Ceramide and ceramide-activated enzymes have been implicated in responses to bacterial lipopolysaccharide (LPS) and the proinflammatory cytokines tumor necrosis factor-α (TNF) and interleukin-1β (IL-1). Although TNF and IL-1 cause elevation of cellular ceramide, which is thought to act as a second messenger, LPS has been proposed to signal by virtue of structural similarity to ceramide. We have investigated the relationship between ceramide and LPS by comparing the effects of a cell-permeable ceramide analog (C2-ceramide) and LPS on murine macrophage cell lines and by measuring ceramide levels in macrophages exposed to LPS. We found that while both C2-ceramide and LPS activated c-Jun N-terminal kinase (JNK), only LPS also activated extracellular signal-regulated kinases (ERKs). C2-ceramide was also unable to activate NF-κB, a transcription factor important for LPS-induced gene expression. Upon measurement of cellular ceramide in macrophage lines, we observed a small but rapid rise in ceramide, similar to that seen upon IL-1 or TNF treatment, suggesting LPS induces an increase in ceramide rather than interacting directly with ceramide-responsive enzymes. We found that C2-ceramide activated JNK and induced growth arrest in macrophages cell lines from both normal mice (Lpsa) and mice genetically unresponsive to LPS (Lpsb), whereas only Lpsa macrophages made these responses to LPS. Surprisingly, LPS treatment of Lpsb macrophages induced a rise in ceramide similar to that observed in LPS-responsive cells. These results indicate that the wild type Lps allele is not required for LPS-induced ceramide generation and suggest that ceramide elevation alone is insufficient stimulus for most responses to LPS.

The Gram-negative bacterial endotoxin lipopolysaccharide (LPS) is a classic and common initiator of inflammation. Macrophages exposed to LPS undergo a differentiation program that involves the up-regulation of genes whose products enhance the ability of macrophages to invade tissue, destroy bacteria, attract other immune system cells, and coordinate their responses. A localized proinflammatory response to LPS promotes host defense against bacterial infection, but if this response becomes systemic, as can occur during bacterial sepsis, it can result in endotoxic shock, which is often fatal.

The principal high affinity receptor for LPS on myeloid cells is CD14, a glycosylphosphatidylinositol (GPI)-linked protein, which recognizes LPS in a complex with LPS-binding protein. Binding of LPS activates a variety of well characterized signaling pathways, possibly through an as yet unidentified coreceptor (1–3). Lipid A, the conserved core structure of LPS, retains the essential biological and signaling properties of intact LPS (4). It has been suggested that the lipid A portion of LPS shares some structural similarity with the cellular lipid ceramide (5) and that LPS may act by mimicking ceramide (6).

Ceramide can be generated by cleavage of membrane sphingomyelin by either acid or neutral sphingomyelinases (7, 8), which remain to be fully characterized, or by de novo ceramide synthesis. Increases in cellular ceramide have been reported in many cell types in response to a variety of stimuli. These include the inflammatory cytokines tumor necrosis factor (TNF) and interleukin-1 (IL-1), as well as environmental stresses, such as UV light, differentiating agents, like vitamin D₃, and other immunomodulatory signals, including Fas and CD28 (9, 10). Micro-organisms have also been shown to stimulate increased cellular ceramide production as follows: binding of P-fimbriated Escherichia coli (11) or internalization of Neisseria gonorrhoeae by nonphagocytic cells reportedly increases ceramide (12). Membrane-permeable ceramide analogs have been used to investigate the function of cellular ceramide, and exposure of mammalian cells to micromolar concentrations of these analogs has profound effects on gene expression, cell growth, and cell survival (10).

One of the earliest signaling events following LPS treatment of macrophages is tyrosine phosphorylation and activation of mitogen-activated protein kinases (MAPKs), including members of the ERK family, as well as the stress-activated MAPKs, JNK and p38 (13–16). Ceramide analogs have likewise been shown to activate ERK and JNK, and more recently p38, although the subset of MAPKs reported to be activated varies and may be cell line-dependent (17–23). Activated MAPKs phosphorylate and regulate multiple transcription factors; among these AP-1/c-Jun and ATF2 have both been shown to be activated by either LPS or ceramide analog treatment (21, 24–26).

The NF-κB/Rel family of transcription factors is also activated by LPS and is critical for induced expression of many proinflammatory genes (3). In some cells, ceramide analogs reportedly activate NF-κB (27, 28), although this is not always observed even in the same cell line (20, 29). The coordinate
activation of NF-κB and other transcription factors by LPS results in the expression of genes encoding adhesion molecules, enzymes involved in the production of oxygen and nitrogen radicals, and inflammatory cytokines, notably TNF, IL-1, and IL-6 (1). These changes in gene expression reflect differentiation to an activated phenotype and are accompanied by growth arrest in bone marrow-derived macrophages (30), as well as in the murine macrophage line RAW 264.7 (31). In some macrophage cell lines and in fibroblasts, ceramide analogs have also been shown to induce expression of cytokines and cell adhesion molecules (32–35), and ceramide treatment causes growth arrest in many eukaryotic cell types, including yeast (36–38).

Direct evidence for a link between LPS and ceramide was initially provided by the observation that LPS up-regulated a 97-kDa serine/threonine protein kinase activity, thought to correspond to ceramide-activated protein kinase (CAPK) (5). This led to the proposal that LPS may act as a structural mimic of ceramide (6). A report that CD14 and LPS-binding protein can transfer LPS into phospholipid bilayers supported the idea that LPS might interact directly with ceramide-responsive enzymes at the plasma membrane (39). The molecular mimicking hypothesis was further strengthened by observations that bacterial sphingomyelinase or synthetic ceramide analogs induced expression of an array of LPS-inducible mRNAs in macrophages from LPS-responsive (Lps⁺) mice but not in those from C3H/HeJ (Lps⁻) mice, which are genetically hyporesponsive to LPS (32). Additionally, the intracellular trafficking of both fluorescently labeled LPS and labeled ceramide analogs is reportedly altered in Lps⁻ macrophages (40), although LPS binding and internalization occur with normal kinetics in the LPS-unresponsive macrophages (41). In contrast, other recent studies suggest that ceramide analogs and LPS, while having some overlapping effects, induced different patterns of gene expression (33) and differed in their ability to prime myeloid cells for superoxide production (42).

In an attempt to clarify the role of ceramide in LPS-induced signaling we have assayed the effects of LPS and the ceramide analog C2₄ on many of the above signaling molecules in murine macrophage lines. We found that both C₂₄- and LPS activated JNK, but only LPS activated the ERK MAPKs. Similarly, NF-κB was not activated by C₂₄ over a range of concentrations, in contrast to rapid activation by LPS treatment, arguing against the molecular mimickry hypothesis of LPS action. Measurement of cellular ceramide in LPS-treated macrophages revealed a rapid increase in ceramide levels, comparable to that induced by IL-1 or TNF. Both C₂₄- and LPS induced growth arrest in a concentration-dependent manner in the RAW 264.7 macrophage line, suggesting that LPS-induced cellular ceramide could contribute to this response. In an Lps⁻ macrophage line, responses to LPS were deficient, but C₂₄- ceramide responses, namely JNK activation and growth arrest, were intact. Elevation of cellular ceramide was also observed in Lps⁻ macrophages treated with LPS, indicating that normal Lps function is not required for this effect and that cellular ceramide increases alone cannot elicit many downstream LPS responses.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Materials**—Murine macrophage cell lines were expanded and maintained in Dulbecco’s modified Eagle’s medium with 5%–10% fetal bovine serum (containing <0.06 units/ml endotoxin) at 37°C in 5% CO₂ for a maximum of 8 weeks of passage. The RAW 264.7 cell line was obtained from the American Type Culture Collection (Rockville, MD). The Lps⁺ and Lps⁻ VN-11 retrovirally transformed lines were the kind gift of Dr. Paula Riccardi-Castagnoli (CNR, Milan, Italy). The latter macrophage lines originated from infection of primary thymic cultures derived from Balb/c and C3H/HeJ mice, respectively. Bone marrow macrophages (BMMO) from C57Bl/6 mice were isolated and cultured as described previously (43).

Cells were stimulated as noted in figure legends and text with wild type LPS from E. coli K-235 (purified by phenol extraction and gel filtration; <1% protein), diphosphoryl lipid A from E. coli F-583, or endotoxin from *Salmonella minnesota* R595 (Genzyme Diagnostics, Cambridge, CA). Lp−C₂₄ (N-acetyl-N-t-erythro-sphingosine) and dihydro-C₂₄- ceramide (dihydro-N-acetyl-t-erythro-sphingosine) obtained from Calbiochem or Bio-Rad (Bio-Rad) were used as substrate for JNK kinase assays; GST-Elk1 (New England Biolabs) was used as substrate for ERK2 activity assays. Kinase reactions were loaded on SDS-PAGE gels and following electrophoresis were either dried down and autoradiographed or re-photographed on X-ray film. The kinase reactions were performed at 30°C for 15 min and terminated by addition of 0.3–1.0 ml of cold lysis buffer to the dish. Lysis buffer consisted of 20 mM Tris (pH 7.9), 137 mM NaCl, 5 mM EDTA, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 10 mM NaF and was supplemented immediately prior to use with 1 mM phenylmethylsulfonyl fluoride or Pefabloc™ (Boehringer Mannheim), 1 mM Na₃VO₄, 1 mM aprotinin, and 1 mM leupeptin. Following high speed centrifugation to remove debris, the protein concentration of extracts was determined using the BCA (Pierce) or Bio-Rad (Bio-Rad) protein assay.

Whole-cell extracts containing 30–50 μg of total protein were separated by electrophoresis in 10% SDS-PAGE gels for Iba1 immunoblotting or in 12% SDS-PAGE gels (acylamide/bis-acrylamide ratio of 120:1) for ERK1/ERK2 and JNK immunoblots (44). The gels were transferred to nitrocellulose, blocked with 4% dried non-fat milk in Tris-buffered saline with 0.5% Tween 20 (TBST), and incubated with specific antibody overnight at 4°C. After extensive washing in TBST, a horseradish peroxidase-coupled sheep (Boehringer Mannheim) or donkey (Amer sham Pharmacia Biotech) anti-rabbit IgG antibody was added to the blots for 1 h. After further washes the peroxidase activity was revealed using Renaissance™ chemiluminescence reagents (NEN Life Science Products). The primary antibodies used for immunoblotting were raised against Iba1 (sc-203, -371, -847) or ERK1-deriv (sc-94) immunogenic peptides (Santa Cruz Biotechnology, Santa Cruz, CA) or against JNK1 (PharMingen, San Diego, CA).

**Immunoprecipitation and in Vitro Kinase Assays**—Whole-cell extracts containing 200–500 μg of total protein were diluted to a 1 ml volume with lysis buffer and immunoprecipitated with 10 μl of specific antibody to JNK1 (sc-474) or ERK2 (sc-154) (Santa Cruz Biotechnology) overnight at 4°C and then for 1–2 h with 15 μl of protein A-Sepharose 400 (Pharmacia Biotech, Piscataway, NJ) or goat anti-mouse IgG (Zyned Laboratories Inc., San Francisco, CA). The resulting immunoprecipitate was washed extensively with lysis buffer and once with kinase assay buffer (25 mM Hepes, pH 7.6, 20 mM MgCl₂, 2 mM dithiothreitol, 20 mM β-glycerol phosphate, 1 mM Na₃VO₄). The kinase reaction was performed in kinase assay buffer with 20 μM unlabeled ATP and 10 μM of [γ-³²P]ATP (Amer sham Pharmacia Biotech) or [γ-³²P]ATP (Amer sham Pharmacia Biotech) overnight at 1–2 h with 15 μl of protein A-Sepharose. The kinase reactions were loaded on SDS-PAGE gels and following electrophoresis were either dried down and exposed or in some cases were transferred for immunoblotting (see above) to verify equivalent kinase precipitation and loading across samples.

**Electrophoretic Mobility Shift Assay**—An Xb oligonucleotide probe (described in Ref. 44) was filled in with the Klenow fragment of DNA polymerase and labeled with [γ-³²P]ATP using T4 polynucleotide kinase (New England Biolabs). Whole-cell extracts were prepared as above, but the salt concentration was increased to 400 mM to ensure complete extraction of nuclear proteins. Specific binding of extract proteins to the Xb probe was assessed by incubation for 15 min at room temperature in a solution containing 7.5 mM Hepes (pH 8.0), 35 mM NaCl, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM dithiothreitol, and 7.5% glycerol, and 0.5 μM probe (difier) (Boehringer Mannheim), followed by electrophoretic separation in a 5% polyacrylamide gel.

**Ceramide Measurement**—Ceramide was labeled in vivo by incubation of cells in [³H]palmitate (10 μCi/well) in 6-well plates for 24–48 h prior to stimulation, using a method similar to that described by Liu and Anderson (45). Cells were stimulated with LPS or IL-1 in triplicate or quadruplicate wells in the same culture medium containing...
Role of Ceramide in Lipopolysaccharide-induced Signaling

[\textsuperscript{3}H]palmitate at 37°C. Unstimulated control wells were included on these plates and were labeled and processed simultaneously with stimulated samples. Following stimulation, cells were washed once with cold PBS, and flash-frozen in a dry-ice/ethanol bath. Water was added to each well, and cells were removed by scraping and transferred to glass tubes. Wells were rinsed with methanol/hydrochloric acid (40:1), and cellular lipids were extracted in chloroform and NaCl (1 N), and the chloroform phase was dried under nitrogen. The resulting labeled lipid mixture was resuspended in chloroform with 5% ethanol and 30 μg of cold C\textsubscript{2}-ceramide from bovine brain as carrier. Samples were separated by thin layer chromatography (TLC) on silica gel plates (19-channel Kieselgel, Whatman) in a solvent of toluene/methanol (85:15) along with lipid standards for phosphatidylethanolamine, phosphatidylserine, sphingomyelin, and C\textsubscript{14}- and/or C\textsubscript{16}-ceramide. The ceramide band was visualized by iodine vapor staining and autoradiography, and the corresponding area of the plate was recovered, scintillation fluid (Countiscint, ICN) was added, and samples were counted in a β-scintillation counter.

Alternatively, ceramide content of RAW 264.7 cells was assessed by diacylglycerol kinase labeling in vitro according to the method of Page et al. (46) with modifications. RAW 264.7 macrophages were seeded in 12- or 6-well tissue culture plates (Corning) 18–24 h prior to stimulation. Following stimulation, total cellular lipids were extracted and dried as described above. Extracted total cellular lipids and ceramide (Avanti Polar Lipids) and diene standards (Sigma) were resuspended in a solution containing 5 mM cardioliulin and subjected to phosphorylation by diacylglycerol kinase (Calbiochem) in a reaction containing 50 mM imidazole, 50 mM MgCl\textsubscript{2}, 2 mM dithiothreitol, 1 mM EGTA, 1 mM ATP, 10 μM diethylthre月中lipantaeic acid (Sigma), and 50 μCi of [\textsuperscript{32}P] or [\textsuperscript{γ-33}P]ATP. Labeled phosphotidic acid and ceramide phosphate were extracted, dried under nitrogen, resuspended in chloroform with 5% ethanol, and separated by TLC in a chloroform/methanol/25:25:1 mixture. Labeled ceramide phosphate signals were quantitated by PhosphorImager analysis (Molecular Dynamics). Phosphorylation of ceramide was linear over the concentration range tested (100–1000 pmol of bovine brain ceramide). Cellular ceramide levels in unstimulated cells were on the order of 100 pmol/10\textsuperscript{5} cells, as judged by signals obtained with ceramide standards.

Flow Cytometry—Flow cytometry was used to determine the cell surface expression of various markers on murine macrophage lines and bone marrow-derived macrophages. Cells (10\textsuperscript{5}/sample) were removed from tissue culture plastic by gentle pipetting following treatment with 6 mM EDTA/PBS, washed, and resuspended in 100 μl of staining buffer (PBS, 2% fetal bovine serum, 0.02% azide) with rat anti-mouse Fc\textsubscript{R} blocking antibody (0.25 μg/ml), and propidium iodide (5 μg/ml) in 1 ml of medium were stimulated with LPS or C\textsubscript{2}-ceramide, as indicated. Supernatants were harvested at various times (5, 9, and 24 h) and centrifuged to remove any cellular debris. TNF-α ELISA—Macrophages growing in 24-well plates (10\textsuperscript{5}/well) in 1 ml of medium were stimulated with LPS or C\textsubscript{2}-ceramide, as indicated. Supernatants were harvested at various times (5, 9, and 24 h) and centrifuged to remove any cellular debris. TNF-α content of these supernatants was assessed by ELISA using an anti-mouse TNF-α Cytooscreen kit (Biosource International, Camarillo, CA).

RESULTS

To elucidate the possible role of ceramide-activated signaling reactions in the response of macrophages to lipopolysaccharide (LPS), we investigated biochemical responses of RAW 264.7 cells, a murine macrophage line, to LPS and the cell-permeable ceramide analog C\textsubscript{2}C. We and others (15, 48) have previously shown that treatment of myeloid cell lines with LPS or lipids activates c-Jun N-terminal kinase (JNK). In several cell types, including the human monoblastic leukemia line U937, ceramide analogs have also been shown to activate JNK (22). We compared JNK activity in whole-cell extracts from RAW 264.7 macrophages treated with LPS or C\textsubscript{2}-ceramide. Dihydro-C\textsubscript{2}-ceramide (dH-C\textsubscript{2}-ceramide) was used as a negative control for C\textsubscript{2}-ceramide, as the dihydro form reportedly lacks biological activity (49). LPS or C\textsubscript{2}-ceramide treatment of RAW 264.7 cells increased JNK activity, as assessed by phosphorylation of a glutathione S-transferase (GST)-c-Jun fusion protein by immunoprecipitated JNK, whereas no increase in JNK activity was detected in extracts from cells treated with dH-C\textsubscript{2}-ceramide (Fig. 1A). Treatment of RAW 264.7 macrophages with the lipid A portion of LPS or with paclitaxel, which is structurally unrelated to LPS but has LPS mimetic properties (50, 51), activated JNK kinase activity to an extent similar to that elicited by LPS treatment (data not shown). Identical results were also obtained in bone marrow-derived macrophages (BMMO), i.e. JNK was activated by both C\textsubscript{2}-ceramide and LPS (data not shown). After 30 min incubation at 37°C, phagocytosis was terminated by removing the plates to ice and washing with cold PBS. Unengaged labeled SRBC were lysed by addition of water (750 μl/well) for 1 min. After a further wash in PBS, adherent macrophages with ingested \textsuperscript{51}Cr-labeled SRBC were lysed in 500 μl of 1% SDS and transferred to tubes for quantitation of radioactivity in a C18-Septa Gamma counter (LKB-Wallac, Finland). Percent phagocytosis was calculated as the mean cpm of 3–4 wells treated as above/mean total cpm of replicate wells treated as described above but without hypotonic lysis of unengaged \textsuperscript{51}Cr-SRBC, i.e. ingested/bound SRBC × 100%.

TNP-α ELISA—Macrophages growing in 24-well plates (10\textsuperscript{5}/well) in 1 ml of medium were stimulated with LPS or C\textsubscript{2}-ceramide, as indicated. Supernatants were harvested at various times (5, 9, and 24 h) and centrifuged to remove any cellular debris. TNP-α content of these supernatants was assessed by ELISA using an anti-mouse TNP-α Cytooscreen kit (Biosource International, Camarillo, CA).

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shown). The activation of JNK in RAW 264.7 cells by C2-ceramide was both concentration- and time-dependent (Fig. 1B). At 30 min JNK activation was evident in cells treated with 50 or 100 μM C2-ceramide; at 1 h JNK was activated in cells treated with a 10 μM (or higher) concentration of C2-ceramide. Activation of JNK by C2-ceramide was considerably weaker (5-fold activation with 100 μM C2 at 1 h than that induced by LPS treatment (25-fold with 1 μg/ml at 1 h) and occurred with slower kinetics (Fig. 1B and see Ref. 15).

We also examined the activity of ERK MAPKs in RAW 264.7 cells treated with LPS or C2-ceramide. LPS activated ERK1 and ERK2 in the RAW 264.7 line, as described previously (13, 52, 53). ERK activation can be detected by a change in mobility of ERK1 and ERK2 to shift (Fig. 2A), treatment of RAW 264.7 cells with LPS, lipid A, or paclitaxel led to increased levels of the active forms of ERK1 and ERK2 within 30 min. Neither C2 nor dH-C2-ceramide caused the mobility of ERK1 or ERK2 to shift (Fig. 2A). ERK2 activity in RAW 264.7 cell extracts was also assayed directly using a GST-Elk1 fusion protein as a substrate. Whereas LPS induced a substantial increase in Elk1 phosphorylation by immunoprecipitated from RAW 264.7 cells treated with the indicated concentration of C2-ceramide, dH-C2-ceramide (100 μM), or LPS (1 μg/ml) for 30 min. The activation of NF-κB by LPS but not by C2-ceramide. A, IκBα is degraded in response to treatment with LPS or lipid A (LA) (20 min; 1 μg/ml), or paclitaxel (TX) (30 min; 100 μM) but not by exposure to C2-ceramide or dH-C2-ceramide (1 h; 100 μM) (lanes 2–6) in RAW 264.7 cells. Lane 1 is an untreated control. B, electrophoretic mobility shift assay with a 32P-labeled κB oligonucleotide probe and high salt extracts from RAW 264.7 cells treated with C2-ceramide or LPS. Cold competitor mutant (lanes 9 and 11) or wild type (lanes 10 and 12) κB oligonucleotides were used to determine specificity of binding.

When RAW 264.7 cells were incubated with the thymidine analog 5-bromo-2-deoxyuridine (BrdUrd) for 8 h, only a small fraction (<10%) of untreated cells failed to incorporate BrdUrd, as measured by flow cytometry, indicating that the majority of the cells passed through S phase during the labeling period. The fraction of BrdUrd-negative, noncycling cells increased significantly when cells were treated for 16 h with 25–50 μM C2-ceramide or 0.1–1 μM C2-ceramide had little effect on cell viability, 100 μM C2-ceramide (100 μM) along with LPS did not detectably lessen ERK2 activity (data not shown).

LPS treatment of macrophages activates binding of NF-κB and other members of the Rel transcription factor family to κB sites in the regulatory regions of many genes encoding proinflammatory molecules (3). Ceramide has been suggested to be an upstream activator of NF-κB in TNF-stimulated cells (27, 28). However, this point is controversial, as others (29, 30) report no effect of ceramide on κB binding. To examine this issue in RAW 264.7 macrophages, we used the following two measures: degradation of a principal inhibitory subunit of Rel proteins, IκBα, and specific κB binding activity. In LPS- or lipid A-treated cells, the majority of IκBα was proteolysed within 20 min (Fig. 3A). Treatment with paclitaxel also induced complete degradation of IκBα, although with slightly slower kinetics (30 min). In contrast, 5–60 min treatment with C2-ceramide, at concentrations from 10 to 100 μM, did not induce any detectable IκBα degradation (Fig. 3A and data not shown). Simultaneous treatment with C2-ceramide (100 μM) and LPS did not appreciably alter the IκBα degradation induced by LPS at 20 min (data not shown).

An electrophoretic mobility shift assay with a 32P-labeled κB oligonucleotide was used to assess directly specific κB binding activity. As seen in Fig. 3B, the constitutive low level κB binding activity present in unstimulated RAW 264.7 cells was not significantly altered by C2-ceramide treatments of up to 1 h, over a range of concentrations. LPS treatment, however, induced a rapid increase in specific κB binding activity (Fig. 3B). Thus LPS induced degradation of IκBα and the appearance of NF-κB binding activity in RAW 264.7 macrophages, but C2-ceramide did not.

In most cases, cells treated with ceramide analogs or agents that provoke an increase in cellular ceramide undergo differentiation, growth arrest, senescence, or apoptosis, rather than proliferation (10). As LPS also induces growth arrest and differentiation in bone marrow macrophages and macrophage lines (30, 31), we compared the effects of C2-ceramide and LPS on DNA synthesis in RAW 264.7 cells.

When RAW 264.7 cells were incubated with the thymidine analog 5-bromo-2-deoxyuridine (BrdUrd) for 8 h, only a small fraction (<10%) of untreated cells failed to incorporate BrdUrd, as measured by flow cytometry, indicating that the majority of the cells passed through S phase during the labeling period. The fraction of BrdUrd-negative, noncycling cells increased significantly when cells were treated for 16 h with 25–50 μM C2-ceramide or 0.1–1 μM C2-ceramide had little effect on cell viability, 100 μM C2-ceramide (100 μM) along with LPS did not detectably lessen ERK2 activity (data not shown).

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Role of Ceramide in Lipopolysaccharide-induced Signaling

Fig. 4. C2-ceramide and LPS induce growth arrest in a significant fraction of RAW 264.7 macrophages. Cells were treated with carrier (EtOH, 0.5%), C2-ceramide, dihydro-C2-ceramide (50 μM), or LPS as indicated for 24 h, and the nucleotide analog BrdUrd was added for the final 8 h of culture. The cells were fixed and stained with a FITC-conjugated anti-BrdUrd antibody, and the BrdUrd-negative, growth-arrested fraction was quantified by flow cytometry. (Representative results of one of 2–4 experiments at each concentration.)

whereas C2-ceramide-treated cells became rounder and less firmly adherent. This observation is consistent with the differences apparent in the signaling pathways activated by the two stimuli, as described above.

We next investigated the ability of macrophages from LPS-hyporesponsive (Lpsd) mice to respond to C2-ceramide. Previous reports suggested the Lpsd mutation altered or abrogated cellular responses to ceramide analogs (32, 40), in addition to the well documented loss of LPS responsiveness and resistance to endotoxic shock (54). It was unclear, however, whether the failure to respond to ceramide analogs was due to the Lpsd defect per se or due to differences in the thioglycollate-elicited macrophage populations of Lpsa and Lpsd mice (see “Discussion”).

To study further the influence of the Lps locus on ceramide-dependent signaling, we obtained a pair of similarly transformed macrophage lines from Lpsa and Lpsd mice (MT2 and MTC, respectively; kind gift of Dr. P. Ricciardi-Castagnoli, CNR, Milan, Italy). Each line was derived from a mouse thymus infected with the VN-11 retrovirus, which bears an myc-env fusion thought to immortalize macrophages via autocrine macrophage colony-stimulating factor expression (55, 56). As previously reported, the Lpsa cell line (MT2) has cell-surface markers and functional characteristics of a macrophage (57, 58). We characterized the expression of a panel of relevant cell-surface proteins by the Lpsd (MTC) cell line; BMMO and RAW 264.7 and Lpsa (MT2) macrophage lines were analyzed in parallel for comparison (Table I). All three macrophage cell lines expressed CD14, Mac1 (CD11b), Fcγ receptor (FcyR), B7.1, and B7.2 constitutively but not major histocompatibility complex class II (I-A), whose expression could be induced by γ-interferon treatment (Table I). Expression patterns for these markers were slightly different in BMMO, which lacked B7.2 but expressed some class II major histocompatibility complex without γ-interferon treatment. All macrophage lines were capable of FcγR-mediated phagocytosis of antibody-coated sheep red blood cells, although with varying efficiencies (Table I). Thus it appears that the retrovirally transformed Lpsa and Lpsd macrophage lines represent a reasonable model system for the study of the role of the Lps gene in LPS signal transduction.

Analysis of the biochemical responses to C2-ceramide in these virally transformed macrophages confirmed our observations in RAW 264.7 cells. Neither the Lpsa nor Lpsd cells exhibited an increase in ERK activity in response to C2-ceramide treatment, even at high concentrations (data not shown). As expected, LPS activated both ERK2 and p38 MAPKs in the Lpsa but not the Lpsd line (data not shown). We observed an increase in JNK activity in both lines in response to C2-ceramide and TNF treatment (Fig. 5A). In contrast, LPS activated JNK substantially only in the Lpsa macrophage cell line, whereas in Lpsd macrophages JNK activity was only slightly elevated above the basal level. Thus the Lps gene product appears not to be required for activation of JNK by C2-ceramide, although its absence notably impaired JNK activation by LPS.

To test the effect of mutation of the Lps locus on a functional effect of ceramide, we assessed the cell cycle status of the virally transformed Lpsa macrophage line. The cells were treated with 10–50 μM C2-ceramide or 1 μg/ml LPS for 24 h with addition of BrdUrd for the final 8 h of culture. Although BrdUrd incorporation in the Lpsa macrophages was not affected by LPS or diH-C2-ceramide treatment, incubation with 50 μM C2-ceramide provoked a dramatic increase in the fraction of BrdUrd-negative cells (Fig. 5B). A fraction of the Lpsa macrophages, like LPS-responsive RAW 264.7 cells (Fig. 4), underwent growth arrest in response to C2-ceramide or LPS but not in response to diH-C2-ceramide, although with both stimuli the arrested fraction was smaller than that observed in RAW 264.7 or Lpsd cells (data not shown). The Lpsd cells also underwent rapid cell death when treated with 100 μM C2- but not dihydro-C2-ceramide. Thus the growth arrest and death responses to C2-ceramide are unaffected by the Lpsd defect.

We assessed TNF production in response to ceramide by ELISA in the Lpsa and Lpsd thymic macrophage lines, as TNF mRNA expression was reportedly induced by C2-ceramide treatment of Lpsa but not Lpsd-elicited peritoneal macrophages (32). Although LPS treatment of the Lpsa cell line or of BMMO or RAW 264.7 cells led to the production of comparable amounts of TNF, no TNF could be detected in any of these cell populations in response to treatment with 10–50 μM C2-ceramide for up to 24 h (Table II and data not shown). Neither was TNF detectable in supernatants from Lpsd cells treated with C2-ceramide or wild type E. coli LPS, whereas these cells produced minimal amounts of TNF in response to a less pure ReLPS preparation (Table II). The genetic defect in the Lpsd cells need not be invoked to account for their lack of TNF expression in response to ceramide, as we did not find this response to be a universal property of murine macrophages.

| Parameter          | BMMO | RAW 264.7 | MT2 (Lpsa) | MTC (Lpsd) |
|--------------------|------|-----------|------------|------------|
| Surface antigens   |      |           |            |            |
| CD11b              | +    | +         | +          | +          |
| CD14               | +    | +         | +          | +          |
| FcγR               | +    | +         | +          | +          |
| B7.1               | +    | +         | +          | +          |
| B7.2               | +    | +         | +          | +          |
| Class II (I-Aαβ)   | +    | +         | +          | +          |
| B1220              | +    | +         | +          | +          |
| CD3                | +    | +         | +          | +          |

% FcγR-mediated phagocytosis

These parameters also exhibited induced or increased expression following IFN-γ treatment (200 units, 24 h).

% phagocytosis = cpm 51Cr-labeled IgG-coated SRBC internalized per cpm bound × 100% in 30 min at 37 °C (see “Experimental Procedures”).
The above results demonstrated important differences in the array of signals elicited by LPS and C₂-ceramide and in their requirement of the wild type \textit{Lps} allele. These findings argue against the idea that LPS exerts its effects by interacting directly with ceramide-dependent enzymes by virtue of a proposed structural similarity to ceramide. To test an alternative hypothesis that ceramide may be generated in response to LPS, we measured cellular ceramide levels in RAW 264.7 macrophages before and after LPS treatment, using IL-1 as a positive control.

Cellular ceramide increased rapidly (within 5 min) in RAW 264.7 cells treated with LPS (1 μg/ml), as assessed by metabolic labeling of cells with [³H]palmitate (Fig. 6A). The LPS-induced increase was modest, representing a maximum near 130% of basal cellular ceramide values but comparable to the increase observed in RAW 264.7 cells treated with IL-1 (125% of basal). The difference between ceramide levels in unstimulated and LPS-stimulated cells was statistically significant at 15–30 min (\(p < 0.05\), two-tailed \(t\) test). We also detected a rapid elevation in ceramide in response to LPS or IL-1, to 116 and 117% of basal levels by 15 min, respectively, when ceramide was measured in RAW 264.7 lipid extracts by an alternative method involving in vitro labeling with bacterial diacylglycerol kinase (data not shown; mean of 5 independent experiments each in triplicate or quadruplicate).

We next asked whether the LPS-stimulated increase in cellular ceramide observed in RAW 264.7 cells was unique to that cell line or a more general phenomenon. As shown in Fig. 6B, a
15-min stimulation with a low or high dose of LPS (50 or 1000 ng/ml) caused an increase in cellular ceramide in the virally transformed Lp$^s$ cell line, MT2, confirming results in RAW 264.7 cells. As in RAW 264.7 cells, the LPS-induced ceramide increase in MT2 cells was similar in magnitude to that measured in response to a 15-min exposure to IL-1 (25 ng/ml) or, additionally, to TNF (10 ng/ml) (Fig. 6B).

Surprisingly, we also observed an increase in cellular ceramide in the Lps$^s$ macrophages, not only upon treatment with IL-1 or TNF, but also in response to LPS, on an order similar to that observed in Lp$^s$ cells (Fig. 6C). This finding indicates that the normal function of the Lps locus is not required for the increase in cellular ceramide observed in LPS-treated cells and suggests that the LPS-induced ceramide increase is not sufficient stimulus on its own for most LPS responses.

**DISCUSSION**

Although most of the known biochemical effects of LPS stimulation, including activation of ERK, JNK, p38, and NF-kB, have also been reported to be activated in some cell lines by ceramide analogs, our comparison of downstream signaling events induced by LPS or C$_2$-ceramide treatment of murine macrophages revealed important differences between the two stimuli. In the RAW 264.7 and MT2 LPS-responsive macrophage lines, we found ERK, JNK, and NF-kB were rapidly activated by LPS but only JNK was activated by C$_2$-ceramide. Rather than supporting the hypothesis that LPS acts primarily by mimicking ceramide, our results demonstrate that, at a minimum, LPS must activate ERK2 and NF-kB via other pathways for which ceramide alone is an insufficient stimulus. Moreover, we found that LPS induced a rapid increase in cellular ceramide levels comparable to that seen with IL-1 or TNF treatment, suggesting that elevation of ceramide may mediate or contribute to some responses to LPS, although the complete LPS-induced differentiation program clearly requires signals in addition to ceramide.

Although evidence links ceramide to activation of both ERK and JNK MAPK cascades, the activation of JNK by ceramide analogs has been more frequently reported. For example, in the human T cell line Jurkat and in rat glomerular mesangial cells, JNK but not ERK was activated by ceramide analogs (20, 23, 59), as we observed in several murine macrophage lines (Figs. 1 and 5B). In RAW 264.7 and MT2 Lps$^s$ macrophages, JNK activation by C$_2$-ceramide was slower and weaker than that induced by LPS. This could reflect inefficiency in delivery of ceramide analogs to the appropriate intracellular compartment. However, it appears more likely that LPS-induced activation of JNK requires signals in addition to ceramide, as suggested by our results in Lps$^s$ macrophages. In those cells, although LPS induces an increase in ceramide equivalent to that seen in wild type macrophages, activation of JNK by LPS was severely impaired.

The immediate upstream activator of JNK in response to LPS treatment is the MAPK kinase MKK4 (48), and a similar pathway has been implicated in activation of JNK by ceramide (22). Several different MAPK kinase kinase (or MEKK) family members appear to be capable of activating the MKK4-JNK pathway. At least one of these, TAK1, has been shown to be activated by C$_2$-ceramide and proinflammatory cytokines in vivo (60). Further characterization of the MEKK(s) involved in activation of JNK by LPS may clarify the possible contribution of ceramide.

LPS had previously been shown to activate a 97-kDa enzyme with the properties of ceramide-activated protein kinase (CAPK) in U937 cells (5). As CAPK, recently identified as KSR (kinase suppressor of activated Ras)(61), reportedly activates the Raf-MEK-ERK MAPK cascade (18), ceramide might be expected to contribute to ERK activation by LPS. However, we found that ceramide analogs did not activate ERK MAPKs (Fig. 2), whereas LPS activates them fully and in a prolonged fashion (13, 44). Currently both the role of ceramide in KSR activation (62, 63) and the effect of KSR on the Raf-MEK-ERK pathway remain unclear, with some recent investigations suggesting KSR can provide a negative rather than positive input to the ERK MAPK pathway (63–65).

Our observation of elevated ceramide in LPS-treated RAW 264.7 and MT2 macrophages suggests that the original observation of CAPK activation by LPS in U937 cells (5) may be a result of ensuing ceramide generation rather than direct interaction of LPS with CAPK. Although Joseph et al. (5) failed to detect an increase in ceramide with the rapid kinetics expected for activation of CAPK in U937 cells, we observed a rise in ceramide within 5 min of LPS treatment in RAW 264.7 murine macrophages (Fig. 6A).

The increase in ceramide levels in LPS-treated macrophages was modest but similar to that induced by IL-1 or TNF in the same cells and on the order of increases reported in the literature for TNF- and IL-1 at early times (i.e. <2-fold) (28, 35, 45, 66, 67). Stimulus-induced increases in ceramide have been proposed to be restricted to a specific subcellular pool, thought to represent 10–20% of total cellular ceramide (68). In IL-1-treated fibroblasts the increase in ceramide is concentrated in caveolae (45), a sphingomyelin-rich plasma membrane domain, and similar findings of localized production of ceramide have been reported for neurotrophin-induced sphingomyelin hydrolysis (69). LPS-induced increases in ceramide may be similarly localized, in which case measurement of total cellular ceramide, as reported here, may understate changes at the site of LPS binding.

CD14 is likely to be an important binding component contributing to the LPS-induced increase in cellular ceramide, as we observed this response with a low dose of LPS (50 ng/ml), which is thought to require CD14 (1). CD14, like other GPI-linked proteins, has recently been shown to localize to the Triton X-100-insoluble membrane fraction (70), which is also rich in ceramide and sphingolipids (71) and may be physically and functionally linked to caveolae (72). It seems likely that LPS-induced ceramide increases occur in these special lipid domains, to which other receptors and signaling molecules have been localized (73). However, in contrast to many GPI-linked receptors, the GPI link of CD14 is not required for most CD14-dependent signaling or activation of cytokine gene expression (70). Thus the functional significance of the physiological localization of CD14 to the Triton X-100-insoluble fraction is unclear at present.

Our results do not address the question of the mechanism of LPS-induced ceramide generation, although it likely involves activation of one or more sphingomyelinases (SMases). TNF and IL-1 have been reported to activate both neutral and acid sphingomyelinases (45, 74, 75), but only the gene for the latter has been cloned in eukaryotes. Interestingly, mice lacking the ASMase gene are less sensitive to a normally lethal dose of LPS, although LPS-induced TNF expression in vivo was reportedly unaffected (76). The reduced lethal endotoxic shock in the ASMase $^{-/-}$ mice was attributed to the failure of TNF to provoke an elevation of ceramide and subsequent apoptosis of endothelial cells. Tissue ceramide levels at earlier times (<1.5 h) were not reported, leaving open the question of the role of ASMase in the initial LPS-induced ceramide increase we identify here.

We found responses to C$_2$-ceramide observed in LPS-responsive macrophages were intact in a C3H/HeJ-derived macrophage cell line (Fig. 5), demonstrating that the wild type Lps...
allele is not required for JNK activation, growth arrest, or cell death elicited by ceramide. This finding contrasts with results obtained in thioglycollate-elicited peritoneal macrophages derived from Lps<sup>d</sup> C3H/HeJ mice (32, 40). One possible explanation for these differing results is that thioglycollate treatment may not elicit identical populations of peritoneal macrophages in Lps<sup>a</sup> and Lps<sup>d</sup> mice because of their different abilities to respond to LPS, a common contaminant of thioglycollate (77). If Lps<sup>a</sup> peritoneal macrophages require prior LPS exposure for ceramide-induced cytokine mRNA expression, then the unresponsiveness of elicited Lps<sup>d</sup> cells to C<sub>2</sub>-ceramide may be explained by their inability to respond normally to LPS, rather than an intrinsic defect in ceramide responses. Alternatively, the Lps<sup>d</sup> mutation could alter some responses to ceramide analogs (i.e. LPS-induced transcription and intracellular trafficking (32, 40)) but not those we observed, namely JNK activation, growth arrest, and apoptosis.

The observation of an LPS-induced increase in ceramide in Lps<sup>a</sup> macrophages was surprising, as our results with C<sub>2</sub>-ceramide suggested the Lps<sup>d</sup> defect did not abrogate ceramide-dependent responses, yet most responses to LPS are profoundly defective in these cells. Taken together, these results suggest that the Lps<sup>d</sup> mutation affects signals other than those which are ceramide-dependent and implies that in the case of LPS stimulation other signals are required to fully activate JNK and induce growth arrest, although ceramide may contribute to these responses.

In addition, the LPS-induced increase in ceramide may participate in LPS-induced events that are normal in Lps<sup>a</sup> macrophages. Specifically, the binding and internalization of LPS via CD14 targets of ceramide remains obscure. In addition, our increase in cellular ceramide, although the identity of downregulated genes is normal in Lps<sup>a</sup> macrophages (41), and the LPS-induced transcription and intracellular trafficking of vesicles at the plasma membrane (78), and in conjunction with phospholipase C treatment, SMase has been shown to promote membrane fusion of vesicles in vitro (79). Moreover, sphingolipid depletion of Caco-2 cells by fumonisin B<sub>1</sub> treatment reportedly explains by their inability to respond normally to LPS, rather than an intrinsic defect in ceramide responses. Alternatively, the Lps<sup>d</sup> mutation could alter some responses to ceramide analogs (i.e. LPS-induced transcription and intracellular trafficking (32, 40)) but not those we observed, namely JNK activation, growth arrest, and apoptosis.

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Role of Ceramide in Lipopolysaccharide-induced Signaling

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