Lipopolysaccharide induces expression of fibronectin $\alpha_5\beta_1$-integrin receptors in human monocytic cells in a protein kinase C-dependent fashion

Jesse Roman, Jeffrey D. Ritzenthaler, Bonnie Boles, Manuel Lois, and Susanne Roser-Page. Lipopolysaccharide induces expression of fibronectin $\alpha_5\beta_1$-integrin receptors in human monocytic cells in a protein kinase C-dependent fashion. Am J Physiol Lung Cell Mol Physiol 287: L239–L249, 2004. First published April 2, 2004; 10.1152/ajplung.00244.2003.—LPS is an outer-membrane glycolipid component of gram-negative bacteria known for its fervent ability to activate monocytes and for its potent proinflammatory capabilities. In addition, LPS triggers the release of cytokines and chemokines as well as cell-cell adhesion molecules. We postulate that LPS may also affect the expression of matrix-binding integrin receptors, thereby modulating cell-adhesive functions in monocytes. To test this hypothesis, we investigated the effects of LPS on the expression of the integrin $\alpha_5\beta_1$, a fibronectin receptor, in a human monocytic cell line (U937) as well as in isolated human peripheral blood mononuclear cells (PBMCs). We found that LPS increased the expression of $\alpha_5\beta_1$ receptors and enhanced the adherence of U937 cells and PBMCs to fibronectin-coated surfaces; this was blocked by anti-$\alpha_5\beta_1$ antibodies. LPS increased $\alpha_5$-subunit mRNA accumulation in a dose- and time-dependent manner. The induction by LPS occurred, at least in part, at the level of gene transcription as indicated by experiments using $\alpha_5$ intact and deletion promoter constructs. LPS-induced $\alpha_5$ gene transcription was associated with rapid induction of conventional PKC-$\alpha$ protein and activity, was blocked by PKC inhibitors, and was mimicked by lipid A. Finally, we found that an anti-CD14 antibody was able to inhibit the LPS response. Overall, the data suggest that LPS stimulates $\alpha_5$ gene transcription via CD14 and PKC-dependent signals to enhance the expression of functional $\alpha_5\beta_1$ receptors in monocytic cells. This process may help stimulate monocytic cell activation and facilitate their migration into fibronectin-containing tissues during infection.

integrins; sepsis; endotoxin; signal transduction

The infection of tissues with gram-negative bacteria triggers a cascade of events that help establish inflammation. If left uncontrolled, infection can lead to grave consequences, including hypotension, disseminated intravascular coagulation, acute respiratory distress syndrome, multiorgan failure, and death (13). The pathophysiological mechanisms that aid in establishing inflammation during gram-negative bacterial infection are not entirely understood. It is known, however, that the interaction between bacteria or bacterial products with host cells (e.g., monocytic cells/macrophages, polymorphonuclear cells, and endothelial cells) is one of the first steps involved in the elicitation of host cell-derived factors (e.g., chemokines, cytokines, eicosanoids) responsible for the initiation and amplification of the inflammatory response (13, 28, 32).

One bacterial product known for its ability to activate mononuclear cells during gram-negative bacterial infection is lipopolysaccharide (LPS). LPSs are immunogenic glycolipids that make up the outer portion of the outer membrane of gram-negative bacteria (32, 47). There are three major domains that comprise the LPS molecule: the lipid A domain (also known as endotoxin), which functions to anchor LPS in the outer membrane and is responsible for the severe systemic inflammatory response associated with severe gram-negative infections; the core domain, made of phosphorylated nonrepeating oligosaccharides that help form the outer membrane and serve as a barrier to antibiotics; and the O-antigen polymer domain, composed of varying lengths of immunogenic repeating oligosaccharides (32). The stimulatory effects of LPS on mononuclear cells appear to be elicited by the binding of lipid A to specific surface receptors expressed by the immune cells termed CD14 (11, 31, 50, 51). Binding to CD14 is facilitated by a specific protein termed lipid A-binding protein (LBP) that is produced by hepatocytes in response to cytokines (42, 47, 48). CD14, in conjunction with LBP and MD-2 accessory protein, presents LPS to a coreceptor (18). This CD14 coreceptor is a member of a growing family of Toll-like receptor proteins (TLRs) responsible for LPS intracellular signaling (18). Once activated, these receptors trigger a number of host responses by mechanisms that remain poorly defined.

An important aspect of the inflammatory response against gram-negative bacteria is the recruitment of immune cells into the affected tissues. This process is dependent on cell-cell adhesion events that mediate the transfer of immune cells from the intravascular space into the interstitium, followed by cell-matrix events responsible for the migration of immune cells into tissues and toward the site of infection (35, 44). In the absence of cell-cell adhesion events, immune cell delivery to infected tissues is inhibited, resulting in unopposed infection. This is observed in children with leucocyte adhesion deficiency, a disease characterized by the absence or malfunction of $\beta_2$-integrin receptors that mediate the interaction between immune cells and the endothelium (3). The role of cell-matrix interactions is considered equally important, but the mechanisms that control these processes are inadequately understood (35).

This report explores the mechanisms by which LPS stimulates the adhesion of monocytic cells to fibronectin, a matrix glycoprotein highly expressed in injured tissues (33, 35, 36). Many of the cellular effects of fibronectin are mediated via the integrin $\alpha_5\beta_1$, a heterodimeric transmembrane glycoprotein.

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capable of signal transduction (33, 37, 40). The interaction of immune cells with fibronectin promotes not only cell adhesion and chemotaxis but also cell activation and the production of proinflammatory cytokines (1, 37, 40). These events are elicited by fibronectin-induced, integrin-mediated signals that induce potent transcription factors (e.g., activator protein-1 and NF-κB) capable of stimulating the transcription of gene products that affect host inflammatory responses (7, 33, 38). Herein, we demonstrate that LPS can modulate monocyteic cell adhesion to fibronectin by affecting the transcription of the α5-integrin gene, thereby stimulating the expression of functional fibronectin α5β1 receptors.

METHODS

Reagents. MEK1 inhibitor PD-98059 was purchased from New England Biolabs (Beverly, MA). The anti-α5 antibody (P1E6; directed to the α5-subunit of the α5β1-integrin), the anti-α3 antibody (P6D6; directed to the α3-subunit of the α5β1-integrin), and the anti-β1 antibody (P4C10; directed to the β1-integrin subunit) were purchased from R&D (Minneapolis, MN). All other reagents were purchased from Sigma Chemical (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA) unless otherwise specified.

Cell culture and treatment. Human monocytic/macrophage cells (U937 cells) were tested on 96-well plates (Costar, Cambridge, MA) or Fisher Scientific (Pittsburgh, PA) unless otherwise specified. U-937 cells (1× 10^6 cells/ml) were treated with 5 µg/ml of LPS for 48 h (α5β1-integrin) or for 4 h (PKC-α), washed with ice-cold PBS, and lysed in 1 ml of homogenization buffer (50 mM NaCl, 50 mM NaF, 50 mM NaPO4·12 H2O, 5 mM EDTA, 5 mM EGTA, 2 mM Na2VO4, 0.5 mM PMSF, 0.01% Triton X-100, 10 µg/ml leupeptin, and 10 mM HEPES, pH 7.4) by repeated passages through a 26-gauge needle. The resulting homogenate was centrifuged at 14,000 rpm for 5 min at 4°C.

Protein concentration was determined by the Bradford method (5). Result of the log-linear phase of the CT method (22).

Detection of α5-integrin and PKC-α by Western blot. U-937 cells (1× 10^6 cells/ml) were treated with 5 µg/ml of LPS for 48 h (α5β1-integrin) or for 4 h (PKC-α), washed with ice-cold PBS, and lysed in 1 ml of homogenization buffer (50 mM NaCl, 50 mM NaF, 50 mM NaPO4·12 H2O, 5 mM EDTA, 5 mM EGTA, 2 mM Na2VO4, 0.5 mM PMSF, 0.01% Triton X-100, 10 µg/ml leupeptin, and 10 mM HEPES, pH 7.4) by repeated passages through a 26-gauge needle. The resulting homogenate was centrifuged at 14,000 rpm for 5 min at 4°C.

Removal of LPS. LPS was removed from samples by an endotoxin affinity resin (Associates of Cape Cod, Woods Hole, MA) followed by the manufacturer’s instructions. All samples were tested after LPS removal (30) and determined to contain <0.06 ng/ml of LPS, which is within the accepted background levels of other endotoxin assays.

Detection of α5-integrin and PKC-α by Western blot. U-937 cells (1× 10^6 cells/ml) were treated with 5 µg/ml of LPS for 48 h (α5β1-integrin) or for 4 h (PKC-α), washed with ice-cold PBS, and lysed in 1 ml of homogenization buffer (50 mM NaCl, 50 mM NaF, 50 mM NaPO4·12 H2O, 5 mM EDTA, 5 mM EGTA, 2 mM Na2VO4, 0.5 mM PMSF, 0.01% Triton X-100, 10 µg/ml leupeptin, and 10 mM HEPES, pH 7.4) by repeated passages through a 26-gauge needle. The resulting homogenate was centrifuged at 14,000 rpm for 5 min at 4°C.

Protein concentration was determined by the Bradford method (5). The protein (100 µg) was mixed with an equal volume of 2× sample buffer (125 mM Tris·HCl, pH 6.8, 4% SDS, 20% glycerol, 5–10% mercaptoethanol, and 0.004% bromphenol blue), boiled for 5 min, loaded onto a 7% (α5-integrin) or a 10% (PKC-α) SDS-polyacrylamide gel with a 5% stacking gel, and electrophoresed for 2 h at 60 mA. The separated proteins were transferred onto nitrocellulose using a Bio-Rad Trans Blot semidry transfer apparatus for 1 h at 25 mA, blocked with Blotto (1× TBS (20 mM Tris, pH 7.5, 150 mM NaCl)), 5% nonfat dry milk, 0.05% Tween 20) for 1 h at room temperature, and washed twice for 5 min with wash buffer (1× TBS, 0.05% Tween 20). Blots were incubated with a polyclonal antibody raised against human α5-integrin (antibody sc-6595; 1:500 dilution) or an antibody against the COOH-terminal region of PKC-α (Sigma antibody P4334; 1:3,000 dilution) for 24 h at 4°C, washed three times for 5 min with wash buffer, and incubated with a secondary antibody raised against goat IgG conjugated to horseradish peroxidase (1:20,000 dilution) for 1 h at room temperature. Identically loaded blots used for loading controls were incubated with either β-actin (1:20,000 dilution) or GAPDH (1:20,000 dilution) primary antibodies. Blots were washed four times for 5 min in wash buffer, transferred to freshly made enhanced chemiluminescence solution (Amersham, Arlington, IL) for 5 min, and exposed to X-ray film.

PKC activity. PKC activity was measured by using the Kinase-Glo Luminescent kinase assay method (Promega) following the manufacturer’s instructions. Briefly, U937 cells (1× 10^5) were grown in suspension at 37°C in a 5% CO2 incubator in the presence or absence of 5 µg/ml of LPS for 4 h. Cells were harvested, washed with ice-cold PBS, resuspended in 200 µl of kinase reaction buffer (40 mM Tris, pH 7.5, 20 mM MgCl2, 0.1 mg/ml BSA), and sonicated. Samples were diluted in 50 µl of PKC reaction buffer (20 mM Tris, pH 7.5, 10 mM MgCl2, 0.1 mg/ml BSA, 250 µM EGTA, 400 µM CaCl2, 0.32 mg/ml phosphatidylethanolamine, 0.032 mg/ml diacylglycerol) with 10 µM ATP and 100 µl of PKCα for 90 min at room temperature. Kinase-Glo reagent (50 µl) was added, samples were incubated at room temperature for 10 min, and light intensity was measured using a ThermoLabsystems Luminoskan Ascent microtiter plate luminometer. Results were recorded as inverse relative light units. Protein
concentrations were determined by the Bradford method using Bio-Rad protein assay reagent (5).

**Electroporation and luciferase assays.** Electroporation of U937 cells was used to introduce the α5 promoter constructs: pα5(-938 bp)LUC, pα5(-178 bp)LUC, pα5(-92 bp)LUC, pα5(-41 bp)LUC, pα5(-27 bp)LUC, and pα5(-1 bp)LUC as previously described (4). Briefly, cells were washed with PBS and added to serum-free media supplemented with 10 mM dextrose and 0.1 mM DTT to a final concentration of 6 × 10^6 cells/ml. U937 cells (4.8 × 10^5 cells) were added to electroporation cuvettes (0.4-cm electrode gap) along with 40 μg of promoter construct plasmid DNA and 20 μg of the β-galactosidase reporter plasmid DNA and were subjected to 400 V and 1075 μF (Gene Pulser II Electroporation System, Bio-Rad). Electroporated cells were pooled, aliquoted into 24-well plates, and incubated with or without LPS (0–10 μg/ml) for 3 h at 37°C and 5% CO_2. In antibody studies, cells were preincubated for 1 h at 37°C and 5% CO_2

**A** U937 cells (1 × 10^5 cells) were exposed to LPS (5 μg/ml) for 3 h at 37°C, washed, and added to fibronectin-coated (50 μg/ml) polystyrene plates for 1 h at 37°C. Nonadherent cells were washed from the plates, and the remaining adherent cells were quantified by using a colorimetric-type assay that detects the intracellular enzyme hexosaminidase (19). Data are presented as means ± SD (n = 9). Note that LPS significantly increased the adhesion of U937 cells (P < 0.001) to fibronectin-coated plates. B: U937 cells or peripheral blood mononuclear cells (PBMCs; 1 × 10^9 cells/ml) were treated with 5 μg/ml of LPS for 48 h, washed with ice-cold PBS, and lysed in 1 ml of homogenization buffer. Proteins (100 μg) were separated on a 7% SDS-polyacrylamide gel, transferred onto nitrocellulose, and incubated with an anti-human α5-integrin antibody (1:500 dilution) for 24 h at 4°C. β-Actin antibody (1:1,000 dilution) was used to control for loading. The blots were incubated with a secondary rabbit anti-goat IgG conjugated to horseradish peroxidase, transferred to freshly made enhanced chemiluminescence solution, and exposed to X-ray film. Protein bands were quantified by densitometric scanning using a GS-800 calibrated laser densitometer (Bio-Rad; n = 4). C: U937 cells (1 × 10^5 cells) were preincubated with anti-α5 antibody (PID6, 100 μg/ml), anti-β1 antibody (P4C10, 100 μg/ml), anti-β2 antibody (MAB1962, 100 μg/ml), or a control IgG (not shown) for 30 min before exposure to LPS (5 μg/ml) for 3 h at 37°C. Cells were added to fibronectin-coated (50 μg/ml) polystyrene plates for 1 h at 37°C. Nonadherent cells were washed from the plates, and the remaining adherent cells were quantified by using the colorimetric-type assay. Data are presented as means ± SD (n = 9). Note that LPS significantly increased the adhesion of U937 cells (P < 0.001) to fibronectin-coated plates and that pretreatment with anti-α5- and anti-β1-integrin antibodies blocked this adhesion (anti-α5, P < 0.001; anti-β1, P < 0.004). Insert: standard curve for U937 adhesion to fibronectin-coated polystyrene plates; r = 0.996. D: PBMCs (1 × 10^6 cells) were preincubated with anti-α5 antibody (PID6, 100 μg/ml), anti-β1 antibody (P4C10, 100 μg/ml), anti-β2 antibody (MAB1962, 100 μg/ml), or a control IgG (not shown) for 30 min before exposure to LPS (5 μg/ml) for 3 h at 37°C. Cells were added to fibronectin-coated (50 μg/ml) polystyrene plates for 1 h at 37°C. Nonadherent cells were washed from the plates, and the remaining adherent cells were quantified by using the colorimetric-type assay. Data are presented as means ± SD (n = 4). Note that LPS significantly increased the adhesion of PBMCs (P < 0.001) to fibronectin-coated plates and that pretreatment with anti-α5- and anti-β1-integrin antibodies blocked this adhesion (P < 0.001). Insert: standard curve for PBMC adhesion to fibronectin-coated polystyrene plates; r = 0.988.

Fig. 1. Integrin α5β1 receptors are involved in LPS-stimulated adhesion to fibronectin. A: U937 cells (1 × 10^5 cells) were exposed to LPS (5 μg/ml) for 3 h at 37°C, washed, and added to fibronectin-coated (50 μg/ml) polystyrene plates for 1 h at 37°C. Nonadherent cells were washed from the plates, and the remaining adherent cells were quantified by using a colorimetric-type assay that detects the intracellular enzyme hexosaminidase (19). Data are presented as means ± SD (n = 9). Note that LPS significantly increased the adhesion of U937 cells (P < 0.001) to fibronectin-coated plates. B: U937 cells or peripheral blood mononuclear cells (PBMCs; 1 × 10^9 cells/ml) were treated with 5 μg/ml of LPS for 48 h, washed with ice-cold PBS, and lysed in 1 ml of homogenization buffer. Proteins (100 μg) were separated on a 7% SDS-polyacrylamide gel, transferred onto nitrocellulose, and incubated with an anti-human α5-integrin antibody (1:500 dilution) for 24 h at 4°C. β-Actin antibody (1:1,000 dilution) was used to control for loading. The blots were incubated with a secondary rabbit anti-goat IgG conjugated to horseradish peroxidase, transferred to freshly made enhanced chemiluminescence solution, and exposed to X-ray film. Protein bands were quantified by densitometric scanning using a GS-800 calibrated laser densitometer (Bio-Rad; n = 4). C: U937 cells (1 × 10^5 cells) were preincubated with anti-α5 antibody (PID6, 100 μg/ml), anti-β1 antibody (P4C10, 100 μg/ml), anti-β2 antibody (MAB1962, 100 μg/ml), or a control IgG (not shown) for 30 min before exposure to LPS (5 μg/ml) for 3 h at 37°C. Cells were added to fibronectin-coated (50 μg/ml) polystyrene plates for 1 h at 37°C. Nonadherent cells were washed from the plates, and the remaining adherent cells were quantified by using the colorimetric-type assay. Data are presented as means ± SD (n = 9). Note that LPS significantly increased the adhesion of U937 cells (P < 0.001) to fibronectin-coated plates and that pretreatment with anti-α5- and anti-β1-integrin antibodies blocked this adhesion (anti-α5, P < 0.001; anti-β1, P < 0.004). Insert: standard curve for U937 adhesion to fibronectin-coated polystyrene plates; r = 0.996. D: PBMCs (1 × 10^6 cells) were preincubated with anti-α5 antibody (PID6, 100 μg/ml), anti-β1 antibody (P4C10, 100 μg/ml), anti-β2 antibody (MAB1962, 100 μg/ml), or a control IgG (not shown) for 30 min before exposure to LPS (5 μg/ml) for 3 h at 37°C. Cells were added to fibronectin-coated (50 μg/ml) polystyrene plates for 1 h at 37°C. Nonadherent cells were washed from the plates, and the remaining adherent cells were quantified by using the colorimetric-type assay. Data are presented as means ± SD (n = 4). Note that LPS significantly increased the adhesion of PBMCs (P < 0.001) to fibronectin-coated plates and that pretreatment with anti-α5- and anti-β1-integrin antibodies blocked this adhesion (P < 0.001). Insert: standard curve for PBMC adhesion to fibronectin-coated polystyrene plates; r = 0.988.
with anti-α2, anti-α5, anti-β1, anti-β2, anti-CD14 antibody, or control antibodies. Cells were harvested, washed, and resuspended in 100 μl of cell lysis buffer, and a 20-μl aliquot was tested for luciferase activity by adding 50 μl of luciferase assay reagent (Promega). Light intensity was measured using a Thermolabsystems Luminoskan Ascent microtiter plate luminometer. Results were recorded as relative luciferase units and standardized for transfection efficiency using β-galactosidase activity.

EMSA. U-937 cells (1 × 10⁶) were grown in suspension at 37°C in a 5% CO₂ incubator in the presence of 5 μg/ml of LPS for 20 h. Cells were washed with ice-cold PBS, and nuclear binding proteins were extracted by a published method (9). Protein concentrations were determined by the Bradford method using Bio-Rad protein assay reagent (5). Double-stranded NF-κB or AP-1 consensus oligonucleotides were radioabeled with [γ-³²P]ATP using T4 polynucleotide kinase enzyme. Nuclear protein (5 μg) was incubated with radioabeled NF-κB or AP-1 (50–100,000 cpm/ng) for 30 min at room temperature as described previously (33). For competition reactions, 50-fold molar excess of double-stranded NF-κB (5'-AgTgAgg-gA CTTCCCAGCg) consensus oligonucleotide or AP-1 consensus oligonucleotide (5'-CgCTTGATgCTAggCgA) or double-stranded mutated NF-κB oligonucleotide (5'-AgTgAggCgACTT-TCCAggC) or AP-1 mutated oligonucleotide (5'-CgCTTgAT-gACTTggCCggAA) was added to the reaction. DNA-protein complexes were separated on 6% native polyacrylamide gel (20:1 acrylamide/bis ratio) in low ionic strength buffer (22.25 mM Tris HCl, pH 8.3, 50 mM NaCl, 5 mM MgCl₂, 1 mM EDTA, 2.5 mM dithiothreitol). Gels were fixed in a 10% acetic acid/10% methanol solution for 10 min, dried under vacuum, and exposed to X-ray film. Radiolaabeled DNA-protein complexes were extracted from gels and quantified by a scintillation counter.

Statistical evaluation. Means ± SD were calculated for all experimental values. Significance was assessed by ANOVA followed by Student’s t-test. All experiments were repeated 4–9 times.

RESULTS

LPS enhances U937 cell and PBMC adhesion to fibronectin via αβ₁. To determine whether LPS could enhance the adhesion of mononuclear cells to fibronectin, U937 cells were exposed to 5 μg/ml of LPS for 3 h and submitted to an adhesion assay using 96-well tissue culture plates coated with fibronectin (50 μg/ml). As shown in Fig. 1A, LPS significantly increased (P < 0.001) the adhesion of U937 cells to fibronectin. This increase in cell adhesion to fibronectin after LPS treatment was associated with an increase in αβ₁-integrin protein in U937 cells as demonstrated by Western blot in Fig. 1B. Freshly isolated PBMCs also showed a similar increase in the amount of αβ₁-integrin protein in response to LPS treatment compared with nonstimulated control cells (Fig. 1B). To further define the role of the αβ₁-integrin in constitutive and LPS-induced adhesion to fibronectin-coated plates, U937 cells and PBMCs were pretreated with blocking anti-α5-, anti-β2-, or anti-β1-integrin antibodies before submission to an adhesion assay. As expected, the increased cell adhesion to fibronectin after exposure to LPS was blocked by the anti-α5- and anti-β1-integrin antibodies; however, an antibody against the β2-integrin subunit failed to block the adhesion of cells to fibronectin-coated plates as shown in Fig. 1.

LPS induces transcription of the α5-integrin mRNA. Because increased adhesion of U937 cells and PBMCs to fibronectin after LPS treatment was associated with an increase in the amount of αβ₁-integrins present in these cells, real-time PCR was used to determine whether this coincided with an increase in endogenous mRNA accumulation for the α5-integrin. We found that the increase in the α5 mRNA was both time and dose dependent. In Fig. 2A, α5-integrin mRNA expression was measured after cells were exposed to increasing doses of LPS, ranging from 500 ng/ml to 10 μg/ml. LPS had the greatest effect on α5-integrin mRNA levels at doses between 1 and 5 μg/ml of LPS. In Fig. 2B, α5-integrin mRNA expression was dose and time dependent. A: total cellular RNA was extracted from U937 cells treated with LPS (0–10 μg/ml), and reverse transcription reactions were performed. Real-time PCR reactions were set up using forward and reverse primers for human α5 mRNA and for human β-actin mRNA. Data are presented as the log-linear phase of the growth curve and analyzed by use of the mathematical equation of the second derivative (n = 6). Note that LPS had the greatest effect at doses between 1 (P < 0.006) and 5 μg/ml (P < 0.001) compared with nontreated cells. B: total cellular RNA was extracted from U937 cells treated with LPS (5 μg/ml) for 0–24 h and reverse transcription reactions were performed. Real-time PCR reactions were set up using the forward and reverse primers for human α5 mRNA and the forward and reverse primers for human β-actin mRNA. Data are presented as the log-linear phase of the growth curve and analyzed by use of the mathematical equation of the second derivative (n = 6). Note that LPS had the greatest effect on α5 mRNA at 8 h (P < 0.005). C: total cellular RNA was extracted from PBMCs treated with LPS (5 μg/ml) for 8 h, and reverse transcription reactions were performed. Real-time PCR reactions were set up using the forward and reverse primers for human α5 mRNA and the forward and reverse primers for human β-actin mRNA. Data are presented as the log-linear phase of the growth curve and analyzed by use of the mathematical equation of the second derivative (n = 4). Note that PBMCs treated with LPS demonstrate an increased expression of α5 mRNA (P < 0.005). CT, cycle threshold.
LPS induces transcription of the α5-integrin gene. The data presented above demonstrate that LPS induces the expression of the α5β1-integrin in U937 cells and PBMCs. To determine whether the stimulatory effect of LPS occurs at the level of gene transcription, experiments were performed in U937 cells transiently transfected with the human α5 gene promoter connected to a luciferase reporter gene. Transfected cells were stimulated with 5–15 μg/ml of LPS for 3–20 h at 37°C and 5% CO2. The cells were harvested, and an aliquot of cell extract was used to measure luciferase activity. We observed that LPS indeed induced the transcription of the α5 promoter construct, which was maximal at a concentration of 5 μg/ml of LPS and after 20 h of incubation (Fig. 3A). To determine the functional significance of LPS in the induction of the α5 promoter, LPS was removed from the cell culture medium using an endotoxin affinity resin. After LPS removal, the medium was determined to contain <0.06 ng/ml of LPS. This resulted in a significant inhibition (P < 0.001) of the LPS-induced transcription of the α5 gene (Fig. 3B).

Induction of α5 gene expression by LPS is dependent on PKC activation. Because LPS has been previously shown to induce the activation of PKC, we set out to investigate whether its stimulatory effect on α5 gene transcription was mediated by this pathway. First, in support for a role of PKC in LPS induction of α5, we demonstrated in Fig. 4A that LPS treatment of U-937 cells enhanced the expression of conventional PKC-α (cPKC-α) protein, which was maximal at 4 h. cPKC-α is one isoformal of PKC shown to regulate selective LPS-induced macrophage functions involved in the host defense and inflammation response (32). Second, to confirm the function of the enhanced expression of PKC by LPS, we measured active PKC with the use of a luminescent kinase assay. U937 cells treated with LPS showed a significant increase (P < 0.001) in PKC activity compared with nontreated control cells as demonstrated in Fig. 4B. Third, we showed that pretreatment of U-937 cells with active calphostin C (CC*) or chelerythrine chloride, two potent inhibitors of PKC, abrogated the increase in α5 gene transcription in response to LPS treatment (Fig. 4, C and D, respectively). In contrast, inactivated PKC inhibitor (CC) did not affect α5 expression (Fig. 4C).

Transcriptional regulation of the α5 gene in response to LPS. To learn more about the possible cis-acting elements involved in the LPS stimulation of the α5 gene transcription, U937 cells were transfected with the 923-bp α5 promoter construct pα5(−923 bp)LUC or various 5′ deletion promoter constructs: pα5(−178 bp)LUC, pα5(−92 bp)LUC, pα5(−41 bp)LUC, pα5(−27 bp)LUC, and pα5(−1 bp)LUC (Fig. 5). As before, LPS stimulated the expression of the pα5(−923 bp)LUC promoter construct >3.7-fold compared with controls not stimulated with LPS. After the deletion of 805 bp of the distal end of the pα5(−923 bp)LUC promoter to create the pα5(−178 bp)LUC construct, the intensity of the LPS response diminished 1.12-fold compared with the pα5(−923 bp)LUC construct. When an additional 86 bp of the 5′ end of the pα5(−178 bp)LUC promoter was deleted to make the pα5(−92 bp)LUC construct, LPS stimulation of the α5 promoter, when analyzed against control, was reduced 2.19-fold compared with the pα5(−923 bp)LUC construct. However, the most drastic reduction [>2.75-fold compared with the pα5(−923 bp)LUC construct] in LPS-stimulated α5 promoter expression was seen with plasmids pα5(−41 bp)LUC, pα5(−26 bp)LUC, and pα5(−1 bp)LUC. This indicates the

μg/ml with cycle threshold (CT) values of 25.19 (P < 0.006) and 26.66 (P < 0.001), respectively, compared with nontreated control cells (CT = 28.31). In Fig. 2B, the effect of LPS (5 μg/ml) was optimal at 8 h (CT = 14.18, P < 0.005) compared with nontreated control cells (CT = 18.10). Similar results were detected in PBMCs as demonstrated in Fig. 2C, with threshold values of 29.0 and 30.56 for LPS-treated cells and nontreated control cells, respectively (P < 0.01).

Fig. 3. LPS induces α5-integrin gene transcription in a time-dependent manner. A: U937 cells (6 × 10⁷ cells/ml) were electroporated with the human α5 promoter pα5(−938 bp)LUC, washed, pooled, and aliquoted into 24-well plates. Cells were treated with or without LPS (5 μg/ml) for 3 or 20 h at 37°C and 5% CO2, followed by testing for luciferase activity as described above. Results were recorded as relative luciferase units and standardized for transfection efficiency using β-galactosidase activity. Data are presented as means ± SD (n = 9). Note that LPS induced maximal α5 gene transcription at 20 h (P < .001). B: U937 cells (6 × 10⁷ cells/ml) were electroporated with the human α5 promoter pα5(−938 bp)LUC, washed, pooled, and aliquoted into 24-well plates. Cells were treated with media that contained LPS (5 μg/ml) or media in which the LPS was removed for 20 h at 37°C and 5% CO2, followed by testing for luciferase activity. Light intensity was measured using a ThermoLabsystems Luminoskan Ascent microtiter plate luminometer. Results were recorded as relative luciferase units and standardized for transfection efficiency using β-galactosidase activity. Data are presented as means ± SD (n = 9). Note that removal of LPS greatly diminishes the induction of the α5 gene transcription (P < 0.001).

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Fig. 4. Role of conventional PKC-α (cPKC-α) in LPS induction of the α5 gene. A: U937 cells (1 × 10^6 cells/ml) were treated with 5 μg/ml of LPS for 4 h, washed with ice-cold PBS, lysed, and processed for Western blot with a polyclonal antibody raised against human cPKC-α (1:3,000 dilution). GAPDH antibody (1:2,000 dilution) was used to control for loading. Protein bands were quantified by densitometric scanning using a laser densitometer. B: PKC activity was measured in U937 cells (1 × 10^7) incubated at 37°C and 5% CO_2 in the presence or absence of 5 μg/ml of LPS for 4 h. Cells were harvested, washed, resuspended kinase reaction buffer, and sonicated. Samples were diluted in 50 μl of PKC reaction buffer and incubated for 90 min at room temperature. Kinase-Glo reagent (50 μl) was added, samples were incubated at room temperature for 10 min, and light intensity was measured using a ThermoLabsystems Luminoskan Ascent microtiter plate luminometer. Results were recorded as inverse relative light units/μg protein (n = 4). Protein concentrations were determined by the Bradford method using Bio-Rad protein assay reagent (5). Note that U937 cells treated with LPS demonstrated a significant increase in PKC activity (P < 0.001) compared with nontreated control cells.

Fig. 5. LPS responsive region is located between -92 and -41 bp downstream from the transcriptional start site of the α5 gene. U937 cells (6 × 10^6 cells/ml) were electroporated with the following human α5 promoter or deletion constructs: pα5(-938 bp)LUC, pα5(-178 bp)LUC, pα5(-92 bp)LUC, pα5(-41 bp)LUC, pα5(-27 bp)LUC, and pα5(1 bp)LUC. Cells were treated with or without LPS (5 μg/ml) for 20 h at 37°C and 5% CO_2 in the presence or absence of the PKC inhibitor calphostin C (CC, inactive; CC*, active) followed by testing for luciferase activity. Light intensity was measured using a plate luminometer, and the results were recorded as relative luciferase units and standardized for transfection efficiency using β-galactosidase activity. Data are presented as means ± SD (n = 9). Note that CHE abrogated the LPS-induced α5 gene transcription (P < 0.001).
presence of a key LPS response element(s) located in the α5 promoter between −92 and −41 bp downstream from the transcriptional start site. This promoter region also appears important for the basal expression of the α5 gene since there was very little promoter activity in cells transfected with the −27-bp and −1-bp deletion constructs.

**Trans-acting factor NF-κB, but not AP-1, is involved in LPS induction of the α5 promoter.** Nuclear binding proteins were isolated from U937 cells stimulated with or without LPS for 20 h and subjected to an EMSA using radiolabeled consensus NF-κB and AP-1 oligonucleotides. Data shown in Fig. 6A show an increase in the bound NF-κB nuclear protein-DNA complex in cells treated with LPS compared with control untreated cells. To demonstrate DNA-protein binding specificity, nonradiolabeled competitor NF-κB or mutated NF-κB oligonucleotide (50-fold molar excess) was added to the binding reaction. The consensus NF-κB, but not the mutated NF-κB competitor, was able to compete for binding. There was also a slight increase in the bound AP-1 nuclear protein-DNA complex in LPS-treated cells (Fig. 6B). Again, to demonstrate DNA-protein binding specificity, nonradiolabeled competitor AP-1 or mutated AP-1 oligonucleotide (50-fold molar excess) was added to the binding reaction. To determine whether NF-κB binding proteins were involved in the function of transcriptional regulation of the α5 gene, consensus NF-κB competing oligonucleotides were cotransfected into U937 cells along with the pα5(−923 bp)LUC promoter and stimulated with or without LPS. Data in Fig. 7A confirm that the NF-κB is an important cis-acting element for the LPS induction of the α5 gene since competing NF-κB is able to abrogate the LPS induction. Figure 7B, on the other hand, demonstrates that the AP-1 cis-acting element is not essential for the LPS induction of the α5 gene since it was unable to abrogate the LPS induction.

**Lipid A mimics the induction of the α5 gene transcription.** Because the lipid A portion of LPS is considered the key component responsible for the pathophysiology of severe gram-negative infections, we set out to determine whether lipid A could mimic the effect of intact LPS on the induction of the α5 gene. Again, U937 cells were transfected with the pα5(−923 bp)LUC promoter construct and treated with or without the lipid A component, and α5 gene transcription was quantified by luminescence. As shown in Fig. 8, lipid A was identical to LPS in its ability to significantly induce α5 gene transcription (P < 0.05).

**LPS induction involves the CD14 receptor.** Data from previous reports identify the CD14 receptor as being a key component in the LPS induction pathway, and to test its importance in our system, U937 cells transfected with the pα5(−923 bp)LUC construct were pretreated with the anti-CD14 antibody and stimulated with or without LPS. Additional antibodies, such as Ab5 or control IgG, anti-integrin α5, anti-CD11b, and anti-integrin β2 were also used. As the data show in Fig. 9, pretreatment of cells with the various antibodies did not significantly alter the baseline induction of the α5 promoter. In contrast, pretreatment of cells with the anti-CD14 antibody completely blocked the LPS induction of the α5 gene transcription (P < 0.001). Treatment with Ab5 or control IgG and the anti-β2 antibody did not inhibit the LPS induction of the α5 promoter (P = 0.9 and P = 0.1, respectively), whereas an anti-CD11b slightly reduced the LPS effect (P < 0.01).

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**Fig. 6.** Role of NF-κB and AP-1 in LPS-induced α5 gene transcription. **A:** U937 cells (1 × 10⁶) were grown in suspension at 37°C in a 5% CO₂ incubator in the presence or absence of 5 µg/ml of LPS. Cells were washed, and nuclear binding proteins were extracted. Radiolabeled double-stranded NF-κB consensus oligonucleotide (75–100,000 cpm/ng) was incubated with extracted nuclear binding proteins for 30 min at room temperature. DNA-protein complexes were separated on 6% native polyacrylamide gel, gels were fixed in a 10% acid acid/10% methanol, dried, and exposed to X-ray film. Note that LPS induced more binding to NF-κB compared with control nontreated cells and that the nonradiolabeled competitor NF-κB, but not mutated NF-κB oligonucleotide (50-fold molar excess), was able to compete for binding. **B:** U937 cells (1 × 10⁶) were grown in suspension at 37°C in a 5% CO₂ incubator in the presence or absence of 5 µg/ml of LPS. Cells were washed, and nuclear binding proteins were extracted. Radiolabeled double-stranded AP-1 consensus oligonucleotide (50–70,000 cpm/ng) was incubated with extracted nuclear binding proteins for 30 min at room temperature. DNA-protein complexes were separated on 6% native polyacrylamide gel, gels were fixed in a 10% acid acid/10% methanol, dried, and exposed to X-ray film. Note that LPS induced slightly more binding to AP-1 compared with control nontreated cells and that the nonradiolabeled competitor AP-1, but not mutated AP-1 (mAP-1) oligonucleotide (50-fold molar excess), was able to compete for binding.
LPS STIMULATES EXPRESSION OF $\alpha_5\beta_1$

During an infection with gram-negative bacteria, host immune cells become activated and migrate into affected tissues where they play a major role in the inflammatory response. LPS has been shown to trigger the release of many host inflammatory factors such as chemokines, cytokines, and cell adhesion molecules. Exposure of host cells to infectious organisms or their cell wall components stimulate the production and release of extracellular matrix molecules, such as collagen and fibronectin, from macrophages, epithelial cells, and fibroblasts in the affected area. These events combine to aid in the migration and activation of immune cells that are capable of detecting insoluble gradients created by the differential expression of matrix components at the site of infection (35, 37). The degradation of these extracellular matrix components via an increase in the expression and activation of matrix-degrading proteases (e.g., matrix metalloproteinases) also releases the chemotactic fragments for attracting immune cells. Fibronectin fragments have been shown to be chemotactic toward monocytes and neutrophils, among other cells (25, 27). This binding of immune cells to fibronectin or fibronectin fragments occurs through the fibronectin receptor or $\alpha_5\beta_1$-integrin. Binding to the $\alpha_5\beta_1$-integrin receptor then activates a cascade of downstream signal transduction events that in turn lead to the activation of transcription factors, including members of the NF-κB/Rel family that activate inflammatory genes like tumor necrosis factor-$\alpha$ and interleukin-1 (7, 38).

In this report, we explore in detail how LPS stimulates the expression of functional $\alpha_5\beta_1$ receptors. Our studies expand on others, demonstrating increased bacterial adherence to fibronectin and endothelial cells in response to LPS (49). We show that LPS treatment of U937 cells and PBMCs increases cell adhesion to fibronectin, a process likely to aid immune cell tropism into infected tissues. Of note, we find that an antibody against another integrin subunit, $\beta_3$, also enhanced cell adhesion to fibronectin. This might be related to IgG-induced receptor clustering and induction of an “outside in” or “inside out” $\beta_2$ receptor signaling that culminates in increased $\alpha_5\beta_1$.

DISCUSSION

During an infection with gram-negative bacteria, host immune cells become activated and migrate into affected tissues where they play a major role in the inflammatory response. LPS has been shown to trigger the release of many host inflammatory factors such as chemokines, cytokines, and cell adhesion molecules. Exposure of host cells to infectious or-
function and/or expression. Further studies of the β2-integrin receptor are needed to resolve this issue. In addition, we show that the lipid A component of LPS stimulates the transcription of the gene coding for the α5-integrin subunit that results in accumulation of α5 mRNA and an increased expression of α5β1-integrin receptors. These events are mediated via CD14-dependent signals that include PKC activation.

Many host molecules have been shown to stimulate the expression of integrins. For example, transforming growth factor-β, a product of the tissue remodeling response triggered after injury, is well known for its ability to stimulate the expression of α5β1 and other integrins (34). Phorbol esters are also capable of this response (4). However, less is known about how pathogenic bacteria can promote this response. Bacterial surface molecules like LPS also show agonistic activity toward integrin expression (16). The ability of LPS to stimulate integrin expression differs depending on the integrin and on the immune cell type and differentiation state being studied. For example, in contrast to alveolar macrophages, monocytes show an increase in the expression of CD11α, CD11b, and CD11c and a decrease in L-selectin when exposed to LPS (15). Others have shown the induction of α5β1-integrin in monocytes exposed to LPS and interferon-γ (41).

In this report, the LPS-induced integrin response was found to be antagonized by anti-CD14 antibodies. This is not surprising since CD14 receptors are known to mediate many of the effects of LPS, at least indirectly. LPS fails to elicit tumor necrosis factor-α and interleukin-6 production in macrophages obtained from CD14-null mice (24). CD14 is a glycosylphosphatidylinositol-linked surface protein found on macrophages and other cells (with a soluble form present in the serum) that is responsible for lipid A recognition (11, 31, 50). The NH2-terminal domain of CD14 contains the LPS binding site and is sufficient for cell activation (31). LBP apparently functions as a lipid transfer protein that delivers LPS to CD14 receptors.

Once bound, CD14 appears to redirect LPS to other transmembrane proteins, such as members of the TLRs like TRL4 (and MD-2 accessory protein) and TLR6 (18). TRL4 signals through myeloid differentiation factor 88 to activate transcription factors NF-κB, ELK-1, and AP-1 (23). Ultimately, LPS induction of these transmembrane proteins generates key intracellular signals that stimulate the production of cytokines, enzymes (such as nitric oxide synthetase), and other non-protein mediators (e.g., platelet-activating factor), many of which are considered important for the development of clinical presentation of endotoxin-induced shock (13, 20, 32). These transcriptional events seem to be related to the rapid activation of specific proteins via tyrosine phosphorylation, specifically mitogen-activated protein kinase (MAPK) and NF-κB (31). Interestingly, β-cells, among other cell types, lack CD14 receptors but nevertheless respond to LPS, suggesting an alternative pathway. It seems that these cells use a soluble CD14 that forms a complex with LPS that is recognized by an unknown component on the cell surface, leading to the release of cytokines and upregulation of adhesion molecules (11, 13, 44, 50, 51). This alternative pathway can be used by CD14-bearing cells in environments where LBP is low (11, 13, 44, 50). This and other observations suggest that CD14 is essential for macrophage responses to free LPS, but that other receptors (e.g., CD11b/CD18) can assist in responding to intact bacteria (39).

Role of PKC and transcription factor activation. Herein, we demonstrate that LPS induces the expression and activity of cPKC-α and that this signaling molecule is important for LPS-induced integrin expression. In monocytes and macrophages, other signaling pathways activated by LPS include phospholipases A (10) and C (6), other PKC isoforms (43), and the Src family tyrosine kinases Hck, Lyn, and Fgr (46). LPS also induces the phosphorylation of Vav (12) and the activation of the MAPK family p42/p44 (21).

Some of the signaling events described above have been shown to induce, among other things, the expression of monocytic genes by activating the NF-κB/Rel transcription factor family. This was found to be true in our system where induction of NF-κB DNA binding by LPS was found to be important for α5 gene transcription. Others have shown in unstimulated monocytes that NF-κB is retained in the cytoplasm by binding to a family of inhibitors (IκB-α, IκB-β, IκB-ε). Because LPS induces IκB kinase activity in human monocytes and THP-1 monocytic cells, it is likely that LPS affects NF-κB activity in more than one way (26).

Implications. Together, our data suggest that the interaction of host mononuclear cells with bacterial-derived LPS triggers intracellular signals that stimulate the expression of α5β1-
integrin receptors. In view of many demonstrated functions of αsβ1 related to cell migration, chemotaxis, and cellular activation, this might represent a mechanism of tissue tropism by which the host enhances its capability of delivering immune cells into tissues during active infection. However, the unopposed stimulation of these signals might lead to an overwhelming inflammatory response that results in tissue destruction and shock. This is consistent with the idea that it is the host inflammatory response that results in tissue destruction and posed stimulation of these signals might lead to an overwhelming, this might represent a mechanism of tissue tropism by

LPS stimulates expression of αsβ1


LPS STIMULATES EXPRESSION OF $\alpha_\delta\beta_1$


