Induction of Bacterial Lipoprotein Tolerance Is Associated with Suppression of Toll-like Receptor 2 Expression*

Received for publication, June 5, 2002
Published, JBC Papers in Press, July 19, 2002, DOI 10.1074/jbc.M205584200

Jiang Huai Wang, Majella Doyle, Brian J. Manning, Qiong Di Wu, Siobhan Blankson, and H. Paul Redmond

From the Department of Academic Surgery, National University of Ireland, Cork University Hospital, Cork, Ireland

Tolerance to bacterial cell wall components including lipopolysaccharide (LPS) may represent an essential regulatory mechanism during bacterial infection. Two members of the Toll-like receptor (TLR) family, TLR2 and TLR4, recognize the specific pattern of bacterial cell wall components. TLR4 has been found to be responsible for LPS tolerance. However, the role of TLR2 in bacterial lipoprotein (BLP) tolerance and LPS tolerance is unclear. Pretreatment of human THP-1 monocytic cells with a synthetic bacterial lipopeptide induced tolerance to a second BLP challenge with diminished tumor necrosis factor-α and interleukin-6 production, termed BLP tolerance. Furthermore, BLP-tolerized THP-1 cells no longer responded to LPS stimulation, indicating a cross-tolerance to LPS. Induction of BLP tolerance was CD14-independent, as THP-1 cells that lack membrane-bound CD14 developed tolerance both in serum-free conditions and in the presence of a specific CD14 blocking monoclonal antibody (MEM-18). Pre-exposure of THP-1 cells to BLP suppressed mitogen-activated protein kinase phosphorylation and nuclear factor-κB activation in response to subsequent BLP and LPS stimulation, which is comparable with that found in LPS-tolerized cells, indicating that BLP tolerance and LPS tolerance may share similar intracellular pathways. However, BLP strongly enhanced TLR2 expression in non-tolerized THP-1 cells, whereas LPS stimulation had no effect. Furthermore, a specific TLR2 blocking monoclonal antibody (2392) attenuated BLP-induced, but not LPS-induced, tumor necrosis factor-α and interleukin-6 production, indicating BLP rather than LPS as a ligand for TLR2 engagement and activation. More importantly, pretreatment of THP-1 cells with BLP strongly inhibited TLR2 activation in response to subsequent BLP stimulation. In contrast, LPS tolerance did not prevent BLP-induced TLR2 overexpression. These results demonstrate that BLP tolerance develops through down-regulation of TLR2 expression.

Lipopolysaccharide (LPS), a predominant glycolipid in the outer membrane of Gram-negative bacteria, activates monocytes and macrophages to produce several pro-inflammatory cytokines such as TNF-α and IL-6. Excessive activation of monocytes and macrophages by LPS with overwhelming production of pro-inflammatory cytokines is thought to be responsible for the clinical manifestation of septic shock (1, 2). Bacterial lipoprotein (BLP) is the most abundant protein in the outer membrane of both Gram-positive and Gram-negative bacteria (3, 4). Recently, BLP has also been shown to activate a variety of host inflammatory cells to produce pro-inflammatory cytokines (5–7) and to induce lethal shock in both LPS-responsive C3H/HeOuJ mice and LPS hypo-responsive C3H/HeJ mice (8).

Pre-exposure to LPS induces a transient state of cellular hypo-responsiveness to subsequent LPS stimulation with a diminished production of pro-inflammatory cytokines and enhanced protection against endotoxic lethality (9). This phenomenon is well established and termed LPS tolerance. LPS tolerance is an adaptive host response and may represent a protective mechanism in response to bacterial infection. For example, pre-exposure of animals to a sub-lethal dose of LPS protects against a subsequent lethal LPS challenge with a significant survival advantage and a reduction in pro-inflammatory cytokine production (10, 11). Although LPS tolerance suppresses TNF-α, IL-1β, and IL-6 production, anti-inflammatory cytokines such as IL-10 are not affected (9). Therefore, LPS tolerance appears to occur as a consequence of alterations in LPS signal transduction pathways rather than a general cellular dysfunction. Several studies (12–14) have shown suppressed activation of the mitogen-activated protein (MAP) kinases including extracellular signal-regulated kinases (ERK), c-Jun NH2-terminal kinases (JNK), and p38 in endotoxin-tolerized cells. Reduced nuclear factor-κB (NF-κB) activation and NF-κB-DNA binding activity accompanied by a decreased degradation of both IκB-α and IκB-β are also found in LPS-tolerized cells in response to a second LPS stimulation (13–16). On the other hand, BLP as well as several other non-LPS bacterial cell wall components have been shown to not only activate host inflammatory cells but also induce tolerance in these cells to the subsequent stimulation (16–18). We have shown previously (19) that pre-exposure of MF-1 mice to a sub-lethal dose of BLP induces BLP tolerance that protects against a subsequent lethal BLP challenge. More interestingly, induction of BLP tolerance also protects against the lethality induced by LPS challenge, indicating a cross-tolerance to LPS (19). Although the

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
‡ Both authors contributed equally to this work.
§ To whom correspondence should be addressed: Dept. of Academic Surgery, National University of Ireland, Cork University Hospital, Wilton, Cork, Ireland. Tel.: 353-21-4901275; Fax: 353-21-4901240; E-mail: jh_wang@ucc.ie.
1 The abbreviations used are: LPS, lipopolysaccharide; BLP, bacterial lipoprotein; MAP kinases, mitogen-activated protein kinases; ERK, extracellular signal-regulated kinases; JNK, c-Jun NH2-terminal kinases; TNF-α, tumor necrosis factor-α; IL-6, interleukin-6; mAb, monoclonal antibody; pAb, polyclonal antibody; FACS, fluorescence-activated cell sorter; PBS, phosphate-buffered saline; EMSA, electromophoretic mobility shift assays; FITC, fluorescein isothiocyanate; ELISA, enzyme-linked immunosorbent assay; Pam3, S-[2,3-Bis(palmitoyloxy)-(2-RS)-propyl]-N-palmitoyl-(R).
signal transduction pathways and the molecular mechanisms of LPS tolerance have been extensively investigated, they are not fully elucidated. Furthermore, little is known in relation to BLP tolerance and the alterations in BLP signaling that are involved in BLP tolerance.

Two pattern recognition receptors, CD14 and Toll-like receptors (TLR), are involved in the recognition of bacteria and their cell wall components by the mammalian innate immune system. CD14 is a 55-kDa glycosylphosphatidylinositol-linked glycoprotein that recognizes and binds to LPS with high affinity (20). LPS can directly activate monocytes and macrophages through its binding to membrane-bound CD14 (mCD14). However, cells that do not express mCD14 also respond to LPS stimulation in a serum-dependent fashion or in the presence of soluble CD14 (sCD14) (21, 22). The presence of CD14 has been shown to facilitate BLP signaling (6, 23, 24). However, a CD14-dependent pathway used by BLP is distinct from that used by LPS (23). Because CD14 lacks a transmembrane region and is incapable of transducing the signaling of bacterial cell wall components including LPS and BLP, a transmembrane protein with potential for intracellular signaling would be required to serve as a primary receptor for BLP and/or LPS signaling and to couple with CD14 as a co-receptor. Recently, a highly conserved family of TLR proteins has been discovered, and each TLR is a type I transmembrane protein and is characterized structurally by an extracellular leucine-rich repeat domain and a cytoplasmic Toll/IL-1 receptor homology domain (25, 26). Among them, TLR4 has been shown to be a primary receptor for LPS signaling, as different mutations in the tlr4 gene found in two mouse strains (C3H/HeJ and C57BL10ScCr) are responsible for an impaired ability to respond to LPS (27, 28). Furthermore, analysis of mice with targeted disruption in their tlr4 gene revealed that TLR4 knockout mice are LPS hypo-responsive (29, 30). TLR2 has also been implicated in mediating LPS signals (31, 32). However, mice with targeted disruption of tlr2 gene, but not TLR4 knockout mice, are hyporesponsive to other non-LPS bacterial cell wall products including BLP (30). TLR2-deficient cells are sensitive to LPS stimulation but have no response to Gram-positive bacterial cell wall components (33). When these cells are transfected with TLR2, they respond to BLP stimulation (24). Furthermore, two recent studies have shown that activation of target cells by highly purified LPS is through TLR4 only and not through TLR2 (34, 35). These data indicate that in contrast to TLR4, TLR2 is the major receptor for BLP and other non-LPS bacterial cell wall products.

Several studies (14, 16, 36, 37) have examined the role of CD14 and TLR in LPS tolerance. It has been reported (16, 36, 37) that the expression of CD14 is not altered in LPS-tolerized monocytes and macrophages, whereas dysregulation of tlr2 and tlr4 gene expression and a reduced surface expression of TLR4 are implicated in LPS tolerance (14, 37). More interestingly, when LPS tolerance in mouse peritoneal macrophages occurs via suppressed TLR4-MD2 surface expression (37), tolerance to LPS induced by mycoplasma lipopolysaccharide, the 2-kDa macrophage-activating lipopolysaccharides (MALP-2), is not through the down-regulation of TLR4-MD2 expression (16). It is unknown whether TLR2, particularly TLR2 protein expression, is associated with the development of BLP tolerance as well as LPS tolerance.

In the present study, we report that a synthetic bacterial lipopeptide induces tolerance in human THP-1 monocytic cells not only to subsequent BLP stimulation but also to LPS, indicating a cross-tolerance. Furthermore, induction of BLP tolerance is CD14-independent. When similar levels of suppressed MAP kinase phosphorylation and NF-kB activation are present in both BLP-tolerized and LPS-tolerized cells, BLP tolerance, but not LPS tolerance, prevents BLP-induced TLR2 overexpression. These results indicate the involvement of TLR2 in the development of BLP tolerance.

**EXPERIMENTAL PROCEDURES**

**Reagents and Polyclonal and Monoclonal Antibodies (pAbs and mAbs)—**RPMI 1640 medium, PBS, fetal calf serum, penicillin, streptomycin, and gentamycin were purchased from Invitrogen. LPS from *Escherichia coli* serotype O55B5 was purchased from Sigma. BLP, a synthetic bacterial lipopeptide (Pam3Cys-Ser-Lys4-OH) derived from the immunologically active LH terminus of bacterial lipopolysaccharides, was purchased from Roche Molecular Biochemicals, which was LPS-free as confirmed by the Limulus amoebocyte lysate assay (Charles River En- dosafe, Charleston, SC). [3H]-TdT (3000 Ci/mmol) and poly(dI-dC) were obtained from Amersham Biosciences. FITC-conjugated mouse anti-human CD14 mAb (anti-Leu-M3) was obtained from BD Biosciences. Rabbit pAb against active (phosphorylated) ERK1 and -2, active JNK1 and -2, and active p38 were obtained from Promega (Madison, WI) and New England Biolabs (Beverly, MA), respectively. Goat pAb against human TLR2 were obtained from Santa Cruz Biotechnol- ogy (Santa Cruz, CA). A specific mouse anti-human CD14 blocking mAb (MEM-18) was obtained from HBT (Uden, Netherlands), and a specific mouse anti-human TLR2 blocking mAb (2392) was a gift from Genen- tech, Inc. (San Francisco, CA).

**Cell Culture and Induction of Tolerance—**THP-1 cells (a human monocytic cell line) were obtained from the American Type Culture Collection (ATCCC, Manassas, VA) and grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, penicillin (100 units/ml), streptomycin sulfate (100 μg/ml), and gentamycin (2 μg/ml) at 37°C in a humidified 5% CO2 atmosphere.

**Tolerance in THP-1 cells was induced as described previously (14). Briefly, cells cultured in a serum-present or serum-free medium were preincubated with culture medium (non-tolerant), 10 ng/ml BLP (BLP tolerant), or 10 ng/ml LPS (LPS tolerant) for 24 h, washed twice with PBS, and incubated in a fresh culture medium for 2 h. Then cells were stimulated with 100 and 1,000 ng/ml BLP or LPS for different time challenges as described above. Cells were then stimulated with 100 and 1,000 ng/ml blocking mAb (MEM-18, 10 μg/ml) (38) or TLR2 blocking mAb (2392, 25 μg/ml) (39) for 30 min to totally block CD14 or TLR2 before they were exposed to BLP or LPS.

**FACS Analysis of Immunofluorescence—**THP-1 cells (1 × 106 cells/100 μl) were stained with 20 μl of FITC-conjugated mouse anti-human CD14 mAb at 4°C for 30 min. FITC-conjugated isotype IgG2b mAb was used as a negative control. mCD14 expression on THP-1 cells was analyzed on a FACScan flow cytometer (BD Biosciences) for detecting the log of the mean channel fluorescence intensity with an acquisition set at 2.5°C.

**Cytokine Measurements—**THP-1 cells (2 × 105 cells/well) incubated in 24-well plates (Falcon, Lincoln Park, NJ) were subjected to different challenges as described above. Cells were then stimulated with 100 and 1,000 ng/ml BLP or LPS for 6 h. Cell-free supernatants were collected by centrifugation, transferred to new tubes, and stored at −70°C until analysis. The levels of TNF-α and IL-6 in cell supernatants were assessed using commercially available ELISA kits (R & D Systems, Minneapolis, MN) according to the manufacturer's instructions.

**Western Blot Analysis—**Non-tolerized and BLP- or LPS-tolerized THP-1 cells were further stimulated with 100 ng/ml BLP or LPS for different time points. After being extensively washed with cold PBS, cells were lysed in ice with lysis buffer (1% Triton X-100, 20 mM Tris, 137 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 2 mM NaVO3, 10 μg/ml leupeptin, 2 μg/ml aprotinin). Protein concentrations were determined using a micro BCA protein assay reagent kit (Pierce). The proteins were denatured at 95°C for 10 min in loading buffer (60 mM Tris, 2.5% SDS, 10% glycerol, 5% mercaptoethanol, 0.01% bromphenol blue). Aliquots containing equal amount of total proteins from each sample were separated in SDS-polyacrylamide gels and transblotted onto nitrocellulose membranes (Schleicher & Schuell). After blocking for 2 h with TBS containing 0.1% Tween 20 and 6% nonfat milk, membranes were probed overnight at 4°C with anti-MAP kinase pAbs or anti-TLR mAb. Blots were further incubated for 1 h with peroxidase-conjugated anti-rabbit IgG (Promega) or anti-goat IgG (Santa Cruz Biotechnology) and developed using an enhanced chemiluminescence detection system (Santa Cruz Biotechnology) according to the manufacturer's instructions.

**Transient Transfection and Luciferase Assays—**Dual transfections of THP-1 cells were accomplished using 12 μl/ml Dmire-C reagent (In-
formed as described previously (41). Briefly, 2.0–

lipopeptides (MALP-2) and LPS on TNF-

LPS stimulation). A synergistic effect of mycoplasmal

tolerance to LPS

versus

freshly added 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl flu-

succeeded in nuclear extract buffer (20 mM Hepes, 25%

0.1% Nonidet P-40, pH 7.9) on ice for 10 min and centrifuged at

13,000 rpm, and supernatants containing the nuclear proteins were collected. All buffers contained

15 min. The lysates were centrifuged at 13,000 rpm, and supernatants

was determined using a micro BCA protein assay

reagent kit (Pierce). Firefly and

nuclei were resuspended in nuclear extract buffer (20 mM Hepes, 25% glycerol, 4% glycerol, 420 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, pH 8.0) on ice for

lysates were centrifuged at 13,000 rpm, and supernatants containing the nuclear proteins were collected. All buffers contained freshly added 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and protease inhibitor mixture (Roche Molecular Biochemicals). Protein concentrations were determined using a micro BCA protein assay reagent kit (Pierce). All nuclear extracts were stored at −70 °C until analysis.

Electrophoretic Mobility Shift Assays (EMSA)—EMSA were per-

formances, as described previously (41). Briefly, 2.0–4.0 μg of nuclear extracts were incubated with 30,000 cpm of double-stranded oligonucleotide, 5′-AGT TGA GGG GAC TTT CCC AGG C-3′/H11032/H9262, containing the NF-κB consensus sequence (underlined) (Promega) that had been labeled previously with [γ-32P]ATP (10 mCi/mmole) by T4 polynucleotide kinase (Promega). The DNA-binding reactions were performed in the presence of 2.0 μg of poly(diC6C6) as nonspecific competitor in binding buffer (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1.0 mM EDTA, 5.0 mM dithiothreitol, 4% glycerol, 100 μg/ml nuclease-free bovine serum albumin) at room temperature for 30 min. For competition experiments, unlabeled double-stranded oligonucleotide, 5′-AGT TGA GGC GAC TTT CCC AGG C-3′, containing the mutated NF-κB consensus sequence (underlined) (Promega) was added to the nuclear extracts 30 min before the addition of the radiolabeled probe. All reaction mixtures were subjected to electrophoresis on native 5% (w/v) polyacrylamide gels, which were subsequently dried and autoradiographed.

Statistical Analysis—All data are presented as the mean ± S.D. Statistical analysis was performed using analysis of variance. Differences were judged statistically significant when the p value was less than 0.05.

RESULTS

BLP Tolerance Is CD14-independent and Shows Cross-toler-

ance to LPS—LPS activates monocytes and macrophages to produce pro-inflammatory cytokines such as TNF-α and IL-6. Fig. 1, A and B, shows that BLP is a more potent stimulus than LPS for human THP-1 monocytic cell activation, as naive (non-tolerant) THP-1 cells exposed to BLP at 100 and 1,000 ng/ml for 6 h produced much higher levels of TNF-α and IL-6 (∗, p < 0.05, versus LPS) in a synergistic effect on cytoplasmic lipopolysaccharide lipopeptides (MALP-2) and LPS on TNF-α and IL-6 production in murine peritoneal macrophages has been reported previously (16). However, there were no further increases in TNF-α and IL-6 production found in THP-1 cells stimulated with combinations of BLP and LPS, when compared with cells stimulated with BLP alone (Fig. 1, A and B).

When THP-1 cells were preincubated with 10 ng/ml BLP for 24 h and then subjected to a second stimulation with high doses of BLP (100 and 1,000 ng/ml), TNF-α and IL-6 production was significantly attenuated (Fig. 1, C and D), indicating induction of BLP tolerance. Most interestingly, BLP-tolerized THP-1 cells had an impaired ability to produce TNF-α and IL-6 in response to LPS stimulation or LPS plus BLP stimulation (Fig. 1, C and D), indicating a novel cross-tolerance to LPS induced by BLP preincubation. Preincubation of THP-1 cells with LPS induced LPS tolerance, as demonstrated by attenuated TNF-α and IL-6 production in response to a second LPS stimulation (Fig. 1, E and F). Notably, LPS-tolerized THP-1 cells exhibited markedly reduced production of TNF-α and IL-6 in response to subsequent BLP or BLP plus LPS stimulation (Fig. 1, E and F). However, unlike BLP-tolerized THP-1 cells, which were effectively unresponsive when challenged with LPS or LPS plus BLP, LPS-tolerized cells still produced significant levels of TNF-α and IL-6 when challenged with BLP or BLP plus LPS (p < 0.05, versus LPS stimulation), indicating an incomplete cross-tolerance to BLP. There were no differences in apoptosis and viability between non-tolerized and BLP plus LPS-tolerized THP-1 cells (data not shown), indicating that the reduced cytokine production observed in tolerated cells was not due to cell death.

CD14 is a 55-kDa glycosylphosphatidylinositol membrane-

anchored glycoprotein that participates in LPS recognition, binding, and signal transduction (17, 42, 43). Recent studies
have shown that CD14 is also involved in BLP signaling (6, 23, 24). Therefore, we sought to examine whether CD14 is required for induction of BLP tolerance. As determined by FACscan analysis, cell surface-expressed mCD14 was undetectable in human THP-1 cells, and BLP stimulation did not induce the expression of mCD14 in this cell line (Fig. 2), indicating the lack of mCD14 in THP-1 cells. Because soluble CD14 (sCD14) has been implicated in mediating LPS signaling in cells that do not possess mCD14 (21, 22), we cultured THP-1 cells in serum-free medium to exclude the influence of sCD14 and other soluble factors such as LPS-binding protein. As shown in Fig. 3, A and B, BLP stimulation of THP-1 cells in the presence or absence of serum induced the release of comparable TNF-α and IL-6 levels. Similar results were also found in cells treated with LPS or LPS plus BLP. More importantly, Fig. 3, C and D, demonstrates that pre-exposure of THP-1 cells to a low dose of BLP (10 ng/ml) in serum-free medium for 24 h induced tolerance not only to BLP, but also to LPS, a cross-tolerance effect that was also induced by BLP pretreatment in medium containing serum. Finally, we incubated THP-1 cells with MEM-18 (10 μg/ml) for 30 min to totally block CD14 (38) before they were exposed to BLP or LPS. As shown in Fig. 4, blockage of CD14 neither prevented BLP- nor LPS-induced TNF-α and IL-6 production in naive cells nor did it abolish tolerance to a second BLP or LPS stimulation in BLP-pretreated cells. These results indicate that induction of BLP tolerance is CD14-independent.

**FIG. 2.** FACS analysis of mCD14 expression on human THP-1 cells. Cells were incubated with culture medium (A) or 10 ng/ml BLP for 6 (B), 12 (C), or 24 h (D). mCD14 expression on THP-1 cells was assessed by flow cytometry using FITC-conjugated anti-Leu-M3 (anti-CD14) mAb (black histograms) and isotype-matched mAb as negative control (gray histograms). The results presented represent one experiment from a total of three independent experiments.

BLP Tolerance Inhibits BLP- and LPS-induced MAP Kinase and NF-κB Activation—Transduction of LPS signals in monocytes and macrophages causes activation of MAP kinases including ERK, JNK, and p38, which subsequently leads to activation of various transcription factors and production of inflammatory cytokines (44). We first investigated the activation of MAP kinases following BLP and LPS stimulation, and we then examined whether BLP tolerance or LPS tolerance affects MAP kinase phosphorylation. Stimulation of naive (non-tolerant) THP-1 cells with BLP at 100 ng/ml for 30 min resulted in ERK1/2, JNK1/2, and p38 activation, whereas similar activation of these three MAP kinases was also found following stimulation with LPS and LPS plus BLP for 30 min (Fig. 5). However, there was almost complete suppression in phosphorylation of ERK1/2, JNK1/2, and p38 in response to a second BLP stimulation in BLP-tolerized THP-1 cells. Furthermore, LPS- and LPS plus BLP-induced activation of these three MAP kinases were also significantly inhibited in BLP-tolerized cells (Fig. 5). A significant inhibition in ERK1/2, JNK1/2, and p38 phosphorylation was observed in LPS-pretreated THP-1 cells in response to a second LPS stimulation. Notably, BLP stimulation did not induce the activation of MAP kinases in LPS-tolerized cells (Fig. 5). These results indicate that the phosphorylation of MAP kinases involved in BLP and LPS signaling is affected by BLP tolerance, as well as by LPS tolerance.

**FIG. 3.** Induction of BLP tolerance in serum-free conditions with suppressed TNF-α and IL-6 production in response to BLP and LPS stimulation. Human THP-1 cells cultured in serum-free medium were pretreated with culture medium (A and B) or 10 ng/ml BLP (C and D) for 24 h. After washing with PBS and incubation in fresh medium for 2 h, the cells were stimulated with 100 and 1,000 ng/ml BLP, LPS, or their combinations for 6 h. The concentrations of TNF-α and IL-6 in the culture supernatants were measured by ELISA. Data are expressed as the mean ± S.D. and are representative of five separate experiments. The statistical significance was compared with the cells stimulated with LPS (*, p < 0.05).

NF-κB is an important transcription factor in relation to LPS and BLP signaling (39, 45). Furthermore, phosphorylation of MAP kinases is associated with subsequent activation of transcription factors including NF-κB (44). As the activation of three MAP kinases was significantly inhibited in either BLP or LPS-tolerized THP-1 cells, we wished to investigate further the effect of BLP tolerance or LPS tolerance on NF-κB activation and NF-κB-DNA binding activity. NF-κB activation in BLP or LPS-tolerized cells was assessed by transfection of THP-1 cells with a pNF-κB-Luc reporter vector. Fig. 6A shows that BLP stimulation in non-tolerized THP-1 cells resulted in a significant increase in luciferase activity, indicating NF-κB activation. This activation was significantly inhibited in BLP-tolerized cells. Results from NF-κB-DNA binding activity assay by EMSA further confirmed these findings (Fig. 6B). On the other hand, LPS-induced NF-κB activation and NF-κB-DNA binding activity were also severely decreased in LPS-tolerized THP-1 cells (Fig. 6, A and B). As expected, LPS stimulation had no effect on NF-κB activation in LPS-tolerized cells, which correlated with the findings in both cytokine production and MAP kinase activation. Notably again, BLP did not cause NF-κB activation in LPS-tolerized THP-1 cells (Fig. 6, A and B).

**FIG. 6.** NF-κB activation and NF-κB-DNA binding activity in BLP-tolerized THP-1 cells. A, NF-κB activation and NF-κB-DNA binding activity in LPS and BLP-stimulated THP-1 cells. The cells were stimulated with 100 and 1,000 ng/ml LPS, BLP, or BLP plus LPS for 24 h. NF-κB DNA binding activity was determined by EMSA after BLP or LPS stimulation. The supershifts were performed with anti-NF-κB-specific antibodies. B, BLP tolerance down-regulates TLR2 expression and suppresses BLP-mediated TLR2 activation. Families of TLR have been shown to be involved in the recognition of bacterial cell wall components. Whereas TLR2 appears to serve as the primary receptor for Gram-positive bacteria and their cell wall components including BLP (24, 30, 33, 46), TLR4 serves as the
primary receptor for LPS signaling (27–30, 34, 35). Furthermore, dysregulation of tlr2 and tlr4 expression and a reduced surface expression of TLR4-MD2 complex have been found in LPS-tolerized murine macrophages (14, 37), whereas tolerance to LPS induced by mycoplasmal lipopeptides (MALP-2) is not through the down-regulation of TLR4-MD2 expression (16). Hence, we sought to investigate the effect of BLP or LPS stimulation on TLR2 activation and to further examine whether BLP tolerance and LPS tolerance affects TLR2 expression in either LPS-tolerized or BLP-tolerized cells.

With reference to the effect of LPS on TLR2 expression in naive THP-1 cells, it is not surprising that LPS stimulation did not prevent TLR2 overexpression induced by BLP stimulation (Fig. 7A). These results indicate that BLP rather than LPS is the ligand for TLR2 engagement and activation. Most importantly, induction of BLP tolerance by pretreatment of THP-1 cells with 10 ng/ml BLP for 24 h strongly inhibited BLP-induced TLR2 activation, whereas induction of LPS tolerance did not prevent TLR2 overexpression induced by BLP stimulation (Fig. 7A). With reference to the effect of LPS on TLR2 expression in naive THP-1 cells, it is not surprising that LPS stimulation did not affect TLR2 expression in either LPS-tolerized or BLP-tolerized cells (Fig. 7A).

**DISCUSSION**

BLP, characterized by a unique, NH₂-terminal lipo-amino acid, is the most abundant protein in the outer membrane of both Gram-positive and Gram-negative bacteria (6, 4, 6, 23). Like LPS, BLP can be released from proliferating E. coli, and treatment of bacteria with antibiotics significantly enhances BLP release (47). BLP has been found to activate monocytes/macrophages and to induce lethal shock in both LPS-responsive C3H/HeOuJ mice and LPS hypo-responsive C3H/HeJ mice (5–8). Using a synthetic bacterial lipopeptide that had been found previously to activate NF-κB through a TLR2-mediated signal transduction pathway (39), we have shown that BLP has

---

**Fig. 4. Induction of BLP tolerance in human THP-1 cells after blockage of CD14.** Cells were incubated with 10 µg/ml MEM-18, a specific CD14 blocking mAb, for 30 min, and then pretreated with culture medium (A and B) or 10 ng/ml BLP (C and D) for 24 h. After washing with PBS and incubation in fresh medium for 2 h, the cells were stimulated with 100 and 1,000 ng/ml BLP, LPS, or their combinations for 6 h. The concentrations of TNF-α and IL-6 in the culture supernatants were measured by ELISA. Data are expressed as the mean ± S.D. and are representative of five separate experiments.

**Fig. 5. Inhibition of BLP-induced and LPS-induced MAP kinase activation in BLP- and LPS-tolerized human THP-1 cells.** Cells were pretreated with culture medium (M), 10 ng/ml BLP (B), or 10 ng/ml LPS (L) for 24 h. After washing with PBS and incubation in fresh medium for 2 h, the cells were stimulated with 100 ng/ml BLP, LPS, or their combinations for 30 min. Cellular extracts were prepared, and MAP kinase phosphorylation was detected by Western blot analysis using pAbs specific for active ERK1/2, JNK1/2, and p38. The results shown represent one experiment from a total of three independent experiments.

**Fig. 6. Suppressed NF-κB activation and NF-κB-DNA binding activity in BLP- and LPS-tolerized human THP-1 cells in response to BLP and LPS stimulation.** A, cells were pretreated with culture medium (M), 10 ng/ml BLP (B), or 10 ng/ml LPS (L) for 24 h. After washing with PBS and incubation in fresh medium for 2 h, the cells were lysed, and the luciferase activity was measured. Data are expressed as the mean ± S.D. and are representative of four separate experiments. The statistical significance was compared with the cells preincubated with medium (*, p < 0.05).
Bacterial Lipoprotein Tolerance and Toll-like Receptor 2

100 ng/ml BLP or LPS for 6 h. The concentrations of TNF-

FIG. 7. Effect of BLP tolerance or LPS tolerance on TLR2 expression in human THP-1 cells. A, cells were pretreated with culture medium, 10 ng/ml BLP, or 10 ng/ml LPS for 24 h. After washing with PBS and incubation in fresh medium for 2 h, the cells were stimulated with 100 ng/ml BLP or LPS for the indicated time points. Cellular extracts were prepared and subjected to Western blot analysis. TLR2 expression was detected using anti-human TLR2 pAb. The results presented represent one experiment from a total of four independent experiments. B, cells were incubated with 25 µg/ml mAb 2392, a specific TLR2 blocking mAb, or control IgG for 30 min and then stimulated with 100 ng/ml BLP or LPS for 6 h. The concentrations of TNF-α and IL-6 in the culture supernatants were measured by ELISA. Data are expressed as the mean ± S.D. and are representative of four separate experiments.

a more potent effect than LPS on human THP-1 cell activation as represented by TNF-α and IL-6 production. A synergistic effect of mycoplasmal lipopeptides (MALP-2) and LPS on TNF-α production in mouse peritoneal macrophages has been reported previously (16). However, co-stimulation of THP-1 cells with BLP and LPS in this study did not result in further increases in TNF-α and IL-6 production, indicating the lack of a synergistic effect between BLP and LPS on human THP-1 cell activation.

In response to LPS stimulation, mononuclear phagocytes produce various inflammatory cytokines such as TNF-α, IL-1β, and IL-6. Although appropriate amounts of these cytokines are essential for monocyte/macrophage microbicidal activity, excessive production by acute bacterial infection during sepsis leads to systemic inflammatory response syndrome and the development of acute respiratory distress syndrome and multisystem organ dysfunction syndrome (48). Endotoxin or LPS tolerance is a well established phenomenon whereby pre-exposure to a sub-lethal dose of LPS blunts the subsequent lethal LPS-induced mortality that has been demonstrated (9–11) to be associated closely with a diminished production of pro-inflammatory cytokines by mononuclear phagocytes. Human neutrophils and monocytes from septic patients show minimal production of inflammatory cytokines in response to further LPS stimulation (49). Therefore, the development of LPS tolerance may represent an adaptive and protective mechanism of host defense during bacterial infection. In addition to LPS tolerance, several other non-LPS bacterial cell wall components including BLP have also been shown to induce tolerance in vitro (16–18). In vivo, we have demonstrated previously that pretreatment of MF-1 mice with a sub-lethal dose of BLP significantly attenuates not only BLP-induced mortality but also LPS-induced mortality, indicating that BLP pretreatment induces a cross-tolerance to LPS (19). In the present study, we investigated the signal transduction pathways and the molecular mechanisms that are involved in BLP tolerance. We have shown the induction of BLP tolerance in human THP-1 cells. When THP-1 cells were pre-exposed to BLP, they became refractory to a second BLP challenge with diminished TNF-α and IL-6 production. Furthermore, BLP-tolerized THP-1 cells produced a novel cross-tolerance to LPS, as these cells no longer responded to LPS stimulation. This effect was not due to BLP contamination by LPS, because we used a synthetic bacterial lipopeptide that was LPS-free. These data further support our in vivo finding of BLP tolerance. MALP-2, synthesized and purified from mycoplasma, is structurally related to BLP and has also been found to induce a cross-tolerance to LPS in mouse peritoneal macrophages (16), which is consistent with the finding of BLP tolerance in human THP-1 cells as shown in the present study.

We were interested to find that the induction of BLP tolerance in human THP-1 cells is CD14-dependent, as evidenced by the following facts. First, the THP-1 cells used in this experiment lacked surface expression of mCD14. Second, when these cells were pretreated with BLP in serum-free medium that excludes sCD14, they also developed BLP tolerance similar to that induced in medium containing serum. Finally, that induction of BLP tolerance is CD14-independent was further confirmed by demonstrating tolerance induction in the presence of a blocking anti-CD14 mAb (MEM-18). Of the various LPS-binding proteins identified over the last decade, CD14 is perhaps the most important receptor for LPS binding and recognition. Accumulated evidence supports the concept that CD14 initiates the transduction of LPS signals. For example, anti-CD14 antibodies block LPS responses in target cells (50), whereas transfection of CD14 into CD14-deficient cells strongly enhances LPS-induced activation (51). LPS, in the presence of sCD14, can also activate CD14-deficient cells (21, 22). However, LPS at high concentrations can induce inflammatory cytokine gene expression and protein synthesis through a CD14-independent pathway (43, 52). Furthermore, the expression of CD14 is not affected by LPS tolerance in monocytes and macrophages (16, 36, 37). In addition to LPS, other non-LPS bacterial cell wall components including BLP appear to be CD14-dependent. BLP from the outer membrane lipoproteins of Borrelia burgdorferi and Treponema pallidum has been shown to induce cell activation via a CD14-dependent pathway that is blocked by specific anti-CD14 antibodies (6, 23). However, it has been noted that Chinese hamster ovary cells transfected with CD14 are exquisitely sensitive to LPS but are unresponsive to BLP and that an LPS receptor antagonist (a deacylated LPS) blocks LPS signaling but fails to antagonize BLP-induced activation (23). These observations suggest that the CD14-dependent pathway used by BLP may be distinct from that used by LPS. The finding that BLP induces tolerance in THP-1 cells via a CD14-independent pathway as demonstrated in the present study suggests that BLP may directly interact with other binding receptors such as TLR2 for its signaling.

The intracellular signal transduction pathways involved in LPS tolerance have been extensively investigated. Several recent studies (12–14) have shown a generally suppressed activation of the MAP kinases including ERK, JNK, and p38 in LPS-tolerized mouse macrophages. As LPS-induced activation of the MAP kinase pathways plays an important role in mediating transcription factor (NF-κB and AP-1) activation, the
down-regulation of MAP kinase activation by LPS tolerance may further affect the translocation of NF-κB and AP-1. Consistently, LPS-tolerized cells are found to have inhibited NF-κB activation and NF-κB-DNA binding activity accompanied by a reduced degradation of both IκB-α and IκB-β, as well as decreased AP-1-DNA binding activity (13–16). In the present study, pretreatment of THP-1 cells with BLP resulted in strongly inhibited phosphorylation of ERK, JNK, and p38 MAP kinases and reduced NF-κB activation and NF-κB-DNA binding activity, which is similar to that seen in LPS-tolerized cells. These results indicate that BLP tolerance and LPS tolerance may share similar intracellular signal transduction pathways. A recent study (16) reported that BLP tolerance induced by MALP-2 produces a cross-tolerance to LPS, which correlates with the suppression of LPS-induced JNK and NF-κB activation in MALP-2-tolerized mouse peritoneal macrophages. However, BLP-induced cross-tolerance to LPS observed in this study cannot be fully accounted for by the inhibitory effect of BLP tolerance on LPS-induced MAP kinase and NF-κB activation, because LPS tolerance also prevented BLP-induced MAP kinase and NF-κB activation but produced an incomplete cross-tolerance to BLP.

Mammalian TLR2 and TLR4 have been identified recently as the pattern-recognition receptors for bacteria and their cell wall components. Over the past 3 years, attempts to investigate differences between TLR2 and TLR4 in the recognition of infectious pathogens and their products have been exhaustive. Results from these studies (27–30) have provided compelling evidence to support the concept that TLR4 serves as the primary receptor for LPS signaling, whereas TLR2 functions as the primary receptor for BLP and other non-LPS bacterial cell wall components (24, 30, 33, 46). Although a number of reports (31, 32) show that TLR2 is also capable of transducing LPS signals, recent work (53) has demonstrated that TLR4 rather than TLR2 imparts ligand-specific recognition of LPS and that highly purified LPS no longer induces cell activation through TLR2 (34, 35). These results indicate that TLR2 does not essentially function as an LPS signal transducer. As TLR2 and TLR4 function differently, it is of interest to investigate the role of TLR2 and TLR4 in the development of LPS tolerance and BLP tolerance. A dysregulation of TLR2 mRNA and TLR4 mRNA has been found in LPS-tolerized mouse macrophages (14). Furthermore, LPS tolerance occurs through the down-regulation of surface expression of TLR4 (37), whereas MALP-2 tolerance does not affect TLR4 surface expression (16). However, it is unclear whether TLR2 is involved in LPS and BLP tolerance. Although one study has shown alterations in TLR2 mRNA in LPS-tolerized mouse macrophages (14), the mRNA expression pattern of TLR2 may not precisely reflect its protein expression level, as evidenced by the lack of correlation between TLR4 mRNA and surface expression in LPS-tolerized cells (37). In the present study, we have shown that BLP, but not LPS, stimulation strongly enhanced TLR2 expression and that blockage of LTR2 significantly attenuated BLP-induced, but not LPS-induced, TNF-α and IL-6 production in naive THP-1 cells, which further demonstrates that TLR2 is an essential receptor for BLP, but not LPS, induced cellular activation. Most importantly, BLP-induced TLR2 activation was totally abrogated in BLP-tolerized cells, whereas LPS tolerance did not prevent BLP stimulation-induced TLR2 overexpression. These results indicate that unlike LPS tolerance, BLP tolerance involves suppression of TLR2 expression.

Taken together, this study shows that pretreatment of human THP-1 monocytic cells with a synthetic bacterial lipopeptide induces BLP tolerance, which is also associated with a cross-tolerance to LPS, and occurs via a CD14-independent pathway. Furthermore, whereas BLP tolerance and LPS tolerance appear to affect similar intracellular signal pathways such as MAP kinase phosphorylation and NF-κB activation, the inhibition of BLP-induced TLR2 activation is observed only in BLP-tolerized cells. These results indicate that induction of BLP tolerance correlates with the down-regulation of TLR2 expression and that the critical difference between BLP tolerance and LPS tolerance may relate to differential expression of TLR2 and TLR4.

REFERENCES

1. Manthey, C. L., and Vogel, S. N. (1992) Rev. Med. Microbiol. 3, 72–81
2. Sessler, C. N., Bloomfield, G. L., and Fowler, A. A. (1996) Clin. Chest Med. 17, 653–655
3. DiRienzo, J. M., Nakamura, K., and Inouye, M. (1978) Annu. Rev. Biochem. 47, 481–532
4. Henderson, B., Poole, S., and Wilson, M. (1996) Microbiol. Rev. 60, 316–341
5. Bierhaus, A., Flach, U. P., Flehe, R., Majetschak, M., Kreuzfelder, E., Dominguez-Fernandez, E., Borgermann, J., Reuter, M., and Oerttace, U. (1999) in Endotoxin in Health and Disease (Brade, H., Opal, S. M., Vogel, S. N., and Morrison, D. C., pp. 55–64, Marcel Dekker, Inc., New York
6. Sanchez-Cantu, L., Rode, H. N., and Christou, N. V. (1989) Arch. Surg. 124, 1432–1436
7. Zeisberger, E., and Both, J. (1998) Ann. N. Y. Acad. Sci. 856, 116–131
8. Kraatz, J., Clair, L., Rodriguez, J. L., and West, M. A. (1999) J. Surg. Res. 83, 158–164
9. Tominaga, K., Saito, S., Matsurra, M., and Nakano, M. (1999) Biochim. Biophys. Acta 1450, 137–144
10. Medvedev, A. E., Kopydovlyk, K. M., and Vogel, S. N. (2000) J. Immunol. 164, 5564–5574
11. Kohler, N. G., and Joly, A. (1997) Biochem. Biophys. Res. Commun. 232, 602–607
12. Sato, S., Numura, F., Kawai, T., Takeuchi, O., Muhratind, P. F., Takeda, K., and Akira, S. (2000) J. Immunol. 165, 7096–7101
13. Libert, D. J., Harr, C. D., and Rosenbai, R. S. (1996) Infect. Immun. 64, 3641–3645
14. Kreutz, M., Ackermann, U., Hauschildt, F., Krause, S. W., Riedel, D., Bessler, W., and Andreesen, R. (1997) Immunity 9, 396–401
15. Doyle, M., Wang, J. H., and Redmond, H. P. (2000) Surg. Forum 51, 191–193
16. Wright, S. D., Ramos, R. A., Tobias, P. S., Ulevitch, R. J., and Mathison, J. C. (1990) Science 241, 1432–1433
17. Frey, E. A., Miller, D. S., Jahr, T. G., Sundan, A., Bazil, V., Espevik, T., Finlay, B. B., and Wright, S. D. (1992) J. Exp. Med. 176, 1665–1671
18. Hazy, A., Rom, G. W., Silver, J., and Goyert, S. M. (1993) J. Immunol. 151, 1500–1507
19. Sellei, T. J., Bouis, D. A., Kitchens, R. L., Darveau, R. P., Ulevitch, R. J., and Goyert, S. M., and Jonsson, D. T. (1999) in Endotoxin in Health and Disease (Brade, H., Opal, S. M., Vogel, S. N., and Morrison, D. C., pp. 4868–4873, Marcel Dekker, Inc., New York
20. Plotz, S. G., Lentschae, A., Behrendt, H., Plotz, W., Hamann, L., Ring, J., and Gu, X. (1999) J. Immunol. 163, 618–622
Bacterial Lipoprotein Tolerance and Toll-like Receptor 2

36075

Rietschel, E. T., Flad, H. D., and Ulmer, A. J. (2001) Blood 97, 235–241
39. Aliprantis, A. O., Yang, R. B., Mark, M. R., Suggett, S., Devaux, B., Rudolf, J. D., Klimpel, G. R., Godowski, P., and Zychlinsky, A. (1999) Science 285, 736–739
40. Bowie, A. G., Moynagh, P. N., and O’Neill, L. A. (1997) J. Biol. Chem. 272, 25941–25950
41. Bourke, E., Kennedy, E. J., and Moynagh, P. N. (2000) J. Biol. Chem. 275, 39996–40002
42. Perera, P. Y., Vogel, S. N., Detore, G. R., Haziot, A., and Goyert, S. M. (1997) J. Immunol. 158, 4422–4429
43. Kitchens, R. L., and Munford, R. S. (1998) J. Immunol. 160, 1920–1928
44. DeFranco, A. L., Crowley, M. T., Finn, A., Hambleton, J., and Weinstein, S. L. (1998) Prog. Clin. Biol. Res. 397, 119–136
45. Vincenti, M. P., Burrell, T. A., and Taffet, S. M. (1992) J. Cell. Physiol. 150, 204–213
46. Takeuchi, O., Kaufmann, A., Grote, K., Kawai, T., Hoshino, K., Marr, M., Muhlradt, P. F., and Akira, S. (2000) J. Immunol. 164, 554–557
47. Zhang, H., Niesel, D. W., Peterson, J. W., and Klimpel, G. R. (1998) Infect. Immun. 66, 5196–5201
48. Bone, R. C. (1996) J. Am. Med. Assoc. 276, 565–566
49. McCall, C. E., Grosse-Wilmoth, L. M., LaRue, K., Guzman, R. N., and Cousart, S. L. (1993) J. Clin. Invest. 91, 853–861
50. Medvedev, A. E., Flo, T., Ingalls, R. R., Golenbock, D. T., Teti, G., Vogel, S. N., and Espevik, T. (1998) J. Immunol. 160, 4535–4542
51. Lee, J. D., Kato, K., Tobias, P. S., Kirkland, T. N., and Ulevitch, R. J. (1992) J. Exp. Med. 175, 1697–1705
52. Haziot, A., Ferrero, E., Kontgen, F., Hijjya, N., Yamamoto, S., Silver, J., Stewart, C. L., and Goyert, S. M. (1998) Immunity 4, 407–414
53. Lien, E., Means, T. K., Heine, H., Yoshimura, A., Kusumoto, S., Fukase, K., Fenton, M. J., Oikawa, M., Qureshi, N., Monks, B., Finberg, R. W., Ingalls, R. R., and Golenbock, D. T. (2000) J. Clin. Invest. 105, 497–504
Induction of Bacterial Lipoprotein Tolerance Is Associated with Suppression of Toll-like Receptor 2 Expression

Jiang Huai Wang, Majella Doyle, Brian J. Manning, Qiong Di Wu, Siobhan Blankson and H. Paul Redmond

J. Biol. Chem. 2002, 277:36068-36075.
doi: 10.1074/jbc.M205584200 originally published online July 19, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M205584200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 52 references, 32 of which can be accessed free at http://www.jbc.org/content/277/39/36068.full.html#ref-list-1