Brief Definitive Report

Ceramide Induces Interleukin 6 Gene Expression in Human Fibroblasts

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Summary

We previously reported that ceramide, the immediate product of sphingomyelin hydrolysis, increases in response to interleukin (IL)-1β and plays a role in modulating IL-1β–mediated prostaglandin E_2 production and cyclooxygenase gene expression in human fibroblasts (Ballou, L. R., C. P. Chao, M. A. Holness, S. C. Barker, and R. Raghow. 1992. *J. Biol. Chem.* 267:20044–20050). Here we describe the effects of ceramide in another IL-1β–mediated process in these cells, the induction of IL-6 production. We found that submicromolar concentrations of C_2-ceramide induced IL-6 gene expression and protein production as effectively as IL-1β. Both D-*erythro*-C_2-ceramide (a cell-permeable analogue of natural ceramide) and D-*threo*-C_2-ceramide were potent inducers of IL-6 production, while neither L isomer of ceramide was effective. Compared with IL-1β–induced IL-6 production, cells treated with ceramide or exogenous sphingomyelinase induced 82 and 50% of maximal IL-1β–induced IL-6 levels by 6 h, respectively; by 24 h all three treatments induced similar levels of IL-6 production. Ceramide-induced IL-6 messenger RNA could be detected within 1 h of treatment and reached maximal levels by 24 h. These findings suggest that ceramide may play a role in the regulation of IL-6 gene expression.

Ceramide has emerged as an important signal transduction molecule that has been shown to play a role in such fundamental biological processes as proliferation, differentiation, receptor function, oncogenesis, and immune and inflammatory responses (1–3). Ceramide is generated by the hydrolysis of sphingomyelin (SM) via the activation of both neutral and acidic sphingomyelinase (SMase) (4). SM hydrolysis is stimulated by specific cytokines such as TNF-α (5–7), IFN-γ (8), and IL-1β (9, 10). Noncytokine inducers of SM hydrolysis include 1α,25-dihydroxyvitamin D_3 (11) and brefeldin A (12).

We and others have shown that IL-1β stimulates SM hydrolysis and the accumulation of ceramide in fibroblasts and EL4 lymphoma cells (9, 10). We also showed that ceramide modulated the effects of IL-1β on PGE_2 production in human fibroblasts via its ability to enhance the expression of the cyclooxygenase-1 gene (9). The ability of ceramide to modulate the effects of IL-1β on PGE_2 production led us to examine the effects of ceramide on IL-1β–mediated IL-6 production. IL-6 is a multifunctional cytokine produced by monocytes and macrophages, endothelial cells, vascular smooth muscle cells, and fibroblasts (13–16). IL-6 is known to activate T cells, promote B cell growth and Ig synthesis, stimulate synthesis of acute phase proteins by hepatocytes, act as an endogenous pyrogen, induce megakaryocyte maturation, stimulate proliferation of keratinocytes, and act as a growth factor for human myeloma and murine plastocytoma (15). IL-6 has been implicated in inflammation (13, 14, 17), viral infection (18), autoimmunity (15), development (19), and malignant cell growth (15).

In an effort to establish the role of ceramide in IL-1β–induced IL-6 production, we treated cells with stereoisomers of C_2-ceramide or exogenous SMase and measured IL-6 protein and messenger RNA (mRNA) levels. Here we show that the treatment of human fibroblasts with ceramide or with exogenous SMase induces IL-6 expression in a time- and dose-dependent manner. D-C_2-ceramide, but not L-C_2-ceramide, induced significant production of IL-6 protein and mRNA at submicromolar concentrations, indicating a high degree of specificity for ceramide and the induction of IL-6 gene expression. Our findings suggest that ceramide may play a role in mediating the effects of IL-1β, and perhaps the effects of other agonists that induce SM hydrolysis, on IL-6 expression in human fibroblasts. Given the many effects of IL-6 on a variety of different cell types, the identification of ceramide as an inducer of IL-6 production represents a potentially new and important role for SM signaling in the regulation of cell function.

Materials and Methods

Materials. IL-6, IL-1β, IL-1α, and TNF-α immunoassay kits were obtained from R&D Systems (Quantikine; Minneapolis, MN). Bacterial SMase (*Staphylococcus aureus*) was obtained from Sigma Chemical Co. (St. Louis, MO) and not purified further; using an in vitro assay system to test the specificity of hydrolysis, phosphatidylcholine was hydrolyzed <5% compared with SM (100%). Ceramide stereoisomers were prepared and analyzed by nuclear magnetic resonance as previously described (20).

Cell Culture. Human foreskin fibroblasts were maintained in culture as previously described (9). Confluent fibroblast cultures were washed and incubated in fresh serum-free medium and treated with IL-1β (50 pgm/ml), ceramide, or bacterial SMase (1 mU/ml) along with appropriate vehicle controls. Ceramide stereoisomers were incubated with fatty acid–free BSA for 1 h as previously described (9) before their addition. The effect of ceramide on cell viability was assessed using trypan blue exclusion and DNA fragmentation; at all concentrations tested, ceramide had no apparent adverse affects.

Immunoassay of IL-6 and IL-1. IL-6, IL-1α, IL-1β, and TNF-α levels in cell supernatants were measured by ELISA (R&D Systems) according to the manufacturer's directions.

RNA Isolation and Northern Analysis of IL-6. Total fibroblast cellular RNA was isolated from treated cells using the RNAzol method (Tel-Test, Inc., Friendswood, TX). RNA was fractionated on 1.2% agarose gels containing formaldehyde and blotted onto nitrocellulose membranes using capillary transfer (21). Blots were hybridized with either a ^{32}P-labeled IL-6 or GAPDH probe. Sequences used as probes were generated by RT-PCR using the following primers derived from human DNA sequences: IL-6 sense 5′-CCA GCT ATG AAC TCC TTC TCC AC-3′/antisense 5′-GGA CTG CAG GAA CTC CTT AAA GC-3′ (22); GAPDH sense 5′-CCA CCC ATG GCA AAT TCC ATG GCA-3′/antisense 5′-TCT AGA CGG CAG GTC AGG TCC ACC-3′(23). The PCR fragments were cloned into pCR II (Invitrogen, San Diego, CA). EcoRI-released inserts were subsequently used as substrates for random primer labeling. Hybridizations were performed for 16–20 h at 42°C in sodium chloride sodium phosphate EDTA buffer containing 50% formamide. Hybridized membranes were autoradiographed on X-OMAT film (Eastman Kodak Co., Rochester, NY) at −80°C.

Results

Ceramide Induces IL-6 Expression. In human foreskin fibroblasts, IL-1β has been shown to induce SM hydrolysis and the accumulation of ceramide within 2 h (9), and IL-1β is also known to be a potent inducer of IL-6 expression in these cells. Therefore, in an effort to establish whether ceramide is involved in the signal transduction pathway by which IL-1β induces IL-6 production, cells were treated with stereoisomers of C_2-ceramide (1 μM final concentration), bacterial SMase (1 mU/ml), or IL-1β. After an overnight incubation, culture media were analyzed for IL-6 by ELISA. As shown in Fig. 1, both D-*erythro*- and D-*threo*-C_2-ceramide induced IL-6 production to a similar extent as did IL-1β, while both L-*erythro*- and L-*threo*-C_2-ceramide were ineffective inducers of IL-6 production. This is not likely to be an artifact of unequal ceramide uptake, as others have shown that these stereoisomers are taken up by cells with equal efficiency (20). Consistent with the hypothesis that SM hydrolysis may be involved in the IL-1β signaling pathway leading to IL-6 synthesis, treatment of cells with bacterial SMase also induced IL-6 production (Fig. 1). When cells were treated with actinomycin D to inhibit transcription, IL-6 production in response to IL-1β, C_2-ceramide, and bacterial SMase was reduced >95%, indicating regulation of IL-6 induction at the transcriptional level (data not shown). Ceramide did not induce IL-1α, IL-1β, or TNF-α production in these cells, which could have resulted in an autocrine induction of IL-6 synthesis. Also, cells pretreated with IL-1 receptor antagonist protein (5 ng/well) inhibited IL-1–induced IL-6 production but had no affect on D-C_2-ceramide–induced IL-6 production (data not shown), suggesting that ceramide is able to induce IL-6 production in the absence of IL-1 receptor stimulation.

Dose-Response for Ceramide-induced IL-6 Expression. Fig. 2 shows that, at the lowest concentration tested in this experiment, only D-*threo*-C_2-ceramide induced significant IL-6

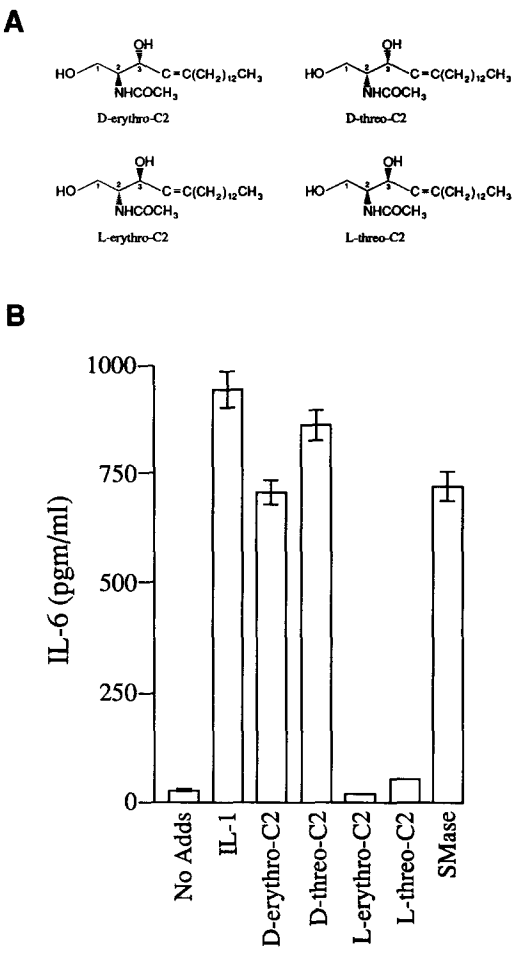

Figure 1. The structures of the stereoisomers of C_2-ceramide (*A*), and the effect of IL-1β, C_2-ceramide isomers, and bacterial SMase on IL-6 induction (*B*). Confluent dermal fibroblasts were treated with 1 μM (final) D- or L-*threo*-C_2-ceramide, D- or L-*erythro*-C_2-ceramide, 50 pgm/ml IL-1β, bacterial SMase (1 mU/ml), or vehicle control in serum-free medium. After 24 h, culture media were collected and IL-6 protein levels were measured by ELISA. All data points depicted were derived from duplicate IL-6 assays from two identically treated wells ± SD. Five independent experiments gave similar quantitative data.

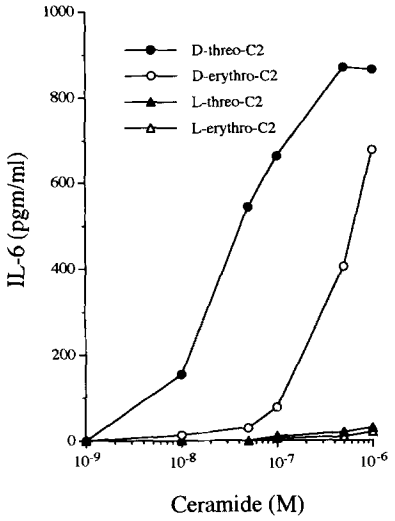

Figure 2. Dose–response of C_2-ceramide–induced IL-6 production. Confluent dermal fibroblast cultures in serum-free medium were treated with the indicated final concentrations of D- or L-*threo*-C_2-ceramide and D- or L-*erythro*-C_2-ceramide. After 24 h, the media were collected from identically treated duplicate wells and analyzed by IL-6 ELISA. The data presented are representative of four separate experiments.

production. D-*erythro*-C_2-ceramide effectively induced IL-6 production, but only at somewhat higher concentrations than the D-*threo* isomer. In contrast, neither of the L forms of ceramide were effective inducers of IL-6 production (Fig. 2). It is noteworthy that the submicromolar concentration of D-C_2-ceramide needed to induce IL-6 gene expression is among the lowest reported for such compounds to elicit a specific biological response.

Time Course for IL-1β, Ceramide, and Bacterial SMase-induced IL-6 Expression. Cells were treated with either IL-1β, D-*threo*-C_2-ceramide (selected for further study because it was the most potent ceramide isomer tested), or bacterial SMase for the times indicated, and IL-6 production was measured (Fig. 3). IL-1β rapidly induced IL-6 production, with ~80% of the maximal stimulation occurring by 2 h of incubation and 100% stimulation by 6 h of incubation. Although both D-*threo*-C_2-ceramide and SMase induced measurable IL-6 production by 2 h of incubation, both treatments were significantly less effective than IL-1β, suggesting the activation of multiple pathways by IL-1β leading to IL-6 production. As the time of incubation increased, both D-*threo*-C_2-ceramide– and SMase-induced IL-6 production continued to increase; by the end of a 24-h treatment, cells treated with either IL-1β, D-*threo*-C_2-ceramide, or SMase produced essentially equivalent amounts of IL-6 (Fig. 3).

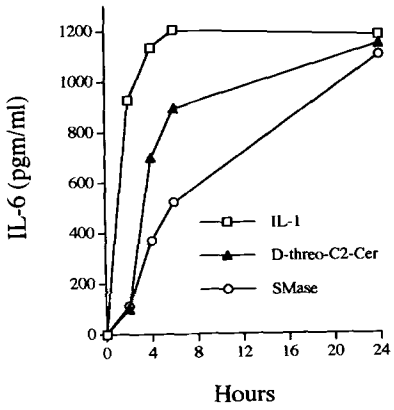

Figure 3. Time course of IL-6 induction in response to IL-1β, D-*threo*-C_2-ceramide, and bacterial SMase. Confluent dermal fibroblast cultures in serum-free medium were treated with 50 pgm/ml IL-1β, 1 μM (final) D-*threo*-C_2-ceramide, or bacterial SMase (1 mU/ml). At the times indicated, the culture media were collected from identically treated wells and analyzed by IL-6 ELISA. The data presented are from duplicate assays of two identically treated wells and are representative of three separate experiments.

Steady State Levels of IL-6 mRNA Induced by D-threo-C_2-Ceramide. Total RNA from cells treated with increasing concentrations of D-*threo*-C_2-ceramide was size fractionated, blotted, and hybridized with an IL-6 cDNA probe. The same Northern blot was also hybridized with a GAPDH cDNA probe to monitor changes in a constitutively expressed mRNA after treatment with C_2-ceramide. Fig. 4 shows the data from a representative dose–response experiment in which cells were treated with D-*threo*-C_2-ceramide at the concentrations indicated for 24 h. There was a clear, dose-dependent increase in IL-6 mRNA in response to ceramide, and significant IL-6 induction was observed at the lowest concentration tested in these experiments, 5×10^{-9} M. The specificity of IL-6 mRNA induction can be readily appreciated from the lack of significant changes in the steady state levels of GAPDH mRNA (Fig. 4).

To establish the time course of IL-6 mRNA induction in response to ceramide, cells were treated with D-*threo*-C_2-ceramide for 0, 1, 3, 6, and 24 h, and total RNA was analyzed by Northern hybridization as above. Ceramide rapidly induced IL-6 mRNA, which was detectable by 1 h and dramatically increased by 3 h (Fig. 5). We consistently observed a slight decrease in IL-6 mRNA levels after 6 h of incubation; however, no corresponding decrease in IL-6 protein levels was seen at 6 h. IL-6 mRNA levels continued to increase over the remaining course of the incubation with ceramide.

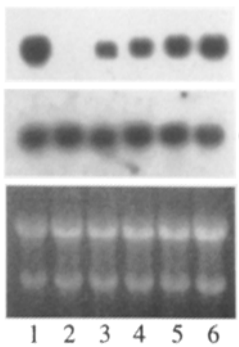

Figure 4. Dose–response for the steady state induction of IL-6 mRNA by D-*threo*-C_2-ceramide. Confluent dermal fibroblasts were treated with the indicated concentrations of D-*threo*-C_2-ceramide, 50 pgm/ml IL-1β, or vehicle control in serum-free medium. Total RNA was isolated after a 24-h incubation, and 20 μg of each sample was analyzed by gel electrophoresis followed by Northern blot. The blotted membrane was hybridized with a ^{32}P-labeled IL-6 probe, stripped, and rehybridized with a GAPDH probe. Lane *1*, IL-1β; lane *2*, no additions; lane *3*, D-*threo*-C_2-ceramide (5×10^{-9} M); lane *4*, D-*threo*-C_2-ceramide (1×10^{-8} M); lane *5*, D-*threo*-C_2-ceramide (1×10^{-7} M); and lane *6*, D-*threo*-C_2-ceramide (1×10^{-6} M).

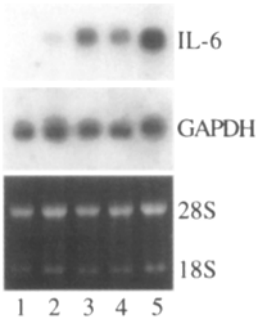

Figure 5. Time course of IL-6 mRNA induction by D-*threo*-C_2-ceramide. Confluent dermal fibroblasts were treated with 1 μM D-*threo*-C_2-ceramide in serum-free medium for the indicated times, after which total RNA was isolated. 10 μg of each sample was analyzed by gel electrophoresis followed by Northern blot. The blotted membrane was hybridized with a ^{32}P-labeled IL-6 probe, stripped, and rehybridized with a GAPDH probe. Incubation times with D-*threo*-C_2-ceramide (1 μM): lane *1*, "zero"; lane *2*, 1 h; lane *3*, 3 h; lane *4*, 6 h; and lane *5*, 24 h.

Discussion

IL-6 is a multifunctional cytokine produced by many different cell types, including fibroblasts. The expression of IL-6 is regulated by a variety of agonists, including IL-1, TNF-α, platelet-derived growth factor, interferons, and glucocorticoids (24, 25). Studies of IL-6 induction in response to TNF-α and IL-1β in human fibroblasts have revealed that both the protein kinase C (26) and cAMP-dependent protein kinase A pathways (27) are involved. However, it is also recognized that these signaling pathways may not completely account for IL-6 induction by IL-1β and TNF-α (28). Because these cytokines also stimulate SM hydrolysis, resulting in increased intracellular ceramide (8, 9), we examined the possibility that ceramide may be involved in the signaling pathway leading to the induction of IL-6 production.

IL-6 gene expression in human fibroblasts was inducible with the addition of nanomolar concentrations of D-*threo*-C_2-ceramide. Maximal induction of IL-6 in response to ceramide required concentrations well below the levels known to result in toxicity or apoptosis in cultured cells (29, 30). Although the kinetics of induction of IL-6 by human fibroblasts was somewhat slower in ceramide-treated cells than in IL-1β–treated cells, equivalent IL-6 levels accumulated by 24 h. The difference in time courses between IL-1β and ceramide could be due to the possibility that the activation of the SM pathway accounts for only a portion of the signaling pathway involved in the IL-1β–mediated induction of IL-6. This is likely, because IL-1β–induced IL-6 production in cultured human fibroblasts has been shown to involve both the cAMP/protein kinase A pathway (27) and the diacylglycerol/protein kinase C pathway (26). Because of the activation of multiple stimulatory pathways in addition to the SM pathway, the more rapid induction of IL-6 production in response to IL-β, compared with the addition of exogenous ceramide by itself, is not an unexpected result. IL-1 causes an increase in cAMP levels more quickly than it causes an increase in ceramide levels. Another possible reason for the time course difference between IL-1β and C_2-ceramide–mediated induction of IL-6 could be a difference in "effective" concentration of ceramide at a specific site of action. Because the IL-1β receptor is most likely linked to the activation of a "specific" SMase, the receptor-coupled action of IL-1β could result in a higher concentration of ceramide at a specific site of action when compared with intracellular levels of ceramide entering the cell by nonspecific diffusion through the plasma membrane. Although SMase-mediated IL-6 induction is somewhat slower than that of C_2-ceramide, the level of IL-6 after 24 h is similar to that induced by IL-1β and C_2-ceramide. This might be due to the rate at which ceramide is produced in response to exogenous SMase treatment. It is also possible that the time course difference between SMase and C_2-ceramide relates to the difference in length of amide-linked acyl chains between C_2-ceramide and the natural ceramide (mostly C_{16}-ceramide), or it may be due to the fact that, unlike natural C_{16}-ceramide, C_2-ceramide is not metabolized and therefore provides a more stable signal.

Our results indicate that the D isomers of ceramide are far better inducers of IL-6 than are the corresponding L isomers. In contrast, the L isomers of ceramide have been shown to be more effective than the D isomers for inhibiting the proliferation of HL-60 cells (20). These unique specificities for ceramide stereoisomers in different cell types remain an enigmatic question and suggest potential differences in the cellular targets for ceramide action. With respect to targets of ceramide action, there have been at least two ceramide-activated enzymes described: a proline-directed serine/threonine protein kinase (5) and a ceramide-activated protein phosphatase (CAPP) (31). At the present time, it is not known whether either of those two enzymes is involved in the induction of IL-6 or whether the activation of another as yet unidentified signal transduction pathway is involved.

Our finding that the D isomers of ceramide are more effective than the L isomers in the induction of IL-6 expression implicates the stereochemistry at carbon 3 as critical for their action. The number 3 carbon was also concluded to be important using structural analogues of ceramide in regulating cellular proliferation (29). The specificity of ceramide for IL-6 gene induction would appear to be different from that of the activation of CAPP, in which all four ceramide stereoisomers were equally effective activators of the purified enzyme (31). These observations would suggest that the signaling pathway involved in the ceramide-mediated induction of IL-6 gene expression may be distinct from that of CAPP activation and the related down-regulation of *c-myc* (31).

The induction of IL-6 by C_2-ceramide appears to be regulated at the transcriptional level, as evidenced by the ability of actinomycin D to completely inhibit ceramide-induced IL-6 production. Further, Northern blot analysis revealed that C_2-ceramide induces IL-6 mRNA in a dose-dependent manner with a time course paralleling the accumulation of IL-6 protein. Studies using two different cell types have shown that ceramide activates NF-κB (7, 32), a transcription factor known to be involved in IL-1–induced IL-6 gene expression (33, 34). Therefore, there is a possibility that ceramide may induce IL-6 gene expression through the activation of NF-κB, and experiments designed to test the hypothesis are currently underway.

IL-6 gene regulation has been extensively studied in a wide variety of experimental systems, and the ability of IL-1β to induce IL-6 production is well established. Here we have implicated signaling via the SM pathway in the regulation of

IL-6 gene expression. The induction of IL-6 by ceramide in human fibroblasts demonstrates the ability of a specific SM metabolite to induce the expression of a distinct gene and will provide an excellent model system for studying the mechanisms involved in the regulation of gene expression by SM metabolites.

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