

Ceramide- and ERK-dependent pathway for the activation of CCAAT/enhancer binding protein by interleukin-1 β in hepatocytes

Natalia V. Giltiy,¹ Alexander A. Karakashian,¹ Alexander P. Alimov,¹ Sandy Ligthle,
and Mariana N. Nikolova-Karakashian²

Department of Physiology, University of Kentucky College of Medicine, Lexington, KY 40536

Abstract Interleukin-1 β (IL-1 β) is a major inducer of liver acute-phase protein expression in response to infection. Several transcription factors, including CCAAT/enhancer binding protein (C/EBP), are known mediators in this process, although the mechanisms by which they modulate IL-1 β 's action are not completely understood. Activation of sphingomyelinase (SMase) and the subsequent generation of ceramide are early steps in the IL-1 β signaling cascade. In this study, we investigate the role of ceramide in the IL-1 β regulation of C/EBP in primary hepatocytes. The C/EBP DNA binding activity was found to increase in a dose-dependent manner after stimulation with IL-1 β and exogenous addition of C₂-ceramide or treatment with SMase. These changes were accompanied by an increase in the nuclear content of C/EBP β . Both IL-1 β and ceramide led to extracellular signal-regulated kinase 1/2 (ERK1/2) activation as early as 15 min after treatment. Furthermore, the increase of cellular ceramide content resulted in increased phosphorylation of C/EBP β at serine 105 at later time points. Concurrently, the cytosolic levels of C/EBP β decreased, suggesting that IL-1 β and ceramide induced nuclear translocation of C/EBP β . Ceramide-induced C/EBP β phosphorylation, translocation, and DNA binding were suppressed by the addition of PD98059, an inhibitor of ERK1/2 phosphorylation. These results suggest that ceramide and ERK mediate a pathway in the IL-1 β signaling cascade, which results in rapid posttranslational activation of C/EBP β .—Giltiy, N. V., A. A. Karakashian, A. P. Alimov, S. Ligthle, and M. N. Nikolova-Karakashian. Ceramide- and ERK-dependent pathway for the activation of CCAAT/enhancer binding protein by interleukin 1 β in hepatocytes. *J. Lipid Res.* 2005. 46: 2497–2505.

Supplementary key words inflammation • sphingomyelinase • liver • acute-phase protein • extracellular signal-regulated kinase

The acute-phase response (APR) of liver is the main host defense to bacterial and viral infections, intoxication, and inflammation. The induction of liver acute-phase pro-

teins (APPs), such as α_2 -microglobulin, fibrinogen, α_1 -acid glycoprotein (AGP), C-reactive protein, and serum amyloid-A, is a hallmark of the APR. Their expression is controlled at the level of transcription by such cytokines as interleukin-1 β (IL-1 β) and IL-6 and by glucocorticoids (1–6). The signaling pathways involved are complex and not completely understood.

Our earlier studies showed that in primary rat hepatocytes, IL-1 β activates sphingomyelin (SM) turnover and induces the generation of ceramide within 15 min of its addition in a dose-dependent manner (7, 8), which correlated with an increased expression of AGP mRNA. This IL-1 β -induced generation of ceramide was likely caused by an activation of neutral sphingomyelinase (nSMase) and played a role in upregulating AGP mRNA levels, because the addition of a cell-permeable ceramide analog, C₂-ceramide, or treatment with sphingomyelinase (SMase) was sufficient to induce the expression of AGP (7). Ceramide was also found to mediate the regulation of another APP, C-reactive protein, in a hepatoma cell line (9).

The mechanisms for ceramide effects on APR expression are not well understood. Ceramide has been shown to regulate several protein kinases and phosphatases that are involved in cytokine signaling. For example, tumor necrosis factor- α -induced activation of SMase and the consequential generation of ceramide was found to activate Raf-1 and the mitogen-activated protein kinase (MAPK) cascade (10). Similarly, Raf-1 has been identified as a target of cer-

Abbreviations: AGP, α_1 -acid glycoprotein; AP-1, Activator Protein-1; APP, acute-phase protein; APR, acute-phase response; APRE, acute-phase response element; C/EBP, CCAAT/enhancer binding protein; ERK, extracellular signal-regulated kinase; IL-1 β , interleukin-1 β ; JNK, c-Jun NH₂-terminal kinase; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase kinase; NF κ B, nuclear factor κ B; nSMase, neutral sphingomyelinase; SerX, serine X; SM, sphingomyelin; SMase, sphingomyelinase.

¹ N. V. Giltiy, A. A. Karakashian, and A. P. Alimov contributed equally to this work.

² To whom correspondence should be addressed.
e-mail: mnikolo@uky.edu

Manuscript received 24 February 2005 and in revised form 1 August 2005.

Published, JLR Papers in Press, August 16, 2005.
DOI 10.1194/jlr.M500337.JLR200

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This article is available online at <http://www.jlr.org>

amide in the IL-1 β signaling pathway, leading to the activation of extracellular signal-regulated kinase 1/2 (ERK1/2) in mesangial cells (11). More recent studies have shown that ceramide might affect targets proximal to the IL-1 β receptor by modulating the phosphorylation pattern of interleukin-1 β receptor-associated kinase-1 (12), transforming growth factor (TGF)- β activating kinase-1 activity (13), and c-Jun NH₂-terminal kinase (JNK) (12).

The induction of AGP expression during APR is regulated through the acute-phase response element (APRE) present in the promoter region of AGP (14–16) and the other major APP (17, 18). The APRE has a functional CCAAT/enhancer binding protein (C/EBP) consensus binding site. Electrophoretic mobility gel shift assays and binding interference methods have shown that C/EBP binds to APRE in vivo, suggesting that the C/EBP family of transcription factors mediates AGP induction during APR (19, 20). The C/EBPs are a family of basic region-leucine zipper motif-containing transcription factors. The six members (C/EBP α , - β , - γ , - δ , - ϵ , and CHOP) form homodimers and heterodimers between each other or with other leucine zipper proteins and bind to DNA to activate or repress transcription (21). C/EBP α , - β , and - δ are the key players in the liver APR and are critical for the upregulation of liver APP. Increased binding of C/EBP β and C/EBP δ are required for lipopolysaccharide (LPS)-induced AGP expression in rats and rabbits in vivo. C/EBPs also interact with other transcription factors, such as nuclear factor κ B (NF κ B), Activator Protein-1 (AP-1), and glucocorticoids, to induce multi-fold increases in APP expression during the early stages of APR (22–24).

The activation of the C/EBP transcription factors during inflammation occurs at transcriptional and posttranslational levels. IL-1 β , IL-6, serum deprivation, and LPS cause an increase in C/EBP δ and C/EBP β mRNA levels in vivo (25–27). Phosphorylation of C/EBP also seems to regulate C/EBP activity either by direct effects on DNA binding and transactivation ability or by inducing nuclear translocation. In vitro phosphorylation of C/EBP may cause either stimulation or repression of the DNA binding affinity, depending on the kinase and the amino acid residue that has been phosphorylated. In the human C/EBP β , phosphorylation at threonine 235 by a Ras/MAPK pathway (28, 29) and at serine 276 (Ser276) by a Ca²⁺/calmodulin-dependent protein kinase (30) stimulates the transactivation potential of C/EBP β . Protein kinase A-mediated phosphorylation of a region between Ser173 and Ser223, and at Ser240, also results in a suppression of DNA binding (31). The rat C/EBP β is phosphorylated at Ser105, a unique site present only in the rat sequence, via a protein kinase C-dependent mechanism. This phosphorylation seems essential for rat C/EBP β activation (32).

Phosphorylation of C/EBP β and C/EBP δ in response to tumor necrosis factor- α correlates with a translocation of the preexisting C/EBP to the nucleus and with an increased DNA binding in hepatocytes (33). Phosphorylation of C/EBP β by protein kinase A in response to oxidative stress also leads to nuclear translocation (34). In macrophages, LPS-induced activation of C/EBP β is mediated by

JNK (35), whereas the ERK1/2 pathway apparently mediates similar response to IFN- γ (36). Interestingly, chronic upregulation of the MAPK increases phosphorylation and activates C/EBP α and C/EBP β (37) independently of a new protein synthesis, thus confirming that ERK can regulate C/EBP posttranslationally.

We have investigated the role of ceramide in the regulation of C/EBP in primary hepatocytes. Our results provide evidence for the existence of a ceramide- and ERK-dependent pathway in the IL-1 β signaling cascade that is responsible for the rapid phosphorylation, nuclear localization, and activation of C/EBP β transcription factor.

MATERIALS AND METHODS

Materials

Male Fisher 344 rats (150–200 g) were purchased from Harlan, Inc. (Indianapolis, IN). Waymouth's MB 752/1 medium and the murine recombinant IL-1 β were from Invitrogen (Carlsbad, CA). Matrigel was purchased from BD Bioscience (Bedford, MA). C/EBP oligonucleotides and polyclonal antibodies, raised against the different isoforms of C/EBP [C/EBP α (14AA), sc-61; C/EBP β (C-19), sc-150; C/EBP β (C-19), sc-150X; and C/EBP δ , sc-636], were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Rabbit anti-rat phospho-C/EBP β (Ser105) was from Cell Signaling Technology, Inc. (Beverly, MA). Antibody against the phosphorylated form of ERK1/2 was purchased from Promega (Madison, WI). The mitogen-activated protein kinase kinase (MEK) inhibitor PD98059 was from Calbiochem (San Diego, CA). All other reagents were from Sigma Chemical Co. (St. Louis, MO).

Cell culture

The tissue culture dishes were treated with Matrigel (6.3 mg/ml) as described previously (7). Hepatocytes were isolated from ether-anesthetized rats by in situ collagenase perfusion, and the cells (3×10^6 /plate; viability >80%) were plated in 3 ml of Waymouth's medium containing insulin (0.15 μ M) as the only hormone. Cultures were maintained for five days at 37°C in a 5% CO₂ atmosphere with replacement of the medium every 48 h, commencing 3 h after the plating. Stock solutions of *N*-acetyl sphingosine (C₂-ceramide), long-chain ceramide, sphingosine, C₂-dihydroceramide, or sphinganine were prepared in absolute ethanol and added to the medium to obtain the desired concentration. The control samples were treated with an equivalent amount of ethanol, not exceeding 0.2% (v/v). Bacterial SMase (Sigma) was used at a final concentration of 0.1 U/ml. nSMase-2 was overexpressed using adenovirus-mediated gene transfer as described recently (12). Hepatocytes were infected at a multiplicity of infection between 2 and 5, and expression was induced for 48 h using 1 μ g/ml doxycycline. For the inhibition studies, the cells were preincubated with PD98059 or vehicle controls for 30 min. The Matrigel was reliquidized by incubating the tissue culture dishes in PBS containing 5 mM EDTA for 30 min at 4°C. Cells were harvested by scraping. The Matrigel was removed by centrifugation at 500 *g* for 4 min, and the cells were washed once again with PBS. The cells were either resuspended directly in 1 \times SDS sample buffer (for Western blot analyses of phosphorylated C/EBP β using antibody directed against phospho-C/EBP β) or treated to isolate nuclear proteins and cytosol.

Isolation of nuclear proteins and cytosol

Cells were incubated in 10 mM HEPES-KOH, pH 7.9, hypotonic buffer on ice for 10 min. After swelling, cells were pelleted

at 4,000 *g* at 4°C and resuspended in 25 mM HEPES-KOH, pH 7.9, 3 mM MgCl₂, 1 mM DTT, and 1 mM PMSE. The cells were lysed in 0.22–0.23% Nonidet P-40 and checked with a microscope for the efficiency of lysis, and intact nuclei were isolated by centrifugation at 3,500 *g* for 15 min. After washing, the nuclei were resolubilized in hypertonic buffer containing 0.4 M KCl and centrifuged at 25,000 *g* for 30 min. The supernatant was divided into aliquots and frozen for further analyses. To isolate cytosol, the cell pellet was resuspended in 200 μl of buffer consisting of 1 mM EDTA, 0.5% Triton X-100, 1 mM Na₂VO₄, 1 mM NaF, 10 μg/ml aprotinin, and 10 μg/ml leupeptin in 10 mM Tris-HCl, pH 7.4. The cells were then incubated on ice for 20 min and centrifuged at 15,000 *g* for 10 min at 4°C.

Electrophoretic mobility gel shift assay

An oligonucleotide (5'-TGC AGA TTG CGC AAT CTG CA-3') corresponding to the C/EBP consensus binding site (Santa Cruz Biotechnology) was labeled with [³²P]ATP using T4 polynucleotide kinase from Invitrogen. The radiolabeled DNA was recovered using Quick Spin G-25 Sephadex columns from Boehringer Mannheim. The binding reaction was performed in 100 mM HEPES, pH 7.6, containing 5 mM EDTA, 50 mM (NH₄)₂SO₄, 5 mM DTT, 1% Tween 20, and 150 mM KCl for 20 min at room temperature in the presence of poly [d(A-T)] to decrease the nonspecific binding. Each incubation mixture contained 10 μg of nuclear proteins and 100,000 dpm of ³²P-labeled C/EBP binding probe. The binding was analyzed by 4% nondenaturing PAGE. For the super-shift assays, the samples were preincubated with antibody against C/EBPβ appropriate for super-gel shift assay [C/EBPβ(C-19), sc-150X] for 1 h at 4°C. After electrophoresis, the gels were dried under vacuum for 1 h and visualized by autoradiography. The intensity of the bands was quantified using NIH Image 2.1 software.

Immunoblot analysis

Protein concentration in the cell extract was measured by Lowry assay. Proteins (25 μg/lane) were resolved by 7.5% or 10% SDS-PAGE and transferred to Immobilon-P™ polyvinylidene difluoride membrane by semidry blotting. The membranes were then blocked in PBS containing 0.5% Tween 20. The membranes were incubated at room temperature with antibodies specific for the dual-phosphorylated, active form of ERK (dilution of 1:4,000, incubation of 2–3 h), C/EBPβ, -δ, or -α (dilution of 1:1,000, incubation of 2–3 h), and antibody specific for the phosphorylated Ser105 of rat C/EBPβ (dilution of 1:1,000, incubation of 16 h), followed by goat anti-rabbit IgG-alkaline phosphatase-conjugated antibody at a dilution of 1:10,000. Protein-antibody interactions were visualized using the ECF kit from Amersham (Piscataway, NJ) and analyzed using a Storm™ 860 fluorescent scanning instrument and Image Quant 5.0 software (Molecular Dynamics, Sunnyvale, CA).

Statistical analyses

After proving the assumption of equal variance across groups, differences were assessed using the Student's *t*-test. The test was used to compare data between two groups, typically, control versus treated cells. Values are expressed as means ± SD.

RESULTS

IL-1β treatment leads to increases in ceramide level followed by activation of C/EBP DNA binding activity

We have previously shown that in primary rat hepatocytes, IL-1β induces SM turnover and the generation of ceramide

that likely mediates the induction of AGP mRNA (7). To decipher the mechanism by which ceramide induced AGP mRNA transcription, we investigated the effects of ceramide on the activity of C/EBP, a transcription factor that is essential for the upregulation of AGP during APR.

DNA binding of C/EBP was measured by gel shift assay using radiolabeled oligonucleotide corresponding to the C/EBP DNA binding site. As anticipated, IL-1β treatment stimulated the C/EBP DNA binding activity at 5 and 10 ng/ml but not at 2.5 ng/ml (Fig. 1A, B). This stimulation was maximal at 2–5 h and disappeared thereafter. The increase in C/EBP DNA binding was inhibited by a non-labeled consensus oligonucleotide in 200-fold excess and was not affected by competing with the consensus oligonucleotide for Sp-1 transcription factor or mutant C/EBP oligonucleotide (data not shown).

As reported earlier (7), IL-1β also induced increases in the level of cellular ceramide at 30 min of treatment (data not shown). The increase in ceramide was pronounced at 5 and 10 ng/ml IL-1β but not at 2.5 ng/ml, which correlated well with the observed activation of C/EBP DNA.

Increases in the cellular level of ceramide are sufficient to stimulate C/EBP DNA binding activity

To test whether cytokine-independent increases in cellular ceramide are sufficient to induce C/EBP DNA binding, the hepatocytes were treated with *N*-acetyl sphingosine

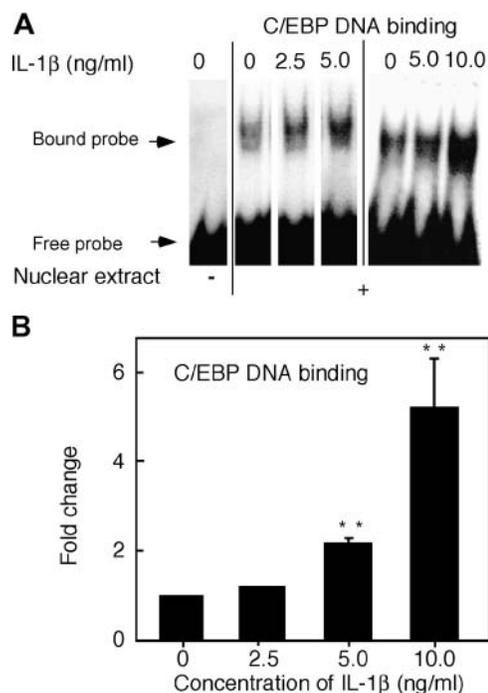


Fig. 1. Interleukin-1β (IL-1β) induces CCAAT/enhancer binding protein (C/EBP) DNA binding activity. Hepatocytes were treated with IL-1β at the indicated concentrations for 5 h. A: Electrophoretic mobility gel shift assay of C/EBP DNA binding in 4% nondenaturing PAGE (10 μg of nuclear protein per lane) using ³²P-labeled consensus binding oligonucleotide. B: Quantification of the intensity of the shifted band. The results are representative of three different experiments. Data shown are means ± SD. Double asterisks indicate statistical significance of *P* < 0.05.

(C₂-ceramide), a membrane-permeable ceramide analog. The ceramide addition led to significant increases in the intensity of the shifted band at 2 and 5 h after treatment in a dose-dependent manner (Fig. 2A, B). This effect was transient, and no differences were observed at 10 h (data not shown). Similar increases in C/EBP binding were observed upon treatment with bacterial SMase, an enzyme that generates ceramide by the turnover of plasma membrane SM (data not shown). Nonlabeled C/EBP consensus binding oligonucleotide (200-fold excess) completely inhibited ceramide-induced binding, whereas nonlabeled Oct1A, NFκB, and AP-1 did not. No binding was found when C/EBP mutant oligonucleotide was used instead of C/EBP (Fig. 2C). These results confirmed that the binding was specific for C/EBP.

To test whether other sphingolipids in addition to ceramide might induce C/EBP DNA binding, hepatocytes were treated with 30 μM C₂-dihydroceramide, sphingosine, or

dihydrosphingosine (sphinganine). As anticipated, treatment with dihydrosphingosine had no effect (Fig. 2D), whereas sphingosine and dihydroceramide induced changes that were significantly smaller than those observed with ceramide, which might be explained by the metabolic conversion of these sphingolipids to ceramide (8, 38).

C₂-ceramide treatment increases nuclear levels of C/EBPβ

The increased C/EBP DNA binding activity could be attributable to increases in the level of active C/EBP in the nucleus and/or to increases in its DNA binding affinity. To study these two possibilities, nuclear extracts were analyzed by Western blotting using antibodies against the three main liver C/EBP forms, C/EBPα, -β, and -δ (Fig. 3A). Anti-C/EBPα recognized multiple C/EBPα forms with molecular masses of 26, 30, and 48 kDa. The 30 and 48 kDa bands were more abundant than the 26 kDa band,

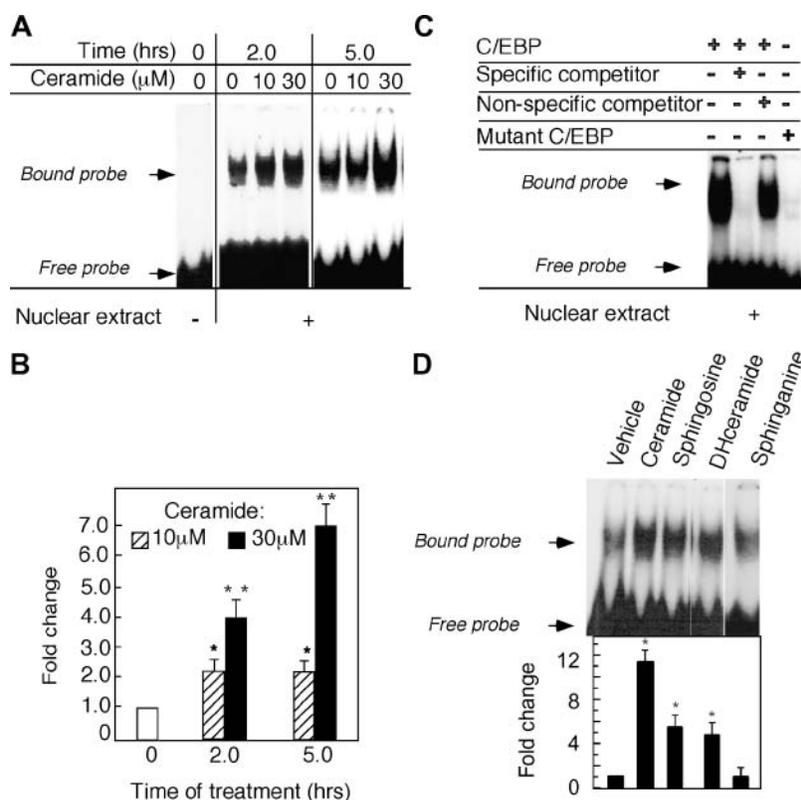


Fig. 2. Increases in the cellular level of ceramide stimulate C/EBP DNA binding activity. Hepatocytes were treated with C₂-ceramide at the indicated concentrations or with 0.1% ethanol for 2 and 5 h. A: Electrophoretic mobility gel shift assay of C/EBP DNA binding in 4% nondenaturing PAGE (10 μg of nuclear protein per lane) using ³²P-labeled C/EBP consensus binding oligonucleotide. B: Quantification of the intensity of the shifted bands. * *P* < 0.05. ** *P* < 0.0025. Data shown are means ± SD (n = 3 replicates per assay) from three independent experiments with comparable results. C: Specificity of C/EBP DNA binding. Binding assays were performed using nuclear extracts from hepatocytes treated with C₂-ceramide (30 μM) for 2 h. For competition studies, ³²P-labeled consensus binding oligonucleotide and nonlabeled C/EBP consensus oligonucleotide (200-fold excess) or nonlabeled, nonspecific (Oct1A) oligonucleotide (200-fold excess) were added to the binding assay (lanes 2 and 3). The ³²P-labeled mutant C/EBP consensus binding oligonucleotide was replaced by ³²P-labeled mutant C/EBP oligonucleotide in lane 4. D: Effect of different sphingolipids on C/EBP binding. Hepatocytes were treated with C₂-ceramide, C₂-dihydroceramide (DHceramide), sphingosine, and dihydrosphingosine (sphinganine; each at 30 μM) for 5 h. Ceramide analogs were delivered in ethanol, and the free sphingoid bases were delivered as a complex with BSA. The results are representative of three different experiments. Data shown are means ± SD. Asterisks indicate statistical significance of *P* < 0.05.

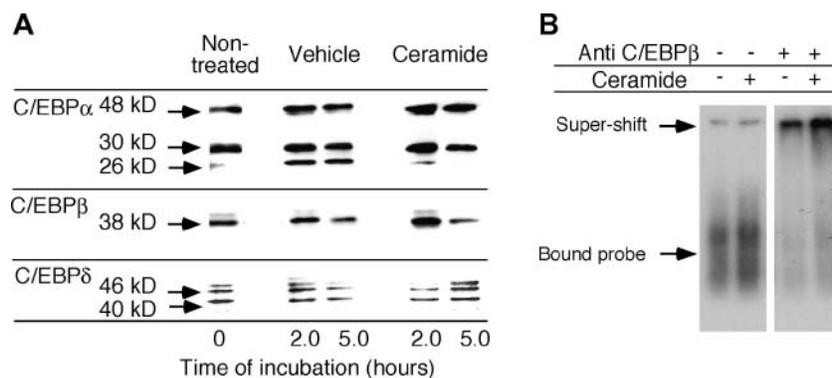


Fig. 3. Ceramide increases C/EBP β nuclear content and DNA binding. Primary hepatocytes were treated with C₂-ceramide (30 μ M) for the indicated times (A) or for 2 h (B). Nuclear proteins were isolated as described. A: Western blot analyses of nuclear extracts using antibodies specific for the major C/EBP isoforms. B: Electrophoretic mobility gel super-shift assay of C/EBP DNA binding using ³²P-labeled C/EBP consensus binding oligonucleotide and antibody specific for the C/EBP β isoform (10 μ g of nuclear protein per lane). The results are representative of two independent experiments.

which is similar to what was reported by Hsieh et al. (39). The levels of the 30 and 48 kDa forms were slightly higher in ceramide-treated cells compared with vehicle-treated cells, although the differences were marginal. Similarly, the levels of the C/EBP δ form remained unchanged.

The most significant changes were observed in the level of C/EBP β , which was detected as a single band with a molecular mass of \sim 38 kDa. This corresponds in size to the liver-activating protein, LAP, the major C/EBP β isoform in liver. Longer exposure times identified 40 and 20 kDa forms, likely representing the mature C/EBP β and the liver-inhibiting protein, LIP, respectively (data not shown). The nuclear content of LAP was 2- to 3-fold higher in ceramide-treated cells compared with controls (Fig. 3A). These increases in the nuclear levels of C/EBP β were further confirmed by a super-shift assay (Fig. 3B). The inclusion of anti-C/EBP antibody in the binding assay caused >90% of the bound probe to be "super-shifted," indicating that C/EBP β is the major component of the C/EBP-DNA complex. Furthermore, the super-shifted band was significantly more intense in the ceramide-treated sample(s), indicating that ceramide addition affected mainly the C/EBP β form. Interestingly, the band that was not super-shifted by the addition of C/EBP β antibody was still more intense in the ceramide-treated samples compared

with the control, indicating that ceramide treatment might also have a minor effect on another C/EBP form, possibly C/EBP α .

Together, these data indicate that C₂-ceramide treatment increased the level of C/EBP β in the nuclei.



Fig. 4. Ceramide increases the nuclear content of phospho-C/EBP β . The ceramide content was increased by two alternative methods: addition of C₂-ceramide (30 μ M) or treatment with bacterial sphingomyelinase (SMase; 0.1 U/ml) for 2 h. The nuclear proteins were isolated, and the phosphorylation of C/EBP β was analyzed by 10% SDS-PAGE and Western blotting using antibody specific for the phosphorylated form of C/EBP β . The results are representative of two independent experiments.

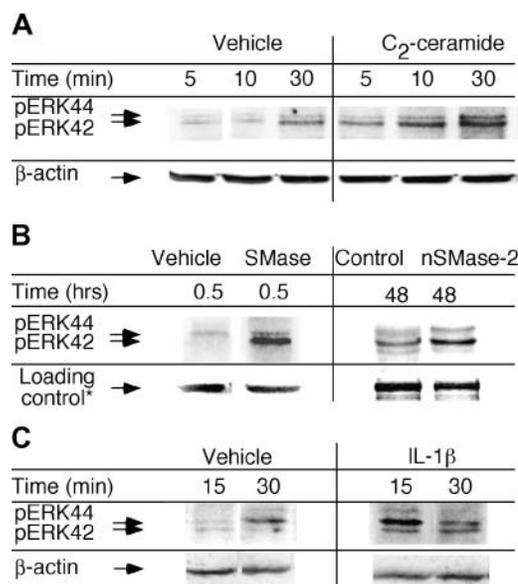


Fig. 5. Ceramide and IL-1 β induce the phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2). After treatment with IL-1 β , ceramide, or SMases, hepatocytes were harvested and cell lysates were prepared. The activation of ERK1/2 was analyzed by 10% SDS-PAGE using Western blotting and antibody against its dual-phosphorylated form. A: Hepatocytes treated with exogenous C₂-ceramide or vehicle control (0.05% ethanol) for the indicated times. B: Hepatocytes treated with bacterial SMase (0.1 U/ml, 30 min) or infected with adenovirus expressing neutral sphingomyelinase (nSMase-2; multiplicity of infection of 3, 48 h). C: Hepatocytes treated with IL-1 β (10 ng/ml) for the indicated times. β -Actin was used as a loading control except in the case of hepatocytes infected with Ad-nSMase-2, for which an irrelevant band from the gel is shown. The results are representative of at least two independent experiments.

Ceramide treatment induced phosphorylation of C/EBP β

Two mechanisms could increase the C/EBP β nuclear content after stimulation with IL-1 β : *i*) increases in mRNA levels, and *ii*) phosphorylation and translocation to the nucleus. In contrast to IL-1 β , ceramide treatment had no effect on the transcription and stability of the C/EBP β mRNA as judged by RT-PCR (data not shown). However, immunoblot analyses of nuclear protein extracts using antibody against the phosphorylated form of C/EBP β showed that both ceramide- and SMase-treated cells had significantly higher content of phosphorylated C/EBP β in the nucleus compared with the controls (Fig. 4), suggesting that increases in cellular ceramide might induce the phosphorylation of C/EBP β .

Ceramide and IL-1 β induce the activation of ERK1/2

Overexpression of ERK1/2 stimulates the phosphorylation of C/EBP β in primary hepatocytes (37). In turn, increases in cellular ceramide content, as well as treatment with IL-1 β , have been found to induce ERK1/2 phosphorylation in various cell types. These observations imply that activation of ERK1/2 might mediate ceramide effects on C/EBP phosphorylation. To test this, ERK activity was measured by immunoblotting using antibodies specific for the dually phosphorylated, active forms of ERK1/2. The addition of exogenous C₂-ceramide led to transient increases in the levels of phosphorylated ERK within 5–10 min (Fig. 5A). Increases of endogenous ceramide levels caused by adding bacterial SMase or by overexpressing nSMase-2,

the plasma membrane-localized form of nSMase (12), also activated ERK (Fig. 5B). As reported earlier, IL-1 β had a similar effect within 15 min of its addition (Fig. 5C). Importantly, activation of ERK preceded the induction of C/EBP DNA binding activity.

ERK1/2 inhibition prevents ceramide-induced C/EBP β phosphorylation, translocation, and DNA binding

To test whether ERK activation mediates the observed ceramide-induced C/EBP β phosphorylation, hepatocytes were treated with ceramide or SMase in the presence and absence of PD98059, a specific inhibitor of ERK. As anticipated, the band corresponding to phospho-C/EBP β was substantially more intense in cells treated with ceramide or SMase compared with the respective controls. These increases in phospho-C/EBP β levels, however, were not observed in the presence of PD98059 (Fig. 6A). Because the antibody used in these assays recognizes C/EBP β phosphorylated at Ser105, these results provide evidence that increases in cellular ceramide cause the phosphorylation of C/EBP β at Ser105 in an ERK-dependent manner.

To further define the role of the ceramide- and ERK-dependent pathway in the IL-1 β signaling cascade, the ability of PD98059 to prevent IL-1 β - or ceramide-induced nuclear translocation of C/EBP β was tested. In these experiments, the cellular content of ceramide was increased by overexpressing nSMase-2 using adenovirus-mediated gene transfer. This treatment led to an increase of endogenous ceramide from 2.35 nmol/mg. Protein (Pr) to 3.75 nmol/mg. Pr. The level of C/EBP β in the cytosolic fraction was

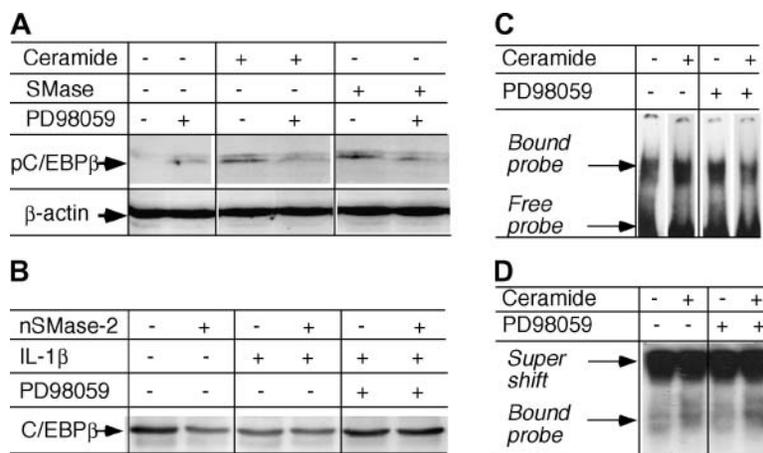


Fig. 6. Requirement for ERK1/2 in ceramide-induced C/EBP β phosphorylation, translocation, and DNA binding. Hepatocytes were treated with IL-1 β (10 ng/ml for 2 h), PD98059 (50 μ M, 30 min pretreatment, 2 h treatment), C₂-ceramide (30 μ M, 2 h), SMase (0.1 U/ml, 2 h), or respective vehicle controls (0.1% ethanol for ceramide, 0.05% DMSO for PD98059). A: Western blot of total cell extracts using antibody specific for serine 105-phosphorylated C/EBP β . B: Western blot of cytosolic cellular fractions using antibody against C/EBP β . Some hepatocytes were infected with Ad-nSMase-2 to increase the ceramide content of the plasma membrane. The expression of nSMase-2 was induced using 2 μ g/ml doxycycline. Infected but not induced cells were used as controls. After 48 h, cells were treated with IL-1 β or PD98059 as indicated. C: C/EBP DNA binding activity analyzed by electrophoretic mobility gel shift assay (10 μ g of nuclear protein per lane) using ³²P-labeled C/EBP consensus binding oligonucleotide. The noninteracting DNA (free probe) and the protein-DNA complex (bound probe) are shown by arrows. D: Super-gel shift assay using antibody against the C/EBP β isoform. The free nonbound probe has run off the gel and hence is not seen. The protein-DNA complex (bound probe) and the antibody-protein-DNA complex (super-shifted band) are shown with arrows. The results are representative of at least two independent experiments.

monitored by Western blot analysis. As anticipated, the cytosolic content of C/EBP β decreased upon treatment with IL-1 β and in response to IL-1 β -independent increases of cellular ceramide by means of nSMase-2 overexpression (Fig. 6B). Treatment with PD98059 completely prevented these decreases, thus confirming that the nuclear translocation of C/EBP β in response to IL-1 β and ceramide requires the activation of ERK1/2.

To test the extent of ERK involvement in ceramide-induced C/EBP DNA binding, we performed electrophoretic mobility gel shift assays in the presence and absence of PD98059. The inhibitor completely blocked the increase in ceramide-induced DNA binding (Fig. 6C), thus lending further support to the proposed role of ERK1/2 in ceramide-induced C/EBP β activation. The C/EBP-DNA complexes were very efficiently (almost completely) super-shifted by C/EBP β antibody in both the presence and absence of PD98059 (Fig. 6D), confirming further that C/EBP β was the major C/EBP form affected by the ceramide and PD98059 treatments. Interestingly, as observed above (Fig. 4), a ceramide-inducible minor complex that apparently did not involve C/EBP β and was not affected by PD98059 could be seen after gross overexposure of the gel (Fig. 6D, lower bands). This suggests that in addition to its ERK-dependent activation of C/EBP β , ceramide could also stimulate another, very minor C/EBP form independently of ERK.

DISCUSSION

IL-1 β and IL-6 are the major mediators of liver APR during inflammation. This study investigated the signaling mechanisms involved in IL-1 β -dependent regulation of APP expression. Our earlier studies have shown that IL-1 β causes transient increases of ceramide and induction of AGP mRNA in hepatocytes. We suggested that ceramide might mediate part of the IL-1 β effects on AGP expression because cytokine-independent increases in ceramide content brought about by adding exogenous ceramide or by treating the cells with bacterial SMase were sufficient to upregulate AGP mRNA.

C/EBP, AP-1, and NF κ B are the main transcription factors that mediate IL-1 β -induced AGP expression. The signaling pathways for the activation of AP-1 and NF κ B are relatively well understood. Those involved in C/EBP regulation are currently being investigated, and both transcriptional and posttranslational mechanisms have been described. We provide evidence that increases in cellular ceramide induce rapid increases in the concentration of C/EBP β in the nucleus without affecting C/EBP β mRNA expression or total protein concentration in the cells. Inhibitor studies indicate that these increases are likely attributable to the activation of ERK1/2 and the phosphorylation of C/EBP β at Ser105 followed by nuclear translocation. Our results thus suggest that ceramide is part of an ERK1/2-dependent pathway for the posttranslational activation of C/EBP β by IL-1 β and does not participate in the transcriptional regulation of C/EBP β .

The role of ceramide in ERK regulation has been studied extensively, and effects of activation (10, 40–42), inhibition (43), as well as a lack of effect (44) have been reported. These different outcomes are likely the result of differences in the subcellular localization of ceramide accumulation. Notably, the activation of ERK has been correlated to the generation of ceramide via the stimulation of SM turnover at the plasma membrane, whereas inhibitory effects are observed when the de novo pathway of ceramide synthesis in the endoplasmic reticulum is engaged (43). In response to IL-1 β , ceramide is apparently generated at the plasma membrane because *i*) IL-1 β activated neutral plasma membrane SMase (8), and *ii*) treatment of the cells with bacterial SMase or overexpression of nSMase-2, both of which generate ceramide at the plasma membrane, mimicked IL-1 β 's ability to activate ERK. Thus, our results concur with those of others (40) in suggesting that the generation of ceramide during the cellular response to inflammation leads to ERK activation.

The mechanism for the activation of ERK by ceramide is unclear; however, a couple of possibilities are likely. Based on observations from different groups, Raf-1, the MEK kinase of ERK, has been identified as a direct target of ceramide and has been shown to undergo phosphorylation in response to ceramide (45). In turn, protein kinase C ζ , which has been found to mediate LPS-induced ERK activation in macrophages and to activate the MEK upstream of ERK (46), is also a well-defined target for ceramide (47). The involvement of protein kinase C in ceramide's effects on C/EBP β is further suggested by the fact

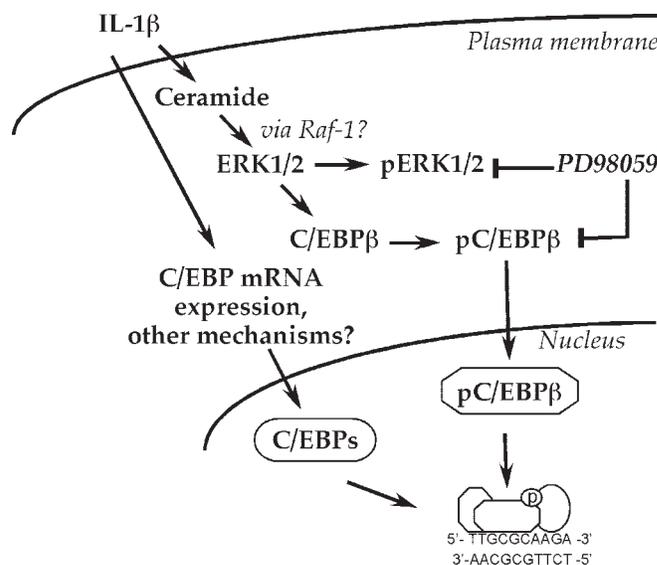


Fig. 7. Role of ceramide in IL-1 β -induced C/EBP DNA binding. In hepatocytes, IL-1 β activates multiple pathways to induce the DNA binding activity of different C/EBP members. Among them, C/EBP β appears to be activated by at least two different mechanisms: *i*) by translocating preexisting C/EBP β from the cytosol to the nuclei, and *ii*) by increasing C/EBP mRNA and protein content. Our data suggest that activation of nSMase, accumulation of ceramide, and activation of ERK1/2 mediate the former. Ceramide-induced activation of the ERK apparently leads to phosphorylation, nuclear translocation, and increased DNA binding of C/EBP β .

that ceramide treatment affected the phosphorylation of Ser105, a unique phosphorylation site in the rat C/EBP, which is required for C/EBP β transcriptional activation and is phosphorylated in a protein kinase C-dependent manner.

Increased liver ceramide content during inflammation is well documented in cell culture studies (7) and in animal models (48, 49). The results presented here emphasize the potential physiological role of these increases and suggest that ceramide may contribute to the upregulation of APP expression by stimulating C/EBP DNA binding. The increases in ceramide content seem to activate mainly the C/EBP β form, although a minor effect on another form (likely C/EBP α) is also evident. The C/EBP β form is required for APP induction, as is evident from studies in C/EBP β knockout mice that have impaired induction of serum amyloid-A, AGP, and complement C3 (50, 51). Therefore, the selective effects of ceramide further support its role in inflammation. At the same time, it is clear that IL-1 β also activates ceramide-independent pathways to increase APP expression in the liver (Fig. 7). As discussed above, IL-1 β has been shown to increase the C/EBP DNA binding activity not only by phosphorylation but also by regulating the rate of C/EBP β and C/EBP δ transcription, apparently in a manner independent of ERK and ceramide.

Two earlier studies had investigated the ability of ceramide to regulate C/EBP β activity. Sprott et al. (52) reported that ceramide is involved in the regulation of C/EBP β nuclear levels during hormonal stimulation of 3T3-L1 preadipocytes. Nevertheless, the addition of ceramide to these cells had a biphasic effect. At an early time (1–4 h), which is within the time period tested in our model, ceramide increased the C/EBP β nuclear content. At later times, however (between 24 and 48 h), C/EBP β was exported from the nucleus and its transcriptional activity declined in a ceramide-dependent manner. Whether or not ceramide has a similar biphasic effect in hepatocytes was not tested in our study. This possibility seems unlikely, however, because those delayed ceramide effects are apparently part of the prolonged signaling processes engaged in adipocyte differentiation.

The addition of exogenous ceramide has been also shown to potentiate the ability of LPS to induce C/EBP β in the RAW macrophage cell line. In good agreement with our study, Cho, Lee, and Kim (35) described a ceramide-mediated increase in C/EBP β phosphorylation by a mechanism involving another member of the MAPK family, JNK. Given that the LPS receptor and the IL-1 β receptor are both members of the Toll-like receptor family and share similar signaling pathways, these data strongly support the role of ceramide in C/EBP β activation during inflammation. At the same time, the findings that different members of the MAPK family mediate C/EBP activation may be attributable to differences in the specific adaptor molecules and kinases involved in transducing the signals downstream of Toll-like receptor-4 (the LPS receptor) and the IL-1 β receptor.

In summary, the results presented here define a cer-

amide- and ERK-dependent pathway that mediates IL-1 β -induced translocation of C/EBP β to the nucleus during the liver APR. 

This work was supported by National Institute for Aging Grant R01 AG-019223 and American Heart Association Scientist Development Grant 01300238N. The authors thank the “cardiovascular research discussion group” for helpful suggestions and criticisms.

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