Decreased activity and enhanced nuclear export of CCAAT-enhancer-binding protein β during inhibition of adipogenesis by ceramide

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To identify novel molecular mechanisms by which ceramide regulates cell differentiation, we examined its effect on adipogenesis of 3T3-L1 preadipocytes. Hormonal stimulation of 3T3-L1 preadipocytes induced formation of triacylglycerol-laden adipocytes over 7 days; in part, via the co-ordinated action of CCAAT-enhancer-binding proteins α , β and δ (C/EBP- α , - β and - δ) and peroxisome-proliferator-activated receptor γ (PPAR γ). The addition of exogenous *N*-acetylsphingosine (C_2 -ceramide) or increasing endogenous ceramide levels inhibited the expression of C/EBP α and PPAR γ , and blocked adipocyte development. C_2 -ceramide did not decrease the cellular expression of C/EBP β , which is required for expression of C/EBP α and PPAR γ , but significantly blocked its transcriptional activity from a promoter construct after 24 h. The ceramide-induced decrease in the transcriptional activity of C/EBP β correlated with a strong decrease in its phosphorylation, DNA-binding ability and nuclear

localization at 24 h. However, ceramide did not change the nuclear level of C/EBP β after a period of 4 or 16 h, suggesting that it was not affecting nuclear import. CRM1 (more recently named 'exportin-1') is a nuclear membrane protein that regulates protein export from the nucleus by binding to a specific nuclear export sequence. Leptomycin B is an inhibitor of CRM1/exportin-1, and reversed the ceramide-induced decrease in nuclear C/EBP β at 24 h. Taken together, these data support the hypothesis that ceramide may inhibit adipogenesis, at least in part, by enhancing dephosphorylation and premature nuclear export of C/EBP β at a time when its maximal transcriptional activity is required to drive adipogenesis.

Key words: dihydroceramide, leptomycin B, nuclear export, 3T3-L1 preadipocyte.

INTRODUCTION

Ceramide is a potent lipid mediator of cell stress responses [1,2]. Depending upon the agonist and cell type, cellular responses to ceramide often culminate in increased apoptosis, decreased proliferative potential or effects on cell differentiation. The molecular mechanisms by which ceramide may activate apoptotic signalling have identified critical roles for stress-activated protein kinases, caspases and changes in mitochondrial function [3]. The anti-proliferative effects of ceramide are mediated, at least in part, by inhibition of cyclin-dependent kinases [4] and decreased phosphorylation of the retinoblastoma tumour suppressor protein [5]. In contrast, the molecular mechanisms by which ceramide affects cell differentiation remain poorly defined.

The process of adipogenesis in 3T3-L1 preadipocytes has proved an excellent model system for identifying molecular mechanisms that regulate terminal cell differentiation [6]. Upon confluence, 3T3-L1 preadipocytes undergo cell cycle arrest and begin to up-regulate the expression of early markers of differentiation, such as lipoprotein lipase [7]. Post-confluent preadipocytes may then be induced to differentiate into cells possessing morphological and biochemical characteristics of mature adipocytes. Although complex, induction of adipogenesis in 3T3-L1 cells is controlled by several basic events. Addition of a cell differentiation 'cocktail' leads to the down-regulation of

preadipocyte factor-1 [8], calpain activation and a subsequent phase of mitotic clonal expansion [9,10]. However, adipogenesis is dependent upon the co-ordinated expression of two classes of transcription factors: CCAAT-enhancer-binding proteins α , β and δ (C/EBP α , C/EBP β , C/EBP δ) [11] and peroxisome-proliferator-activated receptor- γ (PPAR γ) [12,13].

The C/EBP family members are bZIP transcription factors that possess a leucine zipper and a basic DNA-binding domain in their C-terminal region, and an N-terminal transactivation domain [14]. Following hormonal induction of 3T3-L1 cells, C/EBP β and C/EBP δ are rapidly induced and transported to the nucleus [15]. Moreover, phosphorylation of C/EBP β correlates with its ability to bind DNA and competency to transactivate downstream genes necessary to induce adipogenesis [15,16]. The acquisition of DNA-binding ability also correlates with the association of C/EBP β with centromeres and the entry of the quiescent preadipocytes into a phase of mitotic clonal expansion [15]. Although the exact role of clonal expansion in regulating adipogenesis of 3T3-L1 cells is uncertain [17], the activity of C/EBP β and C/EBP δ is required for adipogenesis of 3T3-L1 cells [18] and the development *in vivo* of white and brown adipose tissue [19].

C/EBP β is maximally expressed within the first 48 h following hormonal stimulation, and induces the expression of C/EBP α and PPAR γ , before its own expression begins to diminish rapidly

Abbreviations used: C/EBP- α , - β and - δ , CCAAT-enhancer-binding protein- α , - β and - δ ; C_2 -ceramide, *N*-acetylsphingosine; DIMF, differentiation 'cocktail' containing dexamethasone, insulin, methylisobutylxanthine and fetal calf serum; D-e-MAPP, D-(1S,2R)-erythro-2-(*N*-myristoylamino)-1-phenyl-1-propanol; DMEM, Dulbecco's modified Eagle's medium; EMSA, electrophoretic mobility shift assay; FCS, fetal calf serum; IP, immunoprecipitation; MAPK, mitogen-activated protein kinase; PPAR γ , peroxisome-proliferator-activated receptor γ ; STAT-1, signal transducer of transcription 1; TNF- α , tumour necrosis factor α .

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[11,20]. By 72 h post-stimulation, C/EBP α and PPAR γ are both highly expressed. The continued expression of C/EBP α and PPAR γ is critical for inducing and maintaining the expression of adipocyte-specific genes, such as adipisin and proteins related to adipocyte function, i.e. phosphoenolpyruvate carboxykinase, acyl-CoA synthase, stearoyl-CoA desaturase, etc. [7].

In the present study, we describe the novel finding that increases in endogenous ceramide, or addition of a cell-permeant ceramide analogue to hormonally stimulated 3T3-L1 cells, induced a partial block of mitotic clonal expansion, potentially inhibited the expression of C/EBP α and PPAR γ , and blocked the development of the mature adipocyte phenotype. Although ceramide had no effect on the total cellular expression of C/EBP β , it inhibited the nuclear localization of C/EBP β at 24 h after hormonal stimulation of the 3T3-L1 cells. Decreased nuclear localization of C/EBP β correlated with decreased phosphorylation, DNA binding and its ability to transactivate a reporter construct in differentiating 3T3-L1 cells. Interestingly, an inhibitor of nuclear export, leptomycin B, reversed the ceramide-induced decrease in nuclear levels of C/EBP β , suggesting that the block to adipogenesis by ceramide may arise from a premature export of dephosphorylated forms of C/EBP β from the nucleus. Taken together, our results provide the first evidence that ceramide may act as a negative regulator of adipogenesis; in part, by inhibiting the transactivating capacity of C/EBP β .

EXPERIMENTAL

Materials

3T3-L1 and HEK-293 cells were obtained from the A.T.C.C. [32 P] P_i was obtained from DuPont New England Nuclear (Boston, MA, U.S.A.). *N*-[3 H]Acetylsphingosine ([3 H] C_2 -ceramide; 25 mCi/mmol) was kindly given by Dr A. Bielawska (Medical University of South Carolina, Charleston, SC, U.S.A.). C_2 -ceramide and *D*- and *L*-(1*S*,2*R*)-*erythro*-2-(*N*-myristoylamino)-1-phenyl-1-propanol (*D-e*- and *L-e*-MAPP respectively) were products of Matreya (Pleasant Gap, PA, U.S.A.). Dexamethasone, insulin, methylisobutylxanthine, Oil Red O and leptomycin B were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). The dual reporter luciferase assay was from Promega (Madison, WI, U.S.A.). Polyclonal antibodies raised against C/EBP β , C/EBP α and PPAR γ were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Monoclonal antibody against nucleoporin was from B&D Signal Transduction Laboratories (San Diego, CA, U.S.A.); Alexa-Fluor 488 and 568 secondary antibodies were purchased from Molecular Probes (Eugene, OR, U.S.A.). cDNAs for pMEX-C/EBP β and the firefly luciferase reporter constructs, (DE-I) $_4$ (-35Alb)LUC and (-35Alb)LUC were kindly given by Dr Simon Williams, and have been described previously [21].

Cell lines and differentiation protocol

3T3-L1 preadipocytes were subcultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) calf serum. Adipogenesis was induced using published protocols [20,22]. The cells were grown to confluence and maintained in DMEM/10% (v/v) calf serum for 2 days before the addition of the differentiation cocktail. The cells were induced to differentiate on day 0 by the addition of DMEM containing 2 μ M dexamethasone, 2 μ g/ml insulin, 0.5 mM methylisobutylxanthine and 10% (v/v) fetal calf serum (FCS). We refer to this differentiation cocktail as 'DIMF'. After 2 days, the medium was replaced with DMEM containing 10% FCS supplemented with 2 μ g/ml insulin. Every 2 days, the medium was replaced with DMEM containing 10%

(v/v) FCS without additional insulin. HEK-293 cells were maintained in DMEM containing 10% calf serum.

Ceramide treatment of 3T3-L1 cells and assessment of adipose phenotype

At the time of addition of DIMF, 3T3-L1 preadipocytes were co-treated with either vehicle [0.1% (v/v) DMSO] or the indicated concentration of C_2 -ceramide. Similar to protocols for inhibiting adipogenesis with retinoic acid [20], the C_2 -ceramide remained present during the first 2 days of differentiation and each subsequent media change was supplemented with vehicle or 20 μ M C_2 -ceramide. In experiments using *D/L-e*-MAPP, the cells were co-treated at the time of DIMF addition and the drugs resupplemented with each medium change. After 7 days of differentiation, the development of the mature adipose phenotype was assessed by the formation of lipid-laden droplets using the triacylglycerol-binding dye Oil Red O [20].

Immunoblot analysis and immunoprecipitation (IP)

On the indicated day, the cells were scraped into lysis buffer [60 mM Tris/HCl (pH 8.0)/1% (w/v) SDS], boiled for 2 min, vortex-mixed, and boiled again for 5 min. An equal amount of protein from each day's lysate was subjected to SDS/PAGE using 10% (w/v) acrylamide gels. Proteins were transferred to nitrocellulose membranes, and an equal loading was verified by staining the membrane with 0.5% Ponceau S in 5% (w/v) trichloroacetic acid. The membranes were probed with the indicated primary antibodies and a horseradish-peroxidase-conjugated secondary antibody. Immunoreactive proteins were visualized using the Amersham ECL[®] reagents as described by the manufacturer. For IP, the cells were scraped into IP buffer [50 mM Tris/HCl (pH 7.4)/150 mM NaCl/1 mM EDTA/1% (v/v) Nonidet P40/0.5 mM sodium orthovanadate/40 mM NaF/10 mM β -glycerophosphate/Complete protease inhibitors (Roche Diagnostics, Mannheim, Germany)] and C/EBP β was immunoprecipitated using 4 μ g of primary antibody/mg of cell lysate for 2 h at 4 °C. Immune complexes were formed by the addition of Protein A-Sepharose for 1 h at 4 °C; the isolated immune complexes were then washed three times with IP buffer, and subjected to SDS/PAGE.

Transient transfections and dual luciferase assays

A dual luciferase assay was used to examine the effect of ceramide on C/EBP β -dependent transactivation. The assay permits analysis of the effector-driven transactivation of the reporter construct containing firefly luciferase and the concomitant assessment of transfection efficiency by monitoring the constitutive activity of luciferase from the sea pansy *Renilla reniformis*. 3T3-L1 preadipocytes were grown to confluence and transiently co-transfected with C/EBP β cDNA, the reporter construct (DE-I) $_4$ (-35Alb)LUC and the control reporter plasmid pRL, which contains cDNA encoding luciferase from *R. reniformis*. (-35Alb)LUC was used in separate transfections as a negative control, since it lacks the four DE-I C/EBP β binding sites. The cells were transfected with 2 μ g of total DNA using LIPOFECTAMINE[™]. The proportions of C/EBP β cDNA:firefly luciferase reporter plasmid:sea pansy luciferase reporter plasmid were 50:50:1. After transfection (5 h), the cells were treated with DIMF in the absence or presence of C_2 -ceramide, and cell lysates were prepared 24 h later. HEK-293 cells were transfected similarly, and after 5 h these were treated with vehicle or the indicated concentration

of C₂-ceramide. Luciferase activity was determined 24 h after the addition of C₂-ceramide using the Dual Luciferase Assay Kit (Promega).

Nuclear extraction and electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared essentially as described previously [23]. 3T3-L1 preadipocytes were washed once in ice-cold PBS, treated with trypsin and collected by centrifugation at 250 *g* for 10 min at 4 °C. The supernatant was removed, and the cell pellet was re-suspended in 5 vol. of Buffer A [10 mM Hepes (pH 7.9)/1.5 mM MgCl₂/10 mM KCl/0.5 mM dithiothreitol/0.5 mM PMSF]. The samples were incubated on ice for 10 min, and centrifuged as described above. The pellet was re-suspended in 3 vol. of Buffer A, Nonidet P40 was added to a final concentration of 0.05 % (v/v), and the samples were homogenized to release the nuclei. The nuclei were pelleted by centrifugation at 250 *g* for 10 min at 4 °C, re-suspended with an equal volume of Buffer C [5 mM Hepes (pH 7.9)/26 % (v/v) glycerol/1.5 mM MgCl₂/0.2 mM EDTA/0.5 mM dithiothreitol/0.5 mM PMSF], and NaCl was added to a final concentration of 300 mM. The samples were mixed well, incubated on ice for 30 min, and centrifuged at 24000 *g* for 20 min at 4 °C. The supernatant was quickly frozen in a solid-CO₂/ethanol bath, and stored at -80 °C until use. The EMSA was performed using the Geneka C/EBP- β NuShift kit, as described in the manufacturer's protocol. Protein-DNA complexes were resolved at 200 V on a pre-run 6 % non-denaturing Tris/borate/EDTA ('TBE') gel (1 $\times$ TBE = 45 mM Tris/borate/1 mM EDTA) at 4 °C. The gel was dried, and the bands were visualized by autoradiography.

Immunofluorescence analysis

3T3-L1 preadipocytes were grown on poly(D-lysine)-coated coverslips. After treatment, coverslips were gently washed three times with ice-cold PBS, and fixed with 300 μ l of PBS containing 2 % (w/v) paraformaldehyde, 3 % sucrose and 0.02 % sodium azide for 10 min at 25 °C. The coverslips were again washed three times in PBS, and blocked with 5 % (w/v) BSA in PBS for 10 min at 25 °C. After washing three times in PBS, the cells were permeabilized with 0.2 % (v/v) Triton X-100 in PBS for 5 min at 25 °C. The coverslips were again washed three times in PBS, and then incubated in 5 % BSA in PBS containing the indicated primary antibody for 30 min at 25 °C. The coverslips were washed six times in PBS, and incubated in 5 % BSA in PBS containing either AlexaFluor 488 (green) goat anti-rabbit or AlexaFluor 568 (red) rabbit anti-mouse secondary antibodies for 30 min at 25 °C in the dark. After washing six times in PBS, anti-fade solution (Molecular Probes) was added, and the coverslips were mounted on glass slides. Images were captured on a BioRad MRC 1000 confocal microscope.

RESULTS

C₂-ceramide inhibits adipogenesis

C₂-ceramide is a cell-permeant form of ceramide that has been used extensively to identify ceramide as a mediator of numerous biological responses [24]. To determine the effect of ceramide on adipogenesis, 3T3-L1 preadipocytes were co-treated with vehicle or C₂-ceramide at the time of induction (day 0) with DIMF (see the Experimental section). At 7 days after the initiation of differentiation, the cells were fixed, and the lipid droplets were stained with Oil Red O. Figure 1(A) shows that DIMF induced extensive differentiation of the control cells to mature adipocytes, as shown by the rounded morphology full of red-stained lipid

droplets [6]. In contrast, simultaneous addition of 40–50 μ M C₂-ceramide with DIMF at day 0 strongly decreased the level of differentiated cells at day 7 (Figures 1B and 1C). The inhibitory effect of ceramide on adipogenesis was not limited to focal fields of cells, since macroscopic inspection of the plates of Oil Red O-stained cells revealed extensive inhibition of cell differentiation (Figure 1D).

C₂-ceramide induces apoptosis in many cell systems at the concentrations necessary to inhibit adipogenesis. However, we observed no cell death in the C₂-ceramide-treated preadipocytes, indicating that the block to differentiation is not related to fewer surviving cells. Indeed, C₂-ceramide can be extensively bound by the high concentration of serum proteins present in the differentiation cocktail [25]. To provide a more accurate estimate of the effective concentration of C₂-ceramide required to inhibit adipogenesis, we examined the uptake of 50 μ M [³H]C₂-ceramide (tritium label in sphingoid backbone) by the cells. Preadipocytes were co-treated with DIMF and 50 μ M [³H]C₂-ceramide, and the amount of cell-associated radiolabel present was determined at intervals up to 12 h. The uptake of [³H]C₂-ceramide was rapid, and reached a steady-state level within 2 h (Table 1). The cells incorporated approx. 20 % of the radiolabel at the steady state, suggesting that the effective concentration for inhibiting adipogenesis of the 3T3-L1 cells is approx. 8–10 μ M C₂-ceramide. Importantly, the cell-associated radioactivity at all time points was primarily [³H]C₂-ceramide, and not a metabolite (results not shown). In this regard, other lipids, such as sphingosine, sphingomyelin and phosphatidylcholine, had no effect on adipogenesis (results not shown). Moreover, addition of 50 μ M C₂-dihydroceramide at the time of differentiation was completely ineffective at inhibiting development of the mature adipose phenotype, on the basis of the extent of staining with Oil Red O (see below). C₂-dihydroceramide lacks the 4,5-*trans*-double bond found in ceramide, and, in most cell systems, is unable to mimic the biological responses induced by C₂-ceramide. Taken together, these data suggest that exogenous C₂-ceramide is a potent lipid inhibitor of adipogenesis, similar to retinoic acid [20] and prostaglandin F_{2 α} [26].

C₂-ceramide inhibits expression of C/EBP- α and PPAR- γ

The expression of C/EBP- α and PPAR- γ is necessary for terminal differentiation of adipocytes [7]. Therefore the block to adipogenesis by C₂-ceramide suggested that the expression and/or activity of these transcription factors might be affected by ceramide. Similar to many previous reports, DIMF induced a significant production of C/EBP α and PPAR γ expression, beginning 3 days after the induction of differentiation (Figure 2). However, C₂-ceramide strongly decreased the expression of both C/EBP α and PPAR γ (Figure 2A). The effect of C₂-ceramide on the expression of C/EBP α on day 3 following hormonal stimulation was dose-dependent (Figure 2B, upper panel) and specific, since 50 μ M C₂-dihydroceramide could not inhibit C/EBP- α expression (Figure 2B, lower panel).

To examine the possibility that the inhibition of C/EBP α and PPAR γ expression by C₂-ceramide was purely a pharmacological effect, we increased the levels of intracellular ceramide with the alkaline ceramidase inhibitor, D-*e*-MAPP [27]. Preadipocytes were co-treated with D-*e*-MAPP and DIMF on day 0, the medium was replaced after 2 days (plus fresh D-*e*-MAPP), and cell lysates were prepared on day 3 following DIMF treatment. Treatment of preadipocytes with 50–70 μ M D-*e*-MAPP increased intracellular ceramide levels up to 1.7-fold by 24 h (results not shown). Similar to exogenous C₂-ceramide, D-*e*-MAPP also blocked the expression of C/EBP- α on day 3 after differentiation

Figure 1 C₂-ceramide inhibits development of the adipose phenotype

Post-confluent preadipocytes were co-treated with DIMF in the absence or presence of C₂-ceramide. The cells were differentiated for 7 days and lipid accumulation was determined by staining with Oil Red O. (A) Control cells show extensive lipid accumulation, while cells treated with (B) 40 μM or (C) 50 μM C₂-ceramide accumulate lipid poorly. (D) Macroscopic views of the plates from which microscopic fields in (A)–(C) were chosen. Plates shown are representative of several experiments.

Table 1 Kinetics of [³H]C₂-ceramide uptake by preadipocytes

Post-confluent preadipocytes were co-treated with DIMF and 50 μM [³H]C₂-ceramide (100 000 c.p.m./dish). At the indicated time, the medium was aspirated (after extracting an aliquot), and the cells were washed twice with ice-cold PBS. The cells were scraped into PBS, homogenized and an aliquot was used to determine the amount of cell-associated radiolabel. The amount of cell-associated [³H]C₂-ceramide is presented as a percentage of the total C₂-ceramide recovered from the medium and cells. Data shown are the means ± S.E.M. for three experiments.

Time (h)	Cell-associated [³ H]C ₂ -ceramide (% of the total)
0	4.8 ± 2.6
0.5	16.2 ± 3.7
1.0	18.2 ± 2.6
1.5	20.2 ± 3.7
2	20.6 ± 3.0
4	20.6 ± 3.6
12	18.7 ± 1.0

(Figure 3). To determine the specificity of D-*e*-MAPP on inhibiting the expression of C/EBP-α, the cells were treated with the inactive enantiomer, L-*e*-MAPP [27]. Not surprisingly, L-*e*-MAPP neither increased ceramide levels nor inhibited the expression of C/EBP-α (Figure 3). These data support the notion that C₂-ceramide effectively mimics the effect of endogenous ceramides.

Preliminary studies indicated that addition of C₂-ceramide more than 48 h after differentiation did not block expression of C/EBP-α. To identify the temporal 'window' where ceramide was most effective at inducing downstream changes in the

expression of C/EBP-α, C₂-ceramide was added simultaneously with DIMF (0 h), or 2, 4, 6, 8 or 24 h after the addition of DIMF (Figure 4). Cell lysates were then prepared on day 3 of differentiation to examine the expression of C/EBP-α. The magnitude of C/EBP-α expression at day 3 from cells treated with DIMF plus 0.1 % (v/v) DMSO was used as the control in these experiments (Figure 4, 'Control'). The expression of C/EBP-α was strongly blocked when C₂-ceramide was added within the first 4 h after the induction of differentiation (Figure 4, lanes pertaining to 0–4 h). However, adding C₂-ceramide later than 4 h after DIMF resulted in a gradual loss in the block to C/EBP-α expression. If ceramide was added 24 h following DIMF, it was ineffective at inhibiting the expression of C/EBP-α. These results suggest that C₂-ceramide is affecting an early event in adipogenesis.

Ceramide inhibits DNA binding and C/EBPβ-mediated transcriptional activation

An early event in adipogenesis of 3T3-L1 preadipocytes is the rapid up-regulation and nuclear translocation of C/EBPβ [15]. Moreover, C/EBPβ is known to undergo extensive phosphorylation by various kinases, such as p42/44 mitogen-activated protein kinase (MAPK), p38 MAPK, protein kinase C and Ca²⁺/calmodulin protein kinase [14,28]. Since the expression of C/EBPβ is necessary for mediating transcription of C/EBP-α and PPARγ [20], we examined the effect of ceramide on the expression, phosphorylation and activity of this transcription factor.

C₂-ceramide had no effect on the total cellular expression of C/EBPβ (Figure 5, lower panel), but had a biphasic effect on its

Figure 2 C₂-ceramide inhibits the expression of C/EBPα and PPARγ

(A) Post-confluent preadipocytes were co-treated with DIMF in the absence or presence of 50 μM C₂-ceramide. Immediately following co-treatment (Day 0), and daily thereafter, cell lysates were prepared, 10 μg of protein was separated by SDS/PAGE, and the expression of C/EBPα and PPARγ was determined by immunoblot analysis. (B) Post-confluent preadipocytes were co-treated with DIMF and the indicated concentration of C₂-ceramide (upper panel) or 50 μM C₂-ceramide or C₂-dihydroceramide (lower panel). After the induction of differentiation (3 days), cell lysates were prepared, 10 μg of protein was separated by SDS/PAGE and the expression of C/EBPα was determined by immunoblot analysis. Data shown are representative of experiments performed at least three times.

Figure 3 Increasing endogenous ceramide levels inhibits expression of C/EBPα

Post-confluent preadipocytes were co-treated with DIMF in the absence or presence of the indicated concentration of D-e-MAPP (upper panel), or its inactive enantiomer L-e-MAPP (lower panel). After the induction of differentiation (3 days), cell lysates were prepared, 10 μg of protein was separated by SDS/PAGE and the expression of C/EBPα was determined by immunoblot analysis. Results shown are representative of those obtained from three experiments.

phosphorylation. C₂-ceramide slightly enhanced phosphorylation of C/EBPβ at 4 and 8 h, but strongly decreased its phosphorylation at 24–48 h (Figure 5, upper panel). After normalizing for C/EBPβ expression, ceramide decreased its phosphorylation by $57.8 \pm 1.3\%$ and $67.4 \pm 0.1\%$ ($n = 3$) at 24 and 48 h respectively. Consistent with a previous report that phosphorylation of C/EBPβ at 24–48 h is critical for it to become competent to bind DNA and induce expression of C/EBPα [15], the ceramide-induced decrease in C/EBPβ phosphorylation at 24 h correlated with a decrease in its DNA-

Figure 4 C₂-ceramide is maximally effective at decreasing C/EBPα expression within 4 h after the induction of differentiation

Post-confluent preadipocytes were treated with DIMF in the absence of ceramide and allowed to differentiate for 3 days. Cell lysates were prepared, and the expression of C/EBPα was analysed by immunoblot analysis (lane 1, Control). To determine the time course for inhibition of C/EBPα expression by ceramide, 50 μM C₂-ceramide was added simultaneously with DIMF (time 0) or at the indicated time after the addition of DIMF. The cells were differentiated for 3 days, cell lysates were prepared and the expression of C/EBPα was determined by immunoblot analysis. The extent of C₂-ceramide-induced changes in C/EBPα was semi-quantified by densitometry, expressed as percentages of the control (lane 1), and is featured below each lane. Data shown are from a representative experiment performed three times with similar results.

Figure 5 C₂-ceramide decreases the phosphorylation of C/EBPβ, but not its total cellular expression

Post-confluent preadipocytes were labelled with 0.2 mCi/ml [³²P]P_i overnight, and the medium was supplemented with DIMF in the presence or absence of 50 μM C₂-ceramide. At the indicated time following DIMF addition, the cells were scraped into IP buffer and C/EBPβ was immunoprecipitated. The isolated proteins were resolved by SDS/PAGE, transferred to nitrocellulose and the presence of [³²P]C/EBPβ was determined using a PhosphorImager (upper panels). The total levels of C/EBPβ were then determined by immunoblot analysis (lower panels). Results are representative of those obtained in three experiments.

binding ability (Figure 6; compare the third lane from the left with the lane furthestmost on the right). C/EBPβ bound specifically to the radiolabelled probe, since addition of wild-type unlabelled competitor oligonucleotide completely blocked the formation of DNA–protein complexes (Figure 6, lane 4 from the left), but a mutant oligonucleotide was ineffective (Figure 6, lane 5 from the left). The identity of C/EBPβ as the major component of the protein–DNA complexes was verified by the nearly complete supershift of the complexes by a C/EBPβ antibody (Figure 6, lane 6 from the left). Although C/EBPβ appears to be the major component of these DNA–protein complexes, the remaining unshifted band is likely to be due to the presence of some C/EBPδ in the complexes, as reported previously [15]. Lastly, addition of C₂-ceramide directly to a reaction mix containing recombinant C/EBPβ and the labelled oligonucleotide probe had no effect on C/EBPβ binding (results not shown). These results argue against ceramide directly interfering with the ability of C/EBPβ to bind to the probe.

To examine whether the ceramide-induced decrease in the DNA-binding ability of C/EBPβ correlated with decreased transcriptional activity, confluent 3T3-L1 preadipocytes were transiently transfected with the reporter construct (DE-I)₄(–35 Alb)luc. Following transfection (5 h), the cells were co-treated with DIMF plus vehicle, or 30–40 μM C₂-ceramide; the cells

Figure 6 C₂-ceramide decreases C/EBPβ-mediated DNA binding

Post-confluent preadipocytes were co-treated with DIMF and 50 μM C₂-ceramide. After 24 h, nuclear extracts were prepared and an EMSA for C/EBPβ DNA binding was conducted using 10 μg of each nuclear extract and reagents provided in the NuShift kit. A positive control for C/EBPβ binding was performed using recombinant C/EBPβ (second lane from the left).

Figure 7 C₂-ceramide inhibits C/EBPβ-mediated transcription

Post-confluent preadipocytes were transiently co-transfected with (A) pCDNA or (B) C/EBPβ and the two luciferase reporter constructs. After transfection (5 h), the cells were co-treated with DIMF in the absence or presence of C₂-ceramide. Dual luciferase activity was measured 24 h later as described in the Experimental section. The asterisk indicates statistical significance ($P < 0.05$), as determined using a one-way ANOVA and a Student–Newman–Keuls *post-hoc* test. Data shown are the means $\pm$ S.E.M. from a representative experiment performed in quadruplicate. The experiment was repeated four times with comparable results.

were exposed to less C₂-ceramide, since the transfection protocol rendered the cells sensitive to higher lipid concentrations, resulting in cell death. After differentiation (24 h), cell lysates were prepared, and dual luciferase activity was measured. C₂-ceramide-treated preadipocytes co-transfected with the reporter construct (DE-I)₄(–35 Alb)LUC and pRL showed a statistically significant 20–30 % decrease in transactivation from the DE-I binding sites (Figure 7A). Since little transactivation was apparent using the (–35 Alb)LUC construct in the absence or presence of ceramide, fold activation was calculated on the basis of the very basal level of activity from this construct.

Figure 8 Reconstitution of the inhibitory effect of C₂-ceramide on C/EBPβ-dependent transcription in HEK-293 cells

HEK-293 cells were transiently co-transfected with pcDNA or C/EBPβ and the two luciferase reporter constructs. After 5 h, the cells were treated with 0.1% (v/v) DMSO or the indicated concentration of C₂-ceramide, and dual luciferase activity was measured 24 h later. (A) Expression levels of C/EBPβ in HEK-293 cells transfected with pcDNA (lanes 1–3) or C/EBPβ (lanes 4–5). Cell lysates were prepared 24 h after treatment with vehicle or C₂-ceramide, and the presence of C/EBPβ was determined by immunoblot analysis. (B) Effect of C₂-ceramide on C/EBPβ-driven transcription. Results are expressed as fold control relative to luciferase activity from cells receiving C/EBPβ cDNA, but no C₂-ceramide. The asterisks indicate statistical significance ($P < 0.05$), as determined using a one-way ANOVA and a Student–Newman–Keuls *post-hoc* test. Data shown are the means $\pm$ S.E.M. from four experiments performed in duplicate.

To provide further support that C₂-ceramide was blocking transactivation of luciferase from the DE-I binding sites, preadipocytes were co-transfected with C/EBPβ cDNA in the presence of (DE-I)₄(–35 Alb)LUC and pRL. If C₂-ceramide was inhibiting the transactivating capacity of C/EBPβ, then this inhibition should be more apparent in cells further overexpressing C/EBPβ. Preadipocytes transfected with exogenous C/EBPβ and treated with DIMF in the absence of C₂-ceramide showed a 5-fold increase in baseline transactivation from (DE-I)₄(–35 Alb)LUC (Figure 7B). However, treatment with 30–40 μM C₂-ceramide still significantly diminished transactivation by up to 40 % from the (DE-I)₄(–35 Alb)LUC reporter. Taken together, these results suggest that ceramide, similar to retinoic acid [20], might block adipogenesis at the level of C/EBPβ.

To examine whether the effect of C₂-ceramide on inhibiting transactivation by C/EBPβ was a cell-specific phenomenon, we attempted to reconstitute the response in cells that do not endogenously express C/EBPβ. Immunoblot analysis of whole-cell lysates from HEK-293 cells revealed a very low level of specific immunoreactivity for the presence of endogenous C/EBPβ (Figure 8A). Therefore the HEK-293 cells were transiently co-transfected with C/EBPβ cDNA and the various reporter plasmids. After transfection (5 h), the cells were treated with

Figure 9 C₂-ceramide specifically decreases the nuclear localization of C/EBPβ

(A) Nuclear extracts were prepared from post-confluent preadipocytes co-treated with DIMF and 50 μM C₂-ceramide (upper panel) or C₂-dihydroceramide (lower panel). The nuclear proteins were subjected to SDS/PAGE, transferred to nitrocellulose, and the level of C/EBPβ was determined by immunoblot analysis. The effect of C₂-dihydroceramide on nuclear localization of C/EBPβ was determined only at 24 h. (B) Preadipocytes were grown to 2 days post-confluence on coverslips and treated with DIMF in the absence (Control) or presence of 50 μM C₂-ceramide. The cells were co-stained with an antibody raised against C/EBPβ (green; AlexaFluor 488 anti-rabbit IgG secondary antibody) and the nuclear membrane marker protein, nucleoporin (red; AlexaFluor 568 anti-mouse IgG secondary antibody). The fluorescent signals were analysed by confocal microscopy, and the green and red signals from the same focal fields were merged to clearly demarcate the nucleus. Cells stained only with the AlexaFluor 488 or 568 secondary antibodies showed no specific immunofluorescence (results not shown). (C) Post-confluent preadipocytes were co-treated with DIMF and 50 μM C₂-ceramide at the indicated times, and nuclear extracts were prepared. The nuclear proteins were subjected to SDS/PAGE, transferred on to nitrocellulose and the level of STAT-1 was determined by immunoblot analysis. (D) Preadipocytes were grown to 2 days post-confluence on coverslips, and treated with DIMF in the absence or presence of 50 μM C₂-ceramide. The cells were co-stained with an antibody against STAT-1 (green) and the nuclear membrane marker protein, nucleoporin (red).

C₂-ceramide in 10% (v/v) FCS, and luciferase activity was determined after an additional 24 h. C₂-ceramide caused a significant dose-dependent inhibition of C/EBPβ-mediated transcription in the HEK-293 cells (Figure 8B), which proved less

susceptible to higher concentrations of C₂-ceramide following the transfection protocol. The decreased extent of transcription was not due to differences in the expression of C/EBPβ (Figure 8A) or to ceramide-induced cell death, since no loss of cell

Figure 10 Leptomycin B reverses the C_2 -ceramide-induced decrease in the *in vivo* nuclear localization of C/EBP β

Preadipocytes were grown on coverslips and at 2 days post-confluence were treated with DIMF in the absence (A, C and E) or presence (B, D and F) of 50 μ M C_2 -ceramide for the indicated times. To examine the effect of leptomycin B on the nuclear localization of C/EBP β at 24 h, the cells were treated with 10–15 ng/ml leptomycin B plus DIMF in the absence (G and I) or presence (H and J) of 50 μ M C_2 -ceramide for 24 h. All cells were co-stained with an antibody against C/EBP β (green; AlexaFluor 488 anti-rabbit IgG secondary antibody) and the nuclear membrane marker protein, nucleoporin (red; AlexaFluor 568 anti-mouse IgG secondary antibody). The fluorescent signals were analysed by confocal microscopy, and the green and red signals from the same focal fields were merged so that the nuclear localization of C/EBP β can be clearly demarcated by the staining of the nuclear membrane with nucleoporin.

viability was observed between control and ceramide-treated cells. Importantly, no luciferase activity was observed in the absence of C/EBP β expression (results not shown). These data strongly support the notion that the ability of C_2 -ceramide to inhibit the transactivating capacity of C/EBP β is not necessarily limited to the context of C/EBP β expression during adipogenesis.

Ceramide decreases nuclear levels of C/EBP β and enhances nuclear export of C/EBP β

The differentiation of 3T3-L1 cells induces a rapid expression of C/EBP β and, within 4 h, the total cellular pool is localized to the

nucleus, where its level remains steady for approx. 48 h [15]. Since ceramide did not decrease the total cellular level of C/EBP β , but did decrease its phosphorylation, DNA binding and ability to promote transcription, it is possible that changes in nuclear levels of C/EBP β may account for these effects. To address this issue, cells were treated with DIMF in the absence and presence of C_2 -ceramide, nuclear extracts were prepared and C/EBP β levels were determined by immunoblot analysis. Ceramide slightly increased the nuclear levels of C/EBP β at 4 h but, after 24 h of treatment, its nuclear level was strongly decreased relative to control cells (Figure 9A, upper panel). The ceramide-induced decrease in the nuclear localization of C/EBP β was specific, since

treating the cells with C_2 -dihydroceramide had no effect on its nuclear localization (Figure 9A, bottom panel). This result is very consistent with the lack of effect of C_2 -dihydroceramide on inhibiting the expression of C/EBP α .

To provide a more accurate *in vivo* measure of the effect of ceramide on the nuclear localization of C/EBP β , indirect immunofluorescence and confocal microscopy was performed. As reported previously [15], DIMF induced an intense punctate nuclear staining of C/EBP β (Figure 9B). In agreement with the immunoblot results, C_2 -ceramide strongly decreased the intense nuclear localization for C/EBP β and induced a more diffuse cytosolic staining at 24 h (Figure 9B). Importantly, ceramide did not decrease the nuclear localization *in vivo* of an unrelated transcription factor, signal transducer and activator of transcription 1 (STAT-1; Figures 9C and 9D), suggesting that the lipid was not non-specifically disrupting general nuclear trafficking.

C_2 -ceramide may decrease nuclear levels of C/EBP β by blocking import, increasing export or by enhancing nuclear degradation of the protein. In agreement with immunoblot analysis for the presence of C/EBP β from nuclear extracts, C_2 -ceramide did not alter the nuclear accumulation of C/EBP β after a period of 4 and 16 h, when analysed by indirect immunofluorescence (Figure 10, panels A–D, the green signal). However, ceramide again induced a significant loss of C/EBP β from the nucleus at 24 h (Figure 10, panels E and F). These results suggested that ceramide was not inhibiting the nuclear import of C/EBP β . Alternatively, the rather abrupt decrease in nuclear levels of C/EBP β occurring between 16–24 h raised the possibility that changes in the rate of nuclear export may occur. Nuclear export can be regulated by the interaction of proteins containing a specific leucine-rich nuclear export sequence with CRM1/exportin 1 [29]. Leptomycin B inhibits nuclear export by blocking the interaction of proteins containing a nuclear export sequence with CRM1/exportin 1 [30,31]. Treatment of preadipocytes with 10–15 ng/ml leptomycin B reversed the ceramide-induced decrease in the nuclear levels of C/EBP β at 24 h (Figure 10, panels H and J), but had no effect on the nuclear level of C/EBP β in control cells (Figure 10, panels G and I). Collectively, these results provide a strong temporal correlation that ceramide may block adipogenesis by decreasing phosphorylation of C/EBP β and enhancing its nuclear export at a time when the maximal transcriptional activity of this protein is required to drive adipogenesis [15].

DISCUSSION

The cellular response to ceramide is quite variable, and is likely to be dependent upon cell type, developmental stage and the magnitude and kinetics of the ceramide flux [1]. Although the role of ceramide is poorly defined with respect to adipocyte development and function, our results suggest that elevation of endogenous ceramide levels is sufficient to block adipogenesis. However, these data do not address the necessity/sufficiency of ceramide as a physiological regulator of adipogenesis. Treatment of the cells with D-e-MAPP increased endogenous ceramide levels approx. 1.7-fold, similar to levels induced by numerous ligands, such as interferon- γ , interleukin-1 β and tumour necrosis factor α (TNF- α) [1], which inhibit adipogenesis [32,33]. These results raise the possibility that ceramide production may be sufficient to mediate the inhibitory effects of these ligands on adipogenesis. On the other hand, once adipogenesis is complete, ceramide may contribute to, but not be sufficient to mimic, some of the metabolic effects exerted by cytokines on mature adipocytes. For example, addition of 50 μ M C_2 -ceramide or elevation

of endogenous ceramide with a glucosylceramide synthase inhibitor was not sufficient to inhibit insulin receptor phosphorylation [34]. In contrast, ceramide production was necessary for the effect of TNF α in inhibiting insulin receptor phosphorylation, since partial inhibition of ceramide production reversed the block to insulin receptor phosphorylation by TNF α [34]. Lastly, adipocytes may also affect ceramide metabolism. In obese animals, the release of free fatty acids from triacylglycerol stores in adipocytes may accelerate ceramide synthesis *de novo* in pancreatic islet cells, leading to apoptosis and decreased insulin production [35]. Taken together, these results suggest that ceramide may be important in the regulation of adipocyte differentiation and function, and contribute to the development of diabetes in the obese.

Ceramide is not the first lipid shown to inhibit adipogenesis of 3T3-L1 cells. Indeed, retinoic acid [36] and prostaglandin F2 α [26] both inhibit adipocyte differentiation. Since prostaglandin F2 α inhibits adipogenesis via phosphorylation and inactivation of PPAR γ [26], its mechanism is distinct from ceramide, which blocks PPAR γ expression. Similar to ceramide, retinoic acid partially blocks mitotic clonal expansion, does not affect expression of endogenous C/EBP β and inhibits C/EBP β -mediated transcription [20,22]. In contrast with ceramide, retinoic acid can effectively inhibit adipogenesis when added up to 48 h after hormonal induction [22,36]. Although retinoic acid may increase ceramide levels in certain cells [37], molecular data have strongly suggested that the interaction of the liganded retinoic acid receptor with C/EBP β mediates the block to transcription and inhibits adipogenesis [20]. Ceramide is not known to interact with nuclear hormone receptors for retinoids, and retinoic acid has not been demonstrated to decrease the phosphorylation and nuclear localization of C/EBP β . Thus the mechanisms by which retinoic acid and ceramide inhibit adipogenesis may be fundamentally different, but converge upon the regulation of C/EBP β .

Recent data supports the hypothesis that the phosphorylation of C/EBP β occurs concomitantly with the acquisition of DNA-binding ability and, presumably, increased transcriptional activity towards promoters for genes such as C/EBP α [15]. Indeed, treatment of nuclear extracts from hormonally stimulated preadipocytes with alkaline phosphatase decreased the DNA-binding ability of C/EBP β [15]. These observations correlate very well with the effect of ceramide on decreasing the phosphorylation of C/EBP β and diminishing its DNA-binding ability and transactivation potential at 24 h. Moreover, our data on the punctate nuclear staining of C/EBP β agree with previous results indicating that it co-localizes to centromeres, and that this localization may have functional consequences in controlling mitotic clonal expansion [15]. Our data would be consistent with this hypothesis, since ceramide partially blocked clonal expansion at 24–48 h (20–60%; results not shown), which also correlates with the decreased phosphorylation of C/EBP β and its loss of nuclear localization. Since C/EBP β is phosphorylated on multiple sites, it will be necessary to identify the ceramide-inhibited kinase(s) or ceramide-activated phosphatases involved in regulating C/EBP β to determine whether changes in C/EBP β phosphorylation are a cause or consequence of increased nuclear export. However, we observed that ceramide did not inhibit p42/p44 MAPK or p38 MAPK, both of which may phosphorylate C/EBP β .

A novel and unexpected result from these studies is that ceramide specifically induced a significant change in the nuclear localization of C/EBP β at 24 h after hormonal induction of the preadipocytes. Indeed, C_2 -dihydroceramide had little effect on the nuclear pool of C/EBP β . The loss of nuclear C/EBP β could result from decreased import or enhanced export. In our opinion, several pieces of evidence are supportive of the notion that

enhanced export is a more probable explanation, since: (i) the levels of nuclear C/EBP β were similar between ceramide-treated and control cells at 4 h and 16 h after hormonal induction; (ii) the total cellular levels of C/EBP β did not change between ceramide-treated and control cells, suggesting that increased degradation did not account for the loss of C/EBP β ; and (iii) the ceramide-induced loss of nuclear C/EBP β was reversed by treatment of the cells with leptomycin B. If enhanced nuclear export of C/EBP β was responsible for the ceramide-induced decrease in the expression of C/EBP α , then leptomycin B might be expected to reverse this effect. Unfortunately, treatment of preadipocytes with leptomycin B alone strongly blocked the expression of C/EBP α , suggesting that the nucleocytoplasmic shuttling of various transcription factors may be critical to adipogenesis. Thus, at this point, we cannot determine the exact contribution of the enhanced nuclear export of C/EBP β to the ceramide-induced block to adipogenesis.

Similar to our results, leptomycin B also blocked the nuclear export of C/EBP β from hepatocytes stimulated with TNF α , leading to decreased transcription of the albumin gene [31]. Chojkier and co-workers noted the presence of a putative consensus sequence for nuclear export within the leucine zipper region of C/EBP β [31]. Since leptomycin B inhibits the interaction of proteins containing a nuclear export sequence with CRM1/exportin 1 [29], our results provide additional support for the concept that C/EBP β may contain a nuclear export sequence, and that alterations in the nucleocytoplasmic shuttling of C/EBP β may affect its transcriptional activity. It is interesting that phosphorylation of C/EBP β on Ser²³⁹ enhanced nuclear export and decreased transcriptional activity in hepatocytes [31]. Although we observed a 50–70% decrease in the phosphorylation of C/EBP β by ceramide, it is possible that this may have occurred on residues other than Ser²³⁹. Thus we cannot determine whether enhanced nuclear export of C/EBP β in ceramide-treated adipocytes also requires this specific modification. Alternatively, mechanisms controlling nuclear export of C/EBP β may differ between TNF α -treated hepatocytes and differentiating preadipocytes. For example, other protein modifications of C/EBP β might also be involved in regulating its localization. Conjugation of transcription factors with the small ubiquitin-like molecule SUMO-1 is known to regulate their nuclear localization and transcriptional activity [38]. Moreover, at least in yeast, sumoylation (i.e. post-translational modification by SUMO-1 equivalent to that of ubiquitinylation with ubiquitin) might also regulate interactions between centromeric proteins [39]. Intriguingly, C/EBP β contains a consensus sumoylation sequence in its N-terminal region (Leu-Lys-Ala-Glu) [38], which raises the possibility that changes in phosphorylation and/or sumoylation of C/EBP β might affect its centromeric localization, transcriptional activity and rate of nuclear export.

In summary, our data provide a novel perspective on potential molecular mechanisms by which ceramide may regulate the activity of a transcription factor that is involved in adipogenesis and is up-regulated in response to inflammatory cytokines (e.g. TNF α , interleukin-1 β and interleukin-6), nerve growth factors, lipopolysaccharide, heavy metals, diabetes and age [14,40,41]. Since the activity and localization of C/EBP β are strongly regulated by phosphorylation, ceramide may have diverse effects on its transcriptional activity, depending upon the subset of kinases and/or phosphatases that may be activated by ceramide in a given cell type.

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