Ceramide Generation Is Sufficient to Account for the Inhibition of the Insulin-stimulated PKB Pathway in C2C12 Skeletal Muscle Cells Pretreated with Palmitate*

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We have employed C2C12 myotubes to investigate lipid inhibition of insulin-stimulated signal transduction and glucose metabolism. Cells were preincubated for 18 h in the absence or presence of free fatty acids (FFAs) and stimulated with insulin, and the effects on glycogen synthesis and signaling intermediates were determined. While the unsaturated FFAs olate and linoleate inhibited both basal and insulin-stimulated glycogen synthesis, the saturated FFA palmitate reduced only insulin-stimulated glycogen synthesis, and was found to inhibit insulin-stimulated phosphorylation of glycogen synthase-3 and protein kinase B (PKB). However, no effect of palmitate was observed on tyrosine phosphorylation, p85 association, or phosphatidylinositol 3-kinase activity in IRS-1 immunoprecipitates. In contrast, palmitate promoted phosphorylation of mitogen-activated protein (MAP) kinases. Ceramide, a derivative of palmitate, has recently been associated with similar inhibition of PKB, and here, ceramide levels were found to be elevated 2-fold in palmitate-treated C2C12 cells. Incubation of C2C12 cells with ceramide closely reproduced the effects of palmitate, leading to inhibition of glycogen synthesis and PKB and to stimulation of MAP kinase. We conclude that palmitate-induced insulin resistance occurs by a mechanism distinct from that of unsaturated FFAs, and involves elevation of ceramide by de novo synthesis, leading to PKB inhibition without affecting IRS-1 function.

Skeletal muscle is the most important target of insulin action in terms of post-prandial glucose disposal, and skeletal muscle insulin resistance is a major characteristic of non-insulin-dependent diabetes mellitus (1). The mechanisms by which muscle becomes less sensitive to the hormone are still unclear; however, there is a strong correlation between insulin resistance and increased lipid availability in the tissue (2). Evidence for this has been derived from studies involving obese humans (3, 4) and animals (5), animals fed high-fat diets (6, 7), and the exposure of muscle cells to increased lipid levels (8, 9).

The signaling pathways involved in the metabolic actions of insulin are becoming well characterized. The stimulation of glycogen synthesis from glucose by insulin (reviewed in Ref. 10) involves activation of PI3-kinase through association with IRS molecules that have been tyrosine phosphorylated by the insulin receptor (11). IRS-1 may be the predominant adaptor molecule responsible for metabolic signals, especially in muscle (12), although IRS-2 and further adaptor molecules also play roles (13). PI3-kinase catalyzes the production of PtdIns(3,4,5)P3 which leads to the activation of PKB (also known as Akt or RAC kinase), through the sequential phosphorylation on Thr-308 and Ser-473 by PDK1 and PDK2, respectively (14). PKB in turn phosphorylates and inhibits GSK-3α at Ser-21 (15). GSK-3α is thought to be the most important kinase regulating glycogen synthase activity, through inhibition by phosphorylation (16). Thus the insulin-stimulated increase in glycogen synthesis involves net dephosphorylation of glycogen synthase, largely by decreased activity of GSK-3α, although increased phosphatase activity may also play a role (17). The relative importance of insulin-stimulated glycogen synthesis versus glucose transport, mediated by increased plasma membrane GLUT4 glucose transporter levels, in determining the rate of glucose disposal by muscle remains controversial although it appears that glycogen synthesis is rate-limiting at higher levels of insulin (10, 18). Defects in muscle glycogen synthesis have a dominant role in the insulin resistance that occurs in non-insulin-dependent diabetes mellitus (19).

Insulin also activates MAP kinase pathways, which may be more involved in its mitogenic rather than its metabolic effects. IRS-1 and a further adaptor molecule She appear to be responsible for the activation of p21ras, via Grb2 and mSOS, and hence the kinases downstream leading to ERK MAP kinase activation (13). Evidence is emerging that insulin also stimulates the p38 MAP kinase and the stress-activated protein kinase/JNK MAP kinase although the pathways are less well characterized (20, 21).

Lipids have been well documented to inhibit glucose disposal and reduce insulin sensitivity although the underlying mechanisms are obscure. One possibility is through the Randle glucose-fatty acid cycle, in which lipid oxidation limits glucose metabolism by inhibition of pyruvate dehydrogenase (22). However, this is unlikely to be the full explanation for insulin resistance induced by lipids, and they may also reduce insulin sensitivity through inhibition at the level of signaling compo...

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† The abbreviations used are: P3-kinase, phosphatidylinositol 3-kinase; BSA, bovine serum albumin; CAPP, ceramide-activated protein phosphatase; EMEM, minimum essential medium with Earle's salts; FCS, fetal calf serum; FFA, free fatty acid; GSK-3, glycogen synthase kinase-3; IRS, insulin receptor substrate; JNK, c-Jun N-terminal kinase; KSR, kinase suppressor of Ras; CAPR, caper activated protein kinase; MAP, MAP kinase, mitogen-activated protein kinase; PBS, phosphate-buffered saline; PDK, PtdIns3,4, 5-P2-dependent kinase; PKB, protein kinase B; PKC, protein kinase C; PP1 and PP2A, protein phosphatase 1 and 2A, respectively; PtdIns(3,4,5)P3, phosphatidylinositol (3,4,5)-trisphosphate; TLC, thin layer chromatography; PAGE, polyacrylamide gel electrophoresis.
nents. One possibility, supported by data from animal models of insulin resistance, is inappropriate activation of lipid-dependent PKC isoenzymes (23, 24). It is possible that this leads to interference with insulin action by phosphorylation and inhibition of one or more signaling intermediates. Alternatively, or in addition, lipids may cause attenuation of the insulin signal by mechanisms independent of PKC. For example, recent studies have shown that PKB activation can be reduced in the presence of ceramide (25–27), a lipid second messenger produced by sphingomyelinase activation, which can be also derived from free fatty acids by de novo synthesis (28). The possibility therefore exists that different lipids may affect insulin action in different ways because of the specific signaling pathways which they affect.

To study the effects of lipids on insulin signal transduction, we have developed a model using mouse skeletal muscle C2C12 myotubes. Preincubation of the cells with unsaturated and saturated FFA s led to distinct effects on the insulin sensitivity of glycerogen synthesis, and also on the state of activation of several signaling components. Our observations with the unsaturated FFA palmitate led us to hypothesize that this lipid was acting through elevation of intracellular ceramide and inhibition at the level of PKB. Further investigation confirmed that ceramide levels were indeed elevated in the myotubes and that addition of exogenous ceramide produced palmitate-like effects both on glycerogen synthesis and signaling molecules. In addition to providing insights into the mechanism for palmitate-induced insulin resistance, these results demonstrate that this model will be useful in studying the attenuation of insulin signaling by other lipids or further factors causing insulin resistance.

EXPERIMENTAL PROCEDURES

Materials—EMEM was from Trace Biosciences (Sydney, NSW, Australia). Bovine FCS was from Life Technologies Inc. (Gaithersburg, MD). Gelatin was from Difco Laboratories (Detroit, MI). Fatty acid free bovine serum albumin was from ICN Biomedicals Inc. (Aurora, OH). Protein A-Sepharose, oleic acid, linoleic acid, and palmitic acid were from Sigma Chemical Co. Insulin was from Novo Nordisc (Copenhagen, Denmark). AG 1-X8 resin was from Bio-Rad Laboratories Pty. Ltd. (Sydney, NSW, Australia). RCo2H anti-phosphotyrosine antibodies conjugated to horseradish peroxide were from Transduction Laboratories (Sydney, NSW, Australia). RC20H anti-phosphotyrosine antibodies denatured with 10% (v/v) FCS, 2 mM glutamate, 15 mM Hepes, pH 7.5, 500 mM NaCl, 1 mM MgCl2, 1 mM CaCl2, 2 mM Na2VO4, 10 mM Na3P207, 10 mM NaF, 2 mM EDTA, 1% (v/v) Nonidet P40, 10% (v/v) glycerol, 2 μg/ml aprotinin, 5 μg/ml leupeptin, 0.2 mM phenylmethylosulfonyl fluoride and 2 mM PMSF were from Sigma Chemical Co. or BDH (Merck). Other reagents were from Sigma Chemical Co. or BDH (Merck).

Cell Culture—C2C12 myoblasts were maintained in EMEM supplemented with 10% (v/v) FCS, 2 mM glutamate, 15 mM Hepes, pH 7.5, 500 IU/ml penicillin, and 100 μg/ml streptomycin (here termed 10% FCS-EMEM), in 95% O2, 5% CO2. To obtain fully differentiated myotubes, cells were grown to 90% confluency and incubated for 1 day in the above-described by Svedberg et al. (29). Briefly, FFA s were dissolved in ethanol and diluted 1:25, in either 1% FCS-EMEM or EMEM containing no FCS (here termed SF-EMEM) at 45 °C, each containing 20% (v/v) fatty acid-free bovine serum albumin. Solutions were filter-sterilized and diluted 1:4 with 1% FCS- or SF-EMEM as appropriate. Control media prepared similarly contained ethanol and BSA in the absence of lipid.

The pH of all media was still approximately 7.5. Myotubes were incubated for 16 h in 10 ml/dish or 2 ml/well 1% FCS-EMEM followed by a 2-h period in 5 ml/dish or 1 ml/well SF-EMEM in the absence or presence of FFAs.

Glycogen Assays—Lipid-pretreated myotubes in 6-well plates were incubated for 1 h in 1 ml/well SF-EMEM containing D-[U-14C]glucose (4 μCi/ml) in the absence or presence of 100 nM insulin and FFAs as stated in the figure legends, and glycogen production was assayed by a method adapted from that described by Berti et al. (30). Cells were washed four times with 2 ml of ice-cold PBS and scraped into 300 μl/well 1% KOH.

Extractions were heated to 100 °C for 10 min, and 5 μl aliquots were taken for the determination of protein concentration by the method of Bradford (31). After addition of 40 μl of a saturated solution of Na2SO4, glycogen was precipitated by the addition of 700 μl ice-cold acetone and incubating at −70 °C for 30 min. Samples were centrifuged at 20,000 g and supernatants aspirated. Pellets were washed by resuspension in 50 μl of water followed by addition of 500 μl of ice-cold acetone and reincubation. Final pellets were dissolved in 100 μl of water, mixed with 1 ml of scintillant and counted for radioactivity.

Glucose Uptake/Phosphorylation Assays—Lipid-pretreated myotubes were incubated, washed, and extracted as described for glycogen assays, except that D-[U-14C]glucose (1 μCi/ml) was used. After aliquots were taken for measurement of protein concentration, glucose uptake, and phosphorylation was determined by a method adapted from that described by Perre et al. (32). Unlabeled exogenous glucose was added to all samples except those which were theophylline with the addition of 25% pteroylglutamic acid and centrifuged at 13,000 rpm for 1 min. Supernatants were applied to 0.5 ml of AG 1-X8 columns previously equilibrated with water, for separation of free 2-deoxyglucose from phosphorylated 2-deoxyglucose. Free 2-deoxyglucose was eluted with 5 ml of water and then phosphorylated 2-deoxyglucose with 3 ml of 1 M HCl. Eluates were counted for radioactivity after addition of 25 ml of scintillant. Measurements of free and phosphorylated 2-deoxyglucose indicated that over 90% of total 2-deoxyglucose was recovered as the phosphorylated form.

IRS-1 Immunoprecipitation and PI3-Kinase Assays—Lipid-pretreated myotubes in 10-cm dishes were incubated for 10 min in 5 ml of SF-EMEM in the absence or presence of 100 nM insulin and FFAs, and IRS-1-associated PI3-kinase activity assayed in a method adapted from those described by Follis et al. (33), Goodyear et al. (4), and Bjornholm et al. (34). Cells were washed twice with 5 ml of ice-cold PBS and scraped into 500 μl of extraction buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 1 mM MgCl2, 1 mM CaCl2, 2 mM Na2VO4, 10 mM Na3P207, 10 mM NaF, 2 mM EDTA, 1% (v/v) Nonidet P40, 10% (v/v) glycerol, 2 μg/ml aprotinin, 5 μg/ml leupeptin, 0.2 mM phenylmethysulfonyl fluoride and 2 mM PMSF). After sonication (15 pulses using a Branson 250 Sonifier), the extracts were centrifuged at 20,000 g at 4 °C for 20,000 g for 10 min at 4 °C. Pellets were discarded and 2 μl of IRS-1 antibody was added to supernatants, which were then rocked gently overnight at 4 °C. After addition of 50 μl of a 1:1 suspension of protein A-Sepharose in 20 mM Hepes, pH 7.5, 180 mM NaCl, and further rocking for 1.5 h, lysates were briefly centrifuged at 3000 × g. The immunoprecipitates were resuspended in 200 μl of 20 mM Tris-HCl, pH 7.5, 180 mM NaCl, and split into equal aliquots for assay in triplicate. Aliquots were prewarmed for 5 min at 30 °C, followed by addition of 25 μl of assay buffer (28 mM Hepes, pH 7.5, 50 mM NaCl, 0.15% (v/v) Nonidet P40, 12.5 mM MgCl2, 0.4 mM EGTA, 0.8 mg/ml t-α-phosphatidylinositol-5-diphosphate (10 μg/ml) (ATP (10 μg/ml)). Reactions were terminated after 15 min by addition of 50 μl of 2 M HCl followed by 160 μl CHCl3. Assays were vortexed, briefly centrifuged at 3000 × g and 75 μl of the lower phase transferred to chilled tubes containing 80 μl of CH3OH, 1 M HCl (1:1 v/v). The tubes were vortexed, briefly centrifuged at 3000 × g and 30 μl of the lower phase applied to TLC plates. Plates were developed in CH3OH:CH3OH:CH3OH:H2O (60:47:11:5 by volume). t-α-phosphatidylinositol-4-phosphate (10 μg) was used as a standard and visualized with iodine vapor. Relative radioactivity of lipids co-migrating with the standard was determined either by using a Medical Dynamics 445 Phosphorimagery and densitometry using IP Lab Gel software (Signal Analytics, Vienna, VA). Alternatively, washed immunoprecipitates were subjected to SDS-PAGE after addition of 50 μl of Laemmli sample buffer (35) and immunoblots to assess IRS-1 tyrosine phosphorylation and p85 subunit association.

Immunoblotting—After dilution of 500 μl of the supernatants re-
maining after IRS-1 immunoprecipitation with 100 μl of Laemmli sample buffer and heating at 100 °C for 2 min, 20-μl samples were subjected to SDS-PAGE, immunoblotting, and densitometry as described previously (23). Alternatively, cells in 6-well plates were washed twice with ice-cold 1× NaCl. This extract was quickly added to 3 ml of CHCl₃/CH₃OH (1:2, v/v) and vortex mixed. After addition of 1 ml of 1× NaCl and 1 ml of CHCl₃ and further mixing, phases were separated by centrifugation at 5000×g for 2 min. Lipid extracts in the lower CHCl₃ phase were stored at −20 °C under nitrogen and assayed within 4 days. Ceramide content was determined using a radiometric diacylglycerol assay kit (Amersham Pharmacia Biotech) according to the manufacturer’s instructions, as diacylglycerol kinase can phosphorylate both diacylglycerols and ceramides (36). To improve separation of phosphatidic acid and ceramide-1-phosphate by TLC, plates were first developed with CHCl₃/CH₃OH:NH₄OH (65:35:7.5, v/v/v), dried, and then developed with CHCl₃/CH₃OH:CH₃COOH:(CH₃)₂CO:H₂O (10:2.3:4.1, by volume) (37). ³²P-labeled phosphatidic acid and ceramide-1-phosphate were identified after phosphorimaging by co-migration with authentic standards.

Statistical Methods—All results are expressed as means ± S.E. Statistical calculations using Student’s t test were performed using Statview S.E. + Graphics for Macintosh (Abacus Concepts, Berkeley, CA).

RESULTS

Effects of FFA Preincubation on Insulin-stimulated Glycerogen Synthesis and Glucose Uptake in C2C12 Myotubes—C2C12 myotubes exhibited a 2-fold stimulation of glycogen synthesis in the presence of 100 nM insulin during a 1-h incubation period, from 11 to 22 nmol of glucose units/h·mg of protein (Fig. 1A). This was used as a marker for insulin sensitivity so that the effects of lipids on insulin-signaling steps could be assessed. Three FFAs were used in experiments to induce insulin resistance in this system: the mono-unsaturated FFA oleate (18:1n-9), the di-unsaturated FFA linoleate (18:2n-6), and the saturated FFA palmitate (16:0), which are among the most common fatty acids found in muscle (38). Preliminary experiments (not shown) using lipid concentrations from 0.5 to 2 mM (within the physiological serum range) established that an approximately 50% decrease in total glycogen synthesis in the presence of insulin was obtained using 2 mM oleate, 1 mM linoleate, or 0.75 mM palmitate (Fig. 1A). Interestingly, the unsaturated FFAs also had a significant effect on basal glycogen synthesis, whereas palmitate was without effect, suggesting that the mechanisms involved might be distinct. None of the lipids affected the levels of glycogen synthase protein expressed in the myotubes, as determined by immunoblotting (data not shown).

Because it was possible that the lipid treatments affected glucose uptake and so restricted glucose availability for glycogen synthesis, we also determined glucose uptake and phosphorylation by the cells under identical conditions, using radiolabeled 2-deoxyglucose (Fig. 1B). C2C12 myotubes exhibited little if any stimulation of glucose uptake and phosphorylation in the presence of 100 nM insulin (approximately 250 nmol/h·mg protein in control cells), in agreement with the increase from 1.9 to 2.2 nmol/min·mg protein observed by others (39). Moreover, in contrast to their differing effects on glycogen synthesis, all three lipids gave essentially similar effects on glucose uptake, reducing the uptake and phosphorylation of 2-deoxyglucose by less than 20%. The 10-fold excess of phosphorylated glucose available for glycogen synthesis and the relatively minor reductions observed in the presence of FFAs, taken together with the absence of an effect of palmitate on basal glycogen synthesis, suggest that the observed lipid-specific alterations in glycogen synthesis are unlikely to result from decreased glucose availability.

Investigation of the Effects of FFA Preincubation on the PKB Signaling Pathway—To determine where, in the pathways upstream of glycogen synthase, FFAs were exerting their effects, we first investigated the level of Ser-21 phosphorylation of GSK-3α and Ser-473 phosphorylation of PKB in lysates of myotubes, prepared after FFA treatment and insulin stimulation, using phospho-specific antibodies. A representative experiment is shown in Fig. 2A together with the means of densitometric analysis of five independent experiments in Fig. 2B. While palmitate pretreatment of myotubes resulted in inhibition of the insulin-stimulated phosphorylation of both GSK-3 and PKB, in agreement with its effect on glycogen synthesis, either oleate or linoleate pretreatment affected the phosphorylation state of these kinases (Fig. 2). Similar results were obtained using a phospho-specific antibody directed against the Thr-308 phosphorylation site of PKB (not shown). No effects of FFA pretreatment were seen on basal levels of phosphorylation of either kinase, nor did any treatment affect the total levels of PKB or GSK-3α expression (not shown). These results indicate...
that while the saturated FFA palmitate may inhibit insulin-stimulated glycogen synthesis through effects on the PKB signaling pathway, the unsaturated FFAs do not inhibit insulin action at this site.

**FFA Effects on IRS-1 Tyrosine Phosphorylation, p85 Association, and PI3-Kinase Activation**—Because palmitate pretreatment was found to affect signaling through the PKB pathway, we next determined whether this lipid affected insulin action at the level of IRS-1, including the association and activation of PI3-kinase, because these components lie upstream of PKB activation. IRS-1 was immunoprecipitated from cell lysates, prepared from myotubes that had been pretreated with FFAs, and incubated in the absence or presence of insulin, and the immunoprecipitates were assayed for PI3-kinase activity (Fig. 3). No significant difference in insulin-stimulated PI3-kinase activity was observed between control and palmitate-treated myotubes, while oleate and linoleate pretreatment had minor though statistically significant inhibitory effects (Fig. 3). Basal PI3-kinase activities were similar after all treatments. Immunoprecipitates from palmitate-treated cells were also subjected to SDS-PAGE and immunoblotting, to determine the extent of tyrosine phosphorylation and association of the p85 subunit of PI3-kinase stimulated by insulin (Fig. 4). In agreement with the absence of a palmitate effect on PI3-kinase activity, no significant difference was observed between control and palmitate-treated myotubes in the insulin-stimulated tyrosine phosphorylation of IRS-1 or co-immunoprecipitation with p85.

**FFA Effects on MAP Kinase Phosphorylation**—The palmitate-induced inhibition of insulin signaling from IRS-1 to glycogen synthase appears to originate downstream of PI3-kinase activation but upstream of PKB phosphorylation. While a reduction in the GSK-3-mediated phosphorylation of glycogen synthase appears to be the major mechanism of regulation of glycogen synthesis, there is also some evidence that insulin-stimulated dephosphorylation of the enzyme is mediated through the ERK MAP kinase pathway. We therefore investigated the effects of FFA pretreatment on the insulin sensitivity of ERK1/2 MAP kinase phosphorylation in myotubes to assess whether this pathway is also affected. C2C12 cells were found to express mainly the p42 (ERK1) form of ERK MAP kinase, which exhibited a 4-fold increase in dual Thr-202/Tyr-204 phosphorylation in response to insulin, indicating activation (Fig. 5A). While the unsaturated FFAs oleate and linoleate had little effect on p42 ERK phosphorylation, palmitate pretreatment resulted in a 3-fold increase in basal phosphorylation and a 2.8-fold increase in the insulin-stimulated dual phosphorylation of this kinase, indicating both chronic activation and a potentiation of effects between insulin and the saturated FFA (Fig. 5B).

While the stimulation of the ERK MAP kinase pathway by insulin, through activation of Ras, has been well characterized,
the effects of the hormone on JNK and p38 MAP kinases have
been less studied. Because these kinases lie on parallel signal-
ing pathways which may exhibit cross-talk at the level of up-
stream components, we investigated whether the enhanced
ERK MAP kinase phosphorylation by palmitate pretreatment
was accompanied by activation of JNK or p38 MAP kinases.
Insufficient levels of JNK MAP kinases were detected in im-
munoblots using phospho-JNK MAP kinase antibodies to per-
mit densitometry, and these enzymes were not further inves-
tigated. In contrast, p38 MAP kinase was readily detected:
insulin did not increase phosphorylation of this kinase, nor did
oleate and linoleate. However, lysates from palmitate-treated
myotubes exhibited 7–10-fold increased phosphorylation of p38
MAP kinase (Fig. 5). These results suggest that the oleate and
linoleate effects on glycogen metabolism are not mediated
through the MAP kinase pathways although the possibility
remains that they reduce the phosphatase activity regulating
glycogen synthase.

**Investigation of the Role of Ceramide and MAP Kinases in
Palmitate-induced Insulin Resistance**—Recently, activation of
the Ras-MAP kinase pathway has been demonstrated down-
stream of a ceramide-induced activation of KSR/CAPK, and
was found to inhibit PKB in the absence of an effect on PI3-
kinase (25). Because the data presented here for palmitate-
pretreated cells is in agreement with such a mechanism, we
investigated whether palmitate could be acting through the
elevation of ceramide levels in the myotubes. Ceramide was
measured in cells preincubated in the absence or presence of
FFAs and was found to be elevated 2.1-fold by palmitate (Fig.
6). In contrast, oleate and linoleate were without effect. Fur-
thermore, preincubation of myotubes with increasing concen-
trations of C2-ceramide gave similar results to those obtained
by palmitate pretreatment: both insulin-stimulated glycogen
synthesis (Fig. 7A) and PKB phosphorylation (Fig. 7B) were
inhibited, while ERK MAP kinase phosphorylation was en-
hanced at C2-ceramide concentrations above 10 μM (Fig. 7B).

The results presented in Figs. 5 and 7 had suggested that the
palmitate-induced inhibition of insulin-stimulated PKB, and
hence of glycogen synthesis, may be mediated through overac-
tivation of ERK or p38 MAP kinases by ceramide. There is
some precedence for this, at least in the case of ERK MAP
kinase, from other experimental systems (26, 27). We therefore
examined the effects of the MAP kinase inhibitor
PD98059, which reduces ERK MAP kinase activation, on both
Palmitate- and ceramide-induced insulin resistance in C2C12 myotubes. Cells were preincubated in the absence and presence of palmitate or ceramide as before, in combination with PD98059 treatment, prior to insulin stimulation, and the effects on glycogen synthesis and kinase phosphorylation state were determined. While the inhibitor was confirmed to be effective in the inhibition of ERK1 phosphorylation, there was no improvement in the reduced insulin sensitivity of phosphorylation of PKB after either palmitate or ceramide pretreatment (Fig. 8). Similarly, the inhibition of insulin-stimulated glycogen synthesis was not affected (not shown). Using the p38 MAP kinase-specific inhibitor SB203580, we were also able to assess the contribution of p38 to the insulin resistance caused by palmitate or ceramide. Again, there was no improvement in either the reduced insulin sensitivity of glycogen synthesis, or in the reduced phosphorylation of PKB, in the presence of 50 μM SB203580 (not shown). These results suggest that while palmitate pretreatment may inhibit the PKB pathway through increased production of ceramide, this effect is not mediated through increased activation of the ERK and p38 MAP kinase pathways. However, the close agreement between the results obtained with palmitate (Fig. 8, A and B) and those obtained with ceramide (Fig. 8, C and D) again strongly suggest that the effects of palmitate are mediated by ceramide.

DISCUSSION

The work described here is an extensive study of the mechanisms of lipid-induced insulin resistance of glycogen synthesis in C2C12 skeletal muscle cells, addressing the effects of FFAs on the signaling pathway from IRS-1 to glycogen synthase, as well as on MAP kinases. While we have shown that overnight preincubation with either saturated or unsaturated FFAs can inhibit glycogen synthesis, our results indicate that these lipids probably act via different mechanisms. Palmitate preincubation affected only the insulin-stimulated component of glycogen synthesis, but did not affect immediate signaling from the insulin receptor, in that IRS-1 tyrosine phosphorylation and PI3-kinase recruitment appeared unimpaired. However, it did reduce activation of the downstream PKB pathway, which is a major regulatory input for glycogen synthase. In contrast, palmitate potentiated insulin-stimulated activation of the concurrent ERK MAP kinase pathway. This FFA also activated p38, another MAP kinase family member. Conversely, the un-
saturated FFAs oleate and linoleate had no effect on the PKB or MAP kinase pathways although inhibition of both basal and insulin-stimulated glycogen synthesis was observed, and insulin-stimulated PI3-kinase activity was also slightly reduced. These results, and the observation that the presence of the 

β-oxidation inhibitor etomoxir was unable to protect against the effects of oleate on glycogen synthesis, indicate that inhibition of glucose metabolism by FFAs is not explained purely by Randle glucose-fatty acid cycle effects but that different lipids can exert specific effects at the level of the insulin-signaling cascade. However, it is possible that the observed FFA-induced alterations in glucose uptake and phosphorylation occur as a consequence of basic competition between accumulated lipid and glucose (22). The absence of an effect of insulin on glucose uptake in our system may be because of the fact that C2C12 cells express predominantly the GLUT1 glucose transporter (30) rather than the insulin-sensitive GLUT4 isomform.

The data presented here clearly suggest that palmitate acts downstream of IRS-1 and PI3-kinase. While it might be argued that palmitate affected signaling from the insulin receptor via another adaptor protein such as IRS-2, which might influence the PKB pathway to a greater extent, IRS-1 appears to be the major IRS in skeletal muscle (12). Furthermore, IRS-2 tyrosine phosphorylation in response to insulin appears to be more transient (40), and we have detected relatively little insulin-stimulated tyrosine phosphorylation in IRS-2 immunoprecipitates from C2C12 cells, making such an explanation unlikely. Finally, we have also observed inhibition of PKB phosphorylation in response to EGF in palmitate-pretreated cells. Because PI3-kinase activation by EGF occurs by direct interaction with the EGF receptor rather than through IRS-1, this again suggests that palmitate acts downstream of IRS docking proteins to inhibit stimulation of PKB.

Activation of the Ras-MAP kinase pathway by CAPK, also known as KSR, has been demonstrated concomitantly with the inhibition of the PKB pathway, independently of PI3-kinase (25). Furthermore, palmitate is a precursor of ceramide (28), and ceramide treatment of 3T3-L1 adipocytes and COS-7 cells gave rise to similar inhibition of PKB but not PI3-kinase to that seen here with palmitate (26, 27). Taken together with our findings that palmitate elevates the levels of ceramides in C2C12 myotubes and that exogenously added C_2- ceramide also caused inhibition of PKB phosphorylation but stimulation of MAP kinase phosphorylation, these observations strongly suggest that ceramide synthesis is sufficient to explain the inhibition of insulin-stimulated glycogen synthesis by palmitate.

\[ ^2 \] C. Schmitz-Peiffer, D. L. Craig, and T. J. Biden, unpublished observation.

\[ ^3 \] In keeping with such a proposal, we have observed conversion of H-labeled palmitate into ceramide, as well as diacylglycerol and triglyceride, in C2C12 myotubes, upon TLC separation of lipid extracts.
The increase in ceramides in the myotubes caused by the FFA was approximately 2-fold and similar to that previously reported during the development of skeletal muscle insulin resistance in whole animals (36). Other studies have suggested a role for ceramide in the inhibition of insulin signaling, also downstream of PI3-kinase, including in the induction of insulin resistance by TNFα in 3T3-L1 adipocytes (41). Because, in our system with C2C12 myotubes, inhibition of ERK MAP kinase with PD98059 or of p38 MAP kinase with SB203580 did not protect against palmitate- or ceramide-induced insulin resistance, and since ceramide does not lead to ERK MAP kinase phosphorylation in 3T3-L1 adipocytes while still inhibiting PKB (27), it is likely that ceramide can also lead to reduced PKB phosphorylation independently of these MAP kinases. Studies of ceramide effects indicate that coupling of this lipid to specific signaling cascades is both stimulus and cell-type specific (reviewed in Ref. 42).

While we have established that palmitate acts at the level of PKB, reducing phosphorylation at both Thr-308 and Ser-473, it remains to be determined whether this effect involves inhibition of PDK activity and/or enhanced dephosphorylation. A recent study has shown that PKB inhibition by hyperosmotic stress involves both inhibition of phosphorylation of these regulatory sites and also rapid dephosphorylation by PP2A, while PI3-kinase activity remains unaffected (43). Interestingly, in addition to activation of KSR/CAPK, ceramide may also act through CAPP (44), a member of the PP2A family which undergoes specific (reviewed in Ref. 42).

In summary, we have developed a model of lipid-induced skeletal muscle insulin resistance using mouse C2C12 myotubes. This has led to the identification of specific insulin-signaling steps which are affected by pretreatment with different FFAs. The unsaturated FFAs oleate and linoleate were without effect on PKB despite inhibition of PI3-kinase activation, and the mechanism by which they act remains to be determined. In contrast, the saturated FFA palmitate inhibits insulin activation of the PKB pathway downstream of IRS-1-associated PI3-kinase activation, and its effect is likely to be mediated by the de novo synthesis of ceramide.

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