Bacterial Lipopolysaccharide Activates NF-κB through Toll-like Receptor 4 (TLR-4) in Cultured Human Dermal Endothelial Cells

DIFFERENTIAL EXPRESSION OF TLR-4 AND TLR-2 IN ENDOTHELIAL CELLS*

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A missense mutation in the cytoplasmic domain of the Toll-like receptor-4 (TLR-4) has been identified as the defect responsible for lipopolysaccharide (LPS) hyporesponsiveness in C3H/HeJ mice. TLR-4 and TLR-2 have recently been implicated in LPS signaling in studies where these receptors were overexpressed in LPS non-responsive 293 human embryonic kidney cells. However, the signaling role of TLR-4 or TLR-2 in human cells with natural LPS response remains largely undefined. Here we show that human dermal microvessel endothelial cells (HMEC) and human umbilical vein endothelial cells express predominantly TLR-4 but very weak TLR-2 and respond vigorously to LPS but not to Mycobacterium tuberculosis 19-kDa lipoprotein. Transient transfection of non-signaling mutant forms of TLR-4 and anti-TLR-4 monoclonal antibody inhibited LPS-induced NF-κB activation in HMEC, while a monoclonal antibody against TLR-2 was ineffective. In contrast to LPS responsiveness, the ability of HMEC to respond to 19-kDa lipoprotein correlated with the expression of TLR-2. Transfection of TLR-2 into HMEC conferred responsiveness to 19-kDa lipoprotein. These data indicate that TLR-4 is the LPS signaling receptor in HMEC and that human endothelial cells (EC) express predominantly TLR-4 and weak TLR-2, which may explain why they do not respond to 19-kDa lipoprotein. The differential expression of TLRs on human EC may have important implications in the participation of vascular EC in innate immune defense mechanisms against various infectious pathogens, which may use different TLRs to signal.

Lipopolysaccharide (LPS),¹ or endotoxin, is the major com-

ponent of the outer surface of Gram-negative bacteria. LPS is a potent activator of cells of the immune and inflammatory systems, including macrophages, monocytes, and endothelial cells, and contributes to the systemic changes seen in septic shock (1, 2). Sepsis and septic shock are increasing causes of mortality, with approximately 200,000 deaths annually in the United States (3). The basic paradigm of septic shock is that microbial antigens such as Gram-negative bacterial LPS, Gram-positive bacterial cell wall components, such as soluble peptidoglycan (PGN), lipoteichoic acid (LTA), and bacterial lipoproteins, initiate an uncontrolled network of host-derived proinflammatory mediators, which ultimately lead to cardiovascular shock and death (1, 2, 4). Vertebrates and invertebrates initiate a series of defense mechanisms following infection by various microorganisms by sensing the presence of conserved pathogen-associated molecular patterns, such as bacterial LPS (5). Host organisms have developed a set of receptors that can specifically recognize pathogen-associated molecular patterns and are referred to as pattern recognition receptors (6, 7). A family of such pattern recognition receptors involved in innate immune recognition and cellular activation in response to bacterial LPS, lipoproteins, PGN, and LTA has recently been discovered to be human toll-like receptors (TLRs) (8–17).

TLR family members are transmembrane proteins containing repeated leucine-rich motifs in their extracellular portions, similar to other pattern recognition proteins of the innate immune system (18, 19). TLRs also contain a cytoplasmic domain which is homologous to the signaling domain of the IL-1 receptor, and activation of TLRs result in activation of NF-κB and induction of cytokines and costimulatory molecules required for the activation of the adaptive immune response (18–20). The toll gene controls dorsoventral pattern formation during the early embryonic development of Drosophila melanogaster (21) and initiates a signaling pathway homologous to mammalian NF-κB activation cascade (19, 21). At least eight mammalian Toll-like receptor family members (TLR1–8) have been identified to date, but the function of each individual family member is uncertain.

The C3H/HeJ and C57BL10ScCr mouse strains are hyporesponsi-
vive to LPS, while they respond normally to Gram-positive challenge. As a consequence they have dramatically enhanced susceptibility to Gram-negative pathogens (22). Study of these mice over the past 20 years has shown that hyporesponsiveness

¹ The abbreviations used are: LPS, lipopolysaccharide; EC, endothelial cells; HMEC, human dermal microvessel endothelial cells; HUVEC, human umbilical vein endothelial cells; IRAK, IL-1 receptor-associated kinase; MyD88, myeloid differentiation protein; NF-κB, nuclear factor-κB; PCR, polymerase chain reaction; RT, reverse transcription; TLR, toll-like receptor; TNF, tumor necrosis factor; PGN, peptidoglycan; LTA, lipoteichoic acid; RdPLA, R. sphaeroides diphosphoryl lipid A; mAb, monoclonal antibody; IL, interleukin; bp, base pair(s); β-gal, β-galactosidase; Ab, antibody; FACS, fluorescence-activated cell sorting.

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¹ This paper is available on line at http://www.jbc.org
to LPS maps to a single autosomal locus (lps) (22). Recently, this defect in the LPS response was found to be due to a missense mutation in the cytoplasmic domain of TLR-4 in C3H/HeJ (17, 23, 24) and to an independent mutation at the lps locus in the C57BL/10ScCr strain resulting in the absence of TLR-4 message (17). These genetic studies strongly suggest that TLR-4 is the signal transducing component in the LPS receptor complex (15, 17, 23). Akira and colleagues (25) also showed that mice in which the TLR-4 gene has been deleted have the same defects as mice with mutations in lps.

Transient transfection experiments using LPS non-responsive human embryonic kidney cells (HEK 293) revealed that both TLR-2 (8, 9) and TLR-4 (11) overexpression can enable these cells to respond to LPS, suggesting that perhaps TLR-2 can also be considered a lipopolysaccharide receptor. However, more recent reports have shown that mice lacking TLR-2 can still respond to LPS in the same way as wild type animals (26).

Several studies have identified TLR-2 as the receptor mediating the cellular responses to Gram-positive bacteria (27), PGN, LTA (10, 12), bacterial lipoproteins, Mycobacterium tuberculosis (13, 16), Borrelia burgdorferi lipoproteins (14), and fungi (27). However, the specific role of these TLRs in LPS-responsive human cells is yet undefined. Monocytes and macrophages, dendritic cells, and endothelial cells (EC) play a pivotal role in the development of innate immune responses against invading microorganisms. We therefore investigated the mechanisms involved in bacterial LPS and lipoprotein-induced activation of vascular EC.

We have previously shown that the LPS-induced signal transduction pathway to activate NF-κB shares the IL-1 receptor signaling molecules, including MyD88, IL-1 receptor-associated kinase (IRAK), IRAK-2, and TNF receptor-associated factor-6 (28). Additionally, we have shown that a crucial signaling component in the LPS receptor complex in human dermal microvessel endothelial cells (HMEC) may belong to the IL-1 receptor/TLR superfamily (28). In the current study, we investigated the expression pattern of TLRs in HMEC, HUVEC, monocytes, and THP-1 cells. To investigate the role of TLR-2 and TLR-4 in LPS-induced NF-κB activation in HMEC, we studied the effects of function-blocking mAbs and of transient transfection of C3H/HeJ TLR-4 or an intracellular deletion mutant of TLR-4 together with a NF-κB-responsive reporter gene. Our results indicate that HMEC express TLR-1, TLR-3, TLR-4, and TLR-5 and respond to LPS by TLR-4- and soluble CD14-dependent manner. We observed very weak TLR-2 in HMEC and HUVEC, which explains their lack of response to mycobacterial 19-kDa lipoprotein. Transfection of TLR-2 confers the responsiveness of HMEC to 19-kDa lipoprotein.

**Experimental Procedures**

**Cells and Reagents**

Human THP-1 cells (from ATCC) were cultured in RPMI medium with 10% fetal calf serum. The immortalized HMEC (29) (generous gift of Dr. Candal, Center for Disease Control and Prevention, Atlanta, GA) were cultured in MCDB-131 medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, and 100 µg/ml penicillin and streptomycin in 24-well plates, and used between passages 10 and 14. Tissue culture reagents were from Life Technologies, Inc. Human umbilical vein EC (HUVEC) were isolated and 95% pure) were isolated using a one-step discontinuous Percoll gradient (46%) of the mononuclear cell fraction. Human umbilical vein EC (HUVEC) were isolated and used in intracellular deletion mutant of TLR-4 (0.5 µg) or C3H/HeN TLR-4 (0.5 µg) were co-transfected. After overnight transfection, cells were stimulated for 6 h with 50–100 ng/ml LPS or 10 ng/ml human TNF-α (R & D Systems). Cells were then lysed in 60 µl of reporter lysis buffer (Promega, Madison, WI), and luciferase activity was measured with a Promega kit and a luminometer.

**Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analysis**

Total RNA was isolated from HMEC, HUVEC, and THP-1 cells using a Qiagen kit (Valencia, CA) following manufacturer’s instructions and treated with RNase-free DnaseI. For RT reaction, the MMLV preamplification system (Life Technologies, Inc.) was applied. PCR amplification was performed with Taq gold polymerase (Perkin Elmer, Foster City, CA) for 32 cycles at 95°C for 45 s, 54°C for 45 s, and 72°C for 1 min (for TLR-1, TLR-3, and TLR-4) or 35 cycles at 95°C for 50 s, 50°C for 1 min, and 72°C for 1 min (for TLR-1, TLR-3, and TLR-5). The oligonucleotide primers used for RT-PCR were: TLR-1, 5’-CTATAACACAGATGTTGACC and 5’-GCTCTCAACACTGAAATGTT; TLR-2, 5’-GCCAAAGTCCTGATGTTAGG and 5’-TTGAGATCTCCAGCTTCCT; TLR-3, 5’-GATCTGTCCTCATATAAGTGG and 5’-GACAGATCCGATGCGTGT; for TLR-4, 5’-TGATACACCTTTCCCTATTAG and 5’-GAATGAGGGACCCCTTTC; and for TLR-5, 5’-CTAGCTTCTAATTCTTGAT and 5’-CCATGGTAGATTCTTGG. Glyceraldehyde-3-phosphate dehydrogenase primers were obtained from CLONTECH (Palo Alto, CA).

**Northern Blot Analysis**

Total RNA was isolated by the guanidine isothiocyanate method with minor modifications. Total RNA was analysed by electrophoresis through 1% agarose/formaldehyde gels, followed by northern blot transfer to Gene Screen Plus membranes (NEN Life Science Products). The plasmids containing human TLR-2, TLR-4, or actin cDNAs were labeled with [α-32P]dCTP (3,000 Ci/mmol; Amersham Pharmacia Bio tech, Buckinghamshire, United Kingdom) Membranes were pre-treated and hybridized in 50% formamide (Merek, Rahway, NJ) with 10% dextran sulfate (Sigma) and washed twice with 2× SSC (1× SSC: 0.15 M NaCl, 0.015 M sodium citrate) and 1% sodium dodecyl sulfate (SDS) at 60°C for 30 min, and finally washed twice with 0.1× SSC at room temperature for 30 min. Membranes were exposed for 4–48 h at ~80°C with intensifying screens.

**FACS Analysis and Immunofluorescent Staining**

Flow Cytometry—Surface expression of TLR-2 in HMEC and THP-1 cells was determined using mouse mAb to TLR-2 (2392). The mouse mAb to TLR-2 was a generous gift of Ken-suke Miyake (Saga Medical School, Nabeshima, Saga, Japan) (31). M.
monoclonal TLR-2 antibody was detected with fluorescein isothiocyanate-conjugated goat-antimouse antiserum (CalTag). Samples were viewed and photographed using a Zeiss Axiophot microscope.

**RESULTS AND DISCUSSION**

To investigate the involvement of TLR-2 and TLR-4 in LPS-responsive human vascular EC, we first analyzed the expression pattern of TLRs in HMEC, HUVEC, monocytes, or THP-1 cells by RT-PCR (TLR-1–5) and by Northern blot, immunofluorescence staining, and FACS analysis (TLR-2 and TLR-4). HMEC expressed TLR-1, TLR-3, TLR-4, and TLR-5, but only a weak TLR-2 transcript by RT-PCR (Fig. 1A). TLR-2 cells expressed all five TLRs, including TLR-2 (Fig. 1A). The weak expression of TLR-2 by RT-PCR in HMEC is consistent with our previous observation (28) and was also observed in primary HUVEC (Fig. 1A). Northern blot analysis detected only the 3.4-kilobase pair band consistent with the TLR-4 expression in HMEC and HUVEC, and no TLR-2 message was observed in these two types of EC, while both TLR-2 and TLR-4 mRNA were detected in monocytes (Fig. 1B). Furthermore, immunofluorescence staining and flow cytometric analysis of HMEC with anti-TLR-2 mAb 2392 showed no TLR-2 expression in HMEC, while THP-1 cells showed positive staining (Fig. 1, C and D). We have previously observed that both TLR-4 and TLR-2 were expressed in HMEC by immunostaining using a non-affinity-purified polyclonal antiserum against TLR-2 and TLR-4 (28). However, the availability of a mAb against TLR-2 now allows us to show that there is no TLR-2 expression in HMEC and that the polyclonal anti-TLR-2 antiserum most likely cross-reacts, possibly with another TLR expressed in HMEC (data not shown).

In C3H/HeJ mice, the missense mutation in the intracytoplasmic domain of TLR-4 acts as a dominant negative molecule, possibly by sequestering the downstream adapter and signaling molecules (e.g. MyD88 and IRAK1), and prevents LPS-induced signaling and NF-κB activation (17, 24). To assess the functional role of TLR-4 in LPS-induced signaling and NF-κB activation, while an isotype-matched control Ab and the anti-TLR-4 mAb were ineffective (Fig. 3). Taken together, our findings reveal that LPS signaling in HMEC is mediated specifically via TLR-4 and not TLR-2. Our data do not preclude a contributory role for other TLR family members but concur with genetic evidence in mice and hamsters, in which a mutation in TLR-4 abrogates LPS signaling whereas a mutation in TLR-2 does not (17, 23, 24, 33). Our findings support the hypothesis that TLR-4 is essential in LPS signaling in vivo.

**Bacterial lipoprotein is one of the most abundant proteins in the outer membrane of enteric Gram-negative bacteria (34)** and acts synergistically with LPS to induce proinflammatory cytokine production and lethal shock (35). This suggests that LPS and lipoproteins activate cells via different mechanisms. Recent studies indicate that TLR-2, but not TLR-4, is the required receptor for the cellular responses to microbial lipoproteins (10, 13, 14, 16), to Gram-positive bacteria (27) and cell wall components such as PGN and LTA (10, 12). Since we did not observe TLR-2 expression in HMEC, we next investigated the ability of these cells to respond to M. tuberculosis 19-kDa lipoprotein, which is a potent inducer of NF-κB and IL-12 in monocytes via TLR-2 (13). HMEC were stimulated with increasing concentrations of 19-kDa lipoprotein (10–1,000 ng/ml) for 5 h, and NF-κB activation was measured by ELAM reporter construct luciferase activity. 19-kDa lipoprotein was unable to induce NF-κB activation in HMEC, even at 1,000 ng/ml (Fig. 4). However, expression of wild type TLR-2 cDNA in HMEC conferred responsiveness to 19-kDa lipoprotein (Fig. 4), whereas transfection of vector alone did not. In HMEC transfected with TLR-2, 19-kDa lipoprotein-induced NF-κB activation was inhibited by pre-incubation of the cells with anti-TLR-2 mAb (10 μg/ml) but not with control IgG1 Ab (Fig. 3) or with anti-TLR-4 mAb (data not shown). HUVEC were stimulated with LPS (50 ng/ml) or 19-kDa lipoprotein (50 ng/ml) for 6 h and the supernatants were collected for IL-6 measurement (enzyme-linked immunosorbent assay, BIOSOURCE International, Camarillo, CA). 19-kDa lipoprotein did not induce any IL-6 release from HUVEC while LPS induced a mean of 4-fold increase in IL-6 production compared with resting cells in triplicate experiments (data not shown).

LPS is a complex glycolipid consisting of a proximal hydrophobic lipid A moiety, a distal hydrophilic O-antigen polysaccharide region, and a core oligosaccharide that joins the lipid A and O-antigen structures. The lipid A portion of the molecule is the biologically active portion responsible for LPS responses, and removal of the fatty acid side chains from the lipid A moiety inactivates LPS (36). A special nontoxic pentaacyl diphosphoryl lipid A derived from the LPS of Rhodobacter sphaeroides (RsDPLA) is an LPS antagonist both in vivo and in vitro experimental conditions (37). Based on LPS binding studies, RsDPLA is believed to competitively antagonize LPS activity at its cell surface receptor, leading to inhibition of transmembrane signal transduction (37). Therefore, we next investigated whether RsDPLA blocks LPS- or 19-kDa lipoprotein-induced signaling in native or TLR-2-transfected HMEC, respectively. Pretreatment of HMEC with RsDPLA blocked LPS-mediated NF-κB activation in human vascular endothelial cells.

**To further confirm the functional role of TLR-4 in LPS-induced NF-κB activation in endothelial cells, we pre-incubated HMEC with function-blocking mAbs against human TLR-2 (2392) or TLR-4 and measured LPS-mediated cellular activation. The anti-TLR-4 mAb significantly blocked LPS-induced NF-κB activation, while an isotype-matched control Ab and the anti-TLR-2 mAb were ineffective (Fig. 3). Taken together, our findings reveal that LPS signaling in HMEC is mediated specifically via TLR-4 and not TLR-2. Our data do not preclude a contributory role for other TLR family members but concur with genetic evidence in mice and hamsters, in which a mutation in TLR-4 abrogates LPS signaling whereas a mutation in TLR-2 does not (17, 23, 24, 33). Our findings support the hypothesis that TLR-4 is essential in LPS signaling in vivo.**
LPS-induced NF-κB activation but had no effect on 19-kDa lipoprotein-induced activation of TLR-2-transfected HMEC (Fig. 5). Furthermore, RsDPLA (1 μg/ml) did not affect IL-1- or TNF-α-induced NF-κB gene activation in HMECs (data not shown), indicating that the antagonistic activity of RsDPLA is closely linked to and specific to cell-surface components utilized.
by LPS. Since we showed that TLR-4 is the most likely receptor on HMEC that accounts for LPS responsiveness, our observations suggest that RsDPLA may selectively inhibit LPS-TLR-4 interaction.

We have previously shown that in contrast to monocytes and macrophages, EC do not express membrane CD14, and that LPS-induced EC activation is absolutely dependent on the presence of soluble form of the CD14 receptor (sCD14) present in serum (38). To determine the role of sCD14 in LPS-TLR-4 signaling for EC activation, we tested the response of HMEC to LPS in serum-free media, and serum-free media supplemented with recombinant human sCD14. LPS-induced NF-κB activation in HMEC was serum- and soluble CD14-dependent. LPS (100–1,000 ng/ml) was unable to induce NF-κB activation in serum-free media, and addition of sCD14 (1 μg/ml) was able to restore LPS-induced cellular activation (Fig. 6). However, 19-kDa lipoprotein induced similar levels of NF-κB in TLR-2-expressing HMEC cultured in both serum-containing and in

FIG. 2. Transient transfection of C3H/HeJ TLR-4, or TLR-4 Dicd in HMEC inhibits LPS-induced NF-κB activation in a dose-dependent manner. HMEC were transiently transfected with NF-κB-luciferase and β-galactosidase reporter vectors and various concentrations of C3H/HeJ TLR-4 cDNA (A) or TLR-4 Dicd cDNA (C). The total amount of DNA was kept constant with pCMV vector. The cells were then stimulated with LPS (50 ng/ml) (A and C), or with TNF-α (10 ng/ml) (B) for 5 h. In the TNF-α experiments, 0.5 μg/ml C3H/HeJ TLR-4 cDNA was used (B). Luciferase assay and β-gal assay were performed as described under "Experimental Procedures." Results are shown as mean and standard deviations of three or more independent experiments and are reported as a percentage of LPS-stimulated NF-κB promoter activity co-transfected with a vector control.

FIG. 3. LPS-induced NF-κB activation in HMEC is blocked by anti-TLR-4 mAb, but not by anti-TLR-2 mAb. Native (panel A) or TLR-2-transfected HMEC (panel B) grown on 24-well plates were transfected with NF-κB-luciferase and β-galactosidase reporter vectors overnight and were either grown in media alone, or pre-treated with anti-TLR-4 mAb (10 μg/ml), anti-TLR-2 mAb (10 μg/ml), or isotype-matched control Abs (10 μg/ml) for 1 h, and stimulated with 50 ng/ml LPS (native HMEC) or 50 ng/ml 19-kDa lipoprotein (TLR-2-transfected HMEC) for 5 h. NF-κB luciferase activity was measured by luciferase activity and eventually normalized to β-gal activity. Data shown are mean and standard deviation obtained from three experiments and are reported as a percentage of LPS-stimulated NF-κB promoter activity co-transfected with a vector control.

FIG. 4. HMECs are unresponsive to M. tuberculosis 19-kDa lipoprotein; the response is restored following TLR-2 transfection. Native HMEC were stimulated with different doses of M. tuberculosis 19-kDa lipoprotein for 6 h (A). HMEC were transiently transfected with wild type TLR-2 (0.5 μg of cDNA) and stimulated with 50 ng/ml 19-kDa lipoprotein for 6 h (B). NF-κB luciferase activity was measured with luciferase assay and normalized with β-gal activity. Transfection of flag-tagged wild type TLR-2 construct was confirmed both by anti-flag Western blot and FACS analysis of HMEC with anti-TLR-2 mAb (data not shown). Data are representative of three independent experiments.

FIG. 5. RsDPLA selectively inhibits LPS but not 19-kDa lipoprotein-induced NF-κB activity. HMEC transfected with NF-κB luciferase and β-galactosidase reporter vectors were retreated with RsDPLA (500 ng/ml) for 1 h, and stimulated with LPS (50 ng/ml) for 5 h (A). HMEC co-transfected with wild type TLR-2 were pretreated with RsDPLA (500 ng/ml) and stimulated with 19-kDa lipoprotein (50 ng/ml) for 5 h (B). NF-κB luciferase activity was measured with luciferase assay and normalized with β-gal activity. Data shown are mean and standard deviation obtained from three experiments.
serum-free media (Fig. 6). These findings suggest that, in contrast to LPS, which absolutely requires sCD14 to trigger TLR-4 and activate TLR-2-transfected HMEC, 19-kDa lipoprotein is able to activate TLR-2-transfected HMEC in the absence of serum or sCD14.

Our data demonstrate that human microvascular ECs, which are one of the first lines of defense against invading microbial agents, including Gram-negative bacteria, actively participate in innate immune responses, and that LPS-induced responses in ECs are specifically mediated via TLR-4. LPS in human vascular EC participates in sensing LPS by a mechanism that is soluble CD14-dependent. After detecting the LPS molecules, TLR-4 transmits the information across the cell membrane, leading to the activation of NF-κB and regulation of several responsive genes. Similar to mice where mutations of TLR-4 result in exacerbation of Gram-negative infections, humans who develop severe Gram-negative infections may have TLR-4 mutations at a higher rate.

Our results also indicate a differential expression of TLR-2 and TLR-4 in human vascular endothelial cells. This results in brisk EC responses to Gram-negative LPS but no EC responses to bacterial lipopolysaccharides such as M. tuberculosis 19-kDa lipoprotein or to Gram-positive bacterial cell wall components such as PGN and LTA, which require TLR-2 to signal. The biological relevance of this observation is unclear and needs to be further investigated. Because the mammalian TLR family has many members, it is possible that the innate immune system and its individual cell types use different combinations of TLRs to recognize different groups of microbial pathogens. Finally, our findings indicate that TLR-4 may be a potential target in the development of novel treatment strategies for Gram-negative sepsis and endotoxic shock.

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