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Nitric Oxide Releasing Reagent, S-Nitroso-N-Acetylpenicillamine, Enhances the Expression of Manganese Superoxide Dismutase mRNA in Rat Vascular Smooth Muscle Cells

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Abstract Cultured rat vascular smooth muscle cells (VSMCs) produce nitric oxide (NO) under stimulation by lipopolysaccharide (LPS) and interferon-γ (IFN-γ). NO synthase (NOS) and manganese superoxide dismutase (Mn-SOD) mRNA expressions are simultaneously induced by these stimulants in rat VSMCs. In VSMCs, S-nitroso-N-acetyl penicillamine (SNAP), one of the NO releasing reagents, induces Mn-SOD mRNA which may protect the VSMCs themselves. This suggests that NO itself may enhance the expression of Mn-SOD to protect the VSMC themselves against NO radicals in cultured rat VSMCs.

Key words: nitric oxide, SNAP, Mn-SOD, inducible NOS, vascular smooth muscle cells

INTRODUCTION

Endothelium derived relaxing factor (EDRF) has been identified as nitric oxide (NO) [Moncada et al., 1991]. NO is synthesized from the guanidino nitrogen atom of L-arginine by the catalytic action of NO synthases (NOS) [Moncada et al., 1991]. At least two types of NOS exist; one is constitutive NOS (cNOS), which is Ca^{2+}/calmodulin-dependent and mainly present in brain [Knowles et al., 1989] and endothelial cells [Mayer et al., 1989]; another is inducible NOS (iNOS), which is Ca^{2+}/calmodulin-independent and mainly present in macrophages [Hibbs et al., 1988] and vascular smooth muscle cells (VSMCs) [Busse et al., 1990]. Recently, two isoforms of cNOS have been cloned from rat brain [Bredt et al., 1991] and endothelial cell [Janssens et al., 1992; Sessa et al., 1992]. Moreover, iNOS has been cloned from the activated murine macrophage cell line, RAW264.7 [Xie et al., 1992].

NO has physiological roles in the regulation of blood pressure as vasodilator, neurotransmitter of neural cells and as described above, but at the same time it has been suggested to have pathological roles in endotoxin-induced shock, various ischemic diseases, viral infections, inhibition of cell proliferation, and induction of apoptosis [Albina et al., 1993]. In these pathological roles of NO, it has been suggested that the interactions among NO, O_2 radicals and superoxide dismutases (SODs) might play sensitive modulation. The amount of NO is regulated by NOS activity, while that of O_2 radical is regulated by the SOD activity. Manganese-superoxide dismutase (Mn-SOD) is one of the SODs mainly present in mitochondria. It contains mangane and dismutes O_2 radicals generated from mitochondria. Mn-SOD is always expressed in cells which contain mitochondria, and its transcription is enhanced by several cytokines or lipopolysuccharide [Fujii et al., 1991]. In the present study, we investigated the interactions between the induction of iNOS and that of Mn-SOD in cultured rat vascular smooth muscle cells (VSMCs).

MATERIALS AND METHODS

Animals

Male Jcl:Wistar rats obtained from the Chubu Science Company (Nagoya, Japan) were provided with food and water ad libitum and kept

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on a 12-h light-dark cycle in a temperature- and humidity-controlled environment at the Institute for Laboratory Animal Research (Nagoya University School of Medicine, Nagoya, Japan).

Reagents

LPS from *Escherichia coli* was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Murine interferon-γ (IFN-γ) the kind gift of Shionogi Pharmaceutical Co. (Osaka, Japan). S-nitroso-N-acetyl penicillamine (SNAP) and L-N^G-nitroarginine methyl ester HCL (LNAME) were purchased from Biomol Research Lab. Inc. (Plymouth Meeting, PA, USA).

Cell Culture and Incubation

Rat VSMCs were prepared by the standard explant methods [Hirata et al., 1984]. Briefly, aorta were removed aseptically and placed in phosphate-buffered saline (PBS). The cells were resuspended in Dulbecco's modified Eagle's medium (DMEM, Nissui Pharmaceutical Co., Tokyo, Japan) containing 10% fetal calf serum and antibiotics at 37°C in a humidified atmosphere of 95% air/5% CO_2. Cells were passaged once a week by harvesting with 0.25% trypsin-0.1% EDTA and seeded into 10-cm culture dishes (Falcon Labware, Oxnard, CA). Cells used in the experiments were between passages 5 and 10 after reaching confluence at 7–10 days. They were positively identified as smooth muscle cells by the "hills-and-valleys" growth characteristics and indirect immunofluorescent staining for α-actin, using mouse anti-smooth muscle α-actin antibody (Sigma Chemical Co.) and anti-mouse IgG FITC conjugate (Sigma Chemical Co.).

To study the production of nitrite and nitrate, confluent VSMCs (10^5 cells) in 96-well plates were usually incubated with or without compounds for 48 h. After incubation, media were removed for the nitrite assay.

Nitrite Assay

Nitrite in the culture supernatants was measured by the methods of Ding et al. [1988]. Briefly, 50 μl of supernatants was removed from the 96-well plate and incubated in triplicate with an equal volume of Griess reagent (1% sulfanilamide/0.1% naphthylethylene diamine dihydrochloride/2.5% H_3PO_4) at room temperature for 10 min. The absorbance at 550 nm was determined with a microplate reader. NO concentration was determined using sodium nitrite as a standard, and 0.5 mM LNAME was used as NO competitive inhibitor.

Preparation of cDNA Probes for iNOS and Mn-SOD

Expressions of iNOS and Mn-SOD mRNA were detected by Northern blot analysis. iNOS cDNA probe for Northern blot analysis was donated from Dr. Lowenstein [Lowenstein et al., 1993]. Mn-SOD cDNA probes were prepared by RT-PCR according to the published sequences [Ho et al., 1987].

RNA Preparation and Northern Blot Analysis

Confluent VSMCs in 10-cm culture dishes, preincubated in serum-free DMEM for 12 h, were incubated in 10 ml DMEM containing 10% FCS with or without compounds for indicated times. Total cellular RNA was isolated by the guanidium thiocyanate-phenol-chloroform methods described in the literature [Chomczyski et al., 1987]. For Northern blotting, 20 μg of each RNA preparation was electrophoresed on 1% agarose gels containing 1.1 M formaldehyde. The RNA was transferred to Hybond-N nylon membranes (Amersham). After UV cross-linking, mRNA was detected by ^{32}P-labelled probe (Multiple labeling system, Amersham) by hybridization for 12 h at 42°C in 5 × SSC (1 × SSC was composed of 0.15 M NaCl and 15 mM sodium citrate), 50% formamide, 0.02% sodium dodecyl sulphate (SDS), 0.1% N-lauroylsarcosine, 2% (W/V) solution blocking reagent for nucleic acid hybridization (Boehringer Mannheim, GmbH), 20 mM sodium maleate, and radioactive probe, pH 7.5. Membranes were washed twice for 15 min at 65°C in a solution containing 2 × SSC and 0.1% SDS, then twice in 1 × SSC with 0.1% SDS for 30 min at 65°C, and finally twice for 15 min in 0.1 × SSC with 0.1% SDS at 65°C. The membranes were then autoradiographed at −80°C using Fuji RX film. To verify the relative amount of total RNA, membranes were hybridized with a ^{32}P-labelled β-actin probe. Radioactivity of each band of the hybridized membrane was measured using the image analyzer system (BAS 2000, Fuji, Japan).

MTT Assay

The viability of cells treated with S-nitroso-acetyl penicillamine (SNAP) was examined by MTT assay as described by Mosmann et al.

[Mosmann, 1983]. Briefly, exponentially growing cells were plated in 96-well flat-bottomed microplate at a density of 5×10^4 cells per well and cultured with or without 100 μM SNAP. After indicated times of culture, 50 μl of MTT stock solution (2 mg/ml) was added to each well of the plate, which was incubated for 4 h at 37°C. The formazan crystals generated in viable cells were solubilized in DMSO (200 μl per well) and mixed by a microplate mixer; the OD_{540} was measured, and the inhibition index was calculated by the following formula: Inhibition index = (1 − OD_{540} of treated cells/OD_{540} of control cells).

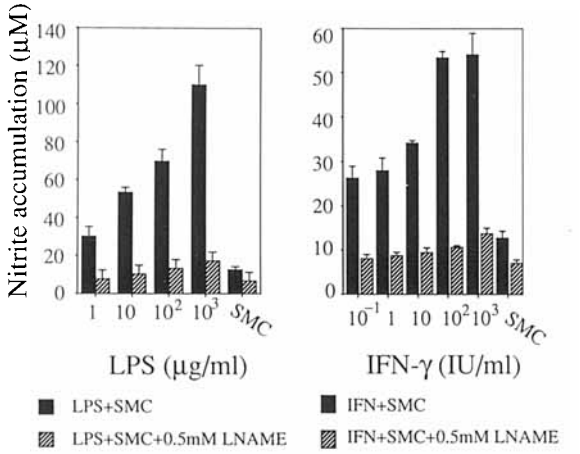


Fig. 1. NO production from 10^5 VSMCs treated with 10^2 μg/ml LPS (**A**) and murine 10^2 IU/ml IFN-γ (**B**) for 48-h cultivations.

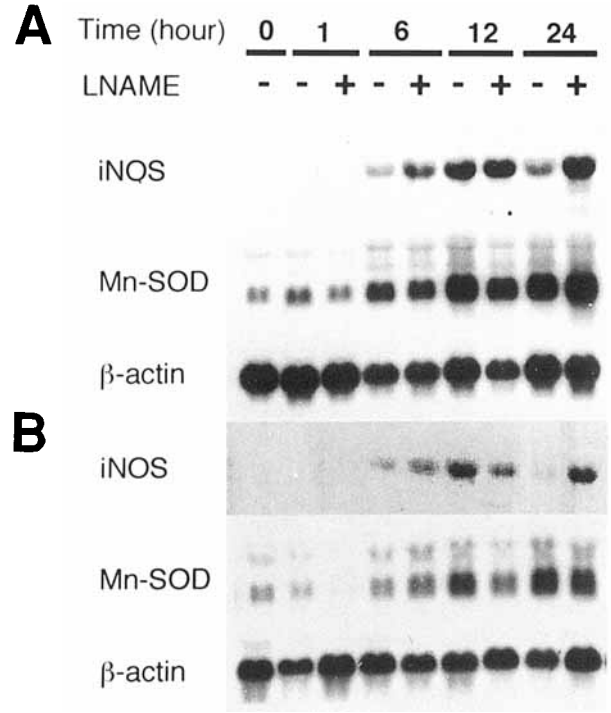
RESULTS

Production of Nitric Oxide From VSMCs Treated With LPS or IFN-γ

As shown in Figure 1, LPS or IFN-γ induced NO production in rat VSMCs in a dose-dependent manner for 48-h cocultivation. The NO production was inhibited by the addition of 0.5 mM LNAME.

Expression of iNOS mRNA and Mn-SOD mRNA in VSMCs Treated With LPS or IFN-γ

As shown in Figures 2A–D, expression of iNOS mRNA was increased in rat VSMCs treated with 10^2 μg/ml LPS or 10^2 IU/ml IFN-γ. iNOS mRNA was expressed from 6- to 24-h cultivation. The expression of iNOS mRNA was increased in VSMCs treated with LPS in a time-dependent



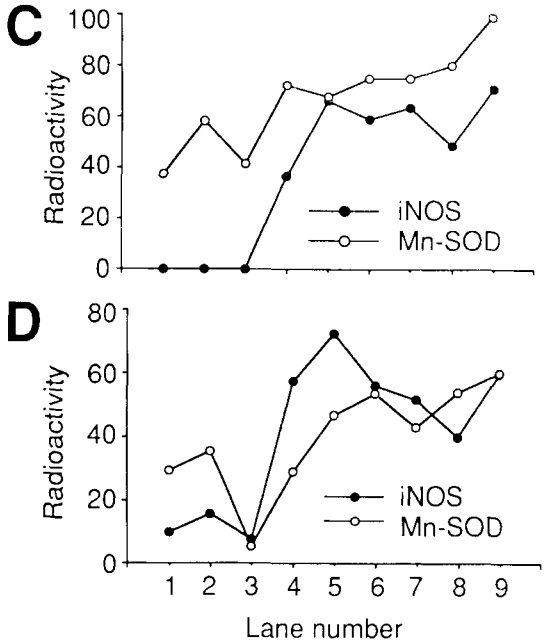


Fig. 2. Effects of LPS and murine IFN-γ on iNOS and Mn-SOD mRNA expressions in VSMCs. Expression of mRNA for iNOS or Mn-SOD was analyzed by Northern blot hybridization. **A:** Cells treated with 10^2 μg/ml LPS for various periods. **B:** Cells treated with 10^2 IU/ml murine IFN-γ for various periods. **C, D:** Radioactivities of iNOS, Mn-SOD, and β-actin bands of the hybridized membranes shown in A and B, respectively.

manner. The expression of iNOS mRNA treated with LNAME tended to increase compared to that without LNAME. In addition, the expression of Mn-SOD mRNA was also increased in the same RNA samples from VSMCs treated with LPS or IFN-γ in a time-dependent manner. The expression of Mn-SOD mRNA treated with LNAME tended to decrease compared to that without LNAME.

Expression of Mn-SOD mRNA in VSMCs Treated With SNAP

Mn-SOD expression has been shown to be increased by O_2 radicals [Wong et al., 1988]. It is of interest to know whether NO also induces Mn-SOD expression. SNAP is one of the NO releasing reagents. As shown in Figures 3A and B, the expression of Mn-SOD mRNA was induced at 1 h after the addition of 10^2 μM SNAP and saturated at 6 h in VSMCs. The radioactivity of each band in the membrane hybridized with Mn-SOD probe was measured. The radioactivity of Mn-SOD band was increased in a time-dependent manner (Fig. 3B), and was correlated with the nitrite accumulation in the supernatants of VSMCs treated with 10^2 μM SNAP (Fig. 3C). The viability of VSMCs treated with 10^2 μM SNAP was confirmed by MTT assay (Fig. 3D).

DISCUSSION

The main finding of this article is that NO itself induces Mn-SOD mRNA expression in rat VSMCs. SNAP, which is one of the NO releasing reagents, was added to the VSMC culture. The expression of Mn-SOD mRNA was induced at 1 h after the addition of SNAP and reached saturation at 6 h.

In this article, we showed that LPS or IFN-γ induced Mn-SOD mRNA together with iNOS mRNA and further demonstrated that NO itself enhanced the expression of Mn-SOD in VSMCs. NO is beneficial as a messenger, modulator or self-protective molecule against infections such

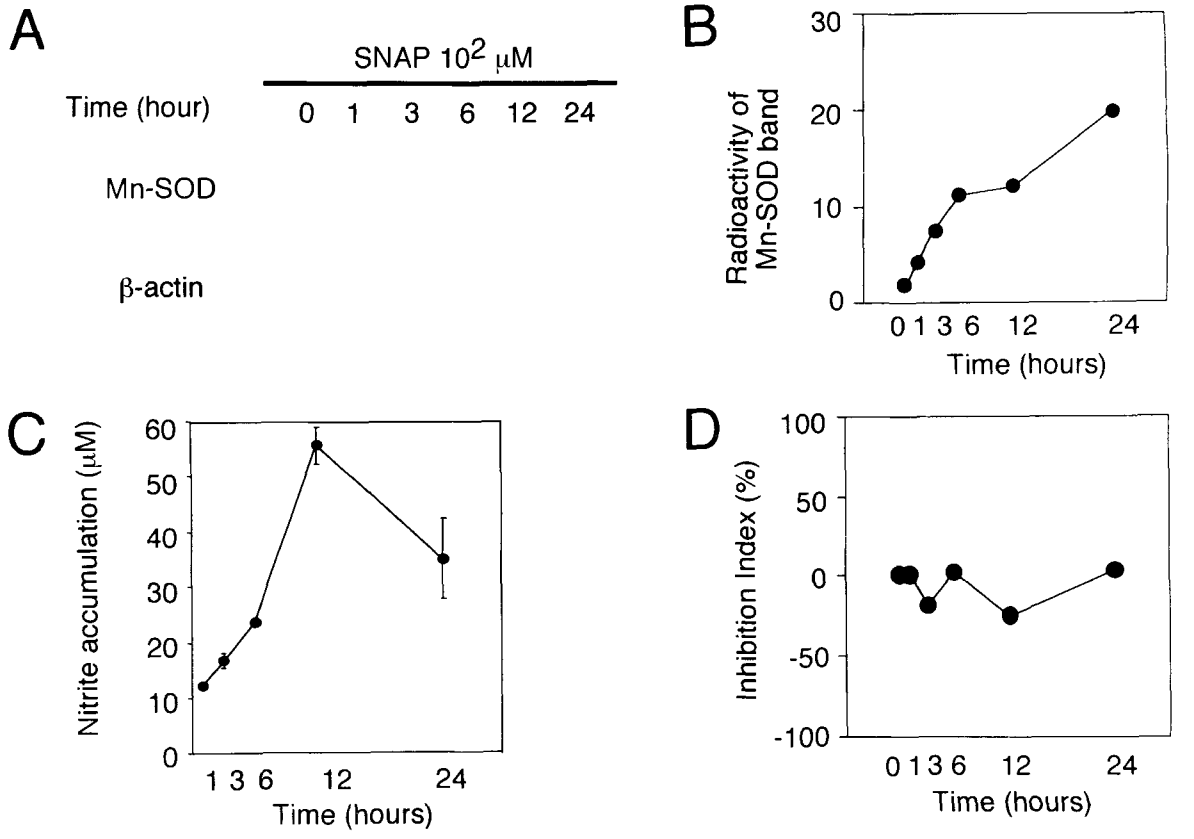


Fig. 3. Effects of 10^2 μM SNAP on Mn-SOD mRNA expression for various periods in VSMCs (**A**) and the radioactivity of each band of the membrane hybridized with Mn-SOD probe (**B**). Expression of mRNA for Mn-SOD was analyzed by Northern blot hybridization. NO production in cultured supernatants from VSMCs treated with 10^2 μM SNAP (**C**). The results of MTT assay of VSMCs treated with 10^2 μM SNAP (**D**).

as viruses, but it has toxic effects on the host. Another possible mechanism by which NO becomes toxic is by generating $ONOO^-$ through the reaction of NO with O_2 radical [Beckman et al., 1990]. $ONOO^-$ may modulate signaling functions of NO [Beckman et al., 1990; Gaston et al., 1994; Moro et al., 1994] and is directly cytotoxic [Beckman, 1991]. It was reported that vascular smooth muscle cells treated with LPS produced O_2 radical in addition to NO [Fujii et al., 1991]. One of the possible explanations is that the expression of Mn-SOD mRNA was stimulated by $ONOO^-$. Although it has been reported that IL-1, IFN-γ, TNF, and LPS induced the expression of both iNOS and Mn-SOD, and that TGF-β inhibited it [Perrella et al., 1994], there have been no reports that NO or NO-derived products directly stimulate the expression of Mn-SOD mRNA. There exist three types of SODs, namely Mn-SOD, Zn-SOD, and Cu-SOD. Mn-SOD mRNA expression was induced by O_2 radicals in human lung cancer cells [Wong et al., 1988]. These SODs may protect the cells from destruction by O_2 radicals. We hypothesized that Mn-SOD mRNA was induced by NO to protect the VSMCs themselves from NO and/or $ONOO^-$. Figure 3D showed that VSMCs were not destroyed by the addition of 10^2 μM SNAP, although a large amount of NO was produced. Nunoshiba et al. reported that various kinds of protective enzymes such as Mn-SOD and glucose-6-phosphate dehydrogenase (G6PD) were induced in bacteria under the stimulation by NO producing reagents [Nunoshiba et al., 1993]. Eukaryocytes also respond to oxidant stress by upregulation on specialized resistant genes such as Mn-SOD [Lewis-Molock et al., 1994], G6PD [Ding et al., 1988], and glutathion S transferase [Bergelson et al., 1994]. In neuronal cells, glutathion S transferase is mediated by AP-1, a transcriptional factor, under control of NO [Peunova et al., 1993].

As shown in Figures 2A and B, the expression of iNOS mRNA was enhanced by the addition of LNAME. Similar results were reported previously [Park et al., 1994]. One possible explanation for these results is that iNOS has an auto-down-regulatory mechanism driven by NO. On the contrary, the expression of Mn-SOD mRNA was decreased by the addition of LNAME, which may support our hypothesis that NO itself enhances the expression of Mn-SOD mRNA, because a small amount of NO was produced from VSMCs treated with LNAME and weakly induced the expression of Mn-SOD mRNA.

The present experiments suggested that infectious stimulants such as LPS (bacteria) or IFN-γ (virus) produced O_2 or NO radicals from VSMCs and that eukaryotic host cells were protected from the destruction caused by O_2 or NO radicals by producing SOD.

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