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Effect of hydrogen peroxide and catalase on rat cerebellum nitric oxide synthase

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Abstract—The effect of H_2O_2 and catalase on isolated rat cerebellum nitric oxide (NO) synthase activity was determined by measuring the conversion of L-[^{3}H]arginine to L-[^{3}H]citrulline. H_2O_2 (1–5 mM) markedly increased NO synthase activity in the presence of endogenous catalase (72 ± 4 U/mL). This effect of H_2O_2 was further increased by exogenous catalase (200 U/mL). Exogenous catalase (0.1 to 1000 U/mL) by itself had no significant effect on NO synthase activity. Nitroblue tetrazolium chloride, an electron acceptor, inhibited NO synthase activity in a concentration-dependent manner. This study suggests that H_2O_2 is not directly involved in NO synthesis and that the H_2O_2/catalase stimulation of NO synthase activity may be due to the excess oxygen produced by the H_2O_2/catalase system.

Key words: nitric oxide; nitric oxide synthase; hydrogen peroxide/catalase

NO* is a novel cell messenger involved in a variety of important physiological processes including vascular relaxation, neuronal transmission and immune responses [1, 2]. NO is synthesized by the oxidation of a guanidino nitrogen atom of L-arginine by NO synthase [1, 2]. Several isoforms of NO synthase from various tissues have been characterized [1, 2]. Although the isoforms of NO synthase differ in molecular structure, cellular localization and cofactor requirement, each produces NO [1, 2]. NO synthase is a dioxygenase and produces both NO and citrulline in equimolar quantities [1]. In addition, NO synthase appears to have multiple catalytic functions such as the oxidation of NADPH [3] and the reduction of cytochrome *c* and synthetic dyes, including nitroblue tetrazolium and dichlorophenolindophenol [4, 5]. At suboptimal concentrations of arginine and tetrahydrobiopterin, NO synthase has been shown to produce H_2O_2 [6, 7] and superoxide [8]. Recently, Mittal [9] has shown that rat brain NO synthase activity is abolished by exogenous catalase, suggesting that H_2O_2 and superoxide are involved in NO synthesis. To determine the effect of H_2O_2 and catalase on NO production and the likelihood of a role for H_2O_2 in NO synthesis, we measured alterations in NO synthase activity in the presence and absence of H_2O_2 and exogenous catalase.

Materials and Methods

Preparation and assay of NO synthase. Rat cerebellum was homogenized in 5 vol. of buffer containing 50 mM HEPES (pH 7.4), 0.5 mM EDTA, 1 mM dithiothreitol, trypsin inhibitor (1 mg/100 mL) and 1 mM phenylmethylsulfonyl fluoride using a glass homogenizer and a Teflon pestle. The homogenate was centrifuged at 20,000 *g* for 20 min, and the supernatant was passed through a Dowex AG50W-X8 (Na^+ form) column to remove endogenous L-arginine. NO synthase activity was determined by measuring the conversion of L-[^{3}H]arginine to L-[^{3}H]citrulline as reported previously [10] with some modifications. The reaction mixture (200 μL) contained 50 mM Tris–HCl (pH 7.4), 100 μL enzyme extract, 0.81 mM calcium, 0.55 mM EDTA, 1 mM NADPH, 20 μM tetrahydrobiopterin and 1 or 50 μM L-[^{3}H]arginine at 37°. To stop the reaction, 50- μL aliquots of the reaction mixture were withdrawn at 10 min and mixed with 2 mL of ice-cold 20 mM 2-(*N*-morpholino) ethane sulfonate (pH 5.5) buffer 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 spectroscopy. Protein concentration of the enzyme extract was determined using the Biorad protein reagent with bovine serum albumin as the standard.

Determination of catalase activity. Catalase activity was determined as reported previously [11]. The reaction mixture (2 mL) contained 50 mM phosphate buffer (pH 7.4), 18 mM H_2O_2 and 20 μL enzyme. The reaction was followed by measuring the decrease in absorbance at 240 nm. One unit of enzyme activity is defined as the amount of enzyme required to oxidize 1 μmol of H_2O_2 in 1 min.

Results

The effect of H_2O_2 and exogenous catalase on NO synthase activity was determined by measuring the conversion of L-[^{3}H]arginine to L-[^{3}H]citrulline. H_2O_2 at concentrations ranging from 1 to 5 mM markedly increased NO synthase activity, determined in the presence of 50 μM arginine (Fig. 1). Addition of exogenous catalase (200 U/mL) increased the effect of H_2O_2 (1–5 mM) on NO synthase activity (Fig. 1). However, H_2O_2 at low concentrations (below 500 μM) produced no significant effect on NO synthase activity (data not shown). H_2O_2/exogenous catalase caused a relatively greater increase in NO synthase activity over control at limiting concentrations of arginine (1 μM), and exhibited a 60 ± 25, 71 ± 15 and 85 ± 28% increase at 1, 2, and 5 mM H_2O_2, respectively.

The effect of catalase alone on NO synthase activity was determined. The rat cerebellum homogenate used as the NO synthase enzyme source in this study was found to contain 144 ± 7 U of catalase/mL. Exogenous catalase in the absence of H_2O_2 over a wide range of concentrations (0.1 to 1000 U/mL) produced no significant effect on NO synthase activity (Fig. 2). At the same time, NBT, an electron acceptor, produced a concentration-dependent (1–50 μM) inhibition of NO synthase activity (Fig. 2).

Discussion

We investigated the effect of H_2O_2 and catalase on a constitutive rat cerebellum NO synthase. H_2O_2 in the presence of endogenous catalase produced a marked increase in NO synthase activity as measured by the conversion of L-[^{3}H]arginine to L-[^{3}H]citrulline. This effect of H_2O_2 on NO synthase activity was further increased by exogenous catalase. This suggests that oxygen produced by the H_2O_2/exogenous catalase system (Reaction 1) may

* Abbreviations: NO, nitric oxide; and NBT, nitroblue tetrazolium chloride.

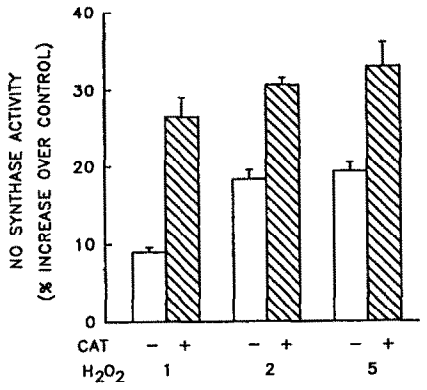

Fig. 1. Effect of H_2O_2 and exogenous catalase (CAT) on NO synthase activity. NO synthase activity was determined at 10 min in the presence or absence of H_2O_2 (1, 2, and 5 mM) with or without exogenous CAT (200 U/mL), as described in Materials and Methods. The control NO synthase activity was 1.2 nmol/mg protein. The data are means ± SEM of three experiments.

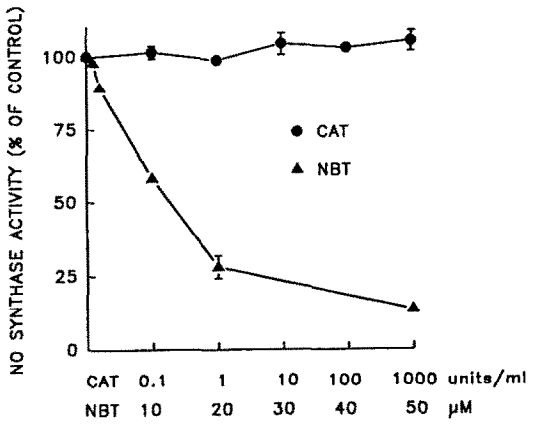

Fig. 2. Effect of exogenous catalase (CAT) and nitroblue tetrazolium chloride (NBT) on NO synthase activity. NO synthase activity was determined at 10 min in the presence or absence of exogenous CAT (0.1 to 1000 U/mL) or NBT (1 to 50 μM) as described in Materials and Methods. The control NO synthase activity was 1.18 nmol/mg protein. The data are means ± SEM of four experiments.

be responsible for the increase in NO synthase activity which utilizes

$$2H_2O_2 \xrightarrow{\text{catalase}} 2H_2O + O_2 \qquad \text{(Reaction 1)}$$

$$\text{L-arginine} + O_2 \xrightarrow{\text{NO synthase}} \text{L-citrulline} + NO \qquad \text{(Reaction 2)}$$

molecular oxygen as one of the substrates (Reaction 2). An increase in oxygen concentration in the reaction mixture is likely to increase NO synthase activity. Indeed, our previous study clearly demonstrated that high oxygen tension (450 ± 12 mm Hg) significantly increased the initial rate of NO synthase activity [12] and that of the enzyme activity at longer time intervals (15–60 min) [12, 13], suggesting that NO synthase activity is enhanced by excess oxygen in the reaction mixture. It is possible that H_2O_2 and hemeproteins oxidize N^{ω}-hydroxy-L-arginine, an intermediate in NO synthesis, and produce NO and citrulline as reported by Boucher *et al.* [14]. Although H_2O_2 and peroxidase caused a significant conversion of N^{ω}-hydroxy-L-arginine to nitrogen oxide and citrulline, H_2O_2 and catalase produced no significant effect [14], suggesting that the increased conversion of L-[^{3}H]arginine to L-[^{3}H]-citrulline observed in the presence of H_2O_2 and catalase in our study is not due to oxidation of N^{ω}-hydroxy-L-arginine.

The ability of NO synthase to produce different products at two different substrate concentrations has been reported [7, 8]. NO synthase catalyzes NO and citrulline production at saturating concentrations of arginine and tetrahydrobiopterin while forming H_2O_2 at limiting concentrations of arginine (below 1 μM) and tetrahydrobiopterin (below 1 nM) [7, 8]. The increase in NO synthase activity observed both at limiting and saturating concentrations of arginine suggests that H_2O_2 formed by NO synthase reaction may have a positive feedback effect on NO production.

A recent study reported that exogenous catalase abolished rat brain NO synthase activity as measured by NO-stimulated cyclic GMP accumulation, suggesting that H_2O_2 is involved in NO synthesis [9]. This conclusion is not supported by our study. In our study, exogenous catalase by itself did not alter NO synthase activity, whereas H_2O_2/exogenous catalase markedly increased the enzyme activity. The discrepancy between these two studies may be due to the difference in the measurement of NO synthase activity. NO synthase activity can be determined by measuring the conversion of L-[^{3}H]arginine to L-[^{3}H]-citrulline, a coproduct stoichiometrically formed in equimolar amounts of NO or by measuring cyclic GMP accumulation caused by NO activation of guanylyl cyclase. In our study, the direct coproduct, L-[^{3}H]citrulline, was measured instead of the indirect product, cyclic GMP. Our observation is also in agreement with a previous study by Stuehr and Griffith [15], showing that catalase does not decrease NO synthesis relative to controls. Although exogenous catalase had no significant effect on NO synthase activity, NBT was effective in inhibiting the enzyme activity, as reported previously [4, 9]. NBT inhibition of NO synthase activity may be due to its ability to compete with molecular oxygen for NADPH-derived electrons [5].

In conclusion, our study demonstrated that H_2O_2 is not directly involved in NO synthesis. This study shows that the H_2O_2/catalase system markedly increased NO synthase activity, suggesting an involvement of excess oxygen produced by the H_2O_2/catalase system.

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