

# Arginine Metabolism: Enzymology, Nutrition, and Clinical Significance

## Enzymes of the L-Arginine to Nitric Oxide Pathway<sup>1,2</sup>

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**ABSTRACT** L-Arginine is the biological precursor of nitric oxide (NO), which serves as an important signal and effector molecule in animals. This review summarizes some structure-function aspects of the mammalian nitric oxide synthases, which are enzymes that catalyze the oxidation of L-arginine to NO and L-citrulline. These include aspects related to: 1) the chemical transformations of L-arginine during enzyme catalysis, 2) binding of L-arginine or its structural analogs to the nitric oxide synthases, and 3) how L-arginine levels may affect product formation by the nitric oxide synthases and how this can be modulated by structural analogs of L-arginine. J. Nutr. 134: 2748S–2751S, 2004.

**KEY WORDS:** • L-arginine • nitric oxide • flavoprotein • reactive oxygen species • heme protein

The widespread interest in nitric oxide (NO) biology can be traced back to nutrition and toxicology research conducted in the early 1980s that established that mammals are indeed capable of NO biosynthesis (1,2). L-Arginine (Arg)<sup>4</sup> was subsequently found to be the precursor of NO and related N-oxides (nitrite, nitrate) that are produced by mammals (3–5). This represents a new metabolic pathway for Arg and is of current interest because of the tremendous biological and medical importance of NO.

### Some general properties of the NO synthases

A family of enzymes called the NO synthases (NOSs, EC 1.14.13.39) catalyze the oxidation of Arg to NO and L-citrulline, with NADPH and O<sub>2</sub> serving as cosubstrates (6). The NOSs first hydroxylate a terminal guanidino nitrogen of Arg to generate N-hydroxy-L-arginine (NOHA) as an enzyme-bound intermediate. NOHA is then oxidized further by the enzyme to generate NO plus L-citrulline (Fig. 1). Three related NOSs are expressed in mammals, along with several splice variants (7). NOS-like enzymes are also coded for in the genomes of most life forms, including bacteria (8,9). The mammalian NOSs have a similar structure and composition. They are all homodimeric, heme-containing flavoproteins. The NOS flavins transfer NADPH-derived electrons to the heme (Fig. 2). This enables the heme to bind and activate dioxygen in both steps of NO synthesis. 6-(R)-Tetrahydro-

biopterin (H4B) is also a required cofactor, and it is tightly bound in NOS next to the heme (10,11). H4B has structural and redox roles in the NOS (11,12). The *K<sub>s</sub>* values for Arg and NOHA binding to NOSs range from 1 to 20 μmol/L (6). The electron transfer reactions of NOS are regulated by a calcium-binding protein (calmodulin), which can bind to some NOSs in a reversible manner (6). This enables cells to couple their NO synthesis to changes in intracellular calcium ion concentration. Calmodulin binds tightly to other NOSs at all cellular calcium ion concentrations; thus, their NO synthesis is regulated primarily through control of gene expression (13).

### Arg binding site in NOS

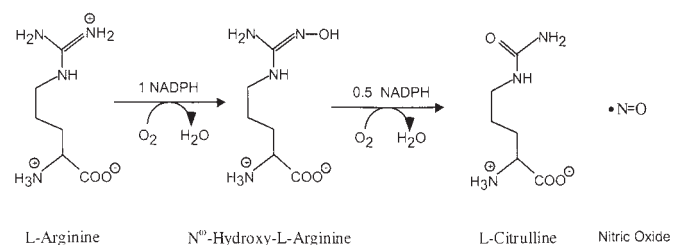
Crystal structures of NOS heme domains that contain bound Arg or NOHA show in detail how either of these substrates bind in the enzyme active site (14–16,10). There is a funnel-shaped entrance to the enzyme active site that allows substrate and dioxygen access to the heme. In general, NOS binds Arg or NOHA in a similar extended conformation, with the guanidino (or hydroxyguanidino) moiety located above the heme iron and the amino acid portion held away from the heme (Fig. 3). The location of the guanidino group above the heme iron is consistent with the heme iron being the site where dioxygen binds and becomes activated in NOS, such that the resulting heme iron-oxy species can react directly with the guanidino moiety. Arg and NOHA are held in place by hydrogen bonds that form between conserved residues of NOS and both the amino acid and guanidino ends of the substrates. Several of the binding residues are located in a substrate-binding helix of NOS (16). The substrate hydrogen-bonding network extends into the H4B binding site, consistent with H4B and Arg binding to NOS in a cooperative manner (12) and with the finding that point mutations in the H4B binding site of NOS often lower the binding affinity toward Arg (17). The α amino groups of Arg and NOHA also

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<sup>4</sup> Abbreviations used: Arg, L-arginine; H4B, 6-(R)-tetrahydrobiopterin; NO, nitric oxide; NOHA, N-hydroxy-L-arginine; NOS, nitric oxide synthase (EC 1.14.13.39).



**FIGURE 1** The two reactions of NO synthesis as catalyzed by NOS. The NADPH and oxygen requirements of each reaction are shown.

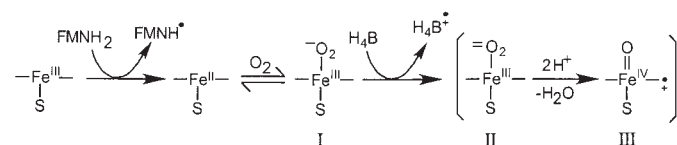
hydrogen bond with a heme propionate group, but the functional significance of this interaction is unclear. Point mutagenesis studies demonstrate the relative importance of some of the NOS substrate binding residues for Arg binding (18–20). In particular, there is a conserved glutamic acid whose side-chain carboxylate forms hydrogen bonds with two of the guanidino nitrogens of Arg or NOHA. Removing this interaction by point mutagenesis completely disables Arg binding to NOS. Mutational substitution at other residues involved in Arg or NOHA binding typically has a less drastic effect.

**Effects of Arg binding on NO synthase**

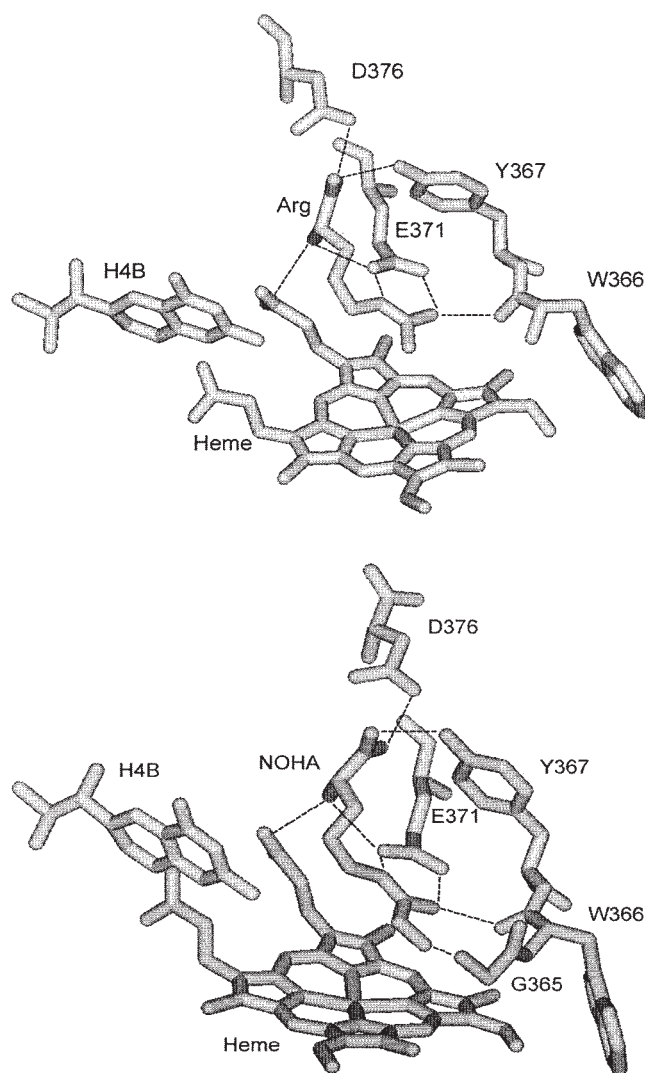
Arg binding causes a number of observable changes in NOSs. It stabilizes the NOS ferric heme iron in a high-spin electronic state, which is typically manifested by the shifting of the heme Soret absorbance band to a lower wavelength (21). Arg binding increases NOS affinity for H4B, consistent with their binding in a cooperative manner (12). Arg binding increases the reduction potential of the NOS heme iron (22). This is important for some NOSs because it makes their heme reduction by the flavins thermodynamically favorable (**Fig. 4**). Arg binding also stabilizes the dimeric structure of some NOSs (23,24), and stabilizes heme-NO complexes that form during NOS catalysis (25,26).

**Substrate analogs of Arg**

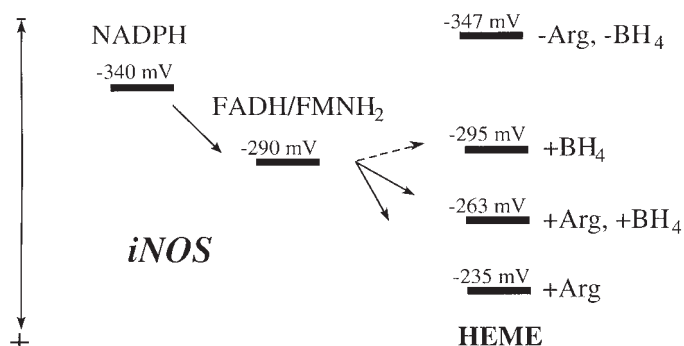
Various Arg derivatives can serve as substrates for NOS, including non-amino acid analogs (**Fig. 5**) (27,28). In general, NOSs display a lower binding affinity toward these analogs, show isoenzyme selectivity, and have slower  $V_{\text{max}}$  values regarding NO synthesis. The *N*-hydroxyguanidine analogs typically bind more tightly than the guanidine analogs and support greater NO synthesis. However, NO synthesis from the guanidine analogs is likely compromised due to dissociation of the *N*-hydroxyguanidine intermediate from NOS during catalysis. These data indicate that NOS interactions with the



**FIGURE 2** Mechanism of oxygen activation by the NOS heme. The ferric heme first receives an electron from the FMN hydroquinone (FMNH<sub>2</sub>) that is located in the NOS flavoprotein domain. This enables dioxygen to bind, forming the ferric-superoxy species (I). This species then receives an electron from tetrahydrobiopterin (H<sub>4</sub>B) to generate heme peroxo (II) and perferryl (III) species that are thought to react with Arg or NOHA.



**FIGURE 3** Arg and NOHA binding in inducible NOS. The crystal structure data indicate how Arg (*top*) or NOHA (*bottom*) binds within the active site of NOS in relation to the enzyme heme and H<sub>4</sub>B groups. Protein residues that make hydrogen bond contacts with Arg or NOHA are indicated, with the hydrogen bonds shown as thin lines. Data are from Crane et al. (10) and Crane et al. (14).



**FIGURE 4** Thermodynamic profile for heme reduction in inducible NOS. Estimated reduction potentials for NADPH and the NOS flavoprotein (FADH/FMNH<sub>2</sub>) are indicated. The right-hand portion of the figure shows how bound Arg or H<sub>4</sub>B increases the reduction potential of the ferric heme iron. Electron transfer toward a more positive potential is favored; arrows indicate the direction of electron transfer. Data are from Presta et al. (22).

Structure	$K_m$ ( $\mu\text{M}$ )	$V_{\text{max}}$ (%)
	5	100
	20	100
	45	6
	55	68
	>1000	0.5
	300	83

**FIGURE 5** Arg analogs that are substrates for NOS. Structures are shown with the corresponding  $K_m$  and relative  $V_{\text{max}}$  values for NO synthesis. Charges are not shown. Data are from Dijols (28).

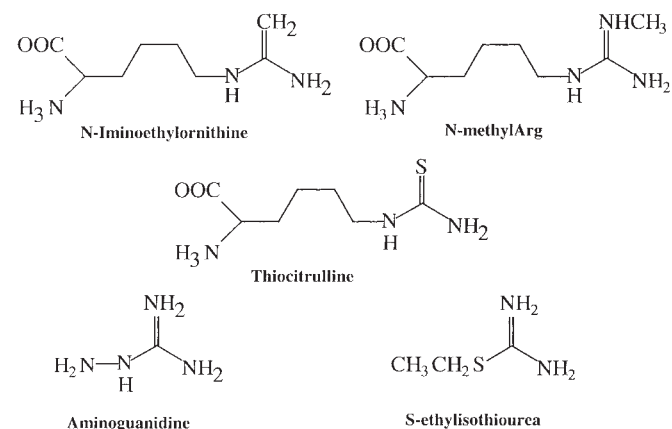
amino acid portion of Arg are not essential for enzyme catalysis.

#### Nonsubstrate analogs of Arg

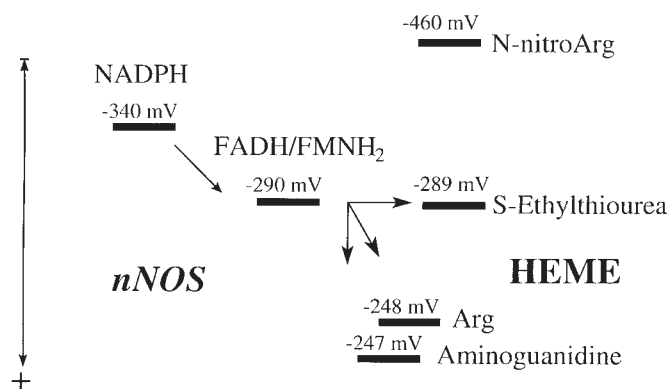
A great number of Arg derivatives are not substrates for NOS and act instead as competitive inhibitors or in some cases as mechanism-based inactivators (29). Many of these compounds no longer contain the guanidino moiety, which is essential for NO synthesis. In some cases, the NOS binding affinity toward these analogs is greater than that toward Arg itself. **Figure 6** depicts the structures of some representative analogs. NOS crystal structures that contain some of the bound inhibitors are available (30).

#### Consequences of suboptimal Arg concentration

At nonsaturating Arg concentrations, a calmodulin-bound NOS will continue to bind and activate dioxygen at its heme



**FIGURE 6** Structures of Arg analogs that are NOS inhibitors. Charges are not shown.



**FIGURE 7** Effect of some NOS inhibitors on the thermodynamics of neuronal NOS heme reduction. Estimated reduction potentials for NADPH and the NOS flavoprotein (FADH/FMNH<sub>2</sub>) are indicated. The right-hand portion of the figure compares how bound Arg and Arg analog inhibitors poise the reduction potential of the ferric heme iron. Electron transfer toward a more positive potential is favored; arrows indicate the direction of electron transfer. Data are from Presta et al. (22).

and subsequently will release reactive oxygen species such as superoxide and H<sub>2</sub>O<sub>2</sub> (31,32). The frequency and biological effect of this process are under scrutiny. An active NOS operating at a subsaturating Arg concentration can generate NO and superoxide at the same time. These products can conceivably react to generate the cellular toxin peroxynitrite (33,34).

Certain Arg analogs can inhibit the production of reactive oxygen species by NOS, whereas others either allow it or enhance it (35,36). Some of the analogs that inhibit reactive oxygen species production by NOS act by lowering the heme midpoint reduction potential, rendering the transfer of electrons from the NOS flavins thermodynamically unfavorable (**Fig. 7**) (22). This represents one strategy to control production of reactive oxygen species by NOS under conditions of low Arg concentration.

#### Regulating Arg availability for the NOSs

In some cells, the expression of cationic amino acid transporters is increased coincident with NOS expression (37,38). Arginase may also be expressed and typically is the chief competitor for Arg in cells and tissues (39–41). In certain environments, arginase expression can deplete Arg levels to the point where NO synthesis by NOS is compromised (42,43). Citrulline can be converted to Arg in many tissues, and this may provide a significant source of Arg in some cases (44). Endogenous methylarginines are also present in tissues at concentrations that may inhibit Arg binding to NOS (45–47). Arg demethylase enzymes may serve to regenerate small amounts of Arg (48). Factors regulating Arg availability are discussed in detail in some of the other papers of this series.

#### LITERATURE CITED

- Green, L. C., Ruiz de Luzuriaga, K., Wagner, D. A., Rand, W., Istfan, N., Young, V. R. & Tannenbaum, S. R. (1981) Nitrate biosynthesis in man. *Proc. Natl. Acad. Sci. U.S.A.* 78: 7764–7768.
- Castillo, L., Beaumier, L., Ajami, A. M. & Young, V. R. (1996) Whole body nitric oxide synthesis in healthy men determined from [<sup>15</sup>N]arginine-to-[<sup>15</sup>N]citrulline labeling. *Proc. Natl. Acad. Sci. U.S.A.* 93: 11460–11465.
- Hibbs, J. B., Jr., Taintor, R. R. & Vavrin, Z. (1987) Macrophage cytotoxicity: role for L-arginine deiminase and imino nitrogen oxidation to nitrite. *Science* 235: 473–476.
- Iyengar, R., Stuehr, D. J. & Marletta, M. A. (1987) Macrophage syn-

thesis of nitrite, nitrate, and nitrosamines: precursors and role of the respiratory burst. *Proc. Natl. Acad. Sci. U.S.A.* 84: 6369–6373.

5. Palmer, R. M., Ashton, D. S. & Moncada, S. (1988) Vascular endothelial cells synthesize nitric oxide from L-arginine. *Nature* 333: 664–666.
6. Alderton, R. K., Cooper, C. E. & Knowles, R. G. (2001) Nitric oxide synthases: structure, function and inhibition. *Biochem. J.* 357 (pt. 3): 593–615.
7. Newton, D. C., Bevan, S. C., Choi, S., Robb, G. B., Millar, A., Wang, Y. & Marsden, P. A. (2003) Translational regulation of human neuronal nitric-oxide synthase by an alternatively spliced 5'-untranslated region leader exon. *J. Biol. Chem.* 278: 636–644.
8. Pant, K., Bilwes, A. M., Adak, S., Stuehr, D. J. & Crane, B. R. (2002) Structure of a nitric oxide synthase heme protein from *Bacillus subtilis*. *Biochemistry* 41: 11071–11079.
9. Bird, L. E., Ren, J., Zhang, J., Foxwell, N., Hawkins, A. R., Charles, I. G. & Stammers, D. K. (2002) Crystal structure of SANOS, a bacterial nitric oxide synthase oxygenase protein from *Staphylococcus aureus*. *Structure (Camb)* 10: 1687–1696.
10. Crane, B. R., Arvai, A. S., Ghosh, S., Getzoff, E. D., Stuehr, D. J., & Tainer, J. A. (2000) Structures of the N(omega)-hydroxy-L-arginine complex of inducible nitric oxide synthase oxygenase dimer with active and inactive pterins. *Biochemistry* 39: 4608–4621.
11. Wei, C. C., Crane, B. R. & Stuehr, D. J. (2003) Tetrahydrobiopterin radical enzymology. *Chem. Rev.* 103: 2365–2383.
12. Werner, E. R., Gorren, A. C., Heller, R., Werner-Felmayer, G. & Mayer, B. (2003) Tetrahydrobiopterin and nitric oxide: mechanistic and pharmacological aspects. *Exp. Biol. Med. (Maywood)* 228: 1291–1302.
13. Guo, Z., Shao, L., Feng, X., Reid, K., Marderstein, E., Nakao, A. & Geller, D. A. (2003) A critical role for C/EBP $\beta$  binding to the AABS promoter response element in the human iNOS gene. *FASEB J.* 17: 1718–1720.
14. Crane, B. R., Arvai, A. S., Ghosh, D. K., Wu, C., Getzoff, E. D., Stuehr, D. J. & Tainer, J. A. (1998) Structure of NO synthase oxygenase dimer with pterin and substrate. *Science* 279: 2121–2126.
15. Raman, C. S., Li, H., Martasek, P., Kral, V., Masters, B. S. & Poulos, T. L. (1998) Crystal structure of constitutive endothelial nitric oxide synthase: a paradigm for pterin function involving a novel metal center. *Cell* 95: 939–950.
16. Fischmann, T. O., Hruza, A., Niu, X. D., Fossetta, J. D., Lunn, C. A., Dolphin, E., Prongay, A. J., Reichert, P., Lundell, D. J. et al. (1999) Structural characterization of nitric oxide synthase isoforms reveals striking active-site conservation. *Nat. Struct. Biol.* 6: 233–242.
17. Ghosh, S., Wolan, D., Adak, S., Crane, B. R., Kwon, N. S., Tainer, J. A., Getzoff, E. D. & Stuehr, D. J. (1999) Mutational analysis of the tetrahydrobiopterin binding site in inducible NO synthase. *J. Biol. Chem.* 274: 24100–24112.
18. Gachhui, R., Ghosh, D. K., Wu, C., Parkinson, J., Crane, B. R. & Stuehr, D. J. (1997) Mutagenesis of acidic residues in the oxygenase domain of inducible NO synthase identifies a glutamate involved in arginine binding. *Biochemistry* 36: 5097–5103.
19. Chen, P. F., Tsai, A. L., Berka, V. & Wu, K. K. (1997) Mutation of Glu-361 in human endothelial nitric-oxide synthase selectively abolishes L-arginine binding without perturbing the behavior of heme and other redox centers. *J. Biol. Chem.* 272: 6114–6118.
20. Chen, P. F., Berka, V., Tsai, A. L. & Wu, K. K. (1998) Effects of Asp-369 and Arg-372 mutations on heme environment and function in human endothelial nitric-oxide synthase. *J. Biol. Chem.* 273: 34164–34170.
21. McMillan, K. & Masters, B. S. (1993) Optical difference spectrophotometry as a probe of rat brain nitric oxide synthase heme-substrate interaction. *Biochemistry* 32: 9875–9880.
22. Presta, A., Weber-Main, A. M., Stankovich, M. T. & Stuehr, D. J. (1998) Spectroelectrochemical determination of nitric oxide synthase heme reduction potentials. *J. Am. Chem. Soc.* 120: 9460–9465.
23. Klatt, P., Schmidt, K., Lehner, D., Glatzer, O., Bachinger, H. P. & Mayer, B. (1995) Structural analysis of porcine brain nitric oxide synthase reveals a role for tetrahydrobiopterin and L-arginine in the formation of an SDS-resistant dimer. *EMBO J.* 14: 3687–3695.
24. Panda, K., Rosenfeld, R. J., Ghosh, S., Meade, A. L., Getzoff, E. D. & Stuehr, D. J. (2002) Distinct dimer interaction and regulation in nitric-oxide synthase types I, II, and III. *J. Biol. Chem.* 277: 31020–30.
25. Migita, C. T., Salerno, J. C., Masters, B. S., Martasek, P., McMillan, K. & Ikeda-Saito, M. (1997) Substrate binding-induced changes in the EPR spectra of the ferrous nitric oxide complexes of neuronal nitric oxide synthase. *Biochemistry* 36: 10987–10992.
26. Huang, L., Abu-Soud, H. M., Hille, R. & Stuehr, D. J. (1999) NO-generated P420 NO synthase: Characterization and roles for tetrahydrobiopterin and substrate in protecting against or reversing the P420 conversion. *Biochemistry* 38: 1912–1920.
27. Jia, Q., Cai, T., Huang, M., Li, H., Xian, M., Poulos, T. L. & Wang, P. G. (2003) Isoform-selective substrates of nitric oxide synthase. *J. Med. Chem.* 46: 2271–2274.
28. Dijols, S., Boucher, J. L., Lepoivre, M., Lefevre-Groboillot, D., Moreau, M., Frapart, Y., Rekkas, E., Meade, A. L., Stuehr, D. J. & Mansuy, D. (2002) First non-alpha-amino acid guanidines acting as efficient NO precursors upon oxidation by NO-synthase II or activated mouse macrophages. *Biochemistry* 41: 9286–9292.
29. Ji, H., Li, H., Flinspach, M., Poulos, T. L. & Silverman, R. B. (2003) Computer modeling of selective regions in the active site of nitric oxide synthases: implication for the design of isoform-selective inhibitors. *J. Med. Chem.* 46: 5700–5711.
30. Li, H., Raman, C. S., Martasek, P., Masters, B. S. & Poulos, T. L. (2001) Crystallographic studies on endothelial nitric oxide synthase complexed with nitric oxide and mechanism-based inhibitors. *Biochemistry* 40: 5399–5406.
31. Stuehr, D. J., Pou, S. & Rosen, G. M. (2001) Oxygen reduction by NO synthases. *J. Biol. Chem.* 276: 14533–14536.
32. Heinzel, B., John, M., Klatt, P., Bohme, E., & Mayer, B. (1992) Ca<sup>2+</sup>/calmodulin-dependent formation of hydrogen peroxide by brain nitric oxide synthase. *Biochem. J.* 281: 627–630.
33. Reiter, C. D., Teng, R. J., & Beckman, J. S. (2000) Superoxide reacts with nitric oxide to nitrate tyrosine at physiological pH via peroxynitrite. *J. Biol. Chem.* 275: 32460–32466.
34. Dedon, P. C. & Tannenbaum, S. R. (2004) Reactive nitrogen species in the chemical biology of inflammation. *Arch. Biochem. Biophys.* 423: 12–22.
35. Abu-Soud, H. M., Feldman, P. L., Clark, P. & D. J. Stuehr, D. J. (1994) Electron transfer in the nitric oxide synthases: Characterization of substrate analogs that block heme iron reduction. *J. Biol. Chem.* 269: 32318–32326.
36. Sennequier, N. & Stuehr, D. J. (1996) Analysis of substrate-induced electronic, catalytic, and structural changes in inducible NO synthase. *Biochemistry* 35: 5883–5892.
37. Mori, M. & Gotoh, T. (2000) Regulation of nitric oxide production by arginine metabolic enzymes. *Biochem. Biophys. Res. Commun.* 275: 715–719.
38. Durante, W. (2001) Regulation of L-arginine transport and metabolism in vascular smooth muscle cells. *Cell. Biochem. Biophys.* 35: 19–34.
39. Wei, L. H., Wu, G., Morris, S. M., Jr. & Ignarro, L. J. (2001) Elevated arginase I expression in rat aortic smooth muscle cells increases cell proliferation. *Proc. Natl. Acad. Sci. U.S.A.* 98: 9260–9264.
40. Bansal, V. & Ochoa, J. B. (2003) Arginine availability, arginase, and the immune response. *Curr. Opin. Clin. Nutr. Metab. Care* 2003 6: 223–228.
41. Hallemeesch, M. M., Lamers, W. H. & Deutz, N. E. (2002) Reduced arginine availability and nitric oxide production. *Clin. Nutr.* 21: 273–279.
42. Albina, J. E., Mills, C. D., Barbul, A., Thirkill, C. E., Henry, W. L., Jr., Mastrofrancesco, B. & Caldwell, M. D. (1988) Arginine metabolism in wounds. *Am. J. Physiol.* 254: E459–E467.
43. Louis, C. A., Reichner, J. S., Henry, W. L., Jr., Mastrofrancesco, B., Gotoh, T., Mori, M. & Albina, J. E. (1998) Distinct arginase isoforms expressed in primary and transformed macrophages: regulation by oxygen tension. *Am. J. Physiol.* 274: R775–R782.
44. Hecker, M., Sessa, W. C., Harris, H. J., Anggard, E. E. & Vane, J. R. (1990) The metabolism of L-arginine and its significance for the biosynthesis of endothelium-derived relaxing factor: cultured endothelial cells recycle L-citrulline to L-arginine. *Proc. Natl. Acad. Sci. U.S.A.* 87: 8612–8616.
45. Cardounel, A. J. & Zweier, J. L. (2002) Endogenous methylarginines regulate neuronal nitric-oxide synthase and prevent excitotoxic injury. *J. Biol. Chem.* 277: 33995–34002.
46. Leiper, J. & Vallance, P. (1999) Biological significance of endogenous methylarginines that inhibit nitric oxide synthases. *Cardiovasc. Res.* 43: 542–548.
47. Leiper, J., Murray-Rust, J., McDonald, N. & Vallance, P. (2002) S-nitrosylation of dimethylarginine dimethylaminohydrolase regulates enzyme activity: further interactions between nitric oxide synthase and dimethylarginine dimethylaminohydrolase. *Proc. Natl. Acad. Sci. U.S.A.* 99: 13527–13532.
48. Tran, C. T., Leiper, J. M. & Vallance, P. (2003) The DDAH/ADMA/NOS pathway. *Atheroscler. Suppl.* 4: 33–40.