Inhibition of Lipopolysaccharide-Induced Transcription Factor Sp1 Binding by Spectrally Pure Diphosphoryl Lipid A from *Rhodobacter sphaeroides*, Protein Kinase Inhibitor H-8, and Dexamethasone

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The transcription factor Sp1 plays a crucial role in the monocyte-specific expression of CD14, a binding site (or putative receptor) for lipopolysaccharide (LPS) complexes with LPS-binding protein (LBP). By using RAW 264.7 macrophages treated with spectrally pure deep-rough-chemotype hexa-acyl LPS from *Escherichia coli* D31m4, three inhibitors were found to block the binding activity of transcription factor Sp1, as measured by electrophoretic mobility shift assays. These inhibitors were diphosphoryl lipid A from *Rhodobacter sphaeroides* (10 µg/ml); the isoquinoline-sulfonamide H-8 (10 and 100 µM), which is thought to be a cGMP-dependent protein kinase inhibitor; and the anti-inflammatory agent dexamethasone (10 µM).

It is believed that early in the course of endotoxic shock, macrophages are activated by lipopolysaccharide (LPS) from gram-negative bacteria. LPS interacts in the serum with the 60-kDa LPS-binding protein (LBP) and then complexes with the 55-kDa membrane-bound CD14 antigen (mCD14) on leukocyte surfaces (10, 25). By a partially understood pathway(s), signals are somehow transduced from mCD14, or another associated protein on the surface (5), via cytosolic kinases, such as mitogen-activated protein kinase (4), to nuclear transcription factors, such as NF-κB (13). Ultimately, gene activation produces the proinflammatory cytokines tumor necrosis factor alpha (TNF-α), nitric oxide (NO), and certain interleukins, including interleukin-1, that lead to shock-like symptoms (2, 9, 24).

Prior to LPS stimulation, NF-κB, a heterodimer of 50- and 65-kDa subunits, is complexed to the inhibitor IκB. After LPS activation of 70Z/3 pre-B cells, IκB dissociates and p50/65 translocates to the nucleus, where it binds to DNA. The LPS-inducible activation of p50/65 in 70Z/3 pre-B cells and macrophages is blocked by the naturally occurring LPS antagonist diphosphoryl lipid A from *Rhodobacter sphaeroides* (RsDPLA). In addition to RsDPLA, there are reports of two other effective disaccharide LPS antagonists (16), but only RsDPLA (12, 18) and RsDPLA-based compounds (3) are active in both humans and in mice. RsDPLA, at high concentrations, induces adrenocorticotrophic hormone and corticosterone within 15 to 30 min of administration in mice (18). In mice and in HeLa cells, dexamethasone, a synthetic corticosteroid, blocks the LPS-induced activation of NF-κB binding by preventing both the dissociation of NF-κB from IκBα and the subsequent translocation of NF-κB to the nucleus (1, 23).

In addition to NF-κB, LPS activates the transcription factor Sp1 (22), which is phosphorylated after binding to DNA enhancer elements. The promoter for the TNF-α gene contains consensus sequence binding sites for Sp1 and NF-κB, among

others (27), suggesting that those transcription factors may also regulate LPS-induced TNF-α production. In human monocytes, there is an Sp1 binding site in the promoter region for the CD14 gene, suggesting that mCD14 expression on the cell surface involves Sp1 binding (26).

We have found three agents that inhibit the ReLPS-initiated Sp1 activity in RAW 264.7 macrophages. They are (i) the LPS antagonist RsDPLA, (ii) H-8, a cyclic GMP (cGMP)-dependent protein kinase (PKG) inhibitor, and (iii) dexamethasone, a synthetic glucocorticoid and potent regulator of gene activation. This is the first report of inhibition, by these three agents, of LPS-induced Sp1 binding. Inhibitors of Sp1 binding might be useful in preventing endotoxic shock in animals.

MATERIALS AND METHODS

Reagents. "Spectrally pure" deep-rough-chemotype hexa-acyl LPS from *Escherichia coli* D31m4 (ReLPS) and RsDPLA were prepared in our laboratory, as described by Qureshi et al., and checked for homogeneity by mass spectrometry (19–21). Without this level of spectral purity, contaminating phospholipids and proteins in LPS preparations may confound the results. The protein kinase inhibitors H-7 and H-8 were obtained from Seikagaku America (Rockville, Md.). Dulbecco's modified Eagle's medium (DMEM, containing glucose plus glutamine) and fetal bovine serum (FBS) were obtained from BioWhittaker. All glassware and plasticware were rendered pyrogen free by heating at 120°C overnight.

Electrophoretic mobility shift assay. RAW 264.7 murine macrophage-like cells were cultured in DMEM supplemented with 10% FBS and 100 µg of gentamicin per ml. At 30 min or more before the experiment, 5×10^5 to 1×10^6 RAW cells/ml were plated at 1 ml in each well of a 12-well plate (Corning) and incubated at 37°C in 5% CO₂. ReLPS or RsDPLA was suspended in pyrogen-free saline at 1 mg/ml, sonicated for 30 s in a sonic water bath, and diluted in DMEM-FBS. A 100-µl portion of 10× ReLPS and/or 10× RsDPLA was added to the cells. Final concentrations (1×) were typically 10 or 100 ng/ml for ReLPS and 1 or 10 µg/ml for RsDPLA. RsDPLA, the inhibitors H-7 and H-8 (prepared as 10 mM stock solutions in pyrogen-free water), and dexamethasone (Lyphomed) were diluted in DMEM-FBS, added to the cells 30 min before ReLPS, and incubated at 37°C.

Cells treated with ReLPS or RsDPLA were scraped free, washed twice with 0.4 ml of cold saline, and resuspended in 5 µl of gel shift buffer [10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 0.5 mM dithiothreitol, 0.5 mM EDTA, 1 mM MgCl₂, 4% glycerol, 50 µg of poly(dI-dC) (Promega) per ml] containing 0.1 mM phenylmethylsulfonyl fluoride prepared from a fresh ethanol stock solution. Adding 5 mM ZnCl₂ to this buffer did not enhance Sp1 binding (data not shown). The cells were lysed by three freeze-thaw cycles and centrifuged at $8,500 \times g$ for 10 min, and the cell supernatant was saved. This method (6) was used for Sp1 electro-

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TABLE 1. Consensus sequence oligonucleotides

Transcription factor	Sequence ^a
NF- κ B	5'-AGT TGA GGG GAC TTT CCC AGG C-3'
Sp1	5'-ATT CGA TCG GGG CGG GGC GAG C-3'
TfIID	5'-GCA GAG CAT ATA AGG TGA GGT AGG A-3'
GRE	5'-TCG ACT GTA CAG GAT GTT CTA GCT ACT-3'
AP1	5'-CGC TTG ATG AGT CAG CCG GAA-3'

^a Obtained from Promega Corp.

phoretic mobility shift assay and measured total cellular transcription factor binding, not nuclear-fraction binding.

Each 10- μ l reaction volume contained 2 μ l of cell supernatant solution, labeled and unlabeled oligonucleotides, and gel shift buffer. For both specific and nonspecific competition, the final concentrations of cold oligonucleotides (Promega) were 175 nM. The final concentrations of radiolabeled oligonucleotides were 3.5 nM. The [γ -³²P]ATP labeling (with T4 polynucleotide kinase) of oligonucleotides containing transcription factor consensus sequences (Table 1), binding reactions, and electrophoresis in 4% native polyacrylamide minigels were carried out as described previously (17a). The gel buffer and electrode buffer were composed of 45 mM Tris-HCl (pH 8.0) with 45 mM borate and 1 mM EDTA. For blockage of Sp1 binding, an anti-Sp1 rabbit polyclonal antibody (Santa Cruz Biotechnology) was preincubated at 100 ng/10 μ l (final concentration) with cell supernatant solution on ice for 30 min before addition of oligonucleotides.

RESULTS

Sanceau et al. (22) reported that 1 μ g of LPS per ml induced Sp1 binding activity. Our spectrally pure ReLPS at 10 ng/ml induced similar Sp1 binding activity (Fig. 1, lane 2). This Sp1 binding was specific, as binding was abolished by preincubation with an anti-Sp1 antibody at 100 ng/10 μ l (lane 4). Lower antibody concentrations gave partial (10 ng/10 μ l) or no (1 ng/10 μ l) inhibition (data not shown). In contrast to ReLPS (Fig. 2, lane 4), 10 μ g of RsDPLA per ml did not induce Sp1 binding (lane 6). After a 30-min preincubation, RsDPLA blocked ReLPS-induced Sp1 binding (lane 8). Lanes 1, 3, 5, and 7 contain the Sp1 oligonucleotide as a cold competitor. In contrast, the TfIID consensus sequence oligonucleotide was often used as a cold, nonspecific competitor for ³²P-labeled

FIG. 1. Induction of Sp1 binding by ReLPS and inhibition by anti-Sp1 antibody. RAW 264.7 cells were incubated with 10 ng of ReLPS per ml for 30 min at 37°C prior to extraction of total cellular proteins for use in a competitive electrophoretic mobility shift assay, as described previously (6). The anti-Sp1 rabbit polyclonal antibody was preincubated at 100 ng/10 μ l (final concentration) with cell extract on ice for 30 min before the addition of oligonucleotides. NF- κ B oligonucleotide was the cold nonspecific competitor. Lanes: 1, unstimulated cells; 2, 10 ng of ReLPS per ml; 3, anti-Sp1 antibody; 4, ReLPS plus anti-Sp1 antibody.

FIG. 2. Induction of Sp1 binding in RAW 264.7 cells by spectrally pure ReLPS and blocking by RsDPLA. Lanes 1, 3, 5, and 7 contain cold Sp1 oligonucleotide as the competitor. Lanes 2, 4, 6, and 8 contain TfIID oligonucleotide as the cold nonspecific competitor. Lanes: 1 and 2, no additions (i.e., cells alone); 3 and 4, 100 ng of ReLPS per ml; 5 and 6, 10 μ g of RsDPLA per ml; 7 and 8, 10 μ g of RsDPLA per ml added 30 min before 100 ng of ReLPS per ml; after ReLPS addition, the cells were incubated for 15 min at 37°C. Arrows indicate the major Sp1-DNA complex.

Sp1, but the same results were obtained with other unrelated, cold competitor sequences (e.g., NF- κ B, AP1, or glucocorticoid response element [data not shown]).

We next sought to determine if Sp1 binding activity could be blocked by inhibitors of PKG, cAMP-dependent protein kinases (PKA), or protein kinase C (PKC). Compound H-8 inhibits PKG most strongly ($K_i = 0.48$ μ M [Seikagaku America]) and also inhibits PKA to a lesser extent ($K_i = 1.2$ μ M) and PKC weakly ($K_i = 6.0$ μ M). On the other hand, H-7 inhibits predominantly PKA ($K_i = 3.0$ μ M) but also PKG ($K_i = 5.8$ μ M) and PKC ($K_i = 6.0$ μ M). Neither 10 μ M (data not shown) nor 100 μ M H-7, the inhibitor predominantly of PKA, had an inhibitory effect on Sp1 binding (Fig. 3, lane 4). It was necessary to use 100 μ M H-7 to ensure a concentration of inhibitor well in excess (33-fold) of the K_i . In our 45-min incubations with the inhibitors at 100 μ M, we found no cell toxicity, as determined by trypan blue dye exclusion. Sp1 binding activity that was induced by 100 ng of ReLPS per ml was blocked by preincubation with either 10 μ M (not shown) or 100 μ M H-8, an inhibitor predominantly of PKG (Fig. 3, lane 6).

Because corticosteroids have been reported to block the translocation of NF- κ B to the nucleus (23) and subsequent DNA binding, we wondered if corticosteroids would also block Sp1 binding activity. We observed that 10 μ M dexamethasone did indeed reverse ReLPS-initiated Sp1 binding activity (Fig. 4, lane 8). Less than 10 μ M dexamethasone did not reproducibly inhibit ReLPS-induced Sp1 binding, and cell viability was not affected by 10 μ M dexamethasone during the 1-h incubations (data not shown). The minor, faster-migrating Sp1 complexes in Fig. 4, for example, are considered by authorities on Sp1 to result from proteolytic degradation products of intact Sp1 (8).

DISCUSSION

Sp1 is a glycosylated transcription factor with three zinc fingers at its C terminus that bind to GC box enhancer elements (GGGCGG) upstream of genes that are transcribed by RNA polymerase II (17). After binding to the GC box, Sp1 is phosphorylated (22). Thus, the universal transcription factor Sp1 is regulated, in part, by phosphorylation. Additionally, Sp1 seems to play a role in the specificity of expression of CD14, a binding site (or putative receptor) for LPS-LBP complexes, on monocytes (26).

FIG. 3. Inhibition of ReLPS-induced Sp1 binding by the PKG inhibitor H-8. ReLPS was incubated with RAW 264.7 cells for 15 min at 37°C. Inhibitors were added 30 min before ReLPS. Lanes: 1, cells alone; 2, 100 ng of ReLPS per ml; 3, 100 μ M inhibitor H-7; 4, 100 μ M H-7 plus 100 ng of ReLPS per ml; 5, 100 μ M inhibitor H-8; 6, 100 μ M H-8 plus 100 ng of ReLPS per ml. TFIID oligonucleotide was the cold nonspecific competitor. Arrowheads indicate the major Sp1-DNA complex.

We have described the induction of Sp1 binding by spectrally pure ReLPS. Thus, we confirmed that it was LPS and not fatty acids or protein that was active in a previous study (22). Here, we note for the first time the inhibition by RsDPLA of ReLPS-initiated Sp1 activity. Furthermore, the PKG inhibitor H-8 and dexamethasone, individually, were also found to block ReLPS-initiated Sp1 binding activity. Thus, we have inhibited Sp1 activity at three separate places in a ReLPS signal transduction pathway. The first place of inhibition was by RsDPLA competition with ReLPS for LBP and mCD14, sCD14, or non-CD14 membrane-associated receptors (5, 11, 14). The second place of inhibition was by H-8 at some step in a kinase cascade that involves ReLPS activation of a PKG and that leads to Sp1 binding activity (Fig. 3). The inhibitor H-7 had no such effect on Sp1 binding (Fig. 3). The amount of kinase inhibitors used, 100 μ M, was enough to be well in excess of each K_i and was not toxic during our short incubations. Thus, Sp1 induction may be

FIG. 4. Inhibition of ReLPS-induced Sp1 binding by dexamethasone. ReLPS was incubated with RAW 264.7 cells for 15 min at 37°C. Dexamethasone was added 30 min before ReLPS. Lanes 1, 3, 5 and 7 contain Sp1 oligonucleotide as the cold competitor; lanes 2, 4, 6, and 8 contain TFIID oligonucleotide as the cold nonspecific competitor. Lanes: 1 and 2, no additions, i.e., cells alone; 3 and 4, 100 ng of ReLPS per ml; 5 and 6, 10 μ M dexamethasone; 7 and 8, dexamethasone and ReLPS.

downstream of one or more PKGs, in the same or a different signalling pathway from that of NF- κ B (13). The third place of inhibition involved dexamethasone, which somehow blocked Sp1 binding (Fig. 4), perhaps by transrepression of *sp1* or by transactivation of other genes (15).

This is the first report of inhibition of LPS-induced Sp1 binding by RsDPLA or H-8. Here we also report that a synthetic corticosteroid, dexamethasone, blocks Sp1 binding activity. Corticosteroids have previously been shown to prevent both the dissociation of NF- κ B from I κ B α and the subsequent translocation of NF- κ B to the nucleus (1, 23). Thus, the mechanism by which corticosteroids affect inflammation may be very complex. As Sp1 seems to be involved in the tissue-specific expression of CD14 (26), and mice that are rendered deficient in CD14 by homologous recombination are 1,000-fold less sensitive to LPS (7), it may be possible to induce tolerance to LPS by blocking Sp1 binding. Two of the agents described here, RsDPLA (18) and dexamethasone (23), may be preventing endotoxic shock in mice by inhibiting Sp1 binding activity.

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