Korean J Physiol Pharmacol
Vol 5: 343−351, August, 2001

Lipopolysaccharide Inhibits Proliferation of the Cultured Vascular Smooth Muscle Cells by Stimulating Inducible Nitric Oxide Synthase and Subsequent Activation of Guanylate Cyclase

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This study was undertaken to investigate the mechanism of lipopolysaccharide (LPS) and nitric oxide (NO) as a regulator of vascular smooth muscle cell (VSMC) proliferation. VSMC was primarily cultured from rat aorta and confirmed by the immunocytochemistry with anti-smooth muscle myosin antibody. The number of viable VSMCs were counted, and lactate dehydrogenase (LDH) activity was measured to assess the degree of cell death. Concentrations of nitrite in the culture medium were measured as an indicator of NO production. LPS was introduced into the medium to induce the inducible nitric oxide synthase (iNOS) in VSMC, and Western blot for iNOS protein and RT-PCR for iNOS mRNA were performed to confirm the presence of iNOS. Inhibitors of iNOS and soluble guanylate cyclase (sGC), sodium nitroprusside (SNP) and L-arginine were employed to observe the action of LPS on the iNOS-NO-cGMP signalling pathway. LPS and SNP decreased number of VSMCs and increased the nitrite concentration in the culture medium, but there was no significant change in LDH activity. A cell permeable cGMP derivative, 8-Bromo-cGMP, decreased the number of VSMCs with no significant change in LDH activity. L-arginine, an NO substrate, alone tended to reduce cell count without affecting nitrite concentration or LDH level. Aminoguanidine, an iNOS specific inhibitor, inhibited LPS-induced reduction of cell numbers and reduced the nitrite concentration in the culture medium. LY 83583, a guanylate cyclase inhibitor, suppressed the inhibitory actions of LPS and SNP on VSMC proliferation. LPS increased amounts of iNOS protein and iNOS mRNA in a concentration-dependent manner. These results suggest that LPS inhibits the VSMC proliferation via production of NO by inducing iNOS gene expression. The cGMP which is produced by subsequent activation of guanylate cyclase would be a major mediator in the inhibitory action of iNOS-NO signalling on VSMC proliferation.

Key Words: Lipopolysaccharide (LPS), Nitric oxide (NO), Guanylate cyclase, Aminoguanidine, LY 83583, cGMP

INTRODUCTION

Nitric oxide is an important signal molecule that regulates several physiological processes in the vasculature, including vasculalr tone (Hollenberg et al, 1993), platelet aggregation (Nguyen et al, 1991), and leukocyte adhesion (Raines & Ross, 1995) in the endothelium. It is generated from the terminal guanidino nitrogen group of L-arginine by the enzyme NOS. Two isoforms of NOS have been identified in the cardiovascular system (Ignarro & Murad, 1995).

Under physiological conditions, a constituitive Ca^{2+}-

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dependent NOS in endothelial cells (eNOS or NOS3) is responsible for the continous release of NO (Farre et al, 1996). In the presence of proinflammatory cytokines, such as interleukin-1 (Bourcier et al, 1995), interferon-γ (Golombo et al, 1994; Hattori, 1995), and bacterial endotoxin (Thiemermann & Vane, 1990), an inducible Ca^{2+}-independent NOS (iNOS or NOS2) is expressed in VSMCs, leading to persistent generation of large quantities of NO.

Injury of vascular segment elicits a complex sequence of events characterized by platelet adhesion and aggregation, leukocyte infiltration, and VSMC proliferation and migration (Ross, 1993). Recently, It has been demonstrated that iNOS is rapidly induced in VSMCs after arterial injury and that the induction of iNOS at the sites of injury is accompanied by significant NO production (Hombach et al, 1996).

Because of its important biological properties, we speculated that NO derived from iNOS in the injured vessel wall may function as a modulator of restenosis and atherosclerosis, although the mechanisms involved in this inhibition by NO are unclear.

Furthermore, NO donors have been shown to inhibit proliferation of cultured VSMCs by different mechanisms (Hecker et al, 1995; Kolpakov et al, 1995; Mooradian et al, 1995), and decreased VSMC proliferation has been noted in vivo after NO augmentation by gene transfer (Moroshita et al, 1994). The effects of NO on processes involved in neointima formation are less clear.

The present study was designed (1) to investigate whether NO derived from VSMCs modulate proliferation of VSMC in vitro, and the mechanisms involved in such inhibition, (2) to determine effect of iNOS and guanylate cyclase inhibition on proliferation of VSMCs and (3) to characterize the expression of iNOS in the cultured VSMCs in response to iNOS and guanylate cyclase inhibition.

METHODS

Cell culture

Rat aortic VSMCs were derived from thoracic aorta of adult male Sprague-Dawley rat by primary explant culture techniques. Briefly, excised thoracic aortas were minced into small pieces and washed by HBSS (Sigma-Aldrich, MO, USA). These pieces were plated onto a culture dish containing 50% fetal bovine serum (FBS) (GibcoBRL®, Life Technologies, NY, USA) amphotericin 2.5 μg/ml and streptomycin 100 μg/ml) (GibcoBRL®, Life Technologies, NY, USA) in DMEM (Sigma-Aldrich, MO, USA). Outgrowthed cells were plated and grown in DMEM supplemented with 10% FBS and antibiotics-antimycotics. Cells were maintained in a humidified 95% O_2-5% CO_2 incubator. Media was changed two or three times a week.

Analysis of cell proliferation

VSMC proliferation was measured by cell count, before experiments 5×10^4 VSMCs were plated on each well of 24 well culture plate (Falcon® Becton Dickinson, NJ, USA). After 24 hours from VSMC plating, media was changed and treated with experimental agents. On days of 1, 2, and 3, VSMCs were trypsinized and counted mannually using a hemocytometer, culture medium was collected to determine nitrite and LDH activity.

Measurement of nitrite

NO production by VSMCs was assesed as nitrite generation. Nitrite contents were measured by using the Griess reagent/reaction. In brief, VSMCs were incubated with LPS (O26 : B6, Sigma-Aldrich, MO, USA) or SNP (Sigma-Aldrich, MO, USA) for 24 hours. The supernatant were recovered; after centrifugation (12,000 g, 5 minutes) nitrite accumulation was measured by mixing equal volumes of supernatants and Griess reagent [1% sulfanilamide and 0.1% N-(1-naphtyl)ethylenediamine dihydrochloride in 2% H_3PO_4]. Nitrite concentrations were determined at an optical density of 550 nm by comparison with standard solutions of sodium nitrite prepared in the same culture medium.

Determination of LDH activity

LDH activity, as a sign of cell death, was assessed in the medium of cultured VSMCs at the end of incubation period by measuring the rate of pyruvate reduction to lactate using a Sigma Diagnostics kit. In the presence of LDH, pyruvic acid and NADH were converted to lactic acid and NAD, therefore LDH activity was inverse proportion to optical density of 460 nm by pyruvic acid hydrazone. LDH activity was then calculated from the change in 460 nm

absorbance.

iNOS protein expression analysis

Confluent VSMC cultures grown in medium supplimented with 10% FBS were treated with LPS or LPS and ohter agents for 24 hours. After stimulation, cells were washed with phosphate buffered saline (PBS) and harvested by scraping. Protein extracts (12,000 g supernatant) were separated by electrophoresis (80 μg protein per lane) on 10% polyacrylamide gels in the presence of sodium dodecylsulphonate and then transferred onto nitrocellulose membranes (Protran, Schleicher & Schuell, NH, USA). The loading and transfer of equal amounts of protein in each lane was verified by staining of the protein bands with Ponceau S (0.2% in 3% trichloroacetic acid). After extensive washing with distilled water to remove the protein stain, blots were blocked with 5% nonfat dry milk (Sigma-Aldrich, MO, USA) in PBS. The immobilized iNOS protein was visualized by subsequent incubation with a polyclonal anti-iNOS antibody (Calbiochem, CA, USA) and a secondary polyclonal peroxldase-conjugated anti-rabbit antibody (Santa Cruz Biochemistry, CA, USA) followed by staining with the enhanced chemiluminescence (ECL) technique developed by NEN life science.

iNOS mRNA expression analysis

Total cellular RNA was isolated using RNAzol B (Tel-Test Inc., TX, USA). RNA concentration was calculated from absorbance at 260 and 280 nm.

For reverse transcriptation, following components were added to the reaction vials: 5 μg of total RNA, both sense and antisense primer, 10× first-strand buffer, RNase inhibitor, dNTPs, MMLV-RT in a total volume of 25 μl. The vials were incubated for 60 min at 37°C, there after the reverse transcriptation was terminated by heating at 95°C for 5 min.

The PCR was carried out with 5 μl of RT reaction mixtures, 10× Taq DNA polymerase buffer, dNTPs, both sense and antisense primer, Taq DNA polymerase, DEPC-treated water in a total volume of 25 μl. The samples were placed in a GeneAmp PCR system 2,400 (PerkinElmer, MA, USA) which was programmed as follows: Pre-PCR; 94°C 1 min, PCR (30 cycle) Denaturation; 94°C 1 min, Annealing; 52°C 1 min, Extension; 72°C 1 min, Post-PCR; 72°C 10 min. The PCR products (10 μl) were size-fractionated by agarose (1.5%) gel electrophoresis, stained with ethidium-bromide (Sigma-Aldrich, MO, USA) and visuallized by use of an ultraviolet transilluminator.

The sequence of the two iNOS-specific primers was 5-ATGCCTTGCCCCTGGA AGTTTCTC-3 (sense) and 5-CCTCTGATGGTGCCATCGGGCATCTG-3 (antisense), and the predominant cDNA amplification product was predicted to be 800 bp in length. The sequence of the glyceraldehyde-3-phosphate-dehydrogenase (GAPDH)-specific primers was 5-GTCATGAGCCCTTCCACGATGC-3 (sense) and 5 GAATCTACT GGCGTCTTTCACC-3 (antisense), and predominant cDNA amplification product was predicted to be 300 bp in length. RT-PCR of GAPDH served as a positive control.

Statistical analysis

Results are reported as mean±S.E.M. Comparisons of the means of the two groups was performed by the paired *t*-test. A value of $P<0.05$ was considered to be statistically significant.

RESULTS

Effects of LPS on the VSMC proliferation, nitrite production, and LDH activity

Control cells incubated in the absence of LPS for up to 24 hours, produced no detectable nitrite. In contrast, when LPS (30 or 150 μg/ml) added, there was a progressive decrease in VSMC proliferation, and this decrease continued for 3 days by a concentration- and time-dependent manner. With the addition of LPS, a marked increase in nitrite concentration was observed. LDH activity was not significantly changed after exposures to LPS of both concentrations (30 μg/ml and 150 μg/ml) (Fig. 1).

Effects of L-arginine, SNP, and 8-Bromo-cGMP on the VSMC proliferation, nitrite production, and LDH activity

L-arginine (10^{-3} M) tended to reduce the proliferation of VSMC, and did not induce any significant change in nitrite production. When SNP (10^{-4} M) added, there was a marked decrease in VSMC proliferation (on 3rd day, 41.9±2.7%) with time dependent manner and increase in nitrite production (on

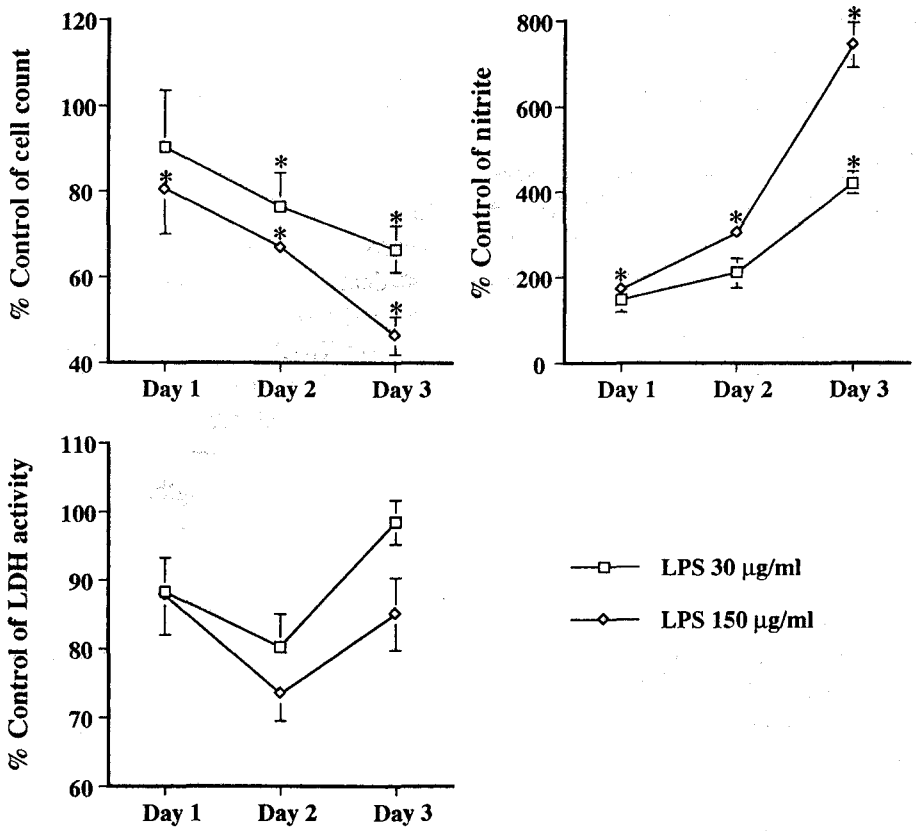

Fig. 1. Effect of lipopolysaccharide (O26 : B6) on the vascular smooth muscle cell proliferation, nitrite production, and LDH activity. Values express mean±S.E.M (n=6 each group). *$P<0.05$: Significantly different from control.

3rd day, 730.4±16.3%) in culture medium. LDH activity in culture medium was not significantly changed after an exposure to L-arginine (10^{-3} M) or SNP (10^{-4} M).

When 8-Bromo-cGMP (10^{-3} or 10^{-4} M) added, there was a decrease in VSMC proliferation (on day 3, 8-Bromo-cGMP 10^{-3} M, 75.3±4.4%) in a dose- and time- dependent manner. We have summarized the levels of cell count and nitrite production after various treatments in Fig. 2.

Effects of aminoguanidine and LY 83583 on the LPS-induced changes

Aminoguanidine (10^{-4} M), an iNOS specific inhibitor, almost completely inhibited the LPS (30 μg/ml)-induced decrease in VSMC proliferation (on 2nd day, 126.3±4.8%) and increase in nitrite production (on 3rd day, 271.0±5.8%). Aminoguanidine was effective whether given 30 minutes before or 30 minutes after the addition of the LPS to the incubation medium.

In contrast to the iNOS inhibition by aminoguanidine, LY 83583, a guanylate cyclase inhibitor, substantially accumulated the nitrite production (on 3rd day, 825.0±14.5%) by LPS. In the presence of LY 83583 (10^{-7} M), LPS (30 μg/ml)-induced decrease in VSMC proliferation was restored to steady state although nitrite accumulation was significantly increased at every interval tested. Untreated cells and cells treated only with LY 83583 produced no nitrite.

Futhermore, aminoguanidine and LY 83583 did not affect LDH activity of the control of LPS-stimulated samples (Fig. 3).

Effect of LY 83583 on the SNP induced changes

To further characterize the restoration by LY 83583 of LPS-induced decrease in VSMC proliferation, VSMCs were incubated for 3 days in the presence of

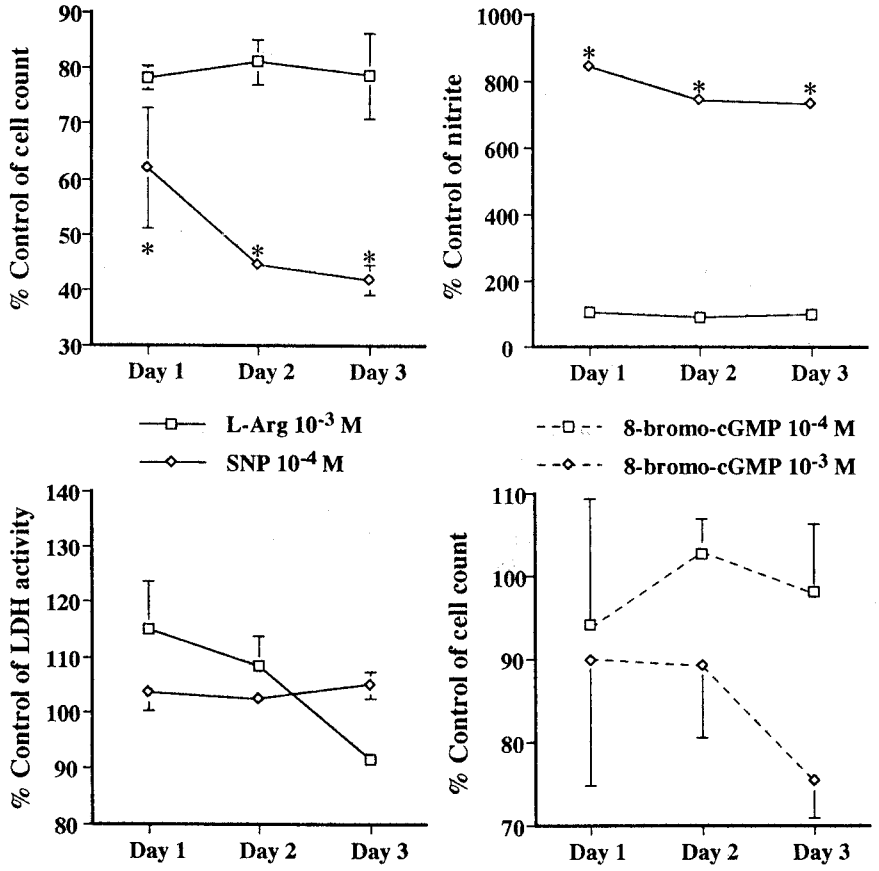

Fig. 2. Effects of L-arginine, sodium nitroprusside and 8-Bromo-cGMP on the vascular smooth muscle cell proliferation, nitrite production, and LDH activity. Values express mean±S.E.M (n=6 each group). L-Arg: L-arginine, SNP: sodium nitroprusside. *$P<0.05$: Significantly different from control.

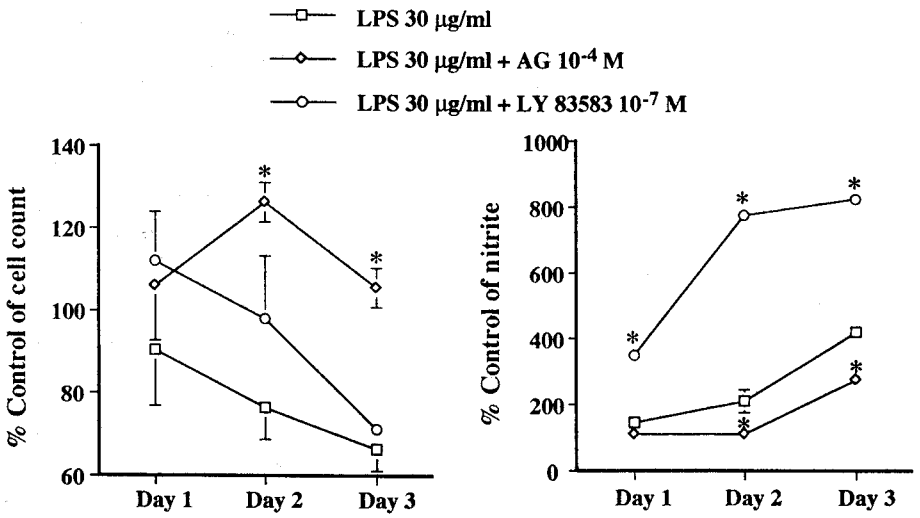

Fig. 3. Effects of aminoguanidine and LY 83583 on the lipopolysaccharide (O26 : B6) induced-inhibition of vascular smooth muscle cell proliferation, and nitrite production. Values express mean±S.E.M. (n=6 each group). LPS: lipopolysaccharide, AG: aminoguanidine *$P<0.05$: Significantly different from LPS 30 μg/ml.

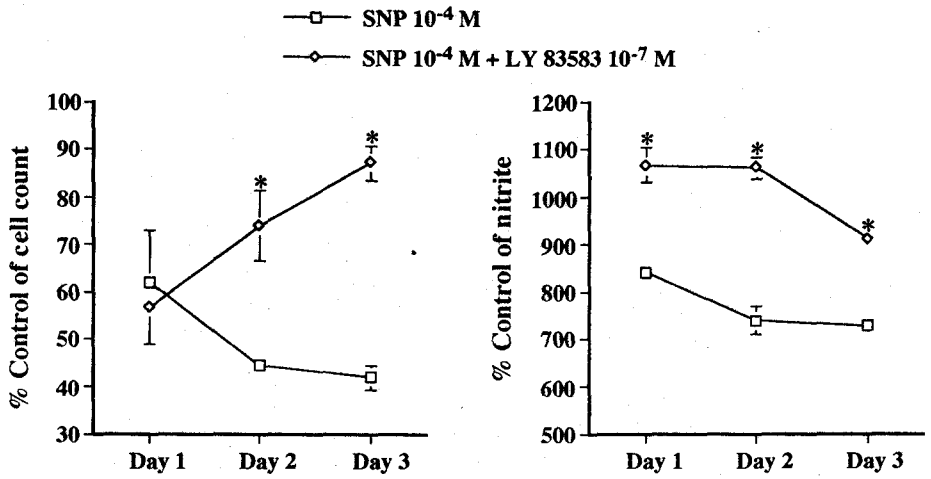

Fig. 4. Effect of LY 83583 on the sodium nitroprusside induced-inhibition of vascular smooth muscle cell proliferation and nitrite production. Values express mean ± S.E.M. (n=6 each group). SNP: sodium nitroprusside. *$P<0.05$: Significantly different from SNP 10^{-4} M.

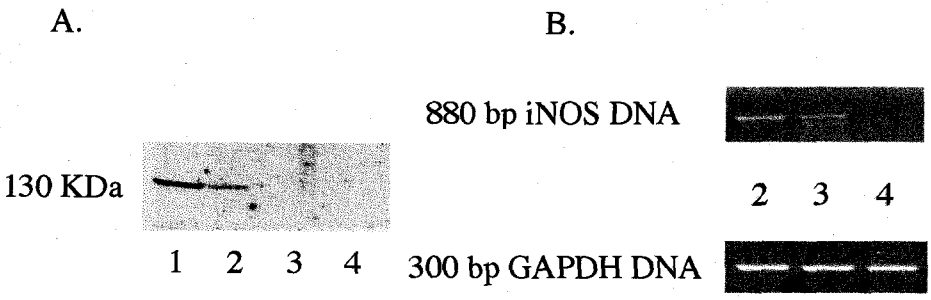

Fig. 5. Dose responses of lipopolysaccharide (O26 : B6) on iNOS protein and mRNA expression in vascular smooth muscle cells. Cells were stimulated with different doses of lipopolysaccharide for 24 hours and then analysed by Westen blot (A) and RT-PCR (B), respectively. Lane 1: Lipopolysaccharide 30 μg/ml, Lane 2: Lipopolysaccharide 3 μg/ ml, Lane 3: Lipopolysaccharide 300 ng/ml, Lane 4: Lipopolysaccharide 30 ng/ml.

SNP (10^{-4} M) and LY 83583 (10^{-7} M). SNP (10^{-4} M) produced marked decrease in VSMC proliferation in a time-dependent manner, and markedly increased the nitrite concentration in media. Whereas LY 83583 progressively inhibited SNP (10^{-4} M)-induced reduction in VSMC proliferation for 3 days (on day 3, 87.1 ±3.7%), it enhanced the increase in nitrite concentration in culture medium (on day 3, 912.7± 15.7%) in this whole period of experiment (Fig. 4).

Effect of aminoguanidine and LY 83583 on the LPS induced iNOS mRNA and protein expression

Western blot and RT-PCR were performed on VSMCs in the presence of LPS. Total RNA and protein preparation were extracted from LPS stimulated VSMCs.

LPS treatment (24 hours) induced iNOS mRNA and protein in VSMCs. At higher concentrations of LPS (3 and 30 μg/ml), iNOS mRNA and protein were induced, the densities of iNOS mRNA and protein band were increased in a concentration-dependent manner. LPS (300 ng/ml) expressed only iNOS mRNA. Low concentration of LPS (30 ng/ml) showed no band of iNOS mRNA or protein (Fig. 5).

To determine if the LY 83583 and aminoguanidine affect iNOS expression, control cells received only LPS (30 μg/ml). For comparison, VSMCs treated

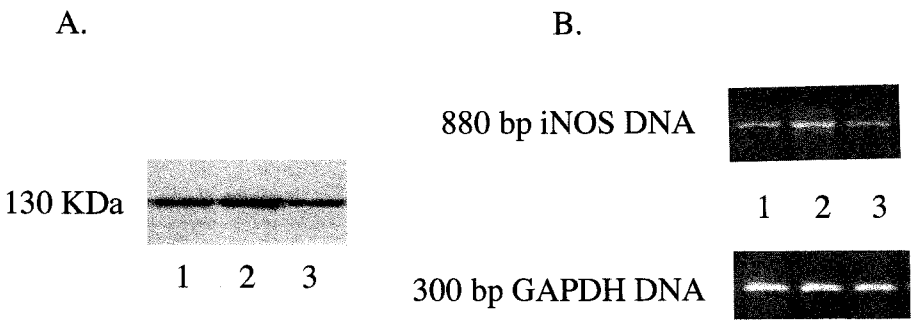

Fig. 6. Effects of LY 83583 and aminoguanidine on the lipopolysaccharide (O26 : B6) induced iNOS protein and mRNA expression in vascular smooth muscle cell. Cells were pretreated with LY 83583 or aminoguanidine before administration of lipopolysaccharide and incubated for 24 hours, then iNOS protein and mRNA were analysed by Westen blot (A) and RT-PCR (B), respectively. Lane 1: Lipopolysaccharide 30 μg/ml only, Lane 2: Lipopolysaccharide 30 μg/ml + LY 83583 10^{-7} M, Lane 3: Lipopolysaccharide 30 μg/ml + Aminoguanidine 10^{-4} M.

with LPS (30 μg/ml) after the LY 83583 (10^{-7} M) or aminoguanidine (10^{-4} M) were present for 1 hours. Between control and aminoguanidine-treated cells, no significant difference in iNOS mRNA and protein expression were observed. There was a clear increase of iNOS mRNA and protein band in LY 83583-treated cells. The GAPDH PCR showed the even amount of RNA was loaded on every lane. We obtained consistent findings in the three separate assays (Fig. 6).

DISCUSSION

Many diverse types of cultured cells can be stimulated to induce iNOS. Previous studies have shown that cytokine-stimulated murine dermal fibroblast (Werner-Felmayer et al, 1990) and rat lung fibroblast (Jorens et al, 1992) upregulate iNOS.

Recently, several reports have suggested that NO donors (Newby et al, 1992; Yang et al, 1994; Thomae et al, 1995) and iNOS gene transfer (Moroshita et al, 1994) can inhibit VSMC proliferation. According to our data, VSMC proliferation seems to be influenced by multiple factors, iNOS and guanylate cyclase. Among various intracellular messengers, NO and cGMP appear to have a critical role in the VSMC proliferation at the injured vessel wall.

Overexpression of iNOS has been postulated as a vasorelaxing factor in septic shock, since NO has been shown to reduce blood pressure (Singh et al, 1996). To test the sensitivity of LPS in the inhibition of VSMC prolifertion, we used LPS of 30 μg/ml through the present study, because of LPS-binding protein (LBP) was not present, in vitro and iNOS induction needed high concentration of LPS than in vivo. But even under the septic conditions, the serum concentration of LPS in vivo can not attain such a high level.

We tested LPS to determine if this could inhibit VSMC proliferation, and the effects of various interventions affecting intracellualr signal transducing pathways on the LPS inhibition of VSMC proliferation. In the present study, we compared the levels of cell count, nitrite production, and LDH activity after 1, 2 and 3 days of each treatment. In vitro, LPS in a dose and time-dependnet manner could inhibit VSMC proliferation with increase in nitrite production. This may have important implications for the treatment of disease such as multiple organ dysfunction syndrome (MODS), which present clinically with the hypotension and multiple organ edema. Additionally, SNP, not L-arginine, could inhibit VSMC proliferation with increase in nitrite production. Thus, we concluded that NO from iNOS activation in VSMCs or NO donor may inhibit VSMC proliferation with the similar pattern.

Generally, cGMP is responsible for circular reaction by NO (Brian et al, 1995), we tested a cell permeable cGMP, 8-Bromo-cGMP also exhibited dose and time-dependnet inhibition of VSMC proliferation in viro.

To examine the interventions of the effects of LPS on VSMC, aminoguanidine and LY 83583 were tested. Aminoguanidine has been reported to reduce NO in culture medium of LPS stimulated VSMCs both before and after induction by LPS. In the present study, aminoguanidine inhibited the LPS-induced decrease in VSMC proliferation and increase in nitrite production. Different preparations of VSMC from separate primary cultures all showed an inhibition of LPS-induced nitrite accumulation by aminoguanidine, with a dose-response pattern

LY 83583 suppressed the effects of LPS and SNP on VSMC, although accelerated the nitrite accumulation in culture medium. LY 83583 by itself did not induce nitrite (Jo et al, 1999), this remains to be investigated.

We postulated that, when drugs were applied for 3 days, some of the pretreatments by themselves affected cellular viability. Although even after LPS exposure, LDH activity was not significantly changed. Moerever, aminoguanidine SNP and LY 83583 did not affect LDH activity of the control of LPS-stimulated samples. This may suggest that LPS, aminoguanidine SNP and LY 83583 concentration used in the present study did not affect VSMC viablity.

Previously, aminoguanidine has been characterized as an specific inhibitor of iNOS, although it is uncertain whether aminoguanidine inhibited the iNOS activity or induction of iNOS mRNA and protein (Yang et al, 1998). LPS (30 μg/ml) stimulation for 24 hours induced iNOS mRNA and protein in VSMCs. We demonstrated that iNOS was induced almost exclusively in VSMCs. Although reduced VSMC proliferation and increased nitrite production by LPS (30 μg/ml) were restored by aminoguanidine (10^{-4} M), aminoguanidine did not affect iNOS mRNA and protein induction by LPS. This may suggest that aminoguanidine acted as a iNOS specific inhibitor. There was a clear increase of iNOS mRNA and protein in LPS stimulated LY 83583-treated cells, although this effect appears to have relationship with accumulated nitrite production, it needs another investigation.

In the present study, we demonstrated that LPS itself induced iNOS in VSMCs, and the inhibitory action of NO on the cell proliferation was via the activation of guanylate cyclase.

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