

PAPER

Cell-permeable ceramides increase basal glucose incorporation into triacylglycerols but decrease the stimulation by insulin in 3T3-L1 adipocytes

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OBJECTIVE: To investigate mechanisms for the regulation of glucose incorporation into triacylglycerols in adipocytes by ceramides, which mediate some actions of tumour necrosis factor- α (TNF α).

DESIGN: The effects of C₂- and C₆-ceramides (N-acetyl- and N-hexanoyl-sphingosines, respectively) on glucose uptake and incorporation into triacylglycerols and pathways of signal transduction were measured in 3T3-L1 adipocytes.

RESULTS: C₆-ceramide increased basal 2-deoxyglucose uptake but decreased insulin-stimulated uptake without changing the EC₅₀ for insulin. Incubating 3T3-L1 adipocytes from 2 to 24 h with C₂-ceramide progressively increased glucose incorporation into the fatty acid and especially the glycerol moieties of triacylglycerol. These effects were accompanied by increased GLUT1 synthesis resulting from ceramide-induced activation phosphatidylinositol 3-kinase, ribosomal S6 kinase and mitogen-activated protein kinase. C₂-ceramide also increased p21-activated kinase and protein kinase B activities. However, C₂-ceramide decreased the insulin-stimulated component of these signalling pathways and also glucose incorporation into triacylglycerol after 2 h.

CONCLUSIONS: Cell-permeable ceramides can mimic some effects of TNF α in producing insulin resistance. However, ceramides also mediate long-term effects that enable 3T3 L1 adipocytes to take up glucose and store triacylglycerols in the absence of insulin. These observations help to explain part of the nature and consequence of TNF α -induced insulin resistance and the control of fat accumulation in adipocytes in insulin resistance and obesity.

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Keywords: fatty acid synthesis; insulin resistance; phosphatidylinositol 3-kinases; triacylglycerol synthesis; tumour necrosis factor- α

Introduction

Insulin resistance is associated with android obesity, type 2 diabetes, hypertension and dyslipidaemia. This Metabolic Syndrome represents a major risk factor for premature cardiovascular disease.^{1,2} Insulin resistance also develops in cancer, sepsis, endotoxaemia, trauma, infection and alcoholism and this may be associated with the production of tumour necrosis factor- α (TNF α).³ This latter cytokine is also produced in the adipose tissue of obese insulin-resistance rodents⁴ and human beings.^{5,6} Neutralization of TNF α with

antibodies in insulin-resistance rodents increases insulin-induced glucose uptake.⁴ TNF α , therefore, could be a key mediator of obesity-linked insulin resistance,^{2–4,7,8} but the mechanisms for this are controversial. TNF α was proposed to increase the serine phosphorylation of insulin receptor substrate (IRS-1) and decrease its tyrosine phosphorylation by insulin.⁹ This in turn decreases the tyrosine phosphorylation of the insulin receptor⁹ and activation of phosphatidylinositol (PI) 3-kinase (3-K).^{10,11} TNF α activates several signalling cascades, which include the stimulation of sphingomyelinases and the production of ceramides through the p55 receptor.^{12–14} Ceramides can then stimulate serine/threonine kinases¹³ and phosphoprotein phosphatases¹² and they inhibit phospholipase D1 and D2.¹⁵ The involvement of ceramides in mediating the effects of TNF α was demonstrated by using cell-permeable ceramides and sphingomyelinase, which were reported to decrease the tyrosine phosphorylation of IRS-1^{17,18} and activated phosphoprotein phosphatase-1.¹⁹ TNF α could also inhibit insulin signalling in human adipocytes through the p80 TNF α receptor.²⁰

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In other work, TNF α did not decrease the tyrosine phosphorylation of IRS-1, but rather it decreased the concentrations of IRS-1 and GLUT4 in adipocytes.^{21,22} This effect was not mediated by signalling through ceramides.²² In fact, ceramides stimulated rather than inhibited PI 3-K activity associated with IRS-1 and the ceramide-induced inhibition of insulin-stimulated glucose probably resulted from decreased insulin-induced transport of vesicles containing GLUT1 and GLUT4 to the cell surface.²² Our later studies with Rat2 fibroblasts demonstrate that TNF α , sphingomyelinase and C₂-ceramide rapidly stimulate tyrosine kinases, production of Ras-GTP and its association with activated PI 3-K.^{23,24} Furthermore, incubation of 3T3-L1 adipocytes with TNF α increases the tyrosine phosphorylation of IRS-1 and its binding to PI 3-K.²⁵ Ceramides also activate Src and PI 3-K in smooth muscle cells.²⁶ In L6 myocytes, ceramides inhibit insulin-stimulated glucose uptake, but this did not depend upon decreased IRS-1 phosphorylation, or PI 3-K activity.²⁷ Ceramides appear to inhibit insulin-stimulated glucose uptake downstream of PI 3-K at the level of protein kinase B (PKB).²⁸

In contrast to these effects on insulin-stimulated glucose uptake, TNF α and ceramides stimulate insulin-independent glucose uptake in the long-term in 3T3-L1 adipocytes through PI 3-K activation.^{22,29,30} The present work was performed to expand this hypothesis and to understand how ceramide-induced changes in insulin sensitivity modify glucose storage as triacylglycerol (TAG). Ceramides rapidly decreased the insulin-stimulated incorporation of glucose into the fatty acid (FA) and glycerol moieties of triacylglycerol. However, in the longer term (2–24 h) ceramides progressively increased basal glucose uptake in the absence of insulin and its incorporation into FA and especially glyceride-glycerol. This was accompanied by increased GLUT1 synthesis that depended on the stimulations of ERK, PI 3-K and pp70^{S6K}.

Materials and methods

Materials

Murine-derived 3T3-L1 fibroblasts were obtained from American Type Culture Collection (Rockville, MD, USA). 2-Deoxyglucose, dexamethasone, bovine insulin, 3-isobutyl-1-methylxanthine, PI, cytochalasin B, aprotinin, leupeptin, protein kinase inhibitor, poor-BSA, and anti-rabbit IgG and anti-mouse IgG antibodies conjugated with horseradish peroxidase were obtained from Sigma (St Louis, MO, USA). C₂- and C₆-ceramides, dihydro-C₂-ceramide, LY294022 and rapamycin were obtained from Biomol (Plymouth Meeting, PA, USA). PD98059 was from Calbiochem (La Jolla, CA, USA). Other reagents were purchased as follows: electrophoresis reagents, Bio-Rad, Mississauga, Canada; polyvinylidene difluoride membranes, Millipore, Bedford, MA, USA; DMEM and foetal bovine serum, Gibco, Gaithersburg, MD, USA; [³H]2-deoxyglucose and [¹⁴C]-(U)glucose, Dupont-New England Nuclear, Boston, MA, USA; enhanced

chemiluminescence detection reagents and [γ -³²P]ATP, Amersham, Oakville, Canada; rabbit polyclonal antibodies for GLUT1, East Acres, Southbridge, MA, USA; PI 3-K (85 kDa subunit), Transduction, Mississauga, Canada; pp70^{S6K}, IRS-1, p42 and p44 ERK polyclonal antibodies against p65PAK and monoclonal antibodies for phosphotyrosine, Santa Cruz, CA, USA; GLUT4, East Acres; and anti-RAC1 Pleckstrin Homology Domain (PKB-PH), Kinetek, Pharmaceuticals Inc., Vancouver, BC, Canada. All other reagents were from Sigma or Fisher Scientific, Nepean, ON, Canada.

Cell culture

Murine 3T3-L1 fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum and then differentiated as described previously.^{22,31} The medium was then changed and the cells were treated with the indicated agents for various times. The final concentrations of C₂- and C₆-ceramides were 50 or 100 μ M as established previously²² and they were added to the albumin or serum containing solution in DMSO (final concentration <0.3%). Albumin binds ceramides but decreases their effective concentration²² and it also has the benefit of binding FA that are released by the adipocytes. Cells were used within three to nine passages.

Lipid synthesis and glucose uptake

3T3-L1 adipocytes were cultured in 35 mm dishes with DMEM containing 10% foetal bovine serum and the agonists or vehicle. At 2 h before the assay, the medium was changed and cells were incubated in DMEM containing 0.5% BSA with agents or vehicle. For the measurement of lipid synthesis, cells were washed with Krebs-Ringer phosphate buffer, and incubated in the same buffer supplemented with [U-¹⁴C]glucose (0.25 μ Ci/ml) and agonists in the presence or absence of 20 nM insulin for 2 h. Cells were scraped from the dishes in 1 ml methanol and lipids were extracted by adding 1 ml chloroform and 0.9 ml of 320 mM HCl. TAG was hydrolysed in 2.67 mM NaOH at 78°C for 2 h and the incorporations of ¹⁴C into FA and glyceride-glycerol were determined.^{32,33} Glucose uptake was measured over 10 min by adding 2-[³H]deoxyglucose (0.5 μ Ci/0.8 ml, 0.1 mM).^{22,29} In the presence of ceramides, >90% of glucose uptake was inhibited by 20 μ M cytochalasin-B.

Cell membrane fractionation and immunoblotting

Cells in 100 mm dishes were incubated with test agents as indicated, and stimulated 100 nM insulin for 5–10 min. Prior to harvesting, cells were rinsed with ice-cold phosphate-buffer saline and scraped in homogenization buffer (10 mM Tris, pH 7.4, 1 mM EDTA, 0.25 M sucrose, 0.5 mM PMSF, 2.5 μ g/ml aprotinin, and 1 μ g/ml leupeptin). Total and plasma membranes were isolated and resuspended

in homogenization buffer (1–5 mg/ml protein) for assay 5'-nucleotidase activity which was used as the plasma membrane marker.^{22,34} Proteins were separated by SDS-PAGE using 7 or 12% gels³⁵, and transferred to nitro-cellulose (Bio-Rad) or Immobilon-P (Millipore) in 25 mM Tris/HCl, pH 7.4, 192 mM glycine and 20% methanol. After transfer, the membrane was blocked in 1% non-fat milk and 1% BSA at 4°C overnight. Results were visualized with anti-rabbit, or anti-mouse IgG antibodies conjugated with horseradish peroxidase and an enhanced chemoluminescence kit.²²

p65PAK, PP70^{S6K}, and p42 and p44 MAP kinases (ERK) were immunoprecipitated from total cell lysates. p65PAK was resolved by SDS-PAGE and the activity determined by an in-gel assay.³⁶ For MAP kinase, cells were harvested in lysis buffer A (1% Nonidet p40, 10% glycerol, 50 mM Hepes, 137 mM NaCl, 1 mM MgCl₂, 1 mM CaCl₂, 2 mM Na₃VO₄, 10 mM Na₄P₂O₇, 100 mM NaF, 5 µg/ml aprotinin, 1 mmol/l PMSF and 1 µg/ml leupeptin). The lysis buffers for PKB were the same as lysis buffer A but with the addition of 1% Triton X-100, 5 mM benzamide and 10 mM dithiothreitol whereas that for pp70^{S6K} contained 1% Triton X-100, 10 µg/ml antipain and 1 µg/ml leupeptin, 1 mM dithiothreitol and 1 µg/ml pepstatin. The activities of PKB^{37,38} and MAP kinase³⁹ were measured by phosphorylation of myelin basic protein, whereas that of pp70^{S6K} was determined by the phosphorylation of the peptide KKRNRLLTK.⁴⁰

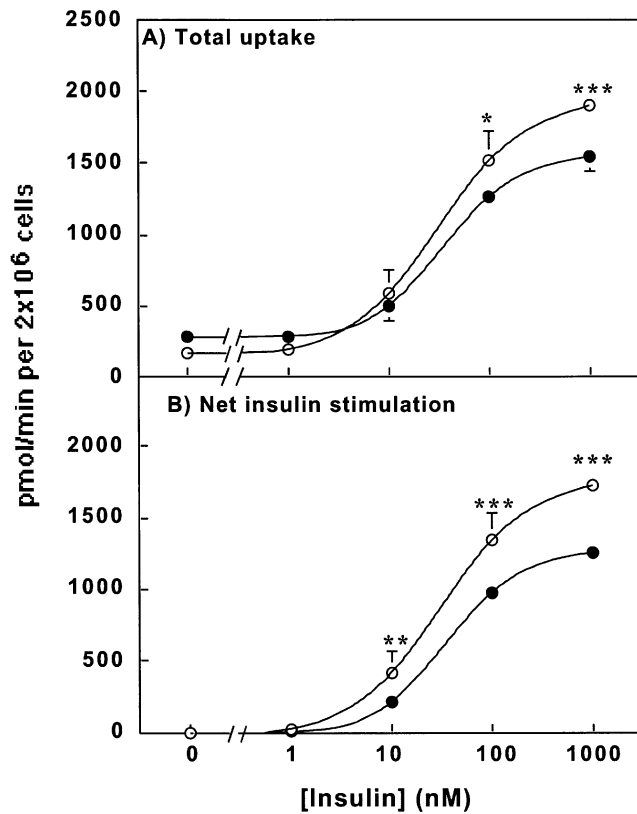


Figure 1 Effect of C₆-ceramide on the stimulation of 2-deoxyglucose transport by insulin. Differentiated 3T3-L1 adipocytes were incubated in DMEM supplemented with 10% foetal bovine serum (○), or the medium containing 100 µM C₆-ceramide (●) for 4 h. Cells were then incubated in DMEM containing 0.5% BSA (w/v) with or without ceramide for 2 h. After washing twice with Krebs-Ringer phosphate buffer, cells were exposed to 0–1000 nM insulin for 30 min. Glucose uptake was measured by adding 0.1 mM 2-[³H]deoxyglucose (0.5 µCi/35 mm dish). After incubation for 10 min, the medium was withdrawn and the cells were washed three times with ice-cold Krebs-Ringer phosphate buffer. Glucose transport was calculated from the radioactivity taken up by the cells (A). Insulin-dependent glucose transport was calculated as the difference between glucose uptake in the presence and absence of insulin (B). Results are means ± s.d. (where large enough to be shown) of duplicate determinations from three independent experiments. Significant differences between control and ceramide-treated cells are indicated by **P* < 0.05, ***P* < 0.01 and ****P* < 0.001.

Statistical analysis

Statistical differences were determined by analysis of variance with appropriate *post hoc* multiple comparison as indicated. Analysis of the effect of insulin concentration on 2-deoxyglucose uptake (Figure 1) was performed using an ALLFIT program.⁴¹

Results

Effects of cell-permeable ceramides on glucose uptake and expression of GLUT1

Our previous work demonstrated that C₂- and C₆-ceramides decreased insulin-stimulated glucose uptake, but increased basal glucose uptake, in 3T3-L1 adipocytes.²² The present work determined whether C₆-ceramide modifies the insulin dose–response curve. 3T3-L1 adipocytes were incubated with 100 µM C₆-ceramide for 6 h and then exposed to various insulin concentrations for 30 min (Figure 1A). C₆-ceramide increased basal 2-deoxyglucose transport by 1.65 ± 0.12-fold in three independent experiments, but decreased total glucose transport in the presence of 100 and 1000 nM insulin (Figure 1A). The insulin-induced component of glucose transport was decreased by ceramide treatment at 10, 100 and 1000 nM insulin (Figure 1B). At the physiological concentration of 10 nM insulin, this inhibition was about 50%. There was no change in the calculated EC₅₀ for insulin (31 and 37 nM, respectively, for control and ceramide-treated cells). This result is compatible with the lack of change in the expression of the insulin receptor, or in its tyrosine phosphorylation in response to insulin stimulation after exposure to ceramides.²² However, the calculated maximum stimulation of glucose uptake was decreased from 1930 pmol/min per 2 × 10⁶ cells to 1590 pmol/min per 2 × 10⁶ cells (Figure 1). These results indicate that the ceramide effects on insulin-stimulate glucose uptake occur on signalling downstream of the activation of the insulin receptor.

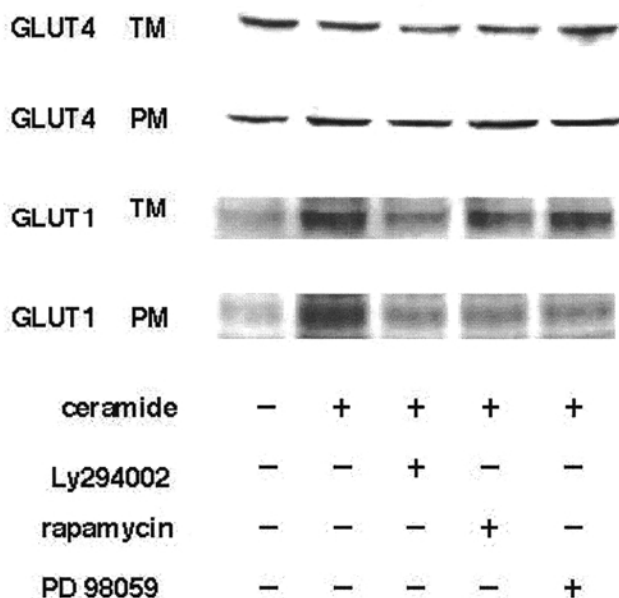


Figure 2 Effects of Ly294002, rapamycin and PD98059 on the distribution of glucose transporters in total and plasma membranes. Differentiated 3T3-L1 adipocytes were incubated with 100 μ M C_2 -ceramide or C_2 -ceramide plus 20 μ M Ly294002, 20 ng/ml rapamycin, or 50 μ M PD98059 for 12 h. After incubation, the cells were fractionated into total membrane (TM) and plasma membranes (PM). GLUT1 and GLUT4 were analysed by immunoblotting using 50 μ g protein per lane. The results are from a representative experiment that was repeated three times.

Effects of ceramides on signalling downstream of the insulin receptor

We postulated that ceramides increase basal glucose uptake progressively up to 24 h mainly by increasing GLUT1 synthesis and its expression at the plasma membrane.²² Increases in

GLUT1 synthesis in L6 myocytes are mediated by increases in PI 3-K activity that subsequently increase the activity of pp70^{S6K}.^{42,43} In 3T3-L1 adipocytes simultaneous stimulation of the Ras-Raf-ERK pathway is also needed for optimum stimulation of GLUT1 synthesis.⁴⁴ The mechanism for the ceramide-induced increase in GLUT1 synthesis was investigated by incubating 3T3-L1 adipocytes for 12 h with C_2 -ceramide, which produces more marked increases in glucose uptake compared with C_6 -ceramide.²² Treatment with C_2 -ceramide increased GLUT1 concentration in the cells by 5.04 ± 0.96 -fold and the expression on the plasma membrane by 9.23 ± 1.6 -fold in three independent experiments (Figure 2). These increases were on average 42, 30 and 25% lower when the cells were treated with Ly 294002, rapamycin or PD 98059, respectively. These inhibitors block the activity of PI 3-K and the activation of pp70^{S6K} and MAP kinase (ERK) respectively. However, GLUT4 concentration were not increased significantly by C_2 -ceramide as expected,²² nor was GLUT4 expression significantly affected by Ly 294002, rapamycin or PD 98059. However, C_2 -ceramide alone did increase the amount of GLUT4 in the plasma membranes by an average of 70% (Figure 2) as expected from our previous studies.²²

We demonstrated that treating 3T3-L1 adipocytes with C_2 -ceramide for 12 h increased the PI 3-K activity associated with IRS-1 by about 2-fold.²² The present studies show that treatment for 2 and 12 h with C_2 -ceramide alone increased pp70^{S6K} activity by 3.7- and 1.7- fold, respectively (Table 1). Treatment with 100 nM insulin alone for 10 min increased pp70^{S6K} activity by 2.9-fold. By comparison, in cells treated with C_2 -ceramide, the additional stimulation of pp70^{S6K} by acute treatment with insulin was much lower. Treatment with C_2 -ceramide for 12 h also increased the activity of p65PAK by about 1.5-fold compared with the 2.1-fold increase in response to acute treatment with 100 nM insulin

Table 1 Effect of C_2 -ceramide on the activation of pp70^{S6K}, p65PAK, PKB and MAP kinase (ERK). Differentiated 3T3-L1 adipocytes were incubated with 100 μ M C_2 -ceramide for 2, 12 or 24 h and the cells were then treated in the presence or absence of 100 nM insulin for 5 or 10 min. Cells were lysed and the kinases were precipitated separately in different experiments using appropriate antibodies. Kinases activities were determined with myelin basic protein as substrate for p65PAK, PKB and MAP kinase and the KKRNRRLTK peptide for pp70^{S6K}. Results are expressed relative to the value of untreated cells and are expressed as means \pm s.e.m. for three or five independent experiments. Statistical differences were calculated by analysis of variance followed by a Fisher multiple range test, * $P < 0.001$, ** $P < 0.0001$, compared with the value for untreated cells. There were no statistically significant differences between results obtained in the presence of C_2 -ceramide vs the corresponding values in the presence of insulin and C_2 -ceramide

Ceramide treatment (h)	Insulin treatment (10 min)	Relative kinase activity			
		pp70 ^{S6K}	PAK	PKB	ERK
0	None	1	1	1	1
2	None	$3.7 \pm 0.6^{**}$	n.m.	$2.0 \pm 0.3^*$	n.m.
12	None	$1.7 \pm 0.3^{**}$	$1.5 \pm 0.3^*$	n.m.	$2.5 \pm 0.6^*$
24	None	n.m.	n.m.	$3.1 \pm 0.9^*$	n.m.
0	Present	$2.9 \pm 0.4^*$	$2.1 \pm 0.09^*$	$2.4 \pm 0.5^*$	$7.9 \pm 3.4^{**}$
2	Present	$4.2 \pm 0.7^{**}$	n.m.	$2.7 \pm 0.6^{**}$	n.m.
12	Present	$2.6 \pm 0.3^{**}$	$1.7 \pm 0.2^*$	n.m.	$7.5 \pm 3.3^{**}$
24	Present	n.m.	n.m.	$2.7 \pm 0.3^{**}$	n.m.

n.m. = not measured.

(Table 1). p65PAK activity in the presence of C₂-ceramide and insulin was 1.7-fold higher than in non-treated cells. Therefore, there was no significant stimulation by insulin above the ceramide effect. Treatment of the adipocytes with C₂-ceramide for 2 or 24 h increased PKB activity by 2.0- and 3.1-fold, respectively (Table 1). However, 100 nM insulin stimulated PKB acutely by about 2.4-fold in control cells. C₂-ceramides decreased the insulin-dependent stimulation of PKB and after 24 h no significant insulin effect was obtained.

The effects of insulin and C₂-ceramide on ERK activity were determined first by using a gel shift assay. As expected, incubation for 5 min with 100 nM insulin caused a gel shift for ERK. Treatment of the cells for 2 h with C₂-ceramide did not produce an obvious gel shift, but this was observed after 12 h incubation (Figure 3). There appeared to be no significant effect of C₂-ceramide on the total activation of ERKs in the presence of insulin. Addition of 20 ng/ml rapamycin or 20 μM Ly294002 did not affect the gel shifts that were produced by C₂-ceramide or insulin at the 12 h point (results not shown). The gel shift results were also verified by determining ERK activity after immunoprecipitation (Table 1). Insulin (100 nM) stimulated the activity of the ERK after 5 min by 7.9-fold in three independent experiments with control cells. Incubation with 100 μM C₂-ceramide for 12 h increased the activity of ERK by an average of 2.5-fold. There was no significant difference in the total stimulation of the ERK in the presence of insulin in control vs ceramide treated cells. Therefore, the insulin-dependent component of the stimulation was decreased by ceramide treatment.

Effects of C₂-ceramide on glucose incorporation into TAGs in the presence and absence of insulin

The major fate of glucose in adipocytes is incorporation into TAG through FA synthesis and incorporation of glycerol phosphate. Treatment of the adipocytes with 20 nM insulin increased [¹⁴C]glucose incorporation into the FA of TAG by about 18-fold (Figure 4A). Preincubation of the cells for 2 h

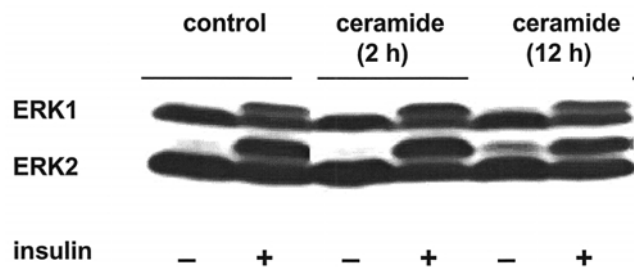


Figure 3 Activation of MAP kinase (ERK) by C₂-ceramide and insulin. 3T3-L1 adipocytes were treated with 100 μM C₂-ceramide for 2 or 12 h, and then exposed to 100 nM insulin for 5 min. The gel shift assays for MAP kinase were performed by Western blotting with a combination of ERK1 and ERK2 antibodies. Results are from a representative experiment that was repeated three times and ERK activities are given in Table 1.

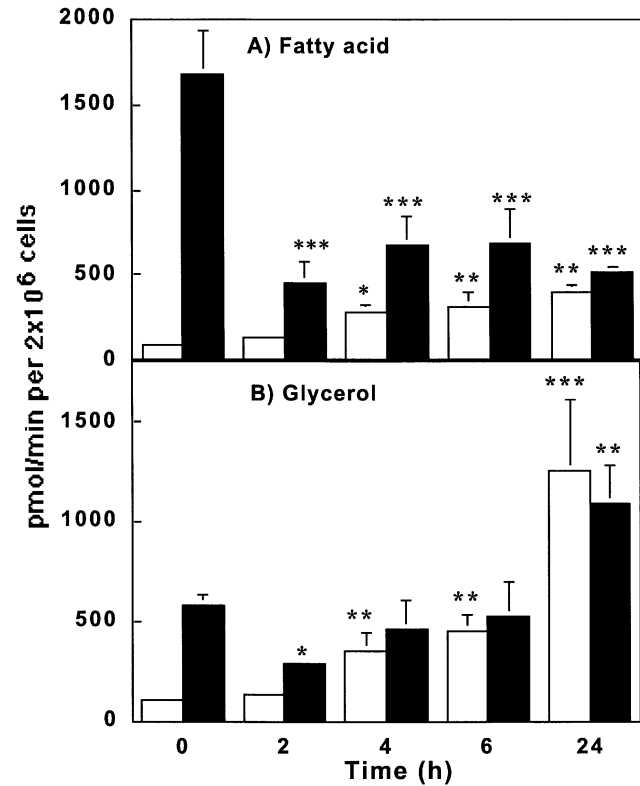


Figure 4 Time-course of the effect of C₂-ceramide on the incorporation of glucose into TAGs. Differentiated 3T3-L1 adipocytes were incubated with 50 μM C₂-ceramide for 0–24 h. At the time indicated, the incorporation of glucose into the fatty acids (A) and glycerol moieties of TAG (B) were measured in the absence (white bars) and presence of 20 nM insulin (black bars). Results are expressed as means ± s.e.m. (where large enough to be shown) from five independent experiments. Significant differences between control (time 0 h) and ceramide-treated cells were established by analysis of variance and *post hoc* multiple comparison by a Bonferroni test. Differences are indicated by: **P* < 0.05, ***P* < 0.01 and ****P* < 0.005.

with 50 μM C₂-ceramide decreased the insulin-dependent component by about 80% and after 24 h incubation with C₂-ceramide there was little insulin-dependent FA synthesis. Ceramide treatment increased glucose incorporation into FA progressively up to 24 h and at this time the increase was about 4.5-fold. Insulin stimulated glucose incorporation into the glycerol moiety of TAG by about 6-fold (Figure 4B). Preincubation with C₂-ceramide for 2 h decreased the insulin-dependent incorporation by about 67% and after the 24 h preincubation there was no significant insulin-dependent incorporation. At this time total glucose incorporation into glyceride-glycerol was higher than in cells that were not exposed to C₂-ceramide because C₂-ceramide caused a 12-fold increase in glyceride-glycerol.

It was predicted that increases in GLUT1 concentrations and glucose uptake after ceramide treatment would be a major contributor to the ceramide-induced incorporation of glucose into TAGs. We therefore determined the effects of

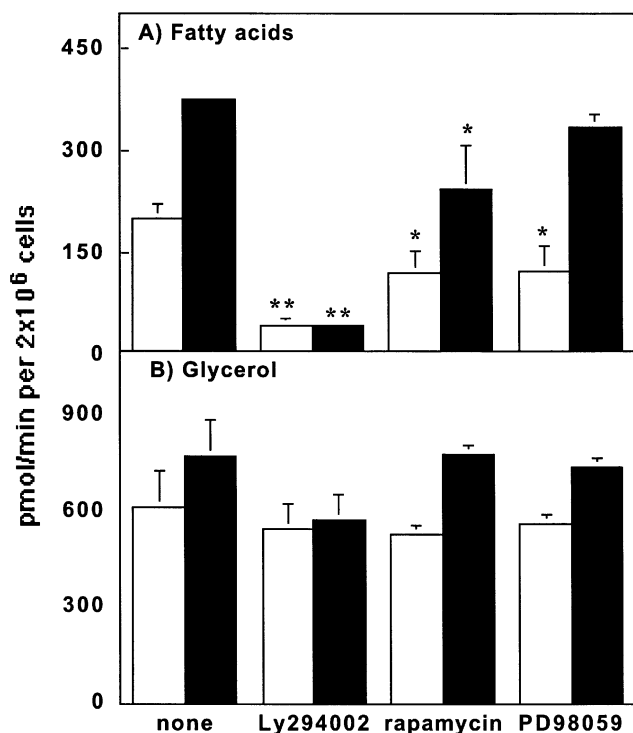


Figure 5 Effect of Ly294002, rapamycin and PD 98059 on the incorporation of glucose into TAG. 3T3-L1 adipocytes were incubated with 50 μ M C₂-ceramide in the presence or absence of the inhibitors, 20 μ M Ly294002, 20 ng/ml rapamycin or 50 μ M PD 98059. The incorporation of glucose into fatty acids (A) and glycerol moieties (B) of triacylglycerol were measured in the absence (white bars) and presence of 20 nM insulin (black bars). Results are expressed as means \pm s.e.m (where large enough to be shown) from four independent experiments. Significant differences between control (absence of inhibitor) and corresponding inhibitor-treated cells were established by analysis of variance and post hoc multiple comparison by a Bonferroni test. Differences are indicated by: * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.005$.

Ly 294002, rapamycin and PD 98059 on TAG synthesis in cells that had been preincubated for 12 h with C₂-ceramide. The ceramide-induced increases in basal glucose incorporations into FA and glyceride-glycerol were 3.2 and 6.0 -fold, respectively. Incubation with Ly 294002, rapamycin and PD 98059 decreased the basal rate of FA synthesis by about 70, 30 and 30%, respectively (Figure 5A). Ly 294002 abolished the effect of insulin in stimulating FA synthesis whereas PD 98059 had no significant effect on the insulin-dependent component. Rapamycin decreased the insulin stimulation of FA synthesis by about 26%. By contrast, Ly 294002, rapamycin and PD 98059 had no significant effects in glucose incorporation into glyceride-glycerol (Figure 5B).

Discussion

C₂- and C₆-ceramides were used for these studies since they are cell-permeable and C₂-ceramide, in particular, is deacylated relatively slowly compared with long-chain ceramides.²³ Therefore, the effects of short-chain ceramides are

mainly direct rather than involving metabolism to sphingosine or sphingosine 1-phosphate.^{23,24} TNF α and ceramides stimulate PI 3-K in 3T3 L1 adipocytes,^{22,25} fibroblasts,^{23,24} smooth muscle cells,²⁶ osteoclasts⁴⁵ and macrophages.⁴⁶ These increases in PI 3-K are associated with protection from apoptosis and increased division rather than cell death. The ceramide concentrations used in the present work were established from previous studies.²²

C₂-ceramide severely inhibited the insulin stimulation of glucose incorporation into the glycerol and particularly the FA moieties of TAG. This effect parallels the inhibition of insulin-stimulated glucose uptake; it occurs after a 2 h preincubation with C₂-ceramide and it is maintained for 24 h. C₆-ceramide did not change the ED₅₀ of insulin for glucose uptake (Figure 1) and C₂-ceramide did not decrease the tyrosine phosphorylation of the insulin receptor. The ceramide effect appeared to be downstream of PI 3-K.²² The inhibitory effects of ceramides on TAG synthesis are, therefore, likely to reflect glucose availability, but there are additional actions on the relative partitioning of glucose into FA and glyceride/glycerol.

The second effect of ceramides is to increase the basal glucose uptake in the absence of insulin through a 5-fold increase in GLUT1 availability and a 9-fold increase at the plasma membrane. TNF α , 10 ng/ml, produced similar increases in GLUT1 concentration in this system after 24 h (our unpublished work), as expected.^{29,30} These effects of TNF α and ceramides resemble those reported for the long-term insulin action on GLUT1 which in 3T3-L1 adipocytes depend upon activation Ras, ERK and pp70^{S6K}.⁴⁴ We demonstrated previously that ceramides increase total and IRS-1-associated PI 3-K activity.²² We now show that ceramides activate pp70^{S6K}, p65PAK and PKB which are downstream of PI 3-K. This explains the long-term insulin mimetic effects of ceramides.²² We did not investigate the mechanism for these effects, but it may be significant that these kinases are activated by small G-proteins: Ras⁴⁷ and Rho⁴⁸ for PI 3-K and Cdc42 and Rac for pp70^{S6K}⁴⁴ and p65PAK.^{36,50,51} Our work with Rat2 fibroblasts demonstrated that TNF α , sphingomyelinase and C₂-ceramide increase Ras-GTP concentrations, the binding of Ras to activated PI 3-K²³ cause the subsequent activation Rac, Cdc42 and the physical interaction of Cdc42 with activated PAK.²⁴ The involvement of PI 3-K, pp70^{S6K} and ERK in increasing GLUT1 concentrations is confirmed by the partial inhibition of the ceramide effect by Ly 294002, rapamycin and PD 98059, which inhibit PI 3-K activity and the activations of pp70^{S6K} and ERK, respectively. Ly294002 could also have inhibited the mammalian target of rapamycin (mTOR).⁵² The effects of the three inhibitors in decreasing GLUT1 expression were also reflected in the decreased stimulation of glucose uptake²² and FA synthesis by C₂-ceramide (Figure 5A).

Constitutively activated PKB stimulates glucose uptake in 3T3-L1 adipocytes and L6 myotubules in the absence of insulin partly through GLUT4 translocation to plasma membranes.^{53,54} Stimulation PKB activity by ceramides (Table 1)

could therefore contribute to the ceramide-induced increase in GLUT1 and GLUT4 concentrations in plasma membranes observed in the absence of insulin.²² Work in other cell types has also demonstrated TNF α and ceramides can activate PKB downstream of PI 3-K.^{45,46,55} However, ceramides can also prevent the translocation of proteins that bind 3-phosphoinositides despite their provision by PI 3-K.⁵⁶ The lack of PKB recruitment to plasma membranes in ceramide treated cells may explain the inhibition of insulin-stimulated glucose uptake.²⁷ It was reported that treatment of 3T2-L1 adipocytes with C₂-ceramide lowered PKB activity in the presence of insulin.²⁸ In this work, C₂-ceramide alone doubled basal glucose uptake as predicted from our previous²² and present work. The ceramide inhibition of PKB activation by insulin is also compatible with our own results. The ceramide effects on PKB appear to be cell-type-specific. Activation of PKB by TNF α prevents apoptosis in osteoclasts⁴⁵ and in fibroblasts TNF α and ceramides can increase cell division rather than leading to cell death.²³ In HMN1 cells C₂-ceramide decreases PKB activity after 15 min and induces apoptosis.⁵⁷

Different cell types also show large variations in the time of response. The increase in PKB activity was observed 2 and 24 h after C₂-ceramide treatment (Table 1). The effects of TNF α in decreasing the tyrosine phosphorylation of IRS-1 took 2–4 days to occur in adipocytes,⁹ whereas there was a decrease in the tyrosine phosphorylation of IRS-1 and its association with PI 3-K after 1 h in rat hepatoma Fao cells.¹¹ Increased tyrosine phosphorylation of IRS-1 and binding to PI 3-K occurred after 20–60 min in 3T3-L1 adipocytes.²⁵ In Rat2 fibroblasts, TNF α , sphingomyelinase and C₂-ceramide increased PI 3-K transiently after 10–20 min,^{23,24} whereas longer incubation times were required in 3T3-L1 adipocytes.²² Furthermore, in Rat2 fibroblasts the stimulation of ERK was inhibited almost completely by Ly 294002, indicating the involvement of PI 3-K.²³ PI 3-K is thought to be particularly important for ERK activation in cells that do not exhibit large stimulations in response to growth factors.⁴⁷ In the present experiments activation of ERK by C₂-ceramide was not inhibited by Ly 294002 and it may rely on increase in Ras-GTP²³ which can then stimulate Raf and MEK.

Glucose incorporation into glyceride–glycerol is less dependent on insulin than that into FA (Figure 4). Furthermore, incubation for 24 h with C₂-ceramide stimulated the incorporation of glucose into glyceride–glycerol 12-fold compared with a 4.5-fold stimulation into FA. Ly294002, rapamycin or PD 98059 did not block the ceramide-induced increase in glyceride–glycerol synthesis (Figure 5B), although glucose uptake was decreased by these inhibitors.²² The present results demonstrate that ceramides produced a rapid and sustained insulin resistance in both glucose uptake and TAG synthesis. However, ceramides stimulate insulin-independent glucose uptake and incorporation into FA and particularly glyceride–glycerol. This is despite the effects of ceramides⁵⁸ and TNF α ⁵⁹ in increasing cAMP concentrations in 3T3 L1 adipocytes due to a time-dependent decrease in phosphodiesterase 3B activity resulting in increased lipolysis.

A ceramide-induced increase in cAMP and FA concentrations should produce a substantial decrease in FA synthesis, whereas the increased FA availability should increase TAG synthesis from glycerol phosphate. Assuming that 1 mol of glucose could provide 2 mol of glyceride–glycerol or acetate for FA biosynthesis, the provision of carbon atoms from glucose alone cannot sustain TAG synthesis. This applies especially after long-term incubation with C₂-ceramide where total FA synthesis is decreased by about 75% compared to the control in the presence of insulin and where glyceride–glycerol production is increased. C₂-ceramide increased total lipolysis after 24 h in our experimental system by 1.8–3.1-fold.⁵⁸ This could contribute to the 12-fold increase in glyceride–glycerol production (Figure 4), but the 7.5-fold increased in glucose uptake²² and the 4.5-fold increase in FA synthesis are also important.

The ceramide-induced changes in TAG synthesis that we observed in cell culture are likely to occur *in vivo*. For example, preferential incorporation of glucose into glyceride–glycerol rather than FA was observed with insulin resistant adipose tissue from JCR:LA rats.³³ In normal Wistar rats FA synthesis predominates.³² Insulin resistance is commonly associated with increased circulating FA and hypertriglyceridaemia² and therefore adipose tissue is able to acquire FA from the circulation in this condition. The observations that TNF α increased GLUT1 concentrations and basal glucose uptake^{22,29,30,62} are also compatible with TNF α stimulating the peripheral glucose uptake *in vivo*.⁶³ Furthermore, GLUT1 expression in plasma membranes from skeletal muscle of obese diabetic SHR rats is increased by 40% compared to the lean genotype.⁶⁴ The idea that ceramides are responsible for part of the TNF α signalling pathway in exacerbating insulin resistance may seem paradoxical if, on their own, ceramides stimulate insulin-independent glucose uptake and TAG synthesis in the long term. However, despite an increase in non-insulin-stimulated (basal) glucose uptake, insulin-resistant tissue is unable to respond with sufficient glucose uptake to dispose of a post-prandial glucose load effectively.

Our work provides further mechanisms for the effects of TNF α and ceramides in decreasing the importance of insulin as a regulator of metabolic balance in adipose tissue. The results also provide insight into the association between obesity and insulin resistance. TNF α and ceramides decrease the effects of insulin in stimulating glucose uptake and TAG synthesis. However, they increase the capacity to store glucose in TAG, especially in the glycerol moiety, in the face of insulin resistance. At the same time lipolysis is increased by TNF α and ceramides. This increased turnover of FA, especially from the increased adipose tissue mass in android obesity, aggravates insulin resistance and the progression of the Metabolic Syndrome.²

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