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TGFβ1 and TNFα potentiate nitric oxide production in astrocyte cultures by recruiting distinct sub-populations of cells to express NOS-2

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Abstract

Nitric oxide (NO) synthase-2 (NOS-2), a key source of NO at sites of neuroinflammation, is induced in astrocyte cultures treated with lipopolysaccharide (LPS) plus interferon-γ (IFNγ). A recent study examining the regulation of astrocytic NOS-2 expression demonstrated that transforming growth factor-β1 (TGFβ1) potentiated LPS plus IFNγ-induced NOS-2 expression via expansion of the pool of astrocytes that express NOS-2. Results in the current report indicate that this population-based mechanism of increasing NOS-2 expression is not restricted to TGFβ1, since it also accounts for the potentiation of NO production in astrocyte cultures by tumor necrosis factor-α (TNFα). In contrast to TGFβ1, which required 24 hr preincubation for optimal potentiation of NO production, TNFα was maximally effective when added concurrently with LPS plus IFNγ. Nevertheless, under conditions that optimally potentiated NO production, both cytokines recruited similar numbers of astrocytes to express NOS-2 (% NOS-2-positive cells after LPS plus IFNγ alone or with TNFα or TGFβ1 was 9.5 ± 1.2, 25.3 ± 2.9, and 32.4 ± 3.0, respectively). Interestingly, stimulation of astrocytes in the presence of both TGFβ1 and TNFα additively increased the number of astrocytes that expressed NOS-2 protein (% NOS-2- positive cells was 61.0 ± 4.2) relative to each cytokine alone. Potentiation of NO production by either TNFα or TGFβ1 was not ablated by neutralizing antibodies to TGFβ1 or TNFα, respectively. Thus, the two cytokines act independently to recruit separate pools of astrocytes to express NOS-2. These results are consistent with the notion that astrocytes possess an innate heterogeneity with respect to responsiveness to these cytokines.

Keywords

LPS; cytokines; heterogeneity; NO; iNOS; nitric oxide synthase

Nitric oxide (NO) is an endogenously derived free radical gas that affects a spectrum of homeostatic and physiologic processes, including blood flow and coagulation, normal brain and heart function, and innate immunity (Belge et al. 2005; Ignarro et al. 1999; MacMicking et al. 1997; Yun et al. 1996). However, it can be harmful under certain pathophysiological conditions. Within the CNS, aberrant NO production has been linked to cerebrovascular dysfunction (Thiel and Audus 2001), pathogenesis of autoimmune-mediated demyelination (Smith and Lassmann 2002), and neurodegeneration associated with cerebral ischemia as well

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as Parkinson and Alzheimer diseases (Iadecola et al. 1997; Liberatore et al. 1999; Nathan et al. 2005). The cytotoxic actions of NO involve oxidative mechanisms that are precipitated by reactive metabolites, such as s-nitrosothiol and/or peroxynitrite (Moncada and Bolanos 2006), which can cause functional alterations in the cytoplasm (Chung et al. 2005; Chung et al. 2004), endoplasmic reticulum (Uehara et al. 2006), mitochondria (Barsoum et al. 2006; Moncada and Bolanos 2006), and nucleus (Dawson and Dawson 2004). Whether NO is beneficial or detrimental depends on the context in which it is produced and on the isoform of nitric oxide synthase (NOS; EC 1.14.13.39) that catalyze its formation (Iadecola 1997).

NO is derived from L-arginine by the catalytic action of three different NOS isoforms (Stuehr 1999). NOS-1 and -3 are constitutively expressed in neurons and endothelial cells, respectively, and thus were initially termed nNOS and eNOS. In contrast, NOS-2 is not constitutively expressed but can be induced in many cell types by inflammatory mediators and, as such, was initially termed inducible NOS or iNOS. NO production by NOS-1 and -3 is coupled to elevated intracellular calcium by transient binding of calmodulin. However, calmodulin is tightly associated with NOS-2 at basal intracellular calcium levels, leading to sustained catalytic activity. The copious amount of NO derived from this isozyme, while an important component of the armamentarium against invading pathogens (MacMicking et al. 1997), accounts for much of the pathophysiology attributed to NO. This notion is supported by results from numerous studies demonstrating that selective inhibition of NOS-2 or NOS-2 gene deletion attenuates the neuropathological sequelae seen in several models of neurological diseases/disorders (Cross et al. 1994; Iadecola et al. 1997; Iadecola et al. 1995; Liberatore et al. 1999; MacMicking et al. 1995; Nathan et al. 2005; Thiemermann et al. 1993).

Astrocytes are among the resident cells of the CNS that express NOS-2 under inflammatory conditions. Inflammatory mediators induce NOS-2 expression in astrocyte cultures *in vitro* (Galea et al. 1992; Hewett et al. 1993; Simmons and Murphy 1992) and NOS-2 has been detected in astrocytes in several CNS inflammatory conditions *in vivo*, including cerebral ischemia and Alzheimer and Parkinson diseases (Acarin et al. 2002; Endoh et al. 1994; Garcion et al. 1998; Katsuse et al. 2003; Kwak et al. 2005; Luth et al. 2002). Importantly, NO from glial NOS-2 has been shown to disrupt the blood-brain barrier (Boveri et al. 2006) and promote or exacerbate excitotoxic neuronal cell death (Bal-Price and Brown 2001; Hewett et al. 1994; Hewett et al. 1996; Vidwans and Hewett 2004), an underlying cause of neuropathology in several neurological diseases (Choi 1988; Coyle and Puttfarcken 1993; Meldrum and Garthwaite 1990). Thus, perturbation of astrocyte function under neuroinflammatory conditions associated with CNS disease or trauma may seriously impinge on the normal CNS function and survival of neurons. These results underscore the need to understand the regulation of NOS-2 expression and function in astrocytes.

Lipopolysaccharide (LPS) in combination with interferon-γ (IFNγ) is a pro-inflammatory stimulus that is frequently used to study the regulation of NOS-2 protein expression and function in cultures of cells, including cortical astrocytes (Bal-Price and Brown 2001; Hewett et al. 1993). Interestingly, previous results demonstrated that only 5–10% of astrocytes in culture could be induced to express NOS-2 protein following exposure to LPS plus IFNγ indicating that these cells were not uniformly responsive to the stimulus (Hamby et al. 2006). Paradoxically, transforming growth factor-β1 (TGFβ1), a cytokine with anti-inflammatory properties, enhanced NO production from astrocytes treated with LPS plus IFNγ and this correlated with a 6-fold increase in the fraction of cells that expressed NOS-2 protein (Hamby et al. 2006). Since TGFβ1 alone did not induce NOS-2, this suggested that TGFβ1 enabled additional astrocytes to respond to LPS plus IFNγ. The question remained whether this population-based upregulation of NOS-2 expression was specific to TGFβ1, or rather, represented a more general mechanism by which NOS-2 expression could be enhanced. Hence, the present study sought to determine if the classical pro-inflammatory cytokine tumor necrosis

factor-α (TNFα) also potentiated NO production by a similar population-based mechanism. The results raise the possibility that the extent to which astrocytes contribute to NO production at sites of neuroinflammation may be dictated not only by the cytokines that stimulate NOS-2 expression but also by an intrinsic heterogeneity among astrocytes in their ability to respond to these cytokines.

EXPERIMENTAL PROCEDURES

Cell Culture

Purified primary astrocyte cultures were prepared from postnatal day 1–3 CD-1 mice as described previously (Hamby et al. 2006) or similarly derived from TNFα gene targeted mutant mice or its recommended wild-type control strain (Stock #s 003008 and 101045, respectively, The Jackson Laboratory). Cerebral cortices were dissected and dissociated aseptically and cells plated in 15 mm 24-well (1–1.5 hemispheres/10 mL/plate; Falcon Primaria, BD Biosciences) or 35 mm 6-well (1.2–1.6 hemispheres/12 mL/plate; Falcon Primaria, BD Biosciences) plates. Plating media consisted of media stock (MS) supplemented with 10% fetal bovine serum (FBS), 10% iron-supplemented calf serum (CS), 50 IU/mL penicillin, 50 μg/mL streptomycin, and 10 ng/mL epidermal growth factor. MS was comprised of modified Eagle's medium (Earle's salt) containing L-glutamine, glucose, and sodium bicarbonate at final concentrations of 2 mM, 25.7 mM, and 28.2 mM, respectively. After ~7 days at 37°C in humidified air containing 6% CO_2, contact-inhibited cell monolayers were cultured with 8 μM cytosine β-D-arabinofuranoside for 5–6 days to antagonize proliferation of microglia. Thereafter, cultures were maintained in MS containing 10% CS, 50 IU/mL penicillin, 50 μg/mL streptomycin and 2 mM L-glutamine and used in studies between 14–31 days after plating. All cultures were treated with 75 mM L-leucine methyl ester for 60 min one day prior to experimentation to further reduce microglia contamination (Hamby et al. 2006). All animal protocols were approved by the Institutional Animal Care and Use Committee of the University of Connecticut Health Center.

Cell Stimulation

The combination of LPS (2 μg/mL; *E. coli* 0127:B8, Difco Laboratories, Inc.) plus IFNγ (3 ng/mL; recombinant mouse; R&D Systems) was used to induce expression of NOS-2 in mouse primary astrocyte cultures since either LPS or IFNγ alone are weak stimuli (Hamby et al. 2006; Hewett et al. 1993; Vidwans et al. 2001). These concentrations were empirically determined to provide a saturating response with respect to astrocytic NO production (Hamby et al. 2006). Stimulation medium consisted of DMEM supplemented with 5% CS, 2 mM L-glutamine, 50 IU/mL penicillin, and 50 μg/mL streptomycin. Some cultures of astrocytes were pre-treated for 24 hr with stimulation medium containing TGFβ1 (3 ng/mL; human recombinant; R&D Systems) or its vehicle (750 ng/mL BSA plus 3 μM HCl) prior to addition of LPS plus IFNγ. To assess the effects of TNFα on NOS-2 expression, cells were stimulated with LPS plus IFNγ concurrently with 0.01–3.0 μg/mL (0.39–116 nM) recombinant mouse TNFα (Alexis Biochemicals). In one study, 3 ng/mL TGFβ1 or 1.0 μg/mL TNFα were administered at various times between 0 and 24 hr prior to LPS plus IFNγ addition. Finally, to examine the possible contribution of endogenous TNFα or TGFβ1 in the potentiation of NO production by TGFβ1 or TNFα, respectively, cultures were treated in the presence of 100 or 30 μg/mL polyclonal anti-TNFα (rabbit IgG fraction, Calbiochem) or monoclonal anti-TGFβ (mouse IgG1, RD Systems) neutralizing antibodies or the appropriate control antibodies.

Assessment of NOS-2 Function

NOS-2-derived nitric oxide (NO) production was assessed indirectly by measurement of nitrite, a stable oxidative breakdown product of NO (Green et al. 1982). Cell culture medium (100 μL) was mixed with an equal volume of Griess Reagent prepared fresh by combining 1 part

0.1% (wt/vol) sulfanilamide in 60% (vol/vol) acetic acid with 1 part 0.1% (wt/vol) napthylenediamine dihydrochloride in distilled H_2O. The absorbance at 550 nm was measured using a microtiter plate reader (Thermolabs).

Assessment of TNFα mRNA Expression

Total RNA was isolated (TRIzol, Life Technologies), purified, and resuspended in 20 μL H_2O. First-strand cDNA was synthesize from 500 ng total RNA using reverse transcriptase and oligo(dT)(12–18) as described previously (Hamby et al. 2006; Hewett et al. 1999). cDNA samples (1 μL) were amplified for 27 (TNFα) or 23 cycles (β-actin) in a Biorad iCycler using Taq DNA polymerase (Invitrogen) and target-specific primers in a total volume of 25 μL. PCR amplimer pairs for mouse TNFα mRNA were 5'-CAGGTCTACTTTGGAGTCATTGC-3' (sense) and 5'-CCAGCATCTTGTGTTTCTGAGTA-3' (antisense). β-actin mRNA was also assessed to control for the amount and the integrity of RNA in each sample. Amplimers for analysis of β-actin mRNA were 5'-GTGGGCCGCTCTAGGCACCAA-3' (sense) and 5'-CTCTTTGATGTCACGCACGATTTC-3' (antisense). PCR products were separated by electrophoresis in 1% agarose along with 1 kb size markers (Gibco/BRL) and visualized by ethidium bromide using a UV transilluminator. Data was recorded using the Kodak Electrophoresis Documentation and Analysis System 120 and processed with Adobe Photoshop.

Quantification of the Percentage of Astrocytes Expressing NOS-2

The number of astrocytes expressing NOS-2 protein was determined by indirect immunofluorescence microscopy as described previously (Hamby et al. 2006). Cultures were fixed for 5 min with a freshly prepared solution of acetone/methanol and then incubated for 7 min with 0.25% Triton X-100 (vol/vol PBS). Cells were washed with PBS and non-specific binding sites blocked with 10% NGS in PBS (25°C, 1 hr). Rabbit polyclonal anti-NOS-2 antibody (2 μg/mL, Upstate Cell Signaling Solutions) was added in PBS containing 5% NGS (4°C overnight). Bound anti-NOS-2 antibody was visualized using Cy3-conjugated goat anti-rabbit IgG antibody (7.5 μg/mL; 25°C, 60 min; Jackson ImmunoResearch). DAPI (28 ng/mL; Molecular Probes) was included during the secondary antibody incubation to label nuclei. Images of three to five microscopic fields (40x magnification final) were acquired from each culture well using a 20x objective (Olympus, 0.4 NA) and a CRX digital camera (Digital Video Camera Co) mounted on an Olympus IX50 inverted microscope outfitted with epifluorescence and processed identically using Adobe Photoshop software. The number of DAPI-labeled nuclei/image was quantified using Scion NIH Image software, while the number of NOS-2 positive cells/image was determined by manual counting as described previously (Hamby et al. 2006). The percent of cells/image that expressed NOS-2 was calculated by dividing the number of NOS-2-positive cells by the total number of cells in each field (i.e. DAPI-labeled nuclei).

GFAP and NOS-2 Co-localization

To assess colocalization of NOS-2 protein with the astrocyte-specific marker, glial fibrillary acidic protein (GFAP), cultures were processed for immunofluorescence microscopy as described above except that cultures were incubated with anti-NOS-2 antibody together with rat monoclonal anti-bovine GFAP antibody (1 μg/mL, Zymed). Primary antibody binding was visualized with Alexa 488-conjugated goat anti-rabbit IgG antibody (10 μg/mL; Molecular Probes) and Cy3-conjugated goat anti-rat IgG antibody (15 μg/mL; Jackson ImmunoResearch), respectively. Nuclei were labeled with DAPI and images were acquired using a 40x objective (Olympus, 0.6 NA) and processed as described above. No cross-reactivity between primary and secondary antibodies from the different species was detected.

Statistical Analysis

Statistics were performed using GraphPad Prism version 4.03 for Windows (GraphPad Software, San Diego). Where necessary, analyses were performed on Log(Y) transformed data. For cell counts, % data were transformed using arcsine square root transformation prior to analysis (Steel and Torrie 1980). Specific tests are indicated in figure legends.

RESULTS

Comparison between TNFα- and TGFβ1-induced potentiation of NO production

Highly enriched primary mouse astrocyte monolayers did not produce NO when treated for 24 hr with 1.0 μg/mL TNFα by itself (nitrite = 0.39 ± 0.05 and 0.20 ± 0.06 μM in untreated and TNFα-treated cultures, respectively). However, when added concurrently with LPS plus IFNγ, TNFα potentiated NO production in a concentration-dependent manner (Figure 1A). Production of NO was enhanced more than 2-fold by 0.01 μg/mL TNFα, whereas 1.0 αg/mL TNFα (38 nM) potentiated NO production nearly to the same extent as a maximally effective concentration of TGFβ1 (3 ng/mL) [Figure 1A, (Hamby et al. 2006)]. The EC_{50} for TNFα was 0.064 ± 0.028 μg/mL (2.5 ± 1.1 nM), which was similar to that reported for C6 glioma cells (Feinstein et al. 1994).

Previous results indicated that optimal potentiation of astrocytic NO production by TGFβ1 required preincubation with the cytokine for 24 hr prior to LPS plus IFNγ administration (Hamby et al. 2006); this was recapitulated herein for the purpose of comparison with TNFα (Figure 1B). In contrast to TGFβ1, potentiation of LPS plus IFNγ-induced NO production by TNFα did not require preincubation. Thus, maximal potentiation was observed when TNFα was added concurrently with LPS plus IFNγ. However, TNFα persisted to effectively potentiate NO production even when administered for up to 24 hr prior to LPS plus IFNγ (Figure 1B).

TNFα expands the pool of astrocytes that express NOS-2

The effect of TNFα on NOS-2 protein expression in astrocyte monolayers was assessed by immunofluorescence microscopy under conditions that optimally potentiated LPS plus IFNγ-induced NO production (identified in Figure 1). TNFα increased the pool of astrocytes that Figure 1 expressed NOS-2 in a manner that closely approximated that elicited by TGFβ1 (Figure 2), in spite of the different exposure conditions for each cytokine (concurrent vs. 24 hr pretreatment, respectively). In this analysis, LPS plus IFNγ induced NOS-2 expression in ~10% of astrocytes and this was augmented to ~25 and 30% by either TNFα or TGFβ1, respectively.

TGFβ1 and TNFα additively increase the population of astrocytes that express NOS-2

Since TGFβ1 and TNFα affect LPS plus IFNγ-induced NO production by a similar population-based mechanism (i.e., augmenting the pool of cells expressing NOS-2), it was of interest to examine whether the cytokines targeted overlapping or separate populations of astrocytes. For example, if they targeted the same population, then treatment with both TGFβ1 and TNFα should not further augment the population of NOS-2-positive cells. On the other hand, if they targeted separate populations of astrocytes, then treatment with both cytokines should further enhance the population of cells expressing NOS-2 to a value that approximates the sum of the two cytokines alone (i.e., be additive). In fact, stimulation of cells with LPS plus IFNγ together with both TNFα and TGFβ1 further augmented the population of NOS-2-positive cells to ~60%. Thus, the effect of the cytokines together was additive relative to each cytokine alone (Figure 2).

While great care was taken to ensure that astrocyte cultures consisted of highly purified GFAP-positive cells (Hamby et al. 2006), it was nevertheless important to confirm that NOS-2 expression after combined treatment with LPS, IFNγ, TGFβ1 and TNFα was localized to

GFAP-positive astrocytes. Results in Figure 3 demonstrate that NOS-2 immunoreactivity in cultures indeed colocalized with GFAP. This, together with the additive nature of TGFβ1 and TNFα, suggests that the two cytokines target different populations of astrocytes within the same monolayer culture.

TNFα and TGFβ1 affect astrocytic NO production independently

The notion that TNFα and TGFβ1 recruit separate populations of astrocytes to respond to LPS plus IFNγ presupposes that TNFα acts independent of endogenous TGFβ1 and visa versa. wever, TNFα has been shown to induce expression of TGFβ1 (Churg et al. 2002; Sullivan et al. 2005). Therefore, it was necessary to determine the extent to which endogenous release of TGFβ1 contributed to the potentiation of LPS plus IFNγ-induced NO production by TNFα. This was assessed using an anti-TGFβ antibody that effectively blocked the potentiation of LPS plus IFNγ-induced NO production by 3 ng/mL exogenous TGFβ1 (nitrite = 42.2 ± 2.75 and 8.24 ± 0.59 μM in the presence of 30 μg/ml control and anti-TGFβ antibodies, respectively). The anti-TGFβ antibody significantly reduced NO production induced by LPS plus IFNγ in either the absence or presence of exogenous TNFα (Figure 4A). Moreover, a slight but significant difference remained after subtracting the responses elicited by LPS plus IFNγ alone from the respective antibody groups in the presence of TNFα (inset, Figure 4A). Although these results suggest a modest contribution of endogenous TGFβ under both stimulation conditions, the potentiation of LPS plus IFNγ-induced NO production by TNFα is largely independent of endogenous TGFβ1.

Conversely, although TGFβ1 can negatively regulate TNFα production both *in vitro* and *in vivo*, perhaps via induction of tristetraprolin expression (Espevik et al. 1987; Ogawa et al. 2003), it can induce expression of TNFα in certain cell types (Chantry et al. 1989; Grammas and Ovase 2002). Therefore, it was also necessary to determine the extent to which endogenous release of TNFα contributed to the potentiation of LPS plus IFNγ-induced NO production by TGFβ1. This was assessed using an anti-TNFα antibody that effectively neutralized the potentiation of LPS plus IFNγ-induced NO production by 30 ng/ml exogenous TNFα (nitrite = 19.3 ± 1.81 and 3.96 ± 0.44 μM in the presence of 100 μg/mL control IgG and anti-TNFα antibody, respectively). Even though the anti-TNFα antibody attenuated NO production stimulated by LPS plus IFNγ without or with TGFβ1 (Figure 4B), no significant difference in the TGF-β1-induced potentiation of NO production was observed between the antibody treatment groups after the respective values for LPS plus IFNγ alone were subtracted (inset, Figure 4B). Thus, endogenous TNFα does not contribute to the potentiation by TGFβ1. In agreement with this result, TGFβ1 potentiation of LPS plus IFNγ-induced NO production and NOS-2 expression was fully retained in astrocyte cultures derived from TNFα null mice (Table 1). These results are consistent with the observation that TGFβ1 did not induce TNFα mRNA in astrocytes cultured from wild-type CD-1 mice (Figure 5). This latter effect was not due to the lack of ability of these cultures to express endogenous TNFα, since LPS plus IFNγ stimulated a time-dependent increase in TNFα mRNA expression (Figure 5). This likely explains the attenuation of LPS plus IFNγ-induced NO production by the anti-TNFα antibody (Figure 4B).

DISCUSSION

The following conclusions can be drawn from the data reported herein. First, although TNFα has been shown to enhance NOS-2-derived NO production induced by LPS or LPS plus IFNγ (Feinstein et al. 1994; MacNaul and Hutchinson 1993; Pahan et al. 1998), results from the present study are the first to demonstrate that TNFα mediates this enhancement, like TGFβ1, by expanding the pool of astrocytes that express NOS-2. Moreover, these results indicate that this mode of regulating gene expression (i.e., population-based) may be a more

general mechanism by which certain cytokines increase NO production from astrocytes. Second, the two cytokines additively increased the number of astrocytes that expressed NOS-2, suggesting that TGFβ1 and TNFα recruit different populations of astrocytes to express NOS-2. It is remarkable that the two cytokines produced an additive effect when administered together in the same culture preparations, given the well documented antagonism between TGFβ1 and TNFα intracellular signal transduction pathways (Bitzer et al. 2000; Schiffer et al. 2001). This further supports the contention that astrocyte monolayers are composed of two subpopulations that are exclusively responsive to only one of the two cytokines, at least with respect to induction of NOS-2 expression. Third, TGFβ1 and TNFα enhance NOS-2 expression by independent and distinct molecular mechanisms. Thus, neither cytokine affected NOS-2 expression via induction of endogenous production of the other, and TGFβ1 but not TNFα required a pretreatment period for optimal enhancement of NOS-2 expression. Take together, the present results raise the intriguing possibility that different populations of astrocytes may be recruited to produce NO at sites of neuroinflammation depending on the cytokines elaborated, and further, imply that the local milieu (i.e., cytokine composition) may determine the strength of the tissue reaction by controlling the number of astrocytes that respond (i.e., express NOS-2). Implicit in this hypothesis is the notion of astrocyte heterogeneity.

Early histochemical analyses of the CNS suggested that astrocytes could be classified by morphological criteria as radial, fibrous or protoplasmic. These anatomical distinctions indicated that astrocytes were structurally heterogeneous *in vivo* (Somjen 1988). Fibrous and protoplasmic astrocytes, which have since been referred to as type 1 and 2 astrocytes, respectively, also exhibit differences in distribution between white and gray matter and can be distinguished by growth characteristics and antigenic properties as well (Raff et al. 1983). It is now becoming clear that astrocytes from various functionally distinct regions of the CNS (e.g., hippocampus, cerebellum, spinal cord, etc.) differ phenotypically (Blomstrand et al. 1999; Cholewinski et al. 1988;Cholewinski and Wilkin 1988; Cholewinski and Wilkin 1988; Hosli and Hosli 1993; Morga et al. 1998; Morga et al. 1999; Porter and McCarthy 1997; Sousa Vde et al. 2004; Stadlin et al. 1998; Wink et al. 2003). For example, astrocytes in the hippocampus and cerebellum express different levels of glutamate transporter isoforms (Lehre et al. 1995). These results suggest that regional astrocyte heterogeneity may serve a functionally important role (Blomstrand et al. 1999; Hosli and Hosli 1993; Porter and McCarthy 1997; Song et al. 2002; Wilkin et al. 1990; Wink et al. 2003). This is supported by the demonstration that astrocytes contribute to the generation of new neurons in the adult hippocampus, where neurogenesis is sustained throughout life, but not in the adult spinal cord (Song et al. 2002), where it is not.

In addition to regional specialization, there is evidence for localized phenotypic diversity among astrocytes even within a given region of the CNS (e.g., within the hippocampus, cerebral cortex, etc.) (Bowman et al. 2003; Conti et al. 1994; Hamby et al. 2006; Hosli and Hosli 1993; Imura et al. 2006; Matthias et al. 2003; Miller and Szigeti 1991; Nikcevich et al. 1997; Porter and McCarthy 1995; Shao et al. 1994; St-Pierre et al. 2000; Swanson et al. 1997; Venance et al. 1998; Whitaker-Azmitia et al. 1993; Zhou and Kimelberg 2001). For example, both *in vitro* and *in vivo* studies show that the expression of glutamate transporter isoforms GLT-1 and GLAST segregate to different hippocampal GFAP-positive astrocytes (Conti et al. 1998; Perego et al. 2000; van Landeghem et al. 2006). Also, within a hippocampal slice culture, Matthias et al. reported the existence of two mutually exclusively subpopulations of astrocytes which exhibit morphologically and functionally distinct properties (Matthias et al. 2003). The results from the current report now suggest that astrocytes cultured from the cerebral cortex are also comprised of different subpopulations, as defined by the ability to express NOS-2 when exposed to either TGFβ1 or TNFα, thus providing additional evidence for the existence of localized astrocyte heterogeneity. The fact that these putative subpopulations accounted for

only approximately twothirds of the total population suggests that there is a third population that is not recruited by either factor.

It has been argued that functional heterogeneity is not an innate property of astrocytes but instead reflects plasticity of a single astrocyte population that is tailored to suit the needs of different environments within the CNS (Walz 2000). In support of this, differences in astrocytic glutamate transporter expression are influenced by neurons, which appear to affect expression levels in an activity-dependent manner (Gegelashvili et al. 1997; Perego et al. 2000; Schlag et al. 1998; Swanson et al. 1997). On the other hand, regional differences in the neurogenic activity of astrocytes (Song et al., 2002), as well as the local differences in NOS-2 expression reported herein, were observed in monolayers of purified primary astrocytes cultured in a homogeneous environment devoid of neurons. These observations suggest that certain forms of astrocyte heterogeneity may indeed be innate or, in any case, are stable in culture.

How the heterogeneity in cytokine responsiveness arises within an astrocyte population remains to be determined. In culture, small numbers of seed cells expand clonally and coalesce to form confluent monolayers of astrocytes. Thus, it might be posited that different precursors could give rise to subpopulations of astrocytes with different phenotypic characteristics. However, preliminary results suggest that heterogeneity in response to cytokines occurs early in culture within individual colonies of astrocytes (unpublished observation). This argues against the role for phenotypic diversity between clonal subpopulations. Indeed, this result demonstrates that cells from individual precursors diverge during the proliferative growth phase in culture prior to becoming contact inhibited. It is possible that this phenotypic divergence within an astrocyte population is analogous to the heterogeneity in antibiotic resistance exhibited by bacteria [referred to as “persisters”, (Balaban et al. 2004)] and may be a manifestation of the postulated “extrinsic noise” by which phenotypic diversity may be generated within a population (Newman and Weissman 2006; Raser and O'Shea 2005). Regardless, further studies are necessary to identify the molecular determinants of this heterogeneity.

One obvious possible molecular explanation for the heterogeneity is the partitioning of TNFα and TGFβ1 cell surface receptors to non-overlapping subpopulations of astrocytes. TNFα signals through type-I (TNFR-I/CD120a) or type-II (TNFR-II/CD120b) cell surface receptors (Baud and Karin 2001). However, whereas rodent astrocytes express TNFR-I, they reportedly lack detectable TNFR-II (Aranguez et al. 1995; Dopp et al. 1997). Intracellular signaling by TGFβ1 in most cells is dependent on a heteromeric complex of type I and II receptors (Lebrin et al. 2005). A preliminary analysis using standard immunofluorescence microscopy found that TNFR-I and both TGFβ1 receptor chains were widely distributed among astrocytes of the monolayer cultures. Indeed, the distribution was much greater than the 25 to 30% of cells recruited to express NOS-2 by the respective cytokines (unpublished observations). This argues that the heterogeneity in cytokine responsiveness is not due simply to the segregation of signaling receptors to distinct astrocyte subpopulations and suggests that the molecular mechanism may be more complex, perhaps involving intracellular signal transduction pathways that enable or silence NOS-2 expression in a given subpopulation. The observation that optimal enhancement of LPS plus IFNγ-induced NO production by TGFβ1 required 24 hr preincubation with the cytokine, whereas TNFα was maximally effective when given simultaneously with the stimulus, is consistent with a fundamental difference in the signaling mechanisms by which these two cytokines recruit their respective populations of astrocytes to respond to LPS plus IFNγ. For example, it is possible that TGFβ1 requires *de novo* protein synthesis to enable a population of astrocytes to express NOS-2, whereas TNFβ does not.

Further studies to elucidate the molecular determinants that underlie the population-based changes in NOS-2 expression reported herein are needed. Such information should contribute to a better understanding of astrocyte biology and will likely lead to the identification of novel molecular markers that could be employed to distinguish subpopulations of astrocytes. It will also be important to determine whether such heterogeneity applies to inducible genes other than NOS-2 and to cell types other than astrocytes. Finally, although the functional significance of this heterogeneity remains to be clarified, it may have relevance at sites of neuroinflammation where NO contributes to neuropathology, including cerebrovascular dysfunction (Boveri et al. 2006; Mark et al. 2004; Mayhan 1998) and neurodegeneration (Hewett et al. 1994; Iadecola et al. 1997). In any case, the results reported herein from cultures of astrocytes may explain, at least in part, the apparent heterogeneous expression of NOS-2 in astrocytes *in vivo* under neuroinflammatory conditions (Iravani et al. 2005; Luth et al. 2001; Oleszak et al. 1998).

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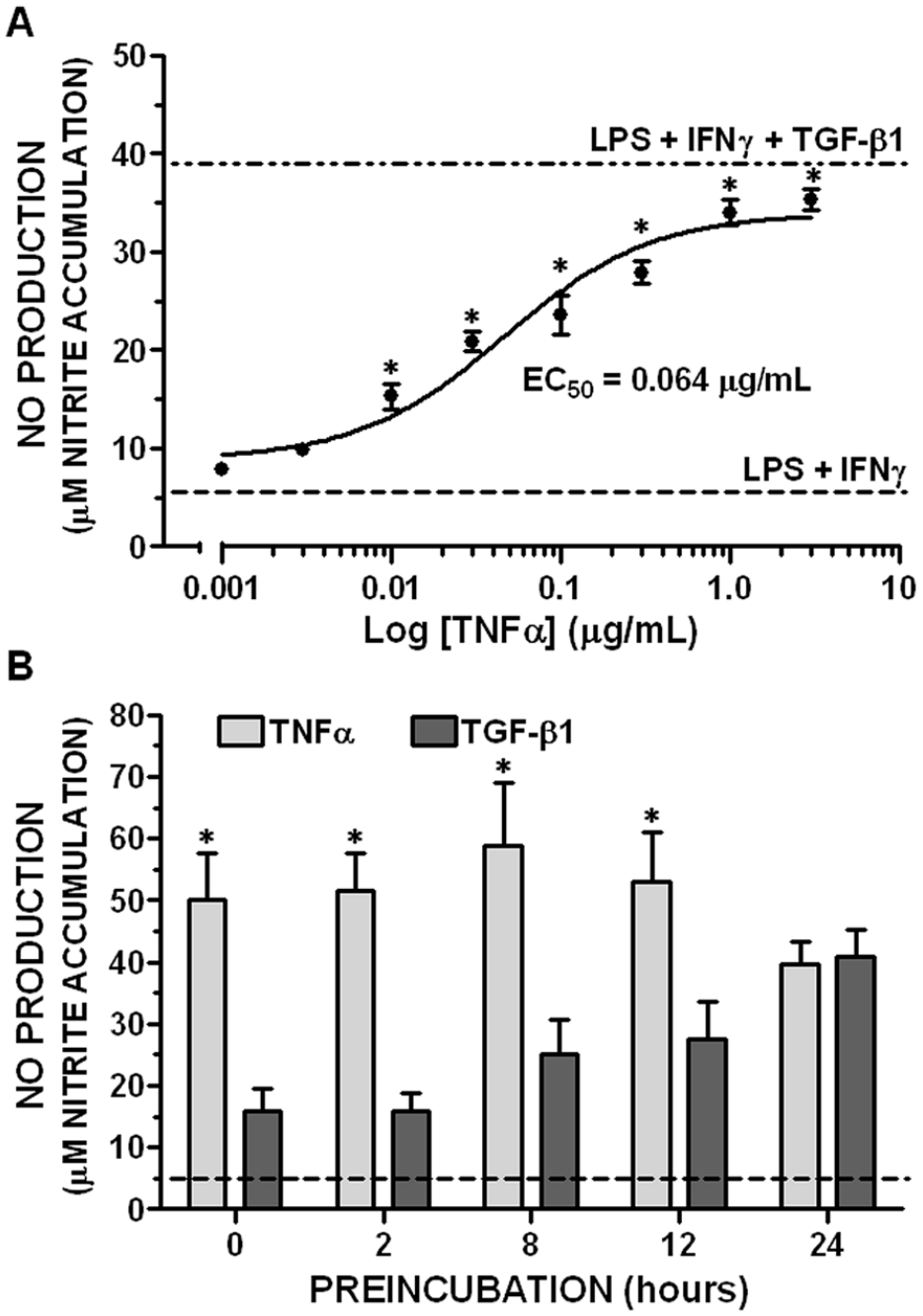

Figure 1. Characterization of TNFα-induced potentiation of astrocytic NO production

A. TNFα concentration response. Primary astrocyte monolayers were treated concurrently with LPS plus IFNγ (2 μg/mL and 3 ng/mL, respectively) and the indicated concentrations TNFα. NO production was assessed by nitrite accumulation 24 hr later as described in "Experimental Procedures" and results expressed as the mean ± SEM; n = 4–5 cultures from 2 separate dissections. Dashed line ("LPS + IFNγ") = NO production induced by LPS plus IFNγ in the absence of TNFα. Dash-dot line ("LPS + IFNγ + TGFβ1") = parallel cultures treated with 3 ng/mL TGFβ1 for 24 hr prior to stimulation with LPS plus IFNγ.

*, significantly different from "LPS + IFNγ" treatment group (one-way ANOVA, Dunnet's post-test, $p<0.05$).

B. Effect of preincubation. Astrocyte monolayers were treated with TNFα (1 μg/mL) or TGFβ1 (3 ng/mL) for the indicated times prior to addition of LPS plus IFNγ. 0 = concurrent administration with LPS plus IFNγ. NO production (nitrite accumulation) was measured 36 hr after LPS plus IFNγ. Results are expressed as mean + SEM; n = 9 cultures from 3 separate dissections. Dashed line = stimulation with LPS plus IFNγ alone.
*, significant difference between cytokine treatment groups (two-way ANOVA, Bonferroni post-test, $p<0.05$).

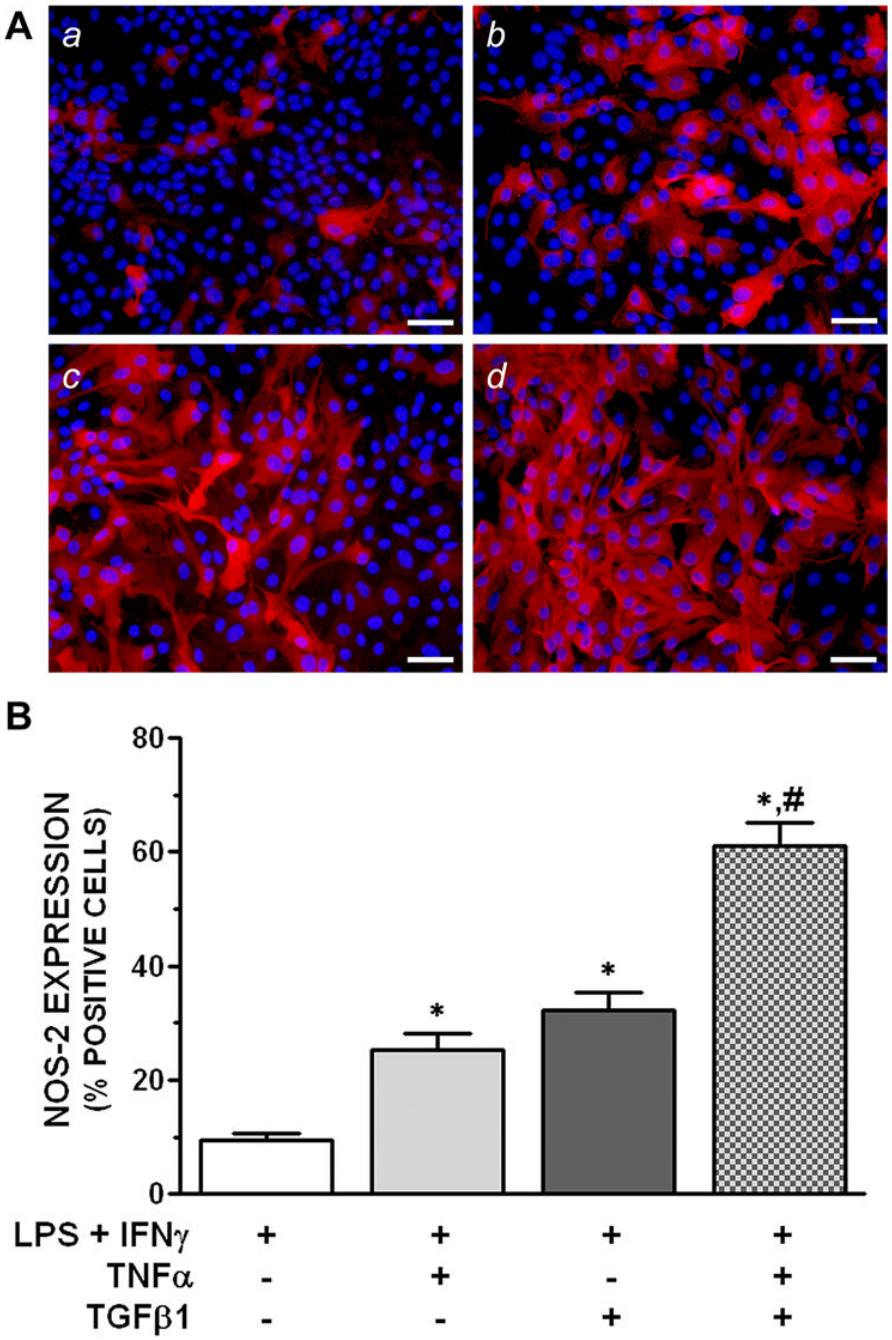

Figure 2. Quantification of the number of astrocytes expressing NOS-2 protein

A. Representative fluorescent images. Astrocyte cultures were treated with LPS plus IFNγ (2 μg/mL and 3 ng/mL, respectively) without or with 1 μg/mL TNFα and/or 3 ng/mL TGFβ1. NOS-2 protein expression was assessed by immunofluorescence microscopy 12 hr after LPS plus IFNγ as described in "Experimental Procedures". TNFα and TGFβ1 were added concurrently with or 24 hr prior to LPS plus IFNγ, respectively. (*a*) LPS + IFNγ alone, (*b*) LPS + IFNγ + TNFα, (*c*) LPS + IFNγ + TGFβ1, (*d*) LPS + IFNγ + TGFβ1 + TNFα. NOS-2 (red) and DAPI (blue) images were obtained from the same microscopic fields and superimposed using Adobe Photoshop. Scale bar = 50 μm.

B. Percent cells expressing NOS-2. The number of cells positive for NOS-2 immunoreactivity is expressed as a % of DAPI-labeled nuclei ("Experimental Procedures"). Results are expressed as means + SEM; n = 9 cultures from 3 separate dissections.
*, significantly different from "LPS + IFNγ" treatment group.
#, significantly different from the effect of each cytokine alone (one-way ANOVA, Student-Newman-Keul's, $p<0.05$).

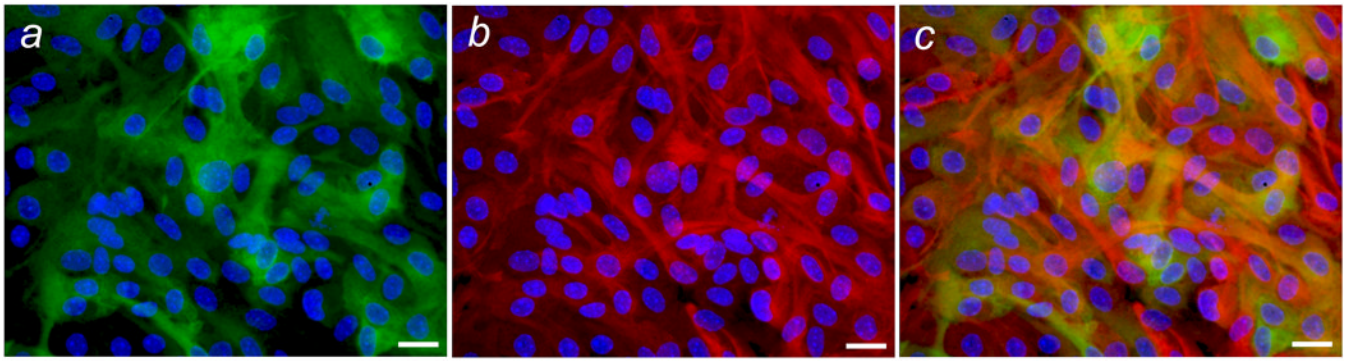

Figure 3. Analysis of colocalization of NOS-2 and GFAP immunoreactivity

Astrocyte cultures were treated with both TNFα and TGFβ1 as described in Figure 2 and exposed to LPS plus IFNγ for 14 hr, after which NOS-2 and GFAP immunoreactivity was assessed by immunofluorescence microscopy. Representative fluorescent images for NOS-2 (green), GFAP (red) and DAPI (blue) were obtained from the same microscopic field and superimposed using Adobe Photoshop. (*a*) NOS-2 and DAPI; (*b*) GFAP and DAPI; (*c*) NOS-2,GFAP, and DAPI. Scale bar = 20 μm.

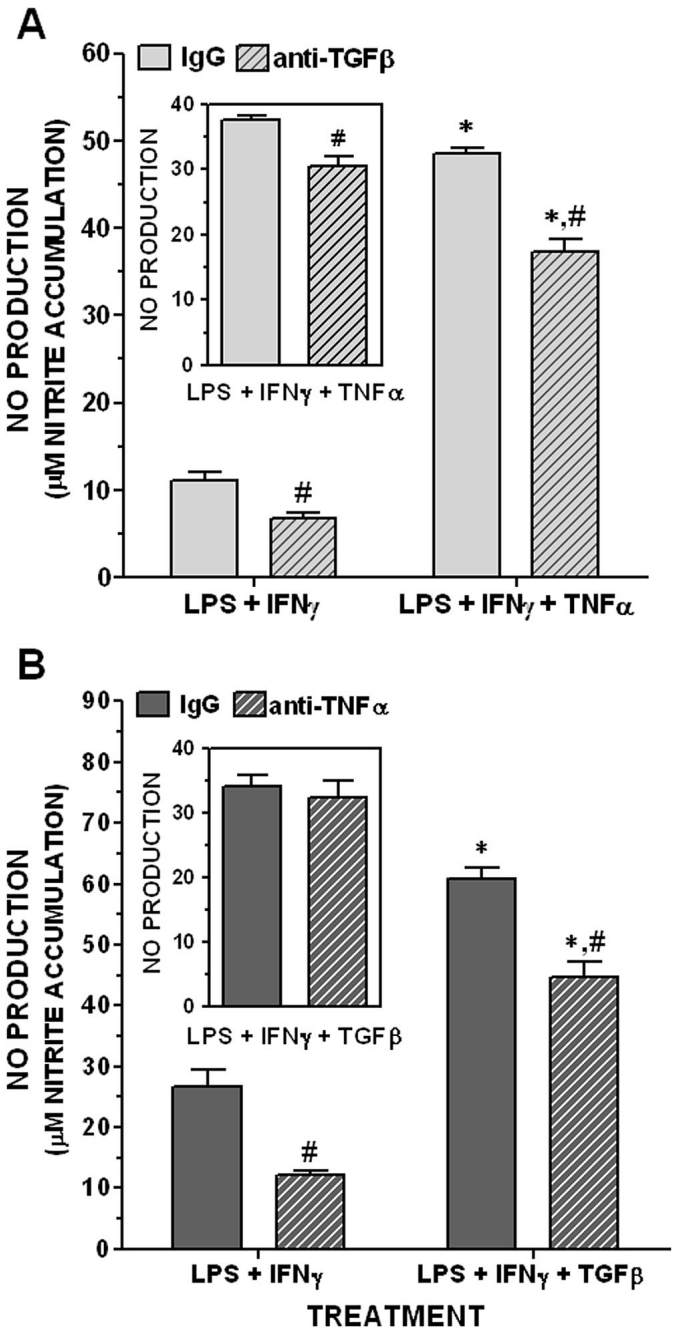

Figure 4. Effect of cytokine neutralizing antibodies on potentiation of NO

A. Effect of TGFβ1 neutralization on TNFα potentiation. Astrocyte cultures were stimulated with LPS plus IFNγ (2 μg/mL and 3 ng/mL, respectively) without or with 1 μg/mL TNFα as described in Figure 2. Stimulation was performed in the presence of 30 μg/mL anti-TGFβ antibody or its non-immune control ("IgG") and NO production was assessed 24 hr later by nitrite accumulation. Inset, values in the presence of TNFα ("LPS + IFNγ + TNFα") after subtracting the respective values in the absence of TNFα ("LPS + IFNγ"). Results are expressed as means + SEM; n = 4 cultures from 2 separate dissections.

*, significant potentiation over respective "LPS + IFNγ" treatment group (two-way ANOVA and Bonforroni post-test, $p<0.05$);

#, significant difference between IgG and anti-TGFβ treatment groups (*t*-test, $p<0.05$).

B. Effect of TNFα neutralization on TGFβ1 potentiation. Astrocyte cultures were pretreated for 24 hrs without or with 3 ng/mL TGFβ1 in the presence of 100 μg/mL of anti-TNFα antibody or its isotype-matched control ("IgG"). Cultures were stimulated 24 hr later with LPS plus IFNγ (2 μg/mL and 3 ng/mL, respectively) and NO production was assessed as in A. Inset, values in the presence of TGFβ1 ("LPS + IFNγ + TGFβ") after subtracting the respective values in the absence of TGFβ1 ("LPS + IFNγ"). Results are expressed as means + SEM; n = 8 cultures.
*, significant potentiation over respective "LPS + IFNγ" treatment group (two-way ANOVA and Bonforroni post-test, $p<0.05$).
#, significant difference between IgG and anti-TNFα treatment groups (*t*-test, $p<0.05$).

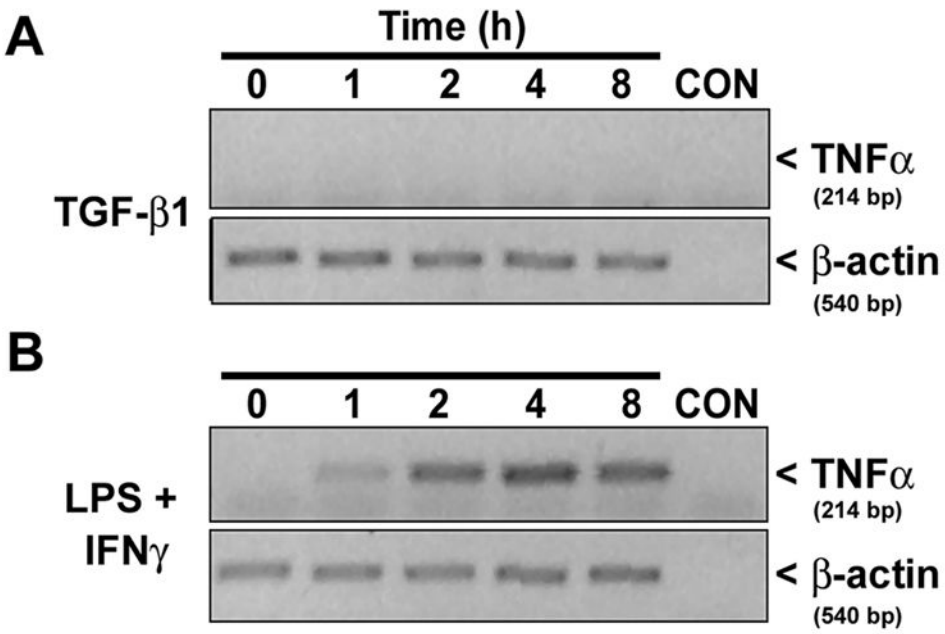

Figure 5. Effect of TGF²1 on TNF± mRNA expression in astrocytes

Astrocyte cultures were treated with either 3 ng/mL TGFβ1 alone (A) or 2 μg/mL LPS plus 3 ng/mL IFNγ (B) and total RNA was harvested at the times indicated. TNFα and β-actin mRNA levels were assessed via RT-PCR (see "Experimental Procedures"). CON = PCR reaction mix minus cDNA. Results are representative of two separate experiments on cultures from independent dissections.

Table 1

Effect of TNFα gene deletion on TGFβ1-induced potentiation

Astrocytes cultured from mice lacking TNFα (TNFα −/−) or their wild-type controls (TNFα +/+) were treated with or without 3 ng/mL TGFβ1 for 24 h prior to stimulation with LPS plus IFNγ (2 μg/mL and 3 ng/mL, respectively). NO production (nitrite accumulation) was quantified and the number of cells positive for NOS-2 immunoreactivity (%) was determined 16 hr later (see “Experimental Procedures”). Data are expressed as the TGFβ1-induced increase in NO production or NOS-2-positive cells relative to LPS plus IFNγ alone (Means ± SEM); n = 3 from 3 separate dissections. Significant differences in NO production or NOS-2 expression between genotypes were assessed using an unpaired *t*-test on data transformed by log or arcsin square root, respectively. No significant differences were detected ($p<0.05$).

Mouse strain	NO PRODUCTION (fold increase by TGF-β1 ± SEM)	NOS-2 Positive Cells (fold increase by TGF-β1 ± SEM)
TNFα +/+	6.18 ± 1.27	9.99 ± 2.08
TNFα −/−	4.79 ± 1.33	13.13 ± 5.35