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NITRIC OXIDE SYNTHASE: INVOLVEMENT OF OXYGEN RADICALS IN CONVERSION OF L-ARGININE TO NITRIC OXIDE

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Summary: The objective of this study was to determine the role of superoxide ion in the formation of nitric oxide by brain NO synthase. NO synthase activity was detected by activation of guanylate cyclase in broken cell preparations. NO synthase activity was dependent on NADPH and was inhibited by EGTA, hemoglobin, N^{ω}-methyl-L-arginine and nitroblue tetrazolium. While the addition of exogenous superoxide dismutase significantly enhanced NO synthase activity, bovine liver catalase completely abolished NO formation. None of these NO synthase modulators, however, altered NO-dependent stimulation of guanylate cyclase activity. These observations indicate that catalytic conversion of L-arginine to nitric oxide by cytosolic, isoform of brain NO synthase requires superoxide ion, hydrogen peroxide and possibly hydroxyl radical.

It is well established that nitric oxide synthase (NOS) exists in different isoform in tissues and catalyzes the formation of endogenous NO from L-arginine in the presence of NADPH and other regulatory factors (1). The nitric oxide has been shown to elevate intracellular levels of guanosine 3',5'-monophosphate (cyclic GMP) by activating the target enzyme guanylate cyclase in various mammalian tissues (2). This makes NOS one of the integral cellular components of the signal transduction mechanisms in the actions of hormones and autacoids (18). Recent studies with purified preparations indicate that NOS is a flavoprotein containing FAD/FMN moieties (19,20). It has been also shown that multiple catalytic activities including NADPH diphorase (3), cytochrome C reductase and cytochrome P-450 (4) reside in the same molecule. The formation of NO from L-arginine requires molecular oxygen (5), and is inhibited by nitroblue tetrazolium (3).

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Earlier studies with endothelium derived relaxing factor (EDRF/NO) have implied that superoxide ion exerts an inhibitory influence on NO formation which can be reversed/stabilized by superoxide dismutase (SOD) (6). The present investigations were undertaken to further characterize the conversion of NO from L-arginine by NOS and study the role of superoxide ion in this process.

MATERIALS AND METHODS

Preparation of Nitric Oxide Synthase: Sprague-Dawley rats (150-200 g) were killed by decapitation. Whole brains were quickly removed, cleaned and immersed in ice-cold 0.25M sucrose. Brains were homogenized in 6 volumes (w/v) of 0.25M sucrose containing 20mM Tris-HCl (pH 8.0), 1mM ethylenediaminetetraacetate (EDTA) and 1mM dithiothreitol (DTT) at 4°C using glass homogenizer with Teflon pestle. The homogenate was centrifuged at 18,000 xg for 15 minutes. The supernatant was stored at -70°C and used as source for nitric oxide synthase.

Assay of NO Synthase Activity: Formation of NO from L-arginine was determined by guanylate cyclase activation assay in brain cytosol. The standard reaction mixture (100µl) contained 50mM Tis-HCl (pH 7.6), 15mM creatine phosphate, 20µg of creatine phosphokinase (120 - 135 units/mg protein), 8mM theophylline, 4mM $MgCl_2$ and 20µl brain cytosol. NADPH, L-arginine and other modifiers were added at concentrations as indicated. Reactions were initiated with the addition of 1mM GTP and were continued for 10 minutes at 37°C before termination by the addition of 50mM sodium acetate (pH 4.0) at 4°C. Tube contents were heated at 90°C for 3 minutes and cyclic GMP formed was determined by radioimmunoassay as described earlier, using polyclonal anti-cyclic GMP antibody (7).

RESULTS AND DISCUSSION

In this study we employed activation of isolated guanylate cyclase as detector system for NO formation. Similar system has been utilized by Moncada et al (8) to study NOS. Cytosolic fraction from whole brain homogenate exhibited significant NOS activity and required both L-arginine and NADPH for NO formation as described earlier (1). In the presence of 100µM L-arginine the apparent Km for NADPH was observed to be approximately 2.5µM (Figure 1) consistent with published reports (21).

The addition of nitroblue tetrazolium (NBT) which interacts with superoxide ion and reduces it to formazan, led to the concentration - dependent inhibition of NOS activity with an apparent Ki of 3-4µM (Figure 2). The inhibitory of effect of NBT was compared with N^{ω}-methyl-L-arginine (NMA). As shown in Figure 3, inclusion

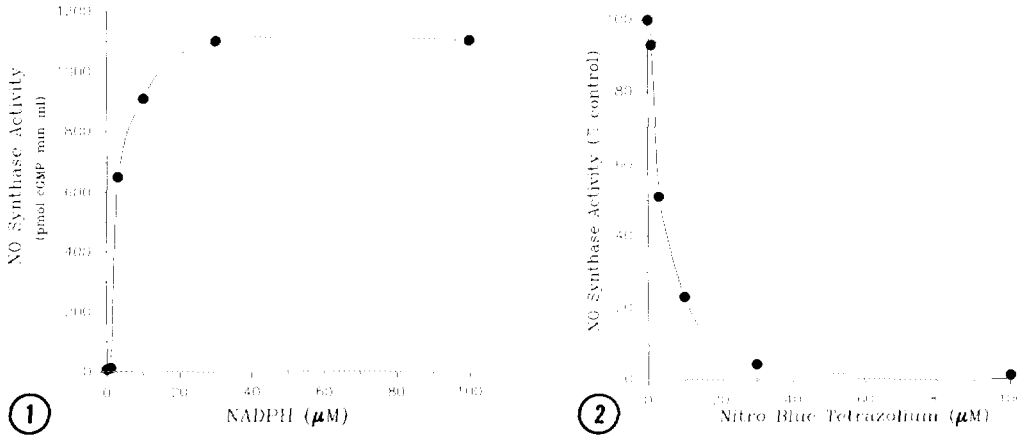

FIG. 1. Requirement of NADPH for nitric oxide formation from L-Arginine by NO sythase. Rat brain cytosolic fraction was incubated with different concentrations of NADPH in a reaction mixture containing 100μM L-arginine and other ingredients as described under "Materials and Methods" and cyclic GMP was determined by radioimmunoassay.

FIG. 2. Effect of nitroblue tetrazolium on NO synthase activity. Enzyme preparation was incubated with L-arginine (100μM) and NADPH (100μM) in the presence of different concentrations of NBT. NO synthase activity was determined by activation of guanylate cyclase in brain cytosol. The results were expressed as percentage of maximal stimulation of guanylate cyclase activation.

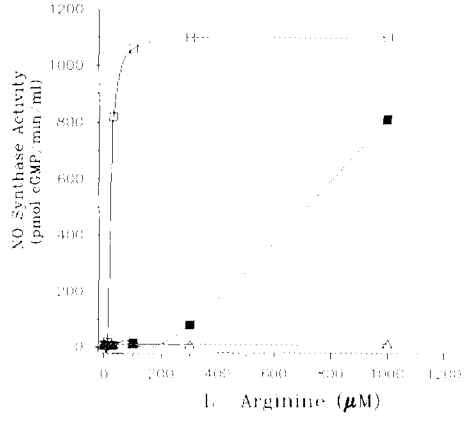

FIG. 3. Effect of substrate (L-arginine) on the inhibition of NO synthase by NMA and NBT. Brain cytosol was incubated with different concentrations of L-arginine (open squares) under standard assay conditions. Some tubes contained 100μM NMA (closed squares) or 100μM NBT (open triangles). Cyclic GMP formed was determined as described above.

of NMA or NBT at 100μM led to the complete inhibition of L-arginine-dependent stimulation of guanylate cyclase activity without any effect on the basal cyclic GMP production. While the inhibitory influence of NMA was overcome by increasing concentrations of L-arginine, inhibition by NBT was not reversed by the substrate under the experimental conditions employed (Figure 3). The apparent Ki for NMA was observed to be approximately 2μM (data not shown). Our results confirm earlier reports which have described competitive inhibition of NOS by NMA. These observations clearly revealed the distinction between the inhibitory mode of NMA and NBT whereby these agents act, and suggest the possibility of superoxide ion being involved in the catalytic conversion of L-arginine to NO. Also, the addition of 10nM hemoglobin inhibited the enzyme activity (data not shown), consistent with earlier reports by this and other investigators (6,17).

The role of superoxide ion was further explored by studying the effects of SOD on NOS activity. Addition of SOD did not affect the basal guanylate cyclase activity but enhanced the L-arginine-dependent stimulation approximately 50%, suggesting an increase in NO production by SOD (Figure 4). In contrast, the addition of bovine liver catalase, which removes H_2O_2 from the system, abolished the arginine effect on cyclic GMP formation. Catalase effect was attributed to its catalytic activity since heat-inactivated enzyme was ineffective in blocking arginine-dependent activation (Figure 4).

In order to characterize and distinguish the effects of SOD and catalase on NO formation by NO synthase versus NO interaction with guanylate cyclase, these two modulators were tested on sodium nitroprusside (NaP)-dependent activation of guanylate cyclase. Addition of NBT, SOD, or catalase did not modify either the basal or the NaP-stimulated cyclic GMP formation (Figure 5). We have earlier reported similar observations on effects of catalase on NaP response using guanylate cyclase preparations from bovine tracheal smooth muscle (9). The data obtained in figure 5 suggest that NBT, SOD or catalase are likely to modify generation of NO by NO synthase rather than prevent the activation of cytosolic guanylate cyclase.

Various reports in the literature attribute enhancement of NO formation by SOD to the removal of inhibitory actions of $O^{\cdot}_2$ on NO (6). This follows that removal of

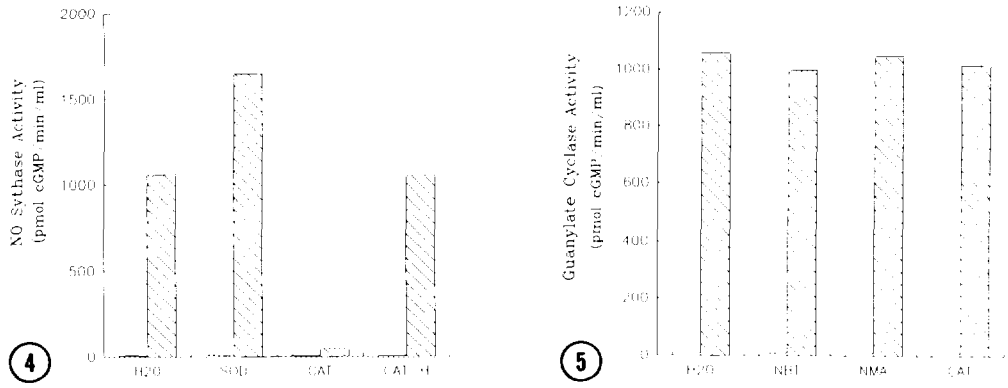

FIG. 4. Effect of SOD and catalase on NO synthase activity. Enzyme preparations were incubated with various modulators in the absence (open bars) and presence of 100μM L-arginine and 100μM NADPH (hatched bars) for 10 minutes at 37°C. Some tubes contained SOD (11 units) or bovine liver catalase (10μg) during incubation. NO synthase activity was determine as increase in cyclic GMP formation.

FIG. 5. Effect of NO synthase modulators on guanylate cyclase activation by nitrovasodilators. Guanylate cyclase activity was determined in the absence (open bars) and presence of 100μM sodium nitroprusside (hatched bars). Some tubes contained NO synthase modulators NBT (100μM), NMA (100μM) or bovine liver catalase (10μg).

$O\cdot^-_2$ should increase NO formation. However, to the contrary our data indicates that NBT which removes $O\cdot^-_2$, in fact, completely blunts the production of NO. This suggests a permissive role for $O\cdot^-_2$ in NO formation. Similar inhibitory effects of NBT on NO synthase have been observed by other investigators using citrulline measurement assays (3). Furthermore, the stimulation of NO production by SOD and blunting action of catalase suggest that both $O\cdot^-_2$ as well as H_2O_2 are required for NO formation by NO synthase. Similar results have been obtained using cytosolic fractions from N1E 115 neuroblastoma cells (data not shown). There are apparent similarities between the requirements for NO formation as observed here and our earlier reports on the SOD-dependent generation of hydroxyl radical which is able to stimulate guanylate cyclase activity (10, 11).

Recently work by Stuehr et al (12) and Leone et a (13) on the mechanism of arginine conversion to NO indicates that L-arginine is first oxidized to N^w-OH-L-arginine which is the first intermediate formed by NOS in the presence of molecular oxygen. This intermediate is then converted to citrulline and NO by

second oxidation step. It is likely that under the experimental conditions employed in our investigations there may be generation of hydroxyl (OH) radical, a process that is

$$O\cdot_2 \xrightarrow{SOD} H_2O_2 \quad .....(1)$$

$$H_2O_2 \xrightarrow{Catalase} H_2O + O_2 \quad .....(2)$$

$$O\cdot_2 + H_2O_2 \xrightarrow{Chelated/Metal} OH + OH + O_2 \quad .....(3)$$

known to require O_2^-, H_2O_2 and cheated iron (Reaction 3), as reported earlier (10, 16). The hydroxyl radical can potentially oxidize L-arginine to NO and citrulline. Alternatively, the hydrogen peroxide formed in the system (Reaction 1) could peroxidatively oxidize the guanidino nitrogen of L-arginine to NO much in the way hydroxylamine and sodium azide are converted to nitric oxide by peroxidases as reported by this and other investigators (14, 15). It is concluded from these studies that in the presence of NADPH and molecular oxygen NO synthase converts L-arginine to NO through a catalytic process which involves oxygen radicals and hydrogen peroxide.

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