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 concentration 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 intramuscular 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 11C/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, 4-morpholinepropanesulfonic acid; TNFα, tumor necrosis factor α.

* This work was supported by Grants R01 DK-40936 and P30 DK-45735 from the National Institutes of Health and a Center grant to the Garvan Institute from the National Health and Medical Research Council of Australia. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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50230 This paper is available online at http://www.jbc.org
C16:0, C18:0) were purchased from Sigma. N-Arachidonyl-1-phosphatidylcholine and N-lignoceroyl-1-phosphatidylcholine 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 Caltag Laboratories (Burlingame, CA). A monocolonal antibody against PCK-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 12-h 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 isotonic saline solution for 5 h. The other groups were infused with a 20% triglycerides emulsion (Liposyn II, Abbott Laboratories, North Chicago, IL) (5 ml/kg/h) combined with heparin (6 units/h) for 1, 3 and 5 h. The wash-out group was infused with saline/heparin for 5 h, which was then discontinued and followed with an isotonic 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 I 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 groups (4 rats/group) with the addition of a 4-h lipid/heparin infusion and 3-h wash-out under conditions identical to those described above, adding a 20-min hyperinsulinemic euglycemic clamp following the lipid/heparin or saline infusion. In these studies an intravenous bolus (150 milliunits/kg for 45 s, 75 milliunits/kg for another 45 s) of insulin (humulin regular insulin, Eli Lilly, Indianapolis, IN) was followed by a constant insulin infusion at 10 milliunits/kg/min, with plasma glucose concentration clamped at 5.5 mM using a variable infusion of glucose (50 g/dl) to maintain euglycemia as described previously (11). At the end of the clamps, rats were anesthetized with pentobarbital (50 mg/kg); soleus muscle samples, rapidly dissected, freeze-clamped in situ, and stored at −70 °C for insulin signaling assays. Rats were euthanized with a lethal dose of pentobarbital.

Extraction of LCACoAs, DAGs, and Ceramides from Tissue Samples—LCACoAs were extracted from frozen tissue samples (~100 mg) and purified using a solid phase extraction method described previously by Deutsch et al. (15) with minor modifications for desalting. A known amount of heptadecanoyl-CoA was added as an internal standard. OPC columns (Applied Biosystems, Foster City, CA) were used for solid phase extraction. Samples were dissolved in 100 µl of methanol/H2O for LC/MS/MS analysis.

DAGs and ceramides were extracted from frozen tissue (~100 mg) with chloroform/methanol (2:1, v/v) containing 0.01% butylated hydroxytoluene. Prior to the extraction, known amounts of 1,3-dipentadecanoyl-2-hexadecanoyl-sn-glycerol and hexadecanoylsphingosine were added as internal standards. Extracted samples were evaporated to dryness and redisolved in 1 ml of hexane-methylene chloride-ethanol ether (95:5:0.5, v/v/v). DAGs were isolated from triglycerides by use of a diol bonded-phase SPE column (Waters, Inc., Milford, MA) under vacuum, as described previously (16). Briefly, the SPE column was preconditioned with 4 ml of hexane. The lipid extract was placed on the column, and triglycerides were eluted with 8 ml of hexane-methylene chloride-ethyl ether (89:10:1, v/v/v). DAGs were eluted with 8 ml of hexane-ethyl acetate (85:15, v/v) into a second set of collection tubes. The solvent was evaporated to dryness under vacuum and redisolved in 0.5 ml of hexane-ethyl acetate (85:15, v/v) for LC/MS/MS analysis. Monitoring for the presence of triheptadecanoin in the DAG fraction assessed the separation of triglycerides from DAGs.

LC/MS/MS Analysis of LCACoAs, DAGs, and Ceramides—A bench-top tandem mass spectrometer, API 3000 (PerkinElmer Life Sciences), interfaced with a Turbolonspray ionization source or atmospheric pressure chemical ionization source was used. Peripherals included a PerkinElmer series 200 micro-pump and an autosampler. LCACoAs were ionized in negative electrospray mode. Doubly charged ions and corresponding product ions were chosen as transition pairs for desalting. A known amount of heptadecanoyl-CoA was added as an internal standard. OPC columns (Applied Biosystems, Foster City, CA) were used for solid phase extraction. Samples were dissolved in 100 µl of methanol/H2O for LC/MS/MS analysis.

For DAG analysis, standard solutions of DAGs were created in methanol containing 0.01% butylated hydroxytoluene. The standards were purified using a solid phase extraction column and redisolved in chloroform/methanol (2:1, v/v) containing 0.01% butylated hydroxytoluene. Known amounts of internal standards (C16:0, C16:1, C18:0, C20:0, C22:0, C24:1, C24:0) were added to the standards. The purified solutions were evaporated to dryness and redisolved in chloroform/methanol (2:1, v/v) containing 0.01% butylated hydroxytoluene. Known amounts of internal standards (C16:0, C16:1, C18:0, C20:0, C22:0, C24:1, C24:0) were added to the standards and the solutions were evaporated to dryness. Known amounts of internal standards were added to the standards and the solutions were evaporated to dryness and redisolved in chloroform/methanol (2:1, v/v) containing 0.01% butylated hydroxytoluene. Known amounts of internal standards (C16:0, C16:1, C18:0, C20:0, C22:0, C24:1, C24:0) were added to the standards and the solutions were evaporated to dryness. Known amounts of internal standards were added to the standards and the solutions were evaporated to dryness and redisolved in chloroform/methanol (2:1, v/v) containing 0.01% butylated hydroxytoluene. Known amounts of internal standards (C16:0, C16:1, C18:0, C20:0, C22:0, C24:1, C24:0) were added to the standards and the solutions were evaporated to dryness. Known amounts of internal standards were added to the standards and the solutions were evaporated to dryness and redisolved in chloroform/methanol (2:1, v/v) containing 0.01% butylated hydroxytoluene.

**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.
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 mm [1-14C]mannitol for an 

**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, stearoyl; O, oleoyl; L, linoleoyl; P, palmitol; Po, palmitoleoyl. Values are means ± S.E. for 3–9 experiments. *, p < 0.05 versus control group.

**Insulin Signaling Assays—**Muscle samples were ground under liquid nitrogen and homogenized in an ice-cold Hepes buffer, pH 7.4, containing 150 mm NaCl 50 mm β-glycerophosphate, 2 mm dithiothreitol, 1 mm NaVO₄, 2 mm EDTA, 1 mm phenylmethylsulfonyl fluoride, 1% Triton, 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 (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 or with 4 μg of anti-IR antibody for IR tyrosine phosphorylation.

**IR and IRS-1 Tyrosine Phosphorylation Assays—**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 semidy 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 reblotted 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-Ser⁹⁶⁷, 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 NaVO₄ 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, leupeptin (200 μg/ml), and 2 mM benzamidine. The homogenate was solubilized by hand for 2 min and centrifuged at 4 °C for 10 min at 100,000 × g. Separation of cytosol and membrane fraction was done as described previously (18). 5 (cytosolic) or 10 μg (particulate) of protein was loaded on SDS-PAGE (10% gel, 187 V). Proteins separated on the gels were electrophotorectically transferred to polyvinylidene difluoride filter membranes (Amersham Biosciences) in 19 mm Tris, pH 8.9, buffer containing 140 mm glycine at 90 V for 90 min. Polyvinylidene difluoride membranes were probed with 0.625 μg/ml anti-PKC-θ antibody (Transduction Laboratories) for 2 h. PKC isozymes visualized by enhanced chemiluminescence reagents and quantitated by densitometry using a Medical Dynamics Personal Densitometer SI and IP Lab Gel H software (Signal Analytics, Vienna, VA). Individual band densities were adjusted for inter-gel variability using the standard, and the amount of PKC-θ in
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 acid 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) and then surprisingly decreased to basal concentrations during the saline wash-out period (Fig. 1C).

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To determine whether higher concentrations of insulin could overcome these lipid-induced defects in insulin signaling and overcome these lipid-induced defects in insulin signaling and specifically activate PKC-ζ,

FIG. 3. Time course for the effects of fatty acids on PKC-ζ activity in soleus muscle in vivo. PKC-ζ protein levels were determined in the cytosolic and membrane fraction by immunoblotting with PKC-ζ specific antibodies. Total PKC-ζ levels were calculated from the sum of cytosolic and membrane-associated amounts, and PKC-ζ distribution was expressed as the ratio of membrane-associated to cytosolic amounts. W/O, without. Values are means ± S.E. for 6–10 experiments. *, p < 0.05 versus control groups.

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. 6). 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

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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 insulin receptor tyrosine phosphorylation, IRS-1 tyrosine phosphorylation, IRS-1-associated PI3-kinase activity, and PKC-θ translocation. We found that during the lipid infusion intramyocellular 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.
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 across all insulin concentrations, 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...
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.

Acknowledgments—We acknowledge the expert technical assistance of Hyegeong Kim, Lynn Croft, Anthony Romanelli, Taca Higashimori, and Theresa Choi.

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