Communication

Rapid Inactivation of NOS-I by Lipopolysaccharide Plus Interferon-γ-induced Tyrosine Phosphorylation*

**Marco Colasanti‡, Tiziana Persichini‡,
Elisabetta Cavalieri§, Cinzia Fabrizi‡,
Sofia Mariotto§, Marta Menegazzi§,
Giuliana M. Lauro‡, and Hisanori Suzuki§¶**

*From the ‡Department of Biology, University of Rome,
ROMA TRE, Viale Marconi 446, I-00146 Rome, Italy
and the §Department of Neuroscience and Vision,
Laboratory of Biological Chemistry, University of
Verona, Strada Le Grazie 8, I-37134 Verona, Italy*

**Human astrocytoma T67 cells constitutively express a
neuronal NO synthase (NOS-I) and, following adminis-
tration of lipopolysaccharide (LPS) plus interferon-γ
(IFNγ), an inducible NOS isoform (NOS-II). Previous re-
sults indicated that a treatment of T67 cells with the
combination of LPS plus IFNγ, by affecting NOS-I activ-
ity, also inhibited NO production in a very short time.
Here, we report that under basal conditions, a NOS-I
protein of about 150 kDa was weakly and partially ty-
rosine-phosphorylated, as verified by immunoprecipita-
tion and Western blotting. Furthermore, LPS plus IFNγ
increased the tyrosine phosphorylation of NOS-I, with a
concomitant inhibition of its enzyme activity. The same
effect was observed in the presence of vanadate, an in-
hibitor of phosphotyrosine-specific phosphatases. On
the contrary, genistein, an inhibitor of protein-tyrosine
kinases, reduced tyrosine phosphorylation of NOS-I, en-
hancing its enzyme activity. Finally, using reverse tran-
scriptase-polymerase chain reaction, we have observed
that a suboptimal induction of NOS-II mRNA expression
in T67 cells was enhanced by vanadate (or L-NAME) and
inhibited by genistein. Because exogenous NO has been
found to suppress NOS-II expression, the decrease of NO
production that we have obtained from the inactivation
of NOS-I by LPS/IFNγ-induced tyrosine phosphorylation
provides the best conditions for NOS-II expression in
human astrocytoma T67 cells.**

Nitric oxide is a major messenger molecule that is generated
by a family of enzymes, termed NO synthases (NOS) (for recent
reviews see Refs. 1–5).[1] There are at least three distinct iso-
forms of NOS; two enzymes (NOS-I and NOS-III, also called
cNOS) are constitutively expressed in neurons, endothelial
cells, and glial cells, and one inducible NOS (NOS-II) is ex-
pressed after stimulation with endotoxin and/or cytokines in a
number of cells including macrophages, neutrophils, hepato-
cytes, and glial cells.

Recently, we have reported that in human microglial cells,
which do not express cNOS, low concentrations of exogenous
NO suppressed the induction of NOS-II expression (6), suggest-
ing an involvement of physiological NO levels, as produced by
cNOS, in preventing the accidental or unfavorable induction of
NOS-II expression. Paradoxically, in cell types (*e.g.* astroglial
cells) expressing both constitutive and inducible NOS, the in-
duction of the latter should be a rare event, unless the NO
produced by constitutive NOS is preventively and quickly
down-regulated. In this respect, we have further demonstrated
that in human astroglial cells, *Escherichia coli* lipopolysaccha-
ride (LPS) plus interferon-γ (IFNγ), two common inducers of
NOS-II expression (7), elicited a decrease in NO synthesis. This
effect was mediated by a very fast inhibition of NOS-I activity,
without affecting the NOS-I mRNA transcription (8).

Here, we used human astrocytoma T67 cells, as an astroglial
model, for a better understanding of the biochemical mecha-
nism of the fast NOS-I inactivation and successive NOS-II
induction. In this respect, we hypothesize that the rapid inhi-
bition of NOS-I activity by NOS-II inducers (*i.e.* LPS plus
IFNγ) in astroglial cells may be mediated by tyrosine phospho-
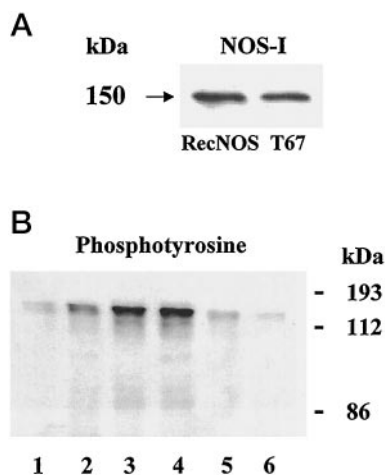rylation of NOS-I.

EXPERIMENTAL PROCEDURES

Materials

Nᵒ-nitro-L-arginine methyl ester (L-NAME) and *E. coli* lipopolysac-
charide (LPS; Serotype 0127:B8) were obtained from Sigma (Milan,
Italy). Human recombinant interferon-γ (IFNγ) was supplied by Biogen
SA (Geneva, Switzerland; specific activity, 2×10^7 IU mg protein^{-1})
and L-2,3,4,5-[^{3}H]arginine by Amersham. Recombinant rat neuronal
NOS was purchased from Calbiochem (La Jolla, CA).

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 (9).

Immunoprecipitation and Western Blot Analysis—For the immuno-
precipitation of NOS-I, T67 cells (1×10^6 cells/sample) were washed
with phosphate-buffered saline and lysed on ice in 10 mM Tris-HCl
buffer, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.5% Nonidet P-40, 1 mM
EDTA, 1 mM EGTA, 1 mM vanadate, 10 mM NaF, 1 mM phenylmeth-
ylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin. Lysates
were cleared by centrifugation, and equal amounts of proteins were
incubated with an anti-NOS-I polyclonal antibody (Santa Cruz Biotech-
nology, Inc., Santa Cruz, CA), diluted 1:30, for 3 h at 4 °C. Protein
A-agarose was added, and the mixture was incubated for additional 30
min. After three washes, the samples were boiled in SDS-polyacryl-
amide gel electrophoresis sample buffer for 5 min. Immunoprecipitated
samples were subjected to electrophoresis in 7.5% polyacrylamide gels
and blotted to nitrocellulose. Membranes were blocked with 5% nonfat
milk for 1 h and incubated for 2 h, with the anti-NOS-I polyclonal
antibody being diluted (1:1000) in the blocking buffer. After extensive
washing, a goat anti-rabbit IgG horseradish peroxidase-conjugated an-
tibody (Santa Cruz Biotechnology, Inc.) was added. In some experi-
ments, a monoclonal anti-phosphotyrosine horseradish peroxidase-con-
jugated antibody (RC20 from Transduction Laboratories, Lexington,

¶ To whom correspondence should be addressed: Dipartimento di
Scienze Neurologiche e della Visione, Sezione di Chimica Biologica,
Università di Verona, Strada Le Grazie 8, I-37134 Verona, Italy. Tel.:
39-45-8098167; Fax: 39-45-8098170.

[1] The abbreviations used are: NOS, NO synthase(s); cNOS, constitutive
Ca²⁺-dependent NOS isoform; LPS, lipopolysaccharide; IFNγ, interfer-
on-γ; L-NAME, Nᵒ-nitro-L-arginine methyl ester; PCR, polymerase chain
reaction.

FIG. 1. **Western blot analysis of NOS-I in human astrocytoma T67 cells.** *A*, NOS-I was immunoprecipitated from 1×10^6 cells/sample and Western blotted using a polyclonal anti-NOS-I antibody. As expected, the resulting band (about 150 kDa) corresponded to that of recombinant rat NOS (*RecNOS*), used as a positive control. *B*, immunoprecipitated NOS-I was Western blotted using a specific antiserum against phosphotyrosine. NOS-I from untreated cells was weakly and partially tyrosine-phosphorylated (*lane 1*). Treatment of cells with LPS (10 μg/ml) plus IFNγ (1000 units/ml) for 30 min increased tyrosine phosphorylation of NOS-I (*lane 2*). The same effect, even more marked, was observed in the presence of vanadate (1 mM for 2 h), an inhibitor of protein-tyrosine phosphatases (*lane 3*). Vanadate was able to increase LPS/IFNγ-induced tyrosine phosphorylation of NOS-I (*lane 4*). Preincubation of both LPS/IFNγ-treated (*lane 5*) and untreated (*lane 6*) T67 cells with an inhibitor of protein-tyrosine kinases, genistein (1 mM for 2 h), completely abolished tyrosine phosphorylation of NOS-I.

KY) was used according to the manufacturer's instructions. Antibody binding was detected by chemiluminescence (ECL, Amersham Italia S.r.l., Milan, Italy).

Assay of Astrocytoma NOS Activity—NOS 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 (10). 2×10^6 human astrocytoma T67 cells were homogenized with Ultra-Turrax homogenizer (5-mm blade) for 60 s in 200 μl of a buffer containing 50 mM HEPES, pH 7.4, 1 mM dithiothreitol, 1 mM EDTA, 10 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor, 10 μg/ml antipain, and 1 mM phenylmethylsulfonyl fluoride. For Ca²⁺-dependent activity, after centrifugation (10,000 $\times$ g for 30 min at 4 °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, 1.2 mM CaCl₂, 1 μg/ml calmodulin, 10 μM FAD, 0.1 mM (6R)-5,6,7,8-tetrahydro-1-biopterin, and 1 mM dithiothreitol. For Ca²⁺-independent activity, 1.2 mM CaCl₂ and 1 μg/ml calmodulin were omitted from and 1 mM EGTA was added to the reaction mixture. The reactions were stopped by adding 0.4 ml (1:1) of slurry of Dowex AG50WX-8 (Bio-Rad, Na⁺ form) in 50 mM HEPES, pH 5.5. and after 15 min of shaking, radioactivity in the supernatant was measured. The enzyme activity was linear up to 15 min of incubation. Specific enzyme activity was calculated as pmol of citrulline formed in 1 min by 1 mg of protein. Values are expressed as the percentage of NOS activity *versus* untreated T67 cells. Protein concentration in the samples was determined by the method of Bradford (11).

Reverse Transcriptase-PCR—Total cellular RNA was purified from 1×10^6 human astrocytoma T67 cells by the method of Chomczynski and Sacchi (12). 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 primer. PCR was carried out with *Taq* DNA polymerase in an automatic DNA thermal cycler (GeneAmp 2400, Perkin-Elmer). NOS-II cDNA amplification was obtained as described previously (6). 10 μl of each final PCR product (450 base pairs) were electrophoresed on 1.5% agarose gel and then visualized after ethidium bromide staining. The bands so obtained were quantified with Fluor-S™ MultImager (Bio-Rad). In addition, the NOS-II-amplified DNA fragment was purified after gel extraction (using Qiaquick gel extraction kit, purchased from Qiagen GmbH, Düsseldorf,
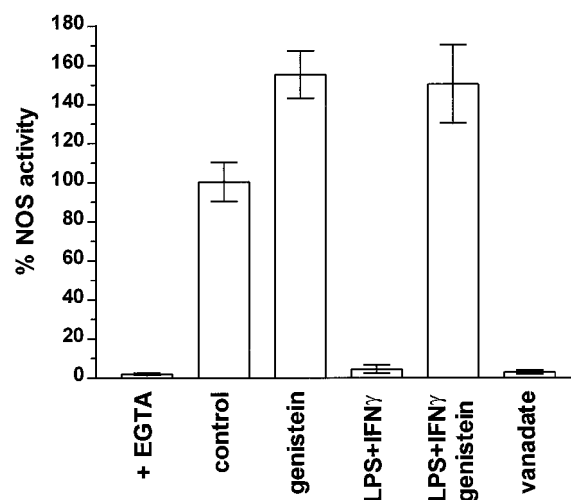
FIG. 2. **Effect of tyrosine phosphorylation on NOS-I activity in human astrocytoma T67 cells.** Homogenates of unstimulated T67 cells exhibited a basal NOS activity (*control*). The presence of EGTA (1 mM) in the assay mixture abolished this activity, demonstrating that the enzyme isoform was a Ca²⁺-dependent NOS. A 30-min treatment of T67 cells with LPS (10 μg/ml) plus IFNγ (1000 units/ml) strongly inhibited NOS-I activity. A preincubation (for 2 h) of LPS/IFNγ-stimulated T67 cells with 1 mM genistein, an inhibitor of protein-tyrosine kinases, completely restored LPS/IFNγ-decreased NOS-I activity. A pretreatment (for 2 h) of T67 cells with 1 mM vanadate, an inhibitor of protein-tyrosine phosphatases, reduced the basal level of NOS-I activity in T67 cells. Values are expressed as the percentage of NOS activity *versus* untreated T67 cells.
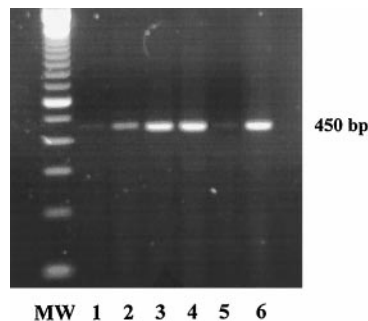
FIG. 3. **Effect of tyrosine phosphorylation on NOS-II mRNA expression in human astrocytoma T67 cells.** *Lane 1,* untreated cells. Treatment of cells with a suboptimal concentration of a mixture of LPS (10 μg/ml) plus IFNγ (100 units/ml) for 8 h induced a less pronounced NOS-II mRNA expression (*lane 2*) than treatment with the optimal concentration of LPS (10 μg/ml) plus IFNγ (1000 units/ml) (*lane 3*). The same sample as in *lane 2* preincubated with vanadate (1 mM) for 2 h showed a NOS-II mRNA overexpression (*lane 4*), the same effect being obtained by preincubation of the cells with the NOS inhibitor L-NAME (1 mM) (*lane 6*). The same sample as in *lane 2* preincubated with genistein (1 mM for 2 h) completely suppressed NOS-II mRNA expression (*lane 5*).

Germany). The nucleotide sequence was determined using the Ampli-Cycle™ sequencing kit (Perkin-Elmer) by direct cycle sequencing with *Taq* DNA polymerase and ³⁵S-labeled dCTP according to the manufacturer's instructions. As expected, we found that the nucleotide sequence of the PCR product from T67 cells displayed 99% identity with NOS-II cDNA (from 3131 to 3580) of human hepatocytes (GenBank™ accession number L09210). The mRNA for the constitutive glycerol-3-phosphate dehydrogenase was examined as the reference cellular transcript. Glycerol-3-phosphate dehydrogenase mRNA amplification products (195 base pairs) were present at equivalent levels in all cell lysates. The reaction was performed using specific primers as described elsewhere (13).

RESULTS AND DISCUSSION

As described previously, a treatment of human astrocytoma T67 cells with the combination of LPS plus IFN-γ strongly inhibited NO production in a very short time, by affecting the

Ca^{2+}-dependent NOS activity but not NOS-I mRNA expression (8). Recently, it has been reported that in endothelial cells, the rapid inhibition of the Ca^{2+}-dependent NOS activity (*e.g.* NOS-III) seems to be associated with the phosphorylation of tyrosine residue(s) of the enzyme (14). Here, we have evaluated the tyrosine phosphorylation state of NOS-I protein in human astrocytoma T67 cells. As shown in Fig. 1*A*, a NOS-I protein from the soluble fractions of T67 cell homogenates was evidenced. The molecular mass was about 150 kDa, as was that from recombinant rat neuronal NOS, used as a positive control. Moreover, under basal conditions, NOS-I protein immunoprecipited from T67 cells was weakly and partially tyrosine-phosphorylated (Fig. 1*B*, *lane 1*). When T67 cells were treated with LPS (10 μg/ml) plus IFNγ (1000 units/ml) for 30 min, tyrosine phosphorylation of NOS-I was enhanced (Fig. 1*B*, *lane 2*). The same effect, even more marked, was observed in the presence of vanadate (1 mM for 2 h), an inhibitor of protein-tyrosine phosphatases (Fig. 1*B*, *lane 3*). Moreover, a pretreatment with vanadate for 2 h was able to increase LPS/IFNγ-induced tyrosine phosphorylation of NOS-I (Fig. 1*B*, *lane 4*). On the contrary, preincubation of both LPS/IFNγ-treated and untreated T67 cells with an inhibitor of protein-tyrosine kinases, genistein (1 mM for 2 h), completely abolished tyrosine phosphorylation of NOS-I (Fig. 1*B*, *lanes 5* and *6*, respectively).

As described previously, homogenates of unstimulated T67 cells exhibited a basal NOS-I activity (8). The elimination of free Ca^{2+} by EGTA (1 mM) from the reaction mixture almost totally abolished this activity (Fig. 2), confirming that the isoform of the enzyme was Ca^{2+}-dependent. To verify the effect of tyrosine phosphorylation state of NOS-I on its enzyme activity, we used genistein, which decreases the tyrosine phosphorylation of NOS-I protein (Fig. 1). In this respect, a preincubation of T67 cells with 1 mM genistein for 2 h enhanced basal NOS-I activity (Fig. 2), the weak, constitutive tyrosine phosphorylation of the enzyme being likely reduced. On the contrary, a 30-min treatment of T67 cells with LPS (10 μg/ml) plus IFNγ (1000 units/ml), which increases the tyrosine phosphorylation of NOS-I protein (Fig. 1), strongly inhibited NOS-I activity (Fig. 2). When LPS/IFNγ-stimulated T67 cells were preincubated with 1 mM genistein for 2 h, NOS-I activity was completely restored to reach the same effect as obtained with genistein alone (Fig. 2). Also, the involvement of tyrosine phosphorylation in modulating NOS-I activity was confirmed using vanadate, which increased tyrosine phosphorylation of NOS-I protein (Fig. 1*B*). In this respect, the treatment of T67 cells with vanadate alone (1 mM for 2 h) reduced the basal level of NOS-I activity, in the same manner as LPS plus IFNγ (Fig. 2).

Taken together, our results suggest that the balance between a native and a tyrosine-phosphorylated form as well as the enzymatic activity of NOS-I can be controlled by the activity of protein-tyrosine kinase(s) and phosphotyrosine-specific phosphatase(s). Moreover, by affecting tyrosine phosphorylation, NOS-II inducers (*e.g.* LPS and IFNγ) quickly inhibit NOS-I activity, thereby providing the best conditions for NOS-II induction. To verify this hypothesis, we have analyzed the effect of the tyrosine phosphorylation on NOS-II mRNA expression as induced by suboptimal concentrations of LPS plus IFNγ in human astrocytoma T67 cells. As shown in Fig. 3, incubation of T67 cells with a suboptimal concentration of LPS (10 μg/ml) plus IFNγ (100 units/ml) for 8 h induced a less pronounced NOS-II mRNA expression (*lane 2*) than observed in the treatment with the optimal concentration of LPS (10 μg/ml) plus IFNγ (1000 units/ml) (*lane 3*). When T67 cells were incubated with the suboptimal concentration of LPS plus IFNγ, a NOS inhibitor L-NAME (1 mM) caused a NOS-II mRNA overexpres-

sion (see *lane 6*), indicating that a rapid NOS inactivation facilitated the induction of NOS-II expression. The present results are in agreement with our previous reports indicating that NO exerts a suppressive effect on NOS-II expression (6, 15, 16).

Furthermore, when T67 cells were stimulated by the suboptimal concentration of LPS plus IFNγ, preincubation (for 2 h) with 1 mM genistein, which inhibited the tyrosine phosphorylation of NOS-I (Fig. 1*B*) causing a NOS-I hyper-activation (Fig. 2), completely suppressed NOS-II mRNA expression (Fig. 3, *lane 5*). On the other hand, when T67 cells were treated with the suboptimal concentration of LPS plus IFNγ, preincubation (for 2 h) with 1 mM vanadate, which enhanced the tyrosine phosphorylation of NOS-I (Fig. 1*B*) causing a NOS-I inhibition (Fig. 2), induced a NOS-II mRNA overexpression (Fig. 3, *lane 4*). Our observations are in agreement with previous results indicating a requirement for protein-tyrosine kinase activation as part of the process of NOS-II induction in several cell types (17–24), and this may reflect the need to switch off cNOS enzyme activity.

As a whole, our results confirm our previous reports suggesting that a change in endogenous NO levels may be a key factor in regulating the induction of NOS-II expression in human astrocytoma cells as well as in rat neutrophils (8, 15). In this respect, affecting the NOS-I tyrosine phosphorylation, NOS-II inducers (*e.g.* LPS and IFNγ) elicit a rapid inactivation of the enzyme, leading to a decrease of basal NO levels (8). Thus, our findings can explain how NOS-II inducers are able to provide the best conditions for the induction of NOS-II expression, thereby resolving an apparent paradox.

REFERENCES

1. Stuehr, D. J. (1997) *Annu. Rev. Pharmacol. Toxicol.* **37,** 339–359
2. Moncada, S., Higgs, A., and Furchgott, R. (1997) *Pharmacol. Rev.* **49,** 137–142
3. Nathan C. (1997) *J. Clin. Invest.* **100,** 2417–2423
4. Geller, D. A., and Billiar, T. R. (1998) *Cancer Metastasis Rev.* **17,** 7–23
5. Marletta, M. A., Hurshman, A. R., and Rusche, K. M. (1998) *Curr. Opin. Chem. Biol.* **2,** 656–663
6. Colasanti, M., Persichini, T., Menegazzi, M., Mariotto, S., Giordano, E., Caldarera, C. M., Sogos, V., Lauro, G. M., and Suzuki, H. (1995) *J. Biol. Chem.* **270,** 26731–26733
7. Xie, Q. W., Whisnant, R., and Nathan C. (1993) *J. Exp. Med.* **177,** 1779–1784
8. Colasanti, M., Cavalieri, E., Persichini, T., Mollace, V., Mariotto, S., Suzuki H., and Lauro, G. M. (1997) *J. Biol. Chem.* **272,** 7582–7585
9. Lauro, G. M., Di Lorenzo, N., Grossi, M., Maleci A., and Guidetti, B. (1986) *Acta Neuropathol.* **69,** 278–282
10. Bredt, D. S., and Snyder, S. H. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 682–685
11. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254
12. Chomczynski, P., and Sacchi, N. (1987) *Anal. Biochem.* **162,** 156–157
13. Genis, P., Jett, M., Bernton, E. W., Boyle, T., Gelbard, H. A., Dzenko, K., Keane, R. W., Reswnick, L., Mizrachi, Y., Volsky, D. J., Epstein, L. G., and Gendelman, H. E. (1992) *J. Exp. Med.* **176,** 1703–1718
14. Garcia-Cardena, G., Fan, R., Stern, D. F., Liu, J., and Sessa, W. C. (1996) *J. Biol. Chem.* **271,** 27237–27240
15. Mariotto, S., Cuzzolin, L., Adami, A., Del Soldato, P., Suzuki, H., and Benoni, G. (1995) *Br. J. Pharmacol.* **114,** 1105–1106
16. Colasanti, M., Persichini, T., Venturini, G., Menegatti, E., Lauro, G. M., and Ascenzi P. (1998) *Biochem. Biophys. Res. Commun.* **246,** 453–456
17. Akarasereenont, P., Mitchell, J. A., Appleton, I., Thiemermann, C., and Vane, J. R. (1994) *Br. J. Pharmacol.* **113,** 1522–1528
18. Feinstein D. L., Galea, E., and Reis, D. J. (1994) *Ann. N. Y. Acad. Sci.* **738,** 325–328
19. Simmons, M. L., and Murphy, S. (1994) *Glia* **11,** 227–234
20. Geng, Y., Maier, R., and Lotz, M. (1995) *J. Cell. Physiol.* **163,** 545–554
21. Paul, A., Pendreigh, R. H., and Plevin, R. (1995) *Br. J. Pharmacol.* **114,** 482–488
22. Tetsuka, T., and Morrison, A. R. (1995) *Am. J. Physiol.* **269,** C55–C59
23. Kong, L. Y., McMillian, M. K., Moronpot, R., and Hong, J. S. (1996) *Brain Res.* **729,** 102–109
24. Faure, V., Courtois, Y., and Goureau, O. (1998) *Am. J. Physiol.* **275,** C208–C215

Rapid Inactivation of NOS-I by Lipopolysaccharide Plus Interferon-γ-induced Tyrosine Phosphorylation

Marco Colasanti, Tiziana Persichini, Elisabetta Cavalieri, Cinzia Fabrizi, Sofia Mariotto, Marta Menegazzi, Giuliana M. Lauro and Hisanori Suzuki

Access the most updated version of this article at http://www.jbc.org/content/274/15/9915

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 24 references, 7 of which can be accessed free at
http://www.jbc.org/content/274/15/9915.full.html#ref-list-1