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Modulation of Lipopolysaccharide-Induced Macrophage Gene Expression by *Rhodobacter sphaeroides* Lipid A and SDZ 880.431

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Rhodobacter sphaeroides **lipid A (*Rs*DPLA) and SDZ 880.431 (3-aza-lipid X-4-phosphate) are prototypic lipopolysaccharide (LPS) antagonists. Herein, we examined the ability of these structures to regulate murine macrophage tumor necrosis factor (TNF) secretion and LPS-inducible gene expression (tumor necrosis factor alpha [TNF-α], interleukin-1β [IL-1β], IP-10, type 2 TNF receptor [TNFR-2], D3, and D8 genes). We report that *Rs*DPLA alone (>1 μg/ml) induced low levels of TNF-α secretion and a selective pattern of gene expression in peritoneal exudate macrophages; SDZ 880.431 alone was completely inactive. When LPS was present at a low concentration (1 ng/ml), *Rs*DPLA and SDZ 880.431 blocked TNF secretion and gene induction in a concentration-dependent fashion. In general, gene induction was measurably reduced by 10 to 30 ng of *Rs*DPLA per ml or 300 ng of SDZ 880.431 per ml, but inhibition could be uniformly overridden by increasing the concentration of LPS. Although induction of all six genes by LPS was suppressed by either inhibitor, effective inhibitor concentrations depended on the gene of interest. Induction of TNFR-2 by LPS was relatively resistant to inhibition by *Rs*DPLA, and induction of TNFR-2 and D3 was relatively resistant to inhibition by SDZ 880.431. When LPS was present at ≥100 ng/ml, correspondingly high concentrations (≥20 μg/ml) of either inhibitor influenced gene expression in a bidirectional manner. Under these conditions, LPS-induced expression of IP-10, D3, and D8 was suppressed regardless of the LPS concentration used (concentrations tested up to 50 μg/ml), while expression of TNF-α mRNA was enhanced about fourfold. In toto, *Rs*DPLA and SDZ 880.431, when present at low concentrations, act in a manner consistent with competitive inhibition of LPS, while at higher concentrations, these structures inhibit certain LPS responses noncompetitively and synergize with LPS for other responses.**

Gram-negative sepsis claims the lives of more than 70,000 people a year in the United States alone (2), and new therapeutic approaches to reduce significantly the mortality associated with sepsis have proven elusive. It is believed that bacterial lipopolysaccharides (LPS) mediate, at least in part, many of the morbid effects of gram-negative infection (18, 32). LPS does not induce these effects directly, but the lipid A portion of the LPS molecule activates leukocytes to secrete inflammatory cytokines and lipids. These mediators, in turn, act on target cells to produce cardiovascular shock, multiorgan failure, and death (18). Thus, intervention in septic shock could theoretically be achieved by blocking the interaction of leukocytes with the lipid A region of LPS.

Towards this end, it has been reasoned that nontoxic lipid A precursors or synthetic analogs that could block LPS-induced toxicity might be prepared. Proctor and coworkers (29), while investigating the biological properties of lipid X, were the first to demonstrate the potential of this approach. In their report, lipid X, a natural monosaccharide precursor of lipid A, was shown to be nontoxic in mice and to reduce markedly the lethality associated with a 100% lethal dose of LPS. Subsequently, several groups have published reports on the LPS inhibitory properties of what is now a large number of lipid A and lipid X-like structures (4, 5, 8, 11, 12, 14, 15, 28, 30, 34, 35, 40, 41, 45). Takayama et al. (35) purified the nontoxic diphosphoryl lipid A of *Rhodobacter sphaeroides* (*Rs*DPLA) and reported it to inhibit LPS-induced macrophage tumor necrosis factor (TNF) secretion, and Qureshi et al. (30) extended this finding to inhibition of LPS-induced TNF production in vivo. Others have recently reported an inhibitory effect of *Rs*DPLA on LPS-induced lethality in mice (45), in vitro-acquired LPS tolerance in macrophages (8), pre-B-cell activation (12), CD18 surface expression on human neutrophils (15), and human monocyte TNF secretion (4). Lipid IV_A, a natural precursor of lipid A, similarly blocks the effects of LPS on a variety of human cells (4, 14, 15, 40), and several synthetic lipid IV_A analogs that retain inhibitory activity have been evaluated (40, 41). Interestingly, treatment of LPS with neutrophil-derived acyloxyacyl-hydrolase removes the 3-acyloxyacyl fatty acids such that the lipid A region of LPS resembles lipid IV_A (5). In doing so, acyloxyacyl-hydrolase not only detoxifies LPS for human cells but renders it capable of blocking the effects of native LPS (28). Finally, a large number of synthetic monosaccharide lipid X analogs have been tested for inhibitor activity (11, 34, 41). Of these, compound SDZ 880.431 (3-aza-lipid X-4-phosphate) was threefold more potent than

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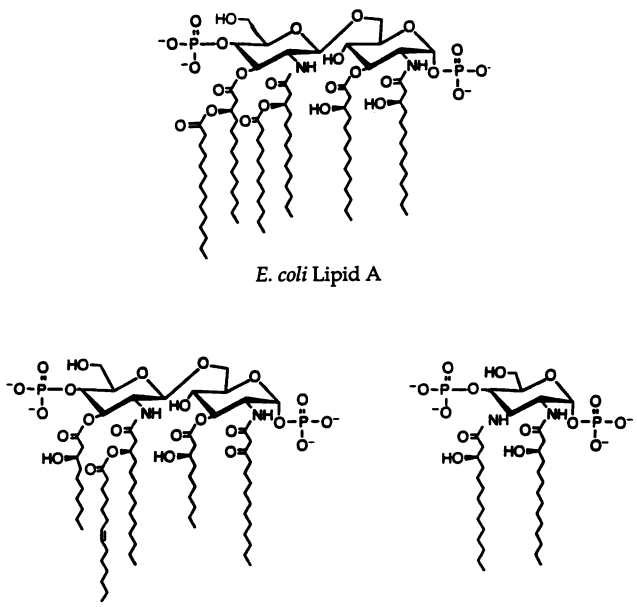

E. coli Lipid A

R. sphaeroides Lipid A

SDZ 880.431

FIG. 1. Structure of *E. coli* lipid A, *Rs*DPLA, and SDZ 880.431.

lipid X at blocking LPS-induced procoagulant activity in cultures of human peripheral blood mononuclear cells.

Because the inhibitors are structurally related to lipid A (Fig. 1), it is generally believed that they act as competitive receptor antagonists. The specificity of the inhibitors for LPS stimulation and the ability of excess LPS to override inhibition has provided strong, albeit indirect, support for this supposition (4, 8, 12, 14, 15, 30, 34, 35, 40, 41). Nonetheless, demonstration of competitive binding to the relevant receptor(s) has not been achieved because the identity of the relevant receptor(s) has not been fully delineated. Identifying receptor mechanisms for LPS signaling is currently an active and emerging area of research (reviewed in reference 19). LPS binds plasma proteins, including a 60-kDa acute-phase LPS-binding protein (33) and septin (42), and association with one or more of these proteins markedly increases the efficiency of subsequent LPS binding to CD14 on the surface of monocytes (38, 43). Binding of LPS to CD14 is a key step in LPS signaling because antibodies against CD14 block both binding and stimulation of monocytes by LPS (9, 13, 43). However, in a recent study, Kitchens et al. (13) used lipid IV_A as a tool to dissociate binding of LPS to CD14 from LPS signaling. This was achieved by demonstrating that concentrations of lipid IV_A that markedly inhibited LPS stimulation of human monocytic THP-1 cells had no effect on LPS binding and cellular uptake through CD14. Thus, binding to CD14 is not sufficient for signaling but may serve to present LPS to an as-yet-unidentified signaling receptor(s). Historically, receptor antagonists have been used as tools to identify receptors and receptor subtypes and characterize their contributions to biological responses. Thus, in addition to representing prototypes of a new class of therapeutics, structural LPS antagonists may prove invaluable in identification of the critical LPS receptor(s) and in a discrimination of receptor subtypes.

In the present study, we have sought to compare the abilities of the disaccharide inhibitor *Rs*DPLA and the monosaccharide inhibitor SDZ 880.431 to block LPS-induced effects on murine macrophages. We evaluated the expression of a panel of LPS-inducible genes, as readouts of LPS stimulation, which included the tumor necrosis factor alpha (TNF-α), interleukin-1β (IL-1β), IP-10, type 2 TNF receptor (TNFR-2), D3, and D8 genes (36). Our objective was to determine whether the inhibitors uniformly blocked LPS-inducible gene expression or whether they could be used to regulate the expression of this panel of genes differentially and thereby identify potentially divergent effects of the inhibitors on LPS signaling. We report that relatively low concentrations of *Rs*DPLA and SDZ 880.431 suppress induction of all six genes by LPS in an apparently competitive fashion. In contrast, high concentrations of *Rs*DPLA and SDZ 880.431 exhibited a bidirectional effect on macrophage responsiveness to LPS; LPS induction of select genes was noncompetitively blocked, while induction of TNF-α was enhanced about fourfold.

MATERIALS AND METHODS

Reagents. The water-soluble bis-Tris salt of SDZ 880.431 was synthesized as described previously (34) by Sandoz Research Institute, Vienna, Austria. *Rs*DPLA was prepared by acid hydrolysis of *R. sphaeroides* LPS and purified by using a DEAE-cellulose acetate column as described previously (31). Protein-free (<0.008%), phenol-water-extracted *Escherichia coli* K235 LPS was prepared by the method of McIntire et al. (20). Heat-killed *Staphylococcus aureus* (HKSA) was prepared from a log-phase culture by boiling and washing it in H_2O. The boiled organisms were collected by centrifugation, dried under vacuum, and resuspended (25 mg/ml) in H_2O (*Limulus* amoebocyte lysate activity, <0.3 endotoxin unit per ml).

Macrophage isolation and culture. Female C3H/OuJ mice (6 to 7 weeks old; Jackson Laboratory, Bar Harbor, Maine) were used as the source of macrophages. Mice were housed in a single-vendor, laminar-flow facility under 12-h light–12-h dark cycles and fed standard mouse chow and acidified water ad libitum. Peritoneal exudate macrophages were isolated by peritoneal lavage 4 days after intraperitoneal injection of 2 ml of sterile 3% thioglycolate broth. Cells were washed and resuspended at 2×10^6 cells per ml in RPMI 1640 supplemented with 2 mM glutamine, 100 U of penicillin per ml, 100 μg of streptomycin per ml, 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), 0.3% sodium bicarbonate, and 2% heat-inactivated fetal calf serum. Cells were plated (4×10^6 cells per well) into six-well plates (Costar, Cambridge, Mass.) and cultured at 37°C and under 6% CO_2. Nonadherent cells were removed by washing 4 to 6 h after plating, and the adherent cells were cultured with stimuli as indicated.

***Rs*DPLA and SDZ 880.431 inhibition of LPS-induced gene expression.** Macrophages were cultured (10 min) with 1.6 ml of medium alone or containing a 1.25× final concentration of *Rs*DPLA or SDZ 880.431. Cultures were then supplemented with 0.4 ml of medium with or without LPS at a 5× final concentration. After 4 h, the cells were lysed in 1 ml of RNAzol B (Tel-Test, Inc., Friendswood, Tex.), and total RNA was isolated as described in the manufacturer's instructions. Northern (RNA) blot analysis was carried out by the method of Maniatis et al. (16). RNA (5 to 10 μg) from each culture condition was fractionated on 1% agarose gels containing formaldehyde, and the RNA was transferred via capillary action to a Nytran filter (Schleicher & Schuell, Inc., Keene, N.H.). The RNA was UV cross-linked to the

filters and hybridized overnight with random-primed ^{32}P-labeled cDNA probes. Probes included a 1,100-bp cDNA recognizing TNF-α (26) (generously provided by Bruce Beutler, Howard Hughes Medical Institute, Dallas, Tex.), a cDNA probe specific for murine β-actin (39) (generously provided by Michael Prystowsky, University of Pennsylvania, Philadelphia), and cDNA probes described by Tannenbaum et al. (36) with specificity for five LPS-inducible genes, C7, D3, D5, D7, and D8. C7, D5, and D7 have been identified as encoding IP-10, IL-1β, and TNFR-2, respectively (23, 25, 37). Hybridized blots were washed three times for 10 min each time at 65°C in 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) containing 0.1% sodium dodecyl sulfate (SDS). Washed blots were exposed to Kodak XAR-5 film with intensifier screens for 18 to 24 h at −70°C. To quantify relative gene expression, phosphor screens were exposed to hybridized blots for 18 to 24 h at room temperature and analyzed by densitometric analysis with a PhosphorImager and Fast Scan program from Molecular Dynamics (Sunnyvale, Calif.). The ratio of the signal of the probe of interest to that of β-actin was used as a measure of relative induction. Between successive hybridizations, cDNA probes were stripped from the Nytran filters by being boiled in distilled water for 5 min.

***Rs*DPLA and SDZ 880.431 inhibition of LPS-induced TNF secretion.** Four-hour culture supernatants (sampled just prior to RNAzol B solubilization of cultures described above) were assayed for TNF-α bioactivity in a standard cytotoxicity assay using actinomycin D-treated L929 cells as described previously (10). One unit of TNF-α was defined as the amount of TNF-α required to lyse 50% of the cells. Human recombinant TNF-α was kindly provided by Cetus Corp. (Emeryville, Calif.), and 1 U of the human recombinant TNF-α was equivalent to ~5 U of activity in our assay system.

Research was conducted according to the principles detailed in *Guide for the Care and Use of Laboratory Animals*, prepared by the Institute of Laboratory Animal Resources, National Research Council.

RESULTS

Effects of *Rs*DPLA alone on gene induction and TNF secretion. We previously reported that very high concentrations of SDZ 880.431 (50 μg/ml) did not induce detectable levels of TNF secretion or macrophage gene expression (27). Before the inhibitory properties of *Rs*DPLA were examined, the effects of this structure alone on macrophages were evaluated. Peritoneal exudate macrophages were cultured for 4 h in the presence of *Rs*DPLA, SDZ 880.431, or LPS, and RNA was harvested for Northern blot analysis of a panel of genes known to be strongly induced by LPS. The panel included genes encoding TNF-α, IL-1β, IP-10, TNFR-2, D3, and D8. Supernatants from these same cultures were concurrently tested for TNF activity. When present at high concentrations (≥1 μg/ml), *Rs*DPLA induced low levels of TNF activity and gene expression (Fig. 2). However, the overall pattern of gene expression stimulated by a high concentration of *Rs*DPLA (20 μg/ml) differed qualitatively from the pattern induced by LPS (Table 1). *Rs*DPLA induced moderate expression of TNFR-2 and TNF-α mRNA but only nominal increases in IL-1β, IP-10, D3, and D8 expression. Consistent with our previous findings, SDZ 880.431 failed to induce TNF-α mRNA and TNF secretion (Fig. 2) or any of the other LPS-inducible genes (not shown).

Inhibition of LPS-induced TNF secretion by *Rs*DPLA and SDZ 880.431. As a first step to compare the antagonist activities of *Rs*DPLA and SDZ 880.431, cells were treated with 1 ng of LPS per ml in the presence of increasing concentrations of the inhibitors. After 4 h, culture supernatants were harvested and assayed for TNF activity (Fig. 3A). *Rs*DPLA was about 30-fold more potent than SDZ 880.431 as an inhibitor of LPS-induced TNF secretion, and *Rs*DPLA was active as an inhibitor at concentrations lower than those required for its weak agonist activity. In a second series of experiments, the ability of LPS to overcome *Rs*DPLA- and SDZ 880.431-mediated suppression was evaluated (Fig. 3B). *Rs*DPLA (300 ng/ml) and SDZ 880.431 (5 μg/ml) blocked TNF induction by 0.1 and 1 ng of LPS per ml. When LPS was present at ≥10 ng/ml, TNF secretion was not inhibited. SDZ 880.431 had no effect on TNF secretion induced by

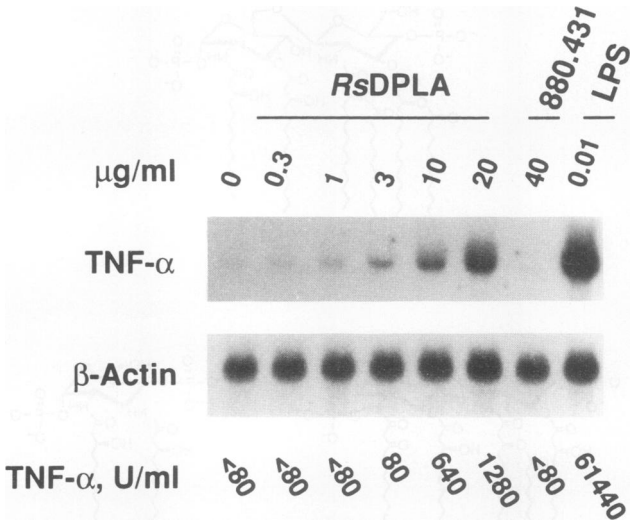

FIG. 2. *Rs*DPLA, but not SDZ 880.431, stimulates murine macrophages to express low levels of TNF-α mRNA and bioactivity. C3H/OuJ macrophages were cultured for 4 h in the presence of the indicated concentrations of *Rs*DPLA, SDZ 880.431, or LPS. Supernatants were assayed for TNF activity (values given below autoradiograms), and RNA was harvested for Northern blot analysis of TNF-α and β-actin gene expression as described in Materials and Methods.

TABLE 1. Macrophage genes induced by *Rs*DPLA[a]

Gene	Steady-state mRNA level (% induced by 10 ng of LPS per ml) in:	
	Medium only	Medium + *Rs*DPLA
TNF-α	2.8 ± 0.8	34.8 ± 3.6
IL-1β	ND[b]	7.5 ± 1.4
IP-10	ND	1.6 ± 0.4
TNFR-2	20.0 ± 3.3	71.3 ± 7.6
D3	5.4 ± 1.6	11.7 ± 2.1
D8	0.5 ± 0.1	1.9 ± 0.7

[a] Macrophages were cultured for 4 h with medium alone or containing 20 μg of *Rs*DPLA per ml or 10 ng of LPS per ml. Steady-state mRNA levels were quantified by Northern blot analysis and phosphor imaging and then corrected for β-actin. Values obtained in the presence of medium alone or with *Rs*DPLA were multiplied by 100 and divided by values obtained in the presence of 10 ng of LPS per ml to obtain a percentage of the level of gene expression induced by LPS alone. Values represent the mean ± standard error of the mean of three to five separate experiments.

[b] ND, below limit of detectability (<0.5%).

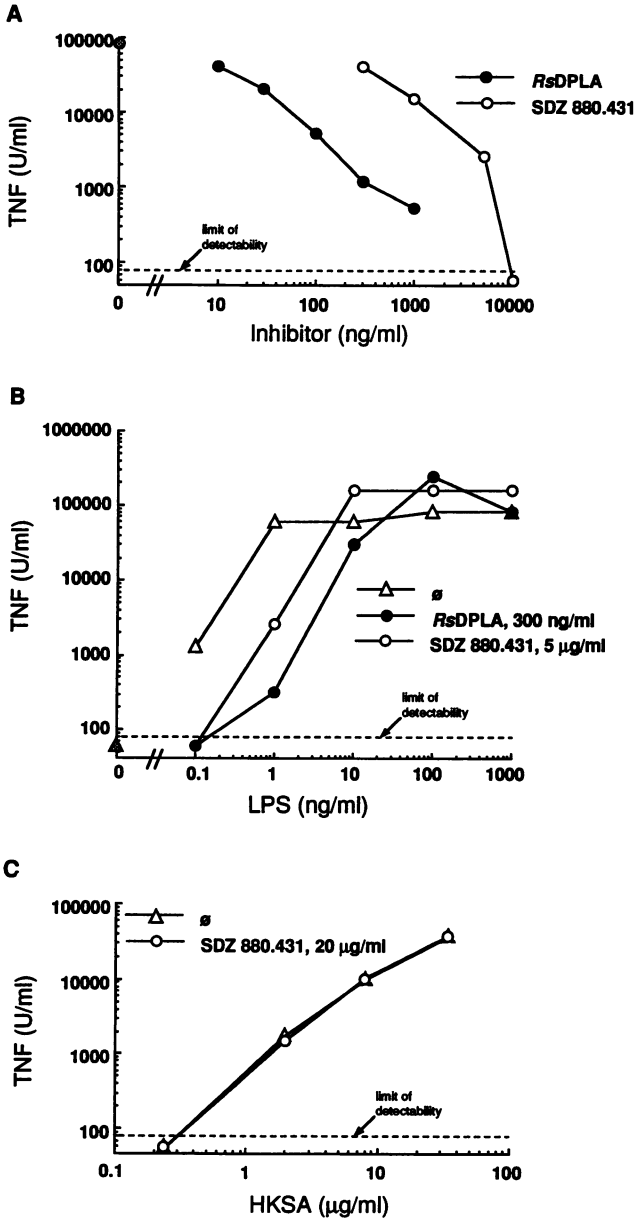

FIG. 3. *Rs*DPLA and SDZ 880.431 inhibit LPS- but not HKSA-induced TNF secretion: concentration dependencies. Macrophages were pretreated (10 min) with or without the indicated inhibitor, and the culture medium was adjusted to contain LPS or HKSA. Supernatants were harvested at 4 h and tested for the presence of TNF activity as described in Materials and Methods. (A) Inhibitor concentration dependence. TNF was induced with 1 ng of LPS per ml in the presence of variable concentrations of inhibitors. (B) LPS concentration dependence. LPS concentration was varied in the presence of medium either alone, with 300 ng of *Rs*DPLA per ml, or with 5 μg of SDZ 880.431 per ml. (C) HKSA is not blocked by SDZ 880.431. HKSA concentration was varied in the presence of medium alone or with 20 μg of SDZ 880.431 per ml. Values represent the means of three separate experiments.

HKSA (Fig. 3C), and similar results were found when *Rs*DPLA was used (not shown).

Inhibition of LPS-induced gene expression by SDZ 880.431 and *Rs*DPLA. We then used the panel of LPS-inducible genes as a tool to test whether *Rs*DPLA- and SDZ 880.431-mediated blockade of LPS signaling was complete (i.e., all LPS-induced genes were suppressed) or selective (i.e., only a subset of genes was suppressed). Towards this end, RNA harvested from cells treated as described in the legend to Fig. 3A and B was submitted to Northern blot analysis (Fig. 4 and 5). As little as 10 ng of *Rs*DPLA per ml inhibited by >40% the induction of TNF-α and IL-1β mRNA by 1 ng of LPS per ml, whereas 300 to 1,000 ng of SDZ 880.431 per ml was required to achieve a similar degree of inhibition. *Rs*DPLA and SDZ 880.431 inhibited the induction of each gene, but the dose dependency for inhibition varied with the gene of interest. For example, it was comparatively more difficult to inhibit, with *Rs*DPLA, induction of TNFR-2, or, with SDZ 880.431, the induction of both TNFR-2 and D3. Figure 5 illustrates that suppression of LPS-induced TNF-α and IP-10 expression (data shown) and IL-1β, TNFR-2, D3, and D8 expression (data not shown) by *Rs*DPLA (300 ng/ml) or SDZ 880.431 (5 μg/ml) was overcome when the concentration of LPS was increased to 100 ng/ml. These data are consistent with a proposed mechanism of competitive inhibition.

Gene induction by ≥100 ng of LPS per ml is influenced in a bidirectional manner by high concentrations of *Rs*DPLA or SDZ 880.431. During preliminary experiments on the inhibitory properties of SDZ 880.431, we observed an apparent paradox. Cells treated with a high concentration (40 μg/ml) of SDZ 880.431 and 1 μg of LPS per ml expressed about four times the levels of TNF-α mRNA as cells treated with LPS alone (Table 2). In contrast, IP-10, D3, and D8 expression remained strongly inhibited by SDZ 880.431 despite the presence of a high concentration of LPS. This bidirectional effect of SDZ 880.431 appeared to be selective for LPS signaling, since SDZ 880.431 did not modulate gene induction by HKSA. To characterize this paradoxical effect further, cells were treated with 20 μg of *Rs*DPLA or SDZ 880.431 per ml with and without a range of concentrations of LPS (Fig. 6). Both inhibitors strongly blocked LPS-induced IP-10 and TNF-α gene expression at LPS concentrations of ≤100 ng/ml. In contrast, at LPS concentrations of ≥100 ng/ml, bidirectional effects of SDZ 880.431 and *Rs*DPLA on gene expression could be seen; TNF-α mRNA was superinduced, whereas IP-10 gene expression remained strongly inhibited by both antagonists. Even 50 μg of LPS per ml could not overcome the inhibitory effects exhibited by 20 μg of *Rs*DPLA or SDZ 880.431 per ml on IP-10 expression. Like IP-10 expression, expression of D3 and D8 also remained strongly suppressed, but the expression of IL-1β and TNFR-2 was restored (not shown).

To clarify the mechanism for the paradoxical effects observed in the presence of high concentrations of inhibitors, two additional experiments were performed. Relatively high concentrations of LPS can stimulate macrophages in the absence of serum, but LPS-binding proteins present in serum markedly increase macrophage stimulation by low concentrations of LPS (33). It remains unclear whether signaling elicited by free versus bound LPS is the same or distinct. We postulated that high concentrations of inhibitors may preferentially block serum protein-LPS interactions while allowing stimulation of macrophages by free LPS. If so, gene induction in the absence of serum should resemble gene induction in the presence of serum and excess inhibitors. However, as shown in Table 3, serum was not required for either the induction of IP-10 or the suppression of IP-10 by SDZ 880.431.

Alternatively, the paradoxical effects of high concentra-

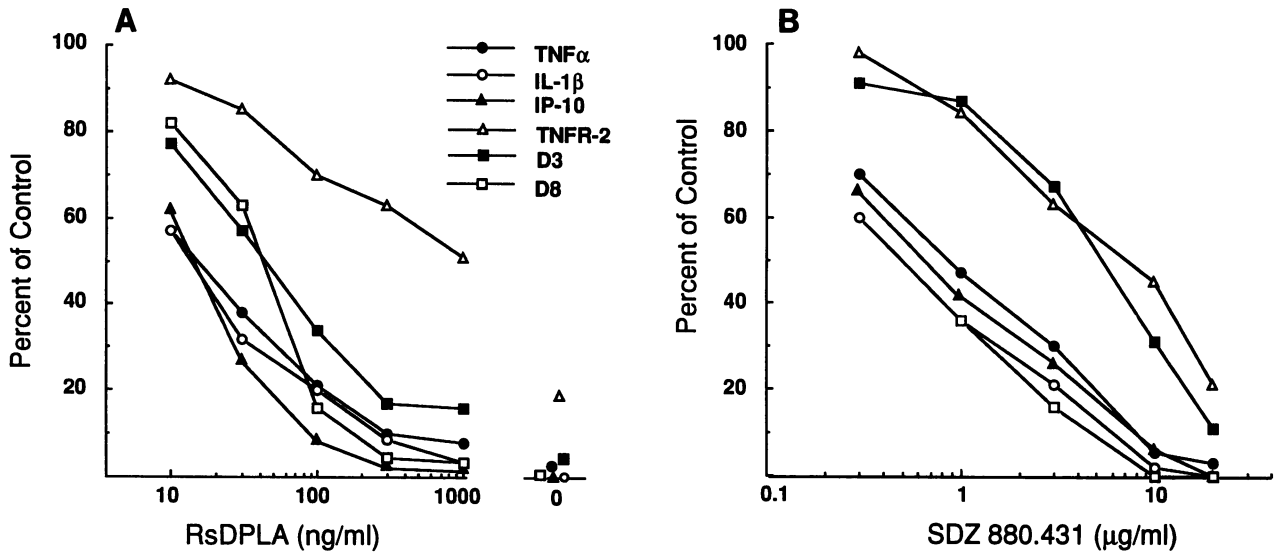

FIG. 4. Inhibition of LPS-induced gene expression by *Rs*DPLA (A) and SDZ 880.431 (B): inhibitor concentration dependence. Macrophages were pretreated (10 min) with or without variable concentrations of *Rs*DPLA or SDZ 880.431. Culture medium was then adjusted to contain 1 ng of LPS per ml. RNA was harvested at 4 h for Northern blot analysis of β-actin mRNA and the expression of the panel of six LPS-inducible genes. Gene expression was quantified by phosphor imaging and normalized for β-actin content. Steady-state levels for samples involving inhibitors were then expressed relative to levels induced with LPS alone (control). Symbols to the right of the bottom of panel A indicate constitutive levels of expression.

tions of inhibitors might be caused by the formation of mixed micelles containing both inhibitor and LPS. In this model, LPS within the mixed micelle would be presented to macrophages in such a fashion as to activate signals that induce TNF-α but not IP-10. To examine this possibility, culture medium containing 40 μg of SDZ 880.431 per ml and 2 μg of LPS per ml was preincubated (30 min, 37°C) to allow for the putative formation of mixed micelles. The solution was then diluted 10-fold with fresh medium and used immediately to stimulate macrophages. If mixed micelles predominated, a dilution of the micelles would be expected to be even less effective at inducing IP-10 than the micelle stock. If mixed micelles did not predominate, the resultant solution would contain too little SDZ 880.431 (4 μg/ml) to block the effects of the LPS (200 ng/ml), and IP-10 would be induced robustly (Fig. 5). Since strong induction of IP-10 was achieved with the 1:10 dilution (Table 4), mixed micelle formation is an unlikely explanation for the bidirectional effects of high concentrations of inhibitors.

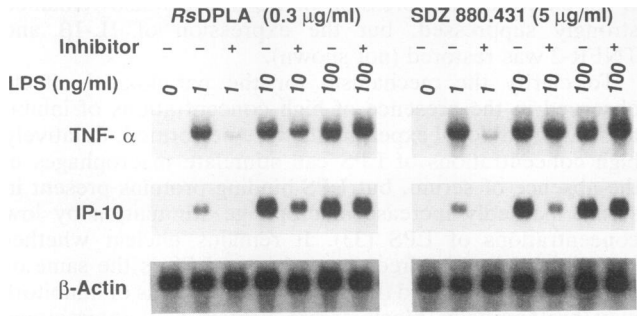

FIG. 5. Inhibition of LPS-induced gene expression by *Rs*DPLA and SDZ 880.431: LPS concentration dependence. Macrophages were pretreated (10 min) with medium either alone, with 0.3 μg of *Rs*DPLA per ml, or with 5 μg of SDZ 880.431 per ml. Culture medium was then adjusted to contain variable concentrations of LPS. RNA was harvested at 4 h for Northern blot analysis of TNF-α, IP-10, and β-actin mRNA as described in Materials and Methods. Results are representative of two separate experiments.

DISCUSSION

*Rs*DPLA and SDZ 880.431 have been documented to block the stimulatory effects of LPS on a variety of cell types (4, 8, 12, 15, 30, 34, 35). However, there is a relative lack of information regarding the effects of LPS inhibitors at the level of LPS-inducible gene expression, and herein we have described the comparative inhibition by *Rs*DPLA and SDZ

TABLE 2. Effects of high concentration of SDZ 880.431 on LPS- and HKSA-induced gene expression[a]

Gene	Steady-state mRNA level (% of that induced by LPS or HKSA alone)[b] in the presence of:	
	LPS (1 μg/ml) + SDZ 880.431	HKSA (125 μg/ml) + SDZ 880.431
TNF-α	398 ± 43	118 ± 15
IL-1β	122 ± 32	100 ± 8
IP-10	5 ± 2	110 ± 13
TNFR-2	143 ± 11	98 ± 18
D3	24 ± 7	NT[c]
D8	13 ± 5	NT

[a] Macrophages were pretreated (10 min) with medium alone or containing 40 μg of SDZ 880.431 per ml. Culture medium was adjusted to contain 1 μg of LPS per ml or 125 μg of HKSA per ml. RNA was harvested at 4 h for Northern blot analysis.

[b] Steady-state mRNA levels were quantified by phosphor imaging and normalized to β-actin. Values obtained in the presence of SDZ 880.431 and either LPS or HKSA were multiplied by 100 and divided by values obtained in the presence of LPS or HKSA alone, respectively. Values represent the means ± standard errors of the means of three to four separate experiments.

[c] NT, not tested.

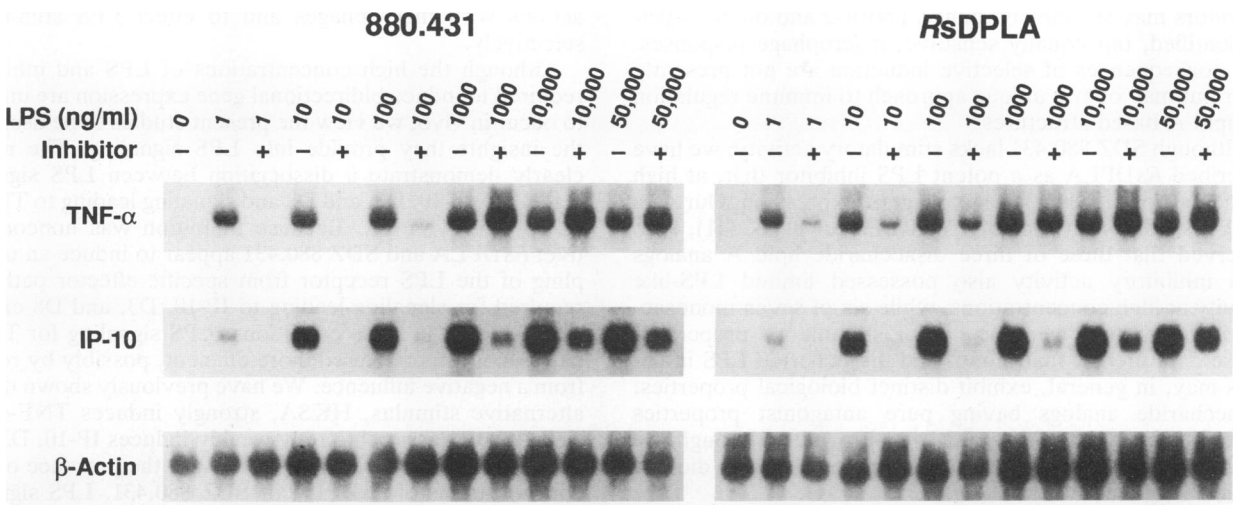

FIG. 6. Bidirectional effects on TNF-α and IP-10 expression by the combination of high concentrations of LPS and *Rs*DPLA or SDZ 880.431. Macrophages were pretreated (10 min) with medium either alone, with 20 μg of *Rs*DPLA per ml, or with 20 μg of SDZ 880.431 per ml. Culture medium was then adjusted to contain variable concentrations of LPS. RNA was harvested at 4 h for Northern blot analysis of TNF-α, IP-10, and β-actin mRNA as described in Materials and Methods. Results are representative of three separate experiments.

880.431 of a panel of six genes strongly induced in murine macrophages by LPS. The six genes were chosen, in part, because their collective regulation was known to be divergent and complex. Specifically, it is known that the LPS-induced expression of select genes within this panel are differentially regulated by calcium (24), gamma interferon (IFN-γ) (16a), IL-4 (21), differentiation (22), and in vitro endotoxin tolerance (7). Furthermore, alternative stimuli such as IFN-β, IFN-γ, IL-2, and HKSA can induce discrete gene subsets within the panel (6, 17, 21). Thus, it seemed reasonable to speculate that induction of these genes by LPS may involve the activation of diverse signaling pathways, possibly emanating from multiple LPS receptors. In the present study, *Rs*DPLA and SDZ 880.431 inhibited the induction of all six genes when LPS was present at low concentrations. Concentrations of *Rs*DPLA and SDZ 880.431 that inhibited gene expression were consistent with concentrations used by others to inhibit other cellular responses (4, 8, 12, 15, 30, 34, 35). Overall, the data are consistent either with a single LPS receptor model or with multiple receptors, each of which can be blocked competitively by either *Rs*DPLA or SDZ 880.431. To date, there is no evidence for LPS-induced cellular responses that are not blocked by lipid A-based inhibitors.

Nonetheless, the inhibitor concentrations needed to suppress induction varied with the gene of interest. We cannot currently explain this finding, but the poor suppression of the gene encoding TNFR-2 may relate, in part, to the finding that TNFR-2 is constitutively expressed and induced by 3- to 100-fold-lower concentrations of LPS than those required for the other five genes in the panel (7, 27). Thus, blockade of minimal receptor occupancy leading to gene induction may be correspondingly more difficult in the case of TNFR-2 expression. Regardless of the mechanism, the results may have important pharmacologic implications. For example, depending on the relative availability of LPS and inhibitor, blockade of LPS-induced functional changes in vivo may, to an extent, be selective. LPS present with lipid A-based

TABLE 3. Effect of serum-free conditions on bidirectional effects of SDZ 880.431[a]

Serum	SDZ 880.431	Steady-state level (% induced by 2 μg of LPS per ml with serum) of:	
		TNF-α	IP-10
+	−	100	100
+	+	356	5
−	−	125	62
−	+	433	0.6

[a] Macrophages were cultured for 4 h in the presence of 2 μg of LPS per ml ± 40 μg of SDZ 880.431 per ml, and RNA was harvested for Northern blot analysis of TNF-α, IP-10, and β-actin mRNA as described in Materials and Methods. Cells treated under serum-free conditions were first washed three times over 30 min (10 min per wash) with 37°C serum-free media.

TABLE 4. Effect of sample dilution on bidirectional effects of SDZ 880.431[a]

Stimulus	Dilution[b] (×)	Steady-state mRNA level (% induced by 2 μg of LPS per ml) of:	
		TNF-α	IP-10
LPS (2 μg/ml)	1	100	100
	0.1	131	107
LPS (2 μg/ml) + SDZ 880.431 (40 μg/ml)	1	356	5
	0.1	122	80

[a] Macrophages were cultured for 4 h with the indicated stimuli, and RNA was harvested for Northern blot analysis of TNF-α, IP-10, and β-actin mRNA as described in Materials and Methods.

[b] Culture medium containing 2 μg of LPS alone per ml or together with 40 μg of SDZ 880.431 per ml was preincubated (30 min, 37°C) to allow for the putative formation of mixed micelles. A portion of each solution was used to stimulate macrophages (1×). The remaining portions of each solution were diluted 10-fold (0.1×) with fresh medium and used immediately to stimulate macrophages.

inhibitors may selectively induce TNFR-2 and other as-yet-unidentified, but equally sensitive, macrophage responses. The consequences of selective induction are not presently clear but may offer a unique approach to immune regulation by lipid A-based structures.

Although SDZ 880.431 lacks stimulatory activity, we have described *Rs*DPLA as a potent LPS inhibitor that, at high concentrations, induces selective gene expression. Our data are consistent with those of Van Dervort et al. (41), who observed that three of three disaccharide lipid A analogs with inhibitory activity also possessed limited LPS-like activity at high concentrations, while six of seven monosaccharide inhibitors were devoid of stimulatory properties. These data predict that mono- and disaccharide LPS inhibitors may, in general, exhibit distinct biological properties. Disaccharide analogs having pure antagonist properties have, however, been described in vitro (40), although the concentrations of inhibitors tested in these studies did not exceed 1 μg/ml.

Other bioactivities of *Rs*DPLA that are distinct from its ability to inhibit LPS have been reported. For example, Baker et al. (1) have shown that *Rs*DPLA partially retains the LPS-like property of reducing suppressor T-cell activity in vivo. Furthermore, Zuckerman and Qureshi (45) have recently reported that *Rs*DPLA protects normal, but not adrenalectomized, mice from LPS-induced serum TNF expression and lethality. High doses of *Rs*DPLA alone induced serum corticosterone in normal mice, and the authors concluded that the protective effects of *Rs*DPLA were a function of its ability to down-regulate LPS responsiveness through the induction and actions of corticosterone, as well as by blocking LPS-macrophage interactions. The mechanism by which *Rs*DPLA induced corticosterone was not delineated but may be related to the stimulatory activity reported herein.

The precise mechanism by which lipid A analogs block LPS effects is unknown, but it is widely assumed that the inhibitors are competitive antagonists of LPS binding to a (as-yet-incompletely-defined) signaling receptor(s). Our data suggest that at low concentrations of inhibitors, competitive inhibition may indeed be a key mechanism, but this model breaks down at high concentrations of inhibitors. Genes induced by high concentrations of LPS are bidirectionally regulated by high concentrations of inhibitors. When *Rs*DPLA or SDZ 880.431 is present at 20 μg/ml, induction of several genes (IP-10, D3, and D8) could not be restored by excess LPS, i.e., gene induction was inhibited noncompetitively. Under these same conditions, inhibitors synergized with LPS to induce TNF-α mRNA. Although not shown herein, IL-1β and TNFR-2 gene expression was also induced synergistically with certain combinations of high concentrations of LPS and inhibitors.

We have not been able to dismiss noncompetitive inhibition or synergy by *Rs*DPLA and SDZ 880.431 as artifacts of toxicity or nonspecific cellular effects. In the presence of LPS, 40 μg of SDZ 880.431 per ml does not significantly reduce 18-h viability as assessed by trypan blue exclusion (16b). Furthermore, the paradoxical effects of high concentrations of inhibitors are specific for LPS stimulation. SDZ 880.431 (40 μg/ml) had no effect on gene induction by high or low concentrations of HKSA or on induction of IP-10, D3, D8, or TNF-α by IFN-γ (Table 2) (16b). Noncompetitive inhibition or synergy by *Rs*DPLA or SDZ 880.431 occurred independently of serum proteins and could not readily be attributed to formation of mixed micelles. Thus, the bidirectional influence of the inhibitors appeared to involve interactions with macrophages and to effect LPS stimulation selectively.

Although the high concentrations of LPS and inhibitors required to induce bidirectional gene expression are unlikely to occur in vivo, we view the present studies to be useful for the insights they provide into LPS signaling. The results clearly demonstrate a dissociation between LPS signaling leading to IP-10, D3, and D8 and signaling leading to TNF-α, IL-1β, and TNFR-2. Because inhibition was noncompetitive, *Rs*DPLA and SDZ 880.431 appear to induce an uncoupling of the LPS receptor from specific effector pathways required for signaling leading to IP-10, D3, and D8 expression. Under the same conditions, LPS signaling for TNF-α expression was rendered more efficient, possibly by release from a negative influence. We have previously shown that an alternative stimulus, HKSA, strongly induces TNF-α and TNFR-2 expression but only weakly induces IP-10, D3, and D8 expression. Thus, it appears that in the presence of high concentrations of *Rs*DPLA or SDZ 880.431, LPS signaling may resemble the subset of effector pathways activated by HKSA. In a recent publication, inhibition of pertussis-toxin-sensitive G proteins blocked LPS-induced nitric oxide production but augmented LPS-induced TNF-α (44). It is tempting to speculate that high concentrations of *Rs*DPLA and SDZ 880.431 may uncouple LPS receptors from specific effector molecules such as G proteins or protein kinases. More work will be required to investigate the influence of *Rs*DPLA and SDZ 880.431 on LPS-induced G-protein activation, protein-tyrosine phosphorylation, and calcium flux. This work will aid in identifying specific signaling pathways leading to gene induction.

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