Mechanism by Which Fatty Acids Inhibit Insulin Activation of Insulin Receptor Substrate-1 (IRS-1)-associated Phosphatidylinositol 3-Kinase Activity in Muscle*

Recent studies have demonstrated that fatty acids induce insulin resistance in skeletal muscle by blocking insulin activation of insulin receptor substrate-1 (IRS-1)-associated phosphatidylinositol 3-kinase (PI3-kinase). To examine the mechanism by which fatty acids mediate this effect, rats were infused with either a lipid emulsion (consisting mostly of 18:2 fatty acids) or glycerol. Intracellular C18:2 CoA increased in a time-dependent fashion, reaching an 6-fold elevation by 5 h, whereas there was no change in the concentration of any other fatty acyl-CoAs. Diacylglycerol (DAG) also increased transiently after 3–4 h of lipid infusion. In contrast there was no increase in intracellular ceramide or triglyceride concentrations during the lipid infusion. Increases in intracellular C18:2 CoA and DAG concentrations were associated with protein kinase C (PKC)-θ activation and a reduction in both insulin-stimulated IRS-1 tyrosine phosphorylation and IRS-1-associated PI3-kinase activity, which were associated with an increase in IRS-1 Ser307 phosphorylation. These data support the hypothesis that an increase in plasma fatty acid concentration results in an increase in intracellular fatty acyl-CoA and DAG concentrations, which results in activation of PKC-θ leading to increased IRS-1 Ser307 phosphorylation. This in turn leads to decreased IRS-1 tyrosine phosphorylation and decreased activation of IRS-1-associated PI3-kinase activity resulting in decreased insulin-stimulated glucose transport activity.

Insulin resistance in skeletal muscle is a major factor in the pathogenesis of type 2 diabetes. Recent studies in animals and humans have demonstrated a strong relationship with increased intramyocellular triglyceride content (1–4) and intramyocellular triglyceride content as assessed by 1H NMR (5–7). In addition, infusions of lipid emulsions with heparin to acutely raise plasma fatty acid concentrations have also been shown to cause profound insulin resistance in rat and human skeletal muscle within 4–6 h (8–11). The mechanism by which fatty acids induce insulin resistance in skeletal muscle remains controversial. Randle et al. (12, 13) first suggested that fatty acids might induce insulin resistance in skeletal muscle by inhibiting pyruvate dehydrogenase activity, resulting in an increase in intracellular citrate concentration, which would then result in inhibition of phosphofructokinase activity leading to an increase in intracellular glucose-6-phosphate; this in turn would inhibit hexokinase activity, resulting in decreased glucose uptake. More recent 31P/13C NMR studies in humans have revealed a very different mechanism of fatty acid-induced insulin resistance whereby an increase in plasma fatty acid concentration was shown to result in lower intramyocellular glucose-6-phosphate (9, 14) and glucose concentrations (10), suggesting that fatty acids inhibit insulin-stimulated glucose transport activity (10). These changes were associated with reduced insulin-stimulated IRS-1 tyrosine phosphorylation (11) and IRS-1-associated phosphatidylinositol 3-kinase (PI3-kinase) activity (10, 11) suggesting that fatty acids cause insulin resistance through inhibition of insulin signaling, which we hypothesized might occur through activation of a serine kinase cascade involving PKC-θ (11). To explore the possible roles of different intracellular fatty acid metabolites such as fatty acyl-CoA, diacylglycerol (DAG), ceramides, and triglycerides in mediating fatty acid-induced insulin resistance in skeletal muscle, we measured these metabolites at different time intervals during a lipid infusion in relation to insulin stimulation: (i) insulin receptor tyrosine phosphorylation, (ii) IRS-1 tyrosine phosphorylation, and (iii) IRS-1-associated PI3-kinase activity as well as PKC-θ translocation. In a separate group of in vitro soleus muscle studies, we also examined whether fatty acid-induced defects in insulin signaling were coupled to defects in insulin-stimulated glucose uptake across a range of insulin concentrations.

**EXPERIMENTAL PROCEDURES**

**Materials**—LCACoA standards (C16:1, C16:0, C17:0, C18:2, C18:1, and C18:0), diacylglyceride standards, and ceramide standards (C6:0, 1 The abbreviations used are: IRS-1, insulin receptor substrate-1; IR, insulin receptor; IRS-1, phosphatidylinositol 3-kinase; PKC, protein kinase C; DAG, diacylglycerol; LCACoA, long-chain acyl-CoA; LC/MSMS, liquid chromatography tandem mass spectrometry; MOPS, 4-morpholinepropanesulfonic acid; TNFα, tumor necrosis factor α.
C16:0, C18:0) were purchased from Sigma. N-Arachidonyl-α-phosphoglycerol and N-lignoceryl-β-phosphoglycerol were purchased from Avanti Polar Lipids (Arlington, AL). Antibody against IRS-1 was purchased from Upstate Biotechnology (Lake Placid, NY). Antibody against the insulin receptor subunit and Zymed phosphotyrosine and rabbit anti-peptide against nPKC-δ were from Santa Cruz Biotechnology (Santa Cruz, CA). Goat anti-mouse IgG antibodies conjugated to horseradish peroxidase were obtained from Coltag Laboratories (Burlington, Canada). A monoclonal antibody against PKC-δ was from Transduction Laboratories (Lexington, KY).

Animals—Male Wistar rats (Charles River, Wilmington, MA) weighing between 250 and 300 g (for the time course study) and 50 and 75 g (for insulin dose response) were housed in an environmentally controlled room with a 2:2 light/dark cycle and fed with regular rat chow diet. The rats were catheterized in the right jugular vein and carotid artery; the catheters were externalized through an incision in the skin flap behind their head. The rats were allowed to recover from surgery until they reached preoperative weight (~5–7 days) and were fasted overnight (~15 h) before the infusion experiment. All procedures were approved by the Yale University Animal Care and Use Committee.

Intralipid Time Course Studies—The rats were divided randomly into five study groups (6–8 rats/group). The control group was infused with 20% triglyceride emulsion (Liposyn II, Abbott Laboratories, North Chicago, IL) (5 mL/kg/h) combined with heparin (6 units/h) for 1, 3, and 5 h. A second wash-out group was infused with lipids/heparin for 5 h, which was then discontinued and followed with an intravenous saline infusion for another 3 h. Identical studies were performed for muscle DAG analysis (3–9 rats/group) with the addition of a 4-h lipid/heparin infusion group (n = 4). At the end of the infusions, rats were anesthetized with pentobarbital (50 mg/kg); soleus muscle samples, rapidly dissected and freeze-clamped in situ, were stored at −70 °C for measurement of fat metabolites. Soleus muscle was selected for all studies because it consists of mostly type 1 fiber, which is highly insulin-responsive and best reflects insulin action in human skeletal muscle (10, 11). To study the effect of fatty acids on insulin signaling in muscle at the same time points, we performed another set of identical parallel studies in five animals (85:15, v/v) into a second set of collection tubes. The solvent was monitored for chemical ionization source was used. Peripherals included a PerkinElmer series 200 micro-pump and an autosampler. LCACoA were ionized in negative electrospray mode. Doubly charged ions and corresponding product ions were chosen as transition pairs for each CoA species (C16:1, C16:0, C18:2, C18:1, and C18:0) for selective reactions monitoring (SRM) quantitation. Total LCACoA concentrations were obtained from the sum of individual species. Methanol/H2O (60/40) was used as continuous flow at 300 μL/min, and 5 μL of sample was injected for analysis. DAGs (derived from C16:1, C16:0, C18:2, C18:1, and C18:0) and ceramides (C16:0, C18:0, C20:0, C22:0, C24:1, C24:0) were ionized in positive atmospheric pressure chemical ionization mode. [M+H+] product ions from corresponding fatty acid moiety were monitored for LC/MS/MS analysis. Total LCACoAs contents were obtained from the sum of individual species. Methanol/H2O (60/40) was used as continuous flow at 300 μL/min, and 5 μL of sample was injected for analysis. DAGs (derived from C16:1, C16:0, C18:2, C18:1, and C18:0) and ceramides (C16:0, C18:0, C20:0, C22:0, C24:1, C24:0) were ionized in positive atmospheric pressure chemical ionization mode. [M+H+] product ions from corresponding fatty acid moiety were monitored for SRM quantitation for DAGs. [M+H+] were monitored for ceramide species for quantitation. The same mobile phase was used for LCACoAs at 300 μL/min with 3 μL of sample injected.

In Vitro Muscle Studies—After a 5-h infusion with glycerol (as control) or lipid/heparin at 85 μL/kg/min, rats were anesthetized with an intravenous injection of sodium pentobarbital (50 mg/kg). Soleus muscles were isolated from the rats and preincubated in oxygenated (95% O2, 5% CO2) Krebs-Henseleit bicarbonate (KHB) buffer containing 2 mM pyruvate, 36 mM mannitol, and 0.1% bovine serum albumin (pre-incubation buffer) to recover for 30 min at 18 °C. The soleus muscles were then incubated at 29 °C in oxygenated preincubation buffer with various concentrations of insulin (0, 50, 1,000, or 10,000 micromolars/mL) for 35 min. After incubation, the muscles were rinsed with ice-cold saline and freeze-clamped in liquid nitrogen for analysis of insulin-stimulated IRS-1 tyrosine phosphorylation and insulin-stimulated IRS-1-associated PI3-kinase activity. To measure the insulin-stimulated

FIG. 1. Time course for plasma fatty acid and intracellular fat metabolite concentrations in soleus muscles during lipid infusion. A, plasma fatty acid concentrations; B, LCACoA concentrations; C, diacylglyceride concentrations; D, ceramide concentrations; E, triglyceride concentrations. Values are means ± S.E. for 6–10 experiments. *p < 0.05 versus control groups; †, p < 0.006, and **, p < 0.001 versus base line.
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Fig. 2. Time course for the concentration profiles of LCACoAs and DAG in soleus muscles during the lipid infusion. A, individual LCACoAs species were quantitated: C16:1, palmitoleoyl-CoA; C16:0, palmitoyl-CoA; C18:2, linoleoyl-CoA; C18:1, oleoyl-CoA; and C18:0, stearoyl-CoA. Values are means ± S.E. for 6–10 experiments. *, p < 0.05 versus control group; **, p < 0.001 versus control group. B, DAG species were abbreviated as two contributing fatty acyl groups. S, stearyl; O, oleyl; L, linoleyl; P, palmitoyl; Po, palmitoleoyl. Values are means ± S.E. for 3–9 experiments. *, p < 0.05 versus control group.

glucose uptake in the muscle, soleus muscles were preincubated at 29 °C with various concentrations of insulin (0, 50, 1,000, or 10,000 microunits/ml) for 35 min followed by incubation in KHB buffer containing 1 mM [3H]2-deoxyglucose and 39 m M [1-14C]mannitol for an 5-h lipid infusion, soleus muscles were freeze-clamped in situ and kept in liquid nitrogen until analysis.

Insulin Signaling Assays—Muscle samples were ground under liquid nitrogen and homogenized in a ice-cold Heps buffer, pH 7.4, containing 1 mM NaCl and 1 mM EDTA. 100 µM Na3VO4 was included in all the wash buffers. Kinase reactions were done as described previously (11). 32P was captured with a storage phosphor-screen, and the screen was scanned with a Storm system. Images were analyzed and quantified using ImageQuant software.

PKC-θ Translocation Assay—100 mg of soleus muscle was homogenized and extracted in 4× (w/v) ice cold 20 mM MOPS, pH 7.5, 250 mM mannitol, 1.2 mM EGTA, 1 mM dithiothreitol, 2 mM phenylmethylsulfonyl fluoride, 1% Triton X-100, 10% glycerol, and 10 µg/ml aprotinin. The homogenates were centrifuged at 20,500 × g for 1 h. Supernatants were collected, and protein concentration was measured with the Bradford protein assay reagent (Bio-Rad). Muscle homogenates (4 mg protein) were immunoprecipitated with 4 µg of anti-IRS-1 antibody for 18 h for IRS-1 tyrosine phosphorylation and PI3-kinase Activity Assay. The immunoprecipitates were washed three times by brief centrifugation and gentle resuspension in ice-cold homogenization buffer plus 0.1% SDS. Immunoprecipitates were subjected to SDS-PAGE on a 4–12% gradient gel. Proteins were transferred to nitrocellulose membrane using a semidry electro-blotter (Owl Separation System, Portsmouth, NH). The membranes were immunoblotted with anti-phosphotyrosine antibody, and bands were visualized using enhanced chemiluminescence (Amersham Biosciences) and quantified by densitometry (Amersham Biosciences). The membrane was stripped with 100 mM glycine, pH 3.0, and rebotted with anti-IRS-1 antibody to determine the amount of IRS-1 proteins.

IRS-1 serine phosphorylation was measured using a site-specific antibody, phospho-Ser307, generated in Dr. Morris White’s laboratory (17). Immunoprecipitation and Western blotting procedures were the same as for IRS-1 tyrosine phosphorylation.

PI3-kinase Activity Assay—The immunoprecipitates were washed twice with phosphate-buffered saline, twice with 100 mM Tris, pH 7.5, containing 500 mM LiCl and twice with 10 mM Tris containing 150 mM NaCl and 1 mM EDTA. 100 µM Na3VO4 was included in all the wash buffers. Kinase reactions were done as described previously (11). 32P was captured with a storage phosphor-screen, and the screen was scanned with a Storm system. Images were analyzed and quantified using ImageQuant software.
each fraction were calculated according to the total amount of protein in the final volume of supernatant extracted.

Analytical Procedures—Plasma fatty acid concentration was determined with an acyl-CoA oxidase-based colorimetric kit (Wako NEFA-C, Wako Pure Chemicals, Osaka, Japan). Tissue triglycerides were extracted by adapting the method described by Storlien et al. (19), and triglyceride content was measured using a kit from Sigma.

Statistical Analysis—Data were expressed as means ± S.E. Analysis of data using analysis-of-variance with one-way post-hoc tests (Fisher’s protected least significant difference) was done to determine the differences between control and different time courses of lipid infusion groups at a minimum p < 0.05 threshold.

RESULTS

Basal plasma fatty concentration increased rapidly following the lipid/heparin infusion and remained constant until the saline wash-out period during which time it returned to baseline concentration (Fig. 1A). This increase in plasma fatty acid concentration in the lipid-infused group resulted in increases in both intramuscular LCACoAs and DAG concentration in the soleus muscle compared with the control group (Fig. 1, B and C). Although the LCACoA continued to increase throughout the lipid infusion, the DAGs reached a peak concentration at 3–4 h and then surprisingly decreased to basal concentrations despite continued lipid infusion (Fig. 1C). In contrast, lipid infusion had no effect on intramyocellular ceramide content (Fig. 1D) or muscle triglyceride (Fig. 1E) content except at the 1-h time point, at which time the concentration decreased compared with baseline. The increase in total LCACoA concentration could be accounted for entirely by a selective increase in C18:2 CoA (major fatty acid composition in liposyn II) (3.86 ± 0.46 nmol/g of weight for control group, 9.30* ± 0.87, 16.17** ± 2.37, and 18.89** ± 2.51 nmol/g of weight after a 1-h, 3-h, and 5-h lipid infusion and 7.22 ± 1.22 nmol/g of weight after wash-out period; *, p < 0.05 versus control; **, p < 0.001 versus control; Fig. 2A). In contrast the transient ~3–4-fold increase in total DAG content at 3–4 h (0.65 ± 0.14 μmol/g of weight for control group, 1.43 ± 0.51, 2.73 ± 0.83*, 2.54 ± 0.79*, 1.36 ± 0.40, 0.96 ± 0.51 μmol/g of weight for 1-h, 3-h, 4-h, 5-h and wash-out groups, respectively; *, p ≤ 0.006 versus control) could be attributed to an increase in virtually all DAG species (Fig. 2B). These increases in intracellular LCACoA and DAG concentrations were associated with PKC-θ activation, as reflected by a significant reduction in the fraction of PKC-θ in the cytosol and a significant increase in the PKC-θ membrane-associated/cytosol fraction after 5 h of lipid infusion (both p = 0.04 versus control group; Fig. 3). There was also a reduction in total PKC-θ content, which is consistent with previous observations in a high-fat fed rat model that had increased intramuscular lipid accumulation (20).

The increase in intracellular fatty acyl-CoA and PKC-θ activation were also associated with a significant impairment in insulin-stimulated IRS-1 tyrosine phosphorylation and IRS-1-associated PI3-kinase activity after 5 h of lipid infusion (Fig. 4). These changes were associated with a 1.6-fold increase (p = 0.002 versus control) in IRS-1 Ser307 phosphorylation following 5 h of lipid infusion (Fig. 4). In contrast lipid infusion did not inhibit insulin-stimulated insulin receptor tyrosine phosphorylation (Fig. 4).

Following the 3-h lipid wash-out period, intracellular 18:2 acyl-CoA returned to base-line concentrations, and PKC-θ activity returned to normal (Figs. 1 and 3). In parallel with these results insulin-stimulated IRS-1 tyrosine phosphorylation and IRS-1-associated PI3-kinase activity also returned to normal.

To determine whether higher concentrations of insulin could overcome these lipid-induced defects in insulin signaling and action, we also examined insulin-stimulated muscle glucose uptake and insulin signaling across a wide range of insulin concentrations (50, 1,000, and 10,000 mIU/ml) in an \textit{in vitro} soleus muscle preparation following 5 h of either lipid or glycerol infusion. Consistent with our previous results, 5 h of lipid infusion induced a profound defect in insulin-stimulated glucose uptake, which occurred across all insulin concentrations (Fig. 5). This reduction in insulin-stimulated glucose uptake was paralleled by similar reductions in insulin-stimulated IRS-1 tyrosine phosphorylation and IRS-1-associated PI3-kinase activity across all insulin concentrations, but there was no change in insulin receptor tyrosine phosphorylation (Fig. 7). Taken together these results demonstrate that fatty acids induce a defect in insulin activation of PI3-kinase at the level of IRS-1 tyrosine phosphorylation that cannot be overcome with supraphysiologic concentrations of insulin.

DISCUSSION

To examine the possible roles of fatty acyl-CoA, diacylglycerol, ceramides, and triglycerides in mediating fatty acid-induced insulin resistance in skeletal muscle, we assessed the intracellular concentration of these metabolites at different
time intervals during a lipid infusion in awake rats. The changes in these fatty acid metabolite concentrations were then compared with changes in insulin-stimulated IRS-1 tyrosine phosphorylation, IRS-1 tyrosine phosphorylation, and insulin-stimulated IRS-1-associated PI3-kinase activity are all expressed as fold increase of insulin stimulation over basal states. Values are means ± S.E. for 5–8 experiments. *, p < 0.05 versus control groups.

We found that during the lipid infusion intracellular C18:2 CoA concentration increased by 6-fold and that it was the only intracellular fatty acyl-CoA to increase. Because the infused intralipid consisted mostly of C18:2 fatty acids, these data strongly suggest that this intracellular fatty acyl-CoA was derived from the infused lipid. Following the increase in intracellular C18:2 CoA, there was a 3-fold increase in intracellular DAG, which peaked at 3–4 h and then surprisingly declined despite persistent elevation in plasma fatty acid concentrations. In contrast to the increases in intracellular fatty acyl-CoA and DAG, there were no significant increases in intracellular ceramides or triglyceride concentrations during the 5-h lipid infusion, which suggests that these metabolites do not play a major role in mediating fatty acid-induced insulin resistance in skeletal muscle.

In parallel with the increases in intracellular fatty acyl-CoA, we observed a ~30% reduction in insulin activation of IRS-1 tyrosine phosphorylation and an ~50% reduction in IRS-1-associated PI3-kinase activity after 5 h of lipid infusion, which coincided with activation of PKC-θ. These data might explain the observed decrease in intramuscular triglyceride content during the first couple of hours of the lipid infusion. In contrast to the increases in intracellular fatty acyl-CoA and DAG, there were no significant increases in intracellular ceramides or triglyceride concentrations during the 5-h lipid infusion, which suggests that these metabolites do not play a major role in mediating fatty acid-induced insulin resistance in skeletal muscle.

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Fig. 4. Time course for the effects of fatty acids on insulin signaling in soleus muscle in vivo. Insulin-stimulated IR tyrosine phosphorylation, insulin-stimulated IRS-1 tyrosine phosphorylation, and insulin-stimulated IRS-1-associated PI3-kinase activity are all expressed as fold increase of insulin stimulation over basal states. Values are means ± S.E. for 5–8 experiments. *, p < 0.05 versus control groups.

Fig. 5. Effects of fatty acids on IRS-1 Ser307 phosphorylation in soleus muscle in vivo. A, IRS-1 Ser307 phosphorylation was detected with a polyclonal antibody raised specifically for phosphorylated Ser307 (upper panel). Nitrocellulose membranes were stripped and reprobed with IRS-1 antibody to ensure equal amount of protein loading (lower panel). IP, immunoprecipitate; INS, insulin; Gly, glycerol; WB, Western blot. B, degree of IRS-1 Ser307 phosphorylation in glycerol- and lipid-infused groups. Values are means ± S.E. from six rats for each group. *, p < 0.05 versus glycerol-infused rats.

Fig. 6. Insulin dose response for the effects of fatty acids on insulin-stimulated 2-deoxyglucose uptake in soleus muscle in vitro. Soleus muscles were isolated from glycerol- or lipid-infused rats. They were then incubated with insulin at 0, 50, 1000, and 10,000 microunits/ml. The rate of 2-deoxyglucose uptake was measured and expressed as fold change over non-insulin-stimulated groups. Values are means ± S.E. from 6–9 experiments. *, p < 0.05 versus glycerol-infused rats.
In contrast, the lipid infusion had no effect on insulin receptor tyrosine phosphorylation. Overall these data demonstrate that increases in plasma fatty acid concentration inhibit insulin activation of IRS-1-associated PI3-kinase at the level of IRS-1, possibly through activation of PKC-θ, a known serine kinase. To gain further insights into this mechanism we assessed IRS-1 Ser307 phosphorylation. Previous in vitro studies by Aguirre et al. (17) demonstrated that IRS-1 Ser307 phosphorylation is a critical site in mediating TNFα-induced insulin resistance in Chinese hamster ovary cells. When IRS-1 Ser307 was mutated to IRS-1 Ala307, these cells were protected from TNFα-induced insulin resistance. Indeed, in the present study we found that after 5 h of lipid infusion there was a 1.6-fold increase in IRS-1 Ser307 phosphorylation in soleus muscle, which suggests that fatty acids may mediate insulin resistance through the same common final pathway as TNFα (21).

To determine whether higher concentrations of insulin could overcome these fatty acid-induced defects in insulin signaling and action, we also examined these parameters in vitro, across a wide range of insulin concentrations, in soleus muscles obtained from rats following 5 h of either lipid or glycerol infusion. Consistent with our current and previous in vivo results, 5 h of lipid infusion induced a profound defect in insulin-stimulated glucose uptake (9–11), which occurred across all insulin concentrations. This reduction in insulin-stimulated glucose uptake was paralleled by similar reductions in insulin-stimulated IRS-1 tyrosine phosphorylation and IRS-1-associated PI3-kinase activity, but there was no change in insulin-stimulated IR tyrosine phosphorylation. Taken together these results demonstrate that the fatty acid-induced inhibition of insulin-stimulated glucose transport activity in muscle can be explained for the most part.
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by decreased activation of PI3-kinase at the level of IRS-1 tyrosine phosphorylation, which cannot be overcome with supraphysiologic concentrations of insulin.

In conclusion, these data provide new insights into the pathogenesis of fat-induced insulin resistance in skeletal muscle and support the hypothesis that an increase in plasma fatty acid concentration results in an increase in intracellular fatty acyl-CoA and DAG concentrations, which then results in activation of PKC-θ leading to increased IRS-1 Ser307 phosphorylation. These changes in turn result in decreased IRS-1 tyrosine phosphorylation and decreased activation of IRS-1-associated PI3-kinase, resulting in decreased insulin-stimulated glucose transport activity.

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