

Induction of Nitric Oxide Synthase mRNA Expression

SUPPRESSION BY EXOGENOUS NITRIC OXIDE*

(Received for publication, July 27, 1995)

Marco Colasanti[‡], Tiziana Persichini[‡], Marta Menegazzi[§], Sofia Mariotto[§], Emanuele Giordano[¶], Claudio M. Caldarera[¶], Valeria Sogos^{||}, Giuliana M. Lauro^{†**}, and Hisanori Suzuki[§]

From the [‡]Department of Biology, III University of Rome, 00154 Rome, Italy, [¶]Department of Biochemistry, University of Bologna, 40126 Bologna, Italy, ^{||}Department of Cytomorphology, University of Cagliari, 09124 Cagliari, Italy, and [§]Institute of Biological Chemistry, University of Verona, 37134 Verona, Italy

The reactive nitrogen species, nitric oxide (NO), plays an important role in the pathogenesis of neurodegenerative diseases. The suppression of NO production may be fundamental for survival of neurons. Here, we report that pretreatment of human ramified microglial cells with nearly physiological levels of exogenous NO prevents lipopolysaccharide (LPS)/tumor necrosis factor α (TNF α)-inducible NO synthesis, because by affecting NF- κ B activation it inhibits inducible Ca²⁺-independent NO synthase isoform (iNOS) mRNA expression. Using reverse transcriptase polymerase chain reaction, we have found that both NO donor sodium nitroprusside (SNP) and authentic NO solution are able to inhibit LPS/TNF α -inducible iNOS gene expression; this effect was reversed by reduced hemoglobin, a trapping agent for NO. The early presence of SNP during LPS/TNF α induction is essential for inhibition of iNOS mRNA expression. Furthermore, SNP is capable of inhibiting LPS/TNF α -inducible nitrite release, as determined by Griess reaction. Finally, using electrophoretic mobility shift assay, we have shown that SNP inhibits LPS/TNF α -elicited NF- κ B activation. This suggests that inhibition of iNOS gene expression by exogenous NO may be ascribed to a decreased NF- κ B availability.

Nitric oxide (NO)¹ is a major messenger molecule playing key roles in many physiological and pathological processes (1). NO production is catalyzed by at least two major forms of the NO synthase (NOS) enzyme: a constitutive Ca²⁺-dependent NOS isoform (cNOS) and an inducible Ca²⁺-independent NOS iso-

form (iNOS), which is expressed after stimulation with *Escherichia coli* lipopolysaccharide (LPS) and cytokines. Recently, we have demonstrated that LPS and/or TNF α are able to induce iNOS in human ramified microglia leading to a high NO output (2). On the other hand, NO release from mouse microglia is thought to play an important role in neuronal cell death (3–5). In a recent work, Meda *et al.* (6) suggested a possible involvement of NO produced by rat microglia after activation with β -amyloid protein and IFN- γ in the pathogenesis of neuronal degradation occurring with age and in Alzheimer's disease.

Preserving iNOS gene from its undesirable induction may be important for neuronal survival. Down-regulation of iNOS expression was reported to be achieved by some factors such as dexamethasone, interleukin-4, transforming growth factor- β , and basic fibroblast growth factor (7–9). Recently, Griscavage *et al.* (10) have indicated that NO may function as a negative feedback modulation of inducible NO synthase by interacting with enzyme-bound heme in activated rat macrophage cell line. Park *et al.* (11) have recently observed that glial cells in rat endogenously produced NO following cytokine stimulation down-regulated iNOS expression.

However, little is known about the regulatory effects on the mechanism by the variable low concentrations of the available NO before iNOS induction. Recently, we have observed that sodium nitroprusside (SNP), a well known NO donor, elicited inhibition of LPS-induced iNOS expression in rat neutrophils, suggesting a possible suppressive effect on iNOS gene expression by exogenous NO (12). The promoter region of human iNOS gene in vascular smooth muscle cells was shown to contain the consensus sequences for the binding of NF κ B, a nuclear transcriptional factor (13), and iNOS transcription has been recently observed as being regulated by NF κ B activation (14).

Here, we used human ramified microglial cells as a model for a better understanding of the molecular mechanism by which NO regulates its own production. To this respect, we hypothesize that a nearly physiological level of exogenous NO may prevent LPS/TNF α -inducible iNOS mRNA expression by affecting NF κ B activation.

EXPERIMENTAL PROCEDURES

Materials—Oxyhemoglobin was prepared by reduction of bovine hemoglobin (Sigma) with sodium hydrosulfite (Aldrich); authentic NO solution was obtained by a 30-min bubbling of distilled and deoxygenated water with >99.5% pure NO gas (SIAD S.p.A., Bergamo, Italy) at 4 °C. This stock solution is in the low millimolar range (about 2 mM at 25 °C); 1% of stock solution was administered 0.5 h before and every 0.5 h during the experiment. N^ω-Nitro-L-arginine methyl ester, SNP, and *E. coli* LPS (serotype 0127:B8) were from Sigma; TNF α (specific activity, >2 × 10⁷ units/mg of protein) was from Boehringer Mannheim, and L-2,3,4,5-[³H]arginine was from Amersham Corp.

Cell Culture—Pure human ramified microglia derived from human fetal brain (8–18 weeks of gestation) primary cultures were obtained as described previously (2). The cells (48th–52nd passages in culture) were characterized for specific microglial markers such as lysozyme, α_1 -chymotrypsin, Fc receptor, GSA1-B₄, and RCA-1 and for phagocytic activity. The negativity of cells for glial fibrillary acidic protein, GalC, Factor VIII, and neurofilaments demonstrated that there were no contaminating astrocytes, oligodendrocytes, endothelial cells, or neurons (2).

Nitrite Analysis—Nitrite (NO₂⁻) was determined by using the Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride, 2.5% H₃PO₄) in supernatants of 5 × 10⁵ human microglial cells. Ab-

* This work was supported by Consiglio Nazionale delle Ricerche (Progetto Finalizzato Biotecnologia ed Invecchiamento) and 40% MURST (to H. S.) and by 40% GNI, 60% MURST (to G. M. L.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

** To whom correspondence should be addressed: Dept. of Biology, III University of Rome, Via Ostiense 173, 00154 Rome, Italy. Tel.: 39 6 49917587; Fax: 39 6 49917594.

¹ The abbreviations used are: NO, nitric oxide; NOS, NO synthase; cNOS, constitutive Ca²⁺-dependent NOS isoform; iNOS, inducible Ca²⁺-independent NOS isoform; LPS, lipopolysaccharide; TNF, tumor necrosis factor; SNP, sodium nitroprusside; RT-PCR, reverse transcriptase-polymerase chain reaction; bp, base pair(s); GAPDH, glyceral-3-phosphate dehydrogenase.

sorbance was measured at 540 nm, and NO_2^- concentration was determined using sodium nitrite as a standard. Since SNP spontaneously generated nitrite, final concentrations of NO_2^- were estimated by the difference between the SNP-produced NO_2^- levels either in the presence or in the absence of cells.

RT-PCR—Total cellular RNA was purified from 1×10^6 human ramified microglial cells by the method of Chomczynski and Sacchi (15). Briefly, a single extraction with an acid guanidinium thiocyanate/phenol/chloroform mixture was performed. Whole RNA was reverse transcribed into cDNA and amplified for the iNOS gene with *Thermus aquaticus* (Taq) DNA polymerase in a thermal cycler (Perkin-Elmer), as described previously (2). Ten microliters of each PCR product (450 bp) were electrophoresed on 1.5% agarose gel and then visualized after ethidium bromide staining. The mRNA for the constitutive glycerol-3-phosphate dehydrogenase (GAPDH) was examined as the reference cellular transcript. GAPDH mRNA amplification products were present at equivalent levels in all cell lysates. The reaction was performed by using specific primers as described elsewhere (16).

Assay of Microglial iNOS Activity—Human microglial iNOS activity was estimated by measuring the conversion of L-2,3,4,5- ^3H arginine to L-2,3- ^3H citrulline according to the modification of the method described by Bredt and Snyder (17). 1×10^7 microglial cells were homogenized with Ultra-Turrax homogenizer (5-mm blade) for 20 s in 1 ml of a buffer containing 50 mM HEPES, pH 7.4, 1 mM DL-dithiothreitol, 1 mM EDTA, 10 $\mu\text{g}/\text{ml}$ leupeptin, 10 $\mu\text{g}/\text{ml}$ soybean trypsin inhibitor, 10 $\mu\text{g}/\text{ml}$ antipain, and 1 mM phenylmethylsulfonyl fluoride. After centrifugation (10,000 $\times g$, 30 min at 4 $^\circ\text{C}$), an aliquot of the supernatant was added to a reaction mixture of a final volume of 100 μl containing 50 mM HEPES, pH 7.4, 20 nM ^3H arginine, 1 μM arginine, 1 mM NADPH, 1 mM EDTA, 1 mM EGTA, 1 $\mu\text{g}/\text{ml}$ calmodulin, 10 μM FAD, 0.1 mM (6*R*)-5,6,7,8-tetrahydro-1-biopterin, 1 mM dithiothreitol. The effect of NO donor SNP on enzyme activity was examined by either preincubating the supernatant for 2 h with SNP (10 μM) or adding it directly to the assay mixture. The mixture was incubated for 1 h at 25 $^\circ\text{C}$. The reaction was stopped by adding 0.4 ml of a 1:1 slurry of Dowex AG 50W-X8 (Bio-Rad, Na^+ form) in 50 mM HEPES pH 5.5, and, after 15 min of shaking, radioactivity in the supernatant was measured. Enzyme activity was expressed as picomoles of citrulline formed in 1 min by 1 mg of protein. Protein concentration in the samples was determined by the method of Bradford (18).

Electrophoretic Mobility Shift Assay—Nuclear extracts of human microglial cells were prepared according to Schreiber *et al.* (19) in the presence of leupeptin (10 $\mu\text{g}/\text{ml}$), antipain (5 $\mu\text{g}/\text{ml}$), pepstatin (5 $\mu\text{g}/\text{ml}$), and phenylmethylsulfonyl fluoride (1 mM). Protein concentrations of nuclear extracts were determined according to Bradford (18). Eight micrograms of nuclear extract were incubated at room temperature for 30 min with 2–5 $\times 10^4$ cpm of ^{32}P -labeled double-stranded oligonucleotides containing the consensus NF- κB DNA binding site (5'-GATCCA-GAGGGGACTTTCCGAGTAC-3') in a 15- μl reaction mixture containing 20 mM HEPES, pH 7.9, 50 mM KCl, 0.5 mM dithiothreitol, 0.1 mM EDTA, 2 μg of poly(dI-dC), 1 μg of salmon sperm DNA, and 10% glycerol. Products were fractionated on a non-denaturing 5% polyacrylamide gel. In competition assays, 100 \times oligonucleotide competitor was added 15 min before addition of the labeled probe. The intensity of the retarded bands was measured by PhosphorImager SF (Molecular Dynamics).

RESULTS AND DISCUSSION

As described previously, human ramified microglial cells were found to release high NO amounts (2). In fact, we observed that stimulation of 5×10^5 microglial cells with a mixture containing LPS (1 $\mu\text{g}/\text{ml}$) + $\text{TNF}\alpha$ (500 units/ml) for 24 h caused a marked production of nitrite (NO_2^-), the breakdown product of NO (from 3.52 ± 0.26 to 15.54 ± 0.66 nmol ml^{-1} ; Fig. 1), as measured by Griess reaction. This increase was abolished by NOS inhibitor *N*^ω-nitro-L-arginine methyl ester (500 μM) (from 15.54 ± 0.66 to 3.07 ± 0.24 nmol ml^{-1}) (Fig. 1). A 0.5-h pretreatment of human ramified microglial cells with SNP (10 μM) decreased NO production induced by LPS/ $\text{TNF}\alpha$ (from 15.54 ± 0.66 to 4.07 ± 0.72 nmol ml^{-1}) (Fig. 1). However, this effect was not accounted for by direct modification of iNOS enzymatic activity, as determined using ^3H citrulline generation from L- ^3H arginine. In fact, we have observed that human microglial cells treated with LPS (1 $\mu\text{g}/\text{ml}$) + $\text{TNF}\alpha$ (500 units/ml) for 24 h expressed a Ca^{2+} -independent NOS activity (680

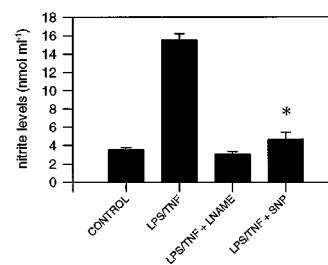


FIG. 1. Effect of SNP on nitrite production in human ramified microglial cells. Nitrite levels were measured using Griess reaction in supernatants of human ramified microglial cells treated with LPS (1 $\mu\text{g}/\text{ml}$) + $\text{TNF}\alpha$ (500 units/ml) for 24 h. A 0.5-h SNP (10 μM) pretreatment of LPS/ $\text{TNF}\alpha$ -treated cells significantly (*, $p \leq 0.01$) decreased nitrite levels. Data are expressed as nmol ml^{-1} . Each bar represents the mean \pm S.E. of four experiments. LNAME, *N*^ω-nitro-L-arginine methyl ester.

pmol/min/mg of protein). When SNP (10 μM) were added directly into the assay mixture or preincubated for 2 h in the enzyme preparations, no significant variations of iNOS activity were observed (data not shown).

The following results provide evidence that NO was able to reduce its own production in human ramified microglial cells by affecting iNOS gene expression. As previously observed (2), using RT-PCR we found that an 8-h treatment of microglial cells with LPS/ $\text{TNF}\alpha$ strongly enhanced iNOS mRNA levels (Fig. 2). When LPS/ $\text{TNF}\alpha$ -treated microglial cells were preincubated with NO donor SNP (10 μM) iNOS gene expression was strongly inhibited (Fig. 2). This effect was significantly reversed by reduced hemoglobin (oxyhemoglobin; 1 μM), a trapping agent for NO. To obtain further evidence that the effect of SNP was mediated by its NO release, SNP was pre-exposed to room light for 2 weeks to prerelease NO, as verified by determination of nitrite levels (data not shown). The resulting cyanide, Fe^{2+} , and nitrite did not interfere with iNOS mRNA induction (Fig. 2). Furthermore, 1% of authentic NO solution was capable of inhibiting iNOS mRNA expression (Fig. 2). Recently, Park *et al.* (11) have observed that NO limited iNOS mRNA expression in rat glial cells when it was produced following stimulation with cytokines. However, we have observed that the early presence (0.5 h) of SNP during cell stimulation with LPS/ $\text{TNF}\alpha$ was essential for iNOS mRNA inhibition. In fact, when SNP was added 4 h after iNOS up-regulation, no inhibition of iNOS mRNA synthesis was observed. Our data indicate that nearly physiological levels of pre-existing exogenous NO may prevent the induction of iNOS gene expression at least in some cell lines such as human microglial cells.

By using electrophoretic mobility shift assay, we found that treatment with LPS/ $\text{TNF}\alpha$ induced the activation of NF- κB , which peaked after a 0.5-h stimulation (Fig. 3), this effect being observed elsewhere for an established mouse macrophage cell line (14). A 0.5-h pretreatment of human microglial cells with SNP (10 μM) was able to inhibit LPS/ $\text{TNF}\alpha$ -elicited NF- κB activation (Fig. 3), suggesting that exogenous NO inhibition of iNOS expression may be due to a decreased NF- κB activation. On the other hand, it has been confirmed recently that in human vascular endothelial cells NO inhibited NF- κB activation, this effect being mediated by induction and stabilization of I- κB (20).

Uncontrolled and massive iNOS-induced NO production was suggested to be potentially detrimental to the tissue integrity (21). The present data indicate that nearly physiological concentrations of NO may keep the iNOS expression suppressed by preventing NF- κB activation. This implies that induction of iNOS expression could be regulated, at least in part, by the endogenous NO level likely to be produced by cNOS enzyme,

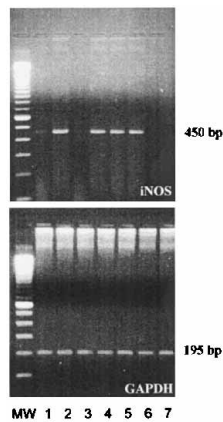


FIG. 2. Effect of NO on iNOS gene expression in human ramified microglial cells. 1.5% agarose gel electrophoresis of the RT-PCR products for iNOS mRNA (450 bp) and for GAPDH mRNA (195 bp) after ethidium bromide staining is shown. *Lane 1*, untreated cells; *lane 2*, an 8-h treatment of 5×10^5 microglial cells with LPS/TNF α strongly enhanced iNOS mRNA levels; *lane 3*, pretreatment of cells with NO donor SNP (10 μ M) inhibited LPS/TNF α -induced mRNA expression; *lane 4*, this effect was reversed by oxyhemoglobin; *lane 5*, sample 2 pretreated with NO-deprived SNP solution; *lane 6*, sample 2 plus SNP added 4 h after iNOS up-regulation; *lane 7*, sample 2 pretreated with 1% of authentic NO solution. PCR products for the GAPDH gene were taken as the reference cellular transcript. Molecular weight is the 100-bp DNA ladder (Life Technologies, Inc.).

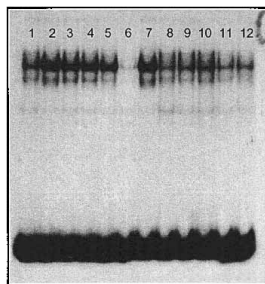


FIG. 3. Effect of SNP on DNA binding activity of NF- κ B transcription factor in human ramified microglial cells. Nuclear extracts of cells were incubated with 32 P-labeled double-stranded oligonucleotide containing the consensus NF- κ B DNA binding site in a reaction mixture. *Lane 1*, untreated cells; *lanes 2-5*, cells treated with LPS/TNF α for 0.5, 1, 2, and 4 h, respectively; *lane 6*, the same samples as for *lane 2* incubated with 100 \times cold NF- κ B as specific competitor; *lane 7*, the same sample as for *lane 2* incubated with 100 \times nonspecific competitor; *lane 8*, control cells pretreated with SNP (10 μ M) for 0.5 h; *lanes 9-12*, LPS/TNF α -treated cells preincubated (0.5 h) with SNP for 0.5, 1, 2, and 4 h, respectively.

although no data concerning this matter are available so far. When the basal level of NO is at a value such as to suppress iNOS expression, the endogenous NO may contribute to avoid

an undesirable and potentially harmful induction of iNOS expression. If the NO level goes down below threshold value, iNOS expression may become easily achievable. In this respect, it seems worthwhile noting that LPS/IFN γ , which induces iNOS gene, is also able to simultaneously decrease cNOS mRNA expression in human monocyte/macrophage cells (22). Furthermore, in endothelial cells TNF α has been shown to regulate cNOS expression by shortening its mRNA half-life (23).

Finally, NO donors could be considered potential drugs for the control of microglial iNOS expression induction. Since the latter seems to play an important role in the pathogenesis of some neurodegenerative diseases, exogenous NO donor could be as potentially active for the prevention of those pathologies.

Acknowledgments—We wish to thank Dr. Marcello Merola for providing oligonucleotides for NF- κ B and L. Mattace for editorial assistance.

REFERENCES

- Moncada, S., Palmer, R. M. J., and Higgs, E. A. (1991) *Pharmacol. Rev.* **43**, 109–142
- Colasanti, M., Persichini, T., Di Pucchio, T., Gremo, F., and Lauro, G. M. (1995) *Neurosci. Lett.*, in press
- Boje, K. M., and Arora, P. K. (1992) *Brain Res.* **587**, 250–256
- Chao, C. C., Hu, S., Molitor, T. W., Shaskan, E. G., and Peterson, P. K. (1992) *J. Immunol.* **149**, 2736–2741
- Dawson, V. L., Dawson, T. M., Bartley, D. A., Uhl, G. R., and Snyder, S. H. (1993) *J. Neurosci.* **13**, 2651–2661
- Meda, L., Cassatella, M. A., Szendrei, G. I., Villalba, M., Ferrari, D., and Rossi, F. (1995) *Nature* **374**, 647–650
- Bogdan, C., Vodovotz, Y., Paik, J., Xie, Q., and Nathan, C. (1994) *J. Leukocyte Biol.* **55**, 227–233
- Vodovotz, Y., Bogdan, C., Paik, J., Xie, Q., and Nathan, C. (1993) *J. Exp. Med.* **178**, 605–613
- Colasanti, M., Di Pucchio, T., Persichini, T., Sogos, V., Presta, M., and Lauro, G. M. (1995) *Neurosci. Lett.* **195**, 45–48
- Griscavage, J. M., Rogers, N. E., Sherman, M. P., and Ignarro, L. J. (1993) *J. Immunol.* **151**, 6329–6337
- Park, S. K., Lin, H. L., and Murphy, S. (1994) *Biochem. Biophys. Res. Commun.* **201**, 762–768
- Mariotto, S., Cuzzolin, L., Adami, A., Del Soldato, P., Suzuki, H., and Benoni, G. (1995) *Br. J. Pharmacol.* **114**, 6–7
- Nunokawa, Y., Ishida, N., and Tanaka, S. (1994) *Biochem. Biophys. Res. Commun.* **200**, 802–807
- Goldring, C. E. P., Narayanan, R., Lagadec, P., and Jeannin, J. F. (1995) *Biochem. Biophys. Res. Commun.* **209**, 73–79
- Chomczynski, P., and Sacchi, N. (1987) *Anal. Biochem.* **162**, 156–157
- Genis, P., Jett, M., Bernton, E. W., Boyle, T., Gelbard, H. A., Dzenko, K., Keane, R. W., Reswnick, L., Mizrahi, Y., Volsky, D. J., Epstein, L. G., and Gendelman, H. E. (1992) *J. Exp. Med.* **176**, 1703–1718
- Bredt, D. S., and Snyder, S. H. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87**, 682–685
- Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
- Schreiber, E., Mattias, P., Muller, M. M., and Schaffner, W. (1989) *Nucleic Acids Res.* **17**, 6419
- Peng, H.-B., Libby, P., and Liao, J. K. (1995) *J. Biol. Chem.* **270**, 14214–14219
- Suzuki, H., Menegazzi, M., Carcereri de Prati, A., Mariotto, S., and Armato, U. (1995) *Adv. Neuroimmunol.*, in press
- Reiling, N., Ulmer, A. J., Duchrow, M., Ernst, M., Flad, H.-D., and Hauschildt, S. (1994) *Eur. J. Immunol.* **24**, 1941–1944
- Yoshizumi, M., Perrella, M. A., Burnett, J. C., and Lee, M. E. (1993) *Circ. Res.* **73**, 205–209

**Induction of Nitric Oxide Synthase mRNA Expression: SUPPRESSION BY
EXOGENOUS NITRIC OXIDE**

Marco Colasanti, Tiziana Persichini, Marta Menegazzi, Sofia Mariotto, Emanuele
Giordano, Claudio M. Caldarera, Valeria Sogos, Giuliana M. Lauro and Hisanori Suzuki

J. Biol. Chem. 1995, 270:26731-26733.
doi: 10.1074/jbc.270.45.26731

Access the most updated version of this article at <http://www.jbc.org/content/270/45/26731>

Alerts:

- [When this article is cited](#)
- [When a correction for this article is posted](#)

[Click here](#) to choose from all of JBC's e-mail alerts

This article cites 21 references, 8 of which can be accessed free at
<http://www.jbc.org/content/270/45/26731.full.html#ref-list-1>