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The effects of oxygen radicals on the activity of nitric oxide synthase and guanylate cyclase

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Abbreviations: NOS, nitric oxide synthase; GC, guanylate cyclase; NBT, nitroblue tetrazolium; MCO, metal-catalyzed oxidation; ABAP, 2'2'-azobis- amidinopropane; SNP, sodium nitroprusside; NAME, L-nitroarginine methyl ester; X/XO, xanthine/xanthine oxidase

Abstract

Reactive oxygen species such as superoxides, hydrogen peroxide (H_2O_2) and hydroxyl radicals have been suggested to be involved in the catalytic action of nitric oxide synthase (NOS) to produce NO from L-arginine. An examination was conducted on the effects of oxygen radical scavengers and oxygen radical-generating systems on the activity of neuronal NOS and guanylate cyclase (GC) in rat brains and NOS from the activated murine macrophage cell line J774. Catalase and superoxide dismutase (SOD) showed no significant effects on NOS or GC activity. Nitroblue tetrazolium (NBT, known as a superoxide radical scavenger) and peroxidase (POD) inhibited NOS, but their inhibitory actions were removed by increasing the concentration of arginine or NADPH respectively, in the reaction mixture. NOS and NO-dependent GC were inactivated by ascorbate/$FeSO_4$ (a metal-catalyzed oxidation system), 2'2'-azobis-amidinopropane (a peroxy radical producer), and xanthine/xanthine oxidase (a superoxide generating system). The effects of oxygen radicals or antioxidants on the two isoforms of NOS were almost similar. However, H_2O_2 activated GC in a dose-dependent manner from 100 μM to 1 mM without significant effects on NOS. H_2O_2-induced GC activation was blocked by catalase. These results suggested that oxygen radicals inhibited NOS and GC, but H_2O_2 could activate GC directly.

Keywords: Oxygen radicals, hydrogen peroxide, NO synthase, guanylate cyclase

Introduction

Oxygen radicals are the reactive oxygen deriviatives with unpaired electrons generated from an oxygen molecule (O_2). Molecular oxygen is reduced to a superoxide radical ($\cdot O_2^-$) and this radical is readily converted to hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD). H_2O_2 is also reduced to a hydroxyl radical ($\cdot OH$) in the presence of a transient metal ion. These reactive oxygen species are considered as endogenous toxicants which cause acute cell demage in a variety of pathological conditions such as ischemia-reperfusion injury, cancer and the aging process (Knight, 1995). However, it has been suggested that oxygen radicals may act as modulators in signal trans-duction (Landle, 1997). Attenuation of coronary artery dilatation by SOD suggested that superoxides may be involved in the control of blood pressure (Burke *et al*., 1991). While hydrogen peroxide also causes dilatation of the cerebral and pulmonary arteries (Ignarro *et al*., 1987, Bredt and Snyder 1989). In addition, reactive oxygens were generated in the processing of signal transduction of growth factors and hormones (Bae *et al*., 1997).

NO, a novel messenger, is involved in a variety of important physiological processes including vascular relaxation, neuronal transmission, immune responses and hemostasis (Palmer *et al*., 1987; Bredt and Snyder, 1989; Kwon *et al*., 1989). NO synthase enzymes are divided into two major isoforms. The constitutive NOS occuring in brain and endothelial cells has bound FAD, FMN and requires Ca^{2+}, calmodulin, NADPH and tetra-hydrobiopterin as cofactors (Mayer *et al*., 1991). The other enzyme is an inducible form found in several cell types including macrophages and vascular smooth muscle in response to cytokines and bacterial toxins. The inducible NOS is different from the constitutive form by a Ca^{2+}/calmodulin-independency on enzyme activity (Stuehr *et al*., 1989).

Mittal (1993) reported that exogenous catalase reduced NOS activity suggesting that hydrogen peroxide and superoxide radical are involved in enhancing NOS activity with the following reasons as the basis of his

proposal. First, NOS catalyzes not only NO formation but also accelerates various oxido-reduction reactions. As NADPH diaphorase NOS has been known to reduced nitroblue tetrazolium (Mayer *et al*., 1991). Secondly, purified NOS can directly catalyze the generation of oxygen radicals at suboptimal concentrations of arginine and tetrahydropterin. NOS from rats brains has been shown to mimic the activity of Ca^{2+}/calmodulin-dependent NADPH oxidase in the production of hydrogen peroxide and superoxide (Heinzel *et al*., 1992). Finally, in the process of arginine conversion to NO, L-arginine is oxidized to N-OH-L-arginine, the first intermediate in the presence of molecular oxygen (Stuehr *et al*., 1991). To the contrary, many reports also (Liu *et al*., 1994; Miyamoto *et al*., 1996) suggested that oxygen radicals could inhibit GC by scavenging NO to produce peroxynitrite.

The present study was undertaken to clarify the effects of various oxygen radicals on the NOS and GC activity with oxygen radical scavengers and oxygen radical-generating systems.

Materials and Methods

Chemicals

L-Arginine, 2'2'-azobis-amidinopropane (ABAP), NADPH, calmodulin, sodium nitroprusside (SNP), L-nitroarginine methyl ester (NAME), creatine phosphate, theophylline, GTP, lipopolysaccharide (LPS), interferon-γ (INF-γ) and creatine phosphokinase were purchased from Sigma (USA). L-[2,3,4-^{3}H]-arginine-HCl and cGMP assay kits were sourced from Amersham (UK), and fetal bovine serum (FBS) and RPMI medium were from Gibco-BRL (USA). All other reagents were of analytical grade.

Preparation of enzyme extracts

Sprague-Dawley rats (150-200 g) were killed by decapitation. Whole brains were quickly removed, cleaned and immersed in ice-cold 0.25 M sucrose. The brains were homogenized in 6 volumes (w/v) of 0.25 M sucrose containing 20 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1 mM dithiothreitol (DTT), phenylmethylsulfonyl fluoride (PMSF, 100 μg/ml), leupeptin (10 μg/ml), pepstatin (10 μg/ml) and soybean trypsin inhibitor (10 μg/ml) at 4˚C using a glass homogenizer with a Teflon pestle. To examine the metal-catalyzed oxidation (MCO) effects on the enzyme activity, EDTA was excluded from the standard homogeni-zing solution. The homogenate was centrifuged at 18,000 *g* for 15 min. The supernatant was stored at -70˚C and used as source for NOS and GC.

Activation of J774 cells by LPS and INF-γ

J774 cells were grown in RPMI 1640 supplemented with 10% FBS, penicillin (100 U/ml) and streptomycin (100 μg/ml) as described by Cunha *et al.* (1993). Cells at a density of 10^5 cells/ml were treated with LPS (10 μg/ml) and INF-γ (100 U/ml) for activation. After incubation for 12 h, cells were harvested in 0.25 M sucrose containing 20 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1 mM DTT, PMSF (100 μg/ml), leupeptin (10 μg/ml), pepstatin (10 μg/ml) and soybean trypsin inhibitor (10 μg/ml) at a cell count of 10^7/ml. After sonication for 20 sec, a supernatant was acquired for iNOS enzyme solution by centrifugation at 18,000 *g* for 15 min.

Determination of NOS activity

NOS activity was determined by the conversion of L-[^{3}H] arginine to L-[^{3}H]citrulline according to Bredt and Snyder (1989). The reaction mixture contained 50 mM Tris-HCl (pH 7.6), 2 mM calcium chloride, 20 μM NADPH, 20 μM L-arginine, approximately 150,000 cpm of L-[2,3,4-^{3}H] arginine-HCl (0.2 μCi) and 100 μg of enzyme protein in a final incubation volume of 100 μl. For the iNOS activity assay, FAD (10 μM) and (6R)-5,6,7,8-tetrahydrobiopterin (0.1 μM) were added and calcium chloride was removed from the reaction mixture. Enzyme reactions were carried out at 37˚C for 8 min and terminated by the addition of 400 μl of Stop Buffer (20 mM sodium acetate, pH 5.5, 2 mM EGTA and 1 mM L-citrulline). The reaction mixture (0.5 ml) generated, was applied over 1 ml of Dowex AG (50W-X8, Na^+ form, 100-200 mesh) which was preequilibrated with Stop Buffer. Columnns were eluted with additional 2 ml of water (4 fractions of 0.5 ml each). To each vial, 2 ml of a scintillation mixture was added prior to assessment in a liquid scintillation counter.

Assay of GC activity

GC activity in brain cytosol was determined according to Mittal (1993). The standard reaction mixture (100 μl) contained 50 mM Tris-HCl (pH 7.6), 15 mM creatine phosphate, 20 μg of creaine phosphokinase (120 units/mg protein), 8 mM theophilline, 4 mM $MgCl_2$ and 100 μg brain cytosolic protein. For activation of GC, NADPH (100 μM) and arginine (100 μM) with or without an NO donor (sodium nitroprusside) was added. Reactions were initiated by the addition of 1 mM GTP and continued for 10 min at 37˚C before termination by the addition of 50 mM of sodium acetate (pH 4.0) at 4˚C. Tube contents were heated in boiling water for 3 min for deproteination and cyclic GMP formed was determined using a radio-immunoassay kit (Amersham Life Sci., U.K.).

Results

Effects of antioxidants and oxygen radicals on NOS

To investigate the involvement of antioxidants and oxygen radicals on NOS, nNOS in rat brains and iNOS of

murine macrophage J774 cell lines were employed. SOD and catalase did not significantly change NOS activity, but POD and NBT inhibited both nNOS and iNOS activity (Figure 1a, b). POD and NBT inhibited NOS activity in a dose-dependent manner (data not shown). NBT was known to interact with superoxide ions and reduce them to formazan (Mittal, 1993). As shown in Figure 2, the inhibitory action of POD or NBT was removed by increasing the concentration of NADPH and arginine, respectively. Oxygen radical generating systems also inhibited NOS to a different degree (Figure 3). Xanthine/xanthine oxidase (X/XO, a superoxide ion generating system) inhibited NOS activity by 90% and MCO and ABAP (a temperature-dependent peroxy radical generator) inhibited it by 33% and 47%, respectively. However, hydrogen peroxide did not significantly alter NOS activity. The effects of antioxidants and oxygen radicals on iNOS were almost similar as to those on nNOS.

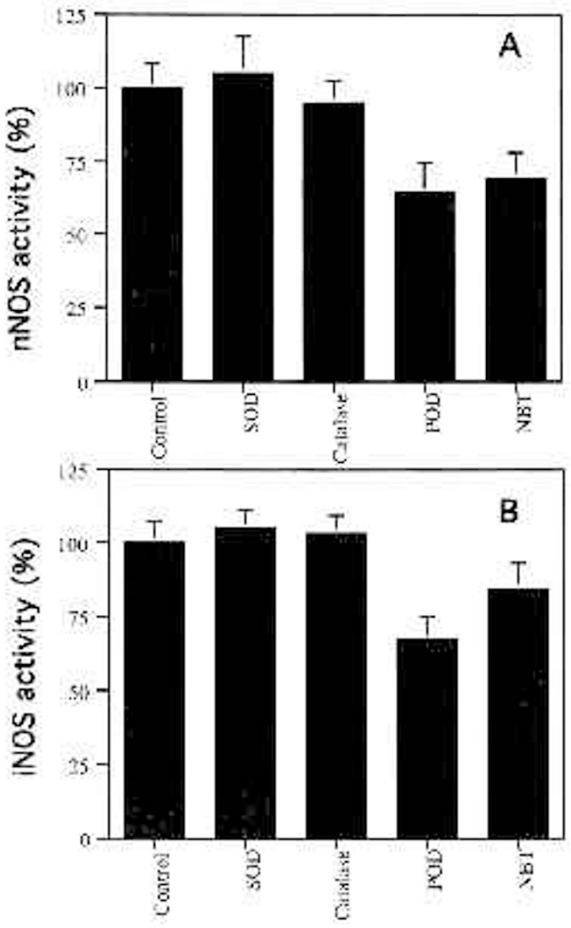

Figure 1. Effects of oxygen radical scavengers on nNOS (A) and iNOS (B) activities. Enzyme preparation (100 μg) was incubated with 20 μM NADPH, 20 mM L-arginine, [^{3}H]arginine (150,000 cpm) and various oxygen radical scavengers in a reaction mixture for 10 min at 37°C as described under 'Materials and Methods'. The [^{3}H]citrulline formation was determined for NOS activity. The oxygen radical scavenging systems were as follows; SOD (100 U), catalase (2,000 U), POD (30 U) and NBT (100 μM). The control neuronal and inducible NOS activity was 0.14 and 0.37 nmole/mg/min, repectively. Results are presented as the means ± SD of triplicate experiments.

Effects of antioxidants and oxygen radicals on GC

Rat brain GC was activated either by NOS reactions or SNP, a NO donor (data not shown). In the NOS-dependent activation of GC, POD inhibited GC activity by 15% and this inhibitory action was blocked by increasing the concentration of NADPH. MCO, ABAP and X/XO systems inhibited NOS-mediated GC activation by 13%, 27% and 57%, respectively (Figure 4). GC activated by SNP was inhibited by MCO and X/XO systems by 15% and 37%, respectively. In these experimental conditions, 100 μM LAME reduced NOS-dependent GC activation to less than 5%, but POD or ABAP did not inhibit SNP-mediated GC activity (Figure 5).

Activation of GC by oxygen radicals

Figure 6 shows that hydrogen peroxide activates rat brain GC in a dose dependent manner, albeit at a supraphysiologic concentration without NO generation systems. To prevent NO formation, NOS was blocked by the

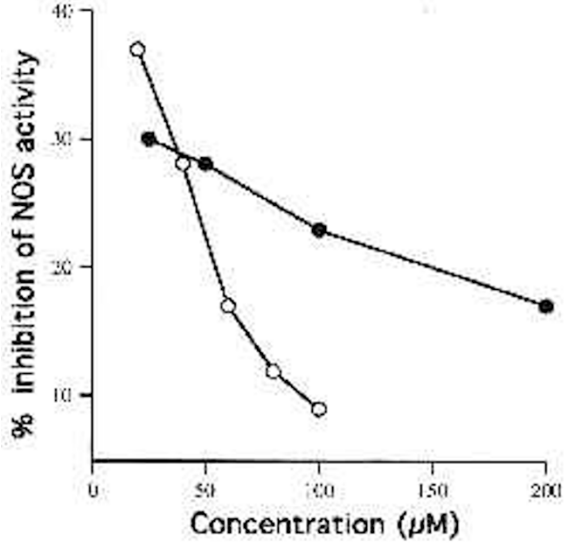

Figure 2. Effects of NADPH and L-arginine on POD (○ — ○)- and NBT (● — ●)-induced NOS inactivation, respectively. Rat brain cytosolic fraction (100 μg) was incubated with 30 U of POD and 100 μM NBT with various concentrations of NADPH and L-arginine in a reaction mixture as described under 'Materials and Methods' and [^{3}H]citrulline formation was determined for NOS activity as Figure 1. Values are means of duplicate experiments.

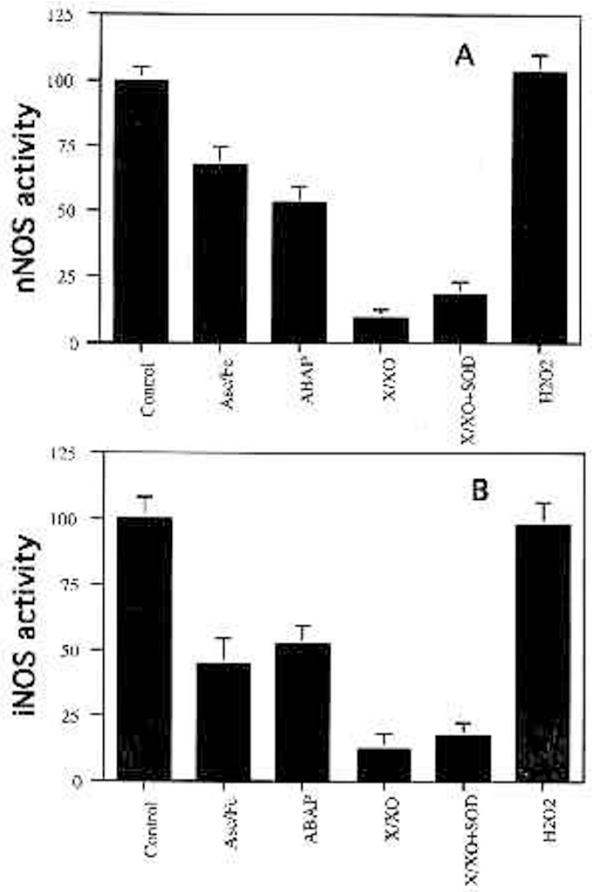

Figure 3. Effects of reactive oxygen radicals on nNOS (A) and iNOS (B) activities. Enzyme preparation (100 μg) was incubated with various oxygen radical generating systems in a reaction mixture as described under 'Materials and Methods' and [^{3}H]citrulline formation was determined for NOS activity as Figure 1. The oxygen radical generation systems are as follows; Asc/Fe (1 mM ascorbate/10 μM Fe), ABAP (5 mM), xanthine/xanthine oxidase (X/XO, 1 mM/5 × 10^{-3} U) with or without SOD (100 U), and H_2O_2 (100 μM). Results are presented as the means ± SD of triplicate experiments.

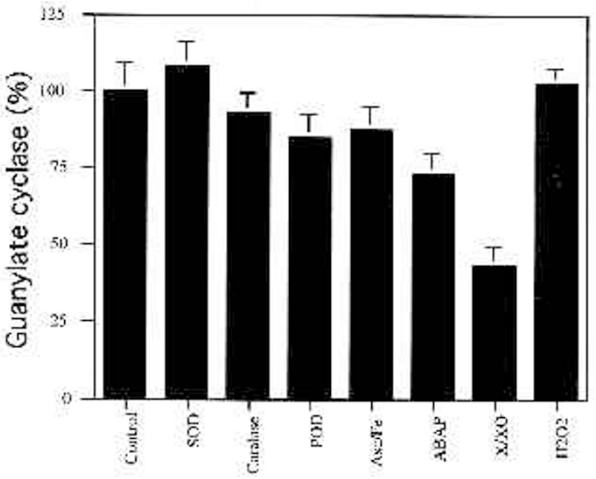

Figure 4. Effects of oxygen radicals and oxygen radical scavengers on NO-mediated GC activation. Rat brain cytosolic fraction (100 μg) was incubated with various oxygen radical generation systems and antioxidant systems in a reaction mixture containing 20 μM L-arginine, 20 μM NADPH, 15 mM creatine phosphatase, 20 μg/ml creatine phosphokinase (120 U/mg protein), 4 mM theophylline, 4 mM $MgCl_2$, and 1 mM GTP for GC as described under 'Materials and Methods' for 10 min at 37°C. The oxygen radical scaverngers and generation systems are as follow; SOD (100 U), catalase (2,000 U), POD (30 U), Asc/Fe (1 mM ascorbate/10 μM Fe), ABAP (5 mM), xanthine/xanthine oxidase (X/XO, 1 mM/5 × 10^{-3} U) and H_2O_2 (100 μM). The cGMP formed was measured for GC activity by radioimmunoassay. Results are presented as the means ± SD of triplicate experiments.

addition of L-NAME, and activation of GC by hydrogen peroxide was prevented by catalase. Oxygen radicals produced by MCO also activated GC, but to a lesser degree than hydrogen peroxide (data not shown).

Discussion

This study investigated the effects of antioxidants and oxygen radicals on NOS and GC activity. Some reports (Burke *et al.,* 1991; Mittal, 1993) suggested that antioxidants could activate NOS, however, they employed the activation of isolated GC or the relaxation of smooth

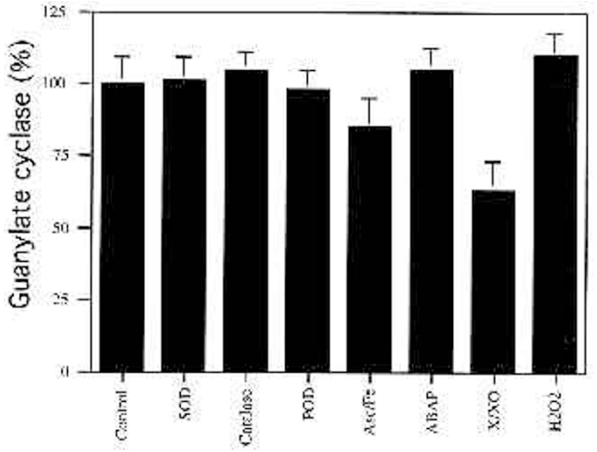

Figure 5. Effects of oxygen radicals and oxygen radical scavengers on SNP-mediated GC activation. Rat brain cytosolic fraction (100 μg) was incubated with various oxygen radical generation systems and antioxidant systems in a reaction mixture containing 100 μM SNP, 100 μM L-NAME, 15 mM creatine phosphatase, 2.4 U creatine phosphokinase, 4 mM theophylline, 4 mM $MgCl_2$, and 1 mM GTP for GC as described under 'Materials and Methods' for 10 min at 37°C. The oxygen radical scavengers and generation systems are as follow; SOD (100 U), bovine liver catalase (2,000 U), POD (30 U), Asc/Fe (1 mM ascorbate/10 μM Fe), ABAP (5 mM), xanthine/xanthine oxidase (X/XO, 1 mM/5 × 10^{-3}U) and H_2O_2 (100 μM). The cGMP formed was measured as described in Figure 4. Results are presented as the means ± SD of triplicate experiments.

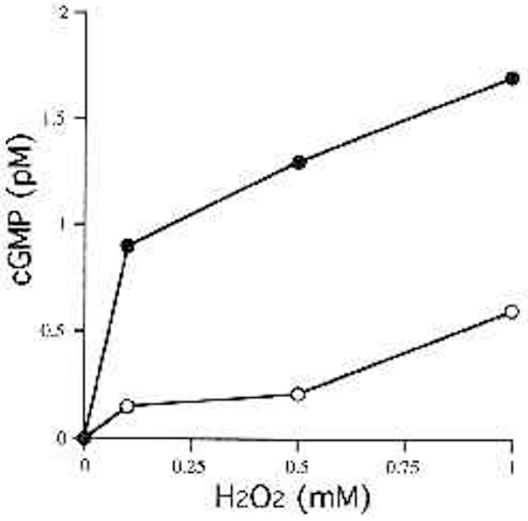

Figure 6. Effects of catalase on H_2O_2-mediated GC activation. Rat brain cytosolic fraction (100 μg) was incubated with various concentrations of H_2O_2 with (○ — ○) or without (● — ●) catalase (2,000 U) and in a reaction mixture containing 100 μM L-NAME, 15 mM creatine phosphatase, 2.4 U creatine phosphokinase, 4 mM theophylline, 4 mM $MgCl_2$, and 1 mM GTP for GC as described under 'Materials and Methods'. The cGMP formed was measured as described in Figure 4. Values are means of duplicate experiments.

muscle as detector systems for NO formation. Therefore, there are some controversies that the direct effects of antioxidant or oxygen radicals on GC or on muscle relaxation rather than on NOS should have been studied. In this study, we detected L-[^{3}H]citrulline from L-[^{3}H] arginine for NOS activity. The amount of formed citrulline is not affected, whereas that of produced NO or NO-mediated GC activation could be affected by oxidants or antioxidant. Superoxide radicals are known to convert NO to peroxy-nitrite (Huie *et al*., 1993). GC activity could be blocked by superoxide or hydroxyl radicals and enhanced by hydrogen peroxide as shown in this study. Exogenous SOD and catalase did not alter NOS activity, but horse-raddish peroxidase (POD) and NBT inhibited NOS activity significantly. The inhibitory effects of POD and NBT were attenuated by increasing the concentration of NADPH and arginine, respectively. POD accepts electrons from non-specific electron donors to dissipate hydrogen peroxide, so it could consume NADPH in the NOS reaction mixture, resulting in decreased NOS activity. The effects of NBT on NOS can be explained by the NADPH diaphorase acitivity of NOS. NBT, the substrate for the NADPH diaphorase reaction (Hope *et al*., 1991), could be able to compete with L-arginine, substrate for NOS. So the NBT in the reaction could act as a substrate competitor rather than as a superoxide scavenger.

Oxygen radicals produced by the MCO system included ABAP and X/XO inhibited NOS. It is well-known that NOS is highly unstable and oxygen radicals can inactivate it. When stored at 0˚C, 50% of the enzyme activity in purified NOS is lost in 2 h, whereas the crude enzyme extracts lose 50% of its activity in 2 days. In this study, the data of non protection of the X/XO induced-inactivation of NOS by SOD suggests that other factors may be involved in inactivation other than superoxides. Molecular oxygen as one of the substrate of NOS may be responsible for enzyme activity rate. According to Rengasamy and Jones (1993), partial oxygen pressure at lower than 35 mmHg decreases NOS activity. Taken together, oxygen radical generation systems could inhibit NOS either by oxidation of NOS or consumtion of molecular oxygen in the reaction.

The effects of antioxidants or oxygen radicals on NOS-mediated GC activity showed similar patterns to those on NOS but with less effects. These results indicate that the change in GC activity results from an alteration of NOS activity. The possibility that oxygen radicals could convert NO to peroxynitrite or other reactive nitrogen species before activating GC could not be ruled out. The inhibition of SNP-mediated GC activation by MCO or X/ XO (Figure 5) supports this assumption.

Mittal (1993) suggested that hydrogen peroxide is involved in enzyme activation by NOS, by showing that exogenous catalase abolished and SOD increased cGMP accumulation in the NOS reaction mixture of rat brains. In this system, exogenous catalase and SOD by themselves did not alter NOS activity. This discrepancy between the two studies may be due to different measuring systems, as mentioned above. Rengasamy and Jones (1994) insisted that exogenous hydrogen peroxide with catalase increased NOS activity by increasing O_2 production without direct effects on NOS itself. In our study, hydrogen peroxide increased GC activity itself without association with NO. The results are consistent with earlier data (Burke *et al*., 1991; Ambrosio *et al*., 1994) which showed that oxygen radicals could increase cGMP and induced vasodilatation, suggesting that reactive oxygen species, especially hydrogen peroxide, might act as second messenger *in vivo*.

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