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Smooth Muscle α-Actin Expression in Rat Hepatic Stellate Cell Is Regulated by Nitric Oxide and cGMP Production

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Mechanism by which lipopolysaccharide (LPS) and interferon γ (IFNγ) downregulate smooth muscle (SM) α-actin expression in rat hepatic stellate cell was studied. Culture-induced SM α-actin expression was suppressed in the presence of LPS ($>$ 5 ng/ml) and/or IFNγ ($>$ 1 U/ml) dose-dependently. Inhibition of nitric oxide (NO) production by Nω-nitro-L-arginine (LNA) canceled such an inhibitory effect of LPS and/or IFNγ. LPS and/or IFN γ increased cellular level of cGMP in an LNA inhibitable manner, and cGMP analogues attenuated SM α-actin expression of the cells. These results indicate that NO-dependent increase of cellular cGMP level mediates the inhibitory effect of LPS and IFNγ on SM α-actin expression in stellate cells.

Hepatic stellate cells (fat-storing cells, Ito cells) in the space of Disse function as a liver specific pericyte and may play a central role in the regulation of hepatic microcirculation (1,2). These cells undergo phenotypic transformation into myofibroblast-like cells having proliferative activity and smooth muscle (SM) α-actin in the process of liver injuries (3-10). Because the SM α-actin expressing stellate cells have potent traction force (11,12), the transformed cells have been speculated to contribute to the tissue shrinkage and the development of microcirculatory disturbance of the chronically damaged liver. However, littele is known about the molecular mechanism that regulate SM α-actin expression in stellate cells.

Nitric oxide (NO) is a gaseous mediator produced by several cell types including hepatic constituent cells (13-15). Stellate cells also generate NO in response to lipopolysaccharide (LPS) and interferon γ (IFNγ) through the induction of NO synthase (15). NO thus produced was reported to regulate DNA synthesis of the cells (16). On the other hand, it was reported that NO production is associated with attenuating the contractility of stellate cells cultured on hydrated collagen lattice (15). This report lets us imagine that NO may regulate the cell traction force through varing SM α-actin level besides inducing a transient relaxation (11). We here show that NO mediates the suppressive effect of LPS and/or IFNγ on SM α-actin expression in stellate cells by some cGMP-dependent mechanism.

MATERIALS AND METHODS

Materials. Dulbecco's modified Eagle's medium (DMEM) and rat recombinant IFN γ were purchased from Gibco (Grand Island, NY). Collagenase was from Wako Pure Chemical Company (Osaka). 3-isobutyl-1-methylxanthine (IBMX), Nω-nitro-L-arginine (LNA), Nω-nitro-L-arginine methyl ester (LNAM), aminoguanidine, L-N^5-(1-iminoethyl)ornithine, dibutyryl cGMP (dBcGMP) and 8-bromo-cGMP were from Sigma (St. Loius, MO). Pronase E was from Merck (Darmstadt, FRG). DNase and monoclonal antibody for SM α-actin were from Boehringer (Mannheim, FRG). EIA kit for cGMP was from Cayman Chemical Company (Ann Arbor, MI). Monoclonal antibody for inducible nitric oxide synthase (iNOS) was from Transduction Laboratories (Lexington, Kentucky). Fetal bovine serum (FBS) was from Gibco BRL (Grand Island, NY). The other chemicals were of analytical grade.

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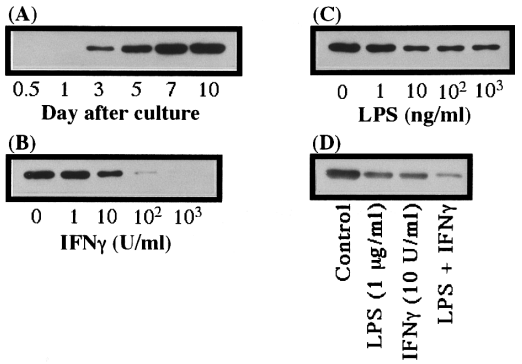

FIG. 1. Effect of LPS and IFNγ on SM α-actin expression in cultured stellate cells. (A) Stellate cells were cultured in the absence of stimulants for indicated days. (B, C, and D) One-day-old stellate cells were further cultured for 4 days in the presence of IFNγ (0 - 10^3 U/ml) (B), LPS (1 - 10^3 ng/ml) (C), or LPS + IFNγ (D). The protein level of SM α-actin was determined by Western blot as described in Materials and Methods. A typical result from four individual experiments is presented here.

Isolation of stellate cells. Stellate cells were isolated from the liver of male Wistar rats (SLC) as described (10). Isolated stellated cells were suspended in DMEM supplemented with 10% FBS and antibiotics (10^5 U/l penicillin G and 100 mg/l streptomycin) and introduced to cell culture wells as described below. The number of stellate cells thus obtained was $1.87 \pm 0.44 \times 10^7$ cells/rat (n=21). The purity of stellate cells was 92 ± 3 % (n = 5) as assessed by their having yellow-colored droplets and desmin immunoreactivity after overnight culture.

Determination of nitric oxide production by stellate cells. Stellate cells were cultured in 10%FBS/DMEM in the presence or absence of test agents for 24 - 48 h. The concentrations of nitrite in the culture medium were determined by the Griess reaction (17).

Western blot analysis of SM α-actin and iNOS. Stellate cells cultured for the indicated days in the presence or absence of test agents were homogenized in SDS sample buffer (62.5 mM Tris, 0.1% glycerol, 2% SDS, 5% 2-β mercaptoethanol, pH 6.8). Ten μg proteins of heat-denatured cell homogenates were analyzed by 10% SDS-PAGE and then transferred onto Immobilon P (Millipore). The membrane was treated first with 5% skim milk and next with monoclonal antibodies for either SM α-actin or inducible NO synthase (iNOS) for 2 h at room temperature. After vigorous washing, the membrane was then incubated with peroxidase-conjugated goat anti-mouse IgG for 1 h at room temperature. Immunoreactive bands were visualized on Kodak XAR5 films by using ECL detection reagent (Amersham, Buckinghamshire, UK). The developed films were scanned by densitometry.

Determination of cellular cGMP. Stellate cells grown in 24 well cell-culture plates (FALCON 3047) for 3 days were further incubated with test agents for 24 h or 48 h. After incubation, 1 mM IBMX was added to the culture medium for 30 min to inhibit phosphodiesterase activity. The reaction was terminated by addition of 5% TCA. After extraction with diethylether, cGMP in a water layer was concentrated by drying under vacuum. Cyclic GMP was determined by the cGMP EIA kit (11).

Statistical analysis. All data are expressed as mean $\pm$ SD. Statistical analysis was carried out according to the Student's *t*-test. Each experiment was performed using cells from a different animal.

RESULTS AND DISCUSSION

Stellate cells cultured on a plastic dish in 10%FBS/DMEM for 1 day contained lipid droplets and extended dendritic processes forming a star-like cell shape (2). These cells underwent spontaneous transformation to myofibroblast-like cells that express SM α-actin (9). This was confirmed in the present study by Western blot as shown in Fig. 1A, which indicates the time-dependent increase of SM α-actin level in stellate cells after culture. IFNγ, an anti-fibrotic cytokine, suppressed dose-dependently the protein expression (Fig. 1B). Densitometric analysis revealed that 10, 10^2, and 10^3 U/ml IFNγ decreased the protein level to 70.8, 17.5, and 5.8% of the control, respectively. LPS at concentrations of more than 10 ng/ml inhibited significantly the protein expression as well (Fig. 1C). Densitometric analysis showed that 10, 10^2, and 10^3 ng/ml LPS suppressed the protein level to 64.1, 52.4, and 47.2% of the control, respectively.

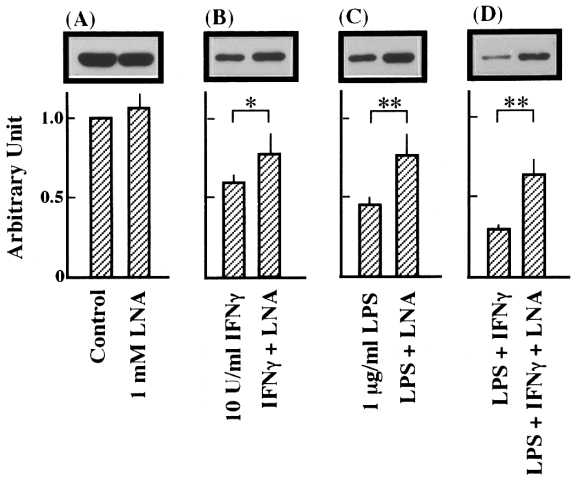

FIG. 2. Effect of a NO synthase inhibitor on SM α-actin expression in stellate cells. One-day-old stellate cells were cultured in the presence or absence of test agents for 96 h. The protein level of SM α-actin was determined by Western blot as described in Materials and Methods. LPS, 1 μg/ml. IFNγ, 10 U/ml. LNA, 1 mM. A typical result from four individual experiments is presented at the top and a result obtained by densitometric analysis is shown at the bottom. Arbitrary unit of SM α-actin bands of untreated stellate cells is calculated at 1. Data are shown as mean $\pm$ SD. * $p < 0.05$. ** $p < 0.01$.

Moreover, as shown in Fig. 1D, co-addition of LPS and IFNγ attenuated synergistically the protein expression.

Because both LPS and IFNγ have been reported to induce NO production of stellate cells (15), role of endogenous NO in SM α-actin expression was investigated here. Before starting experiments, we reconfirmed that LPS and IFNγ dose-dependently increased the concentration of nitrite, a stable degradation product of NO, in our stellate cell culture; when stimulated with either 1 μg/ml of LPS or 10^3 U/ml of IFNγ, nitrite concentration increased from 7.2 $\pm$ 3.4 nmol/ml of untreated cultures to 25.9 $\pm$ 13.4 or 95.9 $\pm$ 16.7 nmol/ml, respectively. A concomitant increase of NO synthase protein level was also observed (data not shown). Such a NO production was restrained by the presence of 1 mM LNA (data not shown). Thus, NO was produced in stellate cells exposed to LPS and/or IFNγ. Although 1 mM LNA had negligible effect on SM α-actin expression of untreated stellate cells (Fig. 2A), it interrupted significantly the decrease in SM α-actin protein level caused by the exposure to LPS and IFNγ (Fig. 2, B, C, and D). The other NO synthase inhibitors, aminoguanidine and L-N^5-(1-iminoethyl)ornithine, were also effective (data not shown). These results indicated that NO may be a negative regulator for SM α-actin protein expression in stellate cells.

Bioaction of NO is mediated at least partially by the activation of soluble guanylate cyclase (18). Hence, we checked the cGMP concentration of LPS and/or IFNγ-stimulated stellate cells. As expected, the cGMP level increased in stellate cells treated with LPS or IFNγ for 24 h (Fig. 3A) or 48 h (data not shown) in their dose-dependent manner. Simultaneous additions of LPS and IFNγ augmented the increase of cGMP (Fig. 3A). Such an increase of cGMP level was almost completely inhibited by the addition of 1 mM LNA (Fig. 3A). These data suggested that the suppressive effect of LPS and/or IFNγ on the expression of SM α-actin in stellate cells might be mediated by the increase of intracellular cGMP concentration caused by NO. Although a specific inhibitor of soluble guanylate cyclase without toxicity to stellate cells was not available commercially, addition of either dBcGMP or 8-bromo-cGMP suppressed the expression of SM α-actin (Fig. 3B) in culture-stimulated stellate cells. Previously, we and

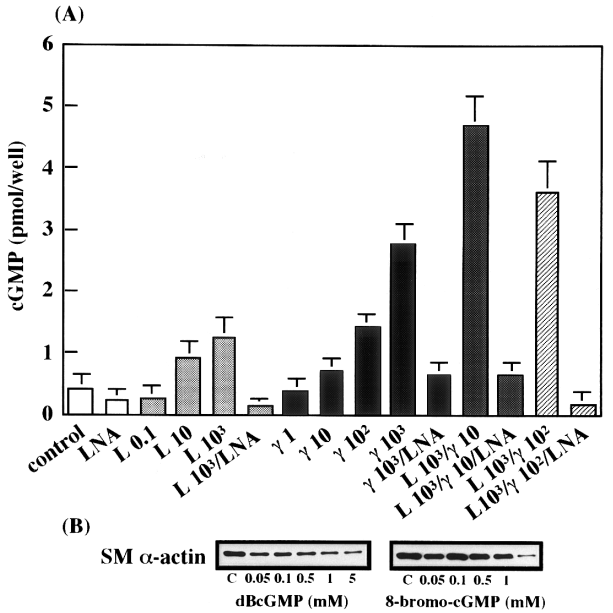

FIG. 3. Change of cGMP level in stellate cells and effect of cGMP analogues on SM α-actin expression. (A) Stellate cells cultured for 3 days were further incubated in the presence of LPS (1-10^3 ng/ml) and/or IFNγ (0-10^3 U/ml) for 24 h. In some experiments, 1 mM LNA was added to the medium. After incubation, cyclic GMP in the stellate cells was measured by a cGMP EIA kit as described in Materials and Methods. The concentration of LPS (L) is expressed as ng/ml and that of IFNγ (γ) as U/ml. Mean $\pm$ SD value of three different preparations is shown. (B) Effect of dBcGMP and 8-bromo-cGMP on SM α-actin expression in stellate cells. One-day-old stellate cells were cultured in the presence or absence of test agents for 96 h. The protein level of SM α-actin was determined by Western blot as described in Materials and Methods. A typical result from three individual experiments is shown here.

the others reported that SM α-actin expression in stellate cells is suppressed by increasing the cellular level of cAMP by using cyclic nucleotide phosphodiesterase methylxanthines and dibutyryl cAMP (19,20). In this regard, G or A-kinase dependent process might down-regulate stellate cell SM α-actin expression.

The present study revealed that NO is a suppressor of SM α-actin expression. This may support a finding by Rockey indicating that NO production by the cells contributes to the reduction in the traction force of the cells (15). Hence, NO seems to be a very important mediator in maintaining the microcirculation and the fluidity of sinusoids in chronically-injured livers. Because Kupffer cells and hepatocytes exposed to LPS and IFNγ are also potential producers of NO (13,14) and NO concentration around stellate cells may therefore be relatively high, such an action of NO should not be disregarded also in vivo.

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