Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 10769–10772, November 1993
Biochemistry

Nitric oxide synthases reveal a role for calmodulin in controlling electron transfer

(flavoprotein/cytochrome P-450/calcium-binding protein/arginine)

HUSAM M. ABU-SOUD* AND DENNIS J. STUEHR*†

*Department of Immunology, NN-1, The Cleveland Clinic, Cleveland, OH 44195; and †Department of Physiology and Biophysics, Case Western Reserve University, Cleveland, OH 44106

Communicated by Robert F. Furchgott, August 23, 1993 (received for review June 16, 1993)

ABSTRACT **Nitric oxide (NO) is synthesized within the immune, vascular, and nervous systems, where it acts as a wide-ranging mediator of mammalian physiology. The NO synthases (EC 1.14.13.39) isolated from neurons or endothelium are calmodulin dependent. Calmodulin binds reversibly to neuronal NO synthase in response to elevated Ca^{2+}, triggering its NO production by an unknown mechanism. Here we show that calmodulin binding allows NADPH-derived electrons to pass onto the heme group of neuronal NO synthase. Calmodulin-triggered electron transfer to heme was independent of substrate binding, caused rapid enzymatic oxidation of NADPH in the presence of O_2, and was required for NO synthesis. An NO synthase isolated from cytokine-induced macrophages that contains tightly bound calmodulin catalyzed spontaneous electron transfer to its heme, consistent with bound calmodulin also enabling electron transfer within this isoform. Together, these results provide a basis for how calmodulin may regulate NO synthesis. The ability of calmodulin to trigger electron transfer within an enzyme is unexpected and represents an additional function for calcium-binding proteins in biology.**

Calmodulin (CAM) regulates a wide range of cell functions through its reversible Ca^{2+}-dependent binding to target enzymes (1, 2). Included in this group are the nitric oxide synthases (NOSs; EC 1.14.13.39), whose product, NO, fulfills vital roles within the immune, circulatory, and central nervous systems (reviewed in refs. 3–6). NOS isoforms purified from brain (7–9) or from cytokine-stimulated macrophages (10, 11) have distinct CAM-binding consensus sequences and therefore possess different affinities for CAM. Neuronal NOS does not contain bound CAM and is inactive regarding NO synthesis, but it binds CAM in the presence of added Ca^{2+} ($K_{0.5}$ = 350 nM) (8). This triggers its conversion of L-arginine to NO (7–9) or its superoxide production in the absence of L-arginine (12, 13). Macrophage NOS, on the other hand, is isolated containing tightly bound CAM (14), which does not dissociate from the enzyme even in the presence of the Ca^{2+}-chelator EGTA. This tightly bound CAM has been proposed to keep macrophage NOS in its tonically active state, enabling NO synthesis to commence immediately when the enzyme is provided with NADPH, O_2, and L-arginine (14). However, the mechanisms by which CAM activates neuronal NOS, or maintains macrophage NOS in its active state, are unknown.

In contrast to their differential CAM requirements, brain and macrophage NOS appear to have a similar composition and biochemistry. They both are homodimers that catalyze a stepwise, NADPH- and O_2-dependent oxidation of L-arginine to form NO (8, 9, 15–17) (Fig. 1), and are two of very few enzymes that contain heme, FAD, and FMN within the same protein (9, 10, 18–22). On the basis of similarities with the cytochrome P-450 monooxygenase system, the NOS flavins are thought to shuttle electrons obtained from NADPH onto the enzyme's heme group, enabling heme-dependent oxygen activation and NO synthesis to take place (18–20):

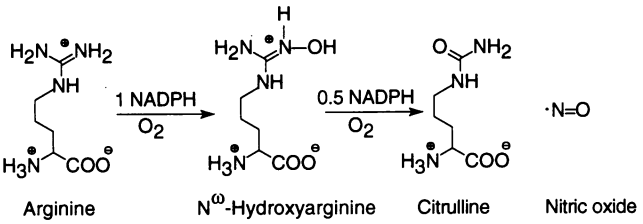

FIG. 1. Reaction catalyzed by the brain and macrophage NOS. The enzymes carry out two distinct oxygenations. NADPH is required both before and after formation of the intermediate N^{ω}-hydroxy-L-arginine, with a total of 1.5 NADPH being oxidized per each NO formed. The oxygen atoms incorporated into NO and citrulline are derived from distinct molecules of O_2.

$$\text{NADPH} \xrightarrow{e^-} \{\text{FAD, FMN}\} \xrightarrow{e^-} \text{heme}$$

However, this electron transfer sequence remains to be demonstrated, and how NOS might control its heme reduction is unclear.

Given the above, we investigated if CAM might regulate NOS by altering electron transfer within the enzyme. Our studies show that CAM binding causes NADPH-derived electrons to transfer onto the heme group of NOS, a process which does not occur in the absence of bound CAM. CAM-triggered heme reduction is associated with commencement of NO synthesis by NOS, is biologically unusual, and provides a basis for how CAM activates NOS at the molecular level.

MATERIALS AND METHODS

Reagents. Human kidney cells expressing cloned rat brain neuronal NOS were a kind gift of Solomon Snyder and David S. Bredt (Johns Hopkins School of Medicine, Baltimore). Interferon-γ was a gift of Genentech. CO gas was obtained from Gas Technics (Cleveland, OH). All other reagents and materials were obtained from Sigma or from sources previously reported (10, 19).

Cell Culture and Enzyme Purification. Kidney cells expressing neuronal NOS were grown in 3-liter spinner flasks in Dulbecco's modified Eagle's medium supplemented with F-12 nutrient mixture (GIBCO) containing 10% calf serum (GIBCO), at 37°C and in a 5% CO_2 atmosphere. Cells were harvested by centrifugation after reaching a density of 1–2 × 10^6 cells per ml. The mouse macrophage cell line RAW 264.7

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: CAM, calmodulin; NOS, nitric oxide synthase.

was used as a source of macrophage NOS and was grown as previously described (10). Eight hours prior to cell harvest, the RAW 264.7 cultures were induced to express NOS by addition of interferon-γ at 100 units/ml and *Escherichia coli* lipopolysaccharide at 1 mg/ml. Neuronal and macrophage NOS were purified from cell supernatants by using a dual-column procedure and Pharmacia FPLC as previously described (19). Column fractions containing purified neuronal or macrophage NOS were concentrated in Centricon-30 microconcentrators (Amicon) at 4°C and stored in 50-μl aliquots at −70°C in 20 mM [bis(2-hydroxyethyl)amino]tris-(hydroxymethyl)methane (Bis-Tris) buffer, pH 7.4, containing 3 mM dithiothreitol, 10% (vol/vol) glycerol, and 2 μM each of FAD and tetrahydrobiopterin. The two-column purification produced macrophage and brain NOS that were ≈90% pure as judged by SDS/PAGE and silver staining, with specific activities that ranged between 750 and 960 nmol of nitrite formed per min per mg of protein at 37°C, in good agreement with previous results (7–10, 19). Protein was assayed with the Bio-Rad kit, using bovine serum albumin as a standard.

NOS Activity. Neuronal NOS activity was monitored during purification by incubating 10 μl of column fractions at 37°C in 40 mM Tris·HCl buffer, pH 7.9, containing protease inhibitors (10), 2 mM L-arginine, 3 mM dithiothreitol, 0.83 mM Ca^{2+}, CAM at 10 μg/ml, 0.6 mM EDTA, and 4 μM each of FAD, FMN, and tetrahydrobiopterin (19). Reactions were started by adding NADPH to give 1 mM and a final volume of 150 μl. Macrophage NOS activity was monitored in a similar system that omitted Ca^{2+}, EDTA, and CAM. After a 2-hr incubation, residual NADPH was removed enzymatically and NO synthesis activity was measured by using the Griess assay for nitrite as previously described (10). Nitrite accumulation was linear over the 2-hr assay period. Nitrite production was found to consistently represent approximately 80% of the total NO generated (determined as nitrite plus nitrate) in this assay system.

The initial rate of NO synthesis by macrophage or neuronal NOS was quantitated at 37°C by using the oxyhemoglobin assay for NO as described (15, 19). Macrophage or neuronal NOS (0.5–2 μg) was added to a cuvette containing 40 mM Tris·HCl buffer, pH 7.8, supplemented with 5–10 μM oxyhemoglobin, 0.3 mM dithiothreitol, 1 mM L-arginine, 0.1 mM NADPH, 4 μM each of FAD, FMN, and tetrahydrobiopterin, catalase at 100 units/ml, superoxide dismutase at 10 units/ml, and bovine serum albumin at 0.1 mg/ml, to give a final volume of 0.7 ml. The NO-mediated conversion of oxyhemoglobin to methemoglobin was monitored over time as an absorbance increase at 401 nm and was quantitated by using an extinction coefficient of 38 $mM^{-1} \cdot cm^{-1}$.

NADPH Consumption by NOS. The initial rates of NADPH oxidation were monitored spectrophotometrically at 340 nm. Cuvettes contained 1 ml of 40 mM Tris·HCl buffer, pH 7.8, with 250 μM NADPH, 0.9 mM EDTA, CAM at 10 μg/ml, 4 μM tetrahydrobiopterin, 1 mM dithiothreitol, 4 μM FAD, 4 μM FMN, and catalase at 10 units/ml. Reactions were run at 37°C. Samples of macrophage or neuronal NOS, L-arginine, and Ca^{2+} were added such that their additions to the cuvette did not increase its final volume by more than 5%.

Optical Spectroscopy. Spectra were recorded with a Hitachi 3110 spectrophotometer at 15°C. Enzyme samples were placed in septum-sealed cuvettes and made anaerobic by repeated cycles of evacuation and purging with high-purity argon gas. The cuvette buffers were purged and gassed with argon in a separate vessel and were saturated with CO gas. Cuvette buffer was then transferred to cuvettes by using gas-tight syringes, and the cuvettes were maintained under a slight positive pressure with argon during the spectral measurements.

RESULTS AND DISCUSSION

Under anaerobic conditions, the visible spectrum of neuronal NOS displayed a broad Soret absorbance peak centered at 398 nm, indicative of a ferric heme, with absorbance shoulders at 456 and 485 nm attributable to oxidized flavins (Fig. 2*A*, trace 1), as reported previously (19, 20). Addition of excess NADPH caused a reduction of the NOS flavins, as indicated by the disappearance of their absorbance peaks at 456 and 485 nm, but did not cause reduction of the NOS ferric heme iron, as indicated by its failure to bind dissolved CO (Fig. 2*A*, trace 2). However, upon Ca^{2+} addition to promote CAM binding to NOS (7–9), electron transfer to the ferric heme iron occurred, as evidenced by the generation of an absorbance peak at 444 nm that indicated binding of dissolved CO to the ferrous (reduced) NOS heme (Fig. 2*A*, trace 3). This Ca^{2+}-dependent electron transfer did not occur when CAM was absent (not shown). Thus, CAM binding to neuronal NOS appeared to trigger electron transfer onto its heme.‡

In cytochromes P-450, electron transfer to heme occurs in response to substrate binding, which induces a change in the heme iron reduction potential (23, 24). Because CAM is thought to activate some target enzymes by enabling substrate to bind within their active sites (1, 2), we examined if CAM would enable L-arginine to bind to neuronal NOS, and whether L-arginine binding could trigger electron transfer onto the NOS heme. Addition of L-arginine to NADPH-reduced NOS under anaerobic conditions caused a shift in its heme Soret absorbance that indicated L-arginine had bound to the enzyme (Fig. 2*B*, traces 1 and 2). This spectral shift is also illustrated in the difference spectrum generated from these two traces, which shows a characteristic type I absorbance increase at 390 nm and decrease at 420 nm (23) upon addition of L-arginine to the enzyme (Fig. 2*B*, *Inset*). This indicated that bound CAM was not required for neuronal NOS to bind L-arginine. Moreover, L-arginine binding did not trigger electron transfer to the NOS heme, as indicated by its failure to bind CO after L-arginine addition (Fig. 2*B*, trace 2). However, electron transfer to the heme of the substrate-bound NOS did occur upon Ca^{2+}-promoted CAM binding (Fig. 2*B*, trace 3), mirroring results obtained with the substrate-free enzyme.

Cytokines induce macrophages to express a distinct NOS which contains tightly bound CAM (14), enabling it to catalyze NO synthesis independent of added Ca^{2+} or CAM (10). Our above results predict that macrophage NOS should always be primed to transfer NADPH-derived electrons onto its heme, due to its constitutively bound CAM. In the absence of added Ca^{2+} or L-arginine, the spectrum of macrophage NOS under anaerobic conditions was similar to that of neuronal NOS and indicated the presence of ferric heme and oxidized flavins (Fig. 3*A*, trace 1) (19). Addition of NADPH did indeed result in spontaneous reduction of the macrophage NOS ferric heme (Fig. 3*A*, trace 2), consistent with the hypothesis that tightly bound CAM had enabled this electron transfer to occur within macrophage NOS. (Definitive proof that CAM also triggers heme reduction in macrophage NOS awaits the generation of a CAM-free species.)

We next measured enzymatic NADPH consumption under various circumstances to compare how CAM binding might influence the *rate* of electron flow through neuronal and macrophage NOS. The results are shown graphically in Figs. 2*C* and 3*B* and are also summarized in Table 1. In oxygenated buffer, neuronal NOS catalyzed a slow oxidation of NADPH in the absence of bound CAM (13.6 nmol/min per mg, $n = 4$),

‡Reduction of the NOS heme iron upon CAM binding has also been observed by using electron paramagnetic resonance spectroscopy (M. Ikeda-Saito, A. Matsuoka, and D.J.S., unpublished results).

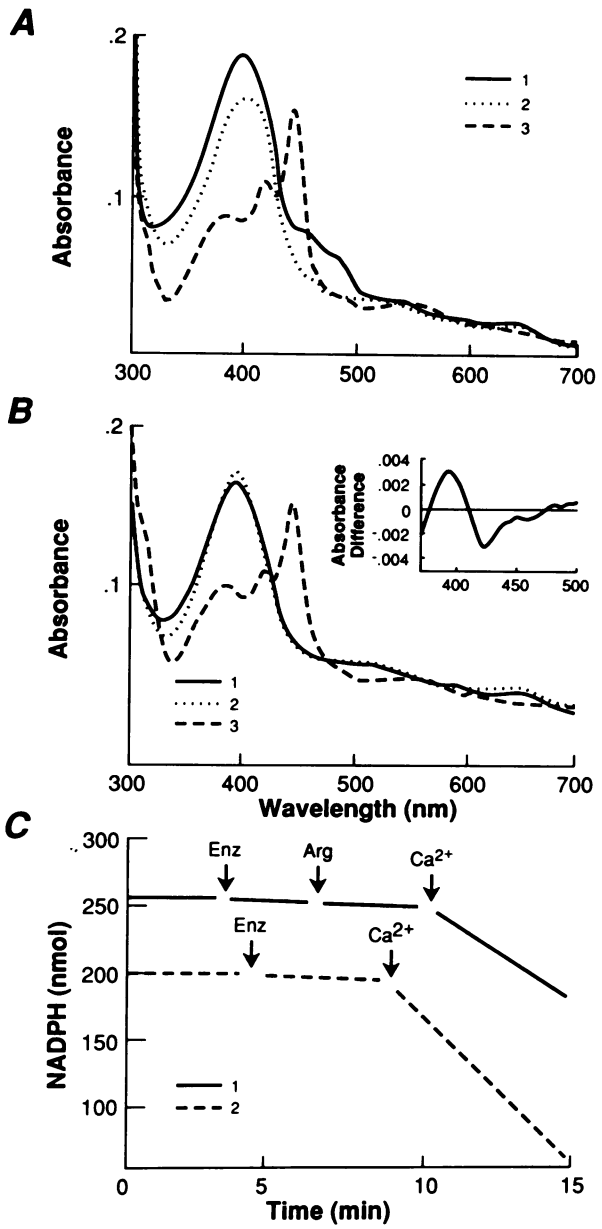

FIG. 2. (*A* and *B*) Light absorbance spectra of purified neuronal NOS, indicating electron transfer to its flavin and heme groups under various conditions. (*A*) Trace 1, neuronal NOS as isolated, under anaerobic conditions and diluted to 3 μM (in heme) in 0.7 ml of 40 mM Tris·HCl buffer, pH 7.8, saturated with CO, and containing 5 μM CAM, 0.9 mM EDTA, 1 mM dithiothreitol, and 4 μM tetrahydrobiopterin. Trace 2, enzyme solution after addition of 10 nmol (14 μM) of NADPH. Trace 3, NADPH-reduced enzyme after addition of 1.2 mM Ca^{2+}. (*B*) Trace 1, neuronal NOS diluted to 3 μM in CO-saturated buffer as described above and after anaerobic reduction with 10 nmol of NADPH. Trace 2, NADPH-reduced enzyme plus 0.5 mM L-arginine. Trace 3, NADPH-reduced, L-arginine-bound enzyme after addition of 1.2 mM Ca^{2+}. (*Inset*) Trace 2 minus trace 1 difference spectrum, which highlights spectral changes caused by L-arginine binding to NOS. (*C*) Rate of NADPH oxidation by neuronal NOS in the presence of O_2 under various conditions. NADPH oxidation at 37°C was measured by the decrease in absorbance at 340 nm over time. Trace 1 is, from the left: in the absence of enzyme, after addition of neuronal NOS to 0.14 μM (in heme), after addition of 0.5 mM L-arginine, and after addition of 1.2 mM Ca^{2+} to trigger CAM binding. Trace 2 is identical to trace 1 except that addition of L-arginine was omitted.

which did not increase upon L-arginine binding (Fig. 2*C*, trace 1) and was not associated with heme reduction (Fig. 2 *A* and *B*, trace 2) or NO synthesis by NOS. We attribute this heme-independent NADPH consumption to a slow transfer of electrons from the neuronal NOS flavins to molecular oxygen. In the presence of L-arginine, CAM binding dramatically increased NADPH oxidation by neuronal NOS to 906 nmol/min per mg (n = 4) (Fig. 2*C*, trace 1), as observed previously (8, 9, 12, 13), and triggered a rate of NO synthesis (660 nmol/min per mg, n = 4) from L-arginine that was consistent with the stoichiometry of the reaction (see Fig. 1). This indicated that NADPH oxidation and NO synthesis by neuronal NOS were controlled through CAM-promoted electron transfer to its heme. In the absence of L-arginine, the Ca^{2+}-promoted CAM binding triggered an even greater rate

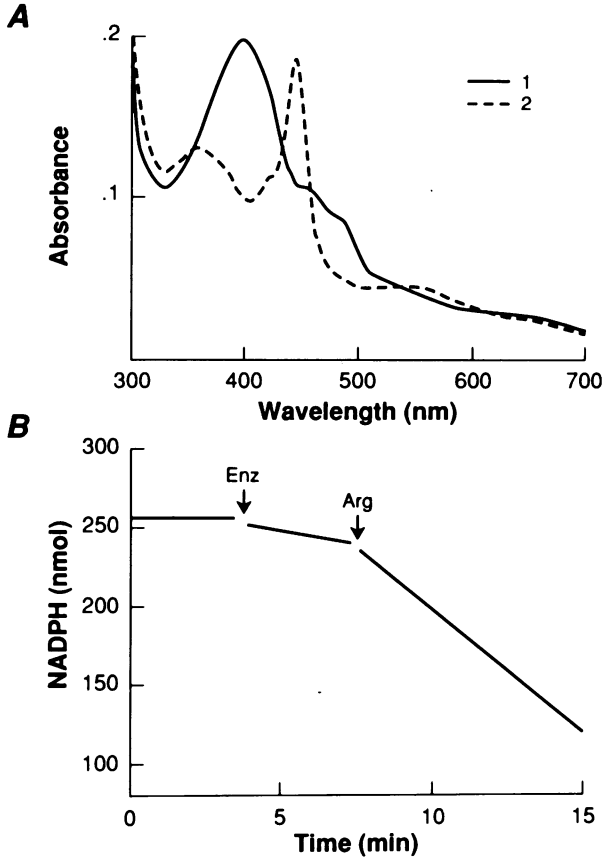

FIG. 3. (*A*) Light absorbance spectra of purified macrophage NOS indicating electron transfer to its heme group. Trace 1, macrophage NOS as isolated, under anaerobic conditions and diluted to 3 μM (in heme) in 0.7 ml of 40 mM Tris·HCl buffer, pH 7.8, saturated with CO and containing 0.9 mM EDTA, 1 mM dithiothreitol, and 4 μM tetrahydrobiopterin. Trace 2, the enzyme after addition of 10 nmol (14 μM) NADPH. (*B*) NADPH oxidation by macrophage NOS over time in oxygenated buffer at 37°C, as measured by the absorbance decrease at 340 nm over time. Traces are, from the left: in the absence of enzyme, after addition of enzyme to 0.10 μM (in heme), and after addition of 0.5 mM L-arginine.

Table 1. Effect of bound CAM and L-arginine on NADPH oxidation by neuronal and macrophage NOS

Condition	NADPH oxidation, nmol/min per mg protein	
	Neuronal NOS	Macrophage NOS
Enzyme alone	14	NA
Enzyme + CAM	1450	229
Enzyme + CAM + L-arginine	906	1390

NA, not available.

of NADPH oxidation by neuronal NOS (1450 nmol/min per mg, $n = 4$) (Fig. 2*C*, trace 2), which was presumably associated with heme-catalyzed reduction of O_2 to superoxide (12, 13). This suggested that in the presence of excess NADPH, CAM binding is sufficient to trigger a high rate of electron transfer to the heme of neuronal NOS both in the presence and in the absence of its substrate, L-arginine.

In oxygenated solution, NADPH consumption by macrophage NOS was 229 nmol/min per mg ($n = 3$) (Fig. 3*B*). This rate was 16-fold greater than that observed for CAM-free neuronal NOS and therefore is consistent with our proposal that tightly bound CAM allows spontaneous transfer of electrons onto the macrophage NOS heme. However, this rate was one-sixth of that obtained with CAM-bound L-arginine-free neuronal NOS (Fig. 2*C*, trace 2; compared in Table 1). In addition, we found that L-arginine increased NADPH oxidation by macrophage NOS 6-fold to 1390 nmol/min per mg ($n = 3$) (Fig. 3*B*), which then was associated with NO synthesis by the enzyme (842 nmol/min per mg, $n = 3$). This contrasted markedly with neuronal NOS, whose transfer of NADPH-derived electrons was actually slowed 37% upon L-arginine binding (traces 1 and 2 of Fig. 2*C*; compared in Table 1).

Thus, clear differences exist regarding how the two NOS isoforms control electron transfer to their heme groups. For neuronal NOS, CAM binding alone appears sufficient to trigger a high rate of electron transfer to its heme. This enables immediate NO production in response to Ca^{2+} but can also lead to high rates of superoxide production in the absence of substrate (12, 13). The apparent lack of substrate control displayed by neuronal NOS contrasts with most cytochrome P-450 enzymes, which require that substrates be bound before their heme can accept electrons from biological electron donors (23, 24). For macrophage NOS, we speculate that its tightly bound CAM may bestow a continuously available but suboptimal means of heme reduction that is increased to a catalytically competent rate (i.e., a rate that is sufficient to support the observed rate of NO synthesis) upon L-arginine binding. Substrate control of heme reduction makes macrophage NOS more similar to the cytochromes P-450, and it provides the macrophage isoform with a means to minimize unwarranted reduction of O_2.

That the NOS isoforms have developed distinct strategies to control electron transfer and uncoupled O_2 reduction may relate to their divergent use in cell signaling versus immunomodulatory pathways. Under physiologic conditions, neurons probably maintain sufficient L-arginine for NOS to function normally (i.e., synthesize NO) in response to transient increases in intracellular Ca^{2+}. Conceivably, NOS might be capable of depleting neuronal L-arginine and generating superoxide during episodes of prolonged Ca^{2+} influx that occur in certain pathologic conditions (25), but this must be rare, given that the enzyme apparently did not evolve a means to control its heme reduction and superoxide generation in the absence of L-arginine. In contrast, macrophage NOS appears capable of continuous heme reduction, possibly by virtue of its bound CAM, but down-regulates this electron transfer unless L-arginine is available. This is consistent with activated macrophages migrating to sites where L-arginine becomes limiting, such as in wounds (26), and indicates a need to govern superoxide generation by NOS in these circumstances.

Although CAM control of electron transfer within NOS is unprecedented, it may not be unique. Evidence suggests that electron transfer within the multimeric heme protein sulfite reductase of *Desulfovibrio gigas* may also be controlled by a distinct Ca^{2+}-binding protein (27). Thus, Ca^{2+}-binding proteins may have previously unexpected but important roles in modulating electron transfer in a variety of biological systems.

We thank Bonnie Thiel and Pam Clark for excellent technical assistance, Drs. Solomon Snyder and David Bredt for providing cells that express cloned neuronal NOS, and Drs. Masao Ikeda-Saito and Tom Hamilton for critical comments on the manuscript. This work was supported by National Institutes of Health Grant 53914 and a grant from the Council for Tobacco Research.

1. Cohen, P. & Klee, C. B., eds. (1988) *Calmodulin: Molecular Aspects of Cellular Regulation* (Elsevier, Amsterdam), Vol. 5.
2. Means, A. R. (1988) *Recent Prog. Horm. Res.* **44,** 223–259.
3. Ignarro, L. J. (1990) *Annu. Rev. Pharmacol. Toxicol.* **30,** 535–560.
4. Moncada, S., Palmer, R. M. J. & Higgs, E. A. (1991) *Pharmacol. Rev.* **43,** 109–142.
5. Stuehr, D. J. & Griffith, O. W. (1992) *Adv. Enzymol. Relat. Areas Mol. Biol.* **65,** 287–346.
6. Nathan, C. F. (1992) *FASEB J.* **6,** 3051–3064.
7. Bredt, D. S., Hwang, P. M., Glatt, C. E., Lowenstein, C., Reed, R. R. & Snyder, S. H. (1991) *Nature (London)* **351,** 714–718.
8. Schmidt, H. H. H. W., Pollock, J. S., Nakane, M., Gorsky, L. D., Förstermann, U. & Murad, F. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 365–369.
9. Mayer, B., John, M., Heinzel, B., Werner, E. R., Wachter, H., Schultz, G. & Bohme, E. (1991) *FEBS Lett.* **288,** 1,2, 187–191.
10. Stuehr, D. J., Cho, H. J., Kwon, N. S., Weise, M. F. & Nathan, C. F. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 7773–7777.
11. Xie, Q.-W., Cho, H. J., Calaycay, J., Mumford, R. A., Swiderek, K. M., Lee, T. D., Ding, A., Troso, T. & Nathan, C. F. (1992) *Science* **256,** 225–228.
12. Heinzel, B., John, M., Klatt, P., Bohme, E. & Mayer, B. (1992) *Biochem. J.* **281,** 627–630.
13. Pou, S., Pou, W. S., Bredt, D. S., Snyder, S. H. & Rosen, G. M. (1992) *J. Biol. Chem.* **267,** 24173–24176.
14. Cho, H. J., Xie, Q.-W., Calaycay, J., Mumford, R. A., Swiderek, K. M., Lee, T. D. & Nathan, C. (1992) *J. Exp. Med.* **176,** 599–604.
15. Stuehr, D. J., Kwon, N. S., Nathan, C. F., Griffiths, O. W., Feldman, P. L. & Wiseman, J. (1991) *J. Biol. Chem.* **266,** 6259–6263.
16. Kwon, N. S., Nathan, C. F., Gilker, C., Griffith, O. W., Matthews, D. E. & Stuehr, D. J. (1990) *J. Biol. Chem.* **265,** 13442–13445.
17. Hevel, J. M., White, K. A. & Marletta, M. A. (1991) *J. Biol. Chem.* **266,** 22789–22792.
18. White, K. A. & Marletta, M. A. (1992) *Biochemistry* **31,** 6627–6631.
19. Stuehr, D. J. & Ikeda-Saito, M. (1992) *J. Biol. Chem.* **267,** 20547–20550.
20. McMillan, K., Bredt, D. S., Hirsch, D. J., Snyder, S. H., Clark, J. E. & Masters, B. S. S. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 11141–11145.
21. Bredt, D. S., Ferris, C. D. & Snyder, S. H. (1992) *J. Biol. Chem.* **267,** 10976–10981.
22. Schmidt, H. H. H. W., Smith, R. M., Nakane, M. & Murad, F. (1992) *Biochemistry* **31,** 3243–3248.
23. Guengerich, F. P. (1990) *Crit. Rev. Biochem. Mol. Biol.* **25,** 97–153.
24. Gonzalez, F. J. (1989) *Pharmacol. Rev.* **40,** 243–288.
25. Dawson, V. L., Dawson, T. M., London, E. D., Bredt, D. S. & Snyder, S. H. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 6368–6371.
26. Albina, J. E., Mills, C. D., Henry, W. L., Jr., & Caldwell, M. D. (1990) *J. Immunol.* **144,** 3877–3880.
27. Liang Chen, L., Liu, M.-Y. & Le Gall, J. (1991) *Biochem. Biophys. Res. Commun.* **180,** 238–242.