Inhibitory Effect of Ceramide on Insulin-Induced Protein Kinase Cζ Translocation in Rat Adipocytes

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Ceramide has been confirmed to be a signal mediator of apoptosis that is induced by tumor necrosis factor-alpha (TNF-α). It has also been reported that ceramide may induce insulin resistance as well as TNF-α. We investigated the effect of ceramide on insulin signaling pathways, such as insulin receptor (IR) β-subunit, insulin receptor substrate 1 (IRS-1), phosphatidylinositol 3-kinase (PI3K), and protein kinase Cζ (PKCζ) in rat adipocytes. We examined insulin-stimulated [^{3}H]2-deoxyglucose (2-DOG) uptake in rat adipocytes pretreated with *N*-hexanoylsphingosine (C_6-ceramide, 10 to 30 μmol/L). Insulin-induced 2-DOG uptake was significantly reduced by C_6-ceramide pretreatment. We also examined the effect of various concentrations of C_6-ceramide pretreatment on insulin-induced autophosphorylation of the IR β-subunit, tyrosine phosphorylation of IRS-1, enzyme activity of PI3K, and membrane-associated PKCζ immunoreactivity. Pretreatment with C_6-ceramide significantly reduced autophosphorylation of the IR β-subunit, tyrosine phosphorylation of IRS-1, and enzyme activity of PI3K. Moreover, membrane-associated PKCζ immunoreactivity and immunoprecipitable PKCζ enzyme activity, downstream of PI3K, were significantly suppressed by C_6-ceramide pretreatment. These results suggest that ceramide may induce insulin resistance via the suppression of IRS-1-PI3K signaling, and subsequent activation of PKCζ.

CERAMIDE is one of the second messengers that is induced by cytokines including tumor necrosis factor-alpha (TNF-α).[1] TNF-α binds its receptor and activates a neutral sphingomyelinase. Activation of the sphingomyelinase results in the hydrolysis of membrane shingomyelin to generate ceramide.[2,3] Recently, 2 major hypotheses regarding TNF-α–induced insulin resistance have been raised. First, treatment of insulin-sensitive cells with TNF-α may downregulate glucose transporter 4 (GLUT4) mRNA levels in cultured cells and rat adipocytes.[4,5] Second, treatment of insulin-sensitive cells with TNF-α has been suggested to lead to serine phosphorylation of insulin receptor substrate 1 (IRS-1) and to convert it into an inhibitor of tyrosine kinase of an insulin receptor (IR) β-subunit.[6,7] We hypothesize that TNF-α–induced insulin resistance may be caused by ceramide production. Moreover, ceramide affects PI 3-kinase–dependent events, including insulin-stimulated glucose uptake via prevention of the recruitment of the phosphatidylinositol 3′4′5′ phosphate (PIP_3)-binding protein Akt/PKB.[8] Although many investigators are interested in the study of apoptosis using ceramide, the exact mechanisms by which ceramide is involved in insulin resistance are still unclear.

On the other hand, protein kinase C (PKC) is a family of structurally related serine/threonine kinases that play an important role in signal transduction in various cell types.[9,10] Recently, it has been reported that atypical PKCζ is activated by insulin in 3T3-L1 cells and rat adipocytes.[11,12] Moreover, it has been previously reported that PKCλ/ζ is located downstream of phosphatidylinositol 3-kinase (PI3K), and may lead to GLUT4 translocation.[13-15] In addition, PKCζ-transfected 3T3-L1 fibroblasts and adipocytes enhanced insulin-induced glucose uptake.[11]

However, no interactions between ceramide and activation of insulin-induced PKCζ in the regulation of insulin resistance have been demonstrated. In this research, we found that (1) ceramide suppresses insulin-induced [^{3}H]2-deoxyglucose uptake; (2) ceramide decreases insulin-induced tyrosine kinase activity, tyrosine phosphorylation of IRS-1, and PI3K activity; and (3) ceramide suppresses insulin-induced activation of membrane-associated PKCζ in rat adipocytes.

In conclusion, this study provided new information that ceramide decreases insulin-stimulated glucose uptake and alters the distribution of PKCζ in addition to inhibiting tyrosine kinase–IRS-1–PI3K signaling.

MATERIALS AND METHODS

Materials

Human insulin was obtained from Novo-Nordisk (Copenhagen, Denmark). [γ-^{32}P]adenosine triphosphate (ATP) was purchased from Amersham (Aylesbury, Buckinghamshire, UK). [1,2-^{3}H]2-deoxyglucose ([^{3}H]2-DOG) was purchased from DuPont-NEN (Boston, MA). C_6-ceramide and C_6-dihydroceramide were purchased from BIOMOL (Plymouth Meeting, PA). Antiphosphotyrosine (anti-PY) antibody was purchased from Transduction Laboratory (Lexington, KY). Anti–IRS-1 and anti–IR β-subunit antibodies were purchased from Upstate Biotechnology (Lake Placid, NY). Anti-PKCζ antibody was purchased from Gibco BRL Products (Gaithersburg, MD). All other chemicals were of reagent grade.

Adipocyte Experiments

Male Wistar rats weighing 150 to 200 g were fed ad libitum and killed by decapitation. Isolated adipocytes were obtained by collagenase digestion of rat epididymal fat pads in a Krebs-Ringer phosphate (KRP) buffer (pH 7.4) containing 127 mmol/L NaCl, 12.3 mmol/L NaH_2PO_4, 5.1 mmol/L KCl, 1.3 mmol/L $MgSO_4$, 1.4 mmol/L $CaCl_2$, 3% bovine serum albumin (BSA), and 2.5 mmol/L glucose.[16] Adipo-

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cytes were washed and incubated at 37°C in a glucose-free KRP buffer containing 1% BSA for 30 minutes.

Glucose Uptake Study

First, isolated rat adipocytes were incubated with or without 1 to 30 μmol/L C_6-ceramide and C_6-dihydroceramide for 10, 30, and 60 minutes, followed by treatment with 10 nmol/L insulin for 30 minutes. Uptake of [^{3}H]2-DOG was measured over 1 minute after treatment with 10 nmol/L insulin as described previously.[17] Second, isolated rat adipocytes were treated with C_6-ceramide alone (0.1 to 30 μmol/L) for 60 min utes to examine its toxic effect. Uptake of [^{3}H]2-DOG was measured over 1 minute after treatment with various concentrations of C_6-ceramide for 60 minutes.

Immunoprecipitation and Western Blot Analysis

Adipocytes were preincubated with or without 10 μmol/L and 30 μmol/L C_6-ceramide for 60 minutes. The samples were treated with 10 nmol/L insulin for 10 minutes, and the reaction was terminated by the addition of an ice-cold buffer A (20 mmol/L Tris-HCl, pH 7.5, 140 mmol/L NaCl, 20 μmol/L phenylmethylsulfonyl fluoride [PMSF], 1% Triton X-100, 1 mmol/L ethylene glycol bis [β-aminoethylether]-N,N,N′,N′-tetraacetic acid (EGTA), 0.5 mmol/L Na_3VO_4, 10% glycerol, 1 mmol/L dithiothreitol [DTT], and 10 μg/mL aprotinin). The samples obtained were immediately homogenized in buffer A, then the homogenates were centrifuged at 1,000 × *g* for 2 minutes, and any floating fatty materials were removed. The protein concentration of the resultant homogenates was determined. Five micorgrams of the anti–IR β-subunit antibody or anti–IRS-1 antibody was added to 500 μg of protein and incubated at 4°C overnight. Thirty microliters of protein A sepharose (Sigma, St Louis, MO) was added and incubation was performed for 2 hours at 4°C. The samples were microcentrifuged, and the pellet was washed twice with washing buffer B (20 mmol/L Tris-HCl, pH 7.5, 140 mmol/L NaCl, 1 mmol/L DTT, and 1% Nonidet P-40) and twice with washing buffer C (10 mmol/L Tris-HCl, pH 7.5, 100 mmol/L NaCl, and 1 mmol/L DTT). The pellet was resuspended in 50 μL of Laemmli sample buffer and boiled for 2 minutes at 100°C. After the supernatants were removed, 25 μL of each sample was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to a nitrocellulose membrane (NC). The NC was incubated with an anti-PY antibody, and subjected to an enhanced chemiluminescence (ECL) Western blotting detection system (Amersham) according to the manufacturer′s protocol. Results were obtained by scanning and analysis with a laser densitometer (UltroScan XL, Pharmacia LKB Biotechnology, Tokyo, Japan).

PI3K Activity

Isolated rat adipocytes were preincubated with or without 10 μmol/L and 30 μmol/L C_6-ceramide for 60 minutes. The samples were treated with 10 nmol/L insulin, and were lysed immediately in a lysis buffer (20 mmol/L Tris-HCl, pH 7.5, 140 mmol/L NaCl, 20 μmol/L PMSF, 0.5 mmol/L Na_3VO_4, 1 mmol/L EGTA, 10% glycerol, and 1% Nonidet P-40). The lysates were centrifuged at 15,000 × *g* for 10 minutes at 4°C. Five micrograms of anti-PY antibody was added to 500 μg of the above resultant supernatant and incubated at 4°C overnight. After the addition of protein A sepharose for 2 hours, the immunoprecipitates were washed twice with buffer B, once with LiCl solution (100 mmol/L Tris-HCl, pH 7.5, 0.5 M LiCl, and 1 mmol/L DTT), and twice with buffer C. The PI3K reaction was started by the addition of a 40-μL solution containing 20 mmol/L Tris-HCl, pH 7.4, 10 μCi [γ-^{32}P]ATP, 250 μmol/L ATP, 10 mmol/L $MgCl_2$, 5 mmol/L EGTA, and 20 μL (1 mg/mL) of sonicated phosphatidylinositol. After a 10-minute incubation at 30°C, the reaction was terminated by the addition of 500 μL 1N HCl. Then, 500 μL of $CHCl_3$/methanol (2:1, vol/vol) was added to the samples, which were then centrifuged. The extracts were washed with 500 μL of $CHCl_3$/methanol/1N HCl (4:2:3, vol/vol), then dried and resuspended in $CHCl_3$. The samples were spotted onto silica gel 60 thin-layer chromatography (TLC) plates (MERCK, Darmstadt, Germany), and visualized using autoradiography (X-OMAT film, Eastman Kodak, Rochester, NY). Results were obtained by scanning and analysis with a laser densitometer.

PKC Studies

The samples were treated with 10 nmol/L insulin for 10 minutes, following preincubation with 10 μmol/L and 30 μmol/L C_6-ceramide for 60 minutes, and reactions were terminated by the addition of ice-cold buffer D (20 mmol/L Tris-HCl, pH 7.5, 0.25 mol/L sucrose, 1.2 mmol/L EGTA, 0.1 mmol/L PMSF, 20 μg/mL leupeptin, 20 mmol/L 2-mercaptoethanol, and 10 μg/mL aprotinin). Each sample was sonicated in buffer D. The homogenates were centrifuged for 60 minutes at 105,000 × *g* to separate the cytosol and membrane fractions. After the membranes were resuspended in lysis buffer (20 mmol/L Tris-HCl, pH 7.5, 0.25 mol/L sucrose, 20 μg/mL leupeptin, 20 mmol/L 2-mercaptoethanol, 5 mmol/L EGTA, 0.1 mmol/L PMSF, 2 mmol/L ethylenediaminetetraacetic acid, and 1% Triton X-100) for 30 minutes at 4°C, they were sonicated, and then centrifuged for 60 minutes at 10,5000 × *g* to obtain solubilized membrane fractions. They were then resuspended in Laemmli sample buffer and boiled for 2 minutes at 100°C. Thirty micrograms of each sample was subjected to SDS-PAGE, transferred to NC, and subjected to Western analysis with the anti-PKCζ antibody as described above. Results were obtained by scanning and analysis with a laser densitometer.

PKC Activity

Endogenous PKCζ was immunoprecipitated with anti-PKCζ antibody and protein A agarose. Immunoprecipitates of PKCζ on agarose beads were rinsed 3 times with reaction buffer I containing 50 mmol/L Tris/HCl (pH 7.5), 5 mmol/L $MgCl_2$, 1μmol/L $CaCl_2$, 100 μmol/L Na_3VO_4, 0.1 mmol/L $Na_3P_2O_7$, 1 mmol/L NaF, and 100 μmol/L PMSF, and its activity was measured by incubation with reaction buffer I containing 40 μg/mL phosphatidylserine (PS), 50 mmol/L [γ-^{32}P]ATP, 40 μmol/L PKCε pseudosubstrate analog, [^{159}Ser]PKCε (AA153-164)-NH2 (Upstate Biotechnology). The reactions were terminated by adding 5% acetic acid, and aliquots of the reaction mixture were stopped on p81 filter paper, and counted as previously described.[18]

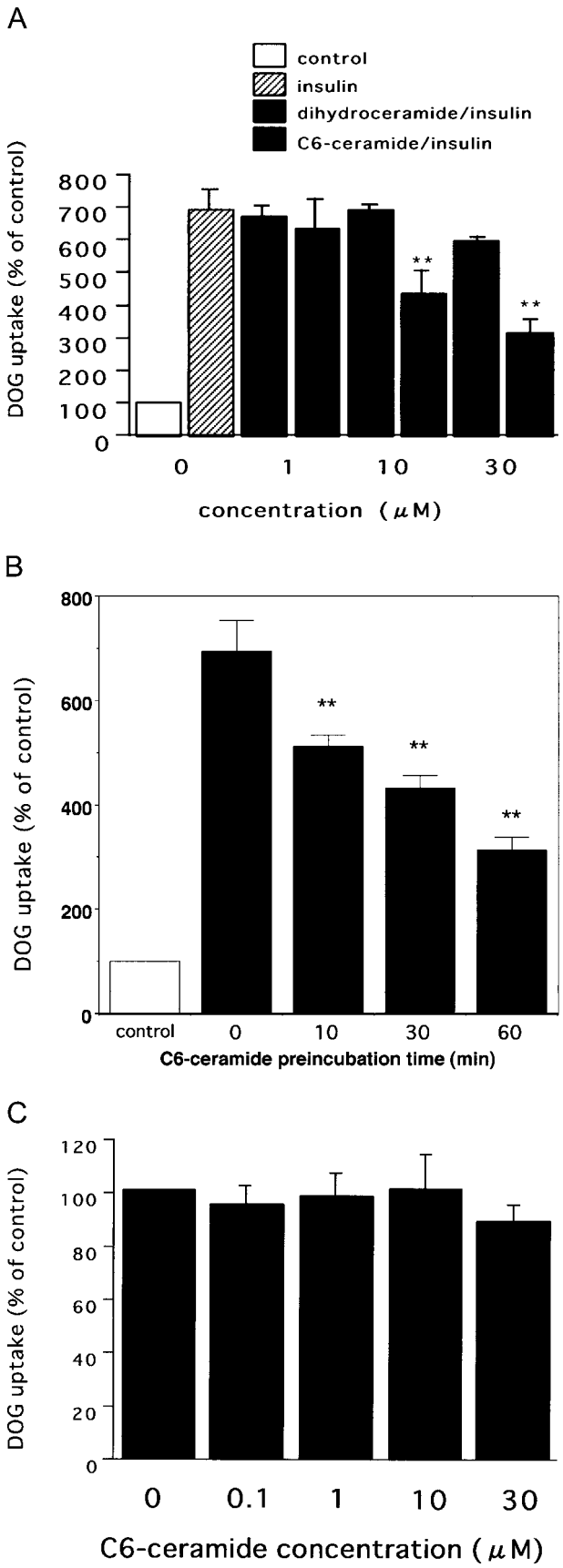
Statistical Analysis

The data are presented as means ± SE unless otherwise stated, and statistical significance was determined by Fisher′s protected least significant difference (PLSD) test.

RESULTS

Effect of C_6-Ceramide Pretreatment on Insulin-Induced [^{3}H]2-DOG Uptake and C_6-Ceramide–Induced [^{3}H]2-DOG Uptake

First, we examined the effect of C_6-ceramide pretreatment for 60 minutes on insulin-induced [^{3}H]2-DOG uptake. In the absence of prior treatment with C_6-ceramide, 10 nmol/L insulin stimulation for 30 minutes provoked an approximately 7-fold increase in [^{3}H]2-DOG uptake (694 ± 59%) when compared with the control (100%). Pretreatment with 10 μmol/L and 30 μmol/L C_6-ceramide for 60 minutes significantly reduced 10 nmol/L insulin-stimulated [^{3}H]2-DOG uptake to 4.3-, and 3.1-



fold increases over the control (100%), respectively (Fig 1A). In addition, 1 to 30 μmol/L C_6-dihydroceramide had no influence on insulin-induced [^{3}H]2-DOG uptake.

Second, we examined the effect of 30 μmol/L C_6-ceramide for 10 and 30 minutes on insulin-induced [^{3}H]2-DOG uptake to show a time-dependent inhibitiory effect of ceramide. Pretreatment with 30 μmol/L C_6-ceramide for 10 and 30 minutes reduced 10 nmol/L insulin-stimulated [^{3}H]2-DOG uptake to 5.1- and 4.3-fold increase over the control, respectively (Fig 1B).

Third, we also examined C_6-ceramide alone–induced [^{3}H]2-DOG uptake in rat adipocytes. C_6-ceramide (1 to 30 μmol/L) treatment for 60 minutes had no significant influence on [^{3}H]2-DOG uptake in this system, when compared with the control (without C_6-ceramide treatment; 100%) (Fig 1C).

Effect of C_6-Ceramide Pretreatment on Tyrosine Phosphorylation of the IR β-Subunit and IRS-1 During Insulin Stimulation

After determination of protein content, equal amounts of protein were immunoprecipitated at 4°C with an antibody against the IR β-subunit or IRS-1, and then immunoblotted with anti-PY antibody. Figure 2 shows tyrosine phosphorylation of proteins at the 95-kd IR β-subunit (Fig 2A) and at the 180-kd IRS-1 (Fig 2B), respectively. In the absence of any prior treatment with C_6-ceramide, 10 nmol/L insulin stimulation for 10 minutes provoked a 7.2-fold increase in insulin-stimulated autophosphorylation of the IR β-subunit (718 ± 142%) (Fig 2A, lane 4), when compared with the control (100%) (Fig 2A, lane 1). In a pretreatment with 10 μmol/L and 30 μmol/L C_6-ceramide for 60 minutes, 10 nmol/L insulin-stimulated autophosphorylation of the IR β-subunit was significantly suppressed (Fig 2A, lanes 5 and 6) when compared with lane 4 (without C_6-ceramide pretreatment).

In the absence of any pretreatment with C_6-ceramide, 10 nmol/L insulin stimulation for 10 minutes provoked a 2.7-fold increase in tyrosine phosphorylation of IRS-1 (269 ± 17%) (Fig 2B, lane 4) when compared with the control (100%) (Fig 2B, lane 1). Pretreatment with 10 μmol/L and 30 μmol/L C_6-ceramide, respectively, for 60 minutes also significantly reduced 10 nmol/L insulin-stimulated tyrosine phosphorylation

Fig 1. (A) Effect of C_6-ceramide and dihydroceramide pretreatment on insulin-induced [^{3}H]2-DOG uptake. Rat adipocytes were incubated with or without 1 to 30 μmol/L C_6-ceramide for 60 minutes, followed by treatment with 10 nmol/L insulin. Control values are indicated as 100%. Insulin-induced percent change (with or without C_6-ceramide) is expressed as the mean ± SE. **$P < .01$, using Fisher's PLSD test. Values are from 6 determinations in 3 separate experiments (ceramide pretreatment) and 3 determinations in 3 separate experiments (dihydroceramide pretreatment). (B) Effect of 30 μmol/L C_6-ceramide pretreatment on 10 nmol/L insulin-induced [^{3}H]2-DOG uptake for 10 and 30 minutes. Results are from 3 separate experiments. (C) C_6-ceramide alone–induced [^{3}H]2-DOG uptake. Isolated rat adipocytes were treated with C_6-ceramide (0.1 to 30 μmol/L) alone for 60 minutes. Uptake of [^{3}H]2-DOG was measured over 1 minute after treatment with C_6-ceramide for 60 minutes. C_6-ceramide–induced [^{3}H]2-DOG uptake exhibited no significant change *v* control (without C_6-ceramide treatment; 100%). Results are from 3 separate experiments. Data are means ± SE.

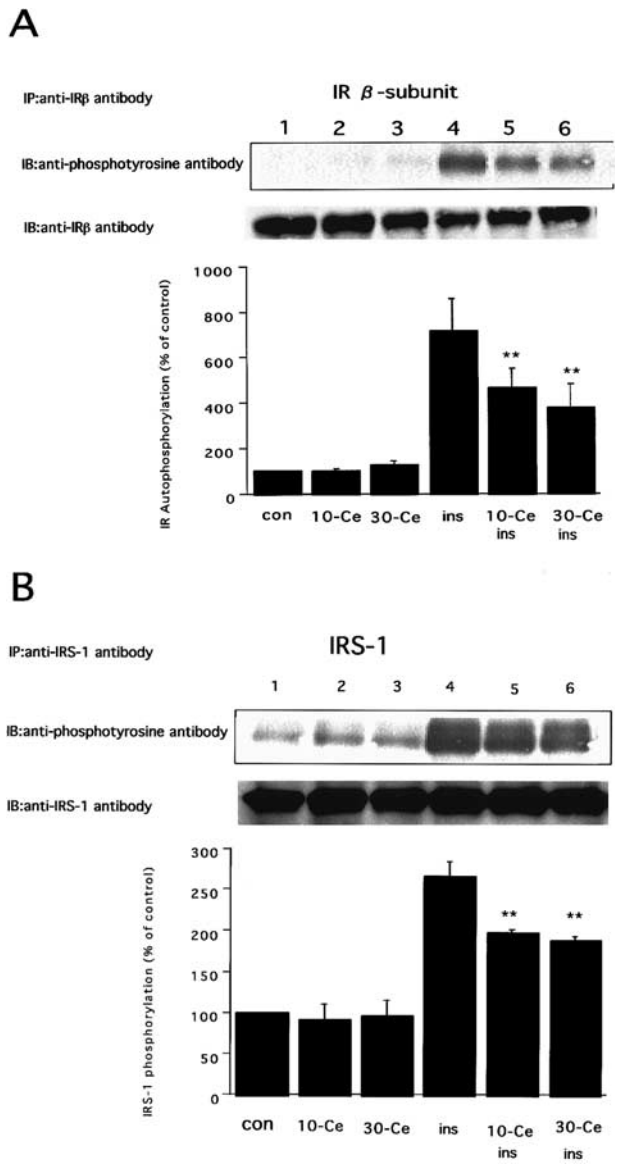


Fig 2. Effect of C_6-ceramide pretreatment on the autophosphorylation of the IR β-subunit and tyrosine phosphorylation of IRS-1. Rat adipocytes were incubated with or without 10 μmol/L and 30 μmol/L C_6-ceramide for 60 minutes, and then stimulated with 10 nmol/L insulin for 10 minutes. Adipocytes were lysed and immunoprecipitated with antibodies against the IR β-subunit or IRS-1. (A) Autophosphorylation of the IR β-subunit. Data are means ± SE of 3 independent experiments. **$P < .01$ using Fisher's PLSD test. Results are representative of 3 separate experiments. (B) Tyrosine phosphorylation of IRS-1. Results are means ± SE of 3 independent experiments. **$P < .01$ using Fisher's PLSD test. Results are representative of 3 separate experiments. All data were obtained with a laser densitometer. IP, immunoprecipitation; IB, immunoblot.

of IRS-1 to 1.97- and 1.88-fold increases over the control (100%), respectively (Fig 2B, lanes 5 and 6). These reductions in tyrosine phosphorylation of IRS-1 were significant when compared with lane 4 (without C_6-ceramide pretreatment).

Effect of C_6-Ceramide Pretreatment on PI3K Activity During Insulin Stimulation

In the absence of any pretreatment with C_6-ceramide for 60 minutes, 10 nmol/L insulin-induced PI3K activity significantly increased (267 ± 17%) (Fig 3, lane 4) when compared with the control (100%) (Fig 3, lane 1). In contrast, pretreatment with 10 μmol/L and 30 μmol/L C_6-ceramide, respectively, for 60 minutes also significantly reduced 10 nmol/L insulin-stimulated PI3K activity to 1.82- and 1.74-fold increases, respectively (Fig 3, lanes 5 and 6), when compared with the control (100%) (Fig 3, lane 1). These reductions in PI3K activity were significant when compared with lane 4 (without C_6-ceramide pretreatment).

Effect of C_6-Ceramide Pretreatment on Insulin-Stimulated Translocation of PKCζ in the Membrane and Immunoprecipitable PKCζ Activity

Without prior treatment with C_6-ceramide, 10 nmol/L insulin-induced PKCζ immunoreactivity in the membrane fractions increased after 10 minutes of stimulation (262 ± 19%) (Fig 4, lane 4), when compared with lane 1 (control: 100%). However, following pretreatment of 10 μmol/L and 30 μmol/L C_6-ceramide for 60 minutes, a significant reduction of insulin-induced increase in membrane-associated PKCζ immunoreactivity was observed (10 μmol/L: 127 ± 13%; and 30 μmol/L:

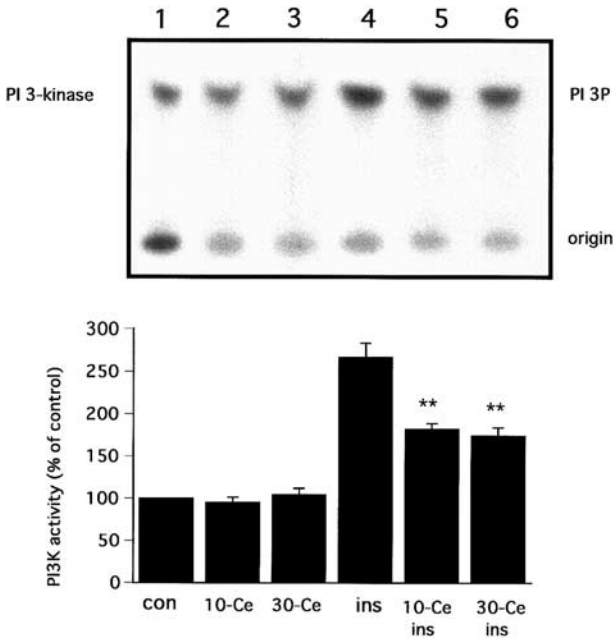


Fig 3. Effect of C_6-ceramide on insulin-induced PI3K activation. PI3K activity was measured as shown in the methods. After immunoprecipitation with anti-PY antibody, adipocyte homogenates were incubated with [γ-^{32}P]ATP and phosphatidylinositol for 10 minutes. The samples were separated on a TLC plate and visualized using autoradiography. Nonradioactive phosphatidylinositol 4-phosphate was chromatographed on the same plate as the standard. Results are means ± SE. **$P < 0.01$, using Fisher's PLSD test. The result shown here is a representative experiment. The values are made from 3 separate experiments. All data were obtained with a laser densitometer.

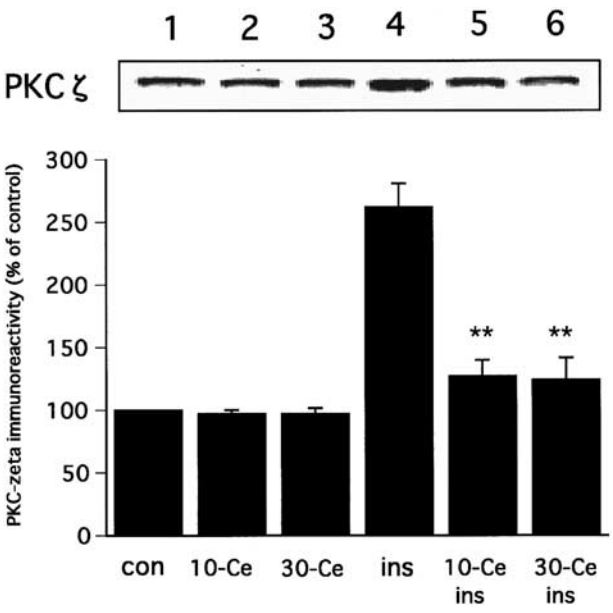


Fig 4. Effect of 10 μmol/L and 30 μmol/L C_6-ceramide pretreatment for 60 minutes on the insulin-stimulated immunoreactivity of membrane-associated PKCζ Rat adipocytes were incubated with or without 10 μmol/L and 30 μmol/L C_6-ceramide for 60 minutes, followed by treatment with 10 nmol/L insulin for 10 minutes. Western blot analysis was performed as described in the methods. Membrane-associated PKCζ immunoreactivities were stimulated by 10 nmol/L insulin for 10 minutes (lane 4). The insulin-induced percentage change (with or without C_6-ceramide pretreatment) is expressed as the mean ± SE ($P < 0.01$, using Fisher's PLSD test). The results are made from 3 different experiments. All data were analyzed with a laser densitometer.**

124 ± 18%, respectively) (Fig 4, lanes 5 and 6). Moreover, we showed the results of immunoprecipitable PKCζ enzyme activity following pretreatment with 10 μmol/L and 30 μmol/L C_6-ceramide for 60 minutes. Without prior treatment with C_6-ceramide, 10 nmol/L insulin-induced PKCζ activity increased after 10 minutes of stimulation (202 ± 18%) (Fig 5). The reduction of insulin-induced increase in PKCζ activity was also observed, as well as the reduction in tyrosine phosphorylation of IRS-1 and IR β-subunit by C_6-ceramide (10 μmol/L: 154 ± 17%; and 30 μmol/L: 146 ± 14%) (Fig 5). Thus, it appears that pretreatment of 10 μmol/L and 30 μmol/L C_6-ceramide for 60 minutes inhibits 10 nmol/L insulin-induced PKCζ activation.

DISCUSSION

In this study, we found that pretreatment with C_6-ceramide suppressed insulin-stimulated DOG uptake in rat adipocytes as shown in Fig 1A. Furthermore, C_6-dihydroceramide, a negative control in the study of C_6-ceramide, did not reduce insulin-stimulated DOG uptake in rat adipocytes as shown in Fig 1A. These results suggest that ceramide suppresses insulin-induced DOG uptake. However, some investigators have previously stated that sphingomyelinase and C_6-ceramide increased glucose uptake in the absence of insulin in skeletal muscle and 3T3-L1 adipocytes.[19, 20] In addition, it was reported that sphingomyelinase, which is activated by TNF-α, had no effect on insulin-induced tyrosine kinase activity of IR or PI3K activity in rat skeletal muscles. They suggested that sphingomyelinase caused translocation of the glucose transporter by an unknown pathway.[19] However, our results indicated that ceramide alone did not elicite DOG uptake, as shown in Fig 1B.

Although ceramide-induced insulin resistance is still controversial, we hypothesize that ceramide may affect insulin signal transduction. In this report, we showed that pretreatment with C_6-ceramide reduced the insulin-induced tyrosine kinase activity of IR, tyrosine phosphorylation of IRS-1, and PI3K activity in rat adipocytes (Figs 2 and 3). Recently, we demonstrated that TNF-α pretreatment suppressed the insulin-induced tyrosine kinase activity of IR, tyrosine phosphorylation of IRS-1, and PI3K activity in rat adipocytes.[21] Moreover, Peraldi et al showed that sphingomyelinase, TNF-α, and C_6-ceramide significantly suppressed the insulin-induced tyrosine kinase activity of IR and the tyrosine phosphorylation of IRS-1, respectively.[22] Thus, it seems that TNF-α– and C_6-ceramide–induced insulin resistance are involved in the same mechanism. It is likely that treatment of insulin-sensitive cells with ceramide leads to the serine phosphorylation of IRS-1, converting it into an autophosphorylation inhibitor of the IR β-subunit. Probably, ceramide-activated protein kinase is relevant to the serine phosphorylation of IRS-1.[22] Recently, it has been reported that salicylate, an inhibitor of Iκβ (IKK-β), a known serine kinase, prevents fat-induced insulin resistance. Therefore, serine phos-

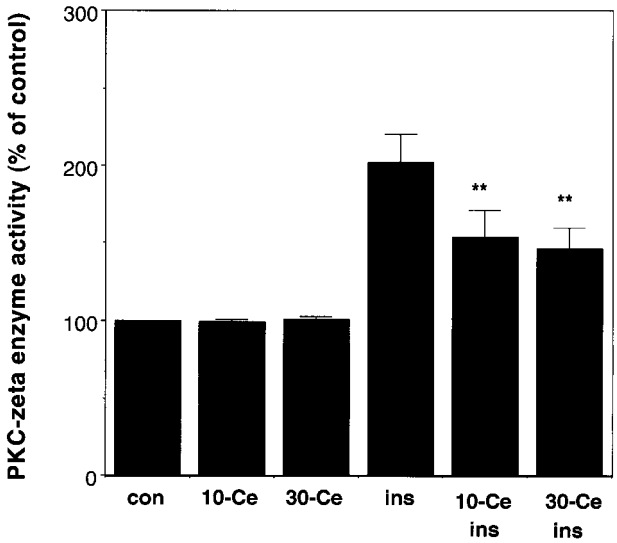


Fig 5. Effect of 10 μmol/L and 30 μmol/L C6-ceramide pretreatment for 60 minutes on insulin-stimulated PKCζ activation. Rat adipocytes were incubated with or without 10 μmol/L and 30 μmol/L C_6-ceramide for 60 minutes, followed by treatment with 10 nmol/L insulin for 10 minutes. Cell lysate obtained in each indicated time was incubated with anti-PKCζ antibody and protein A agarose. Anti-PKCζ immunoprecipitates were washed 3 times with reaction buffer I and incubated with buffer I containing 50 mmol/L [γ-^{32}P]ATP, 40 μmol/L PKCε pseudosubstrate analog, and 40 μg/mL PS as described in the methods. The insulin-induced percentage change (with or without C_6-ceramide pretreatment) is expressed as the mean ± SE ($P < .01$, using Fisher's PLSD test). Results are from 3 different experiments.**

phorylation of IRS-1 may induce insulin resistance.[23] In fact, we already have reported that glucocorticoid-induced mobility shift of IRS-1, which showed serine/threonine phosphorylation of IRS-1, was completely blocked by conventional PKC inhibitor Go6976.[24]

We also examined the effect of C_6-ceramide on the insulin-induced activation of PKCζ, an atypical PKC family. An insulin-induced increase in membrane-associated PKCζ immunoreactivity was inhibited after C_6-ceramide pretreatment. It is clear that PKCζ is activated by PI3K through PIP_3.[25] In the light of this evidence, the reduction of insulin-induced PKCζ immunoreactivity after C_6-ceramide pretreatment may be, in part, a consequence of the inhibition of the C_6-ceramide–mediated IR tyrosine kinase–IRS-1–PI3K pathway.

An objection will no doubt be raised that PKCζ has a binding site for ceramide and subsequent ceramide may activate PKCζ.[26-28] Although ceramide activates PKCζ, this translocation by ceramide occurs towards the nucleus of the cell.[27,28] Insulin-induced (PIP_3-mediated) translocation of PKCζ may be different from ceramide-induced translocation of PKCζ. Conceivably, insulin-induced translocation of PKCζ occurs towards the membrane of the cell[11,12] and may mediate GLUT4 translocation.[14] Our results also suggest that C_6-ceramide does not completely suppress insulin-induced glucose uptake in rat adipocytes. C_6-ceramide suppressed insulin-stimulated IRS-1 tyrosine phosphorylation and PI3K activation at the same level, as well as C_6-ceramide-induced the suppression of insulin-stimulated PKCζ activity. These results suggest that C6-ceramide may suppress insulin-induced glucose uptake. In conclusion, PKCζ translocation in membranes may be correlated with glucose uptake, and ceramide-induced insulin resistance may be associated with a reduction in IRS-1-PI3K signaling in rat adipocytes along with other postreceptor targets of insulin action.

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