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Downregulation of Endothelial Constitutive Nitric Oxide Synthase Expression by Lipopolysaccharide

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Lipopolysaccharide (LPS), a causal agent of sepsis, has been shown to induce systemic nitric oxide (NO) synthesis through complex mechanisms. However, the effect of LPS on endothelial cells is incompletely understood. To investigate the mechanism by which LPS influences the release of NO from endothelial cells, the effect of this compound on endothelial constitutive nitric oxide synthase (ecNOS) was studied in cultured bovine coronary venular endothelial cells. Western and Northern analyses showed that LPS decreased ecNOS expression at the protein and mRNA levels in a time-dependent and dose-responsive manner. Concurrent treatment of the endothelial cells with LPS and a transcription inhibitor, actinomycin D, resulted in decreased ecNOS mRNA within 8 hours. In contrast, treatment with actinomycin D had only a relatively insignificant effect on the ecNOS transcript level. This result suggests that the reduction of ecNOS by LPS resulted from an increased degradation rate of its transcript.

Lipopolysaccharide (LPS), a component of the cell walls of Gram-negative bacteria, has been implicated to be a causal agent of sepsis. Although systemic nitric oxide (NO) production is increased during sepsis (1), the LPS effect on NO release from endothelial cells is incompletely understood. LPS has been reported to decrease NO release by cultured bovine aortic (2) and murine vascular endothelial cells (3). However, LPS treatment of cultured endothelial cells from bovine aortas (4), porcine pulmonary arteries (5), porcine aortas, and human umbilical veins (6) has been reported to stimulate NO release. Furthermore, Walter *et al.* (3) reported that simultaneous treatment of cultured murine endothelial cells with LPS and interferon-γ (INFγ) increases NO production whereas MacNaul and Hutchinson (7) demonstrated that concurrent treatment of human aortic endothelial cells with interleukin-1β (IL-1β), tumor necrosis factor-α (TNFα), IFNγ, and LPS decreased the endothelial constitutive nitric oxide synthase (ecNOS) mRNA level. Recent studies demonstrated that inflammatory mediators, such as TNFα and IL-1β, reduce ecNOS protein (8) and mRNA (7-11) levels. However, the effect of LPS on ecNOS expression has not been demonstrated in the absence of the cytokines. On the basis of the above reports, we hypothesized that LPS alone might also affect the expression of ecNOS in cultured endothelial cells. To test this hypothesis, we used western and northern analyses to detect protein and mRNA levels of ecNOS isolated from cultured bovine coronary venular endothelial cells treated with and without LPS. In addition, an actinomycin D chase experiment was performed to determine the effect of LPS on ecNOS mRNA stability.

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Abbreviations: DMEM, Dulbecco's modified Eagle's medium; DPBS, Dulbecco's phosphate-buffered saline; ecNOS, endothelial constitutive nitric oxide synthase; EDTA, ethylenediaminetetraacetic acid; GAPD, glyceraldehyde-3-phosphate dehydrogenase; IL-1β, interleukin-1β; INFγ, interferon-γ; LPS, lipopolysaccharide; NO, nitric oxide; PSA, penicillin, streptomycin, amphotericin B; PMA, phorbol 12-myristate 13-acetate; PVDF, polyvinylidene fluoride; SDS, sodium dodecyl sulfate; TGFβ, transforming growth factor-β; TNFα, tumor necrosis factor-α.

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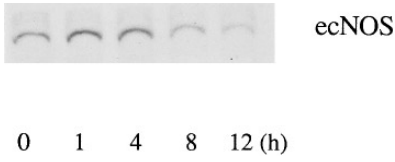

FIG. 1. Western blot (6 μg total protein/lane) showing the time course of the effect of LPS on the protein content of ecNOS in bovine coronary venular endothelial cells.

MATERIALS AND METHODS

Bovine coronary venular endothelial cell culture. Coronary venular endothelial cells were isolated from bovine hearts immediately after the death of the animal using a modification of the method described by Schelling *et al.* (12). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma Chemical Co., St. Louis, MO) supplemented with 10% fetal bovine serum (Summit Biotechnology, Ft. Collins, CO), 2 mM glutamine (Sigma), 2 mM sodium pyruvate (Sigma), 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B (PSA; Sigma). When passaging, the cells were mobilized with 0.25% trypsin (Gibco Laboratories, Grand Island, NY) in Dulbecco's phosphate buffered saline (DPBS; Sigma) with 0.018% ethylenediaminetetraacetic acid (EDTA; Sigma) and plated onto 60 mm sterile plastic tissue culture plates coated with 1.5% (w/v) gelatin (Sigma) in DPBS.

Cells from the 17^{th}-19^{th} passage at 3 days after confluency were used in this study. For the investigation of the time course of LPS effects, media with and without 2.5 μg/ml LPS (*Escherichia coli* serotype 0111:B4; Sigma) were used to treat the cells for 1, 4, 8, and 12 h before mRNA or protein isolation. For the investigation of dose response of LPS, cells were cultured in DPBS containing 0, 0.02, 0.1, 0.5, and 2.5 μg/ml of LPS for 8 h prior to mRNA isolation. In experiments which require inhibition of transcription, cells were concurrently treated with actinomycin D (10 μg/ml) and with or without LPS (2.5 μg/ml) and harvested at 0, 4, and 8 h for mRNA isolation.

Western blot analysis. Protein extraction was conducted as described previously (13). Equal quantities of proteins were separated by electrophoresis on 8% sodium dodecyl sulfate (SDS)-polyacrylamide gels. The separated proteins were transferred to polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, MA), which were then blocked by 5% dry milk in Tris-buffered saline solution (pH 7.6) containing 0.1% Tween. The membranes were then incubated with a 1:2000 dilution of a rabbit polyclonal antibody (Transduction Laboratory, Lexington, KT) against a 20.4 kDa protein fragment corresponding to amino acids 1030-1209 of human ecNOS. The membranes were then incubated with a 1:2500 dilution of a donkey anti-rabbit immunoglobulin conjugated to horse radish peroxidase (Amersham, Arlington Heights, IL). Signals were detected using the ECL detection system (Amersham) and autoradiography film.

RNA isolation. Total RNA was isolated from cultured cells using a method described by Sambrook *et al.* (13) and modified by Lu *et al.* (14). Cells were lysed in 10 mM EDTA (pH 8.0) containing 0.5% SDS. The plates were rinsed with an equal volume of 0.1 M sodium acetate (pH 5.2) and 10 mM EDTA (pH 8.0). After phenol extraction and centrifugation, RNA in the supernatant was precipitated with NaCl and ethanol. The precipitated RNA pellet was redissolved in guanidine-HCl buffer (7.5 M guanidine-HCl, 25 mM Tes-NaOH buffer, 10 mM EDTA, 100 μM 2-mercaptoethanol, pH 7.5). A 7/13 volume of 10 M LiCl was added to precipitate the RNA. The pellet was dissolved in TE buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, pH 8.0) and served as the RNA sample.

Northern blot analysis. Aliquots from each RNA sample (20 μg) were separated by electrophoresis on a 1% (w/v) agarose gel with 0.66 M formaldehyde and 1*X* MOPS buffer (0.2 M MOPS, 50 mM sodium acetate, 10 mM EDTA, pH 7.0). The separated RNA on the gel was transferred to a nylon membrane (Genescreen, NEN Research Products, Boston, MA) by capillary action. The 1.4 kb *Bst*XI fragment at the 5′ end of the coding region of bovine aortic ecNOS (9) was used as the probe for the ecNOS message. As a control, a plasmid containing human glyceraldehyde-3-phosphate dehydrogenase (GAPD) from American Type Culture Collection (ATCC Number: 57090) was used to probe the GAPD message. The DNA fragments were labeled with [α-^{32}P]dCTP using a random primer labeling kit (Gibco Laboratories). Hybridization and washing procedure followed the protocol provided by the membrane manufacturer (NEN Research Products).

RESULTS

LPS decreases ecNOS protein level. To investigate the effect of LPS on ecNOS expression, western blot analysis was used to quantify the ecNOS protein level. Figure 1 shows the time course of the LPS effect on the ecNOS protein level in extracts from endothelial cells treated with 2.5 μg/ml of LPS. The amount of protein detected started to decrease after 4 h of LPS treatment, and the trend continued as time increased. Without LPS treatment, the decrease in

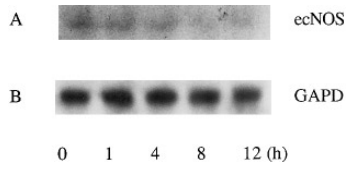

FIG. 2. Northern blot (20 μg total RNA/lane) showing the time course of the effect of LPS on the expression of ecNOS mRNA in bovine coronary venular endothelial cells. (A) ecNOS mRNA. (B) GAPD mRNA as a control.

ecNOS protein was minor and was much less significant than in the presence of LPS. (Data not shown.) This result revealed that LPS reduced the level of ecNOS protein and that the reduction is time-dependent.

Downregulation of ecNOS mRNA level by LPS. To determine whether the effect of LPS on the ecNOS expression is exerted at the mRNA level, northern analysis was used to detect the ecNOS message in cells treated with 2.5 μg/ml of LPS. Figure 2 shows the time course of the effect of LPS on the expression of ecNOS message. The ecNOS transcript diminished with time in the presence of LPS. This observation was consistent with the result at the protein level. Hybridization with labeled GAPD probe showed that LPS had no effect on the GAPD transcript level, suggesting that the reduction of ecNOS transcript level by LPS was specific to some degree. This observed LPS effect on ecNOS transcript level was dose-dependent as shown in Fig. 3 in which cells were treated with different concentrations of LPS (0, 0.02, 0.1, 0.5, and 2.5 μg/ml) for 8 h. The ecNOS mRNA level was reduced significantly by LPS at concentrations higher than 0.1 μg/ml.

LPS reduces the stability of ecNOS mRNA. Since the above result shows that LPS decreases the steady-state level of ecNOS mRNA, the effect may be exerted at the transcription initiation level or the mRNA stability level. To determine the stability of ecNOS mRNA, an actinomycin D chase experiment was performed. Cells were concurrently treated with actinomycin D (10 μg/ml) with or without LPS (2.5 μg/ml), and the total RNA was isolated and analyzed by electrophoresis. Figure 4 shows that the ecNOS mRNA level was reduced significantly after 4 h of LPS treatment in the presence of actinomycin D while GAPD mRNA levels did not change appreciably. Since the RNA synthesis was blocked by actinomycin D, the reduction in ecNOS mRNA by LPS can be attributed mainly to an increased degradation rate of ecNOS transcripts.

DISCUSSION

Although ecNOS has been described as constitutive, its expression can be regulated by mechanical, biological, and pharmacological factors. The positive factors influencing its expression include shear stress (9), cyclic strain (15), proliferation (16), estrogen (17), transforming growth factor-β (TGFβ) (18), lysophosphatidylcholine (19), staurosporine (20), chelerythrine

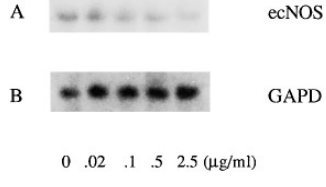

FIG. 3. Northern blot (20 μg total RNA/lane) showing the dose-dependent effect of LPS on the level of ecNOS mRNA in bovine coronary venular endothelial cells. (A) ecNOS mRNA, (B) GAPD mRNA as a control.

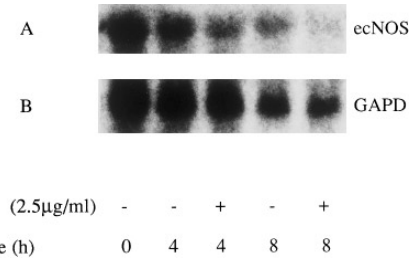

FIG. 4. Northern blot (20 μg total RNA/lane) showing the stability of ecNOS mRNA in bovine coronary venular endothelial cells treated with actinomycin D and with (+) or without (−) LPS (A) ecNOS mRNA. (B) GAPD mRNA as a control.

(20), and phorbol 12-myristate 13-acetate (PMA) (20) whereas the negative factors include hypoxia (21), TNFα (8), IL-1β (10), fibrin monomers (22), fibrin degradation products (22), and the combination of IL-1β, TNFα, INFγ, and LPS (7). Our results indicate that LPS by itself causes decreased ecNOS protein and mRNA levels in cultured bovine coronary venular endothelial cells, and the effect is attributed to the enhanced mRNA degradation in the presence of LPS.

The LPS effect is not observable from the protein and mRNA measurements at 4 h (Fig. 1), but it becomes significant after 8 h of treatment. This finding may explain the result of Walter *et al.* (3), who reported that nitrite production from cultured murine endothelial cells was unchanged at 6 h but decreased after 12 h and remained decreased at 48 h of LPS treatment. However, Myers *et al.* (2) detected the inhibitory effect on NO production from cultured bovine endothelial cells after only 1 h of LPS treatment followed by 1 h of incubation in LPS-free medium. In an earlier report, Salvemini *et al.* (4) used platelet-inhibitory activity as a measurement of NO to showed that LPS enhanced the release of an NO-like factor from cultured bovine endothelial cells within 1 min. In agreement with this rapid response, Fleming *et al.* (5) showed enhanced synthesis of NO within 5 min by the measuring intracellular cGMP concentrations of cultured human umbilical vein and porcine aortic endothelial cells. In contrast to Walter *et al.* (3), Cendar *et al.* (6) showed that LPS increased NO release from cultured porcine pulmonary artery endothelial cells after 24 h and that it remained increased at 48 h. These discrepancies may be attributed to the complex factors that control NO release, such as tetrahydrobiopterin synthesis, L-arginine transport, and the intracellular Ca^{2+} concentration in addition to ecNOS gene expression. It is possible that LPS affects several pathways that are involved in NO release, and the combined effect on these pathways is complex and sensitive to experimental conditions and the vascular location from which the endothelial cells are isolated. For example, LPS has been shown to increase the biosynthesis of tetrahydrobiopterin in human umbilical vein endothelial cells (23). Furthermore, Durante *et al.* (24) reported that while LPS enhanced L-arginine uptake (2.5-fold) from bovine aortic endothelial cells at 24 h of treatment, the NO release from the cells was not affected. In any case, our data demonstrate the direct LPS effect on ecNOS expression for the first time. Detailed LPS effects on the expression and activity of all enzymes that contribute to NO release are needed for a complete explanation of LPS effect on NO release.

MacNaul and Hutchinson (7) reported that simultaneous treatment of human aortic endothelial cells with IL-1β, TNFα, INFγ, and LPS for 8 h resulted in decreased ecNOS mRNA level. Our results are consistent with theirs and further demonstrate that LPS alone causes the downregulation of ecNOS. The mechanism by which the different mechanical, biological, and pharmacological factors regulate ecNOS gene expression have not been elucidated, but roles of Sp1 transcription factor (25,26) and protein kinase C (20) have been suggested to participate

in the regulation of ecNOS transcription. The actinomycin D chase experiment (Fig. 4) indicates that ecNOS protein and mRNA attenuation by LPS is due to an increase in the degradation rate of ecNOS mRNA. This finding is similar to the finding of Yoshizumi *et al.* (10), who suggest that TNFα downregulates ecNOS mRNA by shortening its half-life. However, the mechanism regulating the mRNA stability remains to be investigated.

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