use a Ca^{2+}-sensing receptor (CASR) [Chattopadhyay et al., 2000, Chattopadhyay and Brown, 2003] inhibitor, the "calcilytic" NPS 89636 [Nemeth et al., 2001; Dvorak et al., 2004], to show that the initiation of NOS-2 expression and subsequent NOS-2-activation require that 1.8 mM Ca^{2+} be in the culture medium and that functional CASRs are needed between 24 and 24.5 h after addition of the cytokine triad to start the BH_4-dependent NOS-2-activating second mechanism.

MATERIALS AND METHODS

Isolation and Culturing of Phenotypically Normal Human Astrocytes

For these experiments a small sample of cortical tissue was taken from the temporal lobe of an otherwise normal young male who had suffered a severe head injury in a motorcycle accident. As described in more detail by Chiarini et al. [2005], the cells in the cortical sample were released by mild treatment with 0.25%$^{w/v}$ trypsin (Sigma-Aldrich, Italy) and trituration in Hanks' Balanced Salt Solution (BSS; Eurobio, France) at 18°C.

The isolated cells were planted in culture flasks (BD Biosciences, France) containing a medium consisting of 89%$^{v/v}$ of a 1:1 mixture of Ham's F-12 and MCDB 153 media (Sigma-Aldrich), 10%$^{v/v}$ heat-inactivated (at 56°C for 30 min) fetal bovine serum (FBS; BioWhittaker Europe, Belgium), and 1%$^{v/v}$ of a penicillin-streptomycin solution (Eurobio). Basic fibroblast growth factor (bFGF or FGF-2; 20 ng/ml; PeproTech EC Ltd., England), insulin-like growth factor-I (IGF-I; 20 ng/ml; PeproTech), platelet-derived growth factor (PDGF; 20 ng/ml; PeproTech), and epidermal growth factor (EGF; 10 nM; Sigma-Aldrich) were added to the medium to enhance the initial proliferation and selection of the astrocytes in the mixed cell population. This complete medium was replaced every 2–3 days.

When the primary mixed cultures became 70% "confluent" (1–4 weeks), the cells were detached from the flask surfaces with 0.25%$^{w/v}$ trypsin and 0.02%$^{w/v}$ EDTA (Eurobio) in Hanks' BSS, split 1:4 and planted in new flasks. After

the third subculture a homogeneous population of astrocytes appeared and the growth factors were no longer needed. Just as had occurred with the nerve cells isolated from four other individuals, the cells from the fifth subject became stably "locked into" an astrocyte's phenotype; in fact, they only expressed astrocyte-specific markers such as glial fibrillary acid protein (GFAP) and glutamine synthase (GS). Conversely, none of them expressed neuronal (enolase), oligodendrocytes' (galactocerebroside), microglial (CD-68), or endothelial cell (factor VIII) markers. These astrocytes proliferated slowly without added growth factors in serum-enriched Ham's F-12/MCDB 153 medium. Thus withdrawing growth factors after the third passage did not prevent the astrocytes from proliferating to "confluence" and expressing their characteristic astrocyte markers—they were by now phenotypically "locked in." But they still needed serum and withdrawing it caused them to self-destruct by apoptosis. The proliferatively quiescent cells in confluent astrocyte cultures started cycling again when subcultured. At least 15–18 subcultures could be obtained over 2.5 years from a piece of normal cortex. Only astrocytes from the 4th to 8th subculture were used because the response of the cells to proinflammatory cytokines became erratic with further subculturing.

Experimental Protocol

Since astrocytes are normally not proliferating in the adult human brain when they are assaulted by injury-induced proinflammatory cytokines, we studied the role of Ca^{2+} and CASR in NOS-2 induction and activation by cytokines using confluent, proliferatively quiescent pure astrocyte cultures. Thus, at the experimental "time-0", some such cultures served as untreated controls while others had IL-lβ (20 ng/ml), TNF-α (20 ng/ml), and IFN-γ (70 ng/ml) (all from PeproTech) added to their medium.

We used the specific CASR inhibitor NPS 89636 (from NPS Pharmaceutical, Inc., Toronto, Canada) [Nemeth et al., 2001; Dvorak et al., 2004] to find out whether CASR signaling is needed for NOS-2 expression and/or activation. The inhibitor was first dissolved in dimethylsulfoxide (DMSO) to make a 100 μM stock solution which was finally diluted to 100 nM in the culture medium. In one set of experiments, NPS 89636 (like the U0126 MEK1/MEK2 inhibitor used in our previous study [Chiarini et al., 2005]) was added for 30 min and then washed out just before the cytokines were added, and then added again at 24 h and washed out at 24.5 h. To do this, the cell-conditioned cytokine-free medium was carefully removed and medium containing 100 nM NPS 89636 was put on the cultures for 30 min after which it was removed by washout and the original cell-conditioned NPS 89636-free, but now cytokine-containing medium was put back on the cultures and the experiment began. This was repeated, washout included, for another 30 min at 24 h after adding the cytokines. This was done to block any signaling of the CASR in response to the cytokine triad exposure during the first 24 h of the cells' response to the cytokines. Then to find out when CASR signaling affected the NOS-2/NO responses to the cytokines during the first 24 h, NPS 89636 was added either for 30 min and then washed out just before adding the cytokines or for 30 min at 24 h after adding the cytokines and washed out 30 min later.

Measurement of NO Released Into the Medium

The amount of NO that the cells released was determined from the concentrations of NO's two stable oxidation products, NO_2^- and NO_3^-, in their medium. The fluorometric method we used is based upon the reaction of NO_2^- with 2,3-diamino-naphthalene (DAN; Sigma-Aldrich) to form the fluorescent 1-(H)-naphthotriazole [Misko et al., 1993]. Nitrates in the medium were reduced to NO_2^- by incubation for 30 min with nitrate reductase (0.1 U/ml; Boehringer Mannheim, Germany) in the presence of 10 μM FAD and 100 μM NADPH. Any residual NADPH was then oxidized with lactate dehydrogenase (10 U/ml) in the presence of 10 mM sodium pyruvate. The total NO_2^- concentration was then determined in 50 μl samples of the culture supernatants that were brought up to 100 μl with doubly deionized water. Freshly prepared DAN (10 μl of a 0.05 mg/ml solution in 0.62M HCl) was added to these diluted samples, and after a 10 min incubation at 20°C in the dark, the reaction was stopped with 5 μl of 2.8N NaOH. The amount of 1-(H)-naphthotriazole formed was measured fluorometrically at excitation and emission wavelengths of 365 nm and 450 nm, respectively. Samples of fresh culture medium were used as blanks to correct for background NO_2^- and NO_3^- concentrations in

the medium (~7 μM). NO_2^- concentrations were calculated from a standard curve using $NaNO_2$.

mRNA Isolation and Northern Blot (NB) Detection of NOS-2-Specific mRNA

Astrocytes were lyzed with SV RNA lysis buffer reagent (Promega, Milan, Italy), and their total RNA was isolated according to the manufacturer's instructions for the SV Total RNA Isolation System (Promega). RNA concentration was measured with an Uvikon 922 spectrophotometer equipped with a 5-carat microcell. For NB analysis, samples of total RNA (20 μg/lane) were mixed with ethidium bromide (1–2 μg) and loaded onto a 1.2% denaturing agarose gel containing formaldehyde. After separation by electrophoresis, RNA was transferred to a Hybond-N^+ nylon membrane (Amersham Biosciences, Piscataway, NJ), and UV cross-linked for hybridization at 33°C with double-FITC-labeled probe (HybriProbes; Biognostik GmbH, Göttingen, Germany) specific for the NOS-2 isoform mRNA.

The sequence of this HybriProbe™ for NOS-2 was:

5′-CCTGGCCAGATGTTCCTCTATTTTTGCCTC-3′.

The hybridization reaction was performed with the HybriBuffer N hybridization solution (Biognostik) according to the manufacturer's instructions. After hybridization, each filter was washed three times in 2× SSC (1× SSC: 150 mM NaCl, 15 mM Na_3-citrate · $2H_2O$, pH 7.0 with 1M HCl), 0.1% SDS for 30 min at 20°C, and once in 0.1× SSC, 0.1% SDS solution for 15 min at 40°C. The contents of the hybridized membranes were then analyzed using the Southern Star™ (Tropix, Applied Biosystems, Framingham, MA) chemiluminescent detection system based upon an anti-FITC alkaline phosphatase conjugate antibody and the CDP-STAR substrate (Tropix, Applied Biosystems) following the manufacturer's instructions. The developed membranes were photographed with an Olympus 3300™ digital camera. The membranes were next stripped by boiling in 0.1 × SSC, 0.1% SDS for 20 min and hybridization repeated using a probe for actin's mRNA (Biognostik) as an internal control for the amount of total RNA loaded onto each lane.

Western Immunoblotting (WB)

Seventy-two hours after starting the experiments, control and treated astrocytes were scraped into cold PBS and sedimented at 200*g* for 10 min. The sedimented cells were homogenized in T-PER™ tissue protein extraction reagent (Pierce Chemical Co., Rockford, IL) containing a complete EDTA-free protease inhibitor cocktail (Roche Diagnostics S.p.A., Monza, Italy). The protein contents were assayed with an ad hoc commercial kit (Pierce Chemical Co.). Equal amounts (25–30 μg) of protein from the samples were boiled in buffer (0.0625M Tris-HCl, pH 6.8; 2%$^{w/v}$ SDS; 5.0%$^{w/v}$ β-mercaptoethanol; 10%$^{v/v}$ glycerol; and 0.002%$^{w/v}$ bromphenol blue), electrophoresed in 10%$^{w/v}$ SDS-polyacrylamide gel, and blotted onto nitrocellulose membranes (0.45 μm; Pall Life Sciences, Ann Arbor, MI).

To immunodetect NOS-2, the blots were probed with specific rabbit IgG polyclonal antibodies or mouse monoclonal antibodies at a final dilution of 1 μg/ml (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). CASR was immunodetected using a mouse monoclonal anti-CASR antibody provided by one of the authors (EFN). The blots were next incubated with alkaline phosphatase-conjugated anti-goat or anti-rabbit IgGs (Santa Cruz) and stained with BCTP/NBT liquid substrate reagent (Sigma) and photographed.

Immunofluorescence (IF)

Immunostaining of astrocytes that had been seeded into 24-well plates for primary tissue cultures (Becton Dickinson & Co., Franklin Lakes, NY) was carried out at 4°C. The cells (2.0×10^4/chamber) were washed twice with PBS (phosphate-buffered saline) containing BSA (1.0 %$^{w/v}$) and NaN_3 (0.1%$^{w/v}$), and incubated for 60 min at room temperature with primary antibodies (at 1.0 μg/ml) against NOS-2 or active (i.e., phosphorylated) ERK1/ERK2 (all from Santa Cruz). The cells were washed three times with PBS-BSA solution, and then incubated for 60 min at room temperature with specific secondary antibodies conjugated to Alexa Fluor 555 (Molecular Probes, Inc., Canada). After a final wash with PBS-BSA solution, specimens were examined with an inverted IM35 Zeiss microscope and photographed with an Olympus 3300™ digital camera. Appropriate controls were always run in parallel without primary or secondary antibody. Deconvolved fluorescence images were obtained by using Huygens Essential Software for Windows

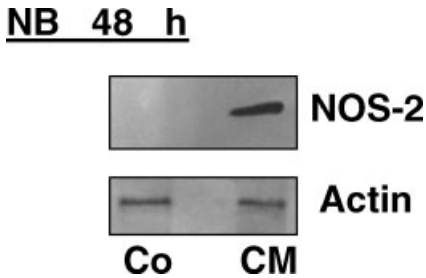

Fig. 1. NOS-2 mRNA was detected in the cytokine triad-treated astrocytes, but not in untreated cells, at 48 h after adding the cytokines. Actin mRNA served as an internal control for the amount of RNA loaded onto the filter. NB, Northern blotting; NOS-2, nitric oxide synthase-2; Co, untreated control cells; CM, cytokine mixture (IL-1β [20 ng/ml], TNF-α [20 ng/ml], and IFN-γ [70 ng/ml]).

(Scientific Volume Imaging b.v., Hilversun, The Netherlands).

Statistical Analysis

A one-way analysis of variance (ANOVA) with post hoc Bonferroni test to compare mean values was applied to the data and $P < 0.05$ was considered to be significant.

RESULTS

Ca^{2+} Requirements for NOS-2 Expression and NO Production in Proliferatively Quiescent Astrocytes

When the normal adult human astrocyte cultures reached a critical density, the cells

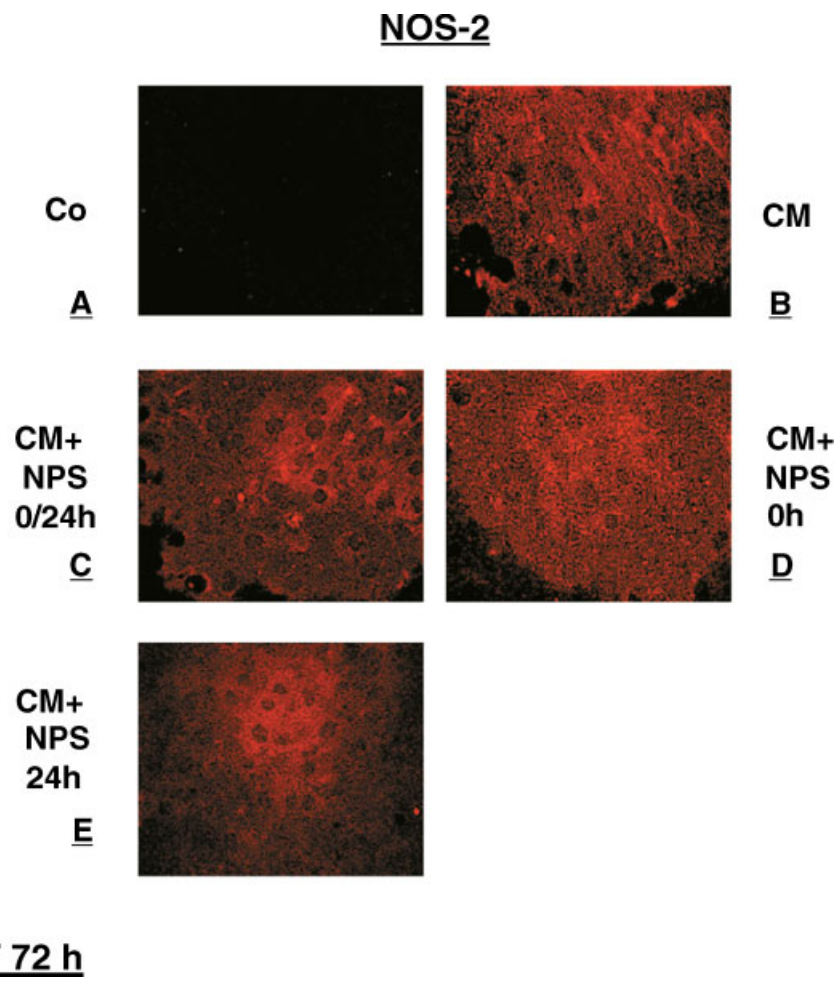

Fig. 2. The induction of NOS-2 protein by an initial exposure to the cytokine triad (CM) (**B**) cannot be prevented by exposing the astrocytes for 30 min either twice (i.e., −0.5 to 0 h and 24 to 24.5 h) or once at either time point to the calcilytic agent NPS 89636 that blocks the calcium-sensing receptor (CASR) (**C**–**E**). Adult human astrocytes were treated as described in the Materials and Methods. Seventy-two hours later the cells were processed for immunocytochemistry with anti-NOS-2 antibody and a secondary antibody labeled with Alexa Fluor 555. Pictures were taken by fluorescence microscopy. Control (Co) cells (**A**) had only a feeble autofluorescence. The deconvolved fluorescent images shown here are typical of at least three distinct experiments and were obtained as indicated in the Materials and Methods. Original magnification: 200×. IF, immunofluorescence; NPS, calcilytic agent NPS 89636.

stopped proliferating and were not expressing their NOS-2 genes, making the enzyme or dumping NO into the medium (Figs. 1, 2A, and 3). If these proliferatively quiescent cultures in 10% FBS-90% Ham's F12/MCDB medium containing 1.8 mM Ca^{2+} were not split, but instead were exposed to the IL-1β + TNF-α + IFN-γ cytokine triad, their cells did not resume proliferating, but by 48 h they had switched on their *NOS-2* genes (Fig. 1), accumulated the enzyme (Fig. 2B), and sharply began dumping NO into the medium between 48 and 72 h (Fig. 3). On the other hand, when the Ca^{2+} concentration in the medium of the quiescent cultures was lowered from 1.8 to 0.75 mM, the cytokine triad could not induce the cells to express NOS-2 (Fig. 4A) or make NO for at least the next 6 days (Fig. 4B and not shown).

Roles of the Ca^{2+}-Sensing Receptor (CASR) in NOS-2 Expression and Activation

When the astrocyte cultures became proliferatively quiescent in the 1.8 mM Ca^{2+}-containing medium, they increased their CASR content about 1.6-fold ($P = 0.002$) without any additional stimulation (Fig. 5). This upsurge of CASR

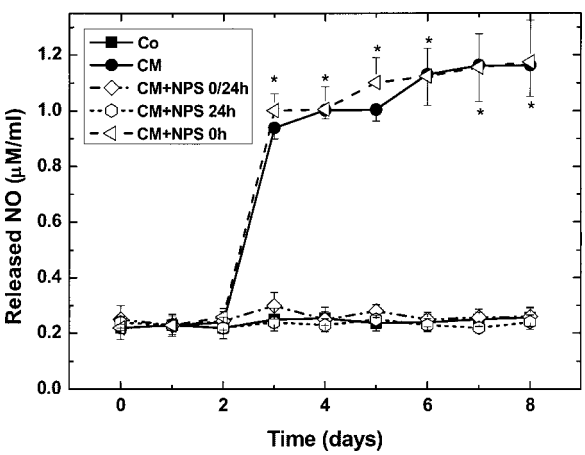

Fig. 3. The release of NO into the growth medium elicited by the cytokine mixture (CM) in adult human astrocyte cultures was prevented by either two brief exposures to, and washouts of NPS 89636 at −0.5 to 0 h and 24 to 24.5 h (CM + NPS 0/24 h) or by a single exposure at 24 to 24.5 h (CM + NPS 24 h), but was not affected by a single treatment at −0.5 to 0 h (CM + NPS 0 h). The concentrations of nitrate and nitrite derivatives of NO in the medium samples were measured to give the NO concentrations as described in Materials and Methods. The points are mean ± SEM of the values from at least three separate experiments (*, $P < 0.001$ for both CM- and CM + NPS 0 h-treated cells). Co, untreated control cells; NO, nitric oxide; NPS, calcilytic agent NPS 89636.

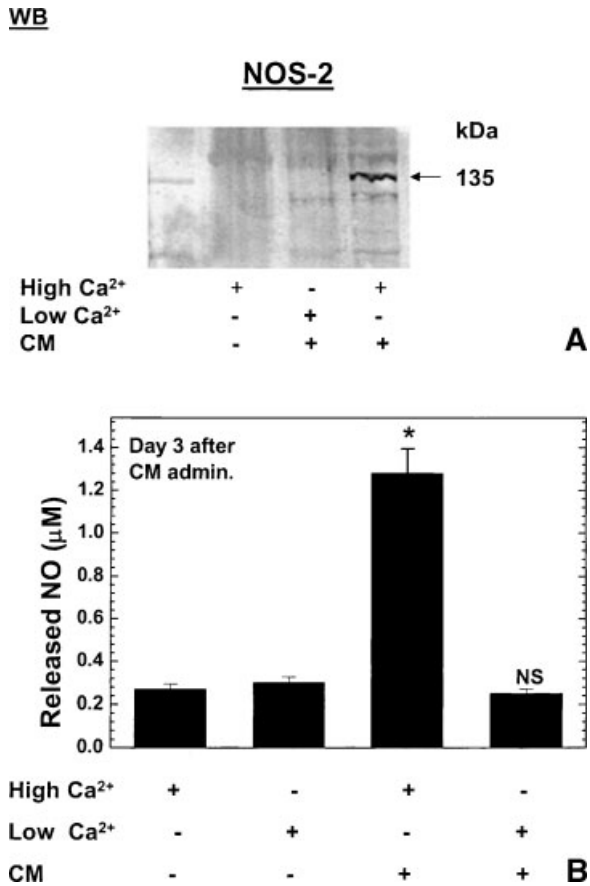

Fig. 4. In proliferatively *quiescent* adult human astrocytes, an exposure to the CM led to the expression of NOS-2 protein (**A**) and to a significant release of NO into the medium (**B**) when the cells were in high-calcium (i.e., 1.8 mM) but not in low-calcium (i.e., 0.75 mM) medium. Bars are mean ± SEM of the values from at least three separate experiments; *, $P < 0.001$ versus all the other treatment; NS, not significant versus both high- and low-Ca^{2+} untreated cells. WB, Western immunoblotting.

expression by unstimulated quiescent astrocytes was not affected by the Ca^{2+} concentration in the medium. Thus, CASR expression by proliferating astrocytes was the same whether they were in medium containing 0.75 or 1.8 mM Ca^{2+} (Fig. 5A,C). And the higher CASR expression by the quiescent cells was also not significantly different in the presence of either Ca^{2+} concentration (Fig. 5B,C).

While 1.8 mM Ca^{2+} was needed, together with the cytokines, in the culture medium to express NOS-2 and make NO (Fig. 4A,B), was the CASR needed to express the enzyme or activate it or both? Of particular interest was the critical stage around 24 h after adding the cytokines, which involves MEK1/MEK2 activity and is needed for the BH_4-dependent activation of the

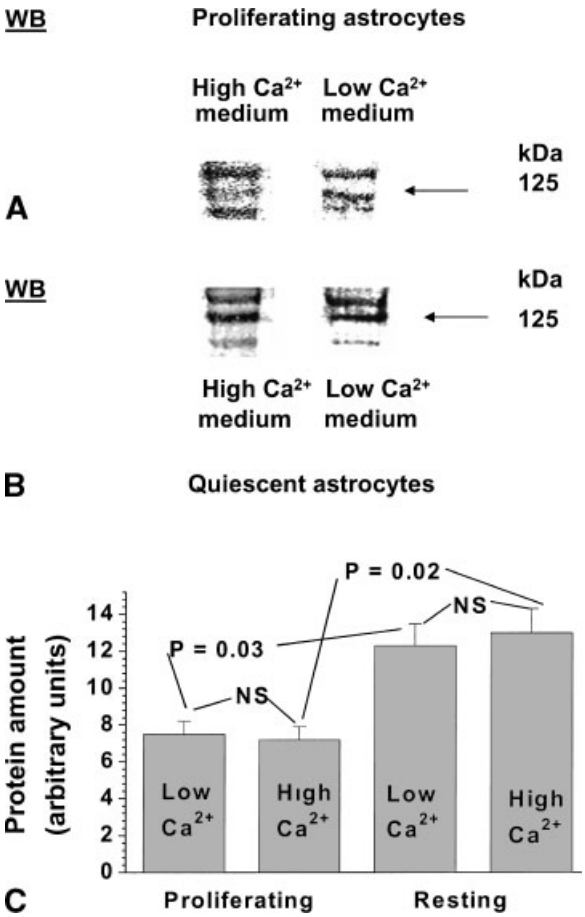

Fig. 5. The actual levels of expression of the CASR depend upon the proliferating (**A**) or quiescent (**B**) condition of the untreated human adult astrocytes, but they are independent from the calcium concentration in the medium. CASR was revealed in the blots using a specific mouse monoclonal antibody. The blots shown are representative of the blots in three experiments. Densitometric analysis (**C**) of CASR-specific bands (125 kDa) was carried out as detailed in the Materials and Methods. Bars are mean ± SEM of the values from three separate experiments.

emerging NOS-2 proteins [Chiarini et al., 2005]. We attempted to answer this question using the CASR-inhibitor, the calcilytic agent NPS 89636 [Dvorak et al., 2004].

As was the case with the selective MEK1/MEK2-inhibiting U0126 [Chiarini et al., 2005], adding 100 nM NPS 89636 to the medium for just 30 min immediately before adding the cytokine triad (i.e., between −0.5 and 0 h) and again between 24 and 24.5 h did not prevent the cells from making NOS-2 (Fig. 2C), but it prevented the emerging NOS-2 from making NO (Fig. 3). Exposing the cells to NPS 89636 only once for 30 min before adding the cytokines did not stop NOS-2 expression (Fig. 2D) or NO production (Fig. 3). But exposing the cytokine-stimulated cells to the same concentration of the inhibitor only once between 24 and 24.5 h after adding the cytokine triad also did not affect the expression of NOS-2 (Fig. 2E), but it prevented the emerging enzyme from making NO (Fig. 3), as did a similarly brief exposure to the selective MEK1/MEK2 inhibitor U0126 in our previous experiments [Chiarini et al., 2005].

In keeping with our previous findings, the exposure to the cytokine triad strongly activated ERK1/ERK2 (Fig. 6A,B), but exposing the cytokine-treated astrocytes to 100 nM NPS 89636 between 24 and 24.5 h after adding the cytokine triad prevented ERK1/ERK2 activation (Fig. 6D,E) just as would have happened with a short exposure to U0126 [Chiarini et al., 2005].

NOS-2 activation requires BH_4 [e.g., Channon, 2004] and, as we showed in our previous study, these cytokine-treated quiescent astrocytes begin rapidly accumulating it between 24 and 36 h and dumping it into the medium along with NO [Chiarini et al., 2005]. And indeed NPS 89636, like the U0126 MEK1/MEK2 inhibitor in our previous study [Chiarini et al., 2005], must have blocked NO production by preventing BH_4 action, because adding 100 μM BH_4 to the medium along with the cytokines prevented exposure to NPS 89636 between 24 and 24.5 h from stopping NO production (Fig. 7) just as it prevented the equally brief exposure to U0126 from stopping NO production [Chiarini et al., 2005].

DISCUSSION

The present experiments have shown that when stimulated by a triad of proinflammatory cytokines (IL-1β + TNF-α + IFN-γ) phenotypically normal adult human cerebral astrocytes switch on their *NOS-2* genes and start making NOS-2 and large amounts of NO, which, if they had been in the brain, could have killed neurons in the non-overlapping microdomains they envelop and control [Beach et al., 1989; Norenberg, 1994; Dawson and Dawson, 1996; McAndrew et al., 1997; Grzybicki et al., 1998; Rah et al., 2001; Estévez and Jordan, 2002; Khaldi et al., 2002; Leong et al., 2002; Liu et al., 2002; Hirsch et al., 2003; Nedergaard et al., 2003; Dokmeci, 2004; Saez et al., 2004; Schultz and Falkenburger, 2004; Zhao, 2005]. It follows that finding ways to block this

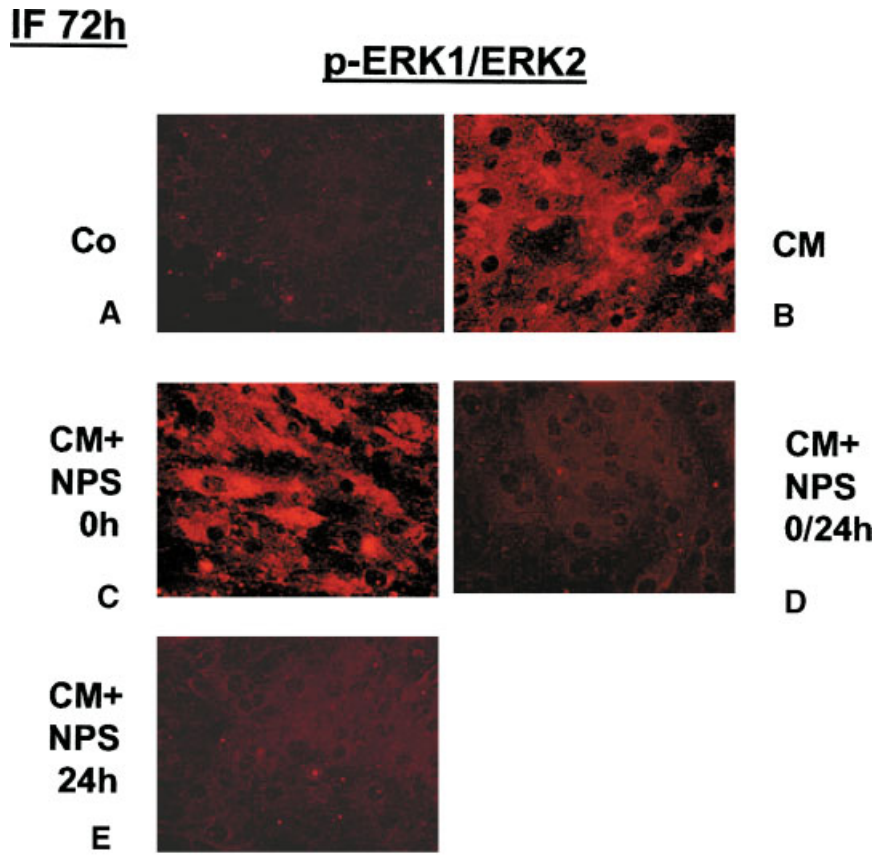

Fig. 6. The functional activation via phosphorylation of ERKs (p-ERK1/ERK2) following the addition of the CM was prevented by exposing adult human astrocytes for 30 min either twice (i.e., −0.5 to 0 h and 24 to 24.5 h) or once at either time points to the calcilytic agent NPS 89636 that blocks the CASR. The inhibition of ERK activation by NPS 89636 is strikingly illustrated by comparing the active p-ERKs-loaded cytoplasms of the CM-treated cells in **panels B** and **C** with the cytoplasms nearly devoid of p-ERK of the NPS 89636/CM-treated cells in **panels D** and **E** and of the untreated control cells (Co) **(panel A)**. The deconvolved fluorescent images shown here are typical of at least three distinct experiments and were obtained as indicated in the Materials and Methods. Original magnifications: 200×.

NOS-2/NO response of astrocytes to proinflammatory cytokines could minimize stroke/reperfusion and traumatic injury, glaucomatous neuropathy, and the progression of diseases such as Alzheimer's disease [Wada et al., 1998; Alessandrini et al., 1999; Akiyama et al., 2000a,b; Piao et al., 2003; Neufeld and Liu, 2003b].

The cytokines-stimulated proliferatively quiescent astrocytes' need for 1.8 mM Ca^{2+} in their 10% FBS-90% Ham's F12/MCDB medium to express and make functional NOS-2 might seem unexpected in view of the fact that the enzyme is itself insensitive to Ca^{2+} increases because of its tightly bound calmodulin [Lincoln et al., 1997; Baltrons and Garcia, 2001]. However, Li et al. [2003] have also reported that an agent such as ATP that triggers a cytosolic Ca^{2+} surge in murine astrocytes also induces NO synthesis. The responsiveness of NOS-2 induction and NO production by our normal human astrocytes to changes in the medium's Ca^{2+} concentration from 0.75 to 1.8 mM is characteristic of processes driven by a cation sensor like the one used by parathyroid chief cells with its 1.0–1.3 mM set-point for maximizing the signal:noise ratio to detect changes in the high ionic Ca^{2+} concentration in the blood [Mithal and Brown, 2003]. And indeed the higher extracellular Ca^{2+} concentration needed by the confluent adult cells to express NOS-2 coincided with an increased expression of a parathyroid-like CASR like that found in normal fetal human astrocytes by Chattopadhyay et al. [2000] and Chattopadhyay and Brown [2003].

The three cytokines probably started the processes leading to the production of NO by the quiescent adult astrocytes with a multipronged attack on the *NOS-2* gene's four up-

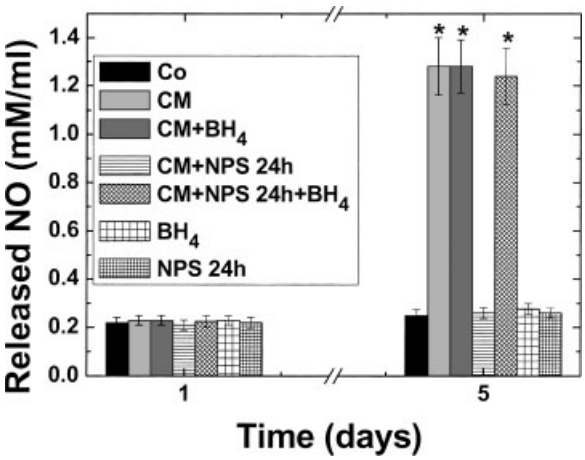

Fig. 7. Adding BH_4 (to a final concentration of 100 μM) enabled the NOS-2 protein accumulating in the astrocytes treated with the CM and a 30 min exposure to NPS 89636 (at 24–24.5 h) (CM + NPS 24 h + BH_4 bar) to release nearly as much NO in their medium by 5 days as did the cells treated with the CM alone (CM bar). The concentrations of nitrate and nitrite derivatives of NO in the medium samples were measured to give the NO concentrations as described in Materials and Methods. The bars are the mean ± SEM of the values from three separate experiments. The level of significance with respect to untreated controls (Co bar) was: *, $P < 0.001$. BH_4, tetrahydrobiopterin.

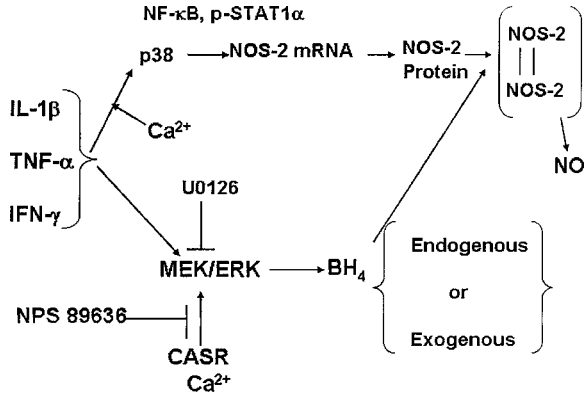

Fig. 8. The NOS-2 expression and activation mechanisms stimulated in normal human cerebral astrocytes by a triad of proinflammatory cytokines commonly found in Alzheimer plaques and other kinds of brain damage and degeneration. The expression of the *NOS-2* gene is Ca^{2+}-dependent and requires a burst of p38 MAPK activity. On the other hand, the BH_4-mediated activation of the accumulating NOS-2 protein requires U0126-inhibitable MEK/ERK activity and NPS 89636-inhibitable CASR signaling. NF-κB, and p-STAT1α are the transcription stimulators activated by IL-1β and TNF-α, and p-STAT1α is the homodimeric transcription stimulator activated by IFN-γ.

stream NF-κB enhancer elements and its STAT-dimer-binding GAS (γ-interferon-activated site) element with: (1) an IL-1β Ca^{2+} surge; (2) a burst of p38 MAP kinase activity; (3) an IL-lβ/TNF-α-triggered liberation of NF-κB from its I-κB inhibitor; and (4) phospho-STAT1α (p-STAT1α) dimers produced by the IFN-γ receptor-stimulated JAK-STAT mechanism [Holliday and Gruol, 1993; Pita et al., 1999; Ganster et al., 2001; Aaronson and Horvath, 2002; Neufeld and Liu, 2003a,b; Chiarini et al., 2005]. But CASR signaling is unlikely to be involved in this first phase because of the inability of the CASR-blocking NPS 89636 to prevent the cells from making NOS-2 and NO, when added for 30 min just before the cytokines as well as the inability of the inhibitor to affect the appearance of the NOS-2 protein, when it was added both for 30 min before and again between 24 and 24.5 h after adding the cytokines. However, CASR signaling is needed to activate the NOS-2 because exposing the cells to NPS 89636 for just 30 min between 24 and 24.5 h prevented NO production without affecting NOS-2 protein expression. This is the time when a process that operates to drive the BH_4-dependent activation of emerging NOS-2 proteins can be stopped by the equally brief exposure to the selective MEK1/MEK2 inhibitor U0126 [Chiarini et al., 2005] (Fig. 8). And it now appears that this is the same mechanism that requires signals from the CASR because NPS 89636 can mimic U0126 by preventing ERK activation, and this inhibition by NPS 89636 can be completely overridden by adding BH_4 to the medium (Fig. 8). Clearly, we must now focus our efforts on finding out how CASR signals and MEK-ERK kinases control the production of BH_4 by GTP-glycohydrolase-1 in human astrocytes activated by proinflammatory cytokines.

ACKNOWLEDGMENTS

The authors thank Prof. A. Bricolo, Head, Neurosurgery Clinic, Major Hospital, Verona, Italy for kindly supplying the fragments of normal human adult temporal cortex, from which were isolated the astrocytes used in the present work.

REFERENCES

Aaronson DS, Horvath CM. 2002. A road map for those who don't know JAK-STAT. Science 296:1653–1655.

Akiyama H, Arai T, Kondo H, Tanno E, Haga C, Ikeda K. 2000a. Cell mediators of inflammation in the Alzheimer disease brain. Alzheimer Dis Assoc Disord 14(Suppl 1): S47–S53.

Akiyama H, Barger S, Barnum S, Bradt B, Bauer J, Cole GM, Cooper N, Eikelenboom P, Emmerling M, Fiebich

BL, Finch CE, Frautschy S, Griffen WS, Hampel H, Hull M, Landreth G, Lue L, Mrak R, Mackenzie IR, McGeer PL, O'Banion MK, Pachter J, Pasinetti G, Plata-Salaman C, Rogers J, Rydel R, Shen Y, Streit W, Strohmeyer R, Tooyoma I, Van Muiswinkel FL, Veerhuis R, Walker D, Webster S, Wegrzyniak B, Wenk G, Wyss-Coray T. 2000b. Inflammation and Alzheimer's disease. Neurobiol Aging 21:383–421.

Alessandrini A, Namura S, Moskowitz MA, Bonventre JV. 1999. MEK1 protein kinase inhibition protects against damage resulting from focal cerebral ischemia. Proc Natl Acad Sci USA 96:12866–12869.

Baltrons MA, Garcia A. 2001. The nitric oxide/cyclic GMP system in astroglial cells. Prog Brain Res 132:325–337.

Barbeito LH, Pehar M, Cassina P, Vargas MR, Peluffo H, Viera L, Estévez AG, Beckman JS. 2004. A role for astrocytes in motor neuron loss in amyotrophic lateral sclerosis. Brain Res Brain Res Rev 47:263–274.

Beach TG, Walker R, McGeer EG. 1989. Patterns of gliosis in Alzheimer's disease and aging cerebrum. Glia 2:420–436.

Blasko I, Stampfer-Kountchev M, Robatscher P, Veerhuis R, Eikelenboom P, Grubeck-Loebenstein B. 2004. How chronic inflammation can affect the brain and support the development of Alzheimer's disease in old age: The role of microglia and astrocytes. Aging Cell 3:169–176.

Boczkowski J, Lisdero CL, Lanone S, Carreras MC, Aubier M, Poderoso JJ. 2001. Peroxynitrite-mediated mitochondrial dysfunction. Biol Signals Recept 10:66–80.

Channon KM. 2004. Tetrahydrobiopterin: Regulator of endothelial nitric oxide synthase in vascular disease. Trends Cardiovasc Med 14:323–327.

Chattopadhyay N, Brown EM. 2003. Calcium-Sensing Receptors. Boston: Kluwer Academic Publishers.

Chattopadhyay N, Evliyaoglu C, Heese O, Carroll R, Sanders J, Black P, Brown EM. 2000. Regulation of secretion of PTHrP by Ca^{2+}-sensing receptor in human astrocytes, astrocytomas, and meningiomas. Am J Physiol Cell Physiol 279:C691–C699.

Chiarini A, Dal Pra I, Gottardo R, Bortolotti F, Whitfield JF, Armato U. 2005. BH_4 (tetrahydrobiopterin)-dependent activation, but not the expression, of inducible NOS (nitric oxide synthase)-2 in proinflammatory cytokine-stimulated, cultured normal human astrocytes is mediated by MEK-ERK kinases. J Cell Biochem 94:731–743 (Epub Nov 23, 2004).

Dawson VL, Dawson TM. 1996. Nitric oxide in neural degeneration. Proc Soc Exp Biol Med 211:33–40.

Dokmeci D. 2004. Ibuprofen and Alzheimer's disease. Folia Med (Plovdiv) 46:5–10.

Dvorak MM, Siddiqua A, Ward DT, Carter DH, Dallas SL, Nemeth EF, Riccardi D. 2004. Physiological changes in extracellular calcium concentration directly control osteoblasts function in the absence of calciotropic hormones. Proc Natl Acad Sci USA 101:5140–5145.

Estévez A, Jordan J. 2002. Nitric oxide and superoxide, a deadly cocktail. Ann NY Acad Sci 962:207–211.

Ganster RW, Taylor BS, Shao L, Geller DA. 2001. Complex regulation of human inducible nitric oxide synthase gene transcription by Stat 1 and NF-kappa B. Proc Natl Acad Sci USA 98:8638–8643.

Grzybicki D, Moore SA, Schelper R, Glabinski AR, Ransohoff RM, Murphy S. 1998. Expression of monocyte chemoattractant protein (MCP-1) and nitric oxide synthase-following cerebral trauma. Acta Neuropathol (Berl) 95:98–103.

Hansson E, Ronnback L. 2003. Glial neuronal signaling in the central nervous system. FASEB J 17:341–348.

Hertz L, Zielke HR. 2004. Astrocytic control of glutamatergic activity: Astrocytes as stars of the show. Trends Neurosci 27:735–743.

Hirsch EC, Breidert T, Rousselet E, Hunot S, Hartmann A, Michel PP. 2003. The role of glial reaction and inflammation in Parkinson's disease. Ann NY Acad Sci 99:214–228.

Holliday J, Gruol DL. 1993. Cytokine stimulation increases intracellular calcium and alters the response to quisqualate in cultured cortical astrocytes. Brain Res 621:233–241.

Hunot S, Boissiere F, Faucheux B, Brugg B, Mouatt-Prigent A, Agid Y, Hirsch EC. 1996. Nitric oxide synthase and neuronal vulnerability in Parkinson's disease. Neuroscience 72:355–363.

Khaldi A, Chiueh CC, Bullock MR, Woodward JJ. 2002. The significance of nitric oxide production in the brain after injury. Ann NY Acad Sci 962:53–59.

Leong SK, Ruan RS, Zhang Z. 2002. A critical assessment of the neurodestructive and neuroprotective effects of nitric oxide. Ann NY Acad Sci 962:161–181.

Li N, Sul JY, Haydon PG. 2003. A calcium-induced calcium influx factor, nitric oxide, modulates the refilling of calcium stores in astrocytes. J. Neurosci 23:10302–10310.

Lin SC, Bergles DE. 2004. Synaptic signaling between neurons and glia. Glia 47:290–298.

Lincoln J, Hoyle CHV, Burnstock G. 1997. Nitric oxide in health and disease. Cambridge: Cambridge University Press.

Liu B, Gao HM, Wang JY, Jeohn GH, Cooper CL, Hong JS. 2002. Role of nitric oxide in inflammation-mediated neurodegeneration. Ann NY Acad Sci 962:318–331.

McAndrew J, Patel RP, Jo H, Cornwell T, Lincoln T, Moellering D, White CR, Matalon S, Darley-Usmar V. 1997. The interplay of nitric oxide and peroxynitrite with signal transduction pathways: Implications for disease. Semin Perinatol 21:351–366.

McGeer PL, McGeer EG. 1995. The inflammatory response system of brain: Implications for therapy of Alzheimer and other neurodegenerative diseases. Brain Res Brain Res Rev 21:195–218.

McGeer EG, McGeer PL. 1999. Brain inflammation in Alzheimer disease and the therapeutic implications. Curr Pharma Des 5:821–836.

McGeer EG, McGeer PL. 2003. Inflammatory processes in Alzheimer's disease. Prog Neuropyschopharmacol Biol Psychiatry 27:741–749.

McGeer PL, McGeer EG. 2004. Inflammation and neurodegeneration in Parkinson's disease. Parkinsonism Relat Disord 10(Suppl 1):S3–S7.

McGeer PL, McGeer EG, Yasojima K. 2000. Alzheimer disease and neuroinflammation. J Neural Transm Suppl 59: 53–57.

Misko TP, Schilling RJ, Salvemini D, Moore WM, Currie MG. 1993. A fluorometric assay for the measurement of nitrite in biological samples. Anal Biochem 214:11–16.

Mithal A, Brown EM. 2003. An overview of extracellular calcium homeostasis and the roles of the CaR in parathyroid and C-cells. In: Chattopadhyay N, Brown EM,

editors. Calcium-sensing receptors. Boston: Kluwer Academic Publishers. pp 1–27.

Mrak RE, Sheng JG, Griffin WS. 1995. Glial cytokines in Alzheimer's disease: Review and pathogenic implications. Hum Pathol 26:816–823.

Nedergaard M, Ransom B, Goldman SA. 2003. New roles for astrocytes: Redefining the functional architecture of the brain. Trends Neurosci 26:523–530.

Nemeth EF, Delmar EG, Heaton WL, Miller MA, Lambert LD, Conklin RL, Gowen M, Gleason JG, Bhatnagar PK, Fox J. 2001. Calcilytic compounds: Potent and selective Ca^{2+} receptor antagonists that stimulate secretion of parathyroid hormone. J Pharmacol Exp Ther 299:323–331.

Neufeld AH, Liu B. 2003a. Comparison of the signal transduction pathways for the induction of gene expression of nitric oxide synthase-2 in response to two different stimuli. Nitric Oxide 8:95–102.

Neufeld AH, Liu B. 2003b. Glaucomatous optic neuropathy: When glia misbehave. Neuroscientist 9:485–495.

Norenberg MD. 1994. Astrocyte responses to CNS injury. J Neuropathol Exp Neurol 53:213–220.

Piao CS, Kim JB, Han PL, Lee JK. 2003. J Neurosci Res 73:537–544.

Pita I, Jelaso AM, Ide CF. 1999. IL-lβ increases intracellular calcium through an IL-1 type 1 receptor-mediated mechanism in C6 astrocytic cells. Int J Dev Neurosci 17:813–820.

Rah JC, Kim HS, Kim SS, Bach JH, Kim YS, Park CH, Seo JH, Jeong SJ, Suh YH. 2001. Effects of carboxyl-terminal fragment of Alzheimer's amyloid precursor protein and amyloid β-peptide on the production of cytokines and nitric oxide in glial cells. FASEB J 15:1463–1465.

Ransom BR, Sontheimer H. 1992. The neurophysiology of glial cells. J Clin Neurophysiol 9:224–251.

Ransom B, Behar T, Nedergaard M. 2003. New roles for astrocytes (stars at last). Trends Neurosci 26:520–522.

Saez TE, Pehar M, Vargas M, Barbeito L, Maccioni RB. 2004. Astrocytic nitric oxide triggers tau hyperphosphorylation in hippocampal neurons. In Vivo 18:275–280.

Schipke CG, Kettenmann H. 2004. Astrocyte responses to neuronal activity. Glia 47:226–232.

Schultz JB, Falkenburger BH. 2004. Neuronal pathology in Parkinson's disease. Cell Tissue Res 318:135–147.

Suzuki H, Menegazzi M, Carcereri de Prati A, Mariotto S, Armato U. 1995. Nitric oxide in the liver: Physiopathological roles. Adv Neuroimmunol 5:379–410.

Wada K, Chatzipanteli K, Kraydieh S, Busto R, Dietrich WD. 1998. Inducible nitric oxide synthase expression after traumatic brain injury and neuroprotection with aminoguanidine treatment in rats. Neurosurgery 43: 1427–1436.

Zamora R, Vodovotz Y, Billar TR. 2000. Inducible nitric oxide synthase and inflammatory diseases. Mol Med 6: 347–373.

Zhao B. 2005. Nitric oxide in neurodegenerative diseases. Front Biosci 10:454–461.