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Ceramide Inhibits IL-2 Production by Preventing Protein Kinase C-Dependent NF-κB Activation: Possible Role in Protein Kinase Cθ Regulation

Nour Abboushi,* Aimee El-Hed,* Wissal El-Assaad,† Lina Kozhaya,* Marwan E. El-Sabban,‡ Ali Bazarbachi,* Rami Badreddine,* Alicja Bielawska,§ Julnar Usta,* and Ghassan S. Dbaibo3*†

The role of the sphingolipid ceramide in modulating the immune response has been controversial, in part because of conflicting data regarding its ability to regulate the transcription factor NF-κB. To help clarify this role, we investigated the effects of ceramide on IL-2, a central NF-κB target. We found that ceramide inhibited protein kinase C (PKC)-mediated activation of NF-κB. Ceramide was found to significantly reduce the kinase activity of PKCθ as well as PKCα, the critical PKC isoforms involved in TCR-induced NF-κB activation. This was followed by strong inhibition of IL-2 production in both Jurkat T leukemia and primary T cells. Exogenous sphingomyelinase, which generates ceramide at the cell membrane, also inhibited IL-2 production. As expected, the repression of NF-κB activation by ceramide led to the reduction of transcription of the IL-2 gene in a dose-dependent manner. Inhibition of IL-2 production by ceramide was partially overcome when NF-κB nuclear translocation was reconstituted with activation of a PKC-independent pathway by TNF-α or when PKCθ was overexpressed. Importantly, neither the conversion of ceramide to complex glycosphingolipids, which are known to have immunosuppressive effects, nor its hydrolysis to sphingosine, a known inhibitor of PKC, was necessary for its inhibitory activity. These results indicate that ceramide plays a negative regulatory role in the activation of NF-κB and its targets as a result of inhibition of PKC. The Journal of Immunology, 2004, 173: 3193–3200.

The sphingolipid ceramide, the backbone of complex sphingolipids, has emerged as a regulator of the stress response by virtue of its ability to induce growth suppression, induction of cell differentiation, cell cycle arrest, apoptosis, and other biologic functions (1). Previous studies had established that the more complex glycosphingolipids derived from tumors or parasites exhibited immunosuppressive activity (2–6). A role for ceramide in the immune response. Its role of ceramide in the immune response. Among the targets of NF-κB, IL-2 is a central regulator of the immune response that is produced by T cells upon antigenic or mitogenic stimulation. Inhibition of IL-2 production achieves significant, clinically useful, immunosuppression. IL-2 is regulated mostly at the level of transcription of its gene (16). Interaction of the TCR/CD3 complex with its specific Ag/MHC together with accessory signals provided by the APC initiate several signaling pathways that result in the induction of the IL-2 gene. These include the activation of protein kinase C (PKC) and mobilization of intracellular calcium. The downstream events that lead to the expression of IL-2 include the activation of a number of specific transcription factors, including NF-κB, NFAT, Oct-1, AP-1, Ets, and a CD28 responsive factor (17). These factors cooperate to form a multifactor complex that binds the enhancer region in a stable manner and initiates transcription. Inhibition of one of these factors is sufficient to markedly depress IL-2 production. This is demonstrated by the ability of two widely used immunosuppressants, cyclosporin A and FK-506, to inhibit IL-2 production by blocking NF-AT activation.

Therefore, we decided to examine the effects of ceramide on the pathways that lead to the activation of NF-κB and its target, IL-2. The results indicate that ceramide is a potent inhibitor of IL-2 induction and that these effects appear to be mediated by the ability of ceramide to specifically inhibit PKC-mediated activation of NF-κB.

Departments of *Biochemistry, †Pediatrics, and ‡Human Morphology, American University of Beirut, Faculty of Medicine, Beirut, Lebanon; and §Department of Biochemistry and Molecular Biology, Medical University of South Carolina, Charleston, SC 29425

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2 N.A. and A.E.-H. contributed equally to this work.

3 Address correspondence and reprint requests to Dr. Ghassan Dbaibo, Department of Pediatrics, American University of Beirut, P.O. Box 1138044/B21, Beirut, Lebanon. E-mail address: gdbaibo@aub.edu.lb

4 Abbreviations used in this paper: PKC, protein kinase C; C2-ceramide, N-acetylsphingosine; C6-ceramide, N-hexanoylsphingosine; FB1, fumonisin B1; OPA, O-phthalaldehyde; PDMP, N,N,N′,N′-tetra-1-phenyl-2-decynylamino-3-morpholino-1-propanol; RPA, RNase protection assay.
Materials and Methods

Cell culture

Jurkat E6-1 leukemic T cells were obtained from American Type Culture Collection (Manassas, VA) and were grown in RPMI 1640 (Invitrogen Life Technologies, Paisley, U.K.) supplemented with 10% FCS (Invitrogen Life Technologies). Jurkat cells were treated at a concentration of 1.5 × 10⁶ cells/ml. PHA blasts were prepared according to standard protocols. All treatments were performed in complete RPMI 1640 medium supplemented with 10% serum, N-acetylphosphingosine (C₄- ceramide), N-hexanoylphosphingosine (C₆- ceramide), and dihydrosphingosine were prepared as described previously (18). Cell viability studies were performed using the trypan blue method. Assessment of apoptosis was by annexin V labeling (Roche, Mannheim, Germany).

Quantification of IL-2

An IL-2 ELISA kit (R&D Systems, Minneapolis, MN) was used to quantify IL-2 production by treated cells, as indicated in the manufacturer’s instructions.

Total cellular RNA extraction

Total cellular RNA from treated or control cells was extracted using the RNeasy Total RNA Mini kit (Qiagen, Hilden, Germany) as instructed by the manufacturer.

Quantification of mRNA by RNase protection assay (RPA)

The RiboQuant Multiprobe RPA (BD Pharmingen, San Diego, CA) was used to detect and quantify IL-2 transcripts. The probe set consisted of a DNA oligonucleotide. Jurkat human cytokines. The templates were used for T7 polymerase-directed synthesis of a high specific activity,[α-³²P]UTP-labeled, antisense RNA. Total RNA extracted from the cells was hybridized with the labeled probe for 16 h in a heat block at 56°C according to the standard assay protocol. The RNA probe hybrid solution was then treated with RNase to digest ssRNA. The protected probe/mRNA hybrids were run on a denaturing polyacrylamide gel and detected by autoradiography. The quantity of each mRNA species was determined by the intensity of the band of the protected probe fragment. A lane was run on the gel with undigested probes that served as markers. From these markers, a standard curve of migration distance vs log nucleotide length was plotted. This curve was used to identify the RNase-protected bands on the gel corresponding to different cytokines, including IL-2.

EMSA

Nuclear extracts and EMSA were performed as described previously (11) using NF consensus oligonucleotides (Santa Cruz Biotechnology, Santa Cruz, CA). The specific bands were determined by competition experiments using mutant oligonucleotides that did not bind to the specific transcription factor (e.g., NF-κB) in question or a 50-fold excess of unlabeled specific oligonucleotide. The specificity of the bands was also confirmed using specific Abs to the transcription factor components, e.g., anti-p50 or anti-p65 for NF-κB or anti-Fos and anti-Jun for AP-1 (Santa Cruz Biotechnology) in the incubation step, which resulted in a supershift of the specific band due to the bound Ab.

Luciferase assay

The pCEFL-PKCβ plasmid was a gift from Dr. S. Shaw (National Cancer Institute, Bethesda, MD). The following luciferase reporter plasmids were used: NF-κB (Strategene, La Jolla, CA), AP-1 and NF-AT (gifts from Dr. T. A. Chatila, Washington University, St. Louis, MO), and IL-2 (a gift from Dr. A. Veillette, McGill University, Montreal, Canada). Cells were seeded at 8 × 10⁵ cells in 0.5 ml of medium with 10% FBS and placed in a 24-well plate. Transfection with the respective plasmids was performed by adding 2 μg of plasmid DNA dissolved with Lipofectamine (Invitrogen Life Technologies) in medium to each well in a dishware fashion while gently shaking the plate. The cells were incubated for 4 h before stimulation with PMA with or without C₄-ceramide for 1.5 h. For IL-2 reporter experiments, cells were transfected by electroporation and treated similarly, except PHA was included with PMA and incubation was performed for 24 h. At the end of the incubation, the Bright Glo luciferase assay reagent (Promega, Madison, WI) was added, and luminescence was measured using a luminometer.

Ceramide measurement

Lipids were extracted from pelleted cells by the method of Bligh and Dyer (19). Ceramide levels were measured by a modified diglycerol kinase assay as described previously (20) and normalized to lipid phosphate content.

Sphingosine measurement

Lipids were extracted by chloroform/methanol (2/1, v/v), and dihydrosphingosine (1 mmol) was added to the extract as an internal standard. The aqueous and cellular layers were re-extracted with chloroform/methanol (2/1, v/v), and chloroform layers from the two rounds of extraction were combined and evaporated to dryness under N₂, then resuspended in 200 μl of chloroform and 800 μl of 0.125 M methanolic KOH and incubated for 75 min at 37°C. The chloroform layer was combined with 1.4 ml of chloroform and washed twice with 400 μl of H₂O, then dried under N₂ before O-phthalaldehyde (OPA) derivatization of sphingosine was performed as previously described (21). After incubation for 20 min at room temperature in the dark, 300 μl of methanol/5 mM sodium phosphate buffer (90/10, v/v), pH 7.0, was added to the sample, and an aliquot of 100 μl was injected into the HPLC. HPLC analyses were conducted using an HP XDB-C18 column (25 cm × 4.6 mm; Beckman Coulter, Fullerton, CA) with a 3-cm Hypersil ODS guard column. A linear gradient system, at a flow rate of 1 ml/min, was used, starting from methanol/5 mM sodium phosphate buffer, pH 7.0 (90/10 (v/v)) to 98/2 (v/v) at 30 min. Elution of the fluorescent OPA-sphingosine peaks was detected using an HP1046A fluorescent detector with an excitation wavelength of 340 nm and an emission wavelength of 455 nm. The retention times of sphingosine and dihydrosphingosine were 13 and 15 min, respectively.

PKC activity

Cells were seeded in complete RPMI 1640 medium at a concentration of 7 × 10⁶ cells/ml. The cells were preincubated with 15 μM C₆-ceramide before stimulation with 15 ng/ml PMA for 20 min. Harvested cells were washed once with 1× cold PBS and suspended in 0.5 ml of cold homogenization buffer (50 mM Tris-HCl, pH 7.5, containing 0.2% (w/v) 2-mercaptoethanol, 5 mM EDTA, 10 mM EGTA, and 50 μg/ml PMSF). Cells were lysed by sonication on ice with four rounds of 15-s duration, vortexed, and then centrifuged at 500 × g for 5 min in 4°C to remove unlysed cells and nuclei (pellet). A fraction of the supernatant was taken as the total lysate, and the rest was centrifuged at 100,000 × g for 40 min at 4°C to separate the cytoskeleton from the membranes and cytosol. PKC isozyme activities were measured by a PKC enzyme assay system purchased from Amersham Biosciences (Arlington Heights, IL) using isozyme-specific peptide substrates from Amersham Biosciences (PKCe and Calbiochem (PKCβ; La Jolla, CA) as recommended by the manufacturer. The phosphorylated peptide was separated on binding paper, and 5% (v/v) acetic acid was used to wash the papers. ³²P incorporated into the synthetic peptides was detected with a scintillation counter, and the counts were normalized to protein levels determined using the Bradford protein assay.

For experiments using immunoprecipitated PKCβ, cells were lysed in 1 ml of lysis buffer containing 20 mM HEPES (pH 7.6), 250 mM NaCl, 1 mM EDTA, 20 mM β-glycerophosphate, 1% Nonidet P-40, 20 mM p-nitrophenyl phosphate, 0.1 mM NaVO₄, 1 mM DTT, 1 mM PMSF, and 1% protease inhibitor mixture (Roche) for 1 h on ice. The lysate was centrifuged at 16,000 × g for 20 min at 4°C. A total of 2 ml of protein was then incubated with 2 μg of PKCβ Ab for 1 h at 4°C, followed by overnight incubation with 30 μl of protein G agarose. Samples were washed four times with lysis buffer and twice with kinase buffer. The beads were then incubated in kinase buffer containing [γ-³²P]ATP and substrate to assess activity.

Statistical analyses

Quantitative data are expressed as the mean ± SD. Significant differences among groups were examined by one-way ANOVA, followed by Bonferroni’s post-hoc test. A value of p < 0.05 was considered significant.

Results

Ceramide inhibits PMA-induced NF-κB activation

Jurkat cells costimulated with PMA and PHA were used to explore the effects of ceramide on IL-2 secretion. Whereas PMA functions mostly by activating several members of the PKC family, PHA provides complementary accessory signals, such as calcium mobilization. Previously, we demonstrated that exogenous cell-permeable ceramides inhibit PMA-induced NF-κB activation in these cells (22). To verify whether PHA cotreatment affected the inhibitory effects of ceramide on PMA-induced NF-κB, cells were stimulated with PMA/PHA in the presence of cell-permeable synthetic C₆-ceramide, and specific NF-κB-DNA binding after 1.5 h was
evaluated. Fig. 1A shows that there was significant dose-dependent inhibition of NF-κB by C6-ceramide. Treatment of cells with PHA alone did not activate NF-κB, whereas PMA alone potently activated it and this was strongly inhibited by C6-ceramide (Fig. 1B). These results suggested that PHA cotreatment did not modulate the inhibitory effects of ceramide on the PMA-induced pathway of NF-κB.

Specific DNA binding of NFAT, AP-1, and Ets after stimulation with PMA was also assayed by EMSA. Comparison of PMA-treated extracts in the presence or the absence of C6-ceramide showed no difference in binding of any of these transcription factors to their specific cognate sequences at 90 min (Fig. 1C). Thus, the effects of ceramide at this early time point (1.5 h) appeared to be limited to the inhibition of PMA-induced NF-κB. This was verified using luciferase reporter plasmid constructs driven by NF-κB, AP-1, or NF-AT. Transient transfection of Jurkat cells with these constructs showed that PMA treatment alone for 1.5 h activated NF-κB-driven luciferase expression, but not that driven by AP-1 or NF-AT (Fig. 1D). Cotreatment with C6-ceramide (20 μM) resulted in significant inhibition of NF-κB-driven activity.

Ceramide inhibits PKCθ and PKCα activities

Several PKC isozymes are involved in TCR-induced stimulation. PKCθ recently emerged as a critical regulator of NF-κB activation after TCR ligation, where it is rapidly recruited to the site of TCR clustering (23). Its loss abrogates TCR-induced NF-κB activation in mature T cells (24). Ceramide differentially modulates various PKC isozymes (14, 25–29), but its effects on PKCθ are unknown. This was explored in Jurkat cells, where PMA-induced PKCθ and PKCα activities were measured in the presence or the absence of ceramide. It was found that ceramide potently inhibited the membrane-associated activation of both isozymes after stimulation with PMA for 20 min (Fig. 2, A and B). These findings indicated that ceramide was able to inhibit the key PKCθ isozyme involved in early T cell activation in addition to its previously reported ability to inhibit classical PKC isozymes.

Fas-induced ceramide results in PKCθ inhibition

Fas is known to play a central role in activation-induced T cell death. Previous studies implicated ceramide as one of the signaling pathways stimulated by Fas during apoptosis (30). To determine whether the observed effects with exogenous ceramide were relevant to normal T cell regulation, ceramide levels and PKCθ activity were measured after T cell activation in the context of Fas stimulation (Fig. 2, C and D). In Fas-stimulated cells, it was found that ceramide levels increased significantly and that treatment with PMA/PHA at 8 or 14 h after Fas stimulation resulted in a slight further increase in ceramide. As shown previously (30), when Fas death agonist B1 (FB1), an inhibitor of de novo ceramide synthesis was added to cells before Fas stimulation, ceramide accumulation was significantly inhibited (Fig. 2C). When PKCθ activity was examined, it was found that PMA/PHA treatment could not induce PKCθ activity 8 and 14 h after Fas stimulation (Fig. 2D). When ceramide synthesis was inhibited by FB1, Fas stimulation no longer inhibited PKCθ activity. These results suggested that Fas-induced ceramide synthesis was necessary for Fas-induced inhibition of PKCθ.

Synthetic or natural ceramides inhibit IL-2 production

The inhibition of PKCθ and NF-κB by ceramide led us to determine the effects of this inhibition on IL-2 secretion. When co-stimulated with PMA and PHA, Jurkat cells produced large amounts of IL-2 in a time-dependent manner, reaching a plateau of 3300 ± 417 pg/ml by 24 h (data not shown). Jurkat cells were treated with vehicle or PMA (10 ng/ml) and PHA (10 μg/ml; P/P), or TNF-α (5 nM) with the indicated concentrations of C6-ceramide for 90 min. EMSA was performed on nuclear proteins using specific NF-κB-labeled oligonucleotide probes. The specific NF-κB band is indicated as well as a nonspecific band (n.s.) and free unbound probe. TNF-α was used as a positive control for NF-κB activation. B, PMA, but not PHA, induced NF-κB activation that was blocked by ceramide. Jurkat cells were treated as described in A, except that PMA and PHA treatments were separate, and C6-ceramide was used at 20 μM. C, Ceramide does not affect DNA binding of AP1, NF-AT, or Ets. Jurkat cells were treated with vehicle or PMA (10 ng/ml) and the indicated concentrations of C6-ceramide for 90 min. EMSAs were performed on equal amounts of nuclear protein extracts using transcription factor-specific oligonucleotide probes. D, Ceramide inhibits PMA-induced NF-κB luciferase reporter activity. Jurkat cells were transiently transfected with reporter constructs, as indicated, before treatment with PMA with (□) or without (□) ceramide for 1.5 h. Luciferase activity in transfected cells was measured as described in Materials and Methods and is presented as relative to activity in control untreated cells.
pretreated with C6-ceramide for 2 h before stimulation with PMA/PHA. Significant, dose-dependent inhibition of IL-2 production was observed (Fig. 3A). Notably, this effect was observed in the absence of ceramide-induced cell death, as assayed at 24 h by both trypan blue uptake and annexin V labeling (data not shown).

To determine whether our observations in Jurkat cells could be extended to normal T cells, PHA blasts were isolated from human PBMCs and subjected to similar treatments. When additionally treated with PMA, PHA blasts produced IL-2 up to a level of 2200 pg/ml within 3–6 h. When PHA blasts were pretreated for 2 h with increasing concentrations of C6-ceramide before stimulation with PMA, profound inhibition of IL-2 production was observed (Fig. 3B). Additionally, there was no ceramide-induced cell death observed in these cells (data not shown). Similar results were obtained when C2-ceramide was used instead of C6-ceramide (data not shown). These findings suggested that our results were not

**FIGURE 2.** Exogenous or Fas-generated ceramide inhibits PKC. A and B, Ceramide inhibits PMA-induced PKCθ (A) and PKCα (B) activation. Jurkat cells were preincubated with 15 μM C6-ceramide before stimulation with 15 ng/ml PMA for 20 min. Specific PKC activity was determined in the total lysate as well as in the cytoplasmic and membrane fractions as indicated. C, Fas-generated ceramide is inhibited by FB1. Jurkat cells were stimulated with Fas Ab for 8 or 14 h before treatment with PMA/PHA for 30 min as indicated. FB1 (100 μM) was added with Fas Ab treatment as indicated. Ceramide levels in cell lipid extracts were measured as described in Materials and Methods and normalized to lipid phosphate. D, Fas-generated ceramide is inhibited by FB1, and this is reversed by FB1. Cells were treated as described in C, and specific PKCθ activity was measured as described in A. Data shown are representative of two or three identical experiments with similar results.

**FIGURE 3.** Inhibition of IL-2 production by C6-ceramide in Jurkat cells. A, Ceramide inhibits IL-2 production. Cells were pretreated with the indicated concentrations of C6-ceramide or ethanol vehicle for 2 h before stimulation with PMA/PHA. IL-2 levels were measured in the supernatant at 24 h and presented as a percentage of PMA/PHA-treated cells. B, Inhibition of IL-2 production by C6-ceramide. PHA blasts from healthy donors were seeded at 1.5 × 106 cells/ml in 10% FCS and treated with PMA (10 ng/ml) in the presence of vehicle or C6-ceramide at the indicated concentrations. IL-2 was measured at 16 h. C, Inhibition of IL-2 production by bacterial sphingomyelinase. Jurkat cells were treated with PMA/PHA in the presence of vehicle or the indicated concentrations of bacterial sphingomyelinase. IL-2 levels were measured and are presented as a percentage of the PMA/PHA-treated control value. Error bars represent the SD. *, Statistically significant difference compared with the control.
unique to the Jurkat cell line, but could also be produced in primary T cells.

The ability of natural ceramide, which is hydrophobic and difficult to deliver to cells, to produce the same effects as synthetic ceramide was examined next. Exogenous bacterial sphingomyelinase, which hydrolyzes membrane sphingomyelin, was used to generate natural ceramide at the cell membrane. PMA/PHA-stimulated Jurkat cells were cotreated with a range of concentrations of sphingomyelinase, as shown in Fig. 3C. Inhibition of IL-2 production occurred at very low concentrations of sphingomyelinase. These studies indicated that the observed effects with synthetic ceramide and Fas-generated, de novo synthesized ceramide could also be produced by natural ceramide generated at the cell membrane.

Ceramide inhibits IL-2 mRNA transcription

The ability of ceramide to inhibit PKC-induced NF-κB activation suggested that ceramide might be interfering with transcription of the IL-2 gene, which is NF-κB dependent. The effects of C₆-ceramide on the level of IL-2 mRNA were examined after 3 h of stimulation. As shown in Fig. 4A, C₆-ceramide inhibited the production of IL-2 mRNA transcripts in a dose-dependent manner without affecting the housekeeping gene GAPDH. Additionally, the transcript of the NF-κB-inducible IL-2Rα gene was potently inhibited (Fig. 4A). Also, the time-dependent production of IL-2 mRNA was inhibited by ceramide at all times examined (Fig. 4B). These experiments indicated that ceramide was inhibiting IL-2 at the level of transcription.

Ceramide inhibits an early event in IL-2 production

Ceramide has been reported to perturb secretory functions in other models (31). Therefore, it was important to determine whether ceramide inhibited the secretion of formed IL-2 in addition to inhibiting IL-2 transcription. Jurkat cells were stimulated with PMA/PHA in the presence or the absence of C₆-ceramide and were harvested 24 h after treatment, lysed with hypotonic buffer to release intracellular IL-2, and combined with their respective medium. Measurement of IL-2 revealed that there was no evidence of intracellular trapping of IL-2 after ceramide treatment (data not shown). This indicated that ceramide did not inhibit the secretion of formed IL-2.

In addition to the early signals that are responsible for initiating IL-2 production, late signals are required for its sustained production, e.g., ERK activation, which also activates NF-κB (32). Therefore, it became important to examine whether ceramide was interfering with these late signals. This was important because ceramide was shown to modulate ERK activity in some systems (33). The effect of delaying the addition of ceramide to stimulated cells was examined. The addition of C₆-ceramide to PMA/PHA-treated cells was made several hours before, simultaneously, or delayed for 1, 2, 4, or 6 h after stimulation. As shown in Fig. 5, inhibition of IL-2 production was maximal when cells were pretreated with ceramide, but also occurred when ceramide was added at the same time as PMA/PHA or when its addition was delayed for up to 2 h. Further delay in the addition of ceramide resulted in the gradual loss of inhibition. These experiments indicated that ceramide was inhibiting an early event (within the first 2 h) important in IL-2 regulation.

Ceramide inhibition of PKC-induced NF-κB is bypassed by TNF-α

NF-κB is activated by many pathways. Some are PKC dependent, whereas others, such as TNF-α, are PKC independent (34, 35). Previously, it was shown that ceramide does not inhibit TNF-α-induced NF-κB activation (22). The ability of TNF-α to overcome the inhibitory effects of ceramide on PMA-induced NF-κB activation was examined. When Jurkat cells treated with PMA and C₆-ceramide for 1.5 h were additionally treated with TNF-α at 2 nM, NF-κB activation was reconstituted, and the ceramide-induced inhibition was bypassed (Fig. 6A). To determine whether the reconstitution of NF-κB activation by TNF-α allowed the production of IL-2 in the presence of ceramide, Jurkat cells were treated with PMA/PHA and C₆-ceramide, with or without TNF-α for 14 h. As shown in Fig. 6B, IL-2 production was partially restored by the addition of TNF-α. These results suggested that ceramide modulated its inhibitory effect on NF-κB mobilization by inhibition of the PKC pathway of NF-κB activation and that TNF-α could partially overcome this inhibition.

FIGURE 4. Ceramide inhibits IL-2 mRNA synthesis. A, Dose-dependent inhibition of IL-2 and IL-2Rα mRNA synthesis by C₆-ceramide. Jurkat cells were treated with PMA/PHA and C₆-ceramide at the indicated concentrations. RNA was collected at 3 h, and the specific mRNA species were detected as described in Materials and Methods. B, Time-dependent inhibition of IL-2 mRNA by ceramide. Cells were treated with PMA/PHA and C₆-ceramide (20 μM). Total RNA was extracted at the indicated time points. GAPDH is a housekeeping gene that serves as a control for technical variations.

FIGURE 5. Ceramide inhibits an early event in IL-2 production. Jurkat cells were prepared as described in Fig. 1. C₆-ceramide (20 μM) was added at the indicated time points relative to PMA/PHA treatment, and IL-2 levels were measured at 16 h. Data are representative of three different experiments. Error bars represent the SD. *, Statistically different values compared with the control.
IL-2 luciferase activity was measured at 24 h, it was found that overexpression of PKC\(\theta\) overcame the inhibition that was induced by ceramide and partially restored IL-2 production.

Effects of ceramide are not due to its metabolism to gangliosides or sphingosine

The previously described immunosuppressive effects of sphingolipids were attributed mostly to complex glycosphingolipids. Therefore, it became important to determine whether the observed IL-2 inhibition by ceramide was due to its metabolism to gangliosides that, in turn, were producing the inhibitory effects. The UDP-glucosyl-ceramide transferase inhibitor, \(\alpha\)-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP), was used. PDMP prevents the synthesis of cerebrosides and gangliosides from ceramide and leads to the accumulation of cellular ceramide (36). Jurkat cells were treated with PDMP (50 \(\mu\)M), resulting in an increase in cellular ceramide levels by 4-fold within 6 h (data not shown). Jurkat cells were pretreated with PDMP, either alone or in combination with \(C_6\)-ceramide, for 2 h before stimulation. PDMP treatment alone prevented IL-2 production (Fig. 7A). This indicated that the elevation of natural cellular ceramide induced by PDMP was sufficient to produce inhibition. When \(C_6\)-ceramide treatment was combined with PDMP, complete inhibition of IL-2 production was still observed. Indeed, PDMP potentiated the inhibitory effects of the lower concentrations of ceramide. These findings indicated that conversion of exogenously added ceramide to gangliosides was not necessary to produce IL-2 suppression and that the natural ceramide that accumulated in response to treatment with PDMP cooperated with the exogenously added synthetic ceramide to produce IL-2 inhibition.

Sphingosine is another sphingolipid that has been demonstrated to inhibit PKC, albeit by a different mechanism (37). Because sphingosine is structurally related to ceramide (it lacks the fatty acyl chain), and both can be converted into one another by single-step biochemical reactions, it became important to determine whether ceramide produced its effects by hydrolysis to sphingosine. First, the ability of sphingosine itself to produce effects on NF-\(\kappa\)B and IL-2 comparable to those of \(C_6\)-ceramide was studied. Jurkat cells were activated with PMA and cotreated with sphingosine. Sphingosine, which is more cell permeable than ceramide, produced inhibition of NF-\(\kappa\)B at high concentrations (Fig. 7B), followed by inhibition of IL-2 at a later time point. Sphingosine, cellular levels after \(C_6\)-ceramide treatment of Jurkat cells were measured. Because the first 6 h after ceramide addition were critical for its inhibitory effects, sphingosine levels were measured during that time frame and were found not to change in response to \(C_6\)-ceramide treatment (Fig. 7C). Thus, although sphingosine was capable of producing effects on IL-2 production similar to those of ceramide by virtue of its ability to inhibit PKC, the effects of ceramide did not appear to be mediated by sphingosine.

**Discussion**

The aim of this study was to clarify the role of ceramide in the regulation of NF-\(\kappa\)B and NF-\(\kappa\)B-dependent IL-2 production. Our findings are in agreement with previous observations showing that ceramide does not activate NF-\(\kappa\)B (11–13). They are also in agreement with a recent study showing that \(C_2\)-ceramide inhibits the production of IL-2 in rat splenocytes, although comparatively higher concentrations of ceramide were used, and significant apoptosis was observed (38). Based on the current study, the inhibitory effects of ceramide on IL-2 and NF-\(\kappa\)B appear to be mediated by inhibition of PKC, particularly PKC\(\theta\). Additionally, and in the context of Fas stimulation, this inhibition might have a role during Fas-induced apoptosis by neutralizing a survival signal for T cells.

**Overexpression of PKC\(\theta\) partially overcomes the inhibitory effects of ceramide**

To determine whether PKC\(\theta\) inhibition by ceramide contributed to the observed effects on IL-2 production, Jurkat cells were transiently transfected with PKC\(\theta\) or vector in addition to either NF-\(\kappa\)B or IL-2 luciferase reporter constructs and were subsequently treated with PMA/PHA in the presence or the absence of ceramide (Fig. 6C). PKC\(\theta\) overexpression resulted in a 2-fold increase in NF-\(\kappa\)B-driven luciferase activity in response to PMA at 3 h compared with vector-transfected cells (data not shown). When
Until recently, the relative importance of the different PKC isoforms in IL-2 production was not known, although both classic (α, β, and γ) and novel (θ and ε) isoforms were implicated (24, 39). Recent evidence has shown that PKCθ is critically important for TCR-mediated NF-κB activation, and that it is the only isoform of PKC that is translocated to the site of contact between T cells and APCs (40). Also, conventional PKC isoforms as well as PKCθ were demonstrated to synergize with calcineurin to activate NF-κB and the IL-2 promoter (41, 42). The mechanisms by which PKC activates NF-κB are not clear, but direct or indirect activation of IκB kinases are possibilities. Our results show that ceramide specifically inhibits PKC-mediated, but not TNF-α-induced, NF-κB activation. Indeed, this inhibition can be bypassed by the overexpression of PKCθ or the addition of TNF-α. This indicates that ceramide does not inhibit the TNF-α-induced IκB kinases and that it has no direct effect on NF-κB, such as modulating its DNA-binding activity or inducing its proteolysis. Whether ceramide inactivates other kinases that are downstream of PKC remains to be determined.

Ceramide appears to differentially modulate various PKC isoforms. A recent study showed that ceramide inactivates PKCα in Molt-4 and Jurkat cells (26). This inactivation appears to be mediated by dephosphorylation of PKCα and without interfering with its ability to translocate from the cytosol to the membrane that normally occurs after its activation. This is in contrast to the direct inhibitory effects of sphingosine on PKC that result from competitive, but reversible, inhibition of the regulatory domain (37). Also, inhibition of PKCβ2 by ceramide was demonstrated in Molt-4 cells (26). In contrast, ceramide has been shown to activate PKCε (25). Our findings in this study show that ceramide, either exogenously added or generated by Fas ligation, inhibits PKCθ activation. This is the first evidence that ceramide regulates this PKC isoform. Additional studies evaluating the effects of ceramide on PKCθ regulation are ongoing in our laboratory.

Our results suggest that the inhibition of PKC-induced NF-κB activation by ceramide is sufficient to significantly disable IL-2 transcription and that this is partially overcome by overexpression of PKCθ. However, other mechanisms, such as the negative regulation of other necessary transcription factors, cannot be completely ruled out based on our experiments. As mentioned above, optimal IL-2 transcription occurs when all the necessary transcription factors are present to form a complex that interacts with the promoter (17). If one of these factors is inhibited, the induction of the gene is down-regulated.

Previous studies have shown that glycosphingolipids cause inhibition of the proliferation of activated T cells. Additionally, several parasites with a rich and complex array of glycosphingolipids in their membranes may evade the immune response not only by immune suppression mediated by the glycosphingolipids, but also by the different ceramides present that may specifically down-regulate T cell activation (5, 6, 43). Our studies suggest that bacterial sphingomyelinas secreted by pathogenic bacteria may induce the generation of endogenous ceramide by host cells, leading to local immunosuppression.

These findings support a new role for ceramide as a putative immunosuppressor lipid that is independent of, but complementary to, its role in the stress response. Additional studies will be needed to explore this role. The emergence of PKCθ as the central PKC isoform involved in TCR signaling and our discovery that ceramide can inhibit its activity provide an opportunity for better understanding the role of ceramide in regulating the immune response.

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References


