Ceramide Mediates Insulin Resistance by Tumor Necrosis Factor-α in Brown Adipocytes by Maintaining Akt in an Inactive Dephosphorylated State

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Tumor necrosis factor (TNF)-α causes insulin resistance on glucose uptake in fetal brown adipocytes. We explored the hypothesis that some effects of TNF-α could be mediated by the formation of ceramide, given that TNF-α treatment induced the production of ceramide in these primary cells. A short-chain ceramide analog, C2- ceramide, completely precluded insulin-stimulated glucose uptake and insulin-induced GLUT4 translocation to plasma membrane, as determined by Western blot or immunofluorescent localization of GLUT4. These effects were not produced in the presence of a biologically inactive ceramide analog, C2-dihydroceramide. Analysis of the phosphatidylinositol (PI) 3-kinase signaling pathway indicated that C2-ceramide precluded insulin stimulation of Akt kinase activity, but not of PI-3 kinase or protein kinase C-ζ activity. C2-ceramide completely abolished insulin-stimulated Akt/protein kinase B phosphorylation on regulatory residues Thr 308 and Ser 473, as did TNF-α, and inhibited insulin-induced mobility shift in Akt1 and Akt2 separated in PAGE. Moreover, C2-ceramide seemed to activate a protein phosphatase (PP) involved in dephosphorylating Akt because 1) PP2A activity was increased in C2-ceramide- treated cells, 2) treatment with okadaic acid concomitantly with C2-ceramide completely restored Akt phosphorylation by insulin, and 3) transient transfection of a constitutively active form of Akt did not restore Akt activity. Our results indicate that ceramide produced by TNF-α induces insulin resistance in brown adipocytes by maintaining Akt in an inactive dephosphorylated state. Diabetes 50:2563–2571, 2001

Brown adipose tissue is a target tissue for insulin action, especially during late fetal development, when insulin promotes both adipogenic and thermogenic differentiation of primary cells, as well as displays survival effects (1–3). Glucose transport in fetal brown adipocytes is maintained mainly by the activity of the insulin-regulated glucose transporter GLUT4, with insulin being involved in increasing both glucose uptake and GLUT4 gene expression (4). Acute insulin treatment stimulates glucose transport in adipocytes and myocytes, largely by mediating translocation of GLUT4 from an intracellular compartment to the plasma membrane, as previously reviewed (5). It is well established that activation of phosphatidylinositol (PI)-3 kinase mediates GLUT4 redistribution to the plasma membrane; overexpression of the catalytic subunit of p110 produces GLUT4 translocation and increases glucose uptake (6,7), and inhibition of PI-3 kinase (by chemical inhibitors, microinjection of blocking p85 protein, or transfection with a dominant-negative mutant of p85) precludes insulin-induced GLUT4 translocation (8,9). The targets of PI-3-kinase action are controversial. Two classes of Ser/Thr kinases have been proposed to act downstream of PI-3-kinase: Akt/PKB and the atypical protein kinase C (PKC) isoform ζ and λ. Expression of a constitutively active, membrane-bound form of Akt, using either stable or inducible expression systems, results in persistent localization of GLUT4 at the plasma membrane and increased glucose uptake in 3T3-L1 adipocytes (10,11), but it does not promote GLUT4 translocation or glucose transport in L6 myotubes (12). Experiments involving expression of a dominant-negative Akt mutant are also controversial, with two different results being reported: either inhibition of insulin-stimulated GLUT4 translocation (13) or no effect (14). Expression of PKC-ζ or λ is also reported to induce GLUT4 translocation, whereas expression of a dominant-interfering PKC-ζ inhibits GLUT4 translocation in 3T3-L1 cells (15,16). Our previous work has shown that PI-3-kinase, PKC-ζ, and Akt could be involved in IGF I/insulin stimulation of glucose uptake in fetal brown adipocyte primary cultures (17–19).

Insulin resistance (a smaller than normal response to a given amount of insulin) is an important contributor to the pathogenesis of type 2 diabetes. Tumor necrosis factor (TNF)-α has been proposed as a link between adiposity and the development of insulin resistance, because 1) the majority of type 2 diabetic patients are obese and show
increased TNF-α expression in fat cells and 2) obese mice lacking TNF-α function have shown protection for developing insulin resistance (20–22). Direct exposure of isolated cells to TNF-α inhibits insulin signaling and induces a state of insulin resistance (23,24) by a mechanism involving Ser phosphorylation of insulin receptor substrate (IRS)-1 that converts IRS-1 into an inhibitor of insulin receptor tyrosine kinase activity in vitro (25). Furthermore, Ser 307 has been identified as a site for TNF-α phosphorylation of IRS-1 (26). We have previously reported that TNF-α causes insulin resistance in brown adipocytes by decreasing IRS-2 phosphorylation and IRS-2–associated PI 3-kinase activity, but that it does not affect IRS-1 signaling (27). Ceramide has been invoked as a mediator of TNF-α, as this cytokine induces the activation of sphingomyelinase in several cell types (28). Ceramide analogs inhibit insulin-stimulated glucose uptake in 3T3-L1 adipocytes and cause apoptosis in motor neuron cells and brown adipocyte cell lines by inhibition of Akt kinase (29–31). Whether ceramide production mediates insulin-resistance by TNF-α and at which step it interferes with insulin signaling remain to be established. In this study, we propose a mechanism for TNF-α induction of insulin resistance on glucose uptake in fetal brown adipocytes mediated by the generation of ceramide, and further propose that this mechanism inhibits Akt activity through a ceramide-activated phosphatase.

RESEARCH DESIGN AND METHODS

Materials. Insulin, bovine serum albumin (BSA; fraction V, essentially fatty acid–free), okadaic acid, and fluorescein-conjugated mouse anti-rabbit IgG were obtained from Sigma (St. Louis, MO). Fetal calf serum (FCS), phosphate-buffered saline (PBS), and culture media were obtained from Imperial Laboratories (Hampshire, U.K.). C2-ceramide and C2-dihydroceramide were purchased from Calbiochem (La Jolla, CA). TNF-α was purchased from Pharma Biotechnologie (Hannover, Germany). The autoradiographic film used was Kodak X-O-MAT/AR (Eastman Kodak, Rochester, NY). The 2-deoxy- D-[1-33]Glc-6-P assay, both DAG and ceramide. To improve the separation of phosphatidic acid and ceramide-1-phosphate by thin-layer chromatography, plates were first developed with CHCl3:CH3OH:NH3:HO (55:35:7.5, by volume), dried, and then developed with CHCl3:CH3OH:CH3COOH:CH3CO2H (10:2.3:4.1, by volume). Ceramide-1-phosphate was identified by comigration with a standard.

Measurement of glucose transport. Glucose transport was measured in duplicate dishes from four independent experiments, as previously described (17).

Subcellular fractionation. Cells were washed with ice-cold PBS and homogenization buffer (20 mmol/l HEPES, pH 7.4), 2 mmol/l EGTA, 2 mmol/l EDTA, 1 mmol/l phenylmethylsulfonyl fluoride (PMSF), 10 mmol/l β-mercaptoethanol, 10 μg/ml aprotinin, and 10 μg/ml leupeptin. After a 10-min incubation, cells were treated with 30 strokes of Dounce homogenization using a tight-fitting pestle. Nuclei were pelleted by centrifugation at 500g for 5 min, and the low-speed supernatant was centrifuged at 100,000g for 30 min. The high-speed supernatant constituted the internal membrane fraction. The pellet was washed three times and extracted in ice-cold homogenization buffer containing 1% Triton X-100 for 60 min. The Triton-soluble component (plasma membrane fraction) was separated from the Triton-insoluble material (cytoskeletal fraction) by centrifugation at 100,000g for 15 min. Internal and plasma membrane fractions were kept at 70°C before protein quantification and Western blotting with GLUT4, GLUT1, and caveolin-1 antibodies.

Western blotting. Cells were lysed as previously described (6), and cellular proteins were subjected to SDS-PAGE, transferred to nitrocellulose membranes (Millipore, Bedford, ME), blocked, and incubated overnight with the primary antibodies in 0.05% Tween-20, 1% nonfat dried milk in 10 mmol/l Tris-HCl, and 150 mmol/l NaCl (pH 7.5). Immune reactive bands were visualized using the enhanced chemiluminescence (ECL) Western blotting protocol (Amersham Pharmacia). Protein determination was performed by the Bradford dye method, using Bio-Rad reagent and BSA as the standard (34).

Immunostaining of GLUT4. Brown adipocytes were seeded onto coverslips in six-well plates, maintained for 4 h in 10% FCS-MEM, and deprived of serum for 20 h before treatments. Cells were treated or not treated with C2-ceramide or C2-dihydroceramide for 6 h, followed by incubation with 10 mmol/l insulin for 30 min. Cells were then rinsed twice with 2% FCS-PBS, fixed with 4% paraformaldehyde-PBS for 10 min at room temperature, and rinsed again. The cells were permeabilized with 0.1% (vol/vol) Triton X-100 in 2% FCS-PBS for 10 min. Coverslips were incubated with anti-GLUT4 antibody (1:500) in 2% FCS-PBS for 1 h at 4°C, rinsed twice with PBS, and incubated with the secondary antibody (fluorescein-conjugated mouse anti-rabbit IgG; 1:100) for 1 h at room temperature, as previously described (35). Then the coverslips were rinsed with 2% FCS-PBS, mounted, and analyzed by fluorescence microscopy. Cells were scored as positive for GLUT4 translocation if they were observed to have a ring of fluorescence at the periphery. Coverslips were viewed by two investigators.

PI 3-kinase activity. PI 3-kinase activity was measured in the anti–IRS-1 and anti–IRS-2 immunoprecipitates by in vitro phosphorylation of PI, as previously described (17).

PKC-ζ and PKB/Akt activities. Fet al brown adipocytes were extracted with lysis buffer (50 mmol/l Tris [pH 7.5], 150 mmol/l NaCl, 1% Triton X-100, 2 mmol/l EDTA, 1 mmol/l EGTA, 1 μmol/l PMSF, 25 μg/ml leupeptin, and 25 μg/ml aprotinin) and immunoprecipitated with an anti–PKC-ζ or an anti–total Akt antiserum (17). Immune complexes were washed five times with ice-cold lysis buffer with 0.5 mol/l NaCl and two times with kinase buffer (35 mmol/l Tris [pH 7.5], 10 mmol/l MgCl2, 0.5 mmol/l EGTA, and 1 μmol/l Na3VO4). The kinase reaction was performed in buffer containing 1 μCi of [γ-32P]ATP, 60 μmol/l MnCl2, and 1 μg of myelin basic protein (MBP) as a substrate for 30 min at 30°C, and it was terminated by the addition of 4 × SDS-PAGE sample buffer and then boiled for 5 min at 95°C (32). Samples were resolved in 12% SDS-PAGE, and gels were dried out and subjected to autoradiography.

Protein phosphatase assay. Protein phosphatase activity was determined by measuring the generation of PO4 using the molybdate-malachite green-phosphate complex assay (36). Cells were lysed in a low-detergent buffer (0.25% Nonidet P-40, 50 mmol/l Tris [pH 7.4], 150 mmol/l NaCl, 1 mmol/l PMSF, 10 μg/ml aprotinin, and 10 μg/ml leupeptin), and 10 μg of isolated protein were used for the phosphatase assay, performed as described by the manufacturer in a PP2A-specific reaction buffer (final concentration 50 mmol/l imidazole [pH 7.2], 0.2 mmol/l EGTA, 0.02% β-mercaptoethanol, and 0.1 mg/ml BSA) using 100 μmol/l phosphopeptide substrate RRA(pT)VA.

Transfection conditions. For transfection experiments, we used the construct pSG5-PKBgag, which encodes a portion of the Moloney murine leukemia virus gag protein, a coactivator that confers a constitutively activated kinase activity (GagAkt). This construct is expressed under the control of the early SV40 promoter for eukaryotic expression. Primary brown adipocytes were cultured for 24 h in the presence of 10% FCS and then transiently transfected according to the calcium
RESULTS

TNF-α inhibited insulin-stimulated glucose uptake in 3T3-L1 cells as well as in human primary adipocytes (23, 24, 37). The fact that glucose transport is induced in fetal brown adipocytes after insulin stimulation (4) prompted us to investigate whether this effect could be blocked by TNF-α in those cells. Cells were deprived of serum for 20 h and further cultured for 24 h in the presence or absence of 0.6 nmol/l TNF-α before being stimulated for 30 min with 10 nmol/l insulin. Glucose uptake was measured during the last 10 min of culture, as described in RESEARCH DESIGN AND METHODS. Results are expressed as disintegrations per minute per 1 × 10⁶ cells. Glucose uptake was higher (30%) in cells pretreated with TNF-α for 24 h compared with untreated cells. Insulin significantly stimulated basal glucose uptake 3-fold, but it produced only a 1.5-fold increase in glucose uptake in TNF-α-treated cells, an effect 50% lower than that observed in TNF-α-untreated cells (Fig. LBA). Because TNF-α can induce the activation of sphingomyelinase, which generates ceramide (38), we determined the production of ceramide after TNF-α treatment of brown adipocytes for different time periods. A representative autoradiogram of phosphorylated ceramide from samples and standard is shown in Fig. 1B, as well as the means ± SE of densitometries from four independent experiments. Statistical significance was tested with a one-way analysis of variance followed by the protected least-significant different test. *P < 0.01 for values in the presence of insulin plus TNF-α vs. insulin alone.

FIG. 1. TNF-α causes insulin resistance and induces ceramide production in cultured fetal brown adipocytes. A: Cells were cultured for 24 h in the absence or presence of 0.6 nmol/l TNF-α and stimulated or not stimulated for 30 min with 10 nmol/l insulin. Glucose uptake was measured the last 10 min by incorporation of 2-DG into the cells. Results are expressed as dpm/1 × 10⁶ cells and are means ± SE (n = 8) of duplicate samples from four independent experiments. Statistical significance was tested with a one-way analysis of variance followed by the protected least-significant different test. *P < 0.01 for values in the presence of insulin plus TNF-α vs. insulin alone. B: Overnight serum-starved cells were incubated or not with 0.6 nmol/l TNF-α for the time indicated. Lipid extracts were assayed for ceramide from samples and standard (St) is shown, as well as the means ± SE of densitometries from four independent experiments. Statistical significance was tested with a one-way ANOVA followed by the protected least-significant different test. *P < 0.01 for values in the presence of TNF-α vs. control.

phosphate-mediated protocol with 10 μg of GagAkt or an empty vector, together with 2 μg of enhanced green fluorescent protein (EGFP) plasmid (to monitor transfection efficiency). After a 4-h incubation, cells were shocked with 3 ml of 15% glycerol-PBS for 2 min, washed, and then fed with 10% FCS-MEM for 24 h. Cells were then deprived of serum for 6 h in either the absence or presence of C2-ceramide.

FIG. 2. Effect of C2-ceramide on glucose uptake in brown adipocytes. A and B: Time course and dosage-response experiments. Brown adipocytes were treated for 2 and 6 h with 100 μmol/l C2-ceramide (A) or for 6 h with increasing concentrations of C2-ceramide (B), followed by stimulation or no stimulation with 10 nmol/l insulin. Results are expressed as percent of insulin-stimulated glucose uptake (basal subtracted) and are means ± SE (n = 8) of duplicate samples from four independent experiments. Statistical significance was tested with a one-way analysis of variance followed by the protected least-significant different test. *P < 0.01 for values in the presence of insulin plus C2-ceramide vs. insulin.
FIG. 3. C2-ceramide inhibits insulin-stimulated GLUT4 translocation. A: Overnight serum-starved fetal brown adipocytes were pretreated or not pretreated with C2-ceramide (C2) for 6 h and stimulated with insulin (Ins) for an additional 30 min. Cells were then harvested and subcellular fractionated. Then, 10 μg of internal and plasma membrane proteins from each condition were submitted to SDS/PAGE; blotted onto nylon membrane; immunodetected with anti-GLUT4, anti-GLUT1, and anti–caveolin-1 antibodies; and developed with ECL. A representative experiment is shown (upper panel). Corresponding autoradiograms for GLUT4 were quantitated by scanning densitometry (lower panel). Results are expressed as arbitrary units and are means ± SE from four independent experiments. IM, internal membrane; PM, plasma membrane. Statistical significance was tested with a one-way analysis of variance followed by the protected least-significant difference test. *P < 0.01 for values in the presence of insulin vs. control; ΔP < 0.01 for values in the presence of insulin plus C2-ceramide vs. insulin.

B: Overnight serum-starved fetal brown adipocytes were pretreated or not pretreated with C2-ceramide (C2) or C2-dihydroceramide (DHC) for 6 h and stimulated with insulin (Ins) for an additional 30 min. Cells were scored as positive for GLUT4 fluorescence at the periphery. The percentage of positive cells are the means ± SE of duplicates coverslips from four independent experiments (lower panel). Statistical analyses were performed as in A. *P < 0.01 for values in the presence of insulin vs. control; ΔP < 0.01 for values in the presence of insulin plus C2-ceramide vs. insulin.

and B). Treatment with 100 μmol/l C2-ceramide for 2 h partially, and for 6 h completely, precluded insulin-stimulated glucose uptake (Fig. 2B); however, this inhibitory effect was not produced in the presence of a biologically inactive ceramide analog, C2-dihydroceramide (Fig. 2C). Moreover, C2-ceramide treatment increased the basal glucose uptake by 30%, in a manner similar to TNF-α (Fig. 2C).

To correlate the data on glucose uptake with translocation of GLUT4 to the plasma membrane, cells were pretreated or not pretreated with C2-ceramide for 6 h and stimulated with insulin for a further 30 min; subcellular fractionating was then performed to obtain plasma membrane and internal membrane. Western blot protein analysis with anti-GLUT4 antibody indicated that insulin increased GLUT4 translocation to the plasma membrane by threefold, with a concomitant decrease in the amount of GLUT4 in the internal membrane. This redistribution of GLUT4 from the internal to the plasma membrane was precluded after C2-ceramide pretreatment (Fig. 3A). However, C2-ceramide increased GLUT1 protein content at the plasma membrane, contributing to the increase in basal glucose uptake. Caveolin-1, an integral protein from caveolae implicated in cellular transport processes (39), was used as a marker protein of plasma membrane; its expression remained essentially unaltered under the different treatments used (Fig. 3A). The translocation of GLUT4 to the cell surface was visualized using a GLUT4 antibody and a fluorescein-conjugated mouse anti-rabbit antibody, in a fluorescence microscope (upper panel). Cells were scored as positive for GLUT4 translocation if they were observed to have a ring of fluorescence at the periphery. The percentage of positive cells are the means ± SE for duplicate coverslips from four independent experiments (lower panel). Statistical analyses were performed as in A. *P < 0.01 for values in the presence of insulin vs. control; ΔP < 0.01 for values in the presence of insulin plus C2-ceramide vs. insulin.
nmol/l) and were then stimulated for 5 min with 10 nmol/l insulin. Control cells were cultured for 6 h in a serum-free medium. PI 3-kinase activity was determined in the immune complexes after immunoprecipitation with anti-IRS-1 or anti-IRS-2 antibodies (Fig. 4A). TNF-α treatment did not modify insulin-induced PI 3-kinase activity associated with IRS-1, but did inhibit the activity associated with IRS-2, as previously reported (27). Meanwhile, insulin stimulation of PI 3-kinase in IRS-1 and IRS-2 immunoprecipitates was not decreased after pretreatment with C2-ceramide, indicating that ceramide did not interfere with the insulin pathway at this level. In addition, cell lysates were analyzed by Western blotting with the anti-phospho Akt antibodies for regulatory residues Ser 473 and Thr 308, as well as with the total and isoform-specific Akt antibodies. After stimulation with insulin, Akt/PKB was highly Ser phosphorylated and, to a lesser extent, Thr phosphorylated, with both effects being completely precluded by pretreatment with C2-ceramide or TNF-α. Pretreatment with C2-dihydroceramide for 6 h did not have any effect. Moreover, antibodies raised against total Akt and the isoforms Akt1 and Akt2 revealed a dramatic mobility shift when cells were stimulated with insulin; this effect was not observed after pretreatment with C2-ceramide when dephosphorylation occurred (Fig. 4B). Immunoprecipitation with the anti-total Akt antibody and determination of the enzymatic activity revealed that insulin stimulated MBP phosphorylation, causing a threefold increase in Akt activity. C2-ceramide and TNF-α prohibited insulin-stimulated MBP phosphorylation (Fig. 4C). All these data indicate that TNF-α and C2-ceramide, but not C2-dihydroceramide, markedly inhibited insulin stimulation of Akt phosphorylation, mobility shift, and kinase activity. In addition, C2-ceramide also caused dephosphorylation of insulin-stimulated p70S6-kinase. However, this enzyme does not seem to have contributed to GLUT4 translocation to the

FIG. 4. Effects of C2-ceramide and TNF-α on insulin-stimulated PI 3-kinase pathway. A: Brown adipocytes were pretreated or not pretreated with 100 μmol/l C2-ceramide (C2) for 6 h or with 0.6 nmol/l TNF-α for 24 h and further stimulated or not stimulated with insulin (Ins) for 5 min. Cell lysates were immunoprecipitated with anti-IRS-1 or anti-IRS-2 antibodies and assayed for PI 3-kinase activity. B: Cells were pretreated or not pretreated with 100 μmol/l C2-ceramide (C2) or 100 μmol/l C2-dihydroceramide (DHC) for 6 h or with TNF-α (0.6 nmol/l) for 24 h, then they were stimulated with insulin (Ins) for 5 min. Cell lysates were analyzed by Western blotting with the corresponding antibodies against phospho-Akt(Ser 473), phospho-Akt(Thr 308), total Akt, Akt1, Akt2, and phospho-p70S6-kinase. C: Cells were treated as in A, lysed, and immunoprecipitated with anti-Akt or anti-PKC-ζ antibodies. The resulting immune complexes were assayed for MBP phosphorylation. Representative experiments are shown as well as the densitometric analysis for Akt activity (results are phosphorylated MBP levels in arbitrary units and are means ± SE from four independent experiments). Statistical significance was tested with a one-way analysis of variance followed by the protected least-significant different test. *P < 0.01 for values in the presence of insulin vs. control; ΔP < 0.01 for values in the presence of insulin plus C2-ceramide or plus TNF-α vs. insulin.
plasma membrane, because direct inhibition of p70S6-kinase phosphorylation with 25 ng/ml rapamycin does not affect insulin stimulation of glucose uptake (19). The brown adipocytes expressed PKC-ζ, and their enzymatic activity was analyzed in the anti-PKC-ζ immune complexes. As shown in Fig. 4C, the level of MBP phosphorylation in the presence of C2-ceramide or insulin individually was higher than that observed in nontreated brown adipocytes. In addition, an additive effect was observed in the presence of combined C2-ceramide and insulin. Moreover, TNF-α treatment strongly induced PKC-ζ activity, with no further additive effect being observed in the presence of insulin.

Because ceramide inhibits insulin induction of Akt but not PI 3-kinase or PKC-ζ activity, we decided to investigate the mechanisms of this inhibition, which could include inhibition of phosphoinositide-dependent kinase (PDK)-1 and PDK-2 activity and/or activation of an Akt phosphatase. The first mechanism was tested by analyzing Akt activity in cells transfected with GagAkt, a construction including the Gag protein fused in-frame to the bovine Akt protein, a configuration that confers a constitutively activated kinase activity (32). Primary brown adipocytes were transiently transfected with 10 μg of GagAkt fusion gene or an empty vector (V). After transfection, cells were incubated for 24 h in 10% FCS-MEM. Next, cells were deprived of serum for 6 h in either the absence (C) or presence of C2-ceramide (C2). Cells were collected and immunoprecipitated with anti-Akt antibody, and the resulting immune complexes were assayed for MBP phosphorylation. Shown is one representative experiment of three, as well as a direct Western blot of total Akt, where both GagAkt and endogenous Akt are detected. B: Brown adipocytes were pretreated or not pretreated with 100 μmol/l C2-ceramide (C2) in the presence of 0.1 μmol/l okadaic acid (OA) for 6 h, followed by stimulation with insulin (Ins) for 5 min. Total protein (10 μg) was used for phosphatase assay, as described under RESEARCH DESIGN AND METHODS. Results are means ± SE from three independent experiments and are expressed in arbitrary units as relative phosphatase activity. Statistical significance was tested with a one-way analysis of variance followed by the protected least-significant different test. *P < 0.01 for values in the presence of C2-ceramide ± insulin or TNF-α ± insulin vs. control; ΔP < 0.01 for values in the presence of C2-ceramide plus insulin plus OA vs. C2-ceramide plus insulin.

FIG. 5. C2-ceramide maintains Akt dephosphorylation through the activation of an okadaic acid–sensitive phosphatase. A: Brown adipocytes were transiently transfected with 10 μg of GagAkt fusion gene or an empty vector (V). After transfection, cells were incubated for 24 h in 10% FCS-MEM. Next, cells were deprived of serum for 6 h in either the absence (C) or presence of C2-ceramide (C2). Cells were collected and immunoprecipitated with anti-Akt antibody, and the resulting immune complexes were assayed for MBP phosphorylation. Shown is one representative experiment of three, as well as a direct Western blot of total Akt, where both GagAkt and endogenous Akt are detected. B: Brown adipocytes were pretreated or not pretreated with 100 μmol/l C2-ceramide (C2) in the presence of 0.1 μmol/l okadaic acid (OA) for 6 h, followed by stimulation with insulin (Ins) for 5 min. Total protein (10 μg) was used for phosphatase assay, as described under RESEARCH DESIGN AND METHODS. Results are means ± SE from three independent experiments and are expressed in arbitrary units as relative phosphatase activity. Statistical significance was tested with a one-way analysis of variance followed by the protected least-significant different test. *P < 0.01 for values in the presence of C2-ceramide ± insulin or TNF-α ± insulin vs. control; ΔP < 0.01 for values in the presence of C2-ceramide plus insulin plus OA vs. C2-ceramide plus insulin.
6 h with 100 μmol/l C2-ceramide, 0.1 μmol/l okadaic acid, or a combination of both, and they were then stimulated for 5 min with 10 nmol/l insulin. Cell lysates were analyzed by Western blotting with anti–phospho Akt and anti–total Akt antibodies. As shown in Fig. 5B, okadaic acid did not affect insulin stimulation of Akt/PKB phosphorylation or mobility shift. However, pretreatment with okadaic acid and ceramide completely restored insulin phosphorylation of Akt at the Ser and Thr regulatory sites, and additionally restored the insulin-induced mobility shift, compared with cells pretreated with ceramide alone. Furthermore, we analyzed phosphatase activity (PP2A) in protein lysates from cells treated with C2-ceramide or TNF-α (Fig. 5C). Both treatments increased PP2A activity by twofold, an effect that was unmodified by insulin stimulation (5 min) but strongly inhibited in the presence of okadaic acid.

**DISCUSSION**

TNF-α produces opposing physiological effects to insulin in the murine 3T3-L1 cell line, human primary adipocytes (23,24,40), and, as described in this study, rat primary brown adipocytes, in which it inhibited insulin-stimulated glucose uptake. Ceramide has been invoked as a mediator of some effects of TNF-α, as this cytokine induces the activation of sphingomyelinase in several cell types (28).

We show in this work that TNF-α treatment induced a peak ceramide generation at 30 min and produced a sustained generation of ceramide after 6 and 24 h of treatment in fetal brown adipocyte primary cultures. The first ceramide peak (30 min) could have been the result of sphingomyelinase activation by TNF-α, as has been previously proposed (38), whereas the increased ceramide levels found after longer TNF-α treatment (6 and 24 h) could have been the result of the synthesis of free fatty acids (33), a consequence of the lipolytic action of TNF-α in fat cells (21). The exogenously added ceramide analog C2-ceramide mimicked TNF-α–induced insulin resistance, producing the 1) complete inhibition of insulin-stimulated glucose uptake, 2) impairment of the redistribution of GLUT4 from the internal to the plasma membrane in response to insulin, and 3) disappearance of the characteristic ring at the cell surface of GLUT4 fluorescent staining induced by insulin. These inhibitory effects of C2-ceramide on insulin action were not produced by the biologically inactive ceramide analog C2-dihydroceramide. These results are in conformity with those reported by Birnbaum and colleagues in 3T3-L1 cells (29,41). Our data indicate that TNF-α–induced insulin resistance in brown adipocytes could be mediated, at least in part, through ceramide generation. Moreover, C2-ceramide increased the basal transport of glucose by increasing the amount of GLUT1 protein at the plasma membrane, thereby mimicking a TNF-α increase in the non–insulin-dependent glucose uptake in brown adipocytes, in a manner similar to that described for 3T3-L1 adipocytes (37,42). TNF-α treatment for 24 h increased GLUT1 mRNA levels (data not shown) and presumably GLUT1 protein content, which could account for the basal effect of TNF-α on glucose transport.

The molecular mechanisms mediating the cross-talk between TNF-α and the insulin-signaling cascade remain incompletely understood; consequently, we investigated the effect of TNF-α in parallel with C2-ceramide on the insulin-stimulated PI 3-kinase pathway, the main route involved in GLUT4 translocation. As previously reported (27), TNF-α inhibited insulin stimulation of IRS-2–but not IRS-1–associated PI 3-kinase activity, whereas C2-ceramide did not decrease insulin-stimulated PI 3-kinase associated with either IRS-1 or IRS-2, a finding that agrees with previous reports in 3T3-L1 cells (29,37). Although TNF-α and C2-ceramide showed different behavior in their actions at the PI 3-kinase level, downstream of PI 3-kinase both C2-ceramide and TNF-α completely inhibited 1) insulin-stimulated Akt kinase activity immunoprecipitated with anti–total-Akt antibody, 2) Akt phosphorylation by insulin at the two regulatory residues Thr 308 and Ser 473, and 3) insulin-induced mobility shift in Akt1, Akt2, and total Akt separated in polyacrylamide gels. These data indicate that besides the increase in ceramide content that mediates the inactivation of Akt, some other signaling pathway responsible for impaired signaling at the IRS-2 level in brown adipocytes is induced in response to TNF-α. Akt inactivation by ceramide presumably would be enough to inhibit insulin-stimulated glucose uptake. In fact, the effect of exogenously added C2-ceramide (100 μmol/l) were similar to that observed in the presence of TNF-α, although intracellular ceramide levels reached by TNF-α treatment might have been lower. This raises the possibility that TNF-α needs both ceramide production and IRS-2–associated PI 3-kinase inactivation to inhibit insulin-stimulated glucose uptake. TNF-α treatment for 24 h increased PKC-ζ activity, which might mediate IRS-2 impairment to activate PI 3-kinase in the same way recently proposed for IRS-1 (43,44). However, treatment with ceramide for 6 h also stimulated PKC-ζ, but it did not impair IRS-2–associated PI 3-kinase activation by insulin, indicating that other kinases activated by TNF-α, but not by ceramide, could mediate this inhibitory effect at the level of IRS-2 in a manner similar to that recently reported for IRS-1 at Ser 307 (26).

Downstream of PI 3-kinase, both Akt and PKC-ζ could be involved in insulin-induced glucose uptake in brown adipocytes (18,19). As we have demonstrated in this study, Akt is a target for inactivation by C2-ceramide and TNF-α action, but they do not have an inhibitory effect on insulin-induced PKC-ζ activation. To the contrary, both signals are activators per se of this atypical PKC (45) (Fig. 4C), and we cannot rule out the possibility that PKC-ζ activation could mediate, to some extent, Akt inhibition (46). However, it has been recently proposed that PKC-ζ overexpression does not mimic the inhibitory effect of palmitate (via ceramide) on PKB (C. Schmitz-Peiffer, personal communication). Both Akt1 and Akt2 isoforms were phosphorylated by insulin in fetal brown adipocytes, although Akt2 was more highly expressed than Akt1, in agreement with previous observations in embryonic brown fat (47). We found a complete inhibition of Akt activity by diphosphorylation at two regulatory residues in the presence of ceramide with insulin. However, it has been reported that ceramide causes partial loss of Akt activation by diphosphorylation at the Ser 473, but not the Thr 308 residue, in TF-1 cell-line treated with the colony-stimulating factor (48). Because ceramide completely inhibits Akt, but not PI 3-kinase or PKC-ζ activities, we decided to investigate the
mechanisms of this inhibition in primary brown adipocytes. Inhibition of Akt activity by C2-ceramide may occur upstream of Akt and may involve intermediate regulators downstream of PI 3-kinase, such as PDK-1 or other PI 3-kinase-independent pathways. Another possibility could be the activation of a ceramide-activated protein phosphatase involved in Akt dephosphorylation, as has recently been proposed for PC-12 cell-line (49). Against the first hypothesis, we have shown that C2-ceramide inhibitory action on Akt kinase activity is not bypassed by the expression of a constitutively active form of Akt (transiently transfected in primary cells). Findings from another recent study, in which the ability of C2-ceramide to inhibit the constitutive phosphorylation and activity of Akt in phosphatase and tensin homolog delete from chromosome 10-negative U87MG cells was demonstrated (50), support these data. Indeed, C2-ceramide-induced dephosphorylation of Akt was prevented by pretreatment with the phosphatase inhibitor okadaic acid, thereby indicating that ceramide inhibits insulin signaling by maintaining Akt in an inactive dephosphorylated state through a mechanism involving an okadaic acid-sensitive phosphatase, such as PP2A or PP1, in brown adipocytes. C2-ceramide has been reported to activate both cytosolic and mitochondrial PP2A (36,49), and, in fact, we have found an increase in okadaic acid-sensitive PP2A activity in C2-ceramide-treated cells. Our results are in conformity with the report from Chen et al. (51) showing dephosphorylation of Akt under osmotic stress conditions, a type of stress that strongly correlates with accumulation of ceramide (52). However, in two related reports on insulin signaling, those investigators could not prevent dephosphorylation of Akt with okadaic acid in 3T3-L1 cells (29,41).

As a general overview, the increased secretion of TNF-α by white adipose tissue from obese humans has been proposed as a link between adiposity and the development of insulin resistance (40). In the short term, this cytokine can activate sphingomyelinases and the concomitant production of ceramide, and in the long term, it produces lipolytic effects on fat cells that could generate free fatty acids needed for de novo ceramide synthesis. These ceramides would be involved in insulin resistance in muscle cells (as shown in the C2C12 cell model) (33), as well as in white adipocytes (as shown in 3T3-L1 cell model) (29,37) and, as we reported in this study, in brown adipocytes. Moreover, because ceramides are apoptotic triggers for several cell types, they could cause apoptosis of β-cells, a cell system in which free fatty acids are deleterious to their survival (53). This complex situation could contribute to the pathogenesis of type 2 diabetes in obese patients.

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