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Mechanisms of Endotoxin Tolerance in Human Intestinal Microvascular Endothelial Cells

Hitoshi Ogawa,* Parvaneh Rafiee,†‡ Jan Heidemann,* Pamela J. Fisher,* Nathan A. Johnson,* Mary F. Otterson,*‡§ Balaraman Kalyanaraman,¶ Kirkwood A. Pritchard, Jr., †‡¶ and David G. Binion*‡§¶

Lipopolysaccharide (endotoxin) tolerance is well described in monocytes and macrophages, but is less well characterized in endothelial cells. Because intestinal microvascular endothelial cells exhibit a strong immune response to LPS challenge and play a critical regulatory role in gut inflammation, we sought to characterize the activation response of these cells to repeated LPS exposure. Primary cultures of human intestinal microvascular endothelial cells (HIMEC) were stimulated with LPS over 6–60 h and activation was assessed using U937 leukocyte adhesion, expression of E-selectin, ICAM-1, VCAM-1, IL-6, IL-8, manganese superoxide dismutase, HLA-DR, and CD86. Effect of repeat LPS stimulation on HIMEC NF-κB and mitogen-activated protein kinase (MAPK) activation, generation of superoxide anion, and Toll-like receptor 4 expression was characterized. LPS pretreatment of HIMEC for 24–48 h significantly decreased leukocyte adhesion after subsequent LPS stimulation. LPS pretreatment inhibited expression of E-selectin, VCAM-1, IL-6, and CD86, while ICAM-1, IL-8, and HLA-DR were not altered. Manganese superoxide dismutase expression increased with repeated LPS stimulation, with a reduction in intracellular superoxide. NF-κB activation was transiently inhibited by LPS pretreatment for 6 h, but not at later time points. In contrast, p44/42 MAPK, p38 MAPK, and c-Jun N-terminal kinase activation demonstrated inhibition by LPS pretreatment 24 or 48 h prior. Toll-like receptor 4 expression on HIMEC was not altered by LPS. HIMEC exhibit endotoxin tolerance after repeat LPS exposure in vitro, characterized by diminished activation and intracellular superoxide anion concentration, and reduced leukocyte adhesion. HIMEC possess specific mechanisms of immunoregulatory hyporesponsiveness to repeated LPS exposure. The Journal of Immunology, 2003, 170: 5956–5964.

Lipopolysaccharide (endotoxin), a major component of the outer surface of Gram-negative bacteria, is a potent activator of both immune and nonimmune cells. Among non-immune cells, vascular endothelial cells are known to undergo activation in response to LPS stimulation, with enhanced expression of cell adhesion molecules (CAM) and cytokines/chemokines. Endothelial activation by LPS leads to enhanced leukocyte adhesion and tissue recruitment from the circulation, a key regulatory step in the inflammatory response. We have reported that cultured human intestinal microvascular endothelial cells (HIMEC) are also strongly activated by bacterial LPS, as demonstrated by significantly enhanced CAM expression and increased leukocyte-binding activity (1). As described in monocytes and macrophages, endothelial cells are also believed to undergo activation through ligation of Toll-like receptor 4 (TLR4), a recently identified receptor and signal transducer of LPS (2–4). Ligation of TLR4 leads to recruitment of MyD88, phosphorylation of IL-1R-associated kinase, oligomerization of TNFR-associated factor 6, and subsequent degradation of IκB. These events lead to the activation of NF-κB, and resultant transcription of immune response genes (2).

Although LPS is a potent cellular activator, repeated endotoxin challenge may lead to a temporary insensitivity of cells toward subsequent LPS stimulation. This phenomenon of LPS tolerance has been described in vivo with reduced LPS lethality in mice pretreated with low-dose LPS (5), as well as in vitro with a reduced capacity of monocytes isolated from septic patients to release proinflammatory cytokines in response to further LPS stimulation (6). Furthermore, repeated LPS stimulation of isolated macrophages or monocytes leads to a reduced production of proinflammatory cytokines such as TNF-α or IL-1β (7, 8). LPS tolerance is considered an adaptive response, which evolves as a protective mechanism to curb the infected host’s continuous response to the invading bacterial organisms, and thereby reduce the autotoxic effect precipitated by overproduction of inflammatory mediators (9, 10).

Because the intestinal mucosal surfaces are in continuous contact with luminal bacteria and their products, it can be expected that the mucosal immune system possesses immunoregulatory mechanisms to avoid an enhanced, destructive inflammatory response toward enteric flora and endotoxin. We hypothesized that microvascular endothelial cells from the human intestine exhibit specific mechanisms for LPS tolerance, given their proximity to the enteric flora and their otherwise strong innate response to LPS.

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3 Abbreviations used in this paper: CAM, cell adhesion molecule; HIMEC, human intestinal microvascular endothelial cell; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; Mn-SOD, manganese superoxide dismutase; TLR4, Toll-like receptor 4.
In this study, we demonstrate endotinotolerance in primary cultures of HIMEC. HIMEC demonstrate a regulated physiologic response to repeat LPS challenge, mediated in part through inhibition of mitogen-activated protein kinase (MAPK) signaling cascades, altered cytokine and CAM expression, enhanced oxynradical defense via increased expression of manganese superoxide dismutase (Mn-SOD), and diminished intracellular superoxide anion, which result in significantly reduced leukocyte adhesion.

Materials and Methods

Isolation and culture of HIMEC

HIMEC isolation was performed, as described previously (1). In brief, surgical specimens obtained from small intestine or colon were rinsed, and mucosal strips were dissected, washed, and digested in a type II collagenase solution (Worthington Biochemical, Freehold, NJ). Microvascular endothelial cells were extruded by mechanical compression and plated onto fibronectin-coated tissue culture dishes in growth medium (MCDB 131 medium; Sigma-Aldrich, St. Louis, MO) containing 20% FBS and endothelial cell growth supplement (Upstate Biotechnology, Lake Placid, NY). After 7–10 days of culture, endothelial cell clusters were physically isolated, and a pure culture was obtained. All experiments were conducted on cultures between passages 8 and 14.

Bacterial LPS

LPS (Escherichia coli 0111:B4; Sigma-Aldrich) was used for both single and repeat LPS challenge, mediated in part through inhibition of HIMEC. HIMEC demonstrate a regulated physiologic response to repeat LPS challenge, mediated in part through inhibition of Mn-SOD, and diminished intracellular superoxide anion, which result in significantly reduced leukocyte adhesion.

RNA preparation and semiquantitative RT-PCR

Total RNA was extracted from HIMEC using TRIzol (Life Technologies, Rockville, MD) and treated with DNase I, amplification grade (Life Technologies), according to the manufacturer’s instruction. The cDNA was generated from 1 μg of total RNA with oligo(dT) primer using Superscript First-Strand Synthesis System for RT-PCR (Life Technologies), according to the protocol, and filled up to 80 μl. A total of 4 μl of cDNA solution was used for RT-PCR in a total volume of 40 μl containing 0.5 μM of sense and antisense primers. The specific primers, the size of PCR products, and the number of PCR amplification cycles for each assay are shown in Table I. PCR amplifications were performed at 94°C for 1 min, 60°C for 1 min, 72°C for 1 min, and followed by final extension for 7 min. A total of 20 μl of PCR product was visualized on 1% agarose gels stained with ethidium bromide.

Assessment of E-selectin, ICAM-1, and VCAM-1 surface expression on HIMEC

HIMEC surface expression of E-selectin, ICAM-1, and VCAM-1 was examined using a RIA, as described previously (1). Briefly, monolayers were incubated with mouse anti-human E-selectin, ICAM-1, or VCAM-1 mAbs (R&D Systems) for 60 min at 4°C, rinsed, then incubated with goat anti-mouse biotinylated Fab’ fragment (Jackson ImmunoResearch Laboratories, West Grove, PA) for 60 min at 4°C. After washing, 125I-labeled streptavidin (5 μCi/ml) (Amersham Bioscience, Piscataway, NJ) was applied to wells to detect adherent secondary Ab. After unbound radioactivity was washed off, cells were lysed with 1.0% (v/v) Triton X-100 in medium, and radioactivity was quantified in a gamma counter. Data from triplicate wells were expressed as a mean of 125I-labeled streptavidin bound (cpm/well) ± SEM.

Measurement of IL-6 and IL-8 in HIMEC culture supernatant by ELISA

IL-6 or IL-8 concentrations in conditioned medium were measured using commercially available ELISA kits (R&D Systems), according to the manufacturer’s protocol. Experiments were conducted in triplicate, and results are shown as mean picograms per milliliter ± SEM.

SDS-PAGE and Western blotting

For Mn-SOD protein expression, cells were lysed in buffer containing 20 mM HEPES, 150 mM NaCl, 1% (v/v) Nonidet P-40, 0.1% (w/v) SDS, 1 mM EDTA, and 0.5% (v/v) protease inhibitor cocktail (protease inhibitor cocktail III; Calbiochem, San Diego, CA), and incubated on ice for 60 min. Lysates were centrifuged for 10 min at 10,000 × g, and resulting supernatants were used. A total of 10 μg of protein per lane was separated by SDS-PAGE, and transferred onto nitrocellulose membranes. After blocking in PBS containing 2% (w/v) BSA and 3% (w/v) nonfat dry milk, blots were washed three times in TBST (10 mM Tris-CI, 150 mM NaCl, 0.1% (v/v) Tween 20, pH 7.4) and probed overnight at 4°C with anti-Mn-SOD polyclonal Ab (Upstate Biotechnology). Following three washes in TBST, membranes were incubated for 1 h at room temperature with secondary HRP-conjugated anti-rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA). Bands were detected using ECL reagents (Amersham Bioscience).

Table I. Specific primers and conditions for semiquantitative RT-PCR

<table>
<thead>
<tr>
<th>Endothelial Gene</th>
<th>Size (bp) of PCR Products</th>
<th>PCR Cycles</th>
<th>LPS Stimulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>E-selectin</td>
<td>610</td>
<td>30</td>
<td>3 h</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>238</td>
<td>35</td>
<td>12 h</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>241</td>
<td>25</td>
<td>3 h</td>
</tr>
<tr>
<td>IL-6</td>
<td>628</td>
<td>28</td>
<td>3 h</td>
</tr>
<tr>
<td>IL-8</td>
<td>157</td>
<td>30</td>
<td>12 h</td>
</tr>
<tr>
<td>Mn-SOD</td>
<td>306</td>
<td>30</td>
<td>12 h</td>
</tr>
<tr>
<td>HLA-DRα</td>
<td>584</td>
<td>35</td>
<td>12 h</td>
</tr>
<tr>
<td>CD86</td>
<td>834</td>
<td>35</td>
<td>12 h</td>
</tr>
<tr>
<td>β-actin</td>
<td>436</td>
<td>25</td>
<td>3 h</td>
</tr>
</tbody>
</table>

Primers (sense and antisense)

E-selectin: 5′-CCAGTGCTTATGTGTCAGC-3′, 5′-CATTCACTGAGGTT-3′

ICAM-1: 5′-TTAGCGACGACCTTCCCT-3′, 5′-CATATCTGAGCTACC-3′

VCAM-1: 5′-CCCTGAGCGGCTGGATT-3′, 5′-CCTGAGAGCAGCATACAATG-3′

IL-6: 5′-ATGACCTCTGCAACACCGC-3′, 5′-GAAGAGCCCTACCGTGAC-3′

IL-8: 5′-ATGTCACAAACCTTTCCACC-3′, 5′-AATCTTCAGCAACCCCTTG-3′

Mn-SOD: 5′-GGTCTGCTGACCACTGACAGT-3′, 5′-GATCTTTCGATGACAATG-3′

HLA-DRα: 5′-CGCAAAGTTTCACCCCCACTG-3′, 5′-CAGAAAGGGCAATAGACAG-3′

CD86: 5′-CTTTTGATGGCCTCTCG-3′, 5′-CCCTCCCCTTCATTGTTG-3′

β-actin: 5′-CCAGAACGAGAGAGCATTCC-3′, 5′-CCTGTTGCGGGAAGCT-3′

$^{5957}$The Journal of Immunology
For the detection of MAPK activation, cells were lysed in buffer containing 10 mM sodium orthovanadate (pH 10), 40 mM β-glycerolphosphate, and 20 mM sodium fluoride, and incubated on ice for 30 min. A total of 15 μg of protein per lane was separated by SDS-PAGE, and Western blotting was performed, as described above, using anti-total or phospho-p44/42, or phospho-p38 MAPK, or stress-activated protein kinase/c-Jun N-terminal kinase (JNK) polyclonal Ab (Cell Signaling Technology, Beverly, MA).

Western blotting for TLR4 protein expression in HIMEC was performed using cell membrane-enriched protein fractions (12). Briefly, cells were washed and homogenized in lysis buffer containing 50 mM Tris-HCl buffer (pH 7.4), protease inhibitor cocktail, and 1 mM EDTA. The homogenate was centrifuged at 10,000 rpm for 10 min. The supernatant was collected and centrifuged at 48,000 × g for 10 min. The resulting pellet (membrane protein-enriched fraction) was resuspended in lysis buffer, and 20 μg of membranes were separated through a 4% polyacrylamide gel. The proteins were transferred to nitrocellulose membranes and immunoblotted using anti-TLR4 polyclonal Ab (H80; Santa Cruz Biotechnology).

Assessment of intracellular superoxide production

HIMEC were grown to confluence in 24-well tissue culture dishes. After stimulation with LPS for 2 h with or without LPS pretreatment, hydroethidine (500 μM; Calbiochem), poly(dI-dC) (Amersham Bioscience), 5 μM Ca2+ (Calbiochem), JNK (SP600125, 10 μM; Calbiochem), p38 MAPK (SB203580, 5 μM; Calbiochem), p44/42 MAPK (Bay11, 2.5 μM; Calbiochem) were added. The homogenate was centrifuged at 48,000 × g for 30 min. Reaction products were separated through a 4% polyacrylamide gel and autoradiographed at 5958 ENDOTOXIN TOLERANCE IN INTESTINAL ENDOTHELIAL CELLS

Effect of LPS pretreatment on HIMEC gene expression

To examine whether intracellular signaling through p44/42 MAPK, p38 MAPK, JNK, or NF-κB is characterized by alterations in gene expression and phenotype. Our next series of experiments were performed to characterize alterations in gene expression in HIMEC exposed to repeated LPS stimulation using semi-quantitative RT-PCR (Fig. 2). E-selectin significantly increased following initial LPS activation, but was attenuated by endotoxin pretreatment 24 and 48 h prior. In marked contrast, ICAM-1 mRNA expression remained significantly decreased by endotoxin pretreatment for 24 and 48 h before assay (Fig. 1, A and B).

Effect of repeated endotoxin stimulation on HIMEC gene expression

LPS stimulation is known to induce endothelial activation, which is characterized by alterations in gene expression and phenotype. Our next series of experiments were performed to characterize alterations in gene expression in HIMEC exposed to repeated LPS stimulation using semi-quantitative RT-PCR (Fig. 2). E-selectin significantly increased following initial LPS activation, but was attenuated by endotoxin pretreatment 24 and 48 h prior. In marked contrast, ICAM-1 mRNA expression remained significantly decreased by endotoxin pretreatment for 24 and 48 h before assay (Fig. 1, A and B).
remained at high levels despite LPS pretreatment. ICAM-1 and IL-8 mRNA were significantly increased following initial LPS activation, but was attenuated by prior LPS exposure. Mn-SOD mRNA was strongly enhanced following 48-h LPS exposure in HIMEC. However, in contrast to HLA-DR, CD86 mRNA was up-regulated by initial LPS stimulation, and the increased expression was not altered by prior LPS exposure. E-selectin expression induced by LPS stimulation was significantly inhibited by LPS pretreatment for 48 h (Fig. 3C). These data confirm that the alterations in gene expression seen at the mRNA level are also demonstrated in protein expression in HIMEC undergoing repeated exposure to LPS. Finally, experiments were performed to confirm that these CAM were involved in the adhesion of U937 monocytes to LPS-activated HIMEC under flow conditions. Anti-E-selectin and VCAM-1 mAbs significantly inhibited U937 binding to LPS-stimulated HIMEC (Fig. 3D).

**Effect of repeated endotoxin stimulation on HIMEC CAM surface expression**

LPS stimulation of HIMEC induced a significant increase in E-selectin expression at 6 h. LPS pretreatment of HIMEC for 24 or 48 h significantly inhibited the E-selectin expression following LPS stimulation (Fig. 3A). In contrast, ICAM-1 expression in HIMEC after LPS stimulation was not significantly altered by LPS pretreatment (Fig. 3B). VCAM-1 surface expression was significantly inhibited by LPS pretreatment for 48 h (Fig. 3C). These data suggest that the decreased inflammatory function demonstrated in HIMEC exposed to repeated LPS did not represent exhausted cells, but instead, was a physiologic result of modulated gene expression.

<table>
<thead>
<tr>
<th>Pre-treatment</th>
<th>LPS stimulation</th>
<th>E-selectin</th>
<th>ICAM-1</th>
<th>VCAM-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPS 0.5 μg/ml</td>
<td>(-) 24 h</td>
<td>(-) 24 h</td>
<td>(+) 48 h</td>
<td>(+) 48 h</td>
</tr>
<tr>
<td>No RT</td>
<td></td>
<td></td>
<td>(+)</td>
<td>(+)</td>
</tr>
</tbody>
</table>

**FIGURE 2.** Effect of repeat LPS stimulation on HIMEC gene expression. Semiquantitative RT-PCR for E-selectin, ICAM-1, VCAM-1, IL-6, IL-8, Mn-SOD, HLA-DRα, and CD86 mRNA expression in HIMEC after repeated LPS stimulation. HIMEC were stimulated with LPS (1 μg/ml) with or without pretreatment with LPS (0.5 μg/ml) for 24 or 48 h, as indicated in the figure. After stimulation, total RNA was extracted and subjected to semiquantitative RT-PCR to assess mRNA expression. To verify that these bands were amplified from cDNA and not from genomic DNA, samples without reverse transcription were used for each PCR and shown in the last lane. E-selectin, IL-6, and CD86 mRNA expression significantly increased following initial LPS activation, but was attenuated by endotoxin pretreatment 24 and 48 h prior. ICAM-1 and IL-8 mRNA were also up-regulated by initial LPS stimulation, and were not affected by prior LPS exposure. VCAM-1 expression decreased following 48-h LPS pretreatment. Mn-SOD and HLA-DRα mRNA were enhanced by LPS, and remained at high levels despite LPS pretreatment. β-actin served as an internal control. These data indicate that specific genes are either increased or diminished following repeated rounds of endotoxin exposure. Representative gels depicting HIMEC gene expression are shown, from three total independent experiments.

Both IL-6 and IL-8 mRNA were up-regulated by initial LPS stimulation. However, the magnitude of IL-6 mRNA induction was attenuated by LPS pretreatment, while increases in IL-8 expression were not affected by prior LPS exposure.

Enzymatic oxyradical defense has been described in endothelial cells and plays an essential role in the down-regulation of activation, as increased intracellular levels of superoxide anion mediate endothelial activation (14). Mn-SOD mRNA was strongly enhanced by LPS, and remained at high levels despite LPS pretreatment.

Endothelial cells are believed to function as nonprofessional APCs (15), and we also examined the effect of sustained LPS activation on expression of established (HLA-DRα) and novel co-stimulatory molecules (CD86) in HIMEC. HLA-DRα mRNA was induced by LPS stimulation, and the increased expression was not altered by prior LPS exposure. CD86 was also induced by initial LPS stimulation in HIMEC. However, in contrast to HLA-DR, CD86 mRNA induction was significantly attenuated by LPS pretreatment. Taken together, these data suggest that a differential response to LPS challenge following endotoxin pretreatment is seen in HIMEC, in which specific genes are either increased or diminished following repeated rounds of endotoxin exposure. These data also suggest that the decreased inflammatory function demonstrated in HIMEC exposed to repeated LPS did not represent exhausted cells, but instead, was a physiologic result of modulated gene expression.
Effect of repeated endotoxin stimulation on cytokine secretion by HIMEC

Both IL-6 and IL-8 secretion from cultured HIMEC monolayers was increased in response to initial LPS stimulation for 6 h. LPS pretreatment significantly diminished the IL-6 secretion by subsequent LPS challenge (Fig. 4A), while IL-8 secretion was not significantly altered (Fig. 4B). Repeated LPS exposure did not completely inhibit the ability of HIMEC to generate IL-6, suggesting that these cells were still able to mount an inflammatory response in the face of prolonged endotoxin exposure.

Repeated endotoxin stimulation enhances HIMEC oxyradical defense

Previous work in endothelial cells has demonstrated that the generation of superoxide anion is integrally linked to activation (14). One of the essential enzymatic intracellular mechanisms for quenching superoxide anion is Mn-SOD. We examined the protein expression of Mn-SOD in HIMEC following repeated exposure to LPS. Mn-SOD protein expression was enhanced by LPS stimulation at 6 h. In marked contrast to the effects seen in CAM, IL-6, and IL-8 production, repeat LPS treatment resulted in a significant increase in Mn-SOD protein expression throughout the evaluation period (Fig. 5A). This data implied that enhanced oxyradical enzymatic defense was a feature of repeated LPS exposure in HIMEC. Unlike the other molecules investigated, the rise in Mn-SOD protein detected in HIMEC cultures repeatedly stimulated with LPS suggests that this enzyme accumulates during chronic endotoxin activation, resulting in a progressive rise in protein, higher than the increase in mRNA that appeared to plateau during the evaluation period.

Repeated endotoxin stimulation diminishes intracellular superoxide generation during subsequent HIMEC activation

We examined the functional sequelae of increased Mn-SOD expression in LPS-activated HIMEC using hydroethidine, an intravital dye used for the detection of superoxide and fluorescence microscopy of live HIMEC monolayers. Hydroethidine passes freely into live cells, and will react rapidly with superoxide anion, resulting in the generation of ethidine, which binds nuclear DNA, generating a nuclear pattern of fluorescence. Unstimulated HIMEC displayed low overall fluorescence intensity when examined using hydroethidine treatment (Fig. 5B). LPS stimulation of HIMEC resulted in bright nuclear staining in a large proportion of cells (Fig. 5C), indicating the generation of superoxide after initial exposure to endotoxin. However, in marked contrast, fluorescence intensity was significantly diminished in the LPS-pretreated HIMEC (Fig. 5D and E). Using NIH Image analysis software, fluorescence intensity returned to baseline in the LPS-pretreated HIMEC at both 24 and 48 h (data not shown). These data suggest that the mechanism of LPS tolerance in HIMEC involves blunting of intracellular superoxide generation during activation, potentially through the action of increased intracellular Mn-SOD.

Early, but not sustained, inhibition of NF-κB activation by subsequent LPS stimulation

HIMEC NF-κB nuclear translocation was detectable at 1 and 2 h after LPS stimulation (1 μg/ml). LPS pretreatment (0.5 μg/ml) for

![FIGURE 4.](image-url) Effect of repeat endotoxin stimulation on cytokine and chemokine production by HIMEC. Assessment of basal and LPS-stimulated IL-6 and IL-8 secretion by HIMEC monolayers using ELISA. HIMEC were treated with LPS, as indicated in the figure, and IL-6 or IL-8 concentrations in culture supernatants were quantified using ELISA. Both IL-6 and IL-8 secretion by HIMEC were enhanced by initial LPS stimulation. However, IL-6 secretion following LPS stimulation was significantly attenuated by endotoxin pretreatment (A), while IL-8 production by HIMEC was not affected (B). Representative data from three individual experiments. *p < 0.05 compared with LPS stimulation without LPS pretreatment using ANOVA.

![FIGURE 5.](image-url) Repeat endotoxin stimulation enhances HIMEC oxyradical defense. A. Western blotting for Mn-SOD expression in HIMEC during repeated LPS treatment. HIMEC were treated with LPS, as indicated in the figure, and Mn-SOD protein in total cell lysates was analyzed by Western blot analysis. Unstimulated HIMEC expressed detectable amounts of Mn-SOD. Mn-SOD expression was markedly increased by 24- and 48-h LPS pretreatment. Representative gel from three independent experiments. B–E, Intracellular superoxide levels in HIMEC are modulated by repeat LPS stimulation. HIMEC monolayer was stimulated with LPS (1 μg/ml) for 2 h after pretreatment with LPS (0.5 μg/ml) for 24 (D) and 48 (E) h before assay. After stimulation, endothelial monolayers were incubated with hydroethidine and observed using a fluorescence microscope. In the presence of intracellular superoxide, hydroethidine is converted to ethidium bromide and detected as bright nuclear staining. B, Unstimulated HIMEC monolayer demonstrates minimal fluorescence with hydroethidine. C, Following LPS stimulation (1 μg/ml) for 2 h, HIMEC loaded with hydroethidine demonstrate a marked increase in nuclear fluorescence staining pattern and intensity. D and E, LPS pretreatment of HIMEC markedly diminishes subsequent increase in superoxide generation and hydroethidine-induced fluorescence. Representative image from triplicate experiments is shown.
6 h abolished the NF-κB activation by the subsequent LPS stimulation (Fig. 6A). However, pretreatment of HIMEC with LPS for 24 or 48 h failed to block NF-κB activation (Fig. 6B). These data suggest that the inhibition of NF-κB in LPS-pretreated HIMEC played a transient early role in LPS tolerance that did not appear to persist after 24–48 h.

**Effect of endotoxin and LPS pretreatment on MAPK activation in HIMEC**

Phosphorylation of p44/42 MAPK, p38 MAPK, and JNK was detected at 30 min after initial LPS stimulation. However, LPS pretreatment for 24 or 48 h exerted an inhibitory effect on MAPK activation by subsequent LPS stimulation (Fig. 7, A–C). Pretreatment with LPS for 6 h also exerted an inhibitory effect on phosphorylation of MAPK (data not shown). These data, in combination with the previous EMSA experiments, suggest that the hyporesponsiveness induced by repeated LPS challenge in HIMEC may involve multiple pathways at different time points, which include an early, transient NF-κB inhibition as well as sustained alterations in MAPK signaling.

The transcription factor NF-κB and MAPK signaling pathways are involved in the proinflammatory activation of HIMEC by LPS

Next, we examined the contribution of NF-κB and the MAPK signaling cascades in the LPS-induced activation of HIMEC, focusing on the mechanisms underlying increased leukocyte adhesion and IL-6 secretion. The increased adhesion of U937 cells to HIMEC monolayers by LPS was attenuated by pretreatment with the selective p44/42 inhibitor PD98059, as well as the NF-κB inhibitor Bay11, but not the selective p38 MAPK inhibitor SB203580 nor the JNK inhibitor SP600125. IL-6 secretion was inhibited by all the MAPK inhibitors, as well as the NF-κB inhibitor Bay11 (Fig. 8). Previous work in our laboratory has demonstrated that MAPK and NF-κB activation play a major role in the expression of CAM in HIMEC (16, 17), which is in concert with published data on the role of these activation pathways in the expression of CAM in HUVEC (18). These data also demonstrate that HIMEC activation in response to LPS is mediated through a complex network of signaling events involving NF-κB as well as MAPK signaling cascades.

**LPS pretreatment does not alter TLR4 expression in HIMEC**

Repeated LPS exposure has been shown to alter the levels of TLR4 in murine peritoneal macrophages, and this decreased receptor expression may be a mechanism underlying endotoxin tolerance in specific cell types (19). We performed a series of experiments to determine the mechanism of activation via LPS in HIMEC, and whether alterations in TLR4 expression might accompany endotoxin tolerance in these cells.

To characterize the mechanism of LPS activation in HIMEC, we initially focused experiments on their expression of CD14, a protein that binds LPS, and facilitates signaling through TLR4. As previously described, the majority of endothelial cell lines will fail to express CD14 in vitro, and CD14 was not detected on HIMEC (data not shown). We performed confirmatory experiments to demonstrate that exogenous, soluble CD14, which is present in culture medium containing FBS, would play a role in LPS-mediated activation of HIMEC. In HIMEC deprived of serum, LPS failed to induce activation of p38 MAPK and p44/42 MAPK (data not shown), demonstrating a need for a serum-derived product in LPS signaling, presumably CD14.

Our next series of experiments were performed to demonstrate the expression of TLR4 in HIMEC. We examined the effect of LPS stimulation on TLR4 mRNA expression at 0, 3, 6, and 24 h after LPS induction, using real-time quantitative RT-PCR. Following LPS treatment (0.5 μg/ml), TLR4 mRNA levels were not changed at any of the time points examined following LPS stimulation (Fig.
as well as LPS-pretreated HIMEC demonstrated no alteration in protein levels, suggesting that no receptor internalization of TLR4 had occurred (Fig. 9B). These data suggest that TLR4 is present in HIMEC, but expression of this LPS receptor does not undergo modulation in response to LPS-induced activation, and receptor density at the cell surface will not mediate the altered signaling that accompanies endotoxin tolerance in this microvascular cell population.

Discussion

The innate immune response to LPS is a critical mechanism for host defense, which will induce potent inflammatory activation. However, sustained inflammatory activation in response to LPS may also lead to damaging effects, and is believed to play a major etiopathogenic role in endotoxic shock, multisystem organ failure, and inflammatory bowel disease (20, 21). In the present study, we have characterized immunoregulatory mechanisms involved in endotoxin tolerance in human gut microvascular endothelial cells. Defining mechanisms of immunoregulation in response to LPS in human gut-specific cell populations represents an essential component in our ability to understand mucosal immune homeostasis. Our report is the first description of endotoxin tolerance in human microvascular endothelial cells from the gut, an area of the body that physiologically coexists with high levels of bacterial products, including endotoxin.

Endotoxin tolerance, the LPS-induced transient impaired inflammatory response to subsequent LPS challenge, has been described at the cellular and molecular level most extensively in monocytes and macrophages. Monocytes/macrophages rendered endotoxin tolerant are characterized by: 1) impaired activation of intracellular signaling pathways, including NF-κB and MAPKs (10); 2) decreased proinflammatory gene transcription and protein production, including TNF-α, IL-1β, IL-6, and IFN-γ (10); and 3) down-regulated proinflammatory cellular function, including Ag presentation (22). At the present time, there is limited information regarding whether cell types other than monocytes or macrophages display LPS tolerance, especially cells originating from the intestinal mucosa whose surface is in continuous proximity to large amounts of endotoxin. A recent report has shown that HUVECs develop LPS tolerance after repeated LPS challenge, demonstrating reduced polymorphonuclear leukocyte adhesion, E-selectin expression, and NF-κB mobilization (23). However, because HIMEC are the resident gut microvascular population possessing unique phenotypic, functional, and physiologic properties distinct from HUVEC (1), we conducted the present study to characterize LPS tolerance in this population.

The most important physiologic finding in our investigation was the demonstration of significantly decreased leukocyte binding in HIMEC undergoing prolonged, repeated activation in response to LPS. The binding of circulating leukocytes to the microvascular endothelium is the initial event in leukocyte emigration and extravasation, and is an early and rate-limiting step in tissue inflammation. Thus, the attenuated binding activity in HIMEC after repeated LPS exposure may represent an important down-regulatory mechanism to prevent excessive inflammation in response to chronic LPS exposure. Simultaneously, the phenomenon of altered HIMEC activation in response to repeated LPS stimulation was selective, as altered E-selectin, VCAM-1, and IL-6 expression, but not ICAM-1 or IL-8, were characterized in our study. The attenuated E-selectin expression after repeated LPS challenge was also reported in HUVEC (23), as well as in mouse gut endothelial cells in vivo (24). These results suggest that this down-regulation of E-selectin expression after repeated LPS stimulation may not be

FIGURE 8. The effect of MAPK or NF-κB inhibitors on HIMEC activation. HIMEC monolayers were pretreated with PD98059 (p44/42 MAPK inhibitor), SB203580 (p38 MAPK inhibitor), SP600125 (JNK inhibitor), or Bay11 (NF-κB inhibitor) for 2 h before LPS stimulation. HIMEC were stimulated with LPS (1 μg/ml) for 6 h, and HIMEC-L937 static adhesion and IL-6 secretion were assessed. A, LPS-induced HIMEC-L937 adhesion was significantly inhibited by SB203580 and Bay11. B, HIMEC IL-6 secretion in response to LPS was significantly diminished by all inhibitors used in this study. These data demonstrate that HIMEC activation in response to LPS is mediated through a complex network of signaling events involving NF-κB as well as MAPK signaling cascades. Representative data from one of a total of three independent experiments. *, p < 0.05 compared with LPS-stimulated HIMEC cultures by ANOVA.

FIGURE 9. TLR4 expression in HIMEC is not affected by LPS treatment. A, HIMEC monolayers were treated with LPS for 0, 3, 6, or 24 h, and TLR4 mRNA expression was determined by real-time quantitative RT-PCR. The relative amount of TLR4 mRNA was expressed as TLR4 mRNA/GAPDH mRNA in each of the LPS-treated samples compared with unstimulated HIMEC. Data from triplicate wells are expressed as mean ± SD. Graph shows representative data from a single experiment, repeated in three independent cell lines. These data demonstrate that LPS treatment did not alter TLR4 mRNA expression. B, TLR4 protein expression in LPS-treated HIMEC cultures detected with Western blotting. Membrane protein-enriched fractions were extracted from HIMEC after 0, 6, and 24 h of LPS treatment (0.5 μg/ml), and TLR4 protein expression was examined by Western blotting. These data demonstrate that LPS treatment did not alter TLR4 protein expression, and also indicate that no receptor internalization was apparent in membrane-enriched protein fractions. Representative gel from two independent experiments.
specific to mucosal endothelial cells, and more importantly, is observed both in vitro and in vivo.

At present, it is uncertain what molecular mechanisms regulate the patterns of differential gene expression in HIMEC undergoing sustained activation in response to LPS. Our examination of mRNA expression suggests that regulation is occurring at the level of transcription and/or mRNA stability. Our investigation of signaling pathways in HIMEC suggests that modulation of NF-κB as well as p44/42 MAPK, p38 MAPK, and JNK activation is involved in LPS tolerance. Because NF-κB is a predominant signal transduction pathway involved in the activation of a wide variety of inflammatory genes, including the CAMs (25), and the inhibition of NF-κB translocation after repeated LPS stimulation has been considered as one potential mechanism of LPS tolerance in monocytes and macrophages (26), we specifically examined NF-κB activation in HIMEC. NF-κB translocation was inhibited by early (6-h) LPS pretreatment, but was not diminished at longer time periods (LPS pretreatment for 24 or 48 h). Because decreased mRNA and/or protein (E-selectin, IL-6, CD86) as well as decreased leukocyte binding were found in the LPS-pretreated HIMEC at distant time periods (24–48 h), we focused on additional signaling pathways that might mediate LPS-induced activation. Activation of MAPK family members is also integrally linked to inflammatory activation in endothelial cells, including HIMEC (13); thus, we also examined MAPK activation after repeated LPS stimulation, and found that p44/42 MAPK, p38 MAPK, and JNK phosphorylation by LPS stimulation were all inhibited by LPS pretreatment. Additionally, we demonstrated that p44/42 MAPK, p38 MAPK, or JNK activation is important for increased leukocyte adhesion and/or IL-6 secretion in HIMEC. These results indicate that the inhibitory effect of LPS pretreatment on MAPK activation may play a mechanistic role in the altered adhesion and protein expression that follow repeated LPS stimulation.

One striking finding in our study was the markedly enhanced Mn-SOD expression in HIMEC exposed to repeated LPS stimulation, which correlated with significant reduction in superoxide anion generation during HIMEC activation. Mn-SOD is a component of the cellular antioxidant defense and catalyzes the conversion of superoxide to hydrogen peroxide, which can be subsequently eliminated either by catalase or glutathione peroxide (27). Several reports have indicated that superoxide radicals increase leukocyte-endothelial cell adhesion, and SOD inhibits increased adhesion mediated by superoxide. In rat mesenteric microvessels, exogenous SOD inhibits leukocyte rolling, adhesion, and emigration induced by angiostatin II (28). In vitro, the adhesion of neutrophils to endothelial cells is increased by hydroxyl radicals, and this enhanced PMN adhesion is blocked by SOD/catalase (29). Another report has demonstrated that the LPS-induced increase in leukocyte-endothelial adhesion in feline mesenteric vessels will be blocked by administration of SOD, indicating that the leukocyte adhesion induced by LPS is in part dependent on superoxide (30). These reports demonstrate that superoxide and the antioxidant defense system are important mediators of endothelial activation and enhanced endothelial-leukocyte adhesion following activation. In the present study, we demonstrate enhanced Mn-SOD expression and decreased superoxide in HIMEC repeatedly stimulated with LPS and have correlated this with diminished leukocyte adhesion. Enhanced Mn-SOD may represent an important mechanism that limits HIMEC intracellular superoxide anion, and may underlie decreased leukocyte adhesion in the setting of repeat LPS activation. Finally, the increased Mn-SOD expression in LPS-tolerant HIMEC is further proof that specific, regulated mechanisms involving increased expression of homeostatic genes as well as decreased expression of molecules involved in inflammatory activation are a component of endotoxin tolerance in HIMEC. Our findings are also the first description of decreased intracellular oxyradical stress occurring within the phenomenon of endotoxin tolerance in endothelial cells.

We have demonstrated that TLR4 mRNA and protein expression were not significantly altered by LPS treatment at the mRNA and protein levels. Although it is unknown whether endotoxin-tolerant human monocytes have a down-regulated expression of TLR4, reduced TLR4 mRNA and surface expression after LPS exposure have been reported in murine macrophages and are considered one of the potential mechanisms underlying impaired NF-κB activation in LPS-tolerant cells (19). However, impaired NF-κB translocation by LPS pretreatment was also observed in TLR4-transfected overexpressing cells (31), suggesting that down-regulation of TLR4 is not a necessary event in impaired signal transduction in LPS-tolerant monocytes. In HIMEC, NF-κB translocation was impaired by LPS pretreatment for 6 h, but not at longer periods (i.e., 24 h), and TLR4 expression was not altered at either of these time points. These results indicate that the altered signaling pathways in repeat LPS-treated HIMEC are not mediated by modulated TLR4 expression or internalization. Finally, preliminary experiments have demonstrated no activation of HIMEC with mycobacterial AraLAM (generous gift of P. Brennan, Colorado State University, Fort Collins, CO), a known ligand of TLR2, which suggests that potential cross-activation of other Toll-like receptors in HIMEC due to our dosage of commercial LPS will not be responsible for the endotoxin tolerance demonstrated in these studies (data not shown).

In summary, we conclude that HIMEC acquire endotoxin tolerance toward repeated LPS stimulation, characterized by reduced leukocyte adhesion, selectively decreased or increased gene/protein expression, impaired NF-κB and MAPK activation, and decreased superoxide production. Microvascular endothelial cells, such as monocytes/macrophages, have precisely defined immunoregulatory responses to repeated LPS exposure. LPS tolerance in HIMEC may represent an important mechanism in controlling gut inflammation during intestinal immune homeostasis in health and disease.

References


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