Mechanism by which Fatty Acids Inhibit Insulin Activation of IRS-1 Associated Phosphatidylinositol 3-Kinase Activity in Muscle

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Running Title: Mechanism of Fatty Acid Induced Insulin Resistance

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Summary

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 (PI 3-kinase). In order 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 a ~6 fold elevation by 5 hours whereas there was no change in the concentration of any other fatty acyl CoAs. Diacylglycerol (DAG) also increased transiently after 3-4 hours 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 PKC-θ activation, a reduction in both insulin stimulated IRS-1 tyrosine phosphorylation and IRS-1 associated PI 3-kinase activity which were associated with an increase in IRS-1 Ser$^{307}$ 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 Ser$^{307}$ phosphorylation. This in turn leads to decreased IRS-1 tyrosine phosphorylation and decreased activation of IRS-1 associated PI 3-kinase activity resulting in decreased insulin stimulated glucose transport activity.
Introduction

Insulin resistance in skeletal muscle is a major factor in the pathogenesis of type 2 diabetes and recent studies in animals and humans have demonstrated a strong relationship with increased intramuscular triglycerides content (1-4) and intramyocellular triglyceride content, assessed by $^1$H 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 hours (8-11). The mechanism by which fatty acids induce insulin resistance in skeletal muscle remains controversial. Randle et al 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 result in inhibition of phosphofructokinase activity leading to an increase in intracellular glucose-6-phosphate which in turn would inhibit hexokinase activity resulting in decreased glucose uptake (12,13). More recent $^{31}$P/$^{13}$C NMR studies in humans have revealed a very different mechanism of fatty acid induced insulin resistance where an increase in plasma fatty acid concentration was shown to result in lower intramyocellular glucose-6-phosphate and glucose concentrations suggesting that fatty acids inhibited insulin stimulated glucose transport activity (9,10,14). These changes were associated with reduced insulin stimulated IRS-1 tyrosine phosphorylation (11) and IRS-1 associated phosphatidylinositol 3-kinase (PI 3-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). In order 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 stimulated: i) insulin receptor tyrosine phosphorylation, ii) IRS-1 tyrosine phosphorylation and iii) IRS-1 associated PI 3-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.
Research Design and Methods

Materials. LCACoAs standards (C16:1, C16:0, C17:0, C18:2, C18:1 and C18:0), diacylglycerides standards, ceramides standards (C6:0, C16:0, C18:0) were purchased from Sigma (St. Louis, MO, USA). N-Arachidoyl-D-Sphingosine, N-Lignoceroyl-D-Sphingosine was 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 were from Santa Cruz Biotechnology (Santa Cruz, CA) and rabbit anti-peptide against nPKC-θ was from Santa Cruz Biotechnology (Santa Cruz, CA). Goat anti-mouse IgG antibodies conjugated to horseradish peroxidase were obtained from Caltag Laboratories (Burlingame, CA). Mouse monoclonal antibody against PKC-θ was from Transduction Laboratories (Lexington, KY).

Animals. Male Wistar rats (Charles River, Wilmington, MA) weighing between 250-300g (for time course study) and 50-75g (for insulin dose response) were housed in environmentally controlled room with 12-hour 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 (approximately 5-7 days) and were fasted overnight (~15 hours) 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 5 study groups (6-8 rats/group). The control group was infused with isotonic saline solution for 5 hours. The other groups were infused with a 20% triglycerides emulsion (Liposyn II, Abbott Laboratories, North Chicago, IL) (5ml/kg/hr) combined with heparin (6U/hr) for 1, 3 and 5 hours. A fifth washout
group was infused with lipid/heparin for 5 hours, which was then discontinued and followed with an isotonic saline infusion for another 3 hours. Identical studies were performed for muscle DAG analysis (3-9 rats/group) with the addition of a 4-hour lipid/heparin infusion group (n=4). At the end of the infusions rats were anesthetized with pentobarbital (50mg/kg); soleus muscle samples were rapidly dissected and freeze-clamped in situ and stored at -70°C for measurement of fat metabolites. Soleus muscle was selected for all studies since it consists of mostly type 1 fibers, which is highly insulin responsive and best reflects insulin action in human skeletal muscle (10,11). In order 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 5 groups (basal, 1, 3, 5 hour lipid/heparin infusion and 3 hour washout) under identical conditions as described above adding a 20 min hyperinsulinemic euglycemic clamp following the lipid/heparin or saline infusion. In these studies an intravenous bolus (150mU/kg for 45 seconds, 75mU/kg for another 45 seconds) of insulin (Humulin regular insulin, Eli Lily, Indianapolis, IN) was followed by a constant insulin infusion at 10mU/kg/min, with plasma glucose concentration clamped at 5.5 mM using a variable infusion of glucose (50g/dl) to maintain euglycemia as previously described (11). At the end of the clamps, rats were anesthetized with pentobarbital (50mg/kg); soleus muscle samples were rapidly dissected and 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 previously described 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 100µl of methanol/H₂O for LC/MS/MS analysis.

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

**LC/MS/MS analysis of LCACoAs, DAGs and ceramides.** A bench top tandem mass spectrometer API 3000 (Perkin-Elmer Scien) interfaced with TurboIonspray ionization source or APCI (atmospheric pressure chemical ionization) source was used. Peripherals included Perkin-Elmer 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 each CoA species (C16:1, C16:0, C18:2, C18:1 and C18:0) for SRM (selective reactions monitoring) quantitation. Total LCACoAs contents were obtained from sum
of individual species. Methanol/H₂O (60/40) was used as continuous flow at 300µl/min, 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 APCI mode. [M+H-18]⁺/product ions from corresponding fatty acid moiety were monitored for SRM quantitation for DAGs. [M+H-18]⁺/264.3 were monitored for ceramide species for quantitation. The same mobile phase was used for LCACoAs at 300µl/min and 3µl of sample was injected.

**In vitro muscle studies.** After 5 hours infusion with glycerol (as control) or lipid/heparin at 85 µl/kg/min, rats were anesthetized with intravenous injection of sodium pentobarbital (50mg/kg). Soleus muscles were isolated from the rats and preincubated in oxygenated (95 % O₂/5 % CO₂) Krebs-Henseleit bicarbonated (KHB) buffer containing 2 mM pyruvate, 36 mM mannitol and 0.1 % BSA (preincubation buffer) to recover for 30 minutes at 18 °C. The soleus muscles were then incubated at 29 °C in oxygenated preincubation buffer with various concentration of insulin (0, 50, 1,000 or 10,000 µU/ml) for 35 minutes. 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 glucose uptake in the muscle, soleus muscles were pre-incubated at 29 °C with various concentrations of insulin (0, 50, 1,000 or 10,000 µU/ml) for 35 minutes followed by incubation in KHB buffer containing 1 mM [³H] 2-DG and 39mM [1-¹⁴C] mannitol for an additional 20 minutes. For IRS-1 serine phosphorylation analysis, after 5 hours 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 Hepes buffer, pH 7.4 containing 150 mM NaCl 50mM β-glycerol
phosphate, 2mM dithiothreitol, 1mM NaVO₄, 2mM EDTA, 1mM PMSF, 1% Triton-100, 10% glycerol, 10 µg/ml aprotinin. The homogenates were solubilized on a nutator for 30 minutes and centrifuged at 20,500 xg for 1 hour. Supernatants were collected and protein concentration was measured with Bradford protein assay reagent (Biorad). Muscle homogenates (4 mg protein) were immunoprecipitated with 4µg anti-IRS-1 antibody for 18 hours for IRS-1 tyrosine phosphorylation and PI 3 Kinase activity assay or 4µg 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 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 Pharmacia Biotech) and quantified by densitometry (Molecular Dynamics). 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.

**PI 3 kinase activity assay.** The immunoprecipitates were washed twice with PBS, twice with 100 mM Tris pH 7.5 containing 500 mM LiCl₂ 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 previously described (11). ³²P was captured with a storage phosphor
screen and the screen was scanned with a Storm system. Images were analyzed and quantified by Images Quant software.

**PKC-θ translocation assay.** 100mg soleus muscle was dismembranated and extracted in 4x (w/v) ice cold 20mM MOPS (pH 7.5), 250mM mannitol, 1.2mM EGTA, 1mM DTT, 2mM PMSF, leupeptin (200µg/ml), and 2mM benzamidine. The homogenate was solubilized by hand for 2mins and centrifuged at 4°C for 10 min at 100,000 x g. Separation of cytosol and membrane fraction was done as described before (18). 5µg (cytosolic) or 10µg (particulate) protein were loaded and subjected to SDS-PAGE (10% gel, 187V). Proteins separated on the gels were electrophoretically transferred to polyvinylidene difluoride (PVDF) filter membranes (Amersham Pharmacia Biotech, city, state) in 19mM Tris, pH 8.9 buffer containing 140mM Glycine at 90V for 90min. PVDF membranes were probed with 0.625µg/mL anti PKC-θ antibody (Transduction Laboratories) for 2h at room temperature, followed by horseradish peroxidase conjugated goat anti-mouse antibody (1:5,000) for 2h. 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 PKC0 in each fraction calculated according to the total amount of protein in the final volume of supernatant extracted.

**Analytical procedures.** Plasma fatty acids concentration was determined with an acyl-CoA oxidase based colorimetric kit (Wako NEFA-C, Wako Pure Chemicals Industries, Osaka, Japan). Tissue triglycerides extraction was adapted from methods described by Storlien et al (19) and TG content was measured using a kit (Sigma Diagnostics, St. Louis, MO).
**Statistical analysis.** Data were expressed as means ± SE. Analysis of data by ANOVA with one-way post hoc tests (Fisher’s protected least significant difference) was done to determine the differences of fat metabolites concentration 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 washout 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 to the control group (Fig 1 B, C). While the LCACoA continued to increase throughout the lipid infusion the DAGs reached a peak concentration at 3-4 hours 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 TG (Fig 1E) content except at the 1-hour time point, which decreased compared to baseline. The increase in total LCACoAs concentration could entirely be accounted for by a selective increase in C18:2 CoA (major FA composition in Liposyn II) (3.86 ± 0.46 nmol/g wt for control group, 9.30* ± 0.87, 16.17** ± 2.37 and 18.89** ± 2.51 nmol/g wt after 1-hr, 3-hr and 5-hr lipid infusion, 7.22 ± 1.22 nmol/g wt after washout period * P<0.05 vs control, ** P<0.001 vs control) (Fig 2A). In contrast the transient ~3-4 fold increase in total DAG content at 3-4 hours (0.65 ± 0.14 µmol/g wt for control group, 1.43 ± 0.51, 2.73±0.83+, 2.54±0.79+, 1.36±0.40, 0.96±0.31 µmol/g wt for 1-hr, 3-hr, 4-hr, 5-hr and washout groups respectively, +P≤0.006 vs control) could be attributed to an increase in virtually all DAG species (Fig 2B). These increases in intracellular LCACoAs and DAG concentration 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-hours of lipid infusion (both P=0.04 vs 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 PI 3-kinase activity after 5 hours of lipid infusion (Fig 4). These changes were associated with a 1.6 fold increase (P=0.002 vs Control) in IRS-1 Ser^{307} phosphorylation following 5 hours of lipid infusion (Fig 5). In contrast there was no effect of lipid infusion to inhibit insulin stimulated insulin receptor tyrosine phosphorylation (Fig 4).

Following the 3 hour lipid washout period intracellular 18:2 acyl CoA returned to baseline 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 PI 3-kinase activity also returned to normal.

To determine if higher concentrations of insulin could overcome this 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, 10,000 µU/ml) in an in vitro soleus muscle preparation following either 5 hours of lipid or glycerol infusion. Consistent with our previous results 5 hours 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 PI 3-kinase activity across all insulin concentrations but no change in insulin receptor tyrosine phosphorylation (Fig 7). Taken together these results demonstrates that fatty acids induce a defect in insulin activation
of PI 3-kinase at the level of IRS-1 tyrosine phosphorylation that cannot be overcome with supraphysiologic concentrations of insulin.
Discussion

In order 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 to changes in insulin stimulated insulin receptor tyrosine phosphorylation, IRS-1 tyrosine phosphorylation, IRS-1 associated PI 3-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. Since 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 hours and then surprisingly declined despite persistent elevation in plasma fatty acid concentrations. In contrast to the fatty acyl CoA, which consisted mostly of C18:2 fatty acids, the increase in DAG consisted of virtually all measured fatty acids. Taken together these data suggest that the increase in intracellular fatty acyl CoA activated a phospholipase that led to production of DAG from endogenous lipid sources which 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 five-hour 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 ~50% reduction in IRS-1
associated PI 3-kinase activity after 5 hours of lipid infusion that coincided with activation of PKC-θ. These data might explain the 3-5 hour delay for fatty acid induced insulin resistance in skeletal muscle resulting from an intralipid/heparin infusion (8,9). In contrast there was no effect of the lipid infusion on insulin receptor tyrosine phosphorylation. Overall these data demonstrate that increases in plasma fatty acid concentration inhibit insulin activation of IRS-1 associated PI 3-kinase at the level of IRS-1 possibly through activation of PKC-θ, a known serine kinase. In order to gain further insights into this mechanism we assessed IRS-1 Ser\(^{307}\) phosphorylation. Previous *in vitro* studies by Aguirre et al demonstrated that IRS-1 Ser\(^{307}\) phosphorylation was a critical site mediating TNFα induced insulin resistance in CHO cells (20). When IRS-1 Ser\(^{307}\) was mutated to IRS-1 Ala\(^{307}\) these cells were protected from TNFα induced insulin resistance. Indeed in the present study we found that after five hours of lipid infusion there was a 1.6 fold increase in IRS-1 Ser\(^{307}\) phosphorylation in soleus muscle, which suggests that fatty acids might mediate insulin resistance through the same common final pathway as TNFα (21).

To determine if 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 either five hours of lipid or glycerol infusion. Consistent with our current and previous *in vivo* results, five hours 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 PI 3-kinase activity across all insulin concentrations but no change in insulin stimulated insulin receptor tyrosine phosphorylation. Taken together these results demonstrate that the fatty acid induced inhibition of insulin stimulated glucose transport activity in muscle...
can mostly be explained by decreased activation of PI 3-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 results in activation of PKC-θ leading to increased IRS-1 Ser\(^{307}\) phosphorylation. These changes in turn result in decreased IRS-1 tyrosine phosphorylation and decreased activation of IRS-1 associated PI 3-kinase resulting in decreased insulin stimulated glucose transport activity.
Acknowledgements

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References


**Figure Legends**

**Figure 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 vs control groups, + P<0.006, ** P<0.001 vs baseline

**Figure 2.** Time course for the concentration profiles of LCACoAs and DAG in soleus muscles during the lipid infusion: (2A) Individual LCACoAs species was 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 vs control group, **P<0.001 vs control group. (2B) DAG species were abbreviated as two contributing fatty acyl groups; S stands for stearoyl, O stands for oleoyl, L stands for Linoleoyl, P stands for palmitoyl and Po stands for palmitoleoyl. Values are means ± S.E. for 3-9 experiments. *P<0.05 vs control group.

**Figure 3.** Time course for the effects of fatty acids on PKC-θ activity in soleus muscle in vivo. PKC-θ protein levels were determined in the cytosolic and membrane fraction by immunoblotting with PKC-θ specific antibodies. Total PKC-θ levels were calculated from the sum of cytosolic and membrane-associated amounts and PKC-θ distribution was expressed as the ratio of membrane-associated to cytosolic amounts. Values are means ± S.E. for 6-10 experiments. * P<0.05 vs control groups.

**Figure 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 PI 3-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 vs control groups.

Figure 5. Effects of fatty acids on IRS-1 Ser\textsuperscript{307} phosphorylation in soleus muscle \textit{in vivo}. 5A. IRS-1 Ser\textsuperscript{307} phosphorylation was detected with a polyclonal antibody raised specifically for phosphorylated serine\textsuperscript{307} (upper panel). Nitrocellulose membranes were stripped and reprobed with IRS-1 antibody to ensure equal amount of protein loading (lower panel). 5B. Degree of IRS-1 Ser\textsuperscript{307} phosphorylation in glycerol and lipid-infused groups. Values are means ± S.E. from 6 rats for each group. * P < 0.05 vs glycerol infused rats.

Figure 6. Insulin dose response for the effects of fatty acids on insulin stimulated 2-deoxyglucose uptake in soleus muscle \textit{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 µU/ml. The rate of 2-DG uptake was measured and expressed as fold changes over non-insulin stimulated groups. Values are means ± S.E. from 6-9 experiments. * P < 0.05 vs glycerol infused rats.

Figure 7. Insulin dose response for the effects of fatty acids on insulin signaling in soleus muscle \textit{in vitro}. 7A. IR tyrosine phosphorylation was detected with phosphotyrosine specific antibody (upper panel). Nitrocellulose membranes were stripped and reprobed with insulin receptor antibody to ensure equal amount of protein loading (lower panel). The bar graph shows the degree of IR tyrosine phosphorylation in glycerol or lipid infused soleus muscle in vitro. 7B. IRS-1 tyrosine phosphorylation was detected with phosphotyrosine specific antibody (upper
Nitrocellulose membranes were stripped and reprobed with IRS-1 antibody to ensure equal amount of protein loading (lower panel). The bar graph showed the degree of IRS-1 tyrosine phosphorylation in glycerol or liposyn infused soleus muscle in vitro. 7C. IRS-1 associated PI3 kinase activity was detected by measuring $^{32}\text{P}$ incorporation into phosphatidylinositol (PIP). The bar graph showed the IRS-1-associated PI 3-kinase activity in glycerol or liposyn infused soleus muscle in vitro. Values are means ± S.E. from 8 independent experiments. * P < 0.05 vs. glycerol infused rats.
Figure 1

Lipid infusion | Washout

A. Plasma fatty acid (mM)

B. C18:2 CoA (nmol/g wt)

C. Diacylglycerides (µmol/g wt)

D. Ceramides (nmol/g wt)

E. Triglycerides (µmol/g wt)

hour
Figure 2B

DAG profile

- Control
- 1 hr
- 3 hr
- 4 hr
- 5 hr
- Washout

μmol/g wt

SS OS LS OO OL LL SP PoS PO PoO PL PoL PP PoP PoPo
Figure 3

Cytosolic PKC-θ

Total PKC-θ

PKC-θ distribution

control 1 hr 3 hr 5 hr W/O
Figure 5

**IP: IRS-1**

- **INS**
  - -
  - +
- **Gly**
- **Lipid**
- **WB**
  - P-ser307
  - IRS-1

**Graph:**

- **Y-axis:** IRS-1 Serine Phosphorylation (arbitrary units)
- **X-axis:**
  - Glycerol
  - Lipid

*Indicates significant difference.
Figure 7A

IP: IR

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<th>Lipid</th>
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WB

P-tyr

IR

---

IR Tyrosine Phosphorylation, fold increase

- Glycerol
- Lipid

---

Insulin (µU/ml)

0 | 50 | $10^3$ | $10^4$
Figure 7B

**IP: IRS-1**

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<th>Lipid</th>
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<td>Insulin (µU/ml)</td>
<td>0  50 10^3 10^4</td>
<td>0  50 10^3 10^4</td>
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WB

P-tyr

IRS-1

![Graph showing IRS-1 Tyrosine Phosphorylation fold increase](http://www.jbc.org/)

- **Glycerol**
- **Lipid**

* indicates statistical significance.
Mechanism by which fatty acids inhibit insulin activation of IRS-1 associated phosphatidylinositol 3-kinase activity in muscle
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