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Ceramide Inhibits Lipopolysaccharide-Mediated Nitric Oxide Synthase and Cyclooxygenase-2 Induction in Macrophages: Effects on Protein Kinases and Transcription Factors

Ya-Wen Hsu,* Kwan-Hwa Chi,† Wan-Chen Huang,* and Wan-Wan Lin‡

The goal of this study was to elucidate whether triggering the sphingomyelin pathway modulates LPS-initiated responses. For this purpose we investigated the effects of N-acetylphosphatidysine (C2-ceramide) on LPS-induced production of NO and PGE2 in murine RAW 264.7 macrophages and explored the signaling pathways involved. We found that within a range of 10–50 μM, C2-ceramide inhibited LPS-elicited NO synthase and cyclooxygenase-2 induction accompanied by a reduction in NO and PGE2 formation. By contrast, a structural analog of C2-ceramide that does not elicit functional activity, C2-dihydroceramide, did not affect the LPS response. The nuclear translocation and DNA binding study revealed that ceramide can inhibit LPS-induced NF-κB and AP-1 activation. The immunocomplex kinase assay indicated that IκB kinase activity stimulated by LPS was inhibited by ceramide, which concomitantly reduced the IκB degradation caused by LPS within 1–6 h. In concert with the decreased cytosolic p65 protein level, LPS treatment resulted in rapid nuclear accumulation of NF-κB subunit p65 and its association with the cAMP-responsive element binding protein. Ceramide coaddition inhibited all the LPS responses. In addition, LPS-induced PKC and p38 mitogen-activated protein kinase activation were overcome by ceramide. In conclusion, we suggest that ceramide inhibition of LPS-mediated induction of inducible NO synthase and cyclooxygenase-2 is due to reduction of the activation of NF-κB and AP-1, which might result from ceramide’s inhibition of LPS-stimulated IκB kinase, p38 mitogen-activated protein kinase, and protein kinase C. The Journal of Immunology, 2001, 166: 5388–5397.

Ceramide is an intracellular second messenger that can be generated by sphingomyelin membrane cleavage using either acid or neutral sphingomyelinases (1, 2). Increases in intracellular ceramide have been reported in many cell types in response to a variety of stimuli, including inflammatory cytokines TNF-α, IL-1, and IFN-γ, as well as apoptosis-inducing stimuli, such as UV light, anti-CD95, anti-CD28, ionizing radiation, glucocorticoid, anti-cancer drugs, and serum deprivation (3–6). Accumulating evidence has linked ceramide to cell growth, differentiation, apoptosis, inflammation, immune responses, and many cellular signals in regulating gene transcription (4). Viewing the crucial roles of ceramide in cell responses, membrane-permeable ceramide analogs have often been used to investigate the function of cellular ceramide.

Endotoxin LPS potently stimulates macrophages to up-regulate genes whose products can enhance the ability of macrophages to invade tissue, destroy bacteria, attract other immune system cells, and coordinate their responses. Macrophages exposed to LPS undergo coinduction of inducible NO synthase (iNOS)3 and cyclooxygenase-2 (COX-2) gene expression, leading to the formation of two multifunctional inflammatory mediators, NO and PGE2 (7–9). Evidence has indicated that secondary to the stimulation by LPS transcription factors, NF-κB and AP-1 are critical and act in a coordinated manner for the induced expression of iNOS and COX-2 (8, 10–12). One of the earliest signaling events following LPS treatment in macrophages is tyrosine phosphorylation and activation of some protein kinases, such as protein kinase C (PKC), extracellular signal-regulated kinase (ERK), p38 mitogen-activated protein kinase (MAPK), and c-Jun N-terminal kinase (JNK) (12, 13). Recently much progress has been made with respect to the protein kinases required in the upstream signaling for inflammatory gene expression. Activation of different kinases ultimately results in either direct or indirect phosphorylation and activation of various transcription factors. In this respect it has been documented that PKC, ERK, p38 MAPK, and JNK are upstream signaling kinases for the induction of AP-1 transcription (14–17), while their roles in NF-κB transcription are still uncertain.

The most common transcriptionally competent form of NF-κB is a heterodimer, primarily composed of a 50-kDa DNA-binding subunit (p50) and a 65-kDa trans-activator (p65 or Rel-A), that is sequestered within the cytosol by association with the NF-κB inhibitors known as IκBαs (18–20). Both the p50 and p65 monomers contain Rel regions, ~300 aa in length, that bind to DNA, interact with one another, and bind the IκB inhibitors (20). In nonstimulated cells, NF-κB proteins are retained in the cytoplasm, because IκBαs mask their nuclear localization sequence. Upon exposure to proinflammatory stimuli, phosphorylation targets IκB for protein ubiquitination and subsequent degradation through a proteasome-dependent pathway, and results in dissociation of NF-κB into the nucleus (21, 22). This process leads to increased levels of NF-κB at specific DNA enhancer sequences (κB binding sites) in the nucleus, resulting in the activation of target gene transcription. A

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3 Abbreviations used in this paper: iNOS, inducible NO synthase; COX-2, cyclooxygenase-2; MAPK, mitogen-activated protein kinase; IKK, IκB kinase; PKC, protein kinase C; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; CBP, CAMP-responsive element-binding protein; IP, immunoprecipitation.

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multisubunit protein kinase complex, IκB kinase (IKK), has recently been shown to phosphorylate IκBα and IκBβ at the sites that mediate their ubiquitination and degradation (21, 23, 24). Moreover, cotreatment of p65 with the coactivator protein cAMP-responsive element binding protein (CBP)/p300 can enhance its trans-activation potential (25–27). Although upstream protein kinases regulating this event have yet to be conclusively identified, recent evidence observed in vivo suggests that p38 MAPK and ERK may play roles in the enhanced trans-activation of p65 in response to stimulation by IL-1 and TNF-α (28, 29).

In macrophage cell lines, ceramide analogs have also been shown to partially mimic LPS-induced cytokine production (30, 31). Notably, because macropages induce ceramide formation in response to a variety of environmental stimuli (13, 32), the effects of ceramide on LPS-induced gene regulation and its upstream signaling cascades have raised much interest.

Materials and Methods

Reagents

Phenol-extracted LPS (L8274) from E. coli was obtained from Sigma (St. Louis, MO), and the protein content measured by Bradford protein assay was 0.07% (w/v). Oligonucleotides were synthesized on a PS 250 CRUAChEM DNA synthesizer (Glasgow, U.K.), using the cyanoethyl phosphoramidite method, and purified using gel filtration. The sequences of the double-stranded oligonucleotides used to detect the DNA-binding activities of NF-κB and AP-1 are as follows (the binding site is underlined): NF-κB, 5′-GATCGAGTCATGAGGGAACTCCAGGCCGGAG-3′; and AP-1, 5′-GATCCGCTTGATGACTCAGCCGGAA-3′; DMEM, FBS, penicillin, and streptomycin were obtained from Life Technologies (Grand Island, NY). Rabbit polyclonal Abs against active (phosphorylated) ERK1/2, JNK1/2, and p38 MAPK were purchased from New England Biolabs (Beverly, MA). HRP-coupled anti-mouse and anti-rabbit Abs and 

Materials and Methods

Preparation of nuclear extracts and EMSAs

Nuclear extracts and EMSAs were prepared according to the method described by Chen et al. (8). Briefly, nuclear extracts from stimulated or nonstimulated macrophages were prepared by cell lysis followed by nuclear lysis. Cells were then suspended in 30 μl of buffer (10 mM HEPES (pH 7.9), 1.5 mM MgCl2, 10 mM KCl, 0.5 mM DTT, and 0.2 mM PMSF), vigorously vortexed for 15 s, left standing at 4°C for 10 min, and centrifuged at 2,000 rpm for 2 min. The pelleted nuclei were resuspended in buffer (20 mM HEPES (pH 7.9), 50 mM NaF, 4 mM MgCl2, 1.5 mM PMSF, 0.2 mM EDTA, 0.5 mM DTT, and 0.2 mM PMSF) for 20 min on ice, then the lysates were centrifuged at 15,000 rpm for 2 min. The supernatants containing the solubilized nuclear proteins were stored at −70°C until used for the EMSA. In EMSA, binding reaction mixtures (15 μl) contained 0.25 μg of poly(dI-dC) (Amersham Pharmacia Biotech) and 20,000 cpm of 32P-labeled DNA probe in binding buffer consisting of 10 μl of 25 mM HEPES (pH 7.5), 1 mM MgCl2, 0.5% Ficoll, 1 mM EDTA, and 75 mM KCl. The binding reaction was started by the addition of cell extracts (10 μg) and was continued for 30 min at room temperature. The DNA-protein complex was resolved from free oligonucleotide by electrophoresis in a 5% polyacrylamide gel. The gels were dried and exposed to x-ray films.

IP and kinase assay

RAW 264.7 cells were cultured on 60-mm dishes. After various stimulation time, cells were washed twice in ice-cold PBS, lysed in 1 ml of lysis buffer containing 20 mM Tris (pH 7.5), 1 mM MgCl2, 125 mM NaCl, 1% Triton X-100, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 25 mM β-glycerophosphate, 50 mM NaF, and 100 μM sodium orthovanadate and centrifuged. To assess PKC isoforms in complex with membrane fraction, cells were lysed with buffer A containing 20 mM Tris-HCl, 0.5 mM EGTA, 2 mM EDTA, 2 mM DTT, 0.5 mM PMSF, and 10 μg/ml leupeptin, pH 7.5, then sonicated and centrifuged. The supernatants and pellets, respectively, represent the cytosolic and membrane fractions. To assess the cellular localization of CBP-p65, cytosol and nuclear extracts were prepared as described below. Samples of equal amounts of protein (50–100 μg) were subjected to SDS-PAGE on 8–12% polyacrylamide gels, then transferred onto a nitrocellulose membrane, which was then incubated in buffer (150 mM NaCl, 20 mM Tris, and 0.02% Tween, pH 7.4) containing 1% nonfat milk, and the protein band was visualized by immunoblotting with specific Abs. Immunoreactivity was detected by ECL following the manufacturer’s instructions.

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Preparation of nuclear extracts and EMSAs

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IP and kinase assay

RAW 264.7 cells were cultured on 60-mm dishes. After various stimulation time, cells were washed twice in ice-cold PBS, lysed in 1 ml of lysis buffer containing 20 mM Tris (pH 7.5), 1 mM MgCl2, 125 mM NaCl, 1% Triton X-100, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 25 mM β-glycerophosphate, 50 mM NaF, and 100 μM sodium orthovanadate. After centrifugation, the supernatant was collected, then anti-IKK or anti-JNK Ab (2 μg) was added with protein A/G-agarose beads (20 μl; Santa Cruz Biotechnology) at 4°C overnight. The precipitates were washed three times with lysis buffer and twice with kinase buffer (25 mM HEPES (pH 7.5), 20 mM MgCl2, 100 μM sodium orthovanadate, and 2 mM DTT). The kinase reactions for the NKJ and IKK complexes were performed by incubating immunoprecipitated proteins in kinase mixture (25 mM HEPES (pH 7.5), 20 mM MgCl2, 100 μM sodium orthovanadate, 2 mM DTT, 10 μM ATP, and 5 μCi of [γ-32P]ATP) respectively containing 1 μg of GST-CB and GST-IκBα as substrates at 30°C for 30 min. Laemmli’s loading buffer was added to stop the reaction, and samples were resolved on SDS-PAGE followed by autoradiography.

IP and Western analysis

To determine the association between CBP and p65, total cell lysates prepared from lysis buffer as previous indicated were incubated with CBP Ab and 20 μl of protein A/G beads for 16 h at 4°C. Complex of proteins, p65, and CBP with beads were washed three times with lysis buffer before addition of SDS loading buffer. Samples were resolved on SDS-PAGE, Immunoreactivities of p65 and CBP were detected by ECL following the manufacturer’s instructions.
Immunocytochemistry
Murine RAW 264.7 cells were cultured in 12-mm coverslips. After stimulation, cells were washed with cold PBS twice and added 10% formaldehyde in PBS to fix the cells. Five minutes later, 1% Triton X-100 was added to the cells followed by 5% nonfat milk for 15 min. To detect the translocation of NF-κB p65, the primary p65 rabbit Ab diluted 1/100 in PBS was added to the cells and incubated for 1 h. Then cells were washed with PBS twice, and fluorescein-conjugated goat IgG fraction was added to rabbit IgG Ab diluted 1/100 in PBS. After 30 min, the cell fluorescein was detected using a Photomicrography Digitalized Integrate System (MGDs, Taipei, Taiwan).

Statistical evaluation
Values are expressed as the mean ± SEM of at least three experiments. Student’s t test was used to assess the statistical significance of the differences; p < 0.05 was considered statistically significant.

Results
C2-ceramide inhibited LPS-mediated iNOS and COX-2 induction
As in our previous reports (9, 12), stimulation of murine macrophages with 0.1 μg/ml LPS for 24 h resulted in the production of NO (from 5 ± 2 to 32 ± 4 μM) and PGE2 (from 0.4 ± 0.1 to 6.7 ± 0.3 ng/ml). C2-ceramide at concentrations up to 50 μM by itself did not affect the release of NO or PGE2 above levels seen in medium-treated macrophages. Coaddition of C2-ceramide with LPS inhibited the formation of NO and PGE2 accompanied by the induction of iNOS and COX-2 (Fig. 1, A and B). Dose-dependent NO production by LPS (0.03–0.1 μg/ml) was significantly reduced by the presence of 10 and 50 μM C2-ceramide. LPS-induced NO production was 30 ± 5% (n = 4) and 69 ± 10% (n = 6) inhibited, and PGE2 production was 28 ± 7% (n = 5) and 54 ± 7% (n = 5) inhibited by 10 and 50 μM C2-ceramide, respectively (Fig. 1A). To confirm the specificity of C2-ceramide, we found that the structural analog of C2-ceramide that did not elicit functional activity, C2-dihydroceramide, did not affect the LPS response at 50 μM (Fig. 1, A and C). To clarify that the inhibitory action of C2-ceramide was not due to cell toxicity, we examined cell viability. We found that C2-ceramide at 50 μM had no significant effect on cell viability with a 24-h incubation as evidenced from MTT, crystal violet, and lactate dehydrogenase release assays (data not shown). Even using the more sensitive annexin V and propidium iodide staining to assess cell viability, no cell toxicity of 50 μM C2-ceramide was found in RAW 264.7 macrophages after a 24-h incubation (data not shown).

In addition to determining the action of C2-ceramide in the murine RAW 264.7 macrophage cell line, we further explored its effect on peritoneal macrophages. In this cell type, as we and others have previously reported (33, 34), LPS itself cannot efficiently induce NO production unless cells have been primed. Here we found that unprimed peritoneal macrophages indeed were less sensitive to LPS, which could not induce NO production until its concentration had increased to 3 μg/ml. However, LPS-induced NO release was markedly potentiated by coinoculation with 30 nM thapsigargin (Fig. 1C), which acts as a macrophage priming signal through increasing the intracellular calcium and by itself does not change the basal NO level. With regard to C2-ceramide action, we intriguingly found that in the absence of thapsigargin priming, C2-ceramide (50 μM) had no significant effect on LPS-induced NO production, whereas 56 ± 7% (n = 3) inhibition was observed in priming cells. Furthermore, C2-ceramide-specific inhibition in priming cells was not mimicked by the same concentration of C2-dihydroceramide. Following 50 μM C2-ceramide and 3 μg/ml LPS incubation for 24 h, the cell viability did not change, as assayed by MTT and lactate dehydrogenase release (data not shown). To explore the mechanism responsible for the inhibitory action of C2-

C2-ceramide inhibited LPS-induced NF-κB and AP-1 activation
To determine whether decreases in iNOS and COX-2 levels were due to inhibition of the essential nuclear translocation of the transcription factors NF-κB and AP-1 (8, 35) and, in turn, their DNA
tracts were treated in vitro with 50 μg/ml extracts were analyzed by EMSA. In a time-dependent manner (Fig. 2B), ceramide was directly added to a nuclear extract prepared with oligonucleotides. The results are representative of three different experiments. NS, Nonspecific binding.

Effects of C2-ceramide on DNA binding by NF-κB and AP-1. Nuclear extracts from cell lysates were assayed for binding activity with specific oligonucleotides containing respective binding sequences for NF-κB and AP-1. A, RAW 264.7 cells were treated with 0.1 μg/ml LPS and/or 50 μM C2-ceramide for 1–3 h. Equivalent amounts of total cellular extracts were analyzed by EMSA. B, LPS (0.1 μg/ml)-treated nuclear extracts were treated in vitro with 50 μM C2-ceramide for 20 min before binding with oligonucleotides. The results are representative of three different experiments. NS, Nonspecific binding.

Ceramide inhibited LPS-induced p65 nuclear translocation

Following inhibitor IκBα degradation, free NF-κB can easily gain access into the nucleus and elicit its trans-activation ability. Next we asked whether C2-ceramide affected nuclear translocation of NF-κB subunit p65. Immunoblot analysis revealed that C2-ceramide abrogated LPS-stimulated p65 nuclear accumulation within 1–6 h, but could not affect this response before 1 h. In parallel to the rapid movement into the nuclear compartment in response to LPS, the initial decrease in cytosolic p65 levels was already detectable after a 10-min incubation. Consistently, C2-ceramide could recover the sustained decrease in cytosolic p65 after LPS incubation for 1–6 h (Fig. 3A).

To confirm the above responses, we performed a further immunocytochemical examination of localized p65 changes. Our results, shown in Fig. 4B, reveal that in the resting state the p65 signal was primarily limited to the cytosol (a), and that ceramide itself did not affect the subcellular distribution of p65 (b–d). Upon LPS stimulation for as long as 6 h, p65 was localized in the nuclei as well as in the cytosol (e–g). In the copresence of C2-ceramide (50 μM), cells exhibited lower p65 nuclear immunointensity (h–j) compared with the LPS-stimulated control (e–g).

Ceramide reduced the interaction of CBP with p65

NF-κB transcriptional competence requires interaction with the transcription cofactor CBP. To assess the physical interactions of p65 with CBP, cells were subjected to coimmunoprecipitation analysis with anti-CBP Ab, followed by Western blot analysis with anti-p65 Ab. Our results indicate that after LPS stimulation, p65 was able to trap CBP from the total cell lysate, suggesting a strong recruitment of cofactor CBP to the NF-κB transcription factor. Upon C2-ceramide (50 μM) addition, LPS-stimulated p65/CBP interactions at 1, 2, 3, and 6 h were attenuated (Fig. 5).

Ceramide inhibited LPS-induced p38 MAPK, but not ERK, activation

Ceramide has been shown to elicit JNK activation in several cell types (13, 35, 36). Based on some reports indicating the involvement of JNK activity in TNF-α (37), iNOS (38), and COX-2 (39) induction, we investigated this possible mechanism. We found that, in contrast to stimulation by anisomycin (0.03 or 0.1 μM), vinblastine (1 μg/ml), UV irradiation (180 J/m2), or taxol (0.3 μM), ceramide at 50 μM could not stimulate JNK within 30 min as assessed by the IP kinase assay (Fig. 6A) as well as by immunoblotting with phosphorylated JNK Ab (Fig. 6B). In contrast, LPS at 0.1 μg/ml only slightly induced transient JNK phosphorylation at 15 min, and this action was unaffected by C2-ceramide (data not shown).
shown). These results rule out the involvement of JNK in LPS-induced or ceramide-regulated iNOS and COX-2 expression in RAW 264.7 cells.

Two other MAPK members, ERK and p38 MAPK, have been shown to mediate many LPS-evoked macrophage responses, including NO and PGE2 production (8, 40, 41). In this study we examined whether LPS-induced kinase activation, as assessed by the amount of phosphorylation, was affected by C2-ceramide. As shown in Fig. 7, LPS and C2-ceramide stimulated p42/p44 ERK and p38 MAPK phosphorylation. LPS and C2-ceramide acted in an additive manner in ERK phosphorylation. On the contrary, their actions on p38 MAPK phosphorylation were reciprocally inhibited.

**Effects of ceramide on LPS-stimulated PKC isoforms**

Because PKC is known as a key kinase for transducing iNOS and COX-2 gene expressions (8, 42), we explored the possible action of C2-ceramide in this respect. As shown in Fig. 8, PKCβ, -δ, -λ, and -ζ, which initially existed more abundantly in the cytosol than in the membrane fraction, showed marked translocation from cytosol to the membrane fraction within 15–60 min of treatment with LPS. C2-ceramide (50 μM) alone also caused the membrane translocation of PKCβ, -λ, and -ζ, with more obvious stimulation seen for PKCβ and -ζ than for PKCα. Although the membrane immunoreactivity of PKCε was barely detected before or after stimulation, decreased levels in the cytosol fraction were observed after LPS treatment. On the contrary, C2-ceramide caused increases in cytosol levels of PKCδ and -ε, both of which increased at 15 min, and PKCζ decreased to near the control levels after 30 min, while PKCε maintained its cytosolic localization for at least 60 min. Interestingly, we found that LPS-stimulated subcellular distribution of all PKC isoforms except PKCζ was diminished by the coaddition of C2-ceramide.

**Discussion**

Ceramide is an important lipid second messenger that can activate a wide variety of cellular responses. To date it has been shown that ceramide is produced by the action of sphingomyelinase, which is triggered by various cytokines, Fas ligand, oxidative stress, and ionizing radiation (1). Bacterial LPS is one of the stimuli known to cause ceramide formation from murine macrophages, despite this effect being transient and weak (13, 32). Although it was once thought that some LPS-triggered signaling events were possibly mediated by ceramide in either macrophages (32) or neutrophils (43), recent comparison studies exploring their cytotoxic effects, cellular signaling, and cytokine-producing ability concluded that, at least in macrophages, LPS and ceramide use divergent signaling pathways, and that a limited role is played by ceramide in LPS signaling (33, 44). Despite not being an intermediate mediator in the signal transduction pathway, ceramide has been shown to directly activate some cellular events, such as apoptosis, cell cycle arrest, and transcription factor activation (45). Therefore, the role of ceramide in LPS signaling pathways remains to be explored further.
LPS signaling, and because it can be efficiently produced by macrophages exposed to some inflammatory cytokines (e.g., TNF-α and Fas ligand) or various stress insults (e.g., irradiation, chemotherapy, and hypoxia/reperfusion), ceramide might influence LPS-elicited signaling and relevant cellular responses when a bacteria-infected host is subjected to inflammation, chemotherapy, or other pathological conditions. To address this issue, we herein examined the effects of the cell-permeable ceramide analog, C₂-ceramide, on LPS-initiated cell signaling and responses in the murine RAW 264.7 macrophage cell line. In this study we not only observed the inhibitory effects of C₂-ceramide on LPS-initiated NO and PGE₂ production, but also investigated its action on the crucial signaling molecules that are required for iNOS and COX-2 expression. Unlike C₂-ceramide, the inactive analog C₂-dihydroceramide did not alter LPS responses at the same concentration. This suggests the specific action of C₂-ceramide as a lipid regulator.

NF-κB is a prerequisite and ubiquitous transcription factor for the expression of many inflammation-related genes, including iNOS, COX-2, TNF-α, IL-6, and GM-CSF. Although ceramide has been reported to activate NF-κB in some cell types (45, 46), this point is still controversial (13, 47, 48). In this study we found that C₂-ceramide could not significantly activate NF-κB in RAW 264.7 cells. On the contrary, inhibition of LPS-induced NF-κB activation by ceramide was observed when ceramide was present in RAW 264.7 cell cultures simultaneously with LPS.

It is well established that the nuclear accumulation of NF-κB relies in large part upon IKK-dependent phosphorylation and subsequent degradation of the cytosolic inhibitor, IκB. In this context, LPS stimulation in RAW 264.7 cells led to rapid IKK activation, IκB degradation, and p65 nuclear translocation. All these sequential upstream signals responsible for NF-κB activation lasted for up to 6 h after the addition of LPS. Moreover, our results show that these sustained events maintained within 1–6 h of LPS incubation were correspondingly reduced by the presence of C₂-ceramide. Following these inhibitory actions of C₂-ceramide, EMSA results consistently showed the ability of C₂-ceramide to inhibit LPS-induced nuclear translocation and DNA binding of NF-κB. This delayed inhibitory action of C₂-ceramide explains the paradoxical results of MacKichn and DeFranco, who reported that C₂-ceramide failed to affect IκB degradation induced by LPS at 20 min (13). For C₂-ceramide action alone, weak IKK stimulation, but not IκB degradation or p65 translocation, was observed. We suggest that the much less pronounced and transient IKK activation compared with LPS can explain the noneffectiveness of C₂-ceramide on IκB degradation and NF-κB activation. A further suggestion drawn from these results is that C₂-ceramide-targeted molecules, either through direct or indirect action, are located in the upstream signaling pathways involved in the sequential activation of IL-1R-associated kinase, TNF receptor-associated factor-6, NF-κB-inducing kinase, and IKK after LPS binding to CD14/toll-like receptors (49, 50). Another intriguing finding is that

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**FIGURE 4.** C₂-ceramide inhibition of the nuclear translocation of NF-κB p65. A, Both cytosolic and nuclear cell lysates were prepared from RAW 264.7 cells treated with 0.1 µg/ml LPS in the absence or the presence of 50 µM C₂-ceramide for the indicated times. The immunoreactivity of NF-κB subunit p65 was determined. B, The nuclear translocation of p65 caused by LPS was shown by cytochemistry and was blocked by C₂-ceramide. The results are representative of three different experiments.
C2-ceramide treatment can transiently reduce IKK activity at 10 min of LPS stimulation. Although the underlying mechanism contributing to this rapid effect is presently unknown, the instantly recovered IKK activation might be the reason why C2-ceramide in RAW 264.7 cells did not appreciably alter IκB degradation or p65 nuclear translocation respectively induced by LPS at 20 and 30 min.

In addition to NF-κB-inducing kinase/IKK signaling transduction, LPS has been reported to stimulate many serine/threonine protein kinases, such as PKC, ERK, p38 MAPK, and JNK in macrophages. The physiological relevance of these kinase signals to macrophage functions has been proven by their crucial roles in iNOS and COX-2 induction (8, 38–40, 51). Recent studies further suggested that ERK (28), p38 MAPK (28, 52), and PKC (53, 54) were responsible for the enhanced trans-activation of NF-κB via an IκB degradation-independent mechanism. In this study we found that the stimulating effects of LPS on p38 MAPK and PKC, but not on ERK and JNK, were also attenuated by C2-ceramide. Thus, we suggest that C2-ceramide inhibition of the LPS response is primarily due to the interruption of LPS signals on IKK, PKC, and p38 MAPK pathways, which play crucial roles in NF-κB- and AP-1-dependent gene expression. Our results do not address the inhibitory mechanism of C2-ceramide on LPS-activated PKC, p38 MAPK, and IKK. Whether any possible intermediate signal transducers are involved in coordinating the signaling network between ceramide and LPS needs further determination.

Relatively little is known about the way in which ceramide acts upon the signaling pathways. Although protein kinases and protein phosphatase are two principal targets in ceramide signaling pathway (55), the effects of ceramide on MAPKs and the mechanism involved are still unclear. The more well-established pathway is that ceramide-activated protein kinase can activate the Raf-MEK-ERK MAPK cascade (56). Consistent with this scenario, we detected increased ERK phosphorylation in C2-ceramide-treated RAW 264.7 cells. Similar findings were reported by Medvedev et al. (35) and Monick et al. (32) in mouse peritoneal and human alveolar macrophages, respectively, but not by MacKichan and DeFranco in the same macrophage cell line that we used (13). We currently can provide no evidence to explain this latter inconsistency.

With respect to the actions of ceramide on the other two MAPKs, our results were supported by those of other studies with macrophages. The ceramide-induced p38 MAPK activation shown in this study is consistent with that observed in peritoneal macrophages (35). The crucial kinase involved in the ceramide-induced apoptosis, JNK (36), was not triggered by 50 μM C2-ceramide during the period examined (i.e., 30 min). Instead, with increasing C2-ceramide concentration to 100 μM, weak JNK activation was detected (data not shown), and these results coincide with previous findings by MacKichan and DeFranco in RAW 264.7 cells (13). In addition, this JNK effect of ceramide might contribute to the weak

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FIGURE 5. Effect of C2-ceramide on the association of p65 with CBP. Cell lysates were made from RAW 264.7 cells after treatment with LPS (0.1 μg/ml) in the presence or the absence of C2-ceramide (50 μM) for the times indicated. CBP was immunoprecipitated using anti-CBP Ab. The washed immunoprecipitates were equally divided into two parts, separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with either anti-NF-κB p65 or anti-CBP Ab. The results are representative of three different experiments.

FIGURE 6. JNK is not activated by ceramide (50 μM) in RAW 264.7 cells. A, GST-c-Jun (1 μg) was phosphorylated by JNK, which was immunoprecipitated from whole-cell lysates of RAW 264.7 macrophages treated with C2-ceramide (10 or 50 μM) or anisomycin (0.03 or 0.1 μM) for 30 min. After drug (0.03 μM anisomycin, 1 μg/ml vinblastine, 0.3 μM taxols, and 50 μM C2-ceramide) treatment or UV irradiation (180 J/m²) for 30 min, levels of phosphorylated JNK were detected by phosphorylation-specific JNK Ab. The results are representative of three different experiments.

FIGURE 7. Effects of LPS and C2-ceramide on the phosphorylation of ERK and p38 MAPK. After RAW 264.7 cells were treated with 0.1 μg/ml LPS and/or 50 μM C2-ceramide for different periods, total cell lysates were subjected to SDS-PAGE, followed by immunoblotting with phosphorylation-specific ERK or p38 MAPK Ab. The results are representative of three different experiments.
cell apoptosis (~30% reduction in MTT assay) caused by 100 μM ceramide treatment for 24 h in RAW 264.7 macrophages. Besides MAPK, ceramide has been demonstrated to inactivate certain members of the PKC family (57, 58), while activating others (5, 54, 59). Consistent with a previous report studying human leukemia cell lines (5), C2-ceramide increased the cytosolic level of PKCd transiently and that of PKCe in a more sustained manner in RAW 264.7 cells. Also supporting previous data indicating ceramide to be an activator of PKCζ (54 59), we observed the membrane translocation of PKCζ after C2-ceramide addition. Interestingly, four of the five PKC isoforms activated by LPS in RAW 264.7 cells (β, δ, λ, and ε, but not ζ) were overcome by ceramide treatment. Certainly, the details and any intermediate steps that contribute to the cross-talk between ceramide and LPS signaling remain to be elucidated.

CBP was originally discovered based on its ability to interact with the cAMP-responsive element binding protein and has been demonstrated to interact with many proteins in a cell signal-regulated manner. It has been shown that phosphorylation of p65 promotes the interaction with CBP and results in the enhanced trans-activation potential of NF-κB based on at least three aspects (25, 26, 60). First, CBP acts as a bridging factor between NF-κB and DNA binding sites. Second, CBP with intrinsic enzyme activity can acetylate histone, which allows the unwinding or loosening of chromatin. Third, CBP can acetylate p65 itself. Thus, it is apparent that CBP acetyltransferase activity is required for the stimulation of NF-κB. To date, upstream kinases, at least protein kinase A, p38 MAPK, and ERK, have been identified as regulating this event (25, 29, 61). In this study we observed the inhibitory effect of C2-ceramide on LPS-induced p65 interaction with CBP and thus suggest that the attenuation of p38 MAPK activity by ceramide can account for this event.

To understand whether the inhibitory action of C2-ceramide is common to different types of macrophages, we performed an NO assay in peritoneal macrophages isolated from BALB/c mice. Intriguingly we found that C2-ceramide failed to alter NO production caused by LPS itself, while abolishing the priming actions of thapsigargin, which transcriptionally up-regulates iNOS gene expression and efficiently enhances NO production in BALB/c macrophages with low sensitivity to LPS (33, 34, 62). These results suggest that C2-ceramide inhibition of LPS-dependent signaling cascades leading to iNOS gene induction is dependent on the types of macrophages. Actually it is known that the iNOS gene is regulated by several coordinated transcription factors (63, 64) and is distinctly induced in different cell types. For example, priming factors, such as IFN-γ, IL-1, TNF-α, and Ca2+, are required for the maximal induction of iNOS expression in peritoneal macrophages (33, 34, 65) and C6-glioma cells (66). Viewing this regulatory complexity, it is our future work to further investigate the action of ceramide on LPS together with cytokine-induced NO formation in other cell types, where the transmission of early signals into the nucleus leading to iNOS expression might be varied. Moreover, the noneffectiveness of ceramide on the LPS response in this study might support the observation by Medvedev et al. that C2-ceramide was unable to change LPS-induced NF-κB activation in peritoneal macrophages from C3H/OuJ mice (35).

In conclusion, this study has identified the mechanisms through which ceramide alters inducible iNOS and COX-2 gene expression in response to LPS. Ceramide inhibition of LPS-mediated IKK, p38 MAPK, PKC, NF-κB, and AP-1 activation as well as the inhibition of p65/CBP interaction may underlie the inhibitory nature of ceramide in macrophages. All these results indicate that...
ceramide functions in a negative regulatory mechanism to attenuate responses to LPS.

References


