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Regulation of Nitric Oxide Synthase by Nitric Oxide

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SUMMARY

Nitric oxide (NO) is a recently discovered messenger for the activation of soluble guanylate cyclase in a wide variety of cell types. Although enzymes involved in NO synthesis have been discovered, the regulation of their action is not clear. The possibility of NO regulating the activity of a crude NO synthase (EC 1.14.23) preparation from bovine cerebellum was investigated. Authentic NO (50–400 μM) produced a marked attenuation of NO synthase activity, as measured by the stoichiometric conversion of L-[^{3}H]arginine to L-[^{3}H]citrulline. This inhibition was mimicked by the nitrovasodilators *S*-nitroso-*N*-acetylpenicillamine, sodium nitroprusside, and glyceryl trinitrate. NO was most potent in inhibiting the enzyme activity, followed by *S*-nitroso-*N*-acetylpenicillamine, sodium nitroprusside, and glyceryl trinitrate. The effects of NO and the nitrovasodilators were concentration dependent and reversible. Oxyhemoglobin (50 μM), a scavenger of NO, partially prevented the inhibition of NO synthase activity by NO. Inorganic nitrite (5 mM), the oxidation product of NO, did not produce any effect on the enzyme activity. The K_m for L-arginine was not significantly changed by NO (200 μM) (from 6.4 ± 0.8 μM to 10.6 ± 1.6 μM), whereas the V_{max} of the enzyme was markedly decreased (from 80 ± 4 to 45 ± 4 pmol/min/mg of protein). This study suggests that NO production may be regulated by a direct effect of NO on the activity of NO synthase.

NO is a novel cell messenger that is produced by the vascular endothelium, cerebellum, and several other cell types and is implicated in diverse cellular functions (1, 2). The action of NO is mediated by the activation of soluble guanylate cyclase, and this effect is mimicked by the nitrovasodilating agents (1, 2). Recent studies have shown that NO and L-citrulline are synthesized by the oxidation of a guanidino nitrogen atom of L-arginine by NO synthase (1–3). Two major forms of NO synthase, a constitutive type and an inducible type, have been described (2, 3). The constitutive enzyme has been shown to be present in cerebellum, endothelium, and several other cells and to require calcium, calmodulin, NADPH, and tetrahydrobiopterin for full activity (2, 3). The enzyme purified from porcine cerebellum has been shown to contain FAD, FMN, and iron in equimolar amounts (4). Macrophages, vascular smooth muscle cells, endothelial cells, and several other cell types are also capable of expressing an inducible form of NO synthase in response to endotoxin and cytokine treatment (2, 3). The inducible enzyme has been shown to be calcium and calmodulin independent but requires NADPH, FAD, FMN, and tetrahydrobiopterin as cofactors for full activity (2, 3). Recent studies demonstrate that macrophage NO synthase has tightly bound calmodulin as a subunit of the enzyme (5).

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In addition to its role as an activator of soluble guanylate cyclase, NO has been shown to have multiple actions that are independent of a cGMP-dependent mechanism. NO activates ADP-ribosyltransferase, which ribosylates a 39-kDa protein in several tissues including brain, heart, and liver (6). Stadler et al. (7) and Hibbs et al. (8) have shown that NO inhibits the activity of mitochondrial enzymes such as aconitase, NADPH-ubiquinone oxidoreductase, and succinate-ubiquinone oxidoreductase. NO has been shown to inhibit DNA and protein synthesis by inhibiting ribonucleotide reductase, an enzyme involved in deoxyribonucleotide synthesis (9, 10). Garg and Hassid (11) have reported that NO decreases the cytosolic free calcium concentration in BALB/c 3T3 fibroblasts by a cGMP-independent mechanism. The ability of NO to inhibit osteoclastic activity suggests a role in osteogenesis and bone remodeling (12). Lipid oxidation by lipoxygenase and cyclooxygenase is inhibited by NO (13). One mechanism of these varied actions of NO is thought to relate to the avid binding of NO to the iron of both heme and non-heme iron proteins (14–16). Because NO synthase is a heme iron-containing enzyme (17), we investigated whether NO has any effect on NO synthase activity. In a preliminary report, we showed that NO and NO donors reversibly inhibited bovine cerebellum constitutive NO synthase activity (18). The current studies were designed to investigate whether NO has any regulatory role in NO synthesis.

ABBREVIATIONS: NO, nitric oxide; SNP, sodium nitroprusside; SNAP, *S*-nitroso-*N*-acetylpenicillamine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; GTN, glyceryl trinitrate.

Materials and Methods

Chemicals. NADPH tetrasodium salt, superoxide dismutase, bovine hemoglobin, SNP, and phenylmethylsulfonyl fluoride were obtained from Sigma Chemical Co. (St. Louis, MO). GTN was obtained from Parke-Davis (Morris Plaines, NJ). L-[^{3}H]Arginine (55 Ci/mmol) was purchased from NEN/DuPont (Boston, MA). Dowex AG50W-X8 cation exchange resin and Bio-Rad protein assay reagent were obtained from Bio-Rad Laboratories (Richmond, CA). NO (99%) was obtained from Matheson Gas Products (East Rutherford, NJ).

Preparation of NO synthase. Crude bovine cerebellum NO synthase was prepared as described previously (19). Briefly, fresh bovine cerebellum was obtained from a slaughterhouse, immediately cleaned free of exogenous blood, and immersed in liquid nitrogen. The cerebellum (10 g) was thawed and homogenized (Polytron homogenizer, setting 5) in 5 volumes of buffer containing 50 mM HEPES, pH 7.4, 0.5 mM EDTA, 1 mM dithiothreitol, and 5 mg phenylmethylsulfonyl fluoride. The homogenate was centrifuged at 20,000 × *g* for 60 min and the supernatant was passed through a Dowex AG50W-X8 (Na^+ form) column to remove endogenous L-arginine. All the procedures described above were carried out at 4°.

Determination of NO synthase activity. Bovine cerebellum NO synthase activity was determined as described previously (19), by measuring the conversion of L-[^{3}H]arginine to L-[^{3}H]citrulline, a product in the enzyme reaction. The reaction mixture (4 ml), containing 3.4 ml of enzyme extract, 0.45 mM calcium, 1 mM NADPH, 20 units/ml superoxide dismutase, and 100 μM L-[^{3}H]arginine, was incubated at 37°. To stop the reaction, 150-μl aliquots of the reaction mixture were withdrawn at various times and mixed with 2 ml of ice-cold 20 mM HEPES buffer, pH 5.5, containing 2 mM EDTA. The mixture was applied to a 1-ml Dowex AG50W-X8 (Na^+ form) column and L-[^{3}H] citrulline was specifically eluted with 2 ml of distilled water. The radioactivity in the eluate was measured by liquid scintillation counting. When the effect of NO and nitrovasodilators on the enzyme activity was studied, the agents were added to the enzyme extract 30 sec before initiation of the reaction with L-[^{3}H]arginine. When the effect of oxyhemoglobin on the inhibition of NO synthase by NO was determined, oxyhemoglobin was added to the reaction mixture just before the addition of NO.

Determination of NO by Greiss reaction. A saturated solution of NO was prepared as described by Ignarro *et al.* (20), with minor changes. NO (50 ml) was passed through a column of KOH pellets and then injected as a fine stream into a 7-ml Vacutainer tube (Becton Dickinson) containing 2 ml of buffer that had been previously deoxygenated by bubbling with nitrogen or helium for 2 hr. Different volumes of NO solutions were injected into the reaction mixtures to obtain various concentrations of NO. The concentration of NO in the stock solution was determined using the Greiss reagent, with $NaNO_2$ as the standard (21).

Determination of NO by chemiluminescence. NO concentration in the gaseous phase (headspace) above the reaction mixture was determined by the chemiluminescence method. Different volumes of saturated NO solution were added to the enzyme solution in a 50-ml tube with a rubber stopper, similar to that used in the enzyme inhibition studies. After 30 sec, the sample was mixed well, aliquots of gaseous phase were withdrawn into a gas-tight syringe, and the sample was injected into a chemiluminescence analyzer (Sievers 270 NOA) connected to an integrator (Hewlett Packard 3390A). The sample was purged with helium and the chemiluminescence was measured. The NO analyzer was calibrated by injecting aliquots of commercially prepared NO gas in nitrogen (Matheson Gas Products, East Rutherford, NJ). NO concentrations are reported as the concentration in solution, calculated based on the partition coefficients for NO at known temperature, the pressure, and the volume of gaseous phase versus aqueous phase.

Preparation of other reagents. Oxyhemoglobin was prepared by mixing hemoglobin with a 10-fold molar excess concentration of sodium dithionite and separating the oxyhemoglobin using a Sephadex G-25 column (30 cm × 2 cm). The fractions containing oxyhemoglobin were pooled and dialyzed for 3 hr against 500 volumes of 10 mM HEPES buffer, pH 7.0, with three changes. Aliquots of oxyhemoglobin were stored at −80° and used within 1 week. The concentration of oxyhemoglobin was determined as described previously (22). SNAP was prepared according to the method of Field *et al.* (23). The partial pressure of oxygen (pO_2) in the reaction mixture was determined by withdrawing the sample into a gas-tight glass syringe and injecting the sample into a Radiometer blood gas analyzer. Protein concentration was determined using the Bio-Rad protein reagent, with bovine serum albumin as the standard.

Results

Inhibition of NO synthase activity by NO. We have investigated the effect of authentic NO on a crude NO synthase preparation from bovine cerebellum by measuring the production of L-[^{3}H]citrulline from L-[^{3}H]arginine. NO (50–400 μM) inhibited the activity of NO synthase in a concentration-dependent manner at all time points tested (Fig. 1). The effects on the activity of NO synthase of NO donors that are widely used for generating NO in different experimental systems were also tested. The NO synthase activity was significantly ($p < 0.01$) inhibited when the enzyme was exposed to SNAP or SNP (each 1 mM) (Fig. 2). SNAP (1 mM) produced 43 ± 4% inhibition of the enzyme activity at 60 min, whereas SNP and GTN caused 25 ± 6% and 12 ± 5% inhibition, respectively. A dose-dependent effect of SNAP on NO synthase activity was determined. At 60 min, the enzyme activity was inhibited by 17 ± 3%, 39 ± 4%, and 49 ± 4% in the presence of 0.5 mM, 1 mM, and 2 mM SNAP, respectively (Fig. 3).

The enzyme mixture treated with SNP and GTN also contained 20 mM cysteine. Cysteine (20 mM) by itself had no significant effect on the enzyme activity (data not shown). Inorganic nitrite (5 mM), a major oxidation product of NO, did not have any effect on NO synthase activity (Fig. 2).

Effect of oxygen concentration on the inhibition of NO synthase activity by NO. Oxygen tension was not a factor in NO synthase inhibition by NO. To rule out an effect of NO interaction with oxygen (and thus limitation of the availability of oxygen, a substrate for the enzyme) as the mechanism of NO

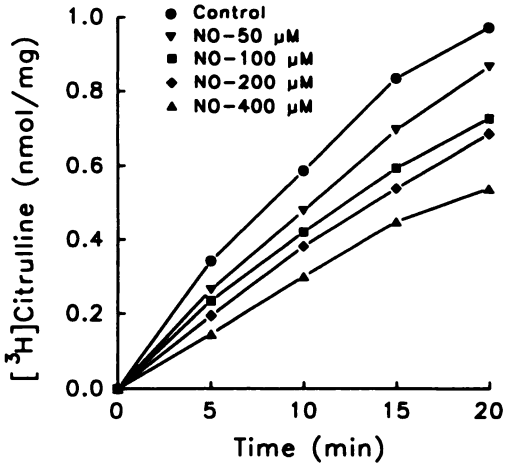

Fig. 1. Inhibition of NO synthase activity by NO. The activity of bovine cerebellum NO synthase was determined by measuring the conversion of L-[^{3}H]arginine to L-[^{3}H]citrulline in the presence of varying concentrations of NO. NO solution was injected into the enzyme mixture 30 sec before initiation of the reaction with L-[^{3}H]arginine.

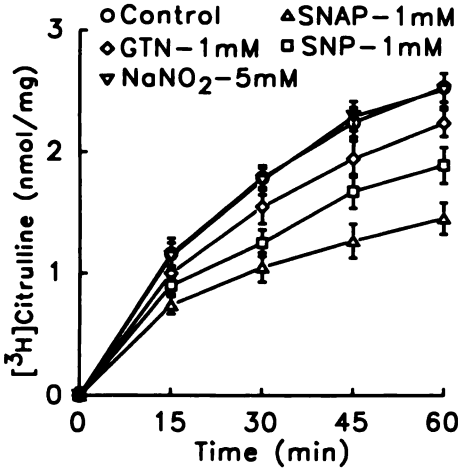

Fig. 2. Inhibition of NO synthase activity by NO donors. The activity of NO synthase was determined in the presence of SNAP, SNP, GTN (1 mM each), or $NaNO_2$ (5 mM) after different time intervals, as described in Materials and Methods.

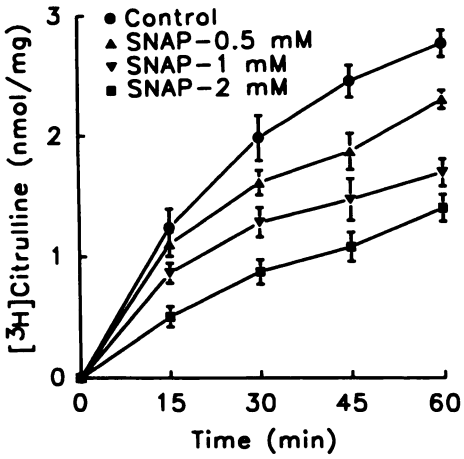

Fig. 3. Concentration dependence of SNAP inhibition of NO synthase activity. NO synthase activity was determined in the presence of varying concentrations of SNAP, as described in Materials and Methods.

synthase inhibition by NO, the effect of SNAP (1 mM) on the enzyme activity was tested by passing no air, air, or oxygen through the reaction mixture at a rate of 2 liters/min. NO synthase activity showed an inhibition of 33 ± 4%, 40 ± 3%, and 36 ± 3% at 15 min when no air (pO_2 = 132 ± 5 mm of Hg), air (pO_2 = 135 ± 4 mm of Hg), or oxygen (pO_2 = 470 ± 12 mm of Hg), respectively, was passed through the reaction mixture.

Effect of oxyhemoglobin on the inhibition of NO synthase activity by NO. To confirm that NO was responsible for the inhibition of NO synthase activity, the effect of oxyhemoglobin, which is known to bind NO, on the inhibition of the enzyme activity was tested. Oxyhemoglobin (50 μM) partially prevented the inhibition of NO synthase activity by NO (50 μM), by 81 ± 10% and 74 ± 9% at 5 and 10 min, respectively.

Kinetic studies. The rate of the enzyme activity (V_{max}) was determined by measuring the initial conversion of L-[^{3}H]arginine to L-[^{3}H]citrulline for 3 min in the presence and absence of 200 μM NO. The K_m for L-arginine was not significantly changed (from 6.4 ± 0.8 μM to 10.6 ± 1.6 μM) by NO, whereas the V_{max} of the enzyme activity was significantly ($p < 0.01$) decreased (from 80 ± 4 to 45 ± 4 pmol/min/mg of protein) (Fig. 4).

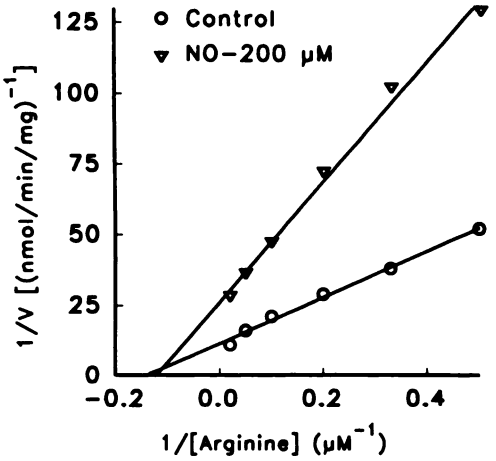

Fig. 4. Effect of NO on the L-arginine concentration dependence of NO synthase activity. NO synthase activity was measured for 3 min in the presence of varying concentrations of L-[^{3}H]arginine, and the rate of conversion to L-[^{3}H]citrulline was determined. The data were analyzed by a Lineweaver-Burk plot. The K_m values for L-arginine were 6.7 (6.4 ± 0.8) μM and 8.3 (10.6 ± 1.6) μM for the control and the 200 μM NO-treated sample, whereas the V_{max} values were 83 (80 ± 4) pmol/min/mg and 38 (45 ± 4) pmol/min/mg, respectively. The numbers in parentheses represent the mean ± SEM of three experiments.

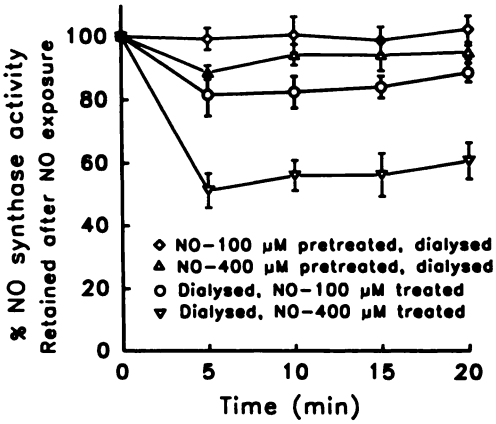

Fig. 5. Reversibility of NO synthase inhibition by NO. The enzyme was mixed with buffer or NO solution (100 or 400 μM), incubated at 4° for 30 min, and then dialyzed for 4 hr. The activity of the dialyzed control enzyme was determined in the presence of 0, 100, or 400 μM NO. The enzyme pretreated with NO (100 or 400 μM) and then dialyzed was assayed for its activity in the absence of NO. The data were normalized to the dialyzed non-NO-treated enzyme activity, which was 0.32 ± 0.04, 0.58 ± 0.06, 0.81 ± 0.07, and 0.99 ± 0.08 nmol/mg at 5, 10, 15, and 20 min, respectively.

Reversibility of NO inhibition of NO synthase activity. To determine whether the inhibition of NO synthase by NO was reversible, the enzyme preparation was treated with either buffer alone or buffer containing NO at two different concentrations (100 and 400 μM). The preparations were dialyzed against buffer without NO. The activity of the NO-treated dialyzed enzyme recovered to 97 ± 6% and to 90 ± 4% of the control enzyme activity in the 100 and 400 μM NO-treated enzyme preparations, respectively (Fig. 5). To confirm that the control dialyzed enzyme was still inhibitable by NO, after dialysis it was exposed to 100 or 400 μM NO, which resulted in an inhibition of 19 ± 3% and 49 ± 5%, respectively.

Determination of NO concentration. The NO concentrations in the reaction mixtures were determined indirectly by

measuring the NO in the gaseous phase by the chemiluminescence method. Based on the known partition coeffient of 1:24.5 for the distribution of NO between aqueous and gaseous phases at 37°, the concentrations of NO in the reaction mixtures were calculated to be 0.16, 0.28, and 0.56 μM after 30 sec when the initially added concentrations were 100, 200, and 400 μM, respectively.

Discussion

The purpose of this study was to investigate whether NO, the bioactive product of L-arginine metabolism by NO synthase, has any regulatory effect on NO synthesis. Our findings demonstrate that NO acts on the crude bovine cerebellum NO synthase to attenuate the production of NO. This suggests that the inhibition of NO synthase by NO may be an important mechanism by which cells regulate NO synthesis.

Authentic NO was found to be a potent inhibitor of NO synthase activity. The NO donors SNAP, SNP, and GTN were also effective in inhibiting the enzyme activity but higher concentrations were required, compared with NO, most likely due to incomplete metabolism of these agents to NO. SNAP was more potent than SNP and GTN and this is likely due to the ability of SNAP to spontaneously release NO, whereas reducing agents or further metabolism is required to produce NO from SNP and GTN (24).

The ability of oxyhemoglobin to attenuate the inhibition of NO synthase activity by NO further confirms that the inhibition of the enzyme activity was mediated by NO. This inhibition of the enzyme activity by NO appears to be independent of oxygen concentration in the reaction mixture, because the percentage of inhibition of the enzyme activity did not vary significantly under different partial pressures of oxygen. The inhibition of NO synthase activity by NO is not due to any irreversible damage to the enzyme, because the enzyme pretreated with NO recovered most of its activity after dialysis to remove the NO.

We have observed inhibition of NO synthase activity at high added concentrations of NO and NO donors. Similar concentrations of NO were required in studies where the susceptibility of ribonucleotide reductase to NO was determined (10). Due to the highly reactive nature of NO, the concentration of NO actually causing inhibition is likely to be significantly less than that added initially. The determination of NO in the reaction mixture after 30 sec reveals a concentration of NO 2–3 orders of magnitude less than that which was initially prepared for addition to the reaction mixture. Furthermore, the sensitivity of NO synthase to NO *in vivo* cannot be compared directly with that measured in this *in vitro* preparation. In addition, the NO added to the crude enzyme preparation reacts with several other heme and non-heme iron proteins in addition to NO synthase, as well as with sulfhydryl groups of proteins, thus reducing the concentration of NO interacting with NO synthase. Also, in the intact system the concentration of NO is highest at its site of production, NO synthase, and the concentration available to bind to the enzyme is higher. Our data have also demonstrated that nitrite, the most abundant oxidation product of NO interaction with oxygen, is not responsible for the observed inhibition. NO is likely to play a significant role in the regulation of NO synthase and thereby the production of NO.

A primary biological function of NO is the activation of soluble guanylate cyclase to increase the level of cGMP in several systems, including vascular smooth muscle and brain. NO is also known to have actions totally independent of the activation of guanylate cyclase. Recently, NO has been shown to inhibit a number of enzymes, including ribonucleotide reductase (9, 10), nitrogenase (25, 26), and mitochondrial enzymes like aconitase, NADH-ubiquinone oxidoreductase, and succinate-ubiquinone oxidoreductase (7, 8). In these studies, the action of NO has been suggested to be a result of its ability to form complexes with iron proteins. NO contains an unpaired electron, which has been shown to react with ferrous iron in aqueous solution containing one or more coordinating anionic ligands to form NO-iron complexes (14). The formation of such a nitrosyl-iron complex by several heme and non-heme iron proteins has been characterized by EPR studies, where NO was shown to bind to both ferrous and ferric iron (15, 16).

The inhibition of NO synthase activity by NO observed in our study may be due to product inhibition or an interaction of NO with the enzyme, cofactors, or other regulatory molecules of NO synthase. The action of NO may be due to direct binding of NO to the enzyme to form a complex. Both constitutive and inducible NO synthases have now been characterized to be cytochrome P450-type hemoproteins, based on their marked inhibition by carbon monoxide and their reduced carbon monoxide binding difference spectra (17). This study suggests that the electrons from NADPH may be shuttled through the flavins in the enzyme, resulting in a perferryl iron-heme complex that could catalyze the formation of N^G-hydroxy-L-arginine. The interaction of NO with heme would prevent its serving as an electron acceptor. The formation of a complex of NO with the iron moiety of NO synthase is possible, because NO forms NO-iron complexes with both heme and non-heme iron proteins. The inhibition of NO synthase by NO observed in our study may be due to the formation of a stable heme iron-NO complex, which has been shown to dissociate at a rate of 10^{-6}/sec (27). Thus, the enzyme that reacts with NO at the initial time points is likely to remain inactive until the end of the reaction. The slow dissociation constant also suggests that NO may have a cumulative inhibitory effect if its mechanism is due to binding to the heme of NO synthase. Low *in vivo* concentrations of NO could effectively inhibit NO synthase activity in this manner.

Nitrogenase, an enzyme containing iron, has been shown to react with NO to form a NO-iron complex, with a resultant inhibition of enzyme activity (25, 26). Recent studies in macrophages demonstrated that endogenous NO derived from L-arginine forms a complex with intracellular proteins (28). Macrophages pretreated with interferon-γ were permeabilized and, on analysis by EPR, a signal in the $g = 2.04$ region, which is characteristic of a nitrosyl-iron-sulfur complex, was observed (28). This EPR signal has been attributed to the formation of a complex of NO with mitochondrial enzymes like aconitase, which showed a concomitant decrease in enzyme activity with an increase in nitrite production (a measurement of NO) (28). Thus, the inhibition of NO synthase activity by NO observed in our study is likely due to formation of a NO-iron complex by NO and the heme iron of the bovine cerebellum enzyme.

The inhibition of NO synthase by NO may be an important intracellular mechanism in the regulation of NO synthesis. Boughton-Smith *et al.* (29) reported that SNAP infusion protected against the acute intestinal damage caused by endotoxin treatment in rats. Recently, Bult *et al.* (30) examined the long

term effects of exposure to exogenous NO in rabbits. The aortae from rabbits that had been repeatedly exposed to molsidomine (a NO-generating compound) exhibited decreased vasodilation in response to acetylcholine, as assessed by the relaxation of a bioassay tissue, thus indicating suppression of NO synthesis by NO itself. This may be a direct effect of NO on NO synthase activity, as observed in our studies.

In summary, we showed that NO and NO donors directly and reversibly inhibited NO synthase activity. This NO inhibition of NO synthase activity may be a significant regulatory mechanism for NO synthesis in intact cells.

Addendum

After submission of this manuscript, similar results were published (31).

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