Formation of free nitric oxide from L-arginine by nitric oxide synthase: Direct enhancement of generation by superoxide dismutase

(chemiluminescence/nitroxyl/superoxide anion)

ADRIAN J. HOBBS, JON M. FUKUTO, AND LOUIS J. IGNARRO*

Department of Pharmacology, Center for the Health Sciences, University of California, School of Medicine, Los Angeles, CA 90024

ABSTRACT Although nitric oxide (NO) appears to be one of the oxidation products of L-arginine catalyzed by NO synthase (NOS; EC 1.14.13.39), past studies on the measurement of NO in cell-free, enzymatic assays have not been based on the direct detection of the free NO molecule. Instead, assays have relied on indirect measurements of the stable NO oxidation products nitrite and nitrate and on indirect actions of NO such as guanylate cyclase activation and oxyhemoglobin oxidation. Utilizing a specific chemiluminescence assay, we report here that the gaseous product of L-arginine oxidation, catalyzed by both inducible macrophage and constitutive neuronal NOS, is indistinguishable from authentic NO on the basis of their physicochemical properties. NO gas formation by NOS was dependent on L-arginine, NADPH, and oxygen and inhibited by N^G-methyl-L-arginine and cyanide anion. Superoxide dismutase (SOD) caused a marked, concentration-dependent increase in the production of free NO by mechanisms that were unrelated to the dismutation of superoxide anion or activation of NOS. These observations indicate that free NO is formed as a result of NOS-catalyzed L-arginine oxidation and that SOD enhances the generation of NO without directly affecting NO itself. SOD appears to elicit a novel biological action, perhaps accelerating the conversion of an intermediate in the L-arginine–NO pathway such as nitroxyl (HNO) to NO.

Nitric oxide (NO) synthase (NOS; EC 1.14.13.39) exists as several different isoforms in mammalian cells and each is believed to catalyze the five-electron oxidation of L-arginine to NO, with the concomitant formation of L-citrulline. NOS activity has been studied extensively by measuring the direct production of L-citrulline. The generation of NO, however, has only been measured indirectly in cell-free systems, as assessed by nitrite/nitrate production (1, 2), guanylate cyclase activation (3, 4), and spectral shifts after reaction with hemoproteins or thiols (5).

As a consequence of this lack of direct evidence for NO production, speculation has often arisen that NOS may catalyze the formation of a related nitrogen oxide, or NO-containing molecule, rather than NO itself (6, 7). Thus, the principal objective of this study was to investigate whether the oxygenation of L-arginine, catalyzed by NOS, does indeed result in the formation of free NO. To accomplish this goal, the technique of chemiluminescence was employed to detect and monitor the formation of NO gas in the head space above buffered (pH 7.4) NOS enzyme reaction mixtures containing L-arginine, oxygen, and the appropriate cofactors.

During the progress of this study, superoxide dismutase (SOD) was discovered to enhance the apparent generation of NO from L-arginine without directly affecting the chemical stability of NO itself. Accordingly, experiments were con-

ducted to elucidate the mechanisms responsible for this novel action of SOD.

MATERIALS AND METHODS

Reagents. All reagents employed in this study were purchased from Sigma, except the following: L-[2,3,4,5-³H]arginine hydrochloride (77 Ci/mmol; 1 Ci = 37 GBq; Amersham); DEAE-Sephacel and 2′,5′-ADP Sepharose 4B (Pharmacia); Dowex AG1-X8 (acetate form), 100–200 mesh (Bio-Rad); interferon γ (rat, recombinant; GIBCO/BRL); NO gas (99%; Matheson); tetrahydrobiopterin (Schircks Laboratories, Jona, Switzerland). N^G-methyl-L-arginine (L-NMA) was synthesized as described (8).

Chemiluminescence Analysis of NO. The experimental setup consisted of two 15-ml glass flasks connected in series to a Dasibi (model 2108; Glendale, CA) chemiluminescence detector (see *Inset* in Fig. 6). NOS reaction mixtures were purged with room air at a constant flow rate of 200 ml/min. The head space above the reaction mixture was thereby swept into the chemiluminescence detector at 200 ml/min. NO may be swept into the detector in either air or N_2, with no apparent difference in sensitivity.

Enzymatic reaction mixtures that were purged for the analysis of free NO were conducted in a final volume of 2 ml containing 1 ml of reaction mixture plus 1 ml of iNOS (inducible macrophage NOS) fraction (3–4 mg of total protein) prepared from homogenates of an activated rat alveolar macrophage cell line (as described below). In the experiments with nNOS (neuronal NOS), a purified enzyme fraction (25 μg of protein) prepared from rat cerebellum was used. Reaction mixtures contained 50 mM triethanolamine (TEA)-HCl (pH 7.4), 1 mM L-arginine, 2 mM NADPH, 100 μM FAD, and 10 μM tetrahydrobiopterin, and, additionally in experiments utilizing nNOS, 2 mM $CaCl_2$ and 5 μg of calmodulin. Reactions were conducted in a conical 15-ml glass reaction flask equipped with three ports. One port was fitted with an inlet tube that was submerged in the reaction mixture, which permitted purging of the solution. A second port provided the gas outflow to trap flasks and the chemiluminescence detector. The third port was fitted with a Teflon septum for injection of components into the reaction mixture. In each experiment, enzyme reactions were conducted in the first (or reaction) flask. The second (or trapping) flask contained 2 ml of reaction mixture lacking enzyme and was used to examine the effect of different agents on the gaseous enzyme reaction product. Both flasks were maintained at 37°C by means of a heating mantle.

NOS Assay. In some experiments, NOS activity was measured by monitoring the formation of L-[³H]citrulline from

---

Pharmacology: Hobbs *et al.*

Proc. Natl. Acad. Sci. USA 91 (1994) 10993

L-[^{3}H]arginine, as described (9). Enzymatic reactions were conducted at 37°C in 50 mM TEA-HCl (pH 7.4) containing 1 mM L-arginine, 2 mM NADPH, 100 μM FAD, 10 μM tetrahydrobiopterin, 50 μl of iNOS fraction (100 μg of total protein), and other test agents as indicated.

Preparation of NOS. The source of iNOS utilized in the assay was the cytosolic fraction from cytokine-activated rat alveolar macrophages obtained by centrifugation of 1% crude homogenates at 100,000 × g for 60 min at 4°C, as described (9, 10). Purified nNOS was derived from the cytosolic fraction obtained from rat cerebellum, as reported (11). The purified nNOS preparations had ≈1000-fold greater specific activity (300–400 nmol per L-citrulline per min per mg of protein) than the starting supernatant fractions.

RESULTS

Fig. 1 illustrates the formation of free NO in reaction mixtures containing iNOS and nNOS. A steady-state level of NO was attained within 60–90 sec of addition of NOS to reaction mixtures at 37°C. Chemiluminescence is a sensitive and direct method for detecting free NO and is suitable for monitoring steady-state levels of free NO generated from L-arginine by NOS but is not suitable for quantifying the initial NO generated prior to attainment of steady-state conditions (Fig. 1). Reaction mixtures were purged at a uniform flow rate of 200 ml/min until a steady-state concentration of NO in the head space was achieved, signifying attainment of partial pressure equilibrium between NO in the head space and that dissolved in the reaction mixture. Thus, changes in the detection signal directly reflect changes in the concentration of NO in the aqueous phase. The steady-state level of free NO generated under assay conditions ranged from 3 to 4 nmol of NO per min for unpurified macrophage iNOS and 2 to 3 nmol of NO per min for purified nNOS. On the basis of specific enzyme activity of the preparations, NO was generated at a steady-state level of 1.1–1.3 nmol/min per mg of protein for unpurified iNOS and 90–120 nmol/min per mg of protein for purified nNOS.

The steady-state levels of chemiluminescence-detectable gaseous product from both iNOS and nNOS were affected neither by passage through concentrated acid (2.5 M H$_2$SO$_4$) or base (5 M NaOH) nor by passage through cationic (Dowex AG50W-X8, Na$^+$ form) or anionic (Dowex AG1-X8, OH$^-$ form) exchange resins, prior to entry into the detector (Fig. 1). Likewise, when authentic NO gas was purged through the same system, to give a chemiluminescence signal similar to that generated by iNOS (≈400 ppb), passage through each of the four trapping agents did not alter NO detection (Fig. 1).

To determine whether the chemiluminescence signal detected was indeed the result of the action of NOS on L-arginine, we examined the effects of several agents, established to modulate the L-arginine–NO pathway, on gaseous product formation. In reaction mixtures deficient in L-arginine or containing D-arginine, addition of NOS did not result in an increase in the chemiluminescence signal (Fig. 2). However, when L-arginine was introduced into the reaction mixture, NO generation proceeded to control levels (Fig. 2). Preincubation of NOS with the arginine analog, L-NMA, caused a significant attenuation of NO production, following the addition of L-arginine (Fig. 2). In an analogous fashion, removal of NADPH or anaerobiosis (purging with pure N$_2$) markedly reduced NO formation until restoration of NADPH levels (2 mM) or oxygen to the carrier gas (not shown). Potassium cyanide (10 mM) also significantly inhibited enzyme activity (Fig. 2), as did lowering the temperature of the mixture to 4°C (not shown).

The two principal products of the NOS reaction are NO and L-citrulline, and they are formed in equimolar quantities per mol of L-arginine oxidized (1, 9, 12). The conversion of L-[^{3}H]arginine to L-[^{3}H]citrulline was monitored to determine
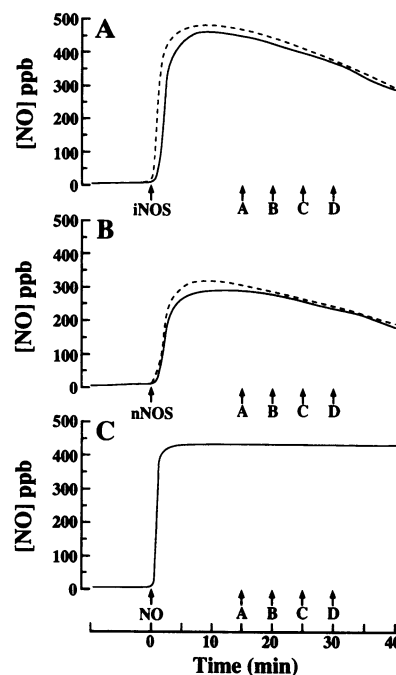

FIG. 1. Influence of various traps on authentic NO gas and free NO generated from L-arginine by iNOS and nNOS. (*A*) One milliliter of iNOS fraction was added to 1 ml of reaction mixture at 37°C. After the NO generated attained a steady-state level, the gas outflow from the reaction flask was purged through four traps, one at a time (5 min each), as indicated. Traps A, B, C, and D were flasks containing 50 ml of 5 M NaOH, 2.5 M H$_2$SO$_4$, 50% slurry of anion-exchange resin (Dowex AG1-X8, OH$^-$ form), and 50% slurry of cation-exchange resin (Dowex AG50W-X8, Na$^+$ form), respectively. The measurement of NO was continued for 10 min after removal of Trap D. The dashed line signifies a similar reaction (control) to that described above without the use of traps. (*B*) One milliliter of nNOS was tested as described for iNOS in *A*. The dashed line signifies a similar reaction (control) to that described above without the use of traps. (*C*) Authentic NO gas (400 ppb) was purged through 2 ml of reaction buffer at 37°C and the gas outflow from the reaction flask was purged through the four traps as indicated. The tracings illustrated are representative of three to five separate experiments.

whether the selected test agents would influence free NO and L-citrulline formation similarly. The rate of generation of L-citrulline from L-arginine by iNOS, under conditions similar to those defined for the measurement of free NO, was 2.5–3.0 nmol/min per mg of protein. Thus, the rate of formation of L-citrulline exceeded the rate of free NO generation by approximately 2- to 3-fold. Paralleling their effects on free NO generation, Fig. 3 illustrates that cyanide and L-NMA inhibited L-citrulline formation and that product formation was negligible in the absence of NADPH or oxygen. A significant difference, however, was whereas SOD enhanced the production of free NO in reaction mixtures containing NOS (discussed below), SOD did not alter (or actually inhibited) the formation of L-citrulline.

Under physiological conditions, one seemingly important pathway leading to the biological inactivation of NO is its reaction with superoxide anion (O$_2^-$), to yield peroxynitrite anion [ONOO$^-$ (13, 14)]. Enhancement of the biological activity of endothelium-derived relaxing factor by SOD has been attributed to removal of O$_2^-$, hence preventing this interaction with NO (15). Having established a method by which it was possible to directly measure the production of free NO by NOS, we determined whether SOD could augment the detectable levels of free NO and thereby account for its enhancing effect on the biological half-life of NO. Fig. 4 demonstrates clearly that SOD markedly increased the NO signal in NOS reaction mixtures in a concentration-
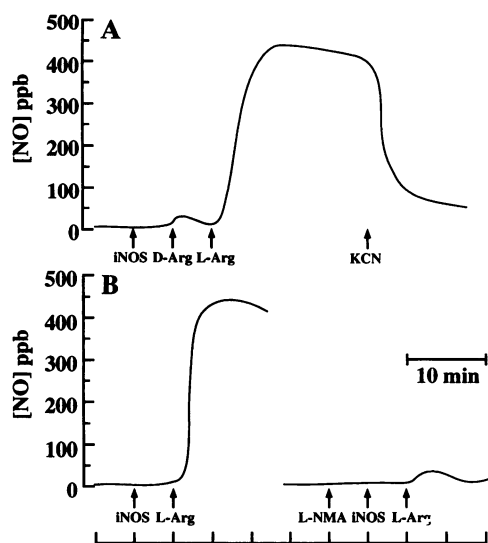
FIG. 2. Factors influencing the generation of free NO by iNOS. (A) One milliliter of iNOS fraction was added to 1 ml of reaction mixture deficient in L-arginine at 37°C. D-Arginine (D-Arg; 1 mM) was added 5 min later. L-Arginine (L-Arg; 1 mM) was added 5 min after addition of D-Arg. KCN (10 mM) was added to the enzyme reaction mixture as indicated. (B) One milliliter of iNOS was added to 1 ml of reaction mixture deficient in L-arginine at 37°C. L-Arg (1 mM) was added to the enzyme reaction mixture as indicated. This served as a control for the subsequent reaction. L-NMA (1 mM) was added to 1 ml of reaction mixture deficient in L-arginine at 37°C, and 1 ml of iNOS was added 5 min later. L-Arg (1 mM) was added 5 min after addition of iNOS. The tracings illustrated are representative of three or four separate experiments.

dependent manner, consistent with the belief that SOD enhances the quantity of NO detected by curtailing its reaction with O_2^-. Thus, SOD should similarly increase the detection signal of authentic NO purged through the same system. In the first of a series of experiments, authentic NO was purged through NOS reaction mixtures deficient in L-arginine at a rate identical to that produced when L-arginine was present (400–500 ppb). NOS is known to generate O_2^- in both the absence and the presence of L-arginine, but studies suggest that O_2^- production may be greater in the absence of

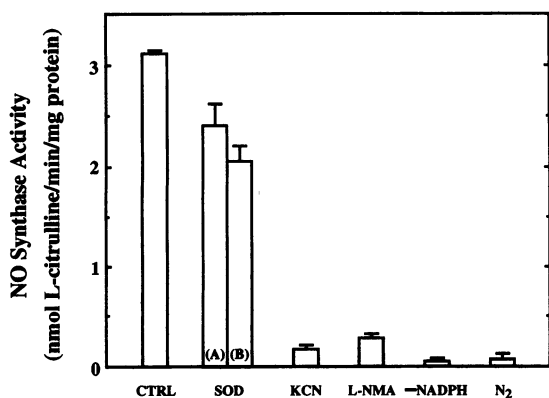

FIG. 3. Effects of SOD, KCN, L-NMA, N₂ atmosphere, and NADPH deficiency on iNOS activity. Enzyme activity was determined by monitoring the formation of L-[³H]citrulline from L-[³H]arginine as described above. SOD [1000 units/ml (A) and 10,000 units/ml (B)], KCN (10 mM), and L-NMA (1 mM) were added to reaction mixtures just prior to addition of enzyme. −NADPH, omission of NADPH from reaction mixtures; N₂, running the reaction in an atmosphere of N₂; CTRL, control. Data represent the mean ± SEM of 6–10 determinations from three to five separate experiments.
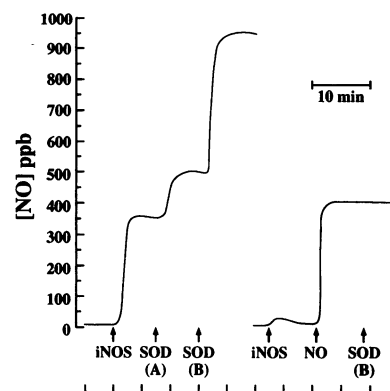
FIG. 4. Actions of SOD on free NO generated by iNOS and authentic NO gas. One milliliter of iNOS fraction was added to 1 ml of reaction mixture at 37°C. After the NO generated attained a steady-state level, SOD (1000 units/ml; A) was added to the reaction mixture. After a second steady-state level of NO was attained, SOD (10,000 units/ml; B) was added as indicated. (Note: the minimally effective concentration of SOD was 100 units/ml.) In the second experiment, authentic NO gas (400 ppb) was purged through 2 ml of iNOS reaction mixture devoid of L-arginine at 37°C. SOD (10,000 units/ml; B) was added to the reaction mixture as shown. The tracings illustrated are representative of three or four separate experiments.

L-arginine (16). Consequently, under such conditions (partial pressure equilibrium), the concentration of dissolved NO in the reaction mixture was identical whether NO was generated by NOS (Fig. 1) or introduced as authentic NO gas (Fig. 4). Clearly, Fig. 4 illustrates that at concentrations that increase enzymatically generated NO by severalfold, SOD had no effect on authentic NO. Thus, the mechanism by which SOD markedly enhanced the signal of enzymatically generated NO may not be attributed merely to the dismutation of O_2^-.

Although nNOS has been shown to catalyze the formation of O_2^- in the absence or presence of L-arginine (16, 17), a similar observation with iNOS has not been shown. However, the oxidation of NADPH by iNOS in the absence of L-arginine has been reported (18). Therefore, the lack of effect of SOD on authentic NO purged through L-arginine-deficient NOS reaction mixtures could be explained if little or no O_2^- was generated by the enzyme under these conditions. Accordingly, we investigated the effect of SOD on the detection of free NO in NOS reaction mixtures that were deficient in L-arginine but contained the artificial O_2^- generating system, xanthine (X)/xanthine oxidase (XO). The concentrations of X/XO employed were calculated to yield O_2^-
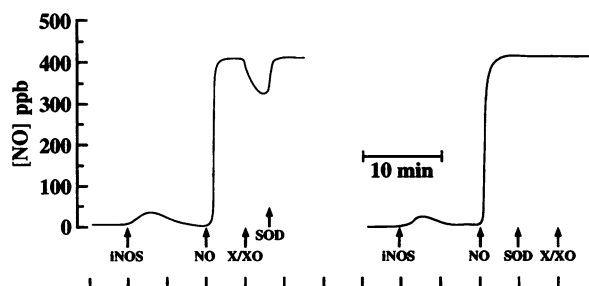

FIG. 5. Influence of O_2^- on authentic NO gas in the presence of iNOS. Authentic NO gas (400 ppb) was purged through 2 ml of iNOS reaction mixture devoid of L-arginine at 37°C. After a steady-state level of NO was attained, X (0.5 mM) plus XO (0.05 units/ml) were added as indicated. Subsequently, SOD (10,000 units/ml) was added as shown. In the second experiment, the protocol was identical, except SOD (10,000 units/ml) was added prior to addition of X (0.5 mM) and XO (0.05 units/ml). The tracings illustrated are representative of three or four separate experiments.
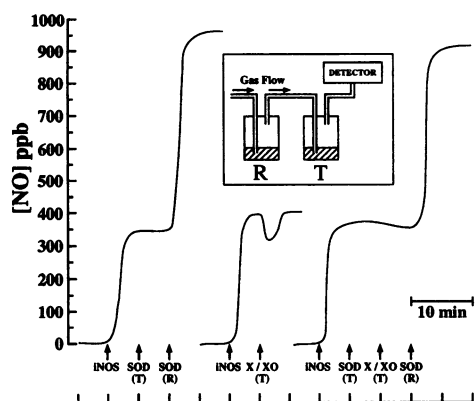
FIG. 6. Effects of SOD on the synthesis of free NO by iNOS versus the chemical stability of free NO already synthesized by iNOS. The enzymatic reaction was conducted in a reaction flask (R), and the gas outflow from R was purged through a trap flask (T) containing 2 ml of reaction buffer (see *Inset*). One milliliter of iNOS fraction was added to 1 ml of reaction mixture at 37°C. After the NO generated attained a steady-state level, SOD (10,000 units/ml) was added to T. Five minutes later SOD (10,000 units/ml) was added to R. In the next experiment, 1 ml of iNOS fraction was added to 1 ml of reaction mixture at 37°C. X (0.5 mM) plus XO (0.05 units/ml) were added to T as shown. In the third experiment, 1 ml of iNOS fraction was added to 1 ml of reaction mixture at 37°C. SOD (10,000 units/ml) was added to T as shown. Five minutes later X (0.5 mM) plus XO (0.05 units/ml) were added to T. Five minutes later SOD (10,000 units/ml) was added to R. The tracings illustrated are representative of three separate experiments.

at a rate of $\approx$0.1 μmol/min under the defined experimental conditions. NO was purged through the solution at a steady-state level equivalent to that which would have been generated enzymatically had the L-arginine been present (400–500 ppb). Thus, under these experimental conditions NOS plus required cofactors were present together with a source of free NO and excess O_2^-. The addition of X/XO caused a decrease in the NO detection signal (Fig. 5). However, despite the presence of excess O_2^-, SOD merely prevented the effect of O_2^- without markedly increasing the quantity of detectable free NO. This action of SOD can be attributed solely to the dismutation of O_2^-, which therefore cannot be used to explain the action of SOD in causing a marked enhancement of the signal for enzymatically generated NO.

In the final series of experiments, we compared the effects of SOD on free NO (formed previously by NOS) versus the enzymatic generation of NO by NOS. As first described above, the addition of SOD to the reaction flask containing NOS caused a marked increase in the NO signal. Addition of SOD to the second or trap flask, however, had no such effect (Fig. 6). This lack of effect of SOD could be attributed to the presence of only small quantities of O_2^- in the trap flask compared to the reaction flask containing NOS. Accordingly, X/XO was added to the trap flask in order to generate excess O_2^-, and this resulted in a decrease in detectable NO (Fig. 6). The addition of SOD to the trap flask just prior to X/XO addition prevented this decrease in the NO signal. The subsequent addition of SOD to the reaction flask, however, caused a marked increase in the NO signal. These observations indicate clearly that the effect of SOD on free NO that had been formed by NOS was different from the effect of SOD on NO generation by NOS. These observations also indicate that free NO generated by NOS behaved similarly to authentic NO with regard to the effects of X/XO and SOD.

DISCUSSION

In this study we demonstrate that NOS in cell-free systems generates free NO from the oxidation of L-arginine. These

observations are consistent with the findings that intact activated macrophages in culture produce a gas with the properties of NO (19). Several observations indicate that the detectable NOS reaction product is free NO. (*i*) The chemiluminescence detector is specific for NO and is insensitive to other nitrogen oxides such as N_2O and NO_2. (*ii*) NO is an uncharged and volatile species at pH 7.4 and is easily swept into the head space. Possible alternative products of L-arginine oxidation, such as nitroxyl (HNO/NO^-; pKa = 4.7), would be charged and nonvolatile at pH 7.4. Only $\approx$1 part per 1000 (0.1%) would exist as HNO at pH 7.4 and be purged into the head space, if volatile. (*iii*) Passage of the gaseous reaction product through strong acid or base traps and cation- or anion-exchange resins failed to alter the recovery of reaction product. These observations indicate that the detectable gaseous reaction product is an uncharged species such as NO. Authentic NO gas, as a mixture in air, adjusted to a steady-state level of NO (400–500 ppb) to mimic the generation of NO by NOS, was not trapped by the above procedures. Thus, the detectable NOS reaction product could not be distinguished from authentic NO on the basis of its chemical and physical properties.

The formation of NO from arginine by NOS is enantiomerically selective for L-arginine, dependent on molecular oxygen and NADPH, and inhibited by certain N^G-substituted analogs of L-arginine and by cyanide anion (20). In the present study, the generation of free NO and L-citrulline by NOS was affected similarly, with one important exception. Whereas SOD markedly enhanced the formation of free NO from L-arginine, it had no effect on L-citrulline production. It is important to note that in the absence of added SOD the steady-state level of free NO, expressed as a specific activity of NOS, was $\approx$3-fold lower than the specific activity of NOS when L-citrulline was measured. When NO was estimated by measuring the formation of nitrite plus nitrate in solution, however, equimolar quantities of NO and L-citrulline were formed from L-arginine by macrophage NOS (9). These observations suggested that either some of the free NO became oxidized prior to detection or there was incomplete conversion of a reaction intermediate to NO. The addition of SOD to NOS reaction mixtures, however, markedly increased the formation of free NO, to a rate equivalent to L-citrulline generation. Thus, SOD appeared to increase the production of free NO without altering the production of L-citrulline.

NO is a radical species that is chemically reactive with and inactivated by other radicals. A primary mechanism of NO degradation under physiological conditions is via its reaction with O_2. However, another pathway leading to NO breakdown, and of particular recent interest, is the reaction of NO with O_2^-, yielding peroxynitrite anion [ONOO$^-$ (13, 14)]. Thus, addition of SOD to aqueous solutions of NO, either generated endogenously or added exogenously, removes O_2^- and often enhances the biological actions of NO. This potentiating effect of SOD, however, has not been consistently observed and the reason for this discrepancy is not known. In this study, SOD markedly increased the quantity of free NO generated by NOS. These observations were consistent with the belief that SOD enhances the quantity of NO detected by curtailing the destruction of NO by O_2^-, especially in view of the findings that NOS catalyzes the formation of O_2^- (16, 17). Additional experiments indicated, however, that this action of SOD could not be adequately explained on the basis of O_2^- dismutation. Moreover, the action of SOD cannot be attributed to activation of NOS because SOD failed to increase the formation of L-citrulline from L-arginine under similar conditions. Thus, the conclusion that SOD increases the quantity of free NO detected in NOS reaction mixtures containing L-arginine by stimulating

the conversion of a precursor to NO rather than via the dismutation of O_2^- is based on the following rationale.

A direct comparison was made between enzymatically generated free NO and authentic NO gas delivered into the reaction mixture. A valid comparison is possible only if the NO concentration in solution under the two conditions is the same, and therefore equally available for reaction with O_2^-. All measurements of free NO were made under steady-state conditions, which signifies partial pressure equilibrium of NO between the head space and the reaction mixture, so that NO concentrations do not change with time. According to Henry's law, the concentration of NO in solution is dependent solely on the partial pressure of NO in the gas phase. As a consequence, the concentration of NO in the reaction mixture must be identical whether authentic or enzymatically generated NO is being measured and so is independent of the source or method of delivery of NO. Furthermore, the marked increase in free NO in the head space caused by addition of SOD to NOS reaction mixtures can result only from an increase in the concentration of NO dissolved in solution. Assuming that similar concentrations of O_2^- are also present in the two systems, SOD should elicit the same effect on NO detection in both systems if the only mechanism of action of SOD is the dismutation of O_2^-. However, SOD increased the NO concentration in reaction mixtures only when the NO was generated by NOS but not when the same concentration of authentic NO was introduced into solution. Therefore, this action of SOD cannot be solely attributed to the dismutation of O_2^-.

The above reasoning is valid only if the concentration of O_2^- in reaction mixtures containing enzymatically generated NO was less than or equal to the O_2^- concentration in reaction mixtures containing authentic NO. In this way, any differential effect of SOD cannot be attributed to O_2^- dismutation. Unpublished observations from our laboratory indicate that macrophage iNOS has an analogous mechanism of catalysis to nNOS (16) in that O_2^- production is inversely proportional to L-arginine concentration. Even so, in the absence of L-arginine, O_2^- generation by macrophage iNOS is minimal since NADPH utilization by the enzyme under such conditions is extremely small (18). Although the concentration of O_2^- was not directly measured in this study, the rate of O_2^- formation in reaction mixtures containing NOS-generated NO was estimated to be almost 2 orders of magnitude lower than that produced by X/XO (0.1 μmol/min) in experiments utilizing authentic NO. Indeed, the generation of O_2^- by NOS should not exceed the production of L-citrulline (3–4 nmol/min) since the rate of NADPH oxidation by NOS is low [1.5 mol of NADPH per mol of L-citrulline (21)] and represents the minimum requirement (in terms of electron donation) for the conversion of L-arginine to L-citrulline. Despite the presence of much lower concentrations of O_2^- in reaction mixtures containing iNOS plus L-arginine, the addition of SOD caused a markedly greater increase in the free NO signal compared with reaction mixtures purged with authentic NO. These observations indicate that this effect of SOD is not attributed to the dismutation of O_2^-.

A plausible explanation for the finding that SOD markedly increased the quantity of free NO, but not L-citrulline, in reaction mixtures containing NOS is that SOD promoted the oxidation of an intermediate nitrogen oxide species to NO. One possible intermediate species is nitroxyl (HNO or NO^-), which can undergo a rapid one-electron oxidation to NO in the presence of SOD, oxygen, or other physiological oxidants (22, 23). Moreover, the oxidation of HNO by SOD and oxygen were shown recently by this laboratory to be additive reactions (23). Thus, even in the aerobic environment used in

this study, SOD is capable of enhancing the conversion of HNO to NO. Hence, the oxidation of enzymatically generated HNO to NO by SOD is the most logical and simplest explanation that can account for all of our observations. SOD often enhances the biological half-life and actions of endogenous and exogenous NO in intact cell systems and tissues (15). In those studies, SOD has access only to membrane-diffusible, extracellular NO, and the likely mechanism involves the dismutation of O_2^- and the consequent protection of NO against oxidative inactivation by O_2^-. In the present study, however, SOD has direct access to the NOS enzyme and its immediate products and exerts an additional effect, possibly by enhancing the formation of NO from an intermediate such as nitroxyl in the L-arginine–NO pathway.

1. Tayeh, M. A. & Marletta, M. A. (1989) *J. Biol. Chem.* **264**, 19654–19658.
2. Kwon, N. S., Nathan, C. F. & Stuehr, D. J. (1989) *J. Biol. Chem.* **264**, 20496–20501.
3. Pollock, J. S., Forstermann, U., Mitchell, J. A., Warner, T. D., Schmidt, H. W., Nakane, M. & Murad, F. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 10480–10484.
4. Mayer, B., John, M. & Bohme, E. (1991) *J. Cardiovasc. Pharmacol.* **17**, S46–S51.
5. Hevel, J. M., White, K. A. & Marletta, M. A. (1991) *J. Biol. Chem.* **266**, 22789–22791.
6. Long, C. J., Shikano, K. & Berkowitz, B. A. (1987) *Eur. J. Pharmacol.* **142**, 317–318.
7. Rubanyi, G. M., Johns, A., Harrison, D. & Wilcox, D. (1990) *Arch. Int. Pharmacodyn. Ther.* **305**, 298 (abstr.).
8. Gold, M. E., Wood, K. S., Byrns, R. E., Fukuto, J. M. & Ignarro, L. J. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 4430–4434.
9. Griscavage, J. M., Rogers, N. E., Sherman, M. P. & Ignarro, L. J. (1993) *J. Immunol.* **151**, 6329–6337.
10. Ignarro, L. J., Fukuto, J. M., Griscavage, J. M., Rogers, N. E. & Byrns, R. E. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 8103–8107.
11. Komori, Y., Chiang, K. T. & Fukuto, J. M. (1993) *Arch. Biochem. Biophys.* **307**, 311–315.
12. Bush, P. A., Gonzalez, N. E., Griscavage, J. M. & Ignarro, L. J. (1992) *Biochem. Biophys. Res. Commun.* **185**, 960–966.
13. Blough, N. V. & Zafiriou, O. C. (1985) *Inorg. Chem.* **24**, 3502–3504.
14. Beckman, J. S., Beckman, T. W., Chen, J., Marshall, P. A. & Freeman, B. A. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 1620–1624.
15. Moncada, S., Palmer, R. M. J. & Higgs, A. E. (1991) *Pharmacol. Rev.* **43**, 109–142.
16. Heinzel, B., John, M., Klatt, P., Bohme, E. & Mayer, B. (1992) *Biochem. J.* **281**, 627–630.
17. Pou, S., Pou, W. S., Bredt, D. S., Snyder, S. H. & Rosen, G. M. (1992) *J. Biol. Chem.* **267**, 24173–24176.
18. Abu-Soud, H. M. & Stuehr, D. J. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 10769–10772.
19. Sung, Y.-J., Hotchkiss, J. H., Austic, R. E. & Dietert, R. R. (1992) *Biochem. Biophys. Res. Commun.* **184**, 36–42.
20. Forstermann, U., Schmidt, H. W., Pollock, J. S., Sheng, H., Mitchell, J. A., Warner, T. D., Nakane, M. & Murad, F. (1991) *Biochem. Pharmacol.* **42**, 1849–1857.
21. Stuehr, D. J., Kwon, N. S., Nathan, C. F., Griffith, O. W., Feldman, P. L. & Wiseman, J. (1991) *J. Biol. Chem.* **266**, 6259–6263.
22. Murphy, M. E. & Sies, H. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 10860–10864.
23. Fukuto, J. M., Hobbs, A. J. & Ignarro, L. J. (1993) *Biochem. Biophys. Res. Commun.* **196**, 707–713.