Superoxide Generation from Endothelial Nitric-oxide Synthase

A Ca<sup>2+</sup>/CALMODULIN-DEPENDENT AND TETRAHYDROBIOPTERIN REGULATORY PROCESS<sup>a</sup>

Yong Xia‡§, Ah-Lim Tsai†, Vladimir Berka‡, and Jay L. Zweier‡‡

From the §Molecular and Cellular Biophysics Laboratories, Department of Medicine, Division of Cardiology and the Electron Paramagnetic Resonance Center, The Johns Hopkins University School of Medicine, Johns Hopkins Bayview Medical Center, Baltimore, Maryland 21224 and the †Division of Hematology, Department of Internal Medicine, University of Texas Houston Medical School, Houston, Texas 77030

It has been previously shown that besides synthesizing nitric oxide (NO), neuronal and inducible NO synthase (NOS) generates superoxide (O<sub>2</sub><sup>-</sup>) under conditions of L-arginine depletion. However, there is controversy regarding whether endothelial NOS (eNOS) can also produce O<sub>2</sub><sup>-</sup>. Moreover, the mechanism and control of this process are not fully understood. Therefore, we performed electron paramagnetic resonance spin-trapping experiments to directly measure and characterize the O<sub>2</sub><sup>-</sup> generation from purified eNOS. With the spin trap 5,5-dimethyl-1-pyrroline-N-oxide (DMPO), prominent signals of O<sub>2</sub><sup>-</sup> adduct, DMPO-OOH, were detected from eNOS in the absence of added tetrahydrobiopterin (BH<sub>4</sub>), and these were quenched by superoxide dismutase. This O<sub>2</sub><sup>-</sup> formation required Ca<sup>2+</sup>/calmodulin and was blocked by the specific NOS inhibitor N-nitro-L-arginine methyl ester (L-NAME) but not its non-inhibitory enantiomer D-NAME. A parallel process of Ca<sup>2+</sup>/calmodulin-dependent NADPH oxidation was observed which was also inhibited by L-NAME but not D-NAME. Pretreatment of the enzyme with the heme blockers cyanide or imidazole also prevented O<sub>2</sub><sup>-</sup> generation. BH<sub>4</sub> exerted dose-dependent inhibition of the O<sub>2</sub><sup>-</sup> signals generated by eNOS. Conversely, in the absence of BH<sub>4</sub>, L-arginine did not decrease this O<sub>2</sub><sup>-</sup> generation. Thus, eNOS can also catalyze O<sub>2</sub><sup>-</sup> formation, and this appears to occur primarily at the heme center of its oxygenase domain. O<sub>2</sub><sup>-</sup> synthesis from eNOS requires Ca<sup>2+</sup>/calmodulin and is primarily regulated by BH<sub>4</sub> rather than L-arginine.

Endogenous nitric oxide (NO)<sup>1</sup> acts as an essential signaling molecule and effector in cardiovascular, neuronal, and immune systems (1). In cells or tissues, NO is derived from the guanidino group of L-arginine in a reaction catalyzed by a family of NO synthases (NOSs) including neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS) (2, 3). Whereas nNOS and eNOS are constitutively present in cells, iNOS expression requires the stimulation of microbial endotoxins or cytokines. Activation of nNOS and eNOS requires Ca<sup>2+</sup>/calmodulin, hence NO production from these two isoforms is initiated and modulated by elevated intracellular free Ca<sup>2+</sup>. Because iNOS has a tightly bound calmodulin and is fully active under basal cytosolic Ca<sup>2+</sup> concentrations, NO formation from iNOS appears to depend primarily on the levels of enzyme transcription (4). Three NOS isoforms have considerable similarity in their structure and catalytic function. They share 50% homology in their amino acid sequences and structurally resemble NADPH cytochrome P-450 reductase. All NOSs use L-arginine, oxygen, and NADPH as substrates to synthesize NO as well as the co-product L-citrulline. Tetrahydrobiopterin (BH<sub>4</sub>), calmodulin, FAD, and FMN are the requisite cofactors for this catalytic process (5).

In addition to synthesizing NO, purified nNOS catalyzes superoxide (O<sub>2</sub><sup>-</sup>) formation in the absence of L-arginine (6, 7). Similar to NO synthesis, O<sub>2</sub><sup>-</sup> generation from nNOS is dependent on the presence of Ca<sup>2+</sup>/calmodulin. In L-arginine-depleted cells, activated nNOS generates both O<sub>2</sub><sup>-</sup> and NO leading to peroxynitrite (ONOO<sup>-</sup>)-mediated cell injury (8). Recently, iNOS was also found to produce O<sub>2</sub><sup>-</sup> as well as ONOO<sup>-</sup> under L-arginine depletion, and it was shown that these oxidants can contribute to the antibacterial activity of macrophages (9). In light of the structural similarity among NOSs, it would be expected that eNOS might also produce O<sub>2</sub><sup>-</sup> just as the other two isoforms. However, there has been controversy regarding whether or not eNOS can also synthesize O<sub>2</sub><sup>-</sup> (10–12). Previous functional studies suggested that eNOS might generate O<sub>2</sub><sup>-</sup> in vasculature under pathological conditions (10, 11). However subsequently it was reported that purified eNOS exhibits only minor uncoupling of NADPH oxidation in the absence of L-arginine or BH<sub>4</sub> (12). It was argued that eNOS does not produce significant amounts of O<sub>2</sub><sup>-</sup> and that O<sub>2</sub><sup>-</sup> synthesis is a unique feature of nNOS. However, those observations were based on an eNOS mutant, and no direct O<sub>2</sub><sup>-</sup> measurement was performed. Recently, another study reported that the reductase domain of eNOS may yield O<sub>2</sub><sup>-</sup>, but this occurred only in the presence of exogenous electron acceptors such as adriamycin. Therefore, controversy has remained regarding whether eNOS itself generates O<sub>2</sub><sup>-</sup>. Furthermore, questions also remain regarding in which domain of the enzyme O<sub>2</sub><sup>-</sup> synthesis occurs and how this process is regulated.

To address these questions, EPR spin-trapping techniques were applied to directly measure and characterize the process of O<sub>2</sub><sup>-</sup> generation from purified eNOS. We observe that eNOS generates O<sub>2</sub><sup>-</sup> and the role of BH<sub>4</sub> and L-arginine in controlling the process of O<sub>2</sub><sup>-</sup> generation from this enzyme is elucidated.

<sup>a</sup>This work was supported by National Institutes of Health Grants HL-38324, HL-53215 (to J. L. Z.), and GM44911 (to A.-L. T.). 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: Johns Hopkins Asthma and Allergy Center, LA-14, 5501 Hopkins Bayview Circle, Baltimore, MD 21224

‡Supported by Grant MDFW3797 from the American Heart Association Maryland Affiliate.

§The abbreviations used are: NOS, nitric-oxide synthase; eNOS, endothelial NOS; iNOS, inducible NOS; nNOS, neuronal NOS; O<sub>2</sub><sup>-</sup>, superoxide; BH<sub>4</sub>, tetrahydrobiopterin; DMPO, 5,5-dimethyl-1-pyrroline-N-oxide; L-NAME, N-nitro-L-arginine methyl ester; SOD, superoxide dismutase; PAGE, polyacrylamide gel electrophoresis; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.
Superoxide Generation from eNOS

EXPERIMENTAL PROCEDURES

Materials—The NADPH, l-arginine, BH_4, calmodulin, N-nitro-l-arginine methyl ester (l-NAME), d-NAME, superoxide dismutase (SOD), catalase, and other reagents were purchased from Sigma, unless otherwise indicated. Sodium cyanide (NaCN) was from Fisher. Cell culture materials were obtained from Life Technologies, Inc. (Gaithersburg, MD). 2',5'-ADP-Sepharose was the product of Amersham Pharmacia Biotech. 1-^{14}C_l-Arginine was purchased from NEN Life Science Products. 5,5-Dimethyl-1-pyrroline-N-oxide (DMPO) was purchased from Aldrich and further purified by double distillation.

eNOS Purification—Recombinant human wide-type eNOS was prepared using a baculovirus expression system as described previously (14, 15). In brief, eNOS-transfected cells were harvested and sonicated in buffer A (20 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 50 mM NaCl, 10% glycerol, 1 mM CHAPS, and 10% glycerol, 1 mM CHAPS, and 10% glycerol). The eluate was concentrated using a Centriprep 100 (Amicon) and then applied to a 10-14% gel (Bio-Rad). The eNOS-containing fractions were pooled, concentrated, and stored in the buffer with 10% glycerol in liquid nitrogen. Protein content was assayed with Bradford reagent (Bio-Rad) using bovine serum albumin as standard (16). The purity of eNOS was determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized with Coomassie Blue staining. eNOS activity was approximately 130 nmol/min/mg at 37 °C assayed by monitoring the conversion of L-^{14}C_arginine to L-^{14}C_citrulline.

EPR Spectroscopy and Spin Trapping—Spin-trapping measurements of oxygen free radicals were performed in 50 mM Tris-HCl buffer, pH 7.4, containing 0.5 mM NADPH, 0.5 mM Ca^{2+}, 10 μg/ml calmodulin, 15 μg/ml purified eNOS, and 50 mM spin trap DMPO. EPR spectra were recorded in a quartz flat cell at room temperature (23 °C) with a Bruker ER 300 spectrometer operating at X-band with a TM 110 cavity using a modulation frequency of 100 kHz, modulation amplitude of 0.5 G, microwave power of 20 milliwatts, and microwave frequency of 9.85 GHz as described (8, 19). The microwave frequency and magnetic field were precisely measured using an EIP 575 microwave frequency counter and Bruker ER 035 NMR gauss meter. NADPH Consumption by eNOS—NADPH oxidation was followed spectrophotometrically at 340 nm (13).

RESULTS

Recombinant human eNOS was expressed in a baculovirus system and isolated using affinity chromatography. The purity and catalytic activity of the preparations were assayed. As shown in Fig. 1A, purified protein preparations exhibited one prominent major band (>90% pure) on SDS-PAGE with a molecular mass of 135 kDa, which is in accordance with the molecular mass for native eNOS as previously reported (4, 5). By monitoring the conversion of L-arginine to l-citrulline, strong NOS activity was measured from this recombinant protein (Fig. 1B). The catalytic activity was dependent on addition of Ca^{2+} and could be blocked by the NOS inhibitor, l-NAME (1 mM), confirming that it was derived from eNOS.

We then performed EPR spin-trapping experiments to determine whether eNOS generates O_2^{-} using the well characterized spin trap DMPO. In the control experiments without the enzyme, no signals were detected from the reaction mixtures containing DMPO and NADPH, as well as Ca^{2+}/calmodulin (Fig. 2A, Control). However, after adding purified eNOS (15 μg/ml), strong EPR signals were seen (Fig. 2A, eNOS). These prominent signals exhibited the characteristic DMPO-OH spectrum (ΔH = 14.2 G, ΔM = 11.3 G, ΔH = 13.3 G), indicative of trapped O_2^{-}. A small DMPO-OH signal (ΔH = 14.9 G), which can be derived from the breakdown of DMPO-OH, was also observed. These signals were totally abolished by SOD (200 units/ml) but not affected by catalase (300 units/ml) (Fig. 2A, SOD and Catalase), demonstrating that O_2^{-} was the primary oxygen radical generated by eNOS and that the small DMPO-OH signals were derived from the decomposition of DMPO-OH (20). EPR signals from eNOS lasted at least 30 min, indicating that sustained O_2^{-} generation occurred. The DMPO-OH adduct has a short half of only 45 s (20, 21), so the lack of decay indicates that superoxide production continues for more than 30 min. The time course of O_2^{-} generation from eNOS is delineated in Fig. 2B. As shown, the signals were detected immediately after the beginning of the reaction and rapidly increased over the first 5–10 min with continued gradual increases over the next 20 min. In the presence of SOD, the process of O_2^{-} generation was totally quenched.

To further demonstrate that the observed O_2^{-} signals were generated by eNOS, the enzyme was treated with the specific NOS blocker l-NAME. In the presence of 1 mM l-NAME, the O_2^{-}-derived signals were decreased by more than 90% (Fig. 3, l-NAME), whereas the non-inhibitory enantiomer d-NAME had no effect on the O_2^{-} signals (spectrum similar to Fig. 3, eNOS, not shown). These
data confirmed that eNOS is responsible for the \( \text{O}_2^\cdot \) formation. To define the role of \( \text{Ca}^{2+}/\text{calmodulin} \) in eNOS-mediated \( \text{O}_2^\cdot \) generation, parallel experiments were carried out without adding \( \text{Ca}^{2+}/\text{calmodulin} \). In the absence of \( \text{Ca}^{2+}/\text{calmodulin} \), no \( \text{O}_2^\cdot \) signals were detected from eNOS (Fig. 3, \( \text{Ca}^{2+}/\text{CaM free} \)). Hence \( \text{O}_2^\cdot \) generation by eNOS is also a \( \text{Ca}^{2+}/\text{calmodulin} \)-dependent process. Because it is known that \( \text{Ca}^{2+}/\text{calmodulin} \) binding with NOS facilitates the electron flow from the reductase domain to the heme of oxygenase domain, the \( \text{Ca}^{2+}/\text{calmodulin} \) dependence of eNOS-mediated \( \text{O}_2^\cdot \) generation suggests that \( \text{O}_2^\cdot \) synthesis occurs at the heme site of the oxygenase domain. To further confirm this, eNOS was treated with the heme blocker NaCN. In the presence of NaCN (100 \( \mu \text{M} \)) \( \text{O}_2^\cdot \) generation from eNOS was decreased by more than 80% (Fig. 3, \( \text{NaCN} \)). Another heme ligand, imidazole (1 mM) also blocked \( \text{O}_2^\cdot \) generation. These data suggest that eNOS-catalyzed \( \text{O}_2^\cdot \) generation occurs primarily at the heme center of its oxygenase domain.

In NOS-catalyzed reactions, the co-substrate NADPH is oxidized and serves as an electron donor for NO or \( \text{O}_2^\cdot \) synthesis (1–5). Therefore, synchronous NADPH consumption always takes place accompanying \( \text{O}_2^\cdot \) generation. Indeed, marked NADPH oxidation was seen in the reaction mixtures containing eNOS in the absence of BH4 and \text{l-arginine} (Fig. 4). Consistent with the \( \text{O}_2^\cdot \) generation measured in the EPR studies, eNOS-mediated NADPH oxidation also depended on the presence of \( \text{Ca}^{2+}/\text{calmodulin} \). \text{l-NAME} but not \text{d-NAME} largely prevented this NADPH oxidation, reconfirming that NADPH oxidation was catalyzed by eNOS. Together, the findings that eNOS consumed \( \text{BH}_4 \) and \text{l-arginine} provided another line of evidence demonstrating that eNOS can catalyze \( \text{O}_2^\cdot \) formation.

Because nNOS produces \( \text{O}_2^\cdot \) only at low levels of \text{l-arginine}, we studied the effect of \text{l-arginine} on the \( \text{O}_2^\cdot \) formation from eNOS. Interestingly, eNOS-catalyzed \( \text{O}_2^\cdot \) formation was not affected by \text{l-arginine}. In the absence of \( \text{BH}_4 \), even high levels of \text{l-arginine} (1 \( \text{mM} \)) did not decrease the \( \text{O}_2^\cdot \) signals generated by eNOS as compared with control (Fig. 5A, \text{Control} and \text{l-arginine}). We then determined the role of \( \text{BH}_4 \) in controlling eNOS-mediated \( \text{O}_2^\cdot \) formation. In contrast to the effect of \text{l-arginine}, \( \text{BH}_4 \) caused a dose-dependent inhibition on the \( \text{O}_2^\cdot \) generation from eNOS (Fig. 5A, \text{BH}_4, 0.1–10 \( \mu \text{M} \)). In the presence of 1 \( \mu \text{M} \) \text{BH}_4, eNOS-mediated \( \text{O}_2^\cdot \) signals were decreased more than 80% (Fig. 5B). Thus, \( \text{O}_2^\cdot \) generation from eNOS was regulated by \( \text{BH}_4 \) rather than \text{l-arginine}.

**DISCUSSION**

Besides synthesizing NO, nNOS and iNOS also generate \( \text{O}_2^\cdot \) under conditions of \text{l-arginine} depletion (6–9). There has been considerable controversy regarding whether or not eNOS also produces \( \text{O}_2^\cdot \) (10–12). Most of the previous data were indirect in nature based on functional assays in complex biological systems such as vascular tissues. Although these functional studies provided important insights, there has been a lack of conclusive information regarding the presence of \( \text{O}_2^\cdot \) generation from eNOS. To definitively establish whether eNOS synthesizes \( \text{O}_2^\cdot \), unambiguous oxygen radical measurements on purified enzyme preparations must be performed. In this study, we applied EPR spin-trapping techniques to directly measure \( \text{O}_2^\cdot \) generation from purified eNOS. With the spin trap DMPO, strong \( \text{O}_2^\cdot \)-derived EPR signals were detected from eNOS in the absence of \( \text{BH}_4 \) and \text{l-arginine}. The fact that SOD quenched the...
signals and catalase had no effect reconfirmed that $O_2^*$ was the primary oxygen free radical generated. This $O_2^*$ formation could be blocked by the NOS inhibitor L-NAME but not by its non-inhibitory enantiomer d-NAME, further proving that $O_2^*$ was synthesized from eNOS.

$O_2^*$ formation was also demonstrated by the fact that eNOS can cause marked NADPH oxidation in the absence of BH$_4$ and L-arginine. These findings are in disagreement with the results reported by List et al. (12). In that study, List et al. (12) reported that eNOS did not catalyze appreciable NADPH oxidation in the absence of L-arginine or BH$_4$, and based on this they presumed eNOS would not produce significant amounts of $O_2^*$. They claimed that $O_2^*$ generation from the uncoupling of oxygen oxidation only occurs in nNOS and is the unique characteristic of this NOS isoform. However, their observations were based on experiments performed with an eNOS mutant, which may have different biochemical properties. In the present study, we found that wild-type eNOS causes NADPH oxidation under conditions of no added BH$_4$ or L-arginine. This NADPH oxidation can be largely prevented by eNOS blockade.

The EPR $O_2^*$ spin-trapping experiments further revealed that the electrons derived from NADPH oxidation are transferred to oxygen molecules leading to $O_2^*$ formation. Considering the preceding reports that both nNOS and iNOS produce $O_2^*$ under L-arginine depletion as well as the current findings, we conclude that $O_2^*$ generation is a general feature of all NOS isoforms.

Although derived from distinct genes and chromosomes, the three NOS isoforms share similarity in their structure and catalytic mechanisms (4, 5). They are all bidomain enzymes consisting of a C-terminal reductase and N-terminal oxygenase domain. The reductase domain contains NADPH, FAD, and FMN binding sites and exhibits 58% homology to NADPH cytochrome P450 reductase (3, 22). Heme, BH$_4$, and L-arginine bind at the oxygenase domain. The catalytic mechanisms of NOSs involve a flavin-mediated electron transport from C-terminal-bound NADPH to the N-terminal heme center where oxygen is reduced and incorporated into the guanidino group of L-arginine giving rise to NO and L-citrulline. Calmodulin binds to a consensus sequence in the NOS enzymes and serves to position the two domains allowing the electron transfer from FMN to heme (5, 23).

In this study, we found that the $O_2^*$ generation from eNOS is dependent on the presence of Ca$^{2+}$/calmodulin, suggesting that $O_2^*$ synthesis requires electron transfer from reductase domain to oxygenase domain. Furthermore, eNOS-catalyzed $O_2^*$ can be prevented by the heme blockers NaCN or imidazole suggesting that $O_2^*$ synthesis occurs primarily at the heme of the oxygenase domain. This finding is different from the report that eNOS reductase domain yields $O_2^*$ in the presence of adriamycin (13). $O_2^*$ formation from that pathway relies on exogenous electron acceptors to deliver electrons. Our current results demonstrated that eNOS can synthesize $O_2^*$ from the heme of its oxygenase domain, and this process does not require additional electron transfer mediators.

The onset of $O_2^*$ generation from eNOS appears to be triggered by a different mechanism compared with the other two NOS isoforms. nNOS and iNOS generate $O_2^*$ under conditions of L-arginine depletion, therefore $O_2^*$ synthesis is triggered by low levels of L-arginine (6–9). Interestingly, we found that $O_2^*$ generation from eNOS is not similarly affected by L-arginine. In the absence of BH$_4$, $O_2^*$ production from eNOS was essentially unchanged even in the presence of high levels of L-argi-
nine (1 mM). Conversely, BH4 blocks this $\text{O}_2^-$ formation in a dose-dependent manner. Thus, eNOS-mediated $\text{O}_2^-$ generation is triggered and controlled by decreased availability of BH4 rather than L-arginine. While the exact role of BH4 in the enzymatic function of NOS is still not fully understood, our observations show that BH4 prevents $\text{O}_2^-$ production. Because BH4 has been found to play a critical role in maintaining eNOS dimerization (24), it is possible that the conversion of eNOS dimer/monomer affects the interchange of NO/$\text{O}_2^-$ generation from this enzyme.

Identification of the determining role of BH4 in controlling $\text{O}_2^-$/NO generation from eNOS is of particular interest in understanding the mechanism of vascular endothelial dysfunction. Impaired endothelial function, represented as declined NO production and elevated oxidant accumulation, plays a fundamental role in the pathogenesis of a number of cardiovascular diseases including hypercholesterolemia, atherosclerosis, hypertension, and ischemia/reperfusion injury. Despite extensive study, it remains poorly understood how this NO/oxidant imbalance takes place. Our current findings suggest that BH4 may play an important role. BH4 is unstable at physiological pH and prone to decompose in oxygenated solutions (25). Oxidants from other enzymatic pathways could also serve to deplete BH4 levels in in vivo tissues. Insufficient BH4 availability will switch eNOS from NO to $\text{O}_2^-$ generation, subsequently leading to NO decline and oxidant accumulation. Indeed, there are functional studies showing that BH4 depletion results in oxidant accumulation and endothelial dysfunction in coronary arterial vessels (10). Considering the controlling role of BH4 in eNOS-catalyzed NO/$\text{O}_2^-$ generation, modulating cytosolic BH4 levels may provide an important therapeutic approach to those diseases associated with endothelial dysfunction.

REFERENCES

1. Moncada, S., Palmer, R. M., and Higgs, E. A. (1991) Pharmacol. Rev. 43, 109–142
2. Marletta, M. A. (1993) J. Biol. Chem. 268, 12231–12234
3. Masters, B. S. S., McMillan, K., Sheta, E. A., Nishimura, J. S., Roman, L. J., and Martasek, P. (1996) FASEB J. 10, 552–558
4. Nathan, C., and Xia, Q.-w. (1994) Cell 78, 915–918
5. Griffith, O. W., and Stuehr, D. J. (1995) Annu. Rev. Physiol. 57, 707–736
6. Pou, S., Pou, W. S., Bredt, D. S., Snyder, S. H., and Rosen, G. M. (1992) J. Biol. Chem. 267, 24173–24176
7. Heinecke, B., John, M., Klatt, P., Bohme, E., and Mayer, B. (1992) Biochem. J. 281, 627–630
8. Xia, Y., Dawson, V. L., Dawson, T. M., Snyder, S. H., and Zweier, J. L. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 6770–6774
9. Xia, Y., and Zweier, J. L. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 6954–6958
10. Cosentino F., and Katzez, Z. S. (1995) Circulation 91, 139–144
11. Pritchard, K. A., Jr., Groszek, L., Smalley, D. M., Sessa, W. C., Wu, M., Villalou, P., Wolin, M. S., and Stemerman, M. B. (1995) Circ. Res. 77, 510–518
12. List, B. M., Klosch, B., Volker, C., Gorren, A. C. F., Sessa, W. C., Werner, E. R., Kukrotz, W. R., Schmidt, K., and Mayer, B. (1997) Biochem. J. 323, 159–165
13. Vasquez-Vivar, J., Martasek, P., Hogg, N., Masters, B. S. S., Pritchard, K. A., Jr., and Kalyanaraman, B. (1997) Biochemistry 36, 11293–11297
14. Chen, P.-F., Tsai, A.-L., and Wu, K. K. (1996) J. Biol. Chem. 271, 14631–14635
15. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
16. Bradin, D. S., and Snyder, S. H. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 9603–9603
17. Xia, Y., and Zweier, J. L. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 12705–12710
18. Xia, Y., Khatchikian, G., and Zweier, J. L. (1996) J. Biol. Chem. 271, 10096–10102
19. Fischerstein, E., Rosen, G. M., and Rauckman, E. J. (1982) Mol. Pharmacol. 21, 262–265
20. Roubaud, V., Sankarapandi, S., Kuppusamy, P., Tordo, P., and Zweier, J. L. (1997) Anal. Biochem. 247, 404–411
21. Bredt, D. S., Hwang P. M., Glatt, C. E., Lowenstein, C., Reed, R. R., and Snyder, S. H. (1991) Nature 351, 714–718
22. Abu-Soud, H. M., Yoho, L. L., and Stuehr D. J. (1994) J. Biol. Chem. 269, 32047–32050
23. Stuehr, D. J. (1997) Annu. Rev. Pharmacol. Toxicol. 37, 339–359
24. Gross, S. S., and Levi, R. (1992) J. Biol. Chem. 267, 25722–25729
25. Gross, S. S., and Levi, R. (1992) J. Biol. Chem. 267, 25722–25729