Free Fatty Acid–Induced Insulin Resistance Is Associated With Activation of Protein Kinase C θ and Alterations in the Insulin Signaling Cascade

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To examine the mechanism by which free fatty acids (FFAs) induce insulin resistance in vivo, awake chronically catheterized rats underwent a hyperinsulinemic-euglycemic clamp with or without a 5-h preinfusion of lipid/heparin to raise plasma FFA concentrations. Increased plasma FFAs resulted in insulin resistance as reflected by a ~35% reduction in the glucose infusion rate (P < 0.05 vs. control). The insulin resistance was associated with a 40–50% reduction in 13C nuclear magnetic resonance (NMR)–determined rates of muscle glycogen synthesis (P < 0.01 vs. control) and muscle glucose oxidation (P < 0.01 vs. control), which in turn could be attributed to a ~25% reduction in glucose transport activity as assessed by 2-[1,2-3H]deoxyglucose uptake in vivo (P < 0.05 vs. control). This lipid–induced decrease in insulin-stimulated muscle glucose metabolism was associated with 1) a ~50% reduction in insulin-stimulated insulin receptor substrate (IRS)-1–associated phosphatidylinositol (PI) 3-kinase activity (P < 0.05 vs. control), 2) a blunting in insulin-stimulated IRS-1 tyrosine phosphorylation (P < 0.05, lipid-infused versus glycerol-infused), and 3) a fourfold increase in membrane-bound, or active, protein kinase C (PKC) θ (P < 0.05 vs. control). We conclude that acute elevations of plasma FFA levels for 5 h induce skeletal muscle insulin resistance in vivo via a reduction in insulin-stimulated muscle glycogen synthesis and glucose oxidation that can be attributed to reduced glucose transport activity. These changes are associated with abnormalities in the insulin signaling cascade and may be mediated by FFA activation of PKC θ.

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Chronic elevation in plasma free fatty acid (FFA) levels is commonly associated with impaired insulin-mediated glucose uptake (1,2) and often coexists with obesity and type 2 diabetes (3). Acute elevations in plasma FFA levels during a triglyceride emulsion infusion impair insulin-mediated glucose uptake in rats (4,5) and humans (6–9). More than 30 years ago, Randle et al. (10,11) demonstrated that FFAs compete with glucose for substrate oxidation in isolated rat heart and diaphragm muscle preparations and speculated that increased fat oxidation may cause the insulin resistance associated with diabetes and obesity.

According to the mechanism proposed by Randle et al., increased FFA levels lead to increased mitochondrial acetyl CoA/CoA ratios, which in turn inhibit pyruvate dehydrogenase activity and increase citrate levels, which in turn inhibit phosphofructokinase activity. This process leads to increased glucose-6-phosphate concentrations, which allosterically inhibit hexokinase, thus reducing glucose transport/phosphorylation activity. However, more recent studies by Boden and colleagues (8,9) and our group (6) have called this mechanism into question. Boden et al. have found that at plasma FFA concentrations ~0.75 mmol/l in humans, there is an increase in intramuscular glucose-6-phosphate concentrations under insulin-stimulated conditions, implying an FFA-induced decrease in glycogen synthase activity (9). In contrast, we have shown using 31P nuclear magnetic resonance (NMR) spectroscopy that insulin resistance induced by FFAs (~1.8 mmol/l) in humans is associated with reduced intramuscular glucose-6-phosphate concentrations, implicating a defect in glucose transport/phosphorylation activity (6). These data suggest that increased FFA levels promote insulin resistance in humans through a mechanism not involving glucose-6-phosphate inhibition of hexokinase (10,11) or FFA inhibition of glycogen synthase activity (9) as previously described.

The mechanisms that underlie these changes in glucose transport/phosphorylation activity in the presence of high circulating FFA levels are unknown, but may include changes in the insulin signaling cascade. The effect of elevated FFAs on insulin binding and postreceptor insulin-mediated signaling is still poorly understood. Fatty acid–rich medium reduced insulin receptor tyrosine kinase (IRTK) activity in rat hepatoma cells compared with cells grown in normal medium (12). Moreover, palmitate incubation reduced insulin-stimulated mitogen-activated protein (MAP) kinase.
activity in rat-1 fibroblasts overexpressing human insulin receptors, but palmitate had no effect on insulin-stimulated phosphatidylinositol (PT) 3-kinase (13). The discrepancies in the published data may be a consequence of the different cell types studied, different fatty acid incubations used, and differing concentrations of fatty acids.

The aim of these studies was to examine rat skeletal muscle glucose metabolism and a number of key proteins involved in the insulin signaling cascade after in vivo insulin stimulation in the presence or absence of high circulating FFAs. To explore possible mechanisms underlying FFA-induced insulin resistance, this study examined protein kinase C (PKC) θ, one of the major PKC isoforms expressed in skeletal muscle, and its potential activation by elevated plasma FFAs.

RESEARCH DESIGN AND METHODS

Materials. PI was purchased from Avanti Polar Lipids (Albany, AL) and PI 4-phosphate from Sigma (St. Louis, MO). Reagents for the detection of Western blots by enhanced chemiluminescence, Rainbow Colored molecular weight markers for SDS-PAGE, and [3H]-ATP (6,000 Ci/mmol) were purchased from Amersham Life Science (Arlington Heights, IL). Protein G-Plus Protein Agarose immunoprecipitation reagent was purchased from Calculbio (Cambridge, MA). Antibodies against insulin receptor substrate (IRS)-ICT (rabbit polyclonal) and PI 3-kinase (p85 subunit, rabbit polyclonal) were purchased from Upstate Biotechnology (Lake Placid, NY), rabbit anti-epitope against nPKCδ from Santa Cruz Biotechnology (Santa Cruz, CA), and nPKCε from Gibco BRL (Mulgrave, Australia). Horseradish peroxidase-labeled anti-mouse and anti-rabbit secondary antibodies were obtained from Rockland (Gilbertsville, PA), horseradish peroxidase-linked don-key anti-rabbit antibody from Jackson ImmunoResearch Materials. (West Grove, PA), and Renaissance enhanced chemiluminescence reagents and 2-[125I]-deoxy-o-glucose ([125I]-2DG) from NEN (Boston, MA).

Animals. Male Sprague-Dawley rats (Charles River, Wilmington, MA) weighing between 250 and 300 g were housed in environmentally controlled conditions with a 12:12-h light/dark cycle and fed a standard rat diet. Rats were caged in the right jugular vein and carotid artery, and the catheters were externalized through an incision in the skin flap behind the head. After surgery, the rats recuperated until they reached preoperative weight (~5–7 days). All rats were fasted 15–18 h before each infusion study.

Hyperinsulinemic-euglycemic clamp. Hyperinsulinemic-euglycemic clamps (10 mU·kg⁻¹·min⁻¹) were performed maintaining glucose concentrations at 5.5 mmol/l using a variable 20% glucose infusion. Humulin regular insulin (Eli Lilly, Indianapolis, IN) was used during the clamps. Blood samples were drawn every 5 min during clamps to assess rates of glucose disposal. Additional blood samples were drawn for insulin, glucose, and FFAs. The time course of the insulin infusion was optimized for each particular study.

13C NMR studies. Two groups of rats were studied using a 5-h preinfusion protocol: one (n = 6) or glycerol (n = 4) followed by a 90-min hyperinsulinemic-euglycemic clamp using a 13C-enriched variable 20% dextrose infusion during which the lipid or glycerol infusion continued. Liposyn II (Abbott Laboratories, North Chicago, IL) and a 20% triglyceride emulsion (continuous infusion 10 ml·kg⁻¹·h⁻¹) combined with heparin (continuous infusion 0.0075 IU/min) or glycerol (1.5 mmol/kg/h) was administered. During the clamp, glycogen synthesis rates were measured using 13C NMR at 7 T in conscious rats as previously described (4). After the infusion, the muscle was freeze-clamped in situ. Steady-state pyruvate dehydrogenase (PDH) flux to tricarboxylic acid (TCA) cycle flux was determined from the ratio of 13C enrichment of C3 alanine to C4 glutamate. The relative amount of intracellular pyruvate derived from plasma glucose was estimated by an equation described in our earlier study (17). Total cellular PKC was determined from lysates prepared independently in a solubilization buffer containing 10 mmol/l MgCl₂, 2 mmol/l EDTA, and 1 mmol/l phenylmethylsulfonyl fluoride [PMSF], 1% Triton X-100, 10% glycerol, 10 mmol/l leupeptin, 3 mmol/l benzamidine, 5 mmol/l pepstatin A, 10 µg/ml aprotinin, 200 µg/ml soybean trypsin inhibitor. The homogenate was allowed to solubilize in a rotating mixer at 4°C for 30–60 min at room temperature and then was centrifuged at 15,000 rpm for 1 h in a 70.1 Ti rotor (Beckman ultratflo; Beckman, Fullerton, CA). The supernatant was collected and assayed for total protein content using the method of Lowry (Hercules, CA) protein assay kit.

PI 3-kinase activity measurements. IRS-1–associated PI 3-kinase activity was measured in immunoprecipitates obtained with antibodies to IRS-1 as previously described (16), with some modifications. A 1-ng aliquot of muscle extract (total protein) was added to the immune complex composed of protein A/G and anti–IRS-1 antibody and allowed to incubate overnight. The immunocomplexes were collected by centrifugation and washed twice with phosphate-buffered saline (PBS) (1× NP-40) containing 0.1 mmol/l Tris (pH 7.5) containing 500 mmol/l LiCl, and 100 mmol/l NaCl, and twice with Tris (pH 7.5) containing 100 mmol/l NaCl, 1 mmol/l EDTA, and 100 µmol/l NaVO₄. The pellets were then resuspended in 50 µl of the final wash buffer, and 12 mmol/l MgCl₂ and 20 µg PI were added. To start the PI 3-kinase reaction, 10 µl of 440 µmol/l ATP containing 30 µCi [32P]-ATP was added to the pellets at room temperature. At 10 min, 20 µl of 8 mmol/l HCl was added to stop the reaction, followed by 160 µl of CH₃CN:MeOH (1:1). The phases were separated by centrifugation, and 50 µl of the lower organic phase was spotted onto a glass-backed silicon thin-layer chromatography (TLC) plate. The lipids were resolved by TLC in MeOH:CH₃OH:H₂O:NH₄OH (60:47:11:3.2) and visualized by autoradiography. The radioactivity that comigrated with the PI 4-phosphate standard was scraped from the TLC plate and quantified by scintillation counting.

Examination of IRS-1 proteins. In a subsequent experiment, rats were preinfused with either glycerol (n = 20) or lipid/heparin (n = 10). After 5 h, 10 of the glycerol-infused animals (basal group) were anesthetized, and muscle was freeze-clamped in situ for evaluation of IRS-1 proteins. The other 10 glycerol-infused rats received an intravenous bolus of insulin (100 mU/kg body wt) and were anesthetized by intravenous infusion of pentobarbital (30 mg/kg); muscle was freeze-clamped in situ at 1 min after the insulin bolus. Two basal and two insulin-treated rats received insulin in an identical manner to the insulin-treated glycerol-infused rats, and muscle was freeze clamped in situ in the same manner. Muscle extracts were prepared as above. To analyze tyrosine phosphorylated IRS-1, muscle homogenates were immunoprecipitated with antiphosphotyrosine antibody. Immunoprecipitates were electrophoresed on 8% SDS-PAGE and transferred to nitrocellulose membranes using a semidyel electroblotter (Owl Separation Systems, Portsmouth, NH). The membranes were immunoblotted using anti-IRS-1 antibody. The proteins were visualized with chemiluminescence reagents according to the manufacturer’s protocol (Amersham, Boston, MA).

PKC activity. PKC translocation from cytosolic fraction to particulate fraction, representative of activation of PKC, was assessed as previously described (17) in two groups of rats, the 1st infused with glycerol for 5 h (n = 7) and the 2nd infused with lipid (n = 5) for 5 h. Briefly, mixed gastrocnemius muscle cytosolic and particulate fractions were prepared as previously described (17). Total cellular PKC was determined from lysates prepared independently in a solubilization buffer containing 20 mmol/l MOPS (pH 7.5), 1.2 mmol/l EGTA, 1 mmol/l EDTA, 2 mmol/l PMSF, 200 µg/ml leupeptin, and 2 mmol/l benzamidine. Fractions were subjected to SDS-PAGE. Proteins were electrophoresed onto polyvinylidene difluoride (PVDF) membrane and were blocked with 3% nonfat dry milk in TBS. Membranes were then probed with rabbit anti-peptide antibodies specific for PKC isoforms θ and ε, followed by horseradish peroxidase–linked donkey anti-rabbit antibody. PKC isoforms were visualized by enhanced chemiluminescence reaction. Densitometry of PKC bands was determined using a Medical Dynamics Personal Densitometer SI and analyzed using IP Lab Gel II software (Signal Analytics, Vienna, VA). Densities were corrected for loading and presented relative to an internal PKC standard that was run in triplicate on each gel.

Statistical comparisons. Groups were compared using analysis of variance with Fischer’s protected least-square difference test.
TABLE 1
Metabolic data from rats undergoing hyperinsulinemic-euglycemic clamps after preinfusion with either glycerol or lipid/heparin during $^{13}$C NMR skeletal muscle and $[^{3}H]$-2DG studies

<table>
<thead>
<tr>
<th></th>
<th>Glycerol</th>
<th>Lipid</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>FFAs during clamp (mmol/l)</td>
<td>0.4 ± 0.1</td>
<td>2.8 ± 0.7</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Glucose infusion rate (µmol · kg$^{-1}$ · min$^{-1}$)</td>
<td>244 ± 22</td>
<td>161 ± 10</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>Glycogen synthesis rate (µmol · kg$^{-1}$ · min$^{-1}$)</td>
<td>147 ± 14</td>
<td>80 ± 11</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>PDH flux/TCA flux</td>
<td>0.49 ± 0.01</td>
<td>0.23 ± 0.01</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>$[^{3}H]$-2DG (µmol · 100 g$^{-1}$ muscle · min$^{-1}$)</td>
<td>11.4 ± 1.0</td>
<td>8.8 ± 0.3</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

RESULTS
Glucose infusion rates were reduced by 34% when plasma FFAs were increased by the lipid infusion (Table 1). NMR studies confirmed insulin resistance at the level of skeletal muscle as reflected by a ~40% reduction in muscle glycogen synthetic rates during the hyperinsulinemic-euglycemic clamp after lipid infusion compared with the control glycerol infusion. The ratio of PDH to TCA cycle flux, reflecting entry of pyruvate relative to other substrates (primarily FFAs) into the TCA cycle, fell from 0.49 ± 0.01 in the control to 0.23 ± 0.01 in the lipid-infused rats ($P < 0.0001$). This decreased PDH/TCA ratio reflects relative decreased muscle glucose oxidation, since most (~70%) of the intracellular pyruvate was derived from plasma glucose in both groups, as reflected by the $^{13}$C3 alanine (tissue)/$^{13}$C1 glucose (plasma) ratio $\times 0.5$ (control, 0.74 ± 0.01; lipid-infused, 0.70 ± 0.01).

Those rats studied during the $[^{3}H]$-2DG clamps were matched with regard to basal levels of FFAs and basal insulin concentrations similar to the NMR experiment. Rats preinfused with lipids had significantly higher levels of FFAs during the clamp than the glycerol-infused rats (3.3 ± 0.8 vs. 0.6 ± 0.2 mmol/l). Again, this elevation in plasma FFA concentrations was associated with insulin resistance, as demonstrated by a 40% reduction in glucose infusion rate (from 237 ± 18 in the glycerol-infused animals to 141 ± 22 µmol · kg$^{-1}$ · min$^{-1}$ in the lipid-infused animals). $[^{3}H]$-2DG uptake in skeletal muscle of lipid-infused rats was decreased (8.8 ± 0.4 µmol · 100 g$^{-1}$ muscle · min$^{-1}$) compared with the glycerol-infused rats (11.4 ± 1.0 µmol · 100 g$^{-1}$ muscle · min$^{-1}$; $P < 0.05$) despite comparable levels of hyperinsulinemia during the clamp (3,324 ± 1,428 vs. 2,814 ± 618 pmol/l).

All three groups of rats studied for PI 3-kinase activity were matched for body weight and had similar levels of fasting FFAs. Equivalent levels of hyperinsulinemia were induced in both groups undergoing the hyperinsulinemic-euglycemic clamp (2.556 ± 540 pmol/l in the group receiving insulin alone compared with 2.076 ± 432 pmol/l in the lipid group). The lipid group had significantly higher plasma FFA levels following the 5 h of infusion as in the previous studies (4.2 ± 0.5 mmol/l vs. 1.2 ± 0.1 mmol/l, $P < 0.05$). Glucose infusion rate (GIR) in the group preinfused with lipid was 30% lower, 188 ± 8 µmol · kg$^{-1}$ · min$^{-1}$ in the insulin-treated group compared with 136 ± 6 µmol · kg$^{-1}$ · min$^{-1}$ in the lipid group ($P < 0.001$). IRS-1-associated PI 3-kinase activity in skeletal muscle increased 4.6 ± 1.2-fold following 30 min of hyperinsulinemia in the control group that received insulin alone ($P < 0.01$ compared with basal). However, IRS-1–associated PI 3-kinase activity increased only 2.1 ± 0.6-fold in the lipid-infused group ($P < 0.05$ versus insulin alone). Thus elevated plasma FFA levels significantly reduced, by >50%, the PI 3-kinase activity associated with IRS-1 (Fig. 1A).

IRS-1 phosphorylation as detected after antiphosphotyrosine immunoprecipitation and Western blot analysis for IRS-1 confirmed insulin-stimulated IRS-1 tyrosine phosphorylation at 1 min in response to a bolus dose of insulin following preinfusion with glycerol, which was 4.0 ± 1.3-fold increased over basal ($P < 0.03$). In the lipid group, this increment was blunted to 1.7 ± 0.3-fold over basal ($P < 0.05$ compared with insulin plus glycerol, $P = 0.51$ compared with basal alone) (Fig. 1B).

In the final study, fasting FFAs (1.3 ± 0.2 mmol/l in the glycerol group vs. 1.3 ± 0.1 mmol/l in the lipid group) and fasting insulin concentrations (89 ± 29 vs. 80 ± 17 pmol/l) were similar. Again, plasma FFA levels were increased with lipid infusion to 4.0 ± 0.6 mmol/l compared with 0.93 ± 0.1 mmol/l in glycerol controls. Membrane PKC $\theta$ levels in rat skeletal muscle increased 4.6 ± 1.2-fold during the hyperinsulinemic-euglycemic clamp conditions. B: IRS-1 phosphorylation in skeletal muscle in response to insulin stimulation, as detected by antiphosphotyrosine immunoprecipitation followed by anti–IRS-1 immunoblot, represented as percentage stimulation over that of basal.
muscle were 0.5 ± 0.1 arbitrary optical density (OD) units in glycerol controls and increased following lipid infusion to 2.2 ± 0.6 (P < 0.03)—or, expressed as percentage at the membrane, 30.6 ± 10.0% in glycerol-infused rats, rising to 66.3 ± 8.0% in lipid-infused rats (P < 0.01) (Fig. 2A i). Total PKC \(_0\) levels did not change, either determined directly (0.87 ± 0.24 vs. 0.49 ± 0.8 arbitrary OD units, glycerol vs. lipid group) or expressed as the sum of the membrane and cytosolic PKC \(_0\) concentrations (2.1 ± 0.5 vs. 3.2 ± 0.5 arbitrary OD units in glycerol vs. lipid group) (Fig. 2A ii). There was no significant increase in membrane PKC \(_0\) (1.0 ± 0.2 vs. 1.7 ± 0.6 OD units in glycerol vs. lipid group or, as membrane percent, 35.2 ± 4.1 vs. 54.6 ± 9.3%) (Fig. 2B).

**DISCUSSION**

Acute elevation in plasma FFA concentrations for 5 h induced insulin resistance in this awake rat model as reflected by a ~35% reduction in the glucose infusion rates during the hyperinsulinemic-euglycemic clamp, compared with the glycerol infusion studies. This insulin resistance could largely be attributed to a ~45% reduction in the rate of muscle glycogen synthesis and is consistent with the recent findings by Chalkley et al. (18). We also found a ~50% reduction in the relative rate of muscle glucose oxidation as reflected by a decrease in the relative \(^{13}\)C enrichment in the C4 position of muscle glutamate versus the C3 position of intramuscular alanine. This combined decrease in both muscle glycogen synthesis and glucose oxidation is consistent with an FFA-induced defect in glucose transport/phosphorylation activity, which was confirmed by lower rates of [\(^3\)H]-2DG uptake in the lipid-infused animals.

PI 3-kinase is a key regulator of GLUT4 translocation in muscle leading to increased glucose transport (19), and its activity is reduced in muscle strips taken from subjects with type 2 diabetes (20) and obesity (21), conditions associated with elevated plasma levels of FFAs. For this reason, we also examined the effect of elevated plasma FFA concentrations on some cardinal proteins of the insulin signaling cascade. We found that FFA-induced insulin resistance is associated with alterations in insulin-stimulated PI 3-kinase activity. Such a reduction in PI 3-kinase activity may occur as a consequence of reduced IRS-1 tyrosine phosphorylation, which was also observed. This in turn would lead to reduced coassociation of PI 3-kinase and IRS-1 and therefore reduced activation of IRS-1-associated PI 3-kinase activity. In support of this hypothesis, muscle strips from subjects with type 2 diabetes (20) and insulin-resistant obese subjects (21) were found to have blunted insulin-stimulated PI 3-kinase activity and reduced insulin-induced insulin receptor and IRS-1 tyrosine phosphorylation, suggesting a defect in the signaling pathway upstream of PI 3-kinase. Recently, Anai et al. (22) reported decreased insulin-stimulated PI 3-kinase activity in the Zucker fatty rat, a model of early-stage type 2 diabetes. Although IRS-1 and IRS-2 phosphorylation were also seen to be reduced, the reduction was comparatively mild, suggesting a direct effect of FFAs on the PI 3-kinase protein, independent of IRS-1 or IRS-2. In contrast to these previous studies, the present study was able to demonstrate these abnormalities in the insulin signaling cascade in response to an acute increase in plasma FFA concentrations consistent with the hypothesis that these abnormalities play a primary role in the pathogenesis of insulin resistance, as opposed to being secondary to chronic hyperinsulinemia or hyperglycemia.

In a recent study, Chalkley et al. (18) reported an increase in muscle triglyceride and long-chain acyl CoA (LCAC) content following a 5-h lipid infusion during a hyperinsulinemic-euglycemic clamp. Such an increase in LCAC could lead to increases in levels of diacyl glycerol (DAG), a known potent activator of PKC. High fat feeding has been shown to both increase LCAC content in muscle and alter the PKC isoen-
zymes θ and ε activity (17). In this study, we found that PKC θ activation, represented either as total membrane PKC θ levels or as percentage membrane bound PKC θ, increased significantly with lipid infusion versus control infusion with glycercer. This raises the attractive hypothesis that increases in plasma FFA concentrations lead to an increase in muscle DAG levels, activating PKC θ, a serine kinase that causes increased serine phosphorylation of IRS-1. Such serine phosphorylation would reduce the ability of IRS-1 to activate PI 3-kinase.

In conclusion, this work implicates alterations in insulin signaling proteins, namely reduced IRS-1 tyrosine phosphorylation and reduced IRS-1-associated PI 3-kinase activity, in the insulin resistance acquired in the presence of acute (>5 h), and possibly chronic, elevations in plasma FFAs. Such alterations may be a consequence of PKC θ activation. This mechanism may play an important role in causing the insulin resistance associated with obesity and type 2 diabetes.

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