

Bacterial Lipopolysaccharide Plus Interferon- γ Elicit a Very Fast Inhibition of a Ca^{2+} -dependent Nitric-oxide Synthase Activity in Human Astrocytoma Cells*

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Previous results indicate that induction of inducible nitric-oxide synthase (iNOS) expression may be kept suppressed by the endogenous NO level as produced by a constitutive NOS (cNOS) enzyme. In cell types possessing both cNOS and iNOS, this may represent an evident paradox. Here, we report that lipopolysaccharide and interferon- γ , which are able to strongly induce iNOS in astrocytoma cells, can rapidly inhibit the NO production generated by the constitutive NOS isoform, thus obtaining the best conditions for iNOS induction and resolving the apparent paradox. In fact, a 30-min treatment of T67 cells with the combination of lipopolysaccharide plus interferon- γ (MIX) strongly inhibits the cNOS activity, as determined by measuring [^3H]citrulline production. In addition, the effect of MIX is also observed by measuring nitrite, the stable breakdown product of NO: a 30-min pretreatment of T67 cells with MIX is able to reduce significantly the *N*-methyl-D-aspartate-induced nitrite production. Finally, using reverse transcriptase-polymerase chain reaction, we have observed that a 30-min treatment of T67 cells with MIX does not affect expression of mRNA coding for the neuronal NOS-I isoform. These results suggest the novel concept of a possible role of a cNOS isoform in astrocytes as a control function on iNOS induction.

Nitric oxide (NO)¹ is an unstable nitrogen radical which is

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¹ The abbreviations used are: NO, nitric oxide; NOS, NO synthase; cNOS, constitutive Ca^{2+} -dependent NOS isoform; iNOS or NOS-II,

generated in different cell types by the concomitant conversion of L-arginine into citrulline through the enzyme NO synthase (NOS) (1). There are at least three distinct isoforms of NOS present in human cells. Two enzymes, the neuronal and the endothelial Ca^{2+} -dependent isoforms (NOS-I and NOS-III, respectively), are constantly expressed and termed constitutive NOS (cNOS). The third enzyme is an inducible Ca^{2+} -independent isoform (iNOS or NOS-II), which is expressed after stimulation with *Escherichia coli* lipopolysaccharide (LPS) and/or cytokines, such as interferon- γ (IFN γ), interleukin-1 β , or tumor necrosis factor- α . The induction of human iNOS occurs at the transcriptional level (2) and is mediated by the early activation of some nuclear transcriptional factors such as NF- κ B (3), and interferon regulatory factor-1 (4).

NO generated at low levels by a cNOS plays an important role in physiological processes, whereas uncontrolled and massive iNOS-induced NO production can be potentially detrimental to the tissue integrity (5). In particular, it has been observed recently that in the central nervous system NO released from activated microglia and/or astrocytes may be involved in the pathogenesis of neurodegenerative diseases (6, 7). Thus, the suppression of an undesirable and potentially harmful iNOS-elicited NO production can be fundamental for neuronal survival.

Recently, we have reported that in human ramified microglial cells, a nearly physiological level of exogenous NO suppresses the LPS/tumor necrosis factor- α -elicited NO production, by preventing induction of iNOS mRNA expression (8). In addition, we have observed that the early presence of NO donors during induction is essential for inhibition of iNOS expression, the latter being due to a suppression of NF- κ B activation (8). Taken together, these data imply that induction of iNOS expression should be prevented by a pre-existing endogenous NO level, as produced by a cNOS enzyme. Therefore, in the cell types possessing both a Ca^{2+} -dependent and a Ca^{2+} -independent NOS isoforms, *i.e.* astroglial, epithelial, smooth muscle, or endothelial cells, iNOS gene expression may be reasonably assumed not to be induced after LPS/cytokines stimulation, unless the cNOS-generated NO level goes down below a threshold value in a short time.

Here, we have used human astrocytoma cells (T67), as a model of astroglia for a better understanding of the molecular mechanisms of the fine regulation of NOS expression. We hypothesize that LPS and IFN γ , which are able to strongly induce iNOS in T67 cells as reported elsewhere (9, 10), may directly and rapidly inhibit the NO production generated by the cNOS isoform, thus obtaining the best conditions for iNOS induction.

EXPERIMENTAL PROCEDURES

Materials

N^ω-Nitro-L-arginine methyl ester (L-NAME), *E. coli* LPS (Serotype 0127:B8), *N*-methyl-D-aspartate (NMDA), L-arginine (L-Arg) were obtained from Sigma (Milan, Italy). Human recombinant interferon- γ (IFN γ) was supplied by Biogen SA (Geneva, Switzerland; specific activity: 2×10^7 IU/mg protein) and L-[2,3,4,5- ^3H]arginine by Amersham Corp.

inducible Ca^{2+} -independent NOS isoform; NOS-I, neuronal or brain cNOS; NOS-III, endothelial cNOS; LPS, lipopolysaccharide; IFN γ , interferon- γ ; NMDA, *N*-methyl-D-aspartate; L-NAME, *N*^ω-nitro-L-arginine methyl ester; PCR, polymerase chain reaction; bp, base pair(s); GAPDH, glycerol-3-phosphate dehydrogenase.

Methods

Preparation of Astrocytoma Cells—Human astrocytoma T67 cells were obtained from explant of III WHO gemistocytic astrocytoma and were characterized in our laboratory, as described previously (11).

Assay of Astrocytoma NOS Activity—NOS activity was estimated by measuring the conversion of L-[2,3,4,5- 3 H]arginine to L-[2,3- 3 H]citrulline according to the modification of the method described by Bredt and Snyder (12). 2×10^6 human astrocytoma cells (T67) were homogenized with Ultra-Turrax homogenizer (5-mm blade) for 20 s in 200 μ l of a buffer containing 50 mM HEPES, pH 7.4, 1 mM DL-dithiothreitol, 1 mM EDTA, 10 μ g/ml leupeptin, 10 μ g/ml soybean trypsin inhibitor, 10 μ g/ml antipain, and 1 mM phenylmethylsulfonyl fluoride. For Ca $^{2+}$ -dependent activity, after centrifugation (10,000 \times g, 30 min at 4 $^{\circ}$ C), an aliquot of the supernatant was added to a reaction mixture of a final volume of 100 μ l containing 50 mM HEPES, pH 7.4, 20 nM [3 H]arginine, 1 μ M arginine, 1 mM NADPH, 1 mM EDTA, 1.2 mM CaCl $_2$, 1 μ g/ml calmodulin, 10 μ M FAD, 0.1 mM (6R)-5,6,7,8-tetrahydro-1-biopterin, and 1 mM dithiothreitol. For Ca $^{2+}$ -independent activity, instead, 1.2 mM CaCl $_2$ and 1 μ g/ml calmodulin were omitted from, and 1 mM EGTA was added to the reaction mixture. The reactions were stopped by adding 0.4 ml of 1:1 slurry of Dowex AG50WX-8 (Bio-Rad, Na $^+$ form) in 50 mM HEPES, pH 5.5, and after 15-min 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 (13).

Nitrite Analysis— 5×10^6 human astrocytoma cells (T67) were resuspended in Krebs' buffer and stirred for 10 min at 1000 rpm in a platelet aggregometer. The intact cells were then centrifuged at 13,000 \times g for 5 min and nitrite (NO $_2^-$) levels were determined in supernatants by the Griess reaction, as described elsewhere (9, 10). Briefly, aliquots of the cell supernatants were mixed with an equal volume of Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride, 2.5% H $_3$ PO $_4$). The absorbance was measured at 546 nm and NO $_2^-$ concentration was determined using sodium nitrite as a standard. Results were expressed as nmol ml $^{-1}$.

Reverse Transcriptase-Polymerase Chain Reaction (PCR)—Total cellular RNA was purified from 1×10^6 human astrocytoma T67 cells by the method Chomczynski and Sacchi (14). Briefly, a single extraction with an acid guanidinium thiocyanate-phenol-chloroform mixture was performed. Whole RNA was reverse-transcribed into cDNA by Moloney murine leukemia virus reverse transcriptase using oligo(dT) $_{12-18}$ as primers. PCR was carried out with Taq DNA polymerase in an automatic DNA thermal cycler (GeneAmp 2400, Perkin-Elmer). For NOS-I cDNA amplification, the gene-specific primers (BN-1, BN-2, and BN-3) were chosen according to Asano *et al.* (15). The cycle program was set as follows: 94 $^{\circ}$ C for 30 s, 68 $^{\circ}$ C for 30 s, and 72 $^{\circ}$ C for 30 s using BN-1 and BN-2 primers for 35 cycles followed by an extension step at 72 $^{\circ}$ C for 7 min. Then, PCR products from BN-1 and BN-2 were reamplified using BN-2 and BN-3 primers for another 30 cycles under the same conditions. Ten μ l of each final PCR product (NOS-I, 204 bp) were electrophoresed on 1.5% agarose gel and then visualized after ethidium bromide staining. The bands so obtained were quantified with Sigma Scan/Image software (Jandel Corp.) after scanner recording from the gel photography. The mRNA for the constitutive glycerol-3-phosphate Dehydrogenase (GAPDH) was examined as the reference cellular transcript. GAPDH mRNA amplification products (195 bp) were present at equivalent levels in all cell lysates. The reaction was performed using specific primers as described elsewhere (16).

RESULTS AND DISCUSSION

As described previously, human cultured astrocytoma cells (T67) possess an inducible Ca $^{2+}$ -independent NO synthase (iNOS) which is stimulated time-dependently by treatments with LPS or cytokines, such as IFN γ (9, 10). Here, we have demonstrated that the homogenates of unstimulated T67 cells also exhibit a basal NOS activity (0.68 ± 0.06 nmol mg protein $^{-1}$ min $^{-1}$), as determined by measuring [3 H]citrulline production (Fig. 1). The elimination of free Ca $^{2+}$ by EGTA (1 mM) from the mixture reaction almost totally abolished this activity (0.68 ± 0.06 to 0.012 ± 0.02 nmol mg protein $^{-1}$ min $^{-1}$), demonstrating that the isoform of the enzyme was a constitutive Ca $^{2+}$ -dependent NOS (cNOS) (Fig. 1).

Interestingly, we found that a quick treatment of T67 cells with the combination of LPS (1 μ g/ml) and IFN γ (1000 units/ml) (MIX) for 30 min strongly inhibited the basal NOS activity

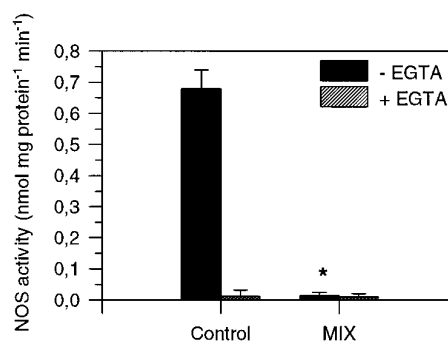


FIG. 1. Effect of MIX on NOS activity in human astrocytoma T67 cells. NOS activity in homogenate of T67 cells was measured both in absence and in presence of EGTA (1 mM) in an assay mixture containing HEPES, [3 H]arginine, arginine, NADPH, EDTA, CaCl $_2$, calmodulin, FAD, (6R)-5,6,7,8-tetrahydro-1-biopterin, and dithiothreitol. A 30-min treatment of T67 cells with combination of LPS (1 μ g/ml) and IFN γ (1000 units/ml) strongly (*, $p \leq 0.001$) inhibited the Ca $^{2+}$ -dependent NOS activity. Values are expressed as nmol mg protein $^{-1}$ min $^{-1}$. Each bar represents the mean \pm S.E. of three experiments.

(0.68 ± 0.06 to 0.015 ± 0.01 nmol mg protein $^{-1}$ min $^{-1}$), as determined in the absence of EGTA (Fig. 1). In MIX-treated T67 cells, NOS activity resulted very low also in the presence of EGTA, thus demonstrating that a 30-min MIX treatment of cells was too short to induce the Ca $^{2+}$ -independent NOS expression (Fig. 1). So, the inhibitory effect on cNOS activity elicited by MIX treatment might be triggered by the direct NOS modifications.

The effect of LPS/IFN γ on cNOS-dependent NO production was also confirmed by measuring nitrite, the stable breakdown product of NO (17), in stirred T67 cells. In the supernatants of stirred unstimulated T67 cells, we have found a basal nitrite (NO $_2^-$) level (about 1.1 nmol ml $^{-1}$). The latter was not abolished by NOS inhibitor L-NAME (500 μ M) (data not shown), indicating that the basal level of NO $_2^-$ was not due to L-arginine metabolism. However, this NO $_2^-$ basal level can somewhat hide the small amount of NO produced by the cNOS enzyme in untreated T67 cells. To enhance cNOS-dependent NO production, we have stimulated T67 cells with NMDA, a typical cNOS activator in the nervous system (18). When T67 cells were incubated with NMDA (500 μ M) for 0.5, 1, and 2 h, a time-dependent increase of nitrite synthesis (above the background of ~ 1.1 nmol ml $^{-1}$) was observed (Fig. 2). The maximal peak was reached after 2 h of treatment (5.0 ± 0.26 nmol ml $^{-1}$), and this effect was inhibited by L-NAME (500 μ M) (5.0 ± 0.26 to 0.2 ± 0.1 nmol ml $^{-1}$), thus demonstrating that the NMDA-increased nitrite generation was associated with the L-arginine-NO pathway (Fig. 2). As already observed, T67 cells possess a NMDA-activated NO pathway. In fact, the treatment of T67 cells with NMDA was able to increase both nerve growth factor mRNA and prostaglandin E $_2$ levels, these effects being mediated by NO synthesis (19, 20).

When T67 cells were preincubated with MIX for 30 min and then washed, the NMDA-induced nitrite production was significantly reduced (5.0 ± 0.26 to 1.0 ± 0.09 nmol ml $^{-1}$; Fig. 2). As described previously, LPS or cytokines such as IFN γ are able to induce a high-output NO in T67 cells (9, 10). In fact, a 24-h stimulation of T67 cells with MIX enhanced iNOS-elicited nitrite production. On the contrary, a 30-min treatment of T67 cells with MIX alone did not affect nitrite synthesis (Fig. 2), thus demonstrating that a short MIX treatment of cells did not induce the iNOS enzyme, in agreement with the NOS activity results shown in Fig. 1.

The swiftness of MIX-elicited inhibition of cNOS pathway would seem to exclude a possible involvement of cNOS mRNA transcript during the first 30 min. To verify this hypothesis, we

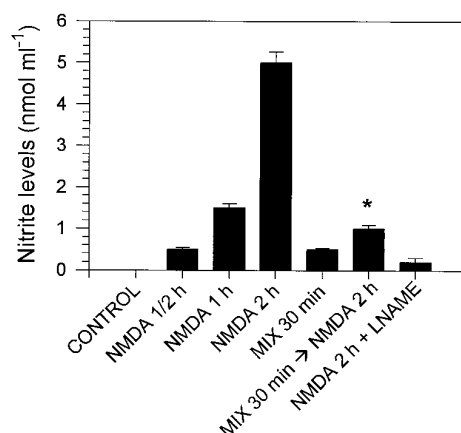


FIG. 2. **Effect of MIX on nitrite production in human astrocytoma T67 cells.** Nitrite levels were measured using Griess reaction in supernatants of stirred T67 cells treated with NMDA (500 μ M) for 0.5, 1, and 2 h. When T67 cells treated with NMDA for 2 h were incubated with LPS (1 μ g/ml) + IFN γ (1000 units/ml) for 30 min, nitrite levels were significantly (*, $p \leq 0.01$) decreased. Data are expressed as nmol ml⁻¹, and all values are obtained by subtraction of background nitrite levels (about 1.1 nmol ml⁻¹) from all corresponding values. Each bar represents the mean \pm S.E. of three experiments.

have analyzed the effect of MIX on cNOS mRNA expression in T67 cells using the reverse transcriptase-PCR.

Here, we have shown that human astrocytoma T67 cell cultures expressed constitutively mRNA coding for the neuronal NOS-I isoform (Fig. 3). As expected, the reverse transcriptase-PCR NOS-I product consisted of a 204-bp fragment amplified using brain NOS-specific primers (BN-1, BN-2, and BN-3), as described elsewhere (15). In agreement with those authors, the first PCR product from BN-1 and BN-2 primers was reamplified by using BN-2 and BN-3 primers. A higher specificity of NOS-I sequence amplification was derived from the use of the BN-3 primer, which was chosen inside the first fragment from BN-1 and BN-2 primers. Our finding is supported by the recent study of Kugler and Drenckhahn (21), which provides immunocytochemical evidence of NOS-I in rat astrocytes, as well as in Bergmann glia.

When 1×10^6 T67 cells were incubated with MIX for 30 min, no inhibition of NOS-I mRNA expression was observed (Fig. 3), thereby demonstrating that MIX-elicited inhibition of the Ca²⁺-dependent NO production was not due to modifications of cNOS mRNA transcription. These findings were confirmed by further experiments carried out using different decreasing template concentrations. In all experiments, untreated and MIX-treated T67 cells showed no significant differences (data not shown).

Some mechanisms explaining the fast inhibitory effect on cNOS enzyme have already been described. As an example, phosphorylation of NOS-I and NOS-III by protein kinase C, which is activated by immunologically active substances including endotoxin, is associated with a reduction of the enzymatic activity (22–24). Alternatively, endotoxin directly inhibits agonist (bradikinin and ADP)-mediated NO production from endothelial cells by affecting cytosolic free Ca²⁺ mobilization mechanisms leading, in turn, to subsequent decreases in the activity of NOS-III (25).

Taken together, our results provide evidence that LPS and IFN γ were able to inhibit NO production in a very short time, by affecting cNOS activity but not cNOS mRNA expression. On the other hand, LPS/cytokines also induce iNOS gene, by quick activation of the transcriptional factor NF- κ B (8). However, the early presence of physiological concentrations of NO can keep the iNOS expression suppressed by preventing NF- κ B activation (8). Thus, a very fast reduction of the cNOS-generated NO

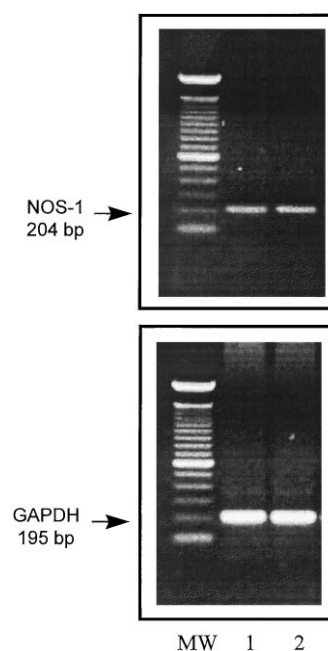


FIG. 3. **Effect of MIX on cNOS gene expression in human astrocytoma T67 cells.** 1.5% agarose gel electrophoresis of the reverse transcriptase-PCR products for NOS-I mRNA (204 bp) and for GAPDH mRNA (195 bp) after ethidium bromide staining. Densitometry of gel photography was used to determine and quantify the levels of mRNA encoding NOS-I. Estimates of the relative NOS-I amounts were obtained dividing the peak densitometry area of NOS-I band by the same area of GAPDH band. PCR products for the GAPDH gene were taken as the reference cellular transcript. Molecular weight (MW) is 100-bp DNA ladder (Life Technologies, Inc.). Lane 1, untreated cells; lane 2, a 30-min treatment of 1×10^6 T67 cells with LPS (1 μ g/ml) + IFN γ (1000 units/ml) did not affect cNOS mRNA levels.

levels below a threshold value could explain how LPS/cytokines are able to provide the best conditions for NF- κ B activation and subsequent iNOS induction. Note that inflammatory stimuli such as LPS/IFN γ have already been reported to trigger endothelial cells to switch from constitutive to inducible NO synthase activity (26, 27).

In conclusion, a novel concept may be introduced that the presence of cNOS isoform in astrocytes, as well as in other cell types possessing both cNOS and iNOS, may have a functional implication as a controller of the iNOS induction, this being consistent with a protective role of physiological NO.

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