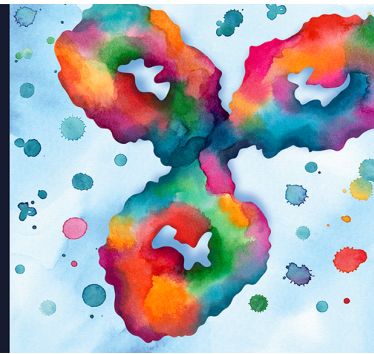


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# Expression of Type II Nitric Oxide Synthase in Primary Human Astrocytes and Microglia

## Role of IL-1 $\beta$ and IL-1 Receptor Antagonist<sup>1</sup>

Judy Liu, Meng-Liang Zhao, Celia F. Brosnan, and Sunhee C. Lee<sup>2</sup>

In this work, we studied the expression of type II nitric oxide synthase (NOS) in primary cultures of human astrocytes and microglia. Cytokine-activated human fetal astrocytes expressed a 4.5-kb type II NOS mRNA that was first evident at 8 h, steadily increased through 48 h, and persisted through 72 h. The inducing signals for astrocyte NOS II mRNA expression were in the order IL-1 $\beta$  + IFN- $\gamma$  > IL-1 $\beta$  + TNF- $\alpha$  > IL-1 $\beta$ . SDS-PAGE analysis of cytokine-stimulated astrocyte cultures revealed an approximately 130-kDa single NOS II band that was expressed strongly at 48 and 72 h (72 h > 48 h). Specific NOS II immunoreactivity was detected in cytokine-treated astrocytes, both in the cytosol and in a discrete paranuclear region, which corresponded to Golgi-like membranes on immunoelectron microscopy. In human microglia, cytokines and LPS failed to induce NOS II expression, while the same stimuli readily induced TNF- $\alpha$  expression. In cytokine-treated human astrocytes, neither NOS II mRNA/protein expression nor nitrite production was inhibited by TGF- $\beta$ , IL-4, or IL-10. In contrast, IL-1 receptor antagonist exerted near complete inhibition of NOS II mRNA and nitrite induction. Monocyte chemoattractant peptide-1 mRNA was induced in TGF- $\beta$ -treated astrocytes, demonstrating the presence of receptors for TGF- $\beta$  in astrocytes. These results confirm that in humans, cytokines stimulate astrocytes, but not microglia, to express NOS II belonging to the high output nitric oxide system similar to that found in rodent macrophages. They also show that the regulation of type II NOS expression in human glia differs significantly from that in rodent glia. A crucial role for the IL-1 pathway in the regulation of human astrocyte NOS II is shown, suggesting a potential role for IL-1 as a regulator of astrocyte activation in vivo. *The Journal of Immunology*, 1996, 157: 3569–3576.

Nitric oxide (NO)<sup>3</sup> has received much attention recently by virtue of the wide range of its biologic effects and of its production by a variety of cell types and tissues (1–3). NO is synthesized from the guanidino-nitrogen of L-arginine and molecular oxygen by nitric oxide synthases (NOS), of which there are two major types. Constitutive NOS isoforms are activated by biologic signals that transiently increase intracellular Ca<sup>2+</sup>, and an inducible form (type II NOS) is Ca<sup>2+</sup> independent and activated by a variety of agents, including cytokines and/or bacterial products. In the nervous system, multiple NOS isoforms have been detected that include expression of the neuronal type I NOS and endothelial type III NOS in astrocytes and in subpopulations of neurons (4, 5); the inducible type II NOS in astrocytes (6, 7) and microglia (8); and perhaps also a subpopulation of neurons (9). Activation of type II NOS results in the sustained release of large

amounts of NO that has been shown to be cytotoxic for both host cells and infectious agents, and that has been implicated in the pathogenesis of several central nervous system (CNS) diseases, including multiple sclerosis, HIV encephalitis, and Alzheimer's disease (10, 11).

One of the central issues in assessing the role of type II NOS in human pathology, however, is the extent to which the data obtained in rodent systems pertain to the regulation of type II NOS in humans. For instance, studies that have attempted to induce type II NOS in human macrophages using a combination of cytokines and bacterial products have generally been unsuccessful, demonstrating only a relatively low level induction, if at all. However, activation of type II NOS in these cells has been found following direct interaction with microorganisms such as HIV-1 (10), *Pneumocystis carinii*, and *Mycobacterium avium* when applied in combination with cytokine stimulation (12). Again, in most of these systems the levels of NO produced do not reach those observed in rodent macrophages and/or microglia.

In contrast to the results obtained with human macrophages, a high output cytokine-inducible NOS can be expressed readily in other human cell types, including hepatocytes, astrocytes, chondrocytes, keratinocytes, and lung epithelial cells (13–17). An inducible NOS has been cloned from human hepatocytes and chondrocytes. While human and murine type II NOS share 80% identity in the coding region, promoter regions from these two species are strikingly different. Analysis of human NOS II promoter demonstrated cytokine-inducible activity in constructs extending –5.8 to –7.0 kb, with further enhancement in a –16-kb construct (18). These results are in marked contrast with the murine macrophage NOS II promoter in which only 1.1 kb of the proximal 5' region is necessary to confer inducibility to LPS and IFN- $\gamma$  (19).

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<sup>3</sup> Abbreviations used in this paper: NO, nitric oxide; NOS, nitric oxide synthase; CNS, central nervous system; DAB, diaminobenzidine; IL-1Ra, interleukin-1 receptor antagonist; macNOS, macrophage nitric oxide synthase; MCP-1, monocyte chemoattractant peptide-1; NADPH, nicotinamide-adenine dinucleotide phosphate.

At present, the mechanisms underlying species- and cell-specific expression of type II NOS are not known, but are likely to involve tissue-specific elements governing transcriptional activation of the NOS gene. We report in this work a detailed study of cytokine regulation of type II NOS expression in two major human glial cell types: astrocytes and microglia. The results show that astrocytes could be induced readily to express a type II NOS by proinflammatory cytokines, whereas a type II NOS could not be induced in microglia following activation with the same group of cytokines. Astrocyte type II NOS expression resembled that found in human hepatocytes in the magnitude of NO produced and the nature of the inducing signals (13, 20). In contrast, the cytokine signals regulating the expression of type II NOS in human astrocytes showed a number of important differences from that found in rodent macrophages and glia.

## Materials and Methods

### Cell culture

Human fetal astrocytes and microglia were isolated and cultivated as described (21). Briefly, cerebral tissue from second trimester fetal abortuses was dissociated by trituration and enzymatic digestion, and plated as mixed cultures comprising astrocytes, neurons, and microglia (21). At 2 wk, culture media containing microglia were pooled, pelleted, and replated in uncoated plastic dishes at high cell densities. Cultures were washed at 1 to 3 h after plating to remove nonadherent cells. Microglia cultures consisted of >99% CD68<sup>+</sup> cells. Astrocyte cultures were purified by two to three cycles of trypsinization, following which 99% of the cells were glial fibrillary acidic protein positive. Culture medium was DMEM with 4.5 g/L of glucose, 4 mM of L-glutamine, and 25 mM of HEPES buffer (BioWhittaker, Walkersville, MD) supplemented with 10% FCS (BioWhittaker) and penicillin (100 U/ml)/streptomycin (100 µg/ml) and fungizone at 0.25 µg/ml (Life Technologies, Bethesda, MD).

### Cell stimulants

Human rIL-1 $\beta$  was a gift from Biologic Modifiers Program at National Cancer Institute (Frederick, MD); human rIFN- $\gamma$ , TNF- $\alpha$ , IL-4, and IL-10 were from Genzyme Corp. (Cambridge, MA); human rTGF- $\beta$  and IL-1Ra were from R&D Systems (Minneapolis, MN); LPS (*Escherichia coli* serotype 055:B5) was from Sigma Chemical Co. (St. Louis, MO); and rHIV-1 gp120 was from Intracel (Cambridge, MA).

### Nitrite assay

Microglia or astrocytes were seeded in 96-well plates at  $4 \times 10^4$  cells/well in triplicate. Cells were stimulated with cytokines or LPS, and culture supernatants were collected at indicated time points. Levels of nitrite were measured by mixing with equal amounts (100 µl) of Griess reagent and determining OD with an ELISA reader at 540 or 570 nm, as described (14).

### Northern blot analysis

Astrocytes grown to confluence in T 75-cm<sup>2</sup> flasks were stimulated, and total cell RNA was isolated by Trizol (Life Technologies). Twenty micrograms of total RNA was separated by electrophoresis in 1% agarose-formaldehyde gels and transferred to Hybond N<sup>+</sup> membranes (Amersham, Arlington Heights, IL). For microglia, RNA extracted from  $1.5$  to  $2 \times 10^6$  cells per sample was loaded in each lane without measuring RNA content. The blots were prehybridized for 1 h and then hybridized overnight at 65°C in  $5 \times$  SSPE (sodium chloride, sodium phosphate, EDTA),  $5 \times$  Denhardt's solution, 0.5% (w/v) SDS, and 20 µg/ml of sonicated salmon sperm DNA. Hybridization solution contained <sup>32</sup>P-labeled probes synthesized using the Megaprime kit (Amersham). Blots were washed to a final stringency of  $0.1 \times$  SSPE and 0.1% SDS at 65°C, and exposed. They were then stripped and reprobed for 18S rRNA. Densitometry was performed using the Molecular Dynamics densitometer and Image Quant software (Sunnyvale, CA).

### DNA probes

Plasmid containing human hepatocyte type II NOS cDNA was a gift from Dr. D. Geller (University of Pittsburgh, Pittsburgh, PA). A human 18S ribosomal cDNA probe was provided from Dr. N. Arnheim (State University of New York, Stony Brook, NY), as described (22). Human TNF- $\alpha$  cDNA was a gift from Genentech (South San Francisco, CA). Human MCP-1 cDNA was obtained from Dr. T. Yoshimura and E. J. Leonard

(National Cancer Institute, Bethesda, MD) (23). cDNA inserts were excised from their plasmid vectors before labeling.

### Immunocytochemistry

Cells were fixed with ice-cold methanol or Trump's fixative (1% glutaraldehyde and 4% paraformaldehyde in PBS) and incubated with 10% normal goat serum in PBS for 1 h. Affinity-purified rabbit Ab against human type II NOS was purchased from Santa Cruz (Santa Cruz, CA) and used at 1/1000 dilution. mAb to murine macrophage type II NOS (IgG2a) was purchased from Transduction Laboratories (Lexington, KY) and was used at 1/50 or 1/100 dilutions. Control Abs were normal rabbit serum and smooth muscle-specific actin (IgG2a; Dako Corp., Carpinteria, CA) (24). An additional control was rabbit type II NOS Ab (50 ng) that was preabsorbed with specific peptide (1 µg) provided from the company (Santa Cruz). Incubation with primary Abs was performed in 10% normal goat serum in PBS for 16 h at 4°C. Secondary Abs were goat anti-mouse IgG2a (1/250) or goat anti-rabbit IgG (1/400) at room temperature for 2 h. The reaction was developed using diaminobenzidine (DAB) as the chromogen.

### Immunoelectron microscopy

Astrocytes were stimulated with IL-1 $\beta$  and IFN- $\gamma$  for 72 h and immunostained using DAB, as described above. The monolayers were postfixed with 2% osmium tetroxide in Millonig's buffer for 1 h at room temperature, and were dehydrated serially in graded ethanol, then acetonitrile, acetonitrile/araldite-epon (1:1), and araldite-epon. BEEM capsules (Electron Microscopy Science, Fort Washington, PA) were then inverted over the cells, filled with araldite-epon, and polymerized at 65°C for 2 days. Ultrathin sections (not counterstained) were examined for DAB deposits with JEOL 100 CX and 1200 EX electron microscopes.

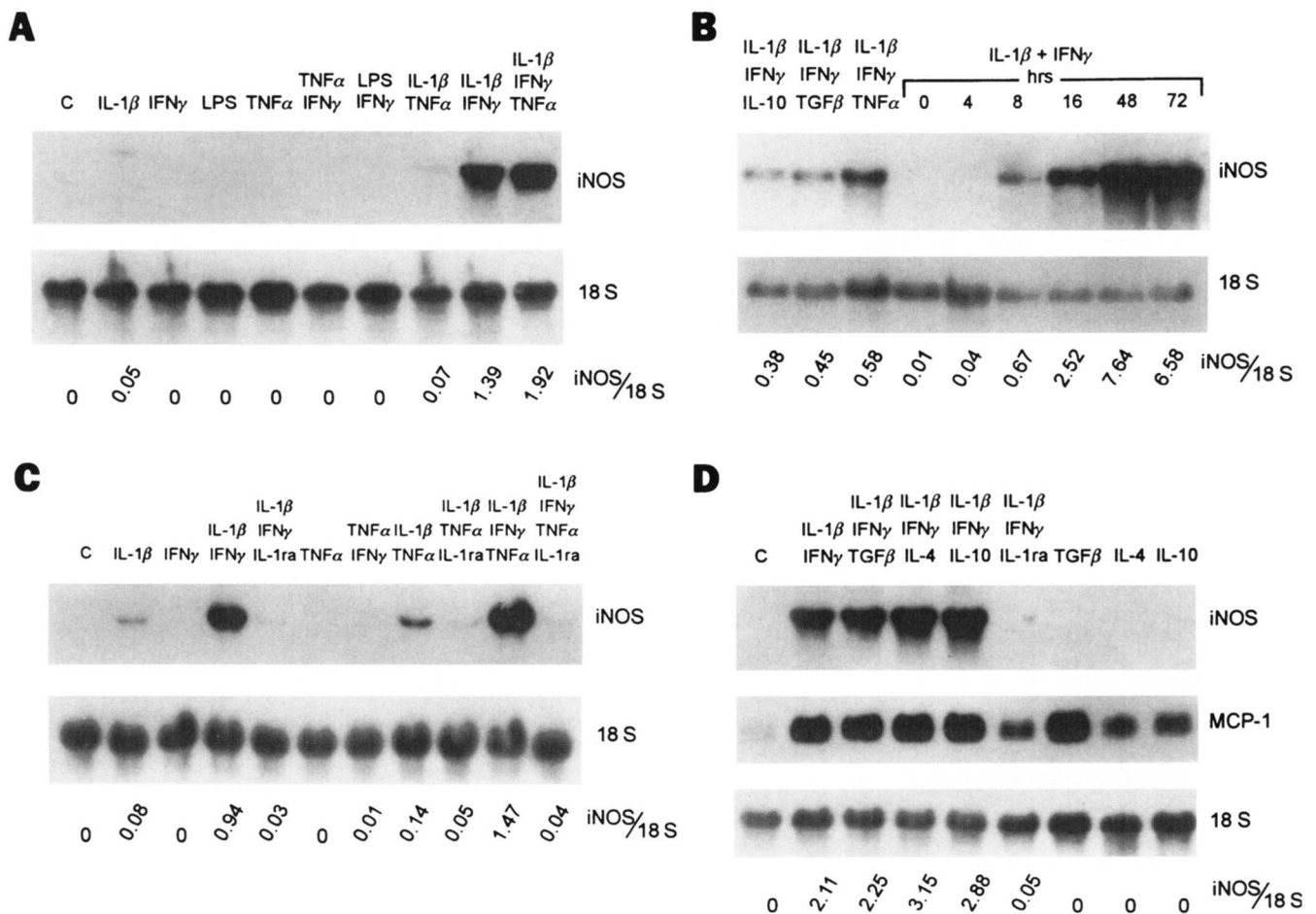
### SDS-PAGE and immunoblotting

Astrocyte monolayers were washed with PBS and lysed in 8 M of urea solution. After passing through 23-gauge needle several times, protein concentrations were determined using the Bradford colorimetric assay. Ten micrograms of protein lysates were loaded in each lane in a sample buffer (2% SDS, 10% glycerol, 0.001% bromophenol blue, 5%  $\beta$ -mercaptoethanol, and 0.05 M Tris-HCl, pH 6.8), separated on 10% SDS-PAGE, and transferred to a nitrocellulose filter. The blots were blocked with 5% nonfat milk in PBS, and then incubated with anti-macNOS Ab (1/400) followed by peroxidase-conjugated goat anti-mouse IgG2a (1/400) and the enhanced chemoluminescence detection system (Amersham).

## Results

### In human fetal astrocytes, type II NOS mRNA and nitrite are induced by cytokines

Human fetal astrocyte cultures stimulated with IL-1 $\beta$  alone or in combination with IFN- $\gamma$  or TNF- $\alpha$  expressed a 4.5-kb mRNA that specifically hybridized with the human hepatocyte cDNA (Fig. 1). Unstimulated astrocyte cultures did not express type II NOS mRNA. IFN- $\gamma$  or TNF- $\alpha$  alone did not induce type II NOS expression in human astrocytes (Fig. 1A). LPS, alone or in combination with cytokines, including IFN- $\gamma$ , had no effect on the induction of type II NOS mRNA in human astrocytes (Fig. 1A). The signals for astrocyte type II NOS mRNA induction were IL-1 $\beta$  < IL-1 $\beta$  + TNF- $\alpha$  < IL-1 $\beta$  + IFN- $\gamma$   $\leq$  IL-1 $\beta$  + IFN- $\gamma$  + TNF- $\alpha$  (Fig. 1, A and C, for values obtained at 16 h; Fig. 1B for values obtained at 8 h poststimulation). These data were also consistent with the rank order of potency of cytokine combinations for the stimulation of nitrite production (14) (see Fig. 2). The kinetic analysis of type II NOS mRNA induction in IL-1 $\beta$  + IFN- $\gamma$ -stimulated astrocytes (Fig. 1B) showed that type II NOS mRNA expression was not detectable at 4 h, but clearly evident at 8 h. The type II NOS mRNA levels increased through 16 and 48 h, persisting through 72 h, the longest time point examined. The blot was then stripped and probed for TNF- $\alpha$  expression. TNF- $\alpha$  mRNA peaked at 8 h and returned to near baseline values by 16 h (data not shown), as shown previously (25). The densitometric values of type II NOS mRNA expression normalized to the values of 18S RNA are shown beneath each panel of Figure 1.



**FIGURE 1.** Northern analysis of cytokine-treated human fetal astrocyte cultures. Astrocyte cultures were treated with cytokines or LPS, as indicated above each lane, and 20  $\mu$ g of total RNA was analyzed from each sample for the expression of type II NOS and 18S. Ratios of densitometric values of NOS II to 18S are given under each lane. IL-1 $\beta$  was used at 5 ng/ml; IFN- $\gamma$  at 200 U/ml; LPS at 1  $\mu$ g/ml; IL-1Ra at 500 ng/ml; and TNF- $\alpha$ , TGF- $\beta$ , IL-4, and IL-10 all at 10 ng/ml. **A**, Astrocyte cultures were treated with cytokines and/or LPS for 16 h before harvesting. **B**, In lanes 1 to 3, astrocytes were treated with IL-10, TGF- $\beta$ , and TNF- $\alpha$  in combination with IL-1 $\beta$  and IFN- $\gamma$  for 8 h. Lanes 4 to 9 show the time course of NOS II mRNA expression following IL-1 $\beta$  + IFN- $\gamma$  stimulation. **C**, Astrocytes were treated with cytokine combinations with and without IL-1Ra for 16 h. **D**, Astrocytes were treated with TGF- $\beta$ , IL-4, or IL-10 for 24 h, then treated with IL-1 $\beta$  + IFN- $\gamma$  for an additional 16 h (lanes 3–5). In lane 6, astrocytes were treated simultaneously with IL-1Ra + IL-1 $\beta$  + IFN- $\gamma$  for 16 h. In lanes 7 to 9, astrocytes were treated with TGF- $\beta$ , IL-4, or IL-10 alone for 40 h.

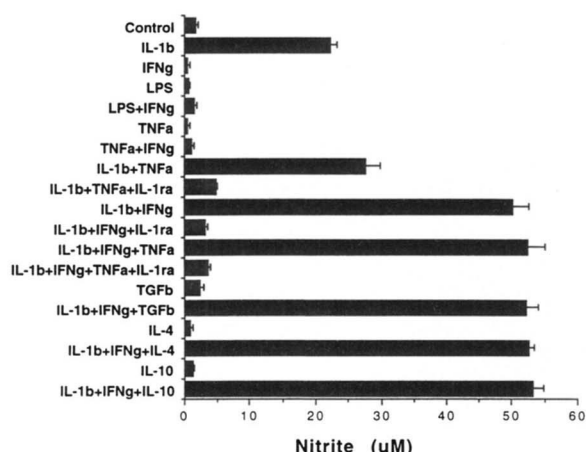
*The expression of type II NOS mRNA and nitrite in cultured human astrocytes is inhibited by IL-1Ra, but not by TGF- $\beta$ , IL-4, or IL-10*

We next examined whether cytokines that have been shown to inhibit mouse macrophage type II NOS exerted a similar inhibitory effect on human astrocyte type II NOS expression. We pretreated astrocytes with TGF- $\beta$ , IL-4, or IL-10 at 10 ng/ml 24 or 0 h before IL-1 $\beta$  + IFN- $\gamma$  stimulation. The results showed that there was no inhibition of type II NOS mRNA accumulation in human astrocytes by any of the three cytokines at any time points tested (Fig. 1D, results from 24-h pretreatment are shown).

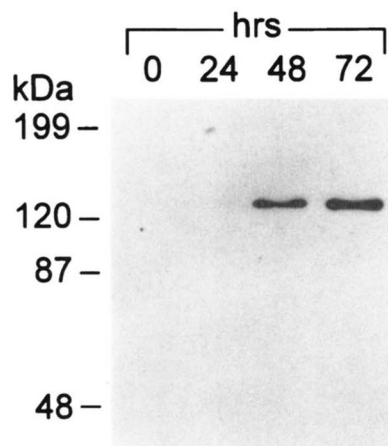
We next examined nitrite levels in astrocyte cultures at 72 h after stimulation with IL-1 $\beta$  + IFN- $\gamma$  or IL-1 $\beta$  + TNF- $\alpha$ , with or without each of the three putative inhibitory cytokines, TGF- $\beta$ , IL-4, and IL-10. The recombinant cytokines at doses of 0.1, 1, or 10 ng/ml were added at 24, 6, or 0 h before addition of stimulatory cytokines in at least three separate experiments. The results revealed that there was no inhibition of nitrite production by any of the three cytokines tested within the range of doses and time periods examined (the results of a representative experiment are shown in Fig. 2).

To test the responsiveness of the cell cultures to TGF- $\beta$ , we probed the same blots for MCP-1 mRNA (Fig. 1D), since TGF- $\beta$  alone can induce MCP-1 expression in human fetal astrocytes (26). As shown in Figure 1D, a specific band that hybridized with the MCP-1 probe was detected in astrocyte cultures treated with TGF- $\beta$ . Lesser induction was also seen after IL-4 and IL-10 treatment. These data exclude the possibility that the lack of inhibitory effect on NOS II expression was due to absence of a response to TGF- $\beta$ , IL-4, or IL-10 in these cultures.

We then explored the effect of IL-1Ra, a naturally occurring IL-1 inhibitor, on the expression of type II NOS mRNA and nitrite in human fetal astrocytes. The dose of IL-1Ra necessary to achieve complete inhibition was greater than a 200-fold excess to IL-1 $\beta$ , consistent with previously reported data (27). As shown in Figure 1, C and D, type II NOS mRNA expression in astrocytes was abrogated almost completely by IL-1Ra used at a 100-fold excess to IL-1 $\beta$ . Nitrite production was also reduced by the addition of IL-1Ra at a 50-fold excess to IL-1 $\beta$  (Fig. 2). At concentrations of IL-1Ra  $\geq$  200-fold excess to IL-1 $\beta$ , nitrite accumulation in culture medium was abrogated completely ( $n = 3$ , data not shown). These results demonstrate that IL-1 is the key cytokine involved in the



**FIGURE 2.** Nitrite production in human fetal astrocyte cultures. Cultures in triplicate wells were treated with cytokines in various combinations for 72 h, and the nitrite concentrations in the culture supernatants were measured using the Griess reaction. Data are expressed as mean  $\pm$  SD. The doses were the same as described in the Figure 1 legend, except that IL-1 $\beta$  was used at 10 ng/ml. TGF- $\beta$ , IL-4, and IL-10 were administered 24 h before IL-1 $\beta$  + IFN- $\gamma$  stimulation. The results shown are representative of at least seven separate experiments testing different time points and cytokine doses.

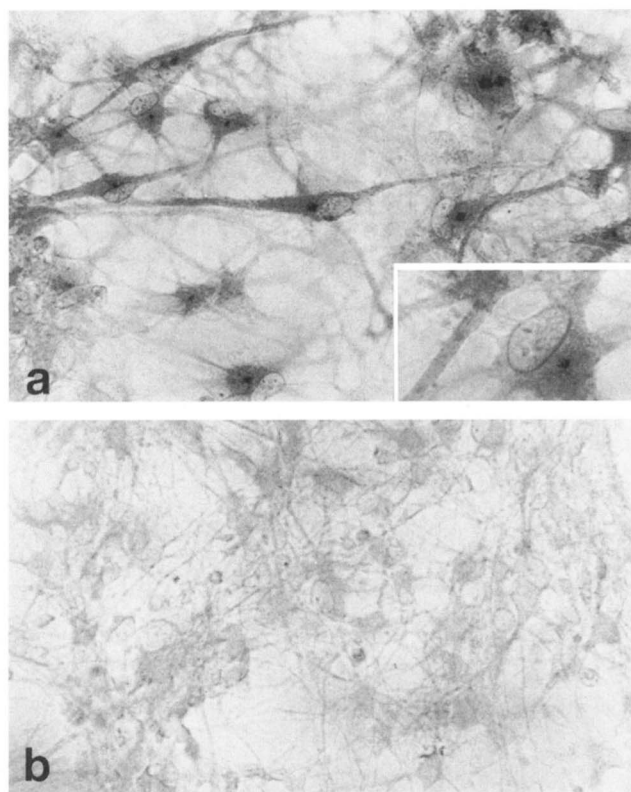


**FIGURE 3.** Immunoblot of NOS II protein in stimulated human fetal astrocyte cultures. Lysates were taken from cells at 0, 24, 48, and 72 h poststimulation with IL-1 $\beta$  + IFN- $\gamma$ , and 10  $\mu$ g of each sample was separated by SDS-PAGE. After transfer, the blot was incubated with anti-macNOS Ab.

induction of the high output nitric oxide system in human fetal astrocytes.

#### Type II NOS protein expression in human fetal astrocytes

The expression of type II NOS protein in cytokine-stimulated astrocyte cultures was examined by immunoblotting and immunocytochemistry. Using a mAb against murine macNOS, a single band of approximately 130 kDa was detected following immunoblotting of cell lysates from IL-1 $\beta$  + IFN- $\gamma$ -stimulated human fetal astrocytes. This band co-migrated with a control murine macNOS sample obtained from the same source as the Ab (data not shown). Unstimulated cultures did not express type II NOS protein (Fig. 3). Kinetic analysis of protein expression in stimulated astrocyte cultures showed barely detectable levels at 24 h, but high levels at 48 and 72 h (72 h > 48 h).



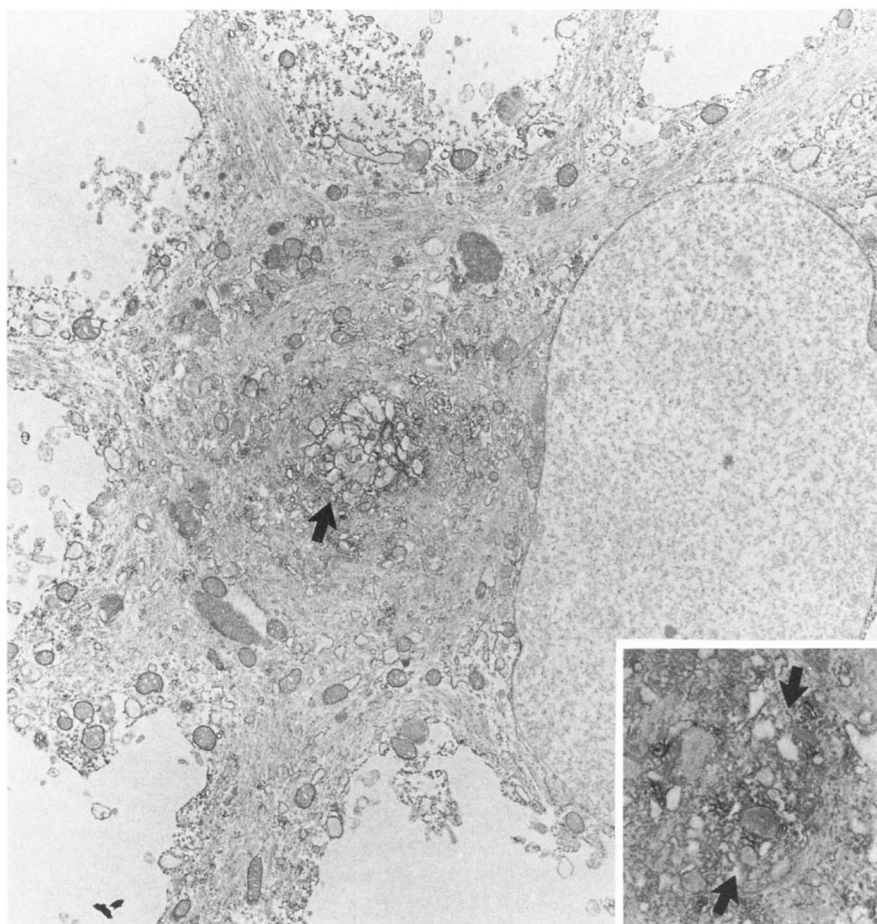
**FIGURE 4.** NOS II immunocytochemistry in primary human astrocytes. Cultures were stimulated with IFN- $\gamma$  + IL-1 $\beta$  for 72 h, and immunostained using rabbit anti-human NOS II Ab (a) or the same Ab preincubated with specific NOS II peptide (b). a, Astrocytes show diffuse staining of cell bodies and processes, as well as intense dot-like paranuclear staining. Inset is a higher magnification of a positively stained cell. b, Preincubation of Ab with NOS II peptide abrogated specific immunoreactivity.

To examine cellular and subcellular localization of type II NOS expression, enriched astrocyte cultures were examined by immunocytochemistry. Both macNOS mAb and a polyclonal Ab raised against a human type II NOS peptide revealed identical results. No specific staining was present in unstimulated astrocytes. In IL-1 $\beta$  + IFN- $\gamma$ -stimulated astrocytes, type II NOS immunoreactivity was localized to the cell body and processes of astrocytes (Fig. 4a). In addition to diffuse cytosolic staining, distinct dot-like paranuclear type II NOS immunoreactivity was evident in stimulated astrocytes (Fig. 4a), which on immunoelectron microscopy was localized to Golgi-like membranes (Fig. 5). Preincubation of Ab with specific type II NOS peptide abrogated immunoreactivity (Fig. 4b).

#### Expression of type II NOS mRNA and nitrite in human fetal microglia

Human fetal microglia obtained from the same sources were examined in parallel with astrocytes for the expression of type II NOS mRNA, protein, or nitrite production. The results from repeated experiments showed that there was no detectable type II NOS expression in human microglia when examined by Northern blot analysis, protein analysis, or nitrite assay. The stimuli used were those that induced type II NOS expression in human fetal astrocytes, as well as murine macrophages. Microglia from at least three different brains were examined at time points from 6 to 16 h after post cytokine stimulation. Human microglia incubated with media alone or with LPS, IFN- $\gamma$  +

**FIGURE 5.** NOS II immunoelectron microscopy in primary human astrocytes. Astrocytes were stimulated with IL-1 $\beta$  + IFN- $\gamma$  for 72 h, and immunostained for NOS II using a peroxidase-conjugated Ab and DAB. Cells were embedded in epon-araldite as a monolayer, and ultrathin sections were examined without counterstaining. Immunoelectron microscopy revealed that the dot-like paranuclear staining (Fig. 4) is associated with a Golgi-like structure (arrows). The nucleus to the right, as well as the intermediate filaments in the cytoplasm, are negative. Inset is a higher magnification of the immunoreactive paranuclear Golgi-like vesicles of a different cell.

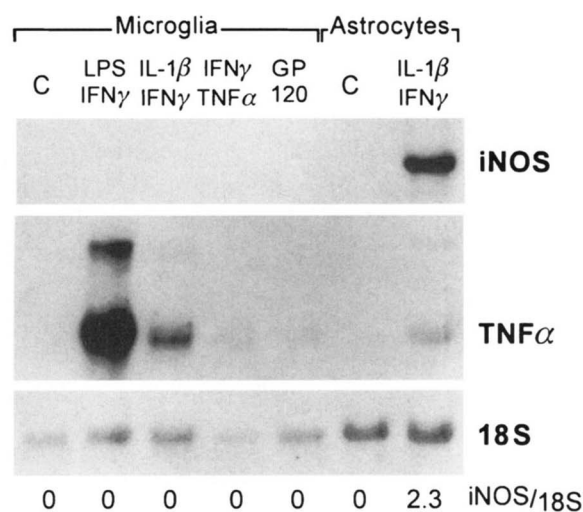


LPS, IFN- $\gamma$  + TNF- $\alpha$ , IL-1 $\beta$  + IFN- $\gamma$ , or HIV-1 envelope protein gp120 did not induce expression of type II NOS mRNA (Fig. 6), whereas human fetal astrocytes stimulated with IL-1 $\beta$  + IFN- $\gamma$  and examined in parallel readily showed expression of type II NOS mRNA (*last lane of Fig. 6*). Repeated experiments with microglia for nitrite assay ( $n = 7$ , time points covering 48 h to 7 days poststimulation) failed to show amounts of nitrite in stimulated cultures that exceeded those in control cultures (1–2  $\mu$ M, lower limit of Griess reaction). Similarly, human microglia stimulated with various combinations of cytokines and LPS and examined for type II NOS protein expression by immunocytochemistry failed to show positive reactivity (data not shown).

To ensure that the microglial cultures were responding appropriately to LPS and cytokine stimulation, the blots were stripped and probed for expression of TNF- $\alpha$ . Previous studies have shown clearly that human fetal microglia (25), like mature peripheral monocytes and macrophages, can be induced by LPS and cytokines to express TNF- $\alpha$  mRNA and protein. As shown in Figure 6, prominent induction of TNF- $\alpha$  mRNA followed exposure to LPS and/or cytokines, indicating that the cells in these cultures were viable and of sufficient maturity to respond appropriately to these induction signals.

## Discussion

In this work, we have assessed the ability of the proinflammatory cytokines IL-1 $\beta$ , TNF- $\alpha$ , and IFN- $\gamma$ , as well as LPS to induce the expression of mRNA, protein, and enzyme activity for the inducible form of NOS (type II NOS) in cultures of human fetal astrocytes and microglia. The results showed that



**FIGURE 6.** Northern analysis of NOS II expression by primary human fetal microglia. Microglia and astrocytes were treated with cytokines (combinations are given above each lane) for 6 h at doses described in Figure 1 legend. Recombinant gp120 was used at 2  $\mu$ g/ml. Total RNA isolated from  $1.5$  to  $2.0 \times 10^6$  cells was loaded in lanes 1 to 5. Twenty micrograms of astrocyte RNA were loaded in lanes 6 and 7. Blots were hybridized with human hepatocyte NOS II probe, human TNF- $\alpha$  probe, and 18S rRNA probe. Ratios of densitometric values for NOS II and 18S are given under each lane.

whereas none of the combinations of cytokines and endotoxin tested was able to induce type II NOS expression in human fetal microglia, astrocytes cultured from the same source could be

induced readily by cytokines to express a high output NO system. Endotoxin either alone or in combination with these cytokines had no demonstrable effect on type II NOS expression. The absence of an endotoxin effect on type II NOS expression in cells of human origin has been noted in other cell types such as pulmonary epithelial cells (17). The lack of effect is in contrast to rat astrocytes (28), a difference that is probably attributable to the absence of the LPS receptor CD14 in human astrocytes and epithelial cells. In addition, neither IFN- $\gamma$  nor TNF- $\alpha$ , alone or in combination, was able to induce type II NOS expression. Together with the data obtained with the IL-1Ra, the results indicate that IL-1 was the key proinflammatory cytokine involved in NOS induction in human astrocytes, with IFN- $\gamma$  acting as the most important synergist. In inflammatory conditions in the human CNS, both resident glia and invading inflammatory cells may be a source for these proinflammatory cytokines. Microglia are a potent source of IL-1 and TNF- $\alpha$  (25, 29). Astrocytes can also be induced to express TNF- $\alpha$  following stimulation with IL-1 and IFN- $\gamma$  (25, 30). Since we have shown that TNF- $\alpha$  further increased the expression of type II NOS in astrocytes, these data suggest that an autocrine loop may further up-regulate type II NOS expression in these cells, a conclusion supported by the observation that Abs to TNF- $\alpha$  reduced the amount of nitrite produced in cultures stimulated with IL-1 $\beta$  + IFN- $\gamma$  (Amaral and Lee, unpublished observations). The expression of IFN- $\gamma$  by astrocytes remains controversial at the present time (31), and the most likely source of this cytokine is from infiltrating T cells and NK cells.

Perhaps the most surprising finding of these studies was the lack of inhibition of human astrocyte type II NOS expression by TGF- $\beta$ , IL-4, or IL-10 when tested over a wide range of doses and with different pretreatment protocols. A strong signal for MCP-1 could be detected in all of the cultures exposed to TGF- $\beta$ , as well as in those exposed to IL-10 and IL-4, excluding the possibility that these cultures do not respond to these cytokines. TGF- $\beta$  has been shown to be an important regulator of type II NOS in murine macrophages (32), as well as in rat astrocytes and microglia (33). IL-4 has also been shown to down-regulate type II NOS expression in activated macrophages (34), rat astrocytes (7), and microglia (35). IL-10 has been shown to inhibit cytokine- and LPS-induced NOS expression in rat astrocytes (7), as well as IgE-mediated NOS induction in normal human keratinocytes (16). Interestingly, in the latter system IL-4 is a potent inducer of type II NOS expression, since this cytokine up-regulates the CD23 receptor involved in IgE-mediated NOS activation. These discrepancies in the pattern of cytokine-mediated regulation, therefore, most likely reflect differences in the activating signals required for type II NOS expression in these different cell types or species-specific differences in the response. In a comparison of the effect of regulatory cytokines on different inducing signals, Simmons and Murphy (7) showed in rat astrocytes that IL-4 and IL-10 differentially inhibited type II NOS expression, depending on whether LPS or cytokines were used in the induction protocol. In murine macrophages, it also has been shown that TGF- $\beta$  and IL-10 differentially inhibited cytokine expression, depending on the induction signals used (36). In a direct comparison of species-specific differences in hepatocyte expression of type II NOS, Nussler et al. (37) showed that whereas TGF- $\beta$  potently down-regulated type II NOS expression in murine cells (~70%), down-regulation of type II NOS expression in rat or human cells by this cytokine was only marginal (~17% and 10%, respectively), and in human cells the values were not statistically significantly different from control cultures not ex-

posed to TGF- $\beta$ . Neither IL-4 nor IL-10 affected type II NOS expression in any of the three species.

In contrast to the lack of regulation by TGF- $\beta$ , IL-4, and IL-10, almost complete inhibition of type II NOS induction could be achieved in these astrocyte cultures by the addition of the IL-1Ra. The IL-1Ra is produced primarily by monocytes/macrophages and neutrophils in response to LPS, cytokines, and immune complexes (27). IL-1Ra binds to the type I and type II IL-1R and inhibits the binding of both IL-1 $\alpha$  and IL-1 $\beta$  (38, 39). The expression and regulation of IL-1Ra in the brain are not known, but preliminary studies have shown that in human microglia, IL-4 and, to a lesser extent, TGF- $\beta$  stimulate expression of IL-1Ra without stimulating IL-1 $\beta$  (Amaral and Lee, unpublished). Thus, it appears that there is a paracrine loop between human astrocytes and microglia in providing signals for activation and deactivation of astrocyte type II NOS expression (25, 40). Factors that regulate the expression of components of the IL-1 system in microglia (which in turn influence the net balance favoring either agonist or antagonist activity in the surrounding CNS environment) will, therefore, determine astrocyte type II NOS expression.

The kinetic studies of astrocyte type II NOS expression suggested that once stimulated by IL-1 $\beta$  and IFN- $\gamma$ , type II NOS mRNA continued to accumulate through 48 h, and significant levels were sustained through 72 h. Total type II NOS protein content also increased through 72 h. Significantly, nitrite levels also steadily rose through 7 days poststimulation (14), the latest time point examined, suggesting that type II NOS protein, once induced, may remain active as long as 7 days. In fact, human fetal astrocytes continued to produce nitrite in the presence of cycloheximide (1  $\mu$ g/ml) when added 48 h after stimulation with IL-1 $\beta$  + IFN- $\gamma$ , the levels reaching 93% of those in control cultures at 96 h in two separate experiments. Interestingly, murine type II NOS has also been shown to be active as long as 5 days in culture if the substrate L-arginine continues to be replenished (41). Factors determining the stability of type II NOS mRNA, protein, and the enzyme activity are not known, but in murine macrophages, TGF- $\beta$  and nitric oxide (32, 41) itself have been implicated. In human astrocytes, neither appears to affect type II NOS expression, as judged by the lack of inhibition of nitrite production by TGF- $\beta$ , and the kinetics of type II NOS expression demonstrating elevated levels of mRNA, protein, and nitrite as long as 72 h, 72 h, and 7 days, respectively (14) (also see *Results*). The latter argues against a role for endogenous nitric oxide as a feedback inhibitor of type II NOS expression in human astrocytes.

It has been demonstrated in this study that immunolocalization of induced NOS II protein in human astrocytes showed intense staining at a paranuclear region, in addition to diffuse cytosolic staining. Immunoelectron microscopy demonstrated the association of paranuclear type II NOS immunoreactivity with Golgi-like membranes and other vesicles. Interestingly, analysis of type II NOS in primary murine macrophages reported particulate NOS, in addition to cytosolic NOS, that localized to the perinuclear *trans*-Golgi network and other vesicles (42). Despite the apparent shift in molecular mass (130–135 kDa) and tight association with membranes, no unique protein modifications or alternative splicing could be detected in the vesicular variant of type II NOS. In primary human astrocytes, a single type II NOS-immunoreactive band was detected on Western blot analysis, suggesting that subcellular compartmentalization of NOS II did not reflect the change in molecular mass. However, further biochemical analysis of astrocyte NOS II is required for confirmation.

The relevance of these observations to human pathology remains to be defined. In multiple sclerosis lesions, Bo et al. detected a strong signal for type II NOS mRNA and identified hypertrophic astrocytes as the major source of NOS using NADPH-diaphorase histochemistry (43). We also detected NADPH-diaphorase reactivity in hypertrophic astrocytes in MS lesions, as well as a diffuse reactivity associated with the lesion center that possibly reflected activity in activated macrophages (44). Bagasra et al. (45) have also examined type II NOS expression in MS lesions. Using *in situ* PCR, they identified macrophages as the source of type II NOS reactivity. These differences in the distribution of NOS activity may reflect differences in the isoform of NOS present in the lesions, or may reflect differences in the age of the lesions examined, since hypertrophic astrocytes (as opposed to fibrous astrocytes) are more a feature of acute rather than chronic lesions. Bukrinsky et al. (10), in a study of patients with AIDS, detected type II NOS in the CNS of patients with severe HIV encephalitis using reverse-transcriptase PCR. Although in that study cellular localization was not performed, *in vitro* studies have shown that human monocytes/macrophages can be induced to express a low output NOS system (2–5  $\mu$ M of nitrite) following infection with HIV, with endotoxin and cytokines acting synergistically. In addition to astrocytes and microglia, immunohistochemical studies using an Ab raised to human type II NOS have also detected the presence of immunoreactivity in a subpopulation of degenerating neurons in patients with Alzheimer's disease (46). Although reactive astrocytes and microglia are elements of senile plaques in Alzheimer brains, neither showed type II NOS immunoreactivity in that study. The results of these studies strongly support the conclusion that type II NOS is up-regulated in the CNS in association with various forms of pathology, and suggest that both astrocytes and inflammatory macrophages may be a significant source of enzyme activity. The results of the experiments reported in this work indicate that the cytokine signals that control type II NOS expression in human astrocytes are different from human macrophages as well as from rodent astrocytes, reflecting both species-specific and cell-type-specific regulatory elements. These data indicate that an understanding of the positive and negative regulatory elements involved in NOS regulation in astrocytes is an important area of research, and that further investigations into these regulatory signals are clearly warranted.

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