

Ceramide impairs the insulin-dependent membrane recruitment of Protein Kinase B leading to a loss in downstream signalling in L6 skeletal muscle cells

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Abstract

Aims/hypothesis. Increased cellular production of ceramide has been implicated in the pathogenesis of insulin resistance and in the impaired utilisation of glucose. In this study we have used L6 muscle cells to investigate the mechanism by which the short-chain ceramide analogue, C₂-ceramide, promotes a loss in insulin sensitivity leading to a reduction in insulin stimulated glucose transport and glycogen synthesis.

Method. L6 muscle cells were pre-incubated with C₂-ceramide and the effects of insulin on glucose transport, glycogen synthesis and the activities of key molecules involved in proximal insulin signalling determined.

Results. Incubation of L6 muscle cells with ceramide (100 µmol/l) for 2 h led to a complete loss of insulin-stimulated glucose transport and glycogen synthesis. This inhibition was not due to impaired insulin receptor substrate 1 phosphorylation or a loss in phosphoinositide 3-kinase activation but was caused by a fail-

ure to activate protein kinase B. This defect could not be attributed to inhibition of 3-phosphoinositide-dependent kinase-1, or to impaired binding of phosphatidylinositol 3,4,5 triphosphate (PtdIns(3,4,5)P₃) to the PH domain of protein kinase B, but results from the inability to recruit protein kinase B to the plasma membrane. Expression of a membrane-targeted protein kinase B led to its constitutive activation and an increase in glucose transport that was not inhibited by ceramide.

Conclusions/interpretation. These findings suggest that a defect in protein kinase B recruitment underpins the ceramide-induced loss in insulin sensitivity of key cell responses such as glucose transport and glycogen synthesis in L6 cells. They also suggest that a stimulated rise in PtdIns(3,4,5)P₃ is necessary but not sufficient for protein kinase B activation in this system. [Diabetologia (2001) 44: 173–183]

Keywords Glucose transport, glycogen synthesis, PI3K, IRS1, TNF-α, muscle, membrane.

The activation of phosphoinositide 3-kinase (PI3K) by insulin represents a key signalling event in the hormonal stimulation of diverse cellular responses

including glucose transport and glycogen synthesis. The activation of PI3K increases the production of 3-phosphoinositides (e.g. phosphatidylinositol 3,4,5 trisphosphate [PtdIns(3,4,5)P₃] and phosphatidylinositol 3,4 bisphosphate [PtdIns(3,4)P₂]), which act as important signalling intermediates in the downstream activation of the serine/threonine kinase, Protein Kinase B (PKB/Akt). Activation of PKB depends upon its phosphorylation on two key amino acid residues, Thr³⁰⁸ and Ser⁴⁷³, with full activation requiring the phosphorylation of both [1]. The N-terminal domain of PKB contains a pleckstrin homology (PH) domain, which is thought to be critical in allowing the kinase to interact with 3-phospho-

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Abbreviations: PKB, protein kinase B; PI3K, phosphoinositide 3-kinase; PDK1, 3-phosphoinositide-dependent kinase 1; mPKB, membrane targeted PKB; IRS1, insulin receptor substrate 1; GSK-3, glycogen synthase kinase-3; SM, membrane sphingomyelin

inositides and possibly other signalling proteins [2–4].

The activation of PKB is preceded by its recruitment to the plasma membrane [5]. How this occurs is not clear but it has been suggested that the binding of PtdIns(3,4)P₂ or PtdIns(3,4,5)P₃ to the PH domain of PKB could be important in inducing conformational changes that expose the regulatory sites, allowing them to be phosphorylated by two upstream kinases. One of these kinases is called 3-phosphoinositide-dependent kinase-1 (PDK1), phosphorylates Thr³⁰⁸ [6, 7], but the identity of the second kinase (putatively termed PDK2) is not known. It has, however, recently been suggested that over-expressing integrin linked kinase in IEC-18 cells leads to the phosphorylation of PKB-Ser⁴⁷³ [8], whereas another study has shown that PDK1 can acquire PDK2-like activity *in vitro* [9].

Activated PKB is known to target glycogen synthase kinase-3 (GSK-3) whose phosphorylation results in its inactivation, a step considered crucial for the concomitant activation of glycogen synthase and the subsequent stimulation in glycogen synthesis [10, 11]. In addition to regulating GSK-3, there is mounting evidence to suggest that PKB also participates in the hormonal activation of glucose transport in adipocytes and skeletal muscle [12–14] and that impaired activation could play a part in the development of insulin resistance [15, 16].

A number of studies have implicated cytokines, in particular TNF α , as regulators of glucose metabolism [17]. Studies in intact tissues as well as isolated cells in culture have shown that TNF α causes a large reduction in insulin-stimulated glucose transport in skeletal muscle and adipocytes [18, 19]. The molecular mechanisms by which TNF α induces insulin resistance in these tissues is not clear but a key event in signal transduction pathways initiated by TNF α could involve the increased turnover of membrane sphingomyelin (SM) and the production of ceramide [20]. The latter is generated from SM by the action of neutral or acidic SMases and its production increases significantly in skeletal muscle during insulin resistance [21] and in response to various cell stresses such as heat shock, UV radiation and oxidants [22]. Interestingly, membrane-permeant analogues of ceramide can abrogate insulin's ability to stimulate the MAP kinase pathway in a manner similar to that of TNF α in muscle cells [23] and can also impair the activation of molecules involved in proximal insulin signalling [24, 25]. Furthermore, chronic exposure to ceramide analogues in 3T3-L1 adipocytes down-regulates substantially mRNA expression of the insulin-regulated glucose transporter, GLUT4 [26]. Taken together, these observations support the idea that ceramide could act as a second messenger mediating the effects of TNF α and that ceramide signalling could play an important part in the disruption of the insulin pathway and the development of insulin resistance.

In this study we used the L6 muscle cell line to investigate the mechanism by which the short-chain ceramide analogue, C₂-ceramide, promotes a loss in insulin sensitivity. We show that incubating L6 cells (for up to 2 h) with C₂-ceramide induces a complete loss in insulin-stimulated glucose transport and glycogen synthesis. This inhibition is not attributable to a down-regulation in IRS-1 tyrosine phosphorylation, PtdIns(3,4,5)P₃ production or a modulation in PDK1 activity but seems to be a consequence of impaired recruitment of protein kinase B to the plasma membrane and subsequent activation.

Materials and methods

Materials. All fine chemicals were from Sigma. The α -minimal essential media (α -MEM), fetal calf serum, antimycotic/antibiotic solution were purchased from Life Technologies (Paisley, Renfrewshire, Scotland). C₂-ceramide was from Tocris (Bristol, UK), C₂-dihydroceramide and okadaic acid were from Calbiochem-Novabiochem (Nottingham, UK). The 2-deoxy-[³H]-D-glucose was from NEN (Boston, Mass., USA) and [γ -³²P]-ATP from ICN (Costa Mensa, Calif., USA). Protein A- and Protein G-sepharose were from Pharmacia Biotech (Uppsala, Sweden). Antibodies against PKB α , PKB γ , PDK1 and GSK-3 α (for immunoprecipitation) and peptide substrates for these kinases were kindly provided by Professor Sir Philip Cohen (MRC Protein Phosphorylation Unit, University of Dundee, Scotland).

Cell culture. L6 muscle cells were grown as described previously [14] in α -MEM containing 2% (v/v) fetal calf serum and 1% (v/v) antimycotic/antibiotic solution (100 U/ml penicillin, 100 μ g/l streptomycin, 250 μ g/l amphotericin B) at 37 °C in an atmosphere of 5% CO₂/95% air. Cells were cultured on six-well plates for uptake studies and on 10 cm dishes for subcellular fractionation and analyses of kinase activities or glycogen synthesis. Confluent L6 myotubes were sub-fractionated following pretreatment with C₂-ceramide and insulin or both to isolate plasma membranes as described previously [14]. In some experiments we used L6 cells that we had transformed for stable expression of a constitutively active membrane-targeted PKB α (mPKB). These cells were grown and maintained as described previously [14].

Glucose transport. L6 myotubes were incubated in serum-free α -MEM for 5 h containing 25 mmol/l D-glucose. During this serum-free incubation muscle cells were exposed to C₂-ceramide, dihydroceramide or insulin or both at the times and concentrations indicated in the Figure legends. Following this pretreatment period carrier-mediated glucose uptake was assayed by measuring cytochalasin B-sensitive 2-deoxy-[³H]-D-glucose transport (2DG; 10 μ mol/l, 1 μ Ci/ml, 26.2 Ci/mmol) as described previously [14]. Cell-associated radioactivity was measured by liquid scintillation counting and protein determined by the method of Bradford [27].

Glycogen synthesis. Following ceramide or dihydroceramide treatment, L6 myotubes were incubated for 30 min with or without insulin in buffer containing [U¹⁴C]-D-glucose (0.1 μ Ci/ml, NEN, Boston, Mass., USA). The incubation was terminated by washing the cells with ice-cold 0.9% (w/v) NaCl prior to lysis in 60% (w/v) KOH. Cellular glycogen was

precipitated from lysates using a method adapted from that described previously [28] and associated radioactivity measured by liquid scintillation counting. Protein was determined using the Bradford method [27].

SDS-PAGE and immunoblotting. Cell lysates (50 µg) were subjected to SDS-PAGE and immunoblotting as described previously [14]. Membranes were probed with antibodies which recognised the phosphorylated or native forms of PKB or p42/p44 MAPK (all used at 1:1000; New England Biolabs, Herts., UK), the $\alpha 1$ subunit of the Na,K-ATPase (1:100; a gift from Dr K Sweadner, Harvard University, Mass., USA), PDK1 (1:500; a gift from Dr D Alessi, University of Dundee, Scotland), anti-p85 (1:100; Upstate Biotechnology, Lake Placid, NY., USA) and anti-PY (1:3000; Upstate Biotechnology Lake Placid, NY., USA). Following primary antibody incubation, membranes were incubated with horseradish peroxidase conjugated anti-rabbit IgG (1:1000, SAPU, Lanarkshire, Scotland), or anti-mouse IgG (1:1000, SAPU, Lanarkshire, Scotland) as appropriate. Immunoreactive bands were visualised by enhanced chemiluminescence (Amersham Pharmacia Biotech, Amersham, UK) upon exposure of Kodak X-OMAT film (Eastman-Kodak, Rochester, UK).

IRS-1 immunoprecipitation. Following treatment with insulin or ceramide or both and dihydroceramide, L6 cells were lysed in lysis buffer (50 mmol/l Tris-HCl pH 7.5, 1 mmol/l EDTA, 1 mmol/l EGTA, 1% (v/v) Triton X-100, 1 mmol/l Na₃VO₄, 10 mmol/l sodium β -glycerophosphate, 50 mmol/l NaF, 5 mmol/l Na₄P₂O₇, 1 µmol/l microcystin-LR, 0.27 mol/l sucrose, 0.2 mmol/l phenylmethylsulphonyl fluoride, 1 mmol/l benzamidine, 10 µg/l leupeptin and 0.1% (v/v) 2-mercaptoethanol). The IRS-1 was immunoprecipitated using an antibody that binds the C-terminal domain of IRS-1 (Upstate Biotechnology Lake Placid, NY., USA) [29]. Immunocomplexes were captured by incubation with protein A-agarose beads and solubilised in Laemmli sample buffer prior to immunoblotting as described above.

Analysis of PI3K activity in L6 cells. Following treatment with insulin or ceramide or both or dihydroceramide, L6 cells were lysed in lysis buffer (composition described above). Cell lysates were incubated with a p85 immunoprecipitating antibody that had been bound to protein-G beads for 1 h at 4°C. Immunoprecipitates were washed and incubated with phosphatidylinositol (0.1 mg/l) for 20 min in buffer containing 50 µmol/l [γ -³²P]ATP, 1.2 mmol/l Na₃VO₄, 5 mmol/l MgCl₂ and 25 mmol/l HEPES, pH 7.4. The reaction was terminated by addition of 20 µl of 8 mol/l HCl and 160 µl CH₃OH/CHCl₃ (1:1; v/v). The products were separated by thin layer chromatography as described previously [30]. Radioactivity associated with ³²P-labelled spots was quantified using a Packard InstantImager and also made visible by exposing Kodak X-OMAT film. In some experiments, the production of PtdIns(3,4,5)P₃ was assessed using a sensitive ligand binding displacement assay [31].

Analysis of protein kinase B α (PKB α) and protein kinase B γ (PKB γ) activities in L6 lysates. The L6 myotubes were extracted from 10 cm dishes using ice-cold lysis buffer (composition as described above). The PKB α and PKB γ were immunoprecipitated from lysates using isoform-specific antibodies and kinase activity assayed using "crosstide" [11]. One unit of PKB was defined as the amount that catalysed the phosphorylation of 1 nmol of substrate in 1 min. Protein concentrations were measured using Bradford method [27].

PDK1 activity. The PDK1 was immunoprecipitated from L6 lysates using a PDK1 specific antibody [9]. The immunoprecip-

itates were washed twice with lysis buffer containing 0.5 mol/l NaCl and twice with a buffer containing 50 mmol/l Tris/HCl (pH 7.5), 0.1 mmol/l EDTA and 0.1% (v/v) 2-mercaptoethanol. Immunoprecipitated PDK1 was used to phosphorylate Δ PH-PKB α and PKB activity was assayed as described previously [6]. One unit of activity was defined as the amount that catalysed the phosphorylation of 1 nmol of substrate in one minute.

Protein-lipid overlay assays. To assess whether C₂-ceramide affects the phospholipid-binding properties of PDK1 and PKB, a protein-lipid overlay assay was carried out using GST-fusion proteins of PDK1 and PKB α as described previously [32]. Briefly, 1 µl of *sn*-2-stearoyl, 3-arachidonoyl D-PtdIns(3,4,5)P₃ (1 pmol) dissolved in chloroform/methanol/water (1:2:0.8) was spotted onto Hybond C Extra membrane and allowed to dry. The membrane was blocked subsequently in 5% BSA (w/v) Tris-buffered saline-Tween (TBST; 10 mmol/l Tris/HCl, pH 8.0, 150 mmol/l NaCl, 0.1% (v/v) Tween 20) for 1 h at room temperature. The membrane was then incubated overnight at 4°C in TBST containing 1 mg/ml of either GST-PDK1 or GST-PKB α (provided generously by Dr D. Alessi, University of Dundee, UK, Scotland) in the absence or presence of C₂-ceramide, at concentrations indicated in the figure legends. Following this incubation period, membranes were washed with TBST before incubation with an anti-GST antibody (used at a dilution of 1:1000, a gift from Dr Alessi). Membranes were washed subsequently and then probed with anti-mouse HRP conjugate and immunoreactivity visualised by enhanced chemiluminescence.

Statistical analysis. Statistical analysis was carried out using a two-tailed Student's *t*-test. In our data *p* values of less than or equal to 0.05 were considered statistically significant.

Results

Ceramide inhibits insulin-stimulated glucose transport and glycogen synthesis. To assess the effects of ceramide on two important cellular responses regulated by insulin, we assayed insulin-stimulated glucose transport and glycogen synthesis in L6 myotubes. Insulin caused a near two-fold stimulation in glucose transport and a four-fold increase in glycogen synthesis (Fig. 1). When muscle cells were, however, pre-incubated with C₂-ceramide for 2 h, we observed a dose-dependent reduction in insulin-stimulated glucose transport, the maximal inhibition being observed at concentrations of 100 µmol/l ceramide. The observed inhibition could not be attributed to a fall in basal glucose transport, which was not affected by ceramide. In contrast, pre-incubation of muscle cells with an inactive ceramide analogue, dihydroceramide had no effect on the hormonal stimulation of glucose transport (Fig. 1). We then examined the effects of ceramide on the insulin-stimulated incorporation of [¹⁴C]-glucose into glycogen. As with glucose transport, pretreatment of muscle cells with 100 µmol/l C₂-ceramide for 2 h abolished completely insulin's ability to stimulate glycogen synthesis (Fig. 1). As expected, dihydroceramide did not have any effect on

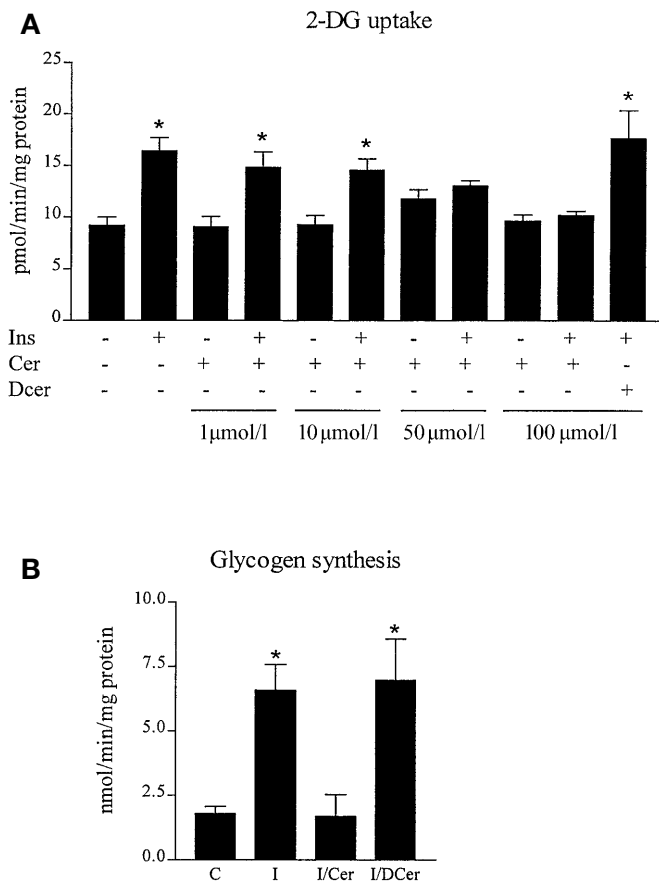


Fig. 1A, B. Effects of insulin (I), ceramide (Cer), and dihydroceramide (Dcer) on glucose transport and glycogen synthesis in L6 myotubes. **(A)** L6 myotubes were pre-incubated with different ceramide (Cer) concentrations (between 1 $\mu\text{mol/l}$ and 100 $\mu\text{mol/l}$), and 100 $\mu\text{mol/l}$ dihydroceramide (Dcer) for 2 h and then with 100 nmol/l insulin (Ins) for 30 min. 2-DG uptake was assayed as described. **(B)** L6 myotubes were pre-incubated with 100 $\mu\text{mol/l}$ Cer for 2 h and then with Insulin (I) for 30 min. Glycogen synthesis was assessed as described. Values represent means \pm SEM from 3 separate experiments. Asterisks indicate a significant change from the control (c) value ($p < 0.05$)

the hormonal stimulation of glycogen synthesis in these cells.

Ceramide does not inhibit insulin-mediated IRS-1 phosphorylation and PI3K activation. In an attempt to establish the mechanism by which ceramide inhibits the hormonal activation of glucose transport and glycogen synthesis, we investigated the effects of this lipid on proximal insulin signalling events. Immunoprecipitation of IRS-1 from L6 lysates revealed that incubation of muscle cells with 100 $\mu\text{mol/l}$ C_2 -ceramide for 2 h neither inhibited the insulin-induced tyrosine phosphorylation nor prevented the insulin-mediated association of IRS-1 with the p85 subunit of PI3K (Fig. 2). Analysis of PI3K activity in p85 immunoprecipitates showed that insulin caused an eight-fold increase in enzyme activity and that this activation was not suppressed by C_2 -ceramide (Fig. 2). An

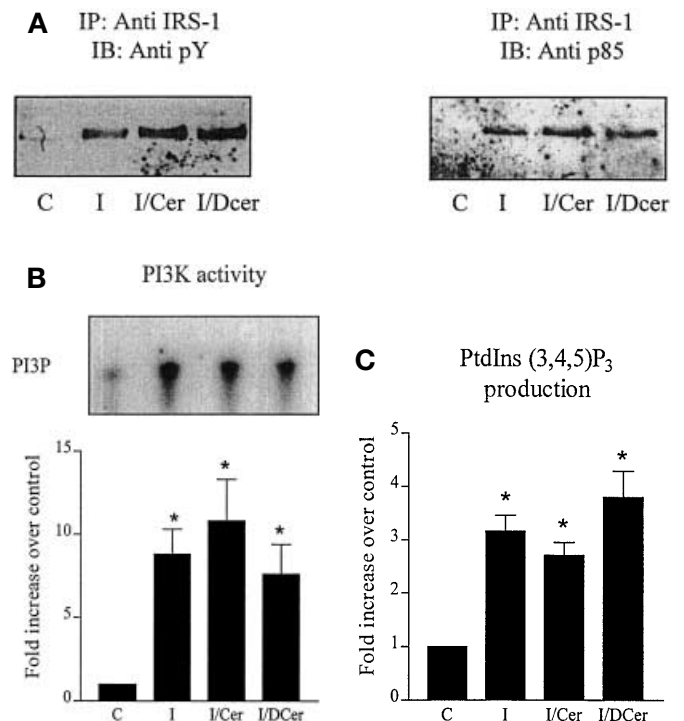


Fig. 2A–C. Effect of insulin, ceramide, and dihydroceramide on IRS-1 tyrosine phosphorylation and association with PI3K, PI3K activity and PtdIns(3,4,5)P₃ production in L6 myotubes. L6 myotubes were pre-incubated in the presence of 100 $\mu\text{mol/l}$ ceramide (Cer) or 100 $\mu\text{mol/l}$ dihydroceramide (Dcer) for 2 h before treatment with 100 nmol/l insulin (Ins) for 10 min. **(A)** Cells were lysed and IRS-1 immunoprecipitated prior to immunoblotting with either anti-phosphotyrosine (left panel) or anti-p85 (right panel) antibodies. **(B, C)** Cells were lysed and prepared for analyses of PI3K activity in p85 immunoprecipitates or PtdIns(3,4,5)P₃ as described. Values represent means \pm SEM from 3 separate experiments. Asterisks indicate a significant change from the control (c) value ($p < 0.05$)

observation consistent with this finding was that the insulin-induced production of PtdIns(3,4,5)P₃, measured using a sensitive ligand binding mass assay [31], was not affected in cells that had been pre-incubated with ceramide (Fig. 2). The inactive ceramide analogue (dihydroceramide) was also without effect in these studies.

Ceramide inhibits the hormonal activation of PKB. Because PKB lies downstream of PI3K and has been implicated in the hormonal regulation of both glucose transport and glycogen synthesis [11, 14], we assessed the effects of ceramide on PKB activation by insulin. Insulin stimulated PKB α activity by \sim 22-fold (Fig. 3). This activation was not altered significantly upon incubation of muscle cells with C_2 -ceramide at concentrations up to 20 $\mu\text{mol/l}$. However, at ceramide concentrations above 20 $\mu\text{mol/l}$, the hormonal activation of PKB was reduced in a dose-dependent manner and was lost completely when muscle

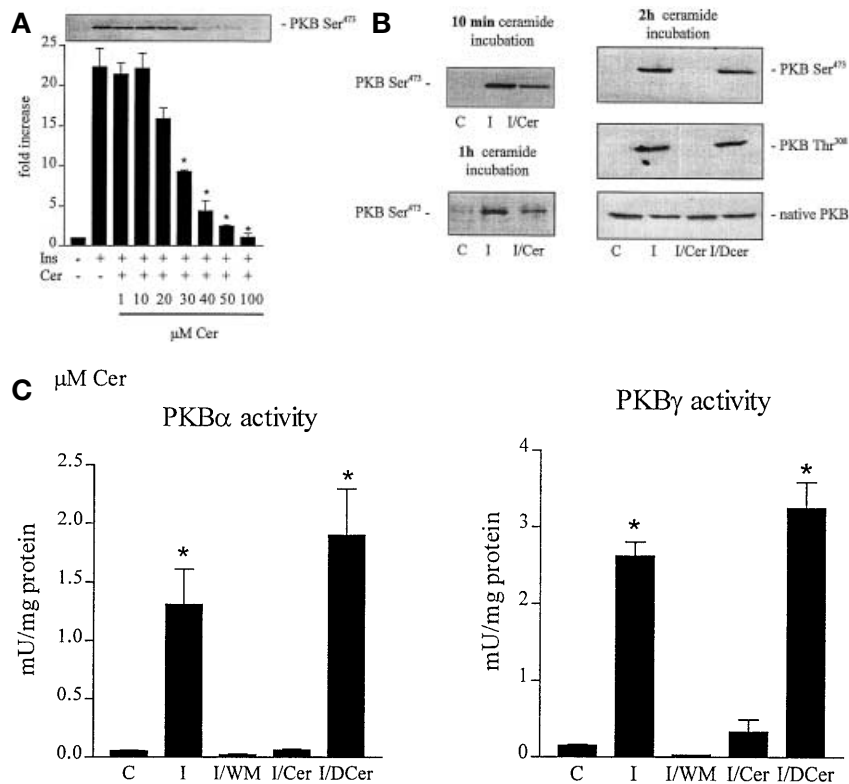


Fig. 3. Effects of insulin, ceramide, and dihydroceramide on PKB phosphorylation and activity in L6 myotubes. **(A)** L6 myotubes were pre-incubated with different ceramide (Cer) concentrations (between 1 $\mu\text{mol/l}$ and 100 $\mu\text{mol/l}$ for 2 h) and then with 100 nmol/l insulin (Ins) for 10 min. Cell lysates were used for either in vitro PKB α activity analysis or immunoblotted with a phospho-specific antibody to PKB-Ser⁴⁷³. **(B)** L6 myotubes were pre-incubated with 100 $\mu\text{mol/l}$ Cer or 100 $\mu\text{mol/l}$ dihydroceramide (Dcer) for times indicated and treated with 100 nmol/l Ins for 10 min. Cell lysates were immunoblotted with a phospho-specific antibody directed to either PKB-Ser⁴⁷³, PKB-Thr³⁰⁸ or a native PKB antibody. **(C)** L6 myotubes were pre-incubated with 100 $\mu\text{mol/l}$ Cer or Dcer for 2 h followed by a 10 min treatment with 100 nmol/l Ins in the presence or not of 100 nmol/l wortmannin (WM). PKB α and PKB γ activities were assessed from lysates as described. Values represent means \pm SEM from at least 3 separate experiments. In panel A the asterisks indicate a significant change from the insulin-stimulated value ($p < 0.05$), whereas in panel C they indicate a significant change from the untreated control ($p < 0.05$).

2 h, with phosphospecific PKB antibodies. The ceramide-induced loss in the hormonal activation of PKB was time-dependent (Fig. 3). Within 10 min the intensity of the immunoreactive phospho-Ser⁴⁷³ PKB band in cells treated with C₂-ceramide was noticeably less than that in cells treated with insulin alone. It was diminished further in cells incubated with C₂-ceramide for 1 h and was undetectable in cells that had been exposed to the lipid for 2 h. Identical results were obtained irrespective of whether we used a phospho-specific antibody directed against the Ser⁴⁷³ or Thr³⁰⁸ PKB sites (Fig. 3). Analysis of in vitro PKB activity showed that there was a corresponding loss in insulin-stimulated kinase activity over the same time period (data not shown). In contrast, dihydroceramide (100 $\mu\text{mol/l}$) had no effect on PKB phosphorylation (Fig. 3).

L6 muscle cells express PKB α and PKB γ and the activity of both isoforms has been shown to be enhanced markedly by insulin [33]. To examine whether ceramide affects the activation of one particular isoform, we measured the effects of C₂-ceramide on the activation of each PKB isoform. Insulin stimulated the activity of the α and the γ PKB isoforms by \sim 26-fold and 18-fold, respectively. The activation of both isoforms was reduced dramatically following incubation of cells with 100 $\mu\text{mol/l}$ ceramide for 2 h but was unaffected in cells treated with dihydroceramide (Fig. 3). In line with previous studies the hormonal activation of both isoforms was also blocked by the PI3K inhibitor, wortmannin [33].

cells were treated with 100 $\mu\text{mol/l}$ C₂-ceramide for 2 h (Fig. 3). Consistent with the in vitro kinase data, immunoblot analysis of L6 lysates using a phosphospecific antibody against the Ser⁴⁷³ residue confirmed that PKB was not phosphorylated (and thus not active) in cells that had been incubated with 100 $\mu\text{mol/l}$ C₂-ceramide (Fig. 3).

To ascertain how quickly C₂-ceramide impairs the hormonal activation of PKB, we immunoblotted cell lysates that were prepared following incubation of cells with the lipid (100 $\mu\text{mol/l}$) for 10 min, 1 h and

Table 1. Effects of insulin, ceramide, and dihydroceramide on PDK1 activation. L6 myotubes were pre-incubated in the presence of 100 $\mu\text{mol/l}$ ceramide (Cer) or 100 $\mu\text{mol/l}$ dihydroceramide (Dcer) for 2 h before treatment with 100 nmol/l insulin (Ins) for 10 min. Cells were lysed and lysates prepared for analysis of PDK1 activity as described. Values are from three experiments (means \pm SEM).

	PDK1 activity (U/mg protein)
Control	68.9 \pm 2.8
Ins	71.2 \pm 1.6
Ins/Cer	75.7 \pm 2.9
Ins/Dcer	70.7 \pm 1.8

To test whether ceramide could inhibit PKB activity directly we prepared phospholipid vesicles (phosphatidylcholine/phosphatidylserine) containing C_2 -ceramide at a final in vitro concentration of 10 $\mu\text{mol/l}$. Then GST-PKB α was incubated with recombinant PDK1 in the presence of Mg-ATP and phospholipid vesicles to allow the activation of GST-PKB α as described previously [6]. This approach showed that ceramide had no direct inhibitory effect on the catalytic activation of PKB in vitro (data not shown).

Ceramide does not modulate PDK1 activity. We speculated that because the activation of PKB α was dependent upon its phosphorylation on Thr³⁰⁸ and Ser⁴⁷³, ceramide could have modulated the activity of the upstream kinases that phosphorylate these sites. We therefore immunoprecipitated PDK1 from L6 lysates following the treatment of cells with C_2 -ceramide or insulin or both and assayed its ability to phosphorylate recombinant $\Delta\text{PH-PKB}\alpha$ in vitro. It is thought that PDK1 is constitutively active and that its activity is not modulated by stimuli that activate PKB through PI3K [34]. In keeping with this view, insulin did not elicit any activation of PDK1 and the incubation of muscle cells with C_2 -ceramide did not cause any inhibition of its constitutive activity (Table 1).

PKB is not inactivated by an okadaic acid-sensitive phosphatase. It is plausible that the impaired hormonal stimulation of PKB could be due to the activation of a protein phosphatase. Indeed, a number of studies have suggested that ceramide activates an okadaic acid-sensitive phosphatase belonging to the PP-2A family [35, 36]. To assess this possibility, we tested whether okadaic acid could prevent the ceramide-induced loss in insulin-mediated PKB activation. Okadaic acid (100 nmol/l) was not able to prevent a loss in PKB activation in cells treated with C_2 -ceramide, based on the analysis of the phosphorylation status of the kinase (Fig. 4). This finding was confirmed by an analysis of PKB activity in cell lysates used for the immunoblot studies (data not shown). In agree-

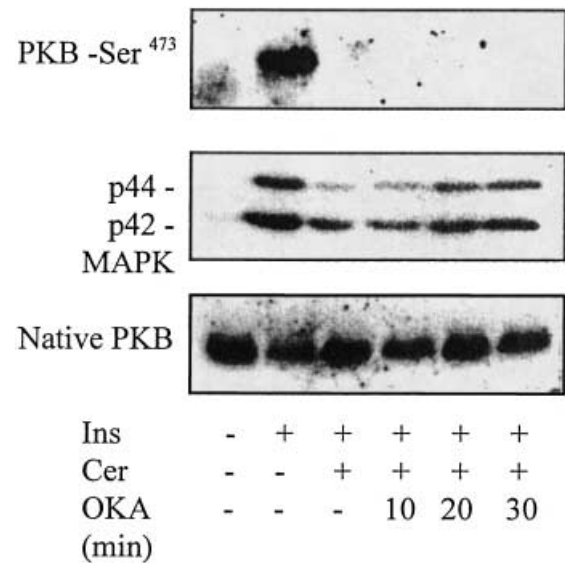


Fig. 4. Effects of insulin, ceramide, and okadaic acid on p42/44 MAP kinase and PKB phosphorylation in L6 myotubes. L6 myotubes were pre-incubated in the presence of 100 $\mu\text{mol/l}$ ceramide (Cer) for 2 h and then incubated with okadaic acid (OKA, 100 nmol/l) for times indicated and with 100 nmol/l insulin (Ins) for 10 min. Cells were lysed and lysates immunoblotted with antibodies against native PKB, specific phospho-antibody directed against p42/p44 MAP kinase, or specific phospho-antibody directed against PKB-Ser⁴⁷³.

ment with a previous study, however [23], okadaic acid reversed the inhibitory effects of ceramide upon the insulin-dependent phosphorylation of p42 and p44 MAP kinases in a time-dependent manner (Fig 4).

Ceramide inhibits PKB translocation to the plasma membrane. A crucial aspect of the mechanism by which PKB is activated involves its recruitment from the cytosol to the plasma membrane, after cell stimulation with insulin [37] or growth factors, such as IGF-1 [5]. Suppressing PKB recruitment in response to insulin thus represents a potential mechanism by which ceramide could block the activation of this kinase. To assess this possibility, we isolated plasma membranes from L6 cells using a well established fractionation procedure [14, 38]. Immunoblot analyses of isolated membranes revealed that treatment of muscle cells with C_2 -ceramide or insulin or both had no effect on the abundance of the α 1-subunit of the Na,K-AT-Pase, the β -subunit of the insulin receptor or PDK1, which are resident plasma membrane proteins (Fig. 5). In contrast, in the same membrane fractions, insulin caused a 2.7-fold increase in PKB abundance that was not observed in muscle cells that had been pre-incubated with 100 $\mu\text{mol/l}$ C_2 -ceramide for 2 h (Fig. 5). Identical findings were obtained when we traced the association of PKB with the plasma membrane using the phospho-specific (Ser⁴⁷³) antibody (Fig. 5).

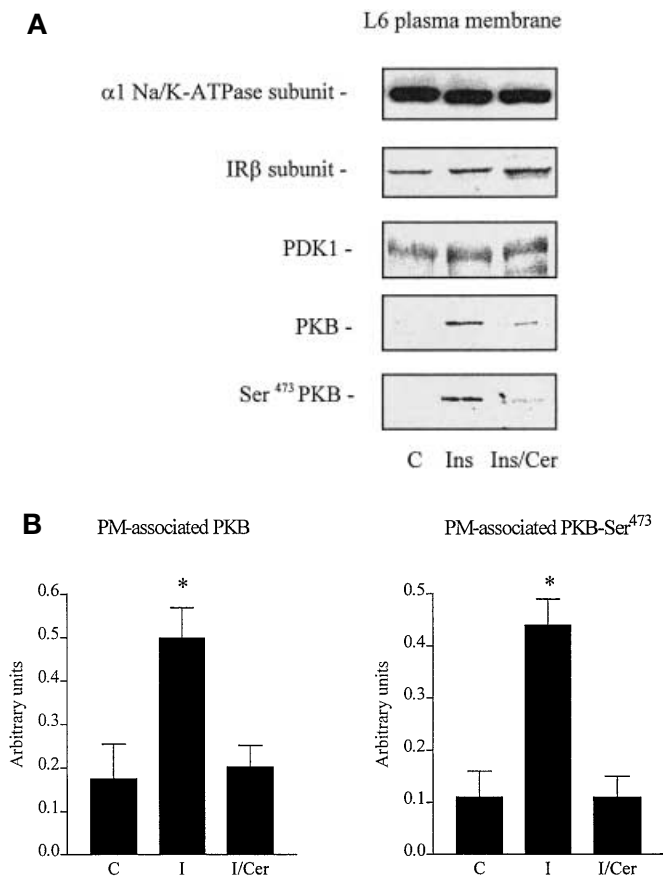


Fig. 5 A, B. Effects of insulin and ceramide on the abundance of PKB, the $\alpha 1$ subunit of the Na/K ATPase, the β subunit of the insulin receptor and PDK1 in the plasma membrane. L6 myotubes were pre-incubated in the presence of 100 $\mu\text{mol/l}$ ceramide (Cer) for 2 h before treatment with 100 nmol/l insulin (Ins) for 30 min, prior to cell harvesting and subcellular fractionation as described. **(A)** Plasma membrane (PM) protein was separated by SDS-PAGE. Immunoblotting was performed using antibodies against the $\alpha 1$ subunit of the Na/K ATPase, the β subunit of the insulin receptor, PDK1, PKB and a specific phospho-antibody directed against PKB-Ser⁴⁷³. **(B)** Quantitative analyses of the amount of PKB associated with the plasma membrane. Values represent means \pm SEM from 3 separate experiments. Asterisks indicate a significant change from the control value ($p < 0.05$)

Expression of a constitutively active membrane targeted PKB overcomes the inhibition exerted by ceramide. We hypothesised that if ceramide does block insulin-stimulated glucose transport by suppressing PKB recruitment to the plasma membrane, then the expression of a membrane targeted PKB (mPKB) should overcome this inhibition. Expression of mPKB α results in its constitutive activation as shown previously. This leads to the stimulation of glucose transport to a level similar to that elicited by insulin in untransfected cells [14]. In line with our previous findings, expression of mPKB led to a ten-fold increase in PKB activity in the absence of any hormonal stimulation (Fig. 6). When cells expressing mPKB were stimulat-

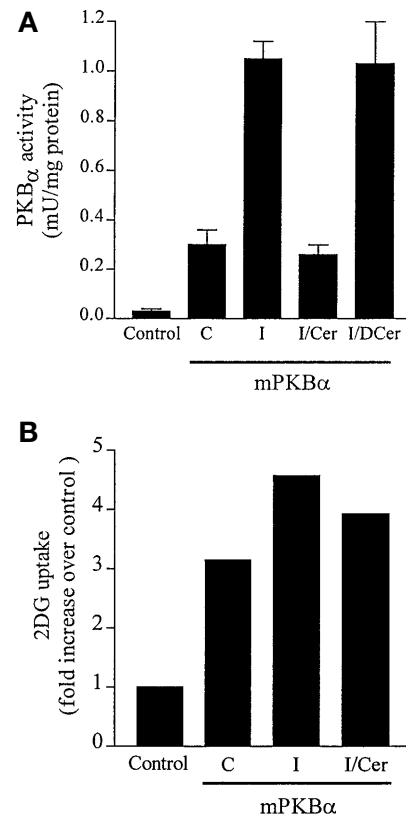


Fig. 6 A, B. Effects of insulin, ceramide, and dihydroceramide on PKB activity and glucose transport in L6 myotubes expressing a constitutively active membrane targeted PKB. **(A)** L6 myotubes expressing or not a membrane targeted PKB α construct were pre-incubated in the presence of 100 $\mu\text{mol/l}$ ceramide (Cer) or 100 $\mu\text{mol/l}$ dihydroceramide (Dcer) for 2 h before treatment with 100 nmol/l insulin (Ins) for 10 min. PKB α activity was assessed from lysates as described. **(B)** L6 myotubes stably transfected with an empty expression vector or a vector containing a membrane targeted PKB α construct were pre-incubated with 100 $\mu\text{mol/l}$ ceramide (Cer) for 2 h before treatment with 100 nmol/l insulin (Ins) for 30 min and 2-DG uptake assayed as described

ed subsequently with insulin, cellular PKB activity was enhanced by a further four-fold. This additional increase in kinase activity most likely reflects the activation of the native PKB α , given that it was inhibited when muscle cells were pretreated with ceramide but not dihydroceramide. In the mPKB α expressing cells ceramide failed, however, to completely inhibit PKB. The residual activity was very similar to that measured in unstimulated cells, suggesting that the lipid has no effect on the constitutive activity of mPKB α (Fig. 6). Analysis of glucose uptake showed that ceramide had no inhibitory effect on the increased glucose transport rate observed following expression of mPKB α (Fig. 6).

Ceramide does not impair or compete with the binding of PtdIns(3,4,5)P₃ to either PDK1 or PKB α in vitro. To determine whether the impaired hormonal re-

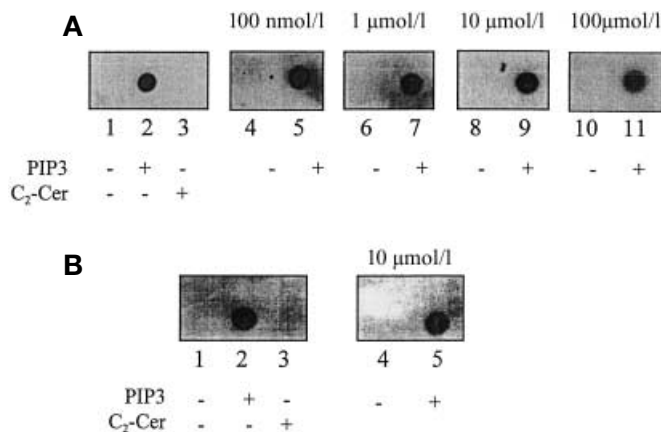


Fig. 7A, B. Effect of ceramide on the binding of PtdIns(3,4,5)P₃ to PKB and PDK1 in vitro. **(A)** Nitrocellulose membranes were spotted with either water (lanes 1, 4, 6, 8 and 10), 1 nmol PtdIns(3,4,5)P₃ (lanes 2, 5, 7, 9 and 11) or 10 nmol ceramide (lane 3). Membranes were then incubated with a GST-PKB fusion protein (1 μ g/l), either alone or in the presence of increasing concentrations of ceramide, as indicated. The membranes were washed and bound PKB was detected by probing with a monoclonal anti-GST antibody, followed by incubation with an anti-mouse IgG HRP conjugate and ECL. Representative blots are shown. **(B)** Nitrocellulose membranes were spotted with either water (lanes 1 and 4), 1 nmol PtdIns(3,4,5)P₃ (lanes 2 and 5) or 10 nmol ceramide (lane 3). Membranes were then incubated with a GST-PDK1 fusion protein (1 μ g/l), either alone or in the presence of 10 μ mol/l ceramide, as indicated. Bound PDK1 was detected by probing with an anti-GST antibody. Blots are representative of three similar experiments

recruitment of PKB seen in muscle cells incubated with ceramide was attributable to a loss in PtdIns(3,4,5)P₃ binding to the kinase PH domain, we carried out a protein-lipid overlay assay using a GST-PKB α fusion protein (Fig. 7). This analysis showed that, unlike PtdIns(3,4,5)P₃ (Fig. 7), spotting C₂-ceramide on the nitrocellulose membrane did not result in the adherence of PKB α to the membrane (Fig. 7), signifying that this lipid does not bind to the kinase. Moreover, when membranes spotted with PtdIns(3,4,5)P₃ were incubated with GST-PKB α in the presence of increasing concentrations of C₂-ceramide, the binding of PKB to the phosphoinositide was not diminished (Fig. 7). Similarly, ceramide was found not to associate or interfere with the binding of PtdIns(3,4,5)P₃ to GST-PDK1 (Fig. 7), consistent with our previous finding that ceramide has no effect on the kinase activity of PDK1 (Table 1).

Discussion

The pathogenesis of insulin resistance in skeletal muscle is poorly understood, but one factor that could be important in its development is the increased production of ceramide. Intramuscular con-

centrations of this lipid are increased in the skeletal muscle of insulin resistant rats [39] and a number of studies have shown that cell-permeant analogues of ceramide, as well as saturated free fatty acids (e.g. palmitate), from which ceramide can be generated [40], promote cellular insulin resistance [25, 26, 41, 42]. How this membrane lipid induces a loss in insulin sensitivity is not clear, but some studies indicate that ceramide or agonists that induce ceramide production, such as TNF α , impair the hormonal activation of IRS1 [43] and PI3K [24, 44, 45]. Other reports, however, suggest that the inhibitory effect of ceramide is mediated downstream of PI3K, at the level of PKB [25, 40, 46]. The present study attempted to understand the mechanism by which ceramide induces insulin resistance in skeletal muscle and focused upon the effects of this lipid on early-insulin signalling events participating in the control of glucose transport and glycogen synthesis.

Our results show that ceramide inhibits insulin-stimulated glucose transport and glycogen synthesis in L6 rat muscle cells. We also show that this inhibition occurs as a result of a dramatic reduction in insulin's ability to activate both PKB α and PKB γ . This effect takes place without any detectable disruption in IRS-1 and PI3K function. Our data also shows, for the first time, that the changes observed cannot be accounted for by an inhibition of PDK1. The loss in insulin-stimulated PKB phosphorylation could reflect increased dephosphorylation of the kinase. Previous work supported this possibility, showing that ceramide stimulates the activity of a cytosolic protein phosphatase that is sensitive to okadaic acid [23, 47]. Moreover, other investigators [48] have recently shown that C₂-ceramide prevents NGF-induced stimulation of PKB in neuronal PC12 cells through activation of an okadaic acid-sensitive phosphatase. In line with previous work [23], our data indicate that okadaic acid prevents the loss in the insulin-dependent phosphorylation of p42/p44 MAP kinases initiated by ceramide. The inhibitor fails, however, to suppress the effects of ceramide on PKB activity/phosphorylation in our experimental system. This discrepancy could therefore be explained by a difference in cell type given that our findings are consistent with similar data obtained in 3T3-L1 adipocytes [25]. We cannot, however, discount the possibility that an okadaic acid-insensitive protein phosphatase could be involved in mediating ceramide's effect on PKB in insulin-responsive cell types.

Our data indicate that the more likely mechanism by which ceramide could exert its inhibitory effects on PKB is by suppressing its translocation to the plasma membrane. This proposition is supported by our finding that ceramide reduces substantially the amount of PKB that associates with the plasma membrane upon treatment of muscle cells with insulin as well as by recent data showing that ceramide inhibits

translocation of a GFP-tagged PKB in response to PDGF in NIH3T3 cells [49]. The observation that ceramide fails to inhibit the increase in glucose transport elicited by expressing a membrane-targeted PKB in L6 cells (Fig. 6), and in 3T3-L1 adipocytes also supports our proposition [25]. The latter finding, made in two different cell types, implies that the inhibitory effects of ceramide on insulin signalling can be bypassed when PKB is delivered directly to the plasma membrane.

The exact mechanism by which ceramide inhibits PKB recruitment is not known but it is possible that the lipid affects the characteristics of the PKB-PH domain. The PH domain is thought to function as a membrane-targeting module that helps direct the kinase to the plasma membrane in response to cell stimulation with insulin and growth factors [5, 37]. The PH domain also binds $\text{PtdIns}(3,4,5)\text{P}_3$ and $\text{PtdIns}(3,4)\text{P}_2$, and such interactions are considered important in enabling conformational changes that allow for its activation by the two upstream kinases [10]. It is possible that ceramide could interfere with the interaction of 3-phosphoinositides with PKB thus preventing it from adopting the appropriate conformation for recruitment and activation. Our data do not, however, support this because we could not detect any direct effects of ceramide on the catalytic activation of PKB *in vitro* or on the binding of PKB to $\text{PtdIns}(3,4,5)\text{P}_3$. Whether additional signalling/trafficking molecules are involved in the insulin-induced recruitment of PKB to the plasma membrane is not known at present. Evidence does, however, exist that proteins such as GRP1 and cytohesin-1, which bind 3-polyphosphoinositides through their PH domains, act to link receptor-activated PI3K pathways with molecules involved in protein sorting and trafficking [50]. If ancillary proteins are involved in the trafficking of PKB to the plasma membrane, then they could serve as potential targets for ceramide action.

Ceramide is generated in response to various cell stresses [20] and is known to target a number of kinases, including members of the classical stress-signalling pathways [51–53]. Thus, it is possible that the loss in insulin-stimulated glucose transport could, in part, be a consequence of ceramide signalling through a stress-activated pathway. Indeed, ceramide activates the SAPK2/p38 MAP kinase pathway in L6 cells (unpublished results). We have also recently shown that the activation of this pathway by oxidants, such as hydrogen peroxide, inhibits insulin signalling to glucose transport [54]. Although pretreatment of muscle cells with SB 203580, an inhibitor of p38 MAP kinase, blocks p38 activation by ceramide, the inhibitor fails to prevent the loss in activation of glucose transport (data not shown). Thus, at least in the present studies, activation of p38 does not seem to participate in impaired insu-

lin signalling to glucose transport. Because other stress kinases, such as SAPK1/JNK, are also activated in response to ceramide treatment in L6 cells (unpublished data), we are not able to exclude the possibility that these could be involved in mediating the inhibitory effects of this lipid on glucose transport.

A number of studies have shown that the expression of constitutively active or dominant negative forms of PKB, in muscle and adipocytes, induce changes in GLUT4 translocation and glucose transport that are consistent with its involvement in the insulin signalling pathway regulating glucose transport [12–14, 55]. An interesting observation to emerge from our study which supports this idea is the very close correlation between the relative activation of PKB and glucose transport achieved by insulin, following treatment of muscle cells with different ceramide concentrations (Fig. 1, 3). At concentrations below 50 $\mu\text{mol/l}$, ceramide had no significant impact on insulin's ability to stimulate glucose transport and insulin is still capable of eliciting an increase in PKB activity. When muscle cells were, however, exposed to 100 $\mu\text{mol/l}$ ceramide, a loss in insulin-stimulated glucose transport occurred. This was associated with a corresponding loss in the hormonal activation of PKB. Moreover, the finding that ceramide fails to inhibit glucose transport in muscle cells expressing a membrane-targeted PKB, which is not inhibited by ceramide, is further evidence that PKB is involved in the hormonal regulation of glucose transport.

Our findings indicate that ceramide inhibits insulin-stimulated glucose transport and glycogen synthesis in L6 muscle cells. This inhibition is not mediated at the level of the insulin receptor, IRS1, PI3K or PDK1 but is due to a targeted disruption in the hormonal activation of PKB. This stems from a failure to recruit the kinase to the plasma membrane which occurs in the presence of a stimulated rise in cellular $\text{PtdIns}(3,4,5)\text{P}_3$. In addition to its role in insulin signalling, PKB participates in many diverse cell functions, including the control of cellular proliferation, differentiation and survival. Indeed, the kinase is generally perceived as an anti-apoptotic signalling kinase [56, 57]. Thus it is possible that, by suppressing PKB recruitment and activation, increased ceramide generation could also shift the balance from anti-apoptotic to pro-apoptotic signalling. Identifying the mechanism by which ceramide blocks PKB recruitment is therefore likely to have important implications, not only for our conceptual understanding of how the lipid participates in the pathogenesis of insulin resistance but also for developing new insights into how ceramide promotes apoptosis.

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