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CONSTITUTIVE NITRIC OXIDE SYNTHASE FROM CEREBELLUM IS REVERSIBLY INHIBITED BY NITRIC OXIDE FORMED FROM L-ARGININE

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SUMMARY: The objective of this study was to determine whether constitutive nitric oxide (NO) synthase from rat cerebellum could be regulated by the two products of the reaction, NO and L-citrulline, utilizing L-arginine as substrate. NO synthase activity was determined by monitoring the formation of ^{3}H-citrulline from ^{3}H-L-arginine in the presence of added cofactors. The rate of citrulline formation in enzyme reaction mixtures was non-linear. Addition of superoxide dismutase (SOD; 100 units) inhibited NO synthase activity and made the rate of product formation more non-linear, whereas addition of oxyhemoglobin (HbO_2; 30 μM) increased NO synthase activity, made the rate of product formation linear and also abolished the effect of SOD. Added NO (10 μM) inhibited NO synthase activity and this inhibition was potentiated by SOD and abolished by HbO_2. Added L-citrulline (1 mM) did not alter NO synthase activity. The two NO donors, S-nitroso-N-acetylpenicillamine (200 μM) and N-methyl-N'-nitro-N-nitrosoguanidine (200 μM) mimicked the inhibitory effect of NO and inhibition of NO synthase activity by NO was reversible. These observations indicate clearly that NO formed during the NO synthase reaction or added to the enzyme reaction mixture causes a reversible inhibition of NO synthase activity. Thus, NO may function as a negative feedback modulator of its own synthesis.

NO synthase catalyzes the five-electron oxidation of L-arginine to NO plus citrulline (1-3). The cytosolic constitutive isoform of NO synthase, one of three principal enzyme isoforms, from rat cerebellum has a relatively high specific activity in unpurified cytosolic tissue fractions (4) and has been shown to synthesize equimolar quantities of NO and citrulline from L-arginine (5). In previous experiments designed to quantify the synthesis of NO from L-arginine, we noted that the rates of formation of both NO and citrulline were non-linear (5,6). One explanation for these observations was enzyme substrate utilization and partial depletion over time and a consequent deviation from initial velocity conditions, although enzyme substrate was present in excess. In more recent preliminary experiments, however, we noted that the addition of hemoglobin or oxyhemoglobin, but not methemoglobin or albumin, to enzyme reaction mixtures restored the rate of product formation to linearity. These observations suggested that the NO formed during the enzymatic reaction was inhibiting NO synthase activity.

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The objective of this study was to determine whether NO and L-citrulline, generated enzymatically or added exogenously, could inhibit or modulate constitutive NO synthase activity. The cytosolic constitutive isoform of NO synthase from rat cerebellum was studied because of its relatively high specific activity in catalyzing the conversion of L-arginine to both NO and citrulline, and the knowledge that this enzyme isoform catalyzes the formation of equimolar quantities of NO and citrulline from L-arginine (5).

MATERIALS AND METHODS

Chemicals and solutions: L-Arginine, L-citrulline, NADPH, calmodulin, N-methyl-N'-nitro-N-nitrosoguanidine, hemoglobin (human), superoxide dismutase (bovine erythrocytes), dithiothreitol, phenylmethylsulfonyl fluoride, pepstatin A, leupeptin, EDTA, and EGTA were purchased from Sigma Chemical Co. Tetrahydrobiopterin was purchased from Schircks laboratories (Jona, Switzerland). NO gas (99%; Matheson) was purified further by passage through distilled water to remove any NO_2 and then through a column of potassium hydroxide to remove any traces of water from the NO. The NO was collected in a glass bulb fitted with a rubber septum. Aliquots of 40 ml of gas were bubbled through one ml of distilled water contained in a small test tube that had been vacuum evacuated for 15 min followed by venting with oxygen-free nitrogen for 15 min. This procedure yields a nearly saturated solution of NO in water at 25°C and 1 atmosphere (approximately 1 mM). Serial dilutions were then made in oxygen-free water with the aid of gas-tight syringes. S-Nitroso-N-acetylpenicillamine (SNAP) was prepared, stored, and used as described previously (7). Oxyhemoglobin was prepared from hemoglobin as described previously (8). Dowex AG50W-X8 (H^+ form) 100-200 mesh, Dowex AG 1-X8, OH^- form, 100-200 mesh, and Tris base (electrophoresis grade) were purchased from Bio-Rad Laboratories. Sodium nitrite and sodium nitrate were obtained from Fisher Chemical Co. Aquasol-2 was purchased from Du Pont Company/NEN Research Products.

Citrulline determination: Citrulline was determined by monitoring the formation of ^{3}H-citrulline from ^{3}H-L-arginine as described previously (5). Briefly, samples (2 ml) were applied to columns (1 cm diameter) containing 1 ml of Dowex AG50W-X8, Na^+ form (prepared from the H^+ form), that had been pre-equilibrated with 20 mM sodium acetate, pH 5.5, containing 1 mM L-citrulline, 2 mM EDTA, and 0.2 mM EGTA (Stop Buffer). The eluate (2 ml) was collected into a liquid scintillation vial. Columns were eluted with 2 ml of water and collected into another vial. Aquasol-2 (10 ml) was added to each vial and samples were counted in a Beckman LS 3801 liquid scintillation spectrometer. Citrulline was recovered in the first 4 ml of Dowex column eluate to the extent of 96%, and data were corrected to account for such recovery.

Protein determination: Protein concentrations in cerebellum supernatant fractions were determined by the Bradford, Coomassie brilliant blue method as described by Bio-Rad. Bovine serum albumin was used as the standard.

NO synthase assay: Rat cerebellum was used as the source of NO synthase. Rats (Sprague Dawley, males, 150-175 g) were sacrificed by decapitation and the cerebella were excised, rinsed and stored frozen at -75°C. Homogenates (25% w/v) of cerebellum were prepared in 50 mM Tris HCl, pH 7.4, containing 0.1 mM EDTA, 0.1 mM EGTA, 0.5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 μM pepstatin A, and 2 μM leupeptin at 0-4°C with the aid of a tissue grinder fitted with a ground glass pestle. Homogenates were centrifuged at 20,000 x g for 60 min at 4°C and the supernatant was used as the source of NO synthase. Further centrifugation of the above supernatant at a force of 100,000 x g

for 60 min yielded a supernatant fraction that was nearly devoid of NO synthase activity (5). Thus, the supernatant derived from centrifugation of homogenates at 20,000 x g for 60 min was used in this study. Enzymatic reactions were conducted at 37°C in 50 mM Tris HCl, pH 7.4, containing 50 μM L-arginine (approximately 200,000 dpm of L-[2,3,4,5-^{3}H]arginine HCl; 77 Ci/mmol; Amersham), 100 μM NADPH, 2 mM $CaCl_2$, 1 μg calmodulin, 10 μM tetrahydrobiopterin, 0.17 - 0.26 mg supernatant protein, and other test agents as indicated, in a final incubation volume of 100 μl. The L-[2,3,4,5-^{3}H]arginine HCl was previously purified by anionic exchange chromatography on columns of Dowex AG 1-X8, OH^- form, 100-200 mesh in order to remove traces of ^{3}H-citrulline. Enzymatic reactions were initiated at 37°C by addition of enzyme-containing supernatant. Enzymatic reactions were terminated by addition of 2 ml of ice-cold Stop Buffer, and samples were chromatographed as described above.

RESULTS AND DISCUSSION

Previous experiments revealed that the constitutive NO synthase from rat cerebellum catalyzes the formation of equimolar quantities of NO and citrulline from L-arginine (5). In the present study, NO formation could not be monitored because many of the experiments entailed the addition of NO or NO-donor compounds to enzyme reaction mixtures. Therefore, citrulline formation was used to monitor NO synthase activity. Under the experimental conditions employed, the rate of citrulline formation was non-linear up to 20 min of incubation (Fig. 1). This non-linearity of enzymatic activity with respect to time

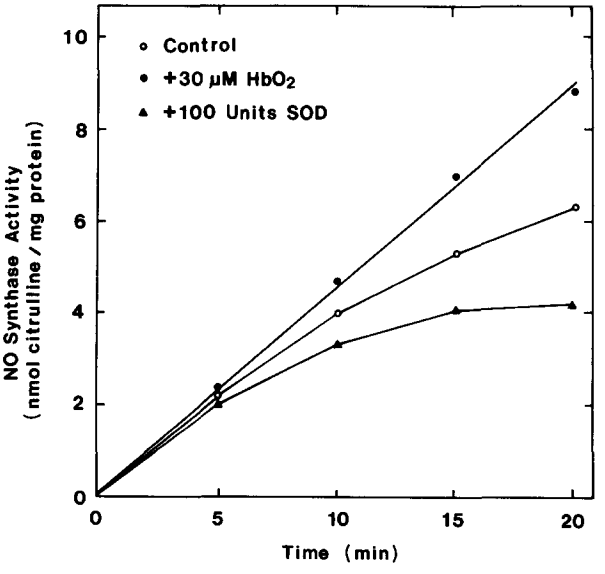

FIG. 1. Influence of oxyhemoglobin (HbO_2) and superoxide dismutase (SOD) on the time course of citrulline formation from L-arginine by NO synthase. Enzymatic reactions were conducted at 37°C in 50 mM Tris HCl, pH 7.4, containing 50 μM L-arginine (200,000 dpm), 100 μM NADPH, 2 mM $CaCl_2$, 1 μg calmodulin, 10 μM tetrahydrobiopterin, and supernatant containing 0.21 - 0.24 mg protein in a final assay volume of 100 μl. HbO_2 and SOD were added to reaction mixtures just prior to initiation of reactions by addition of supernatant. Data points represent mean values of 6 - 8 determinations from 3 - 4 separate experiments.

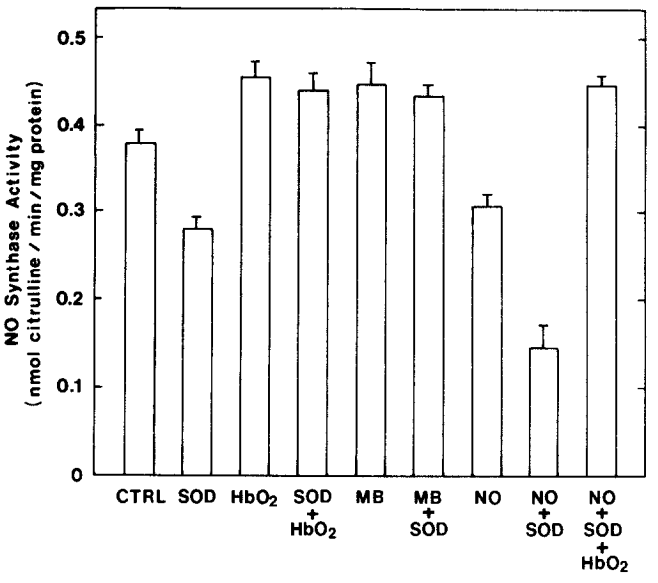

FIG. 2. Inhibition of NO synthase activity by NO and superoxide dismutase (SOD) and prevention by oxyhemoglobin (HbO_2) and methylene blue (MB). Enzymatic reactions were conducted at 37°C in 50 mM Tris HCl, pH 7.4, containing 50 μM L-arginine (200,000 dpm), 100 μM NADPH, 2 mM $CaCl_2$, 1 μg calmodulin, 10 μM tetrahydrobiopterin, and supernatant containing 0.17 - 0.21 mg protein in a final assay volume of 100 μl. SOD (100 Units), HbO_2 (30 μM), and MB (1 μM) were added to reaction mixtures just prior to initiation of reactions by addition of supernatant. NO (10 μM) was added to reaction mixtures immediately after addition of supernatant. CTRL signifies control. Data represent the mean ± S.E.M. of 6 - 10 determinations from 3 - 5 separate experiments.

was not attributed to substrate depletion, as reaction rates remained non-linear after increasing the L-arginine concentration 10-fold from 50 μM to 500 μM. Addition of 100 units of SOD to enzyme reaction mixtures inhibited NO synthase activity and made the rate of citrulline formation more non-linear (Fig. 1). On the other hand, addition of 30 μM oxyhemoglobin to enzyme reaction mixtures increased NO synthase activity and made the rate of citrulline formation linear (Fig. 1). Since oxyhemoglobin avidly binds and inactivates NO, whereas SOD increases the biological half-life of NO by destroying any superoxide anion that might otherwise chemically inactivate NO, one plausible explanation of these observations is that the NO generated during the enzymatic reaction inhibits NO synthase activity. This interpretation is supported by the finding that methemoglobin, which has a lower binding affinity than oxyhemoglobin for NO, was completely without effect when tested at the same concentration as oxyhemoglobin. Moreover, the inhibitory effect is not attributed to either of the two spontaneous oxidation products of NO as neither 1 mM nitrite nor 1 mM nitrate was inhibitory.

In order to ascertain whether the inhibitory effect of SOD may have been mediated by enzymatically generated NO, oxyhemoglobin was tested for its capacity to counteract the effect of SOD. Oxyhemoglobin abolished the inhibitory effect of SOD (Fig. 2). Moreover,

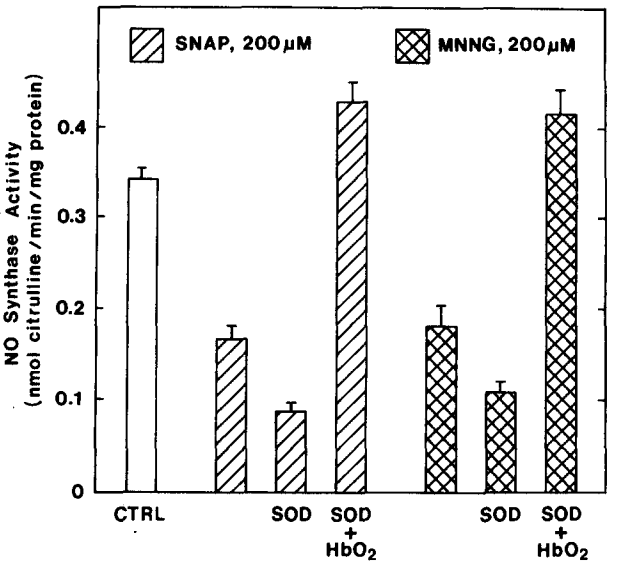

FIG. 3. Inhibition of NO synthase activity by NO donor compounds, enhancement by superoxide dismutase (SOD) and abolition by oxyhemoglobin (HbO_2). Enzymatic reactions were conducted at 37°C in 50 mM Tris HCl, pH 7.4, containing 50 μM L-arginine (200,000 dpm), 100 μM NADPH, 2 mM $CaCl_2$, 1 μg calmodulin, 10 μM tetrahydrobiopterin, and supernatant containing 0.18 - 0.22 mg protein in a final assay volume of 100 μl. SOD (100 Units) and HbO_2 (30 μM) were added to reaction mixtures just prior to initiation of reactions by addition of supernatant. S-Nitroso-N-acetylpenicillamine (SNAP) and N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) were added to reaction mixtures immediately after addition of supernatant. CTRL signifies control. Data represent the mean ± S.E.M. of 6 determinations from 3 separate experiments.

methylene blue, which generates superoxide anion from oxygen-containing solutions (9), increased NO synthase activity and abolished the inhibitory effect of SOD (Fig. 2). These observations are consistent with the view that preservation of NO causes further inhibition, whereas sequestration or destruction of NO causes an increase, in NO synthase activity.

Authentic NO as well as two NO donor compounds were tested for their inhibitory action on NO synthase. NO (10 μM) caused a slight inhibition of NO synthase activity that was markedly enhanced in the presence of SOD and abolished by oxyhemoglobin (Fig. 2). In the presence of SOD, inhibitory effects on NO synthase were elicited by concentrations of NO ranging from 0.1 μM (15% inhibition) to 100 μM (100% inhibition). S-Nitroso-N-acetylpenicillamine and N-methyl-N'-nitro-N-nitrosoguanidine are chemically labile compounds that release NO in aqueous solution (10). S-Nitroso-N-acetylpenicillamine and N-methyl-N'-nitro-N-nitrosoguanidine each inhibited NO synthase activity in a manner that was enhanced by SOD and abolished by oxyhemoglobin (Fig. 3). L-Citrulline at concentrations ranging from 1 μM to 1 mM failed to modify NO synthase activity. These observations further support the view that it is the NO species generated from L-arginine

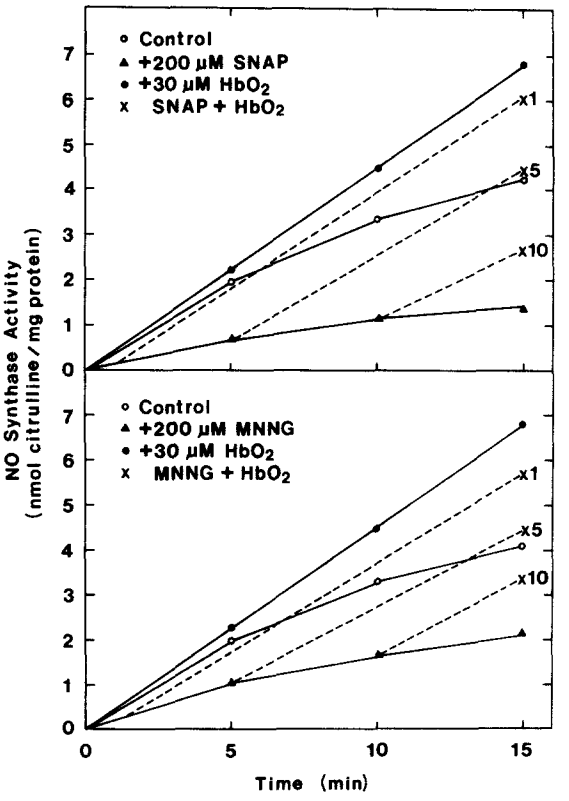

FIG. 4. Reversible inhibition of NO synthase activity by S-nitroso-N-acetylpenicillamine (SNAP) and N-methyl-N'-nitro-N-nitrosoguanidine (MNNG). Enzymatic reactions were conducted at 37°C in 50 mM Tris HCl, pH 7.4, containing 50 μM L-arginine (200,000 dpm), 100 μM NADPH, 2 mM $CaCl_2$, 1 μg calmodulin, 10 μM tetrahydrobiopterin, 100 Units superoxide dismutase, and supernatant containing 0.21 - 0.26 mg protein in a final assay volume of 100 μl. SNAP or MNNG was added to reaction mixtures immediately after addition of supernatant. Oxyhemoglobin (HbO_2) was added to reaction mixtures just prior to initiation of reactions by addition of supernatant. **X 1**, **X 5**, and **X 10** signify the addition of HbO_2 to incubating reaction mixtures at 1 min, 5 min, and 10 min, respectively, after initiation of enzymatic reactions. Dashed lines from the **X** points are extrapolated back to the corresponding times of addition to reaction mixtures. Data points represent mean values of 6 - 8 determinations from 3 - 4 separate experiments.

by NO synthase that is responsible for the inhibitory influence on catalytic activity and, therefore, the non-linear rate of product formation in the absence of added oxyhemoglobin.

In order to determine whether the inhibitory effect of NO is reversible, oxyhemoglobin was added to enzyme reaction mixtures at various times after prior addition of the NO generating compounds, S-nitroso-N-acetylpenicillamine and N-methyl-N'-nitro-N-nitrosoguanidine. Reversible inhibition by NO would be indicated by recovery of enzymatic activity from inhibition starting at the time of addition of oxyhemoglobin. The reversible nature of the inhibitory action of NO is illustrated in Fig. 4. Oxyhemoglobin was added at 1, 5, or 10 min after addition of NO donor compounds to enzyme reaction mixtures. The dashed lines shown in Fig. 4 were extrapolated from the 15 min (termination of reaction)

point back to the earlier time point at which the oxyhemoglobin was added, for the purpose of highlighting the reversible characteristics of the inhibitory action of NO. The recovery of enzymatic activity from NO-elicited inhibition indicates that NO is not causing irreversible inactivation of NO synthase or of any of its required cofactors.

Although the mechanism by which NO reversibly inhibits NO synthase activity is presently unknown, a selective binding site for NO on the enzyme protein could serve to modulate its catalytic activity. One of the most selective binding sites for NO is heme-iron, which is present in many proteins (hemoproteins). Indeed, cytosolic guanylate cyclase is a hemoprotein in which the heme-iron allows NO to bind and activate the enzyme (11,12). The inducible isoform of NO synthase from activated macrophages has been identified as a hemoprotein containing approximately one mole of heme per mole of 130-kDa monomer (13). Carbon monoxide, which binds to heme, was shown to inhibit NO synthase activity. In view of the similar molecular mechanisms of L-arginine conversion to NO plus L-citrulline shared by the constitutive and inducible isoforms of NO synthase, it is plausible that the constitutive isoform, like the inducible isoform, contains heme-iron. Indeed, preliminary findings indicate that the cytosolic constitutive NO synthase from rat cerebellum contains bound heme (13). One of the functions of enzyme-bound heme may be to act as the terminal electron acceptor in the oxidation of L-arginine to the intermediate N^G-hydroxy-L-arginine (13). The results of the present study indicate that another function of the heme prosthetic group may be to bind the NO reaction product and inhibit catalytic activity.

There are at least several biological implications of a negative feedback modulatory effect by NO on constitutive NO synthase. In nonadrenergic-noncholinergic transmission, where NO is believed to be the inhibitory neurotransmitter (14-16), NO could serve to regulate its own synthesis and, therefore, the neurotransmission process. Excess NO production may be undesirable because of its potential to induce cytotoxicity (17). Reduced hemoproteins such as hemoglobin, myoglobin, and/or their oxygen adducts could block the inhibitory effect of NO by sequestering the NO as it is formed. The biological actions of NO would also be blocked. Superoxide anion generated in the vicinity of newly formed NO could block the inhibitory effect of NO by destroying the NO as soon as it is formed. However, the simultaneous production of NO and superoxide anion could enhance the cytotoxic action of NO by reacting with one another to generate peroxynitrite anion, a lipid peroxidizing species (18). If the membrane-bound isoform of NO synthase in vascular endothelial cells is also inhibited by NO, then either excessive formation of NO or the presence of exogenously added NO in the form of nitrovasodilator drugs could diminish the

vasodilator responses to endothelium-dependent relaxants and flow or shear stress. If the inducible isoform of NO synthase in macrophages is also inhibited by NO, then the concomitant generation of superoxide anion by factors that activate macrophages could block the negative feedback modulatory action of NO and increase the quantity of NO generated by the activated macrophages. In this case, the increased quantities of NO formed could react with superoxide anion to generate cytotoxic quantities of peroxynitrite anion. These possible biological consequences of a negative feedback modulatory effect of NO on NO synthase are presently under investigation in this laboratory.

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