Enhancement of CREB$^{Serine-133}$ Phosphorylation through Nitric Oxide-Mediated Signaling Induced by Bacterial Lipopolysaccharide in Vascular Smooth Muscle Cells from Rats

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Abstract

Nitric oxide (NO) induced by bacterial lipopolysaccharide (LPS) plays a critical role in various patho-physiological implications, such as atherosclerosis, vasculitis and septic shock. In addition, cAMP-responsive element binding protein (CREB), an important transcription factor for cell differentiation, has been shown to be involved in atherosclerogenesis in VSMCs. Here we investigated the possibility whether LPS-induced NO signaling led to phosphorylation of cAMP-responsive element binding protein on Serine-133 (CREB$^{Ser-133}$) in cultured vascular smooth muscle cells (VSMCs) from rats. Addition of LPS (1-10 μg/ml) for 48 hours increased not only the production NO, but also the phosphorylation of CREB$^{Ser-133}$. The use of NOS inhibitor (100-500 μM L-NAME) blocked the magnitudes of both LPS-induced NO production and CREB$^{Ser-133}$ phosphorylation. In addition, either a guanylyl cyclase (GC) inhibitor (30 μM ODQ) or a cGMP-dependent protein kinase (PKG) inhibitor (20 μM (Rp)-8-pCPT-cGMPs) significantly attenuated the magnitudes of LPS-induced CREB$^{Ser-133}$ phosphorylation, suggesting the involvement of NO-GC-PKG signaling. Thus, the present study suggests that NO-mediated signaling activated by bacterial LPS, at least in part, enhance CREB$^{Ser-133}$ phosphorylation in cultured VSMCs. The findings here may provide not only signaling pathway involved in VSMC differentiation during inflammatory response, but also new insight into possible therapeutic intervention.

Key Words: atherosclerosis, differentiation, smooth muscle cell, CREB

Introduction

The bacterial lipopolysaccharide (LPS)-mediated inflammatory response was not only an important component in the defense against pathogens, but also an essential contributor to certain pathological processes such as atherosclerosis, autoimmune diseases and endotoxic shock (5, 21, 23, 30). For instance, LPS, the mediator of Gram-negative endotoxic shock, was associated with the development of atherosclerotic lesions (17). Both clinical and biochemical studies for the understanding of atherosclerosis suggested that lesion development within the vessel wall could be accelerated by locally inflammatory responses (10, 11, 29). Previous studies indicated that an enhanced production of nitric oxide (NO) induced by LPS, principally by the calcium-independent inducible isoform of NO synthase, was frequently associated with a generalized and/or localized inflammatory response deriving from infection or tissue injury (12, 15, 17-20, 27, 31).

Phosphorylation of cAMP-responsive element binding protein (CREB) on Serine 133, an important transcription factor for cell differentiation (2, 6-8, 14, 25), could play a role in atherosclerotic vascular smooth muscle cells (VSMCs) (16). Previous studies

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showed that various kinases were capable of phosphorylating CREB, including cAMP-dependent protein kinase (PKA), PKC, Ras-dependent p105 kinase, Rsk-2, and Ca^{2+}/Calmodulin-dependent protein kinases (6-8). However, the signaling pathways to CREB in arterial smooth muscle remain to be determined, especially during certain pathological conditions (e.g., bacterial LPS-induced insults). Thus, the major purpose of the present study was to investigate the possibility whether an increased production of NO during bacterial LPS insults could trigger a cascade of NO-mediated events leading to an enhancement of $CREB^{Ser\text{-}133}$ phosphorylation in cultured VSMCs form rats.

Materials and Methods

Cell Culture

Vascular smooth muscle cells (VSMCs) were isolated from the medial layer of the thoracic aorta of male Sprague-Dawley rats (250-300 g). Cell cultures were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (DMEM/10% FBS), 50 U/ml penicillin, and 50 μg/ml streptomycin. The cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Cells (3×10^6) from passages 3 were seeded for the present study.

Western Blotting

After a 48 hours exposure to LPS, the cells were collected and stored at -80°C until assayed. The frozen cells were sonicated briefly in ice-cold homogenization buffer (HB) [containing (in mM): 50 Tris-HCl, pH7.5, 50 NaCl, 10 EGTA, 5 EDTA, 2 sodium pyrophosphate, 4 paranitrophenylphosphate (pNPP), 1 sodium orthovanadate, 0.5 phenylmethylsulfonyl fluoride (PMSF), 20 μg/ml leupeptin, and 20 μg/ml aprotinin]. The protein concentrations were measured by the use of the Bio-Rad DC protein assay kits (Cat. No. 500-0112, Bio-Rad Laboratories, Hercules, CA, USA). A volume of 2× sample buffer was added to the homogenate, and the sample was incubated in 95°C water bath for 10 min. Samples were loaded on the 10% SDS-polyacrylamide gels and resolved by standard electrophoresis (Novex, Carlsbad, CA, USA). Then the gels were transferred on PVDF filters. The filters were incubated with polyclonal anti-phospho-$CREB^{Ser\text{-}133}$ (1:1000; Upstate Biotechnology, Lake Placid, NY, USA) or polyclonal anti-iNOS (1: 1000; Santa Cruz, CA, USA) and were visualized by chemiluminescence. Then the filters were stripped and reprobed with anti-CREB (1:1000, Upstate Biotechnology) to detect total CREB. For quantitation of immunoblot signals, the band intensity was measured with Kodak Digital Science 1D program (Rochester, NY, USA). Each phospho-$CREB^{Ser\text{-}133}$ band intensity was equalized to the total CREB (1: 1000, Upstate Biotechnology) signal in the same lane. The increase in phosphorylation of $CREB^{Ser\text{-}133}$ was normalized to the basal level and expressed as fold increase. In addition, expression of iNOS in each band was normalized to the α-actin in the same lane.

Drugs

Unless otherwise stated, LPS (*Escherichia coli 0111:B4*) and other drugs were purchased from Sigma Chemicals Company (St Louis, MO, USA). Dulbecco's modified Eagle's medium, FBS, and a mixture of penicillin and streptomycin were all obtained from GIBCO-BRL (Gaithersburg, MD, USA).

Nitrite Assay

The production of nitric oxide (NO) was assessed as the accumulation of nitrite in the culture supernatants, using a colorimetric reaction with the Griess reagent (28, 32). The culture supernatants were collected after an application of LPS and mixed with equal volumes of the Griess reagent (0.1% N-[1-naphthyl]ethylenediamine dihydrochloride, 1% sulfanilamide, and 2.5% H_3PO_4). The absorbance at 540 nm was measured with UV microplate reader (Model ELX-800, Bio-Tek Inc., VT, USA). The nitrite concentration was determined from a sodium nitrite standard curve. The threshold for detection with this assay was approximately 0.5 μM.

MTT Assay

Mitochondria function of VSMCs, an index of cell survival, was determined by colorimetric assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Cat. NO. M2128, Sigma) (28). MTT was diluted to 200 μM in DMEM/F12 solution and added to cultures for 2 hours at 37°C. The MTT formazan product was released from microglial cells by addition of dimethylsulphoxide (Sigma) and measured at 570 nm against the 630 nm reference with an UV microplate reader. Cell viability was represented as the optical density values in comparison to vehicle control.

Statistics

Data are given as mean ± standard error of mean (SEM) of *n* observations. Statistical differences were first determined by one-way ANOVA followed by

Bonferroni *t*-test for *post hoc* multiple comparisons. A significant level of ($\alpha = 0.05$ and $P < 0.05$ was applied to all tests.

Results

To characterize NO production and cell cytotoxicity on VSMCs following LPS application, cultured VSMCs were exposed with various concentrations of LPS (1-100 μg/ml). Figure 1A shows a concentration- and time-dependent increase in nitrite production in cultured VSMCs. In addition, the cell viability indicated by the MTT level was decreased by LPS at the concentrations ranging between 30 and 100 μg/ml. It was likely that LPS (below 10 μg/ml) used in the present study could be a non-cytotoxic level to cultured VSMCs for 48 hours. We therefore applied 10 μg/ml LPS to study the modulation of LPS-induced NO signaling on CREB$^{Ser-133}$ phosphorylation throughout the present study, without introducing significant cytotoxic effects on cultured VSMCs.

To determine whether LPS (1-10 μg/ml) altered iNOS expression and CREB$^{Ser-133}$ phosphorylation in cultured VSMCs, experiments were performed to measure iNOS expression and CREB$^{Ser-133}$ phosphorylation from the same cultures. As illustrated in Figure 2, both the magnitudes of iNOS expression (Fig. 2A) and CREB$^{Ser-133}$ phosphorylation (Fig. 2B) were significantly increased by a 48-hr exposure of LPS, as compared to the vehicle-control cultures.

To further determine whether NOS expression (e.g., inducible NOS) following LPS exposure was involved in the enhancement of CREB$^{Ser-133}$ phosphorylation seen in Figure 2, a NOS inhibitor, N^{ω}-nitro-L-arginine methyl ester (L-NAME) (3, 4), was applied to the cultures. The use of 100-500 μM L-NAME blocked not only LPS-induced NO production (Fig. 3A), but also CREB$^{Ser-133}$ phosphorylation (Fig. 3B).

It has been showed that signaling pathways resulted from NO effects on cells appeared to stimulate guanylyl cyclase (GC), and hence led to consequent activation of cGMP-dependent protein kinase (PKG) (13, 19). To investigate whether the sequential mechanism, NO-GC-PKG, was involved in LPS-induced CREB$^{Ser-133}$ phosphorylation seen in the present study, we applied a guanylyl cyclase inhibitor (1*H*-[1,2,4] oxadiazolo[4,3-a]quinoxalin-1-one methyl ester; ODQ) or a PKG inhibitor (Rp-8-[(4-Chlorophenyl) thio]-cGMPs Triethylamine; (Rp)-8-pCPT-cGMPs), into the cultured VSMCs (3, 4). Figure 4 shows that either 30 μM ODQ (Fig. 4A) or 20 μM (Rp)-8-pCPT-cGMPs (Fig. 4B) significantly attenuated the magnitudes of LPS-induced CREB$^{Ser-133}$ phosphorylation.

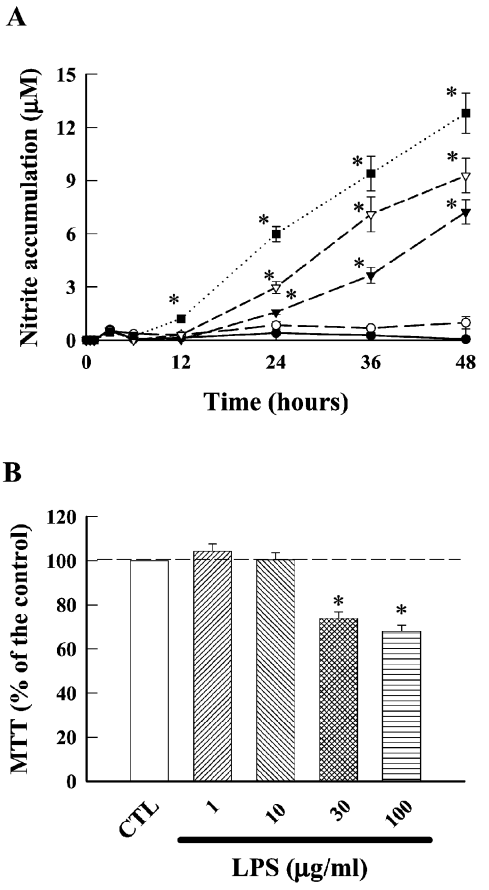

Fig. 1. Concentration-dependent and time-dependent effects of LPS-induced NO and cytotoxicity in cultured VSMCs. (A) Cultures were treated with vehicle-control medium or indicated concentrations of LPS. Culture supernatants were collected at indicated time points and were assayed for the production of nitrite (n = 18 experiments). LPS concentrations are as the follows: ●: vehicle-control; ○: 1 μg/ml; ▼: 10 μg/ml; ▽: 30 μg/ml; ■: 100 μg/ml. (B) Following a 48-hr exposure of various concentrations of LPS (1- 100 μg/ml), the damaged levels of VSMCs were determined by the measurement of MTT (n = 18 experiments). The results are the mean ± SEM. *, $p < 0.05$ compared to values for cultures treated with vehicle-control cultures.

Discussion

The present study shows that 1. A concentration-dependent increase in both iNOS expression and NO production are induced by bacterial LPS in cultured VSMCs. 2. An enhancement of CREB$^{Ser-133}$ phosphorylation activated by a sequential pathway, NO-GC-PKG, in cultured VSMCs exposed to bacterial LPS.

Nitric oxide is a highly effective autacoid with multitude of biological effects ranging from vasodilatation to cytotoxicity. Expression of the inducible NO synthase (iNOS) in vascular smooth muscle cells following exposure to microbes or microbial products, such as bacterial LPS, is frequently associated with a

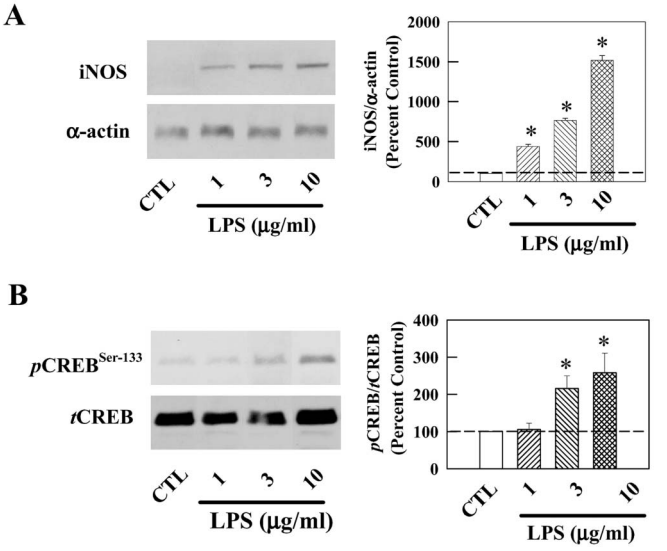

Fig. 2. Effects of LPS on iNOS expression and CREB$^{Ser\text{-}133}$ phosphorylation in cultured VSMCs. Cells were treated with the vehicle or the indicated concentrations of LPS for 48 hours. Representative immunoblots and averaged data of iNOS expression (A) and CREB$^{Ser\text{-}133}$ phosphorylation (B) are shown in graph forms (n = 9 experiments). Phospho-CREB$^{Ser\text{-}133}$ was immunoblotted with phospho-specific antibodies. Total CREB (*t*CREB) was immunoblotted with specific antibodies that are not sensitive to a phosphorylation state, as described in *Materials and Methods*. The results are the mean ± SEM. *, $p < 0.05$ compared to values for cultures treated with vehicle-control cultures.

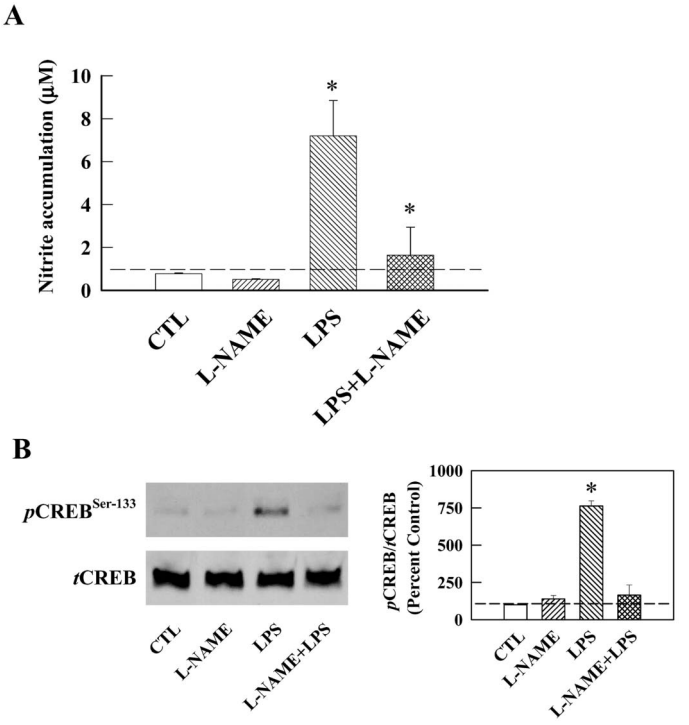

Fig. 3. LPS-induced NO production and CREB$^{Ser\text{-}133}$ phosphorylation in the absence or presence of an iNOS inhibitor in cultured VSMCs. (A) A summary of experiments in which L-NAME (100-500 μM) blocked NO production by indicated concentrations of 10 μg/ml LPS (n = 10 experiments). (B) Representative immunoblots and averaged data of CREB$^{Ser\text{-}133}$ phosphorylation are shown in graph forms (n = 10 experiments). L-NAME (100-500 μM) was added to the culture medium at lease 1 hr before 10 μg/ml LPS application and throughout the entire experiment. *, $p < 0.05$, compared to values for cultures treated with vehicle-control cultures.

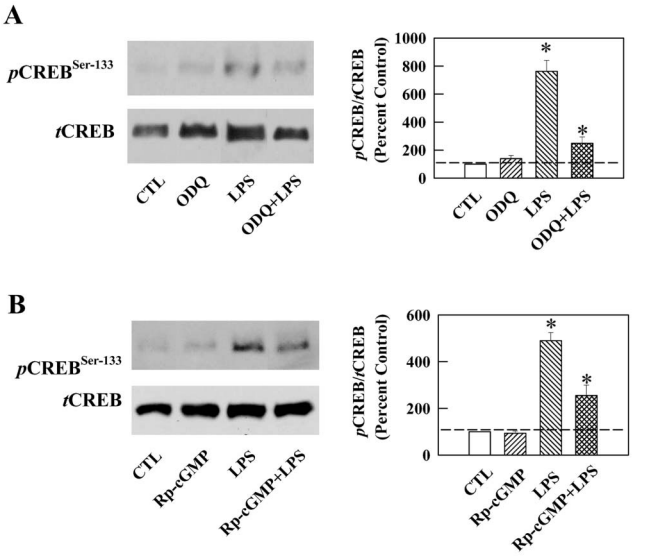

Fig. 4. Involvement of NO-GC-PKG signaling in LPS-induced CREB$^{Ser-133}$ phosphorylation in cultured VSMCs. LPS (10 μg/ml) was added into cultures in the presence of a guanylyl cyclase inhibitor (30 μM ODQ; n = 9 experiments; A) or a PKG inhibitor (20 μM (Rp)-8-pCPT-cGMPs; n = 7 experiments; B). Representative immunoblots and averaged data of CREB$^{Ser-133}$ phosphorylation are shown in graph forms. *, $p < 0.05$, compared to values for cultures treated with vehicle-control cultures.

generalized or localized inflammatory response resulting from infection or tissue injury (12, 15). As shown in the present study (Figs. 3 and 4), an enhancement of phosphorylated CREB$^{Ser-133}$, an important transcription factor for cell differentiation (2, 6-8, 14, 25), induced by NO-mediated signaling (NO-GC-PKG) following LPS exposure seemed to support a possibility regarding of an abnormal control of VSMCs differentiation under certain pathological status, such as bacterial infections that is of interest to the present study.

Previous observations suggest that differentiation of VSMCs could be highly dependent on local environmental signals, which while altered, could lead to quick alterations in the differentiated phenotype of VSMCs (10, 11, 15, 20, 22, 23, 29). Furthermore, vascular smooth muscle cell differentiation is an important component of vessel wall remodeling in response to injury, for example, after angioplasty or vein grafting, during atherosclerosis formation and inflammatory vasculitis (15, 17, 20, 23). Various kinases are capable of phosphorylating CREB, including cAMP-dependent protein kinase (PKA), PKC, Ras-dependent p105 kinase, Rsk-2, and Ca^{2+}/Calmodulin-dependent protein kinases (6-8). However, it remains unclear whether the sequential mechanism, NO-GC-PKG, induced by bacterial LPS is involved in the enhanced CREB$^{Ser-133}$ phosphorylation in cultured VSMCs. Indeed, as shown in the present study, L-NAME blocked not only the production of NO (Fig. 3A), but also the phosphorylation of CREB$^{Ser-133}$ (Fig. 3B). It is worth of noting that the use of ODQ or (Rp)-8-pCPT-cGMPs to selectively block NO-mediated signaling (13, 19) attenuated, but not blocked, the development of CREB$^{Ser-133}$ phosphorylation in cultured VSMCs, suggesting a possible involvement of NO-GC-PKG-independent mechanisms. Taken together, an enhanced CREB$^{Ser-133}$ phosphorylation was mediated by, at least in part, LPS-induced NO-GC-PKG signaling, and presumably altered VSMC differentiation (1, 9, 15, 19).

In summary, the present study underscores the possible importance of elucidating cellular and molecular mechanisms that may contribute a pathophysiological differentiation of VSMCs during bacterial inflammation as a prelude to understanding how these control processes might be altered in vascular diseases. Thus, the findings here may provide not only signaling pathway involved in VSMC differentiation during inflammatory response, but also new insight into possible therapeutic intervention.

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