C2-Ceramide Influences the Expression and Insulin-Mediated Regulation of Cyclic Nucleotide Phosphodiesterase 3B and Lipolysis in 3T3-L1 Adipocytes

Jie Mei,1 Lena Stenson Holst,1 Tova Rahn Landström,1 Cecilia Holm,1 David Brindley,2 Vincent Manganiello,3 and Eva Degerman1

Cyclic nucleotide phosphodiesterase (PDE) 3B plays an important role in the antilipolytic action of insulin and, thereby, the release of fatty acids from adipocytes. Increased concentrations of circulating fatty acids as a result of elevated or unrestrained lipolysis cause insulin resistance. The lipolytic action of tumor necrosis factor (TNF)-α is thought to be one of the mechanisms by which TNF-α induces insulin resistance. Ceramide is the suggested second messenger of TNF-α action, and in this study, we used 3T3-L1 adipocytes to investigate the effects of C2-ceramide (a short-chain ceramide analog) on the expression and regulation of PDE3B and lipolysis. Incubation of adipocytes with 100 μmol/l C2-ceramide (N-acetyl-sphingosine) resulted in a time-dependent decrease of PDE3B activity, accompanied by decreased PDE3B protein expression. C2-ceramide, in a time- and dose-dependent manner, stimulated lipolysis, an effect that was blocked by H-89, an inhibitor of protein kinase A. These ceramide effects were prevented by 20 μmol/l troglitazone, an antidiabetic drug. In addition to downregulation of PDE3B, the antilipolytic action of insulin was decreased by ceramide treatment. These results, together with data from other studies on PDE3B and lipolysis in diabietic humans and animals, suggest a novel pathway by which ceramide induces insulin resistance. Furthermore, PDE3B is demonstrated to be a target for troglitazone action in adipocytes. Diabetes 51:631–637, 2002

A dipose tissue lipolysis is to a large extent determined by the intracellular concentration of cAMP (1), which is controlled at both the level of synthesis by adenylate cyclases and the level of degradation by phosphodiesterases (PDEs). Eleven PDE families have been identified, differing in their primary structures, affinities for cAMP and cGMP, responses to specific effectors and inhibitors, and mechanisms whereby they are regulated (2). The PDE3 gene family contains two members, PDE3A and PDE3B (3); PDE3B is mainly expressed in insulin-sensitive cells such as adipocytes (4), hepatocytes (5), and pancreatic β-cells (6). We have previously demonstrated that insulin-induced phosphorylation and activation of PDE3 is the major mechanism by which insulin antagonizes catecholamine-induced lipolysis in primary rat adipocytes (7–9) and 3T3-L1 adipocytes (10), leading to reduced release of free fatty acids (FFAs). This stimulation of PDE3B involves activation of phosphatidylinositol 3-kinase (PI3K) (11) and, most likely, protein kinase B (PKB) (12–14).

Elevated or unrestrained lipolysis leading to increased levels of circulating FFAs has been suggested to play an important role in the development of insulin resistance (15). The induction of systemic insulin resistance by FFAs has been explained by their ability to stimulate hepatic gluconeogenesis (16), increase very low–density lipoprotein (VLDL) secretion in hepatocytes (17), decrease insulin binding to hepatocytes (18), and inhibit insulin-induced glucose uptake in skeletal muscle cells (19). Furthermore, increased concentrations of circulating FFAs impair insulin secretion from pancreatic β-cells (20).

The role of PDE3B in the development of insulin resistance has not been unraveled, although previous studies indicate that downregulation of PDE3B protein (21) and/or dysregulation of PDE3B activity by insulin (22,23) correlates with insulin resistance or diabetic states in humans as well as animals (24–26). Downregulation of PDE3B has been suggested to contribute to the mechanism by which tumor necrosis factor (TNF)-α induces lipolysis and excess release of FFAs (27). Stimulation of 3T3-L1 adipocytes by TNF-α results in decreased PDE3B activity, increased activity of protein kinase A (PKA), and lipolysis. Although the mechanisms whereby TNF-α induces these...
changes are only partly understood, it has been indicated that TNF-α, via the p55 TNF receptor, can activate sphingomyelinase, resulting in production of ceramide (28–30).

Intramuscular levels of ceramide are elevated in skeletal muscle from diabetic rats (31,32). In 3T3-L1 adipocytes, ceramide inhibits insulin-induced glucose uptake and translocation of GLUT4 to the plasma membrane (33). Furthermore, in these cells, ceramide inhibits the insulin signaling cascade downstream of P3K (33,34). On the other hand, other reports demonstrate that ceramide can antagonize early events in insulin signaling, such as tyrosine phosphorylation of insulin receptor substrate (IRS) proteins and recruitment/activation of P3K (30,35). Very little is known regarding the effect of ceramide on the regulation of adipocyte lipolysis.

In the present study, we investigated the effects of ceramide on PDE3B expression/activity and lipolysis in 3T3-L1 adipocytes. We found that long-term treatment with ceramide induced downregulation of PDE3B, an effect associated with increased lipolysis. Expression of hormone-sensitive lipase (HSL), the rate-limiting enzyme of triglyceride hydrolysis, was, however, slightly decreased. The ceramide effects were reversed by troglitazone, an antidiabetic drug. Finally, the ability of insulin to activate PDE3B and inhibit lipolysis was diminished in 3T3-L1 adipocytes exposed to ceramide.

RESEARCH DESIGN AND METHODS

Materials. Murine-derived 3T3-L1 cells were from American Type Culture Collection (Manassas, VA). Polyclonal PDE3B antibodies were generated by immunizing rabbits with the peptide LRRSSGASGLLTSEHHSR, and the affinity-purified affinity-purified. Polyclonal HSL antibodies were generated by immunizing rabbits with recombinant rat HSL (36). Affinity purification was performed against the same protein coupled to cyanogen bromide (CNBr)-activated Sepharose CL-4B. Rabbit polyclonal pexoxisome proliferator-activated receptor-γ (PPAR-γ) antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-Akt (Ser473) antibody was from Cell Signaling Technology (Beverly, MA). Anti-PKB antibody was from Upstate Biotechnology (Lake Placid, NY). Anti-rabbit IgG antibody conjugated with horseradish peroxidase was from Amersham (Buckinghamshire, U.K.). Supersignal West Pico Luminol/Enhancer Solution was from Pierce (Rockford, IL). C2-ceramide and dihydro-C2-ceramide were from Biomol (Plymouth Meeting, PA). Dulbecco’s modified Eagle’s medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco (Gaithersburg, MD). NAD, glycerol-3-phosphate dehydrogenase, and glycerokinase were purchased from Boehringer Mannheim (Mannheim, Germany). ATP, bovine insulin, dexamethasone, 3-isobutyl-1-methylxanthine, aprotinin, leupeptin, pepstatin, and protein kinase inhibitor were from Sigma (St. Louis, MO).

Cell culture. 3T3-L1 fibroblasts were cultured in DMEM supplemented with 10% FBS and then differentiated as described previously (37). Adipocytes were used 9–14 days after induction of differentiation when >90% of the cells expressed the adipocyte phenotype. Cells cultured in 35-mm dishes were used for assay of lipolysis, and 60-mm dishes were used for the analysis of PDE3B activity. C2-ceramide, dihydro-C2-ceramide, and troglitazone were dissolved in DMSO and added to the cells (final concentration of DMSO, ≤0.2%).

Lipolysis. 3T3-L1 adipocytes were incubated with test agents or DMSO (control) in 10% FBS-DMEM. Glycerol released into the medium was determined as previously described (27). To determine the effect of ceramide on insulin-induced lipolysis, the cells were incubated with C2-ceramide or DMSO for 16 h in 10% FBS-DMEM and then in serum-free DMEM for 8 h. After two washes with Krebs-Ringer HEPES buffer containing 100 mmol/l NaCl, 5 mmol/l KCl, 1 mmol/l KH₂PO₄, 1 mmol/l MgSO₄, 2.5 mmol/l CaCl₂, and 25 mmol/l HEPES supplemented with 1% BSA, 2 mmol/l glucose, and 200 mmol/l adenosine, the cells were exposed to 10 nmol/l insulin for 45 min. The medium was collected and glycerol determined by the method of Dole and Neimertz (38).

Western blot analysis. For PDE3B, PPAR-γ, HSL, and PKB. Cells cultured in 100-mm dishes were incubated with or without test agents. Before harvesting, cells were rinsed twice with ice-cold PBS, pH 7.4. For the detection of PDE3B, cells were scraped in homogenization buffer (25 mmol/l Tris, pH 8.0, 250 mmol/l sucrose, 5 mmol/l MgCl₂, 0.2 mmol/l EDTA, 20 mmol/l glycerol 2-phosphate, 20 mmol/l phenyl phosphate, 20 μg/ml leupeptin, 2 μg/ml aprotinin, and 1 μg/ml pepstatin) and a crude membrane fraction was prepared (39). To determine the levels of PPAR-γ, cells were lysed in buffer (150 mmol/l NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mmol/l Tris, pH 8.0, 1 mmol/l phenylmethylsulfonyl fluoride [PMSF], and 10 μg/ml aprotinin) (40). To determine the level and phosphorylation status of PKB, cells were harvested in lysis buffer containing 50 mmol/l TES (N-tris [hydroxymethyl]-methyl-2-aminoethane sulfonic acid) (pH 7.4), 2 mmol/l EGTA, 1 mmol/l EDTA, 250 mmol/l sucrose, 40 mmol/l phenyl phosphate, 1 mmol/l MgCl₂, 2 mmol/l Na VO₄, 10 mmol/l Na P₄O₆, 100 mmol/l NaF, 5 μg/ml aprotinin, 1 mmol/l PMSF, 1 μg/ml leupeptin, 5 mmol/l benzamidine, and 10 mmol/l dithiothreitol. Protein (40–80 μg) was separated by 7% (PDE3B), 10% (PPAR-γ), 5% (HSL), or 9% (PKB) SDS-polyacrylamide gel in the separation buffer. Western blot analysis using anti-PDE3B, anti-PPAR-γ, anti-HSL, anti-phospho (Ser473) PKB, and anti-PKB polyclonal antibodies was performed, and the proteins were detected using the Supersignal chemiluminescence system.

Enzyme activity measurements. Membrane-associated PDE3B was prepared and PDE3 activity determined in a total volume of 300 μl in the presence and absence of a selective PDE3 inhibitor (OPC 9021) as reported previously (39). The lipase activity of homogenized cells was measured using a phospho-lipid-stabilized emulsion of (1:3)-mono[14C]-Holeoyl-2-oleylglycerol (MOME), a diacylglycerol ether analog not hydrolysable at position 2 (36). HSL activity is expressed in millinits (1 mU = 1 nmol fatty acid released per min at 37°C using MOME as substrate). Specific activity is expressed as units per milligram of protein. The protein concentration in cell homogenates was measured using the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA).

Statistical analysis. Results are means ± SE. Statistical significance was determined by one-way ANOVA followed by Fisher multiple range tests.

RESULTS

C2-ceramide induces downregulation of PDE3B and stimulation of lipolysis. To investigate effects of C2-ceramide on PDE3B expression in 3T3-L1 adipocytes, the present study, cells were treated with or without a short-chain ceramide analog (C2-ceramide, 100 μmol/l) for 6, 12, and 24 h. Incubation with C2-ceramide for 12 and 24 h decreased PDE3B activity by 40 and 51%, respectively (Fig. 1A). This was accompanied by decreased PDE3B protein expression, detected by Western blot analysis (Fig. 1B). By contrast, treatment of cells with dihydro-C2-ceramide, a nonactive ceramide analog, did not decrease PDE3B activity or protein expression (data not shown).

Next, we examined whether ceramide-induced downregulation of PDE3B was associated with increased lipolysis. As shown in Fig. 2A, treatment of 3T3-L1 adipocytes with C2-ceramide (50 and 100 μmol/l) for 24 h increased glycerol release by 1.3- and 3.1-fold, respectively. However, in cells treated for 24 h with 100 μmol/l dihydro-C2-ceramide, glycerol release was not increased (Fig. 2A). As shown in Fig. 2B, after incubation of 3T3-L1 adipocytes with 100 μmol/l C2-ceramide, accumulated glycerol release was only slightly increased after 6 h, but significantly increased (by 1.2- and 1.8-fold) after 12 and 24 h, respectively (P < 0.01).

PPAR-γ, an important nuclear transcription factor in adipocyte differentiation, has been suggested to be involved in TNF-α-mediated dedifferentiation of 3T3-L1 adipocytes (41). As shown in Fig. 1C, C2-ceramide did not decrease PPAR-γ protein expression, suggesting that the effects of ceramide on PDE3B and lipolysis are specific events and not due to dedifferentiation of adipocytes (Fig. 1C).

C2-ceramide-stimulated lipolysis is blocked by H-89, a PKA inhibitor. The level of cAMP and the activity of PKA determine the rate of lipolysis under most conditions (1). Furthermore, incubation of adipocytes with specific
cell-permeable PDE3 inhibitor results in an increase in basal and/or isoproterenol-stimulated lipolysis (9,42,43). Thus, it is logical to reason that a ceramide-induced downregulation of PDE3B, the major cAMP-hydrolyzing enzyme in the adipocyte, would lead to increased lipolysis, at least in the absence of any compensatory mechanisms. However, direct effects of ceramide on more downstream targets in the lipolytic cascade, such as HSL, cannot be excluded and could contribute to a PKA-independent lipolysis. As shown in Fig. 3, treatment of cells with the PKA inhibitor H-89 (10 μmol/l) for 6, 12, and 18 h reduced ceramide-induced lipolysis by 53% (P < 0.05), 94% (P < 0.001), and 61% (P < 0.05) (Fig. 3). H-89 alone slightly decreased lipolysis but had no significant effect compared with the control (untreated) cells. We also tested the effect of C2-ceramide on HSL protein expression and activity. As shown in Fig. 4B, treatment with C2-ceramide decreased HSL protein levels after 12 and 24 h of treatment. The decreased HSL protein levels were paralleled by similar changes in the HSL activity, as measured using MOME as substrate (Fig. 4A). Phosphorylation (activation) of HSL by PKA does not cause any significant changes in its activity against MOME. Accordingly, MOME activity is a measure of the total enzyme concentration. There were no significant changes in the HSL levels in cells treated with ceramide for 2 and 6 h (Fig. 4).

Troglitazone inhibits the ability of C2-ceramide to downregulate PDE3B and induce lipolysis. It has previously been reported that thiazolidinediones (TZDs), a new class of antidiabetic drugs, antagonize TNF-α-induced lipolysis (44) and inhibition of insulin signaling in 3T3-L1 adipocytes (45). To investigate the ability of TZDs to modulate the effect of C2-ceramide on PDE3B activity and lipolysis, cells were incubated for 16 h with C2-ceramide in the presence or absence of troglitazone (20 μmol/l), a
member of the TZD family of compounds. The C2-ceramide–induced decrease in PDE3B activity (38%) was blocked in the presence of troglitazone, which restored PDE3B activity to 95% (Fig. 5A). The changes in PDE3 activity correlated with changes in PDE3B protein expression (Fig. 5B). Furthermore, C2-ceramide–induced lipolysis was blocked by troglitazone (Fig. 5C). Troglitazone alone had no effect on lipolysis, PDE3 activity, or PDE3B protein expression compared with control cells.

C2-ceramide reduces the ability of insulin to activate PDE3B and antagonize lipolysis. PDE3B is a key enzyme in insulin's antilipolytic signaling pathway (9,10,46). Thus, the observed ceramide-induced downregulation of PDE3B would be expected to lead to reduced capacity of the cell to lower cAMP and to respond antilipolytically to insulin. In addition, ceramide could influence the signaling pathways leading to activation of PDE3B. To test the effect of ceramide on insulin-induced activation of PDE3B and inhibition of lipolysis, 3T3-L1 adipocytes were pretreated with or without C2-ceramide and then stimulated with insulin for 10 min. Insulin increased PDE3B activity by 44% in the absence of ceramide. By contrast, pretreatment of the cells with C2-ceramide significantly blocked the insulin-mediated activation of PDE3B (Fig. 6A). Incubation with insulin at 10 nmol/l decreased lipolysis by 54% (Fig. 6B). In contrast, insulin caused only a 10% reduction of lipolysis in adipocytes preincubated with C2-ceramide and was not able to reverse the ceramide-induced lipolysis (Fig. 6B).

In several cell lines, ceramide treatment has been demonstrated to inhibit insulin-mediated activation of PKB (33,34), a kinase that most likely is involved in insulin-mediated activation of PDE3B (12–14). To test the possibility that reduced insulin-mediated activation of PDE3B by ceramide could be due to reduced insulin-mediated activation of PKB, we pretreated cells with C2-ceramide for 24 h and analyzed insulin-mediated Ser 473 phosphorylation of PKB. As shown in Fig. 7, ceramide pretreatment markedly inhibited the insulin-mediated Ser 473 phosphorylation of PKB, whereas the expression of PKB protein was not changed.

**DISCUSSION**

The central findings in this study are that treatment of 3T3-L1 adipocytes with C2-ceramide results in decreased PDE3B expression and, concomitantly, increased lipolysis. The ceramide-induced effects on PDE3B and lipolysis...
and inhibition of lipolysis. 3T3-L1 adipocytes were treated with C2-ceramide (100 μmol/l) or DMSO (control) for 24 h. After two washes with PBS, the cells were stimulated with insulin (10 nmol/l) for 10 min and then homogenized. Total cell lysate protein (80 μg) was subjected to 9% SDS-PAGE and analyzed by Western blotting with anti-phospho-PKB-Ser^473 and anti-PKBα antibody. The bands corresponding to PKB were detected using the Supersignal chemiluminescence system. The result is representative of three independent experiments.

FIG. 6. C2-ceramide decreases insulin-mediated activation of PDE3B and inhibition of lipolysis. 3T3-L1 adipocytes were treated with C2-ceramide (C2-cer) (100 μmol/l) or DMSO (control) for 24 h. After two washes with PBS, cells were stimulated with insulin (10 nmol/l) for 10 min and then homogenized. A: Membrane fractions were prepared and PDE3B activity was measured as described in RESEARCH DESIGN AND METHODS. Data are expressed as % of control (80 pmol·min⁻¹·mg⁻¹ protein) ± SE from six independent experiments. B: Cells were pretreated with or without C2-ceramide for 24 h and then exposed to insulin (10 nmol/l) for 40 min. Glycerol released into medium was measured. Data are means ± SE (n = 6). *P < 0.05 and **P < 0.01 between ceramide-treated and control cells.

were reversed by troglitazone, an antidiabetic drug that was previously suggested to act by lowering systemic fatty acid levels (47). In addition, we demonstrated that pre-treatment of cells with ceramide blocks the insulin-mediated activation of PDE3B and the antilipolytic action of insulin. Thus, two alterations related to PDE3B are observed as a result of ceramide treatment, both of which could give rise to increased lipolysis.

Downregulation of PDE3B has previously been suggested to contribute to the development of insulin resistance linked to obesity and type 2 diabetes. For example, PDE3B activity and gene expression are decreased in adipose tissue in the JCR:LA-cp rat, a strain that develops obesity, insulin resistance, and vasculopathy (48); and in obese, insulin-resistant, diabetic KKAy mice (49). Downregulation of PDE3 activity also occurs in spontaneously diabetic BB rats with full-blown insulin deficiency (21) and in their prediabetic littermates (22–25). It is well documented that increased levels of circulating FFAs can induce systemic insulin resistance (15). Thus, our data from 3T3-L1 adipocytes showing downregulation of PDE3B and stimulation of lipolysis by ceramide may identify one potential mechanism for the pathogenesis of insulin resistance.

The importance of downregulation of PDE3B in ceramide-induced lipolysis is supported by our finding that ceramide-induced lipolysis is to a large extent inhibited by H-89, a PKA inhibitor. However, H-89 as well as other cell-permeable PKA inhibitors have been shown to efficiently inhibit other kinases as well (50), making it impossible to exclude a role for these other kinases. Taking into account that 1) cAMP/PKA has a key role in the regulation of lipolysis (1) and 2) a lowering of PDE3B activity in adipocytes using another strategy (cell-permeable PDE3 inhibitors) results in increases in basal and/or isoproterenol-mediated lipolysis (9,42,43) makes it likely that the effect observed using H-89 is mediated by inhibition of PKA. A PKA-independent effect would have indicated that the major targets for the ceramide effect were to be found downstream of PKA, for example at the level of HSL, the rate-limiting enzyme in the hydrolysis of stored triglycerides. We also tested the effect of C2-ceramide on the expression of HSL. We found that treatment with C2-ceramide decreased rather than increased HSL protein levels. In agreement with what has been previously found for TNF-α (44), these findings suggest that in ceramide-induced lipolysis, the amount of HSL protein is less important than its phosphorylation and activation by PKA, and that downregulation of the cAMP-hydrolyzing enzyme, PDE3B, contributes to the activation of PKA. However, modulation of other components in the lipolytic/antilipolytic signaling cascades by long-term incubation of ceramide is likely to occur. Before reliable hypotheses regarding the mechanisms for ceramide-induced lipolysis can be made, further basic knowledge of ceramide signaling is necessary. Although ceramide has been identified as an important second messenger for TNF-α, this cytokine may act through other pathways (51,52). Nevertheless, referring to previous work showing TNF-α–induced downregulation of PDE3B (27), the observation of similar effects in C2-ceramide-stimulated cells makes it plausible that C2-ceramide acts as a second messenger for TNF-α to regulate the expression of PDE3B. Furthermore, the con-
tribution of TNF-α action to elevated FFA levels and development of insulin resistance is well documented (53,54) and, in adipocytes, proteins such as perilipin (55), Gi proteins (56), adrenergic receptors (57), and PKA (27) are known to be modulated in pathways induced by TNF-α. Recent studies in FDCP2 cells revealed that TNF-α blocks interleukin-4–induced activation of PDE3 and PDE4 (58). Whether these modulations are due to TNF-α signaling that involves ceramide is, however, not known.

Since activation of PDE3B plays a key role in the antilipolytic action of insulin (9,10,46), it is conceivable to reason that downregulation of PDE3B would lead to a reduced ability of insulin to antagonize lipolysis. This hypothesis is demonstrated by our findings that ceramide pretreatment reduces insulin-mediated antilipolysis. Furthermore, we also demonstrate that the ability of insulin to activate PDE3B is inhibited under these conditions. We do not know how ceramide interferes with the insulin-mediated activation of PDE3B. The proposed mechanism whereby insulin activates PDE3B involves insulin receptor (IR) tyrosine kinase–mediated phosphorylation of IRS proteins, leading to activation of PI3K and PKB. In studies on insulin-mediated tyrosine phosphorylation of IR and IRS-1 and activation of PI3K following ceramide treatment of 3T3-L1 adipocytes, no inhibitory effects of ceramide were detected (33). Recently, however, it was revealed that pretreatment of 3T3-L1 adipocytes with ceramide for 2 h results in inhibited ability of insulin to activate PKB (34), and the generation of ceramide might account for the inhibition of the insulin-induced activation of PKB in C2C12 skeletal muscle cells pretreated with palmitoleate (59). In agreement with those results, we found that pretreatment of 3T3-L1 adipocytes with C2- ceramide inhibited insulin-induced phosphorylation of PKB, suggesting that ceramide pretreatment results in impaired insulin-mediated activation of PKB and thereby impaired activation of PDE3B.

Interestingly, adipocytes obtained from insulin-resistant diabetic KK mice (49) were reported to contain less PDE3B and were defective with regard to insulin-mediated activation of PDE3B and antilipolysis. These defects were normalized after treatment of the rats with pioglitazone, results which are in agreement with early studies in diabetic patients (26), showing that reduced PDE activity in adipocytes is normalized after treatment with insulin or oral antidiabetic agents (26). Consistent with these studies, our data demonstrate that troglitazone can block the ability of ceramide to stimulate lipolysis and induce down-regulation of PDE3B in 3T3-L1 adipocytes. The mechanisms by which troglitazone and other members in the TZD family of antidiabetic compounds exert their actions seem to involve activation of PPAR-γ, a nuclear hormone receptor expressed at high levels in adipose tissue (41), although in some reports PPAR-γ-independent effects of TZZDs have been reported (44,47). Also, TZZDs have been shown to decrease circulating FFA levels (60), supporting an important role for adipose tissue as a target tissue for TZD action. Specifically, our finding that troglitazone antagonizes ceramide action on both PDE3B and lipolysis places PDE3B on the list of interesting adipocyte targets for TZD action.

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