Stimulation of the Ceramide Pathway Partially Mimics Lipopolysaccharide-Induced Responses in Murine Peritoneal Macrophages

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Recent studies have suggested that lipopolysaccharide (LPS) stimulates cells by mimicking the second-messenger function of ceramide, a lipid generated in the cell by the action of sphingomyelinase (SMase). To examine this possibility further, we compared the abilities of LPS, SMase, and/or ceramide analogs to induce cytokine secretion, modulate gene expression, and induce endotoxin tolerance in macrophages. SMase and LPS induced secretion of tumor necrosis factor alpha (TNF- α) to comparable degrees; however, unlike LPS, SMase failed to stimulate detectable interferon activity. Cell-permeable analogs of ceramide induced the expression of many LPS-inducible genes; however, the expression of interferon-inducible protein 10 (IP-10) and interferon consensus sequence-binding protein (ICSBP) mRNAs was significantly lower than that induced by LPS. Both SMase-induced TNF- α secretion and LPS-induced TNF- α secretion were inhibited by pretreatment with a serine/threonine phosphatase inhibitor, calyculin A. Macrophages preexposed in vitro to LPS to induce a well-characterized state of endotoxin tolerance secreted little or no TNF- α upon secondary challenge with either LPS or SMase, whereas macrophages preexposed to SMase secreted high levels of TNF- α upon secondary stimulation with LPS or SMase. Collectively, these results suggest that ceramide activates a subset of LPS-induced signaling pathways in murine peritoneal exudate macrophages.

Lipopolysaccharide (LPS)-stimulated macrophages produce cytokines and other inflammatory mediators that are largely responsible for the pathophysiology associated with gram-negative septic shock. Stimulation of macrophages by LPS is primarily mediated by serum proteins, such as LPS-binding protein (LBP), and CD14 (9, 22, 33). The intracellular signaling pathways activated in response to LPS, although not yet fully elucidated, involve src-related kinases lyn and hck, protein kinase C, mitogen-activated protein (MAP) kinases, raf-1, MEK-1, phosphatidylinositol 3-kinase, G proteins, and NF- κ B (1, 7, 8, 10, 13, 15, 26, 29, 31, 32).

Ceramide is a lipid second messenger generated from membrane sphingomyelin by the action of sphingomyelinase (SMase) (reviewed in reference 19). The signaling pathway activated by ceramide involves a cytosolic ceramide-activated protein phosphatase (6) and a membrane-associated ceramide-activated protein kinase (CAK) (23), recently shown to phosphorylate raf-1 (40). The ceramide pathway is thought to participate in tumor necrosis factor (TNF)-, interleukin-1 (IL-1)-, and gamma interferon (IFN- γ)-induced signaling and to initiate intracellular events that lead to apoptosis (11, 19). Exogenous SMase and/or cell-permeable analogs of ceramide have been shown to induce some of the cellular responses previously associated with LPS. For example, ceramide and SMase have been shown to activate MAP kinases (28) and to induce IL-6 gene expression (21). Controversy exists in the literature, how-

ever, as to the ability of ceramide or SMase to induce the nuclear translocation of NF- κ B (5, 16, 20, 30). Recently, a structural similarity between a portion of the lipid A region of LPS and ceramide was revealed (17). Further, LPS was found to activate CAK, in a manner that was CD14 dependent and enhanced by LBP yet independent of ceramide production (17). Thus, it has been suggested that LPS stimulates cells by mimicking the second-messenger function of ceramide.

The ability of mice to respond to LPS in vivo, as well as the ability of cells to respond to LPS in vitro, has been mapped genetically to the *Lps* locus on mouse chromosome 4 (reviewed in references 35 and 37). Macrophages derived from LPS-hyporesponsive C3H/HeJ (*Lps^d*) mice fail to respond to LPS to produce the cytokines and other inflammatory mediators characteristic of LPS-responsive C3H/OuJ (*Lpsⁿ*) mice (35). To explore the possibility that LPS stimulates macrophages by mimicking the second-messenger function of ceramide, previous studies were designed to evaluate the effect of *Lps^d* versus *Lpsⁿ* allelic expression on the ability to activate the ceramide signaling pathway. C3H/OuJ (*Lpsⁿ*), but not C3H/HeJ (*Lps^d*), macrophages responded to ceramide to express a number of LPS-inducible genes. Thus, the ability of macrophages to respond to ceramide, as to LPS, requires expression of the *Lpsⁿ* allele (4).

To investigate further the possibility that LPS stimulates macrophages by mimicking the second-messenger function of ceramide, the present studies were designed to examine the following questions. (i) Does SMase, like LPS, induce the secretion of both TNF- α and IFN by *Lpsⁿ* macrophages? (ii) Does activation of the ceramide pathway result in the induction of a well-characterized panel of LPS-inducible genes? (iii) Is activation of the ceramide pathway dependent on serine/threonine phosphatase activity analogous to the LPS signaling pathway? (iv) Does prior exposure to LPS (previously shown to

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TABLE 1. Comparison of levels of TNF- α and IFN production induced by LPS and SMase^a

Treatment	TNF- α (pg/ml)	IFN bioactivity (U/ml)
Medium only	<35	<14.3
LPS (100 ng/ml)	1,910	119
SMase (1 U/ml)	1,600	<14.3

^a Supernatants were collected from macrophages stimulated for 4.5 h with either LPS (100 ng/ml) or SMase (1 U/ml). Results represent the geometric means for four to six separate experiments per treatment. For the TNF- α ELISA, the lowest concentration of rTNF- α included on the standard curve was 35 pg/ml. For the IFN bioassay, the lowest detectable concentration was 12.5 U/ml.

render macrophages transiently hyporesponsive to secondary challenge with LPS [34]) render macrophages transiently hyporesponsive to secondary challenge with SMase, and, conversely, does prior exposure of macrophages to SMase render macrophages transiently hyporesponsive to LPS?

To address the first question, it was hypothesized that if the signaling pathways elicited by ceramide and LPS are identical, then the pattern of cytokine secretion resulting from SMase stimulation should mimic that induced by LPS. Conversely, if the signaling pathways are divergent (i.e., diverging after the requirement for the *Lps*ⁿ gene product), then a different pattern of cytokine secretion might be expected. Five- to 6-week-old female C3H/OuJ (*Lps*ⁿ) mice (Jackson Laboratories, Bar Harbor, Maine) were reared according to the principles set forth in *Guide for the Care and Use of Laboratory Animals* (14). Thioglycollate-elicited peritoneal macrophages prepared from the mice as previously described (2) were cultured for 4.5 h with 100 ng of *Escherichia coli* K235 LPS (prepared by phenol-water extraction [25]) per ml or 1 U of exogenous SMase (from *Staphylococcus aureus*; Sigma Chemical Co., St. Louis, Mo.) per ml. Supernatants were collected and analyzed for the production of TNF- α (mouse TNF- α enzyme-linked immunosorbent assay [ELISA] kit; Genzyme, Cambridge, Mass.) and IFN- α , - β , and - γ bioactivity as previously described (36). Importantly, only trace levels of LPS were detected in the SMase used in this study (0.03 ng/ml in 1 U of SMase per ml), as measured by the *Limulus* amoebocyte lysis assay. Previous studies have demonstrated that maximal LPS-induced gene expression results from treatment with 100 ng of LPS per ml (2, 32), and preliminary experiments established that maximal TNF- α secretion results from treatment with 1 U of SMase per ml (data not shown). In addition, that SMase treatment resulted in the generation of ceramide under each experimental condition was verified by using a diacylglycerol kinase assay (Amersham, Inc., Arlington Heights, Ill.) followed by thin-layer chromatography (data not shown).

The data shown in Table 1 indicate that TNF- α , but not IFN, was detected in supernatants derived from C3H/OuJ macrophages treated with SMase. In contrast, LPS induced significant production of both TNF- α and IFN. Even after 19 h of stimulation, with as much as 2 U of SMase per ml, no IFN bioactivity was detected, indicating that the inability to detect SMase-induced IFN cannot be overcome by a higher dose of SMase or a longer incubation period (data not shown).

As a second approach to compare LPS- and ceramide-induced signaling pathways, LPS or various concentrations of cell-permeable analogs of ceramide that differ in hydrocarbon side chain length (as indicated by their subscript designation, e.g., C₂, C₆, or C₁₆) (BIOMOL Research Laboratories, Inc., Plymouth Meeting, Pa.) were used to stimulate macrophages for 4.5 h, and total cellular RNA was extracted and analyzed by

Northern (RNA) blot analysis of TNF- α , IL-1 β , interferon regulatory factor 1 (IRF-1), IRF-2, TNF receptor 2 (TNFR-2), interferon-inducible protein 10 (IP-10), and interferon consensus sequence-binding protein (ICSBP) mRNAs as described previously (4, 24). Analysis of data from five or six separate experiments is shown in Fig. 1, in which the data are expressed as the percentage of the maximum LPS-induced response for each gene. Ceramide-induced levels of IL-1 β and TNFR-2 mRNAs were generally higher than levels induced by LPS, and the levels of TNF- α , IRF-1, and IRF-2 mRNAs were similar. In contrast, levels of IP-10 and ICSBP mRNAs induced by ceramide analogs were consistently lower than levels induced by LPS. None of the cell-permeable analogs of ceramide contained detectable *Limulus* amoebocyte lysis activity (<0.025 ng of LPS per ml at the highest concentration used in these studies). Thus, it appears that activation of the ceramide pathway effectively induces only a subset of LPS-inducible genes.

Interestingly, macrophages derived from mice with a targeted disruption in CD14 (i.e., the mice express neither membrane CD14 nor soluble CD14) respond to high concentrations of LPS (1.0 μ g/ml) to induce normal levels of TNF- α and IL-1 β mRNAs in comparison with levels induced by control macrophages but comparatively low levels of IP-10 mRNA (27). Thus, our observations that activation of the ceramide pathway induces normal levels of TNF- α and IL-1 β mRNAs but low levels of IP-10 mRNA are consistent with the possibility that high concentrations of LPS (shown previously to activate cells independently of CD14 [18, 39]) may intercalate directly into the membrane, resulting in the activation of membrane-associated CAK and, thus, a pattern of gene expression like that induced by ceramide.

Previous studies have shown that prior incubation of macrophages with the serine/threonine phosphatase inhibitor calyculin A inhibits the LPS-induced expression of immediately genes, suggesting that Ser/Thr phosphatase activity is required very early in the LPS signaling pathway (3). To test the dependence of the ceramide signaling pathway on Ser/Thr phosphatase activity, macrophages were pretreated with 25 nM calyculin A for 1 h prior to stimulation with LPS or SMase, after which supernatants were collected and analyzed for the presence of TNF- α . The results in Table 2 indicate that TNF- α secretion induced by LPS or SMase requires Ser/Thr phosphatase activity. In this regard, the ceramide pathway is similar to the LPS pathway, although there is currently no way to discern if the required phosphatase activities are shared by the two pathways. It should be noted that this concentration of calyculin A was shown previously not to diminish cellular viability or adherence and in fact potentiated TNF secretion when added after LPS administration (3), indicating that it does not interfere with the ability of the macrophage to secrete cytokines.

Macrophages preexposed to endotoxin exhibit a transient hyporesponsive phenotype following subsequent challenge with LPS (34). This phenomenon has been referred to as endotoxin tolerance. If LPS stimulates cells by mimicking the second-messenger function of ceramide, as suggested by Joseph et al. (17), then macrophages preexposed to endotoxin might also be tolerant of subsequent challenge with SMase and, conversely, macrophages preexposed to SMase might be tolerant of subsequent challenge with LPS. To address these possibilities, macrophages were pretreated for 19 h (a time previously shown to induce a maximal LPS-hyporesponsive phenotype in murine peritoneal macrophages [34]) with medium only, LPS (100 ng/ml), or SMase (1 U/ml), after which the cells were washed twice with medium and restimulated with either medium alone, LPS (100 ng/ml), or SMase (1 U/ml). Supernatants were

FIG. 1. Ceramide-induced gene expression measured as a percentage of LPS-induced gene expression. Macrophages were stimulated with LPS (100 ng/ml) or with various concentrations of ceramide analogs (C_2 , C_6 , and C_{16} ; 6.25 to 100 μ M) for 4.5 h. PhosphorImager data were obtained (as described elsewhere [2]) from five to six independent Northern blot experiments. The data are expressed as the geometric mean of the percent maximum LPS-induced expression for each gene and were derived from the concentration of each ceramide analog that induced the highest level of expression for each gene within each experiment. The error bars show the range represented by the lower and upper values defined by 1 standard error of the mean.

collected and analyzed for the secretion of TNF- α . The data shown in Table 3 indicate that macrophages "tolerized" to LPS secreted very low levels of TNF- α in response to challenge with either LPS or SMase. Thus, the molecular mechanisms required for the induction of endotoxin tolerance compromise the functions of critical molecules involved in the activation of the ceramide pathway. In contrast, macrophages preexposed to SMase failed to develop transient hyporesponsiveness to secondary challenge with LPS or SMase. Thus, the signals elicited by SMase activation of the ceramide pathway alone do not lead to the development of a tolerant state, while the signals initiated by LPS are sufficient to render macrophages tolerant of both LPS and SMase.

In summary, three findings from our laboratory demonstrate strong similarity between ceramide- and LPS-induced signaling pathways: (i) both signaling pathways are dependent upon the expression of the *Lps*ⁿ allele (4), (ii) both signaling pathways are dependent on serine/threonine phosphatase activity, and (iii) the molecular mechanisms that maintain the state of en-

dotoxin tolerance compromise the functions of critical molecules involved in activation of the ceramide pathway (Table 3). In light of the first and third observations, it would not be unreasonable to suspect that the common critical molecule is the *Lps*ⁿ gene product or a tightly associated molecule. However, results from the present study also suggest that LPS stimulation of macrophages cannot result entirely from mimicking of the second-messenger function of ceramide since activation of the ceramide pathway does not lead to IFN production or the induction of IP-10 and ICSBP mRNAs to levels comparable to those induced by LPS. Moreover, despite the observation that 1 U of SMase per ml induced TNF- α secretion to a degree comparable to that induced by 100 ng of LPS per ml (Table 1), SMase-induced activation of the ceramide pathway did not lead to a state of desensitization (tolerance) that is characteristic of that induced by LPS. Collectively, these data suggest that despite a common dependence on the expression of the *Lps*ⁿ gene product, activation of the ceramide

TABLE 2. Effect of calyculin A on TNF- α production induced by LPS or SMase^a

Pretreatment	Amt of TNF- α produced (pg/ml) with treatment:		
	Medium	LPS	SMase
Medium	<35	2,125	2,053
Calyculin A (-1 h)	<35	<35	<35

^a Supernatants were collected from macrophages that were pretreated for 1 h (-1 h) with medium only or with calyculin A (25 nM) prior to stimulation with medium, LPS (100 ng/ml), or SMase (1 U/ml) for 4.5 h and analyzed for the presence of TNF- α . For the TNF- α ELISA, the lowest concentration of rTNF- α included on the standard curve was 35 pg/ml. The data are representative of four separate experiments.

TABLE 3. TNF- α production in macrophages pretreated with LPS or SMase^a

Pretreatment	Amt of TNF- α produced (pg/ml) after challenge with:		
	Medium	LPS	SMase
Medium	<35	2,065	1,782
LPS	<35	<35	127
SMase	<35	2,291	1,349

^a Macrophages were pretreated with medium only, LPS (100 ng/ml), or SMase (1 U/ml) for 19 h as described in the text. Supernatants collected from macrophages after subsequent restimulation with medium only, LPS, or SMase for 4.5 h were analyzed for the presence of TNF- α . For the TNF- α ELISA, the lowest concentration of rTNF- α included on the standard curve was 35 pg/ml. Results represent the geometric means for three separate experiments.

pathway does not mimic entirely that of the LPS signaling pathway; rather, the ceramide pathway may contribute a portion of the LPS response. Further, these data suggest that although the function of the *Lps* gene product (or a closely associated molecule) may be compromised during endotoxin tolerance (since neither LPS nor SMase can stimulate endotoxin-tolerized macrophages) (Table 3), the intracellular mechanisms required to invoke the tolerant state occur after the two pathways diverge, i.e., downstream of the common requirements for the *Lps* gene product (since SMase does not induce tolerance [Table 3]).

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