Leptin, skeletal muscle lipids and lipid-induced insulin resistance

by

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ABSTRACT

Leptin-induced increases in insulin sensitivity are well established and may be related to the effects of leptin on lipid metabolism. However, the effects of leptin on the levels of lipid metabolites implicated in pathogenesis of insulin resistance and the effects of leptin on lipid-induced insulin resistance are unknown. The current study addressed in rats the effects of hyperleptinemia (HL) on insulin action and markers of skeletal muscle (SkM) lipid metabolism in the absence or presence of acute hyperlipidemia induced by an infusion of a lipid emulsion. Compared to controls (CONT), HL increased insulin sensitivity, as assessed by the hyperinsulinemic-euglycemic clamp (~15%), and increased SkM Akt (~30%) and GSK3α (~52%) phosphorylation. These improvements in insulin action were associated with decreased SkM triglycerides (TG, ~61%), elevated ceramides (~50%), and similar diacylglycerol (DAG) levels in HL compared to CONT. Acute hyperlipidemia in CONT decreased insulin sensitivity (~25%), and increased SkM DAG (~33%) and ceramide (~60%) levels. However, hyperlipidemia did not induce insulin resistance or SkM DAG and ceramide accumulation in HL. SkM total FAT/CD36, FABPpm, ACC phosphorylation and fatty acid oxidation were similar in HL compared to CONT. However, HL decreased SkM PKCθ, a kinase implicated in mediating the detrimental effects of lipids on insulin action. We conclude that increases in insulin sensitivity induced by HL are associated with decreased levels of SkM TG and PKCθ, and increased SkM insulin signaling, but not with decreases in other lipid metabolites implicated in altering SkM insulin sensitivity (DAG and ceramide). Furthermore, insulin resistance induced by an acute lipid infusion is prevented by HL.
INTRODUCTION

Leptin, the adipocyte derived hormone, has potent effects on lipid metabolism. Of these, leptin-induced stimulation of fatty acid oxidation and decreases in circulating and tissue levels of triglyceride are best described (11, 25, 26, 36, 37, 43, 49, 55). In addition to a role in the regulation of lipid metabolism, the beneficial effects of leptin on insulin sensitivity are well established. Thus, insulin resistance in leptin deficient states resulting from leptin gene mutations or lipodystrophy is markedly improved by leptin administration in humans (29, 52) and rodents (5, 21, 38, 50). A primary site of leptin action appears to be skeletal muscle, since leptin increases insulin-stimulated glucose uptake into this tissue (3, 11, 43, 54, 60, 61). Leptin-induced increases in insulin sensitivity are also associated with substantial improvements in the dyslipidemia associated with leptin deficient/resistant states (5, 11, 16, 29, 38, 43, 49, 52). Arising from these observations, it has been proposed that the beneficial effects of leptin on lipid metabolism may contribute to improvements in insulin sensitivity, a hypothesis supported by studies demonstrating detrimental effects of lipids on insulin action (2, 7, 12, 20, 22, 27, 31, 39, 41, 58, 62).

In humans and rodents there is a strong negative correlation between plasma and tissue triglyceride levels and insulin sensitivity (22, 31, 34, 51). Furthermore, acute infusions of lipid induce skeletal muscle insulin resistance in vivo (2, 6, 7, 12, 15, 20, 27, 62) and fatty acids induce insulin resistance in skeletal muscle cell lines in vitro (13, 48, 53). In addition to triglycerides, a number of other lipid metabolites have been proposed to contribute to the detrimental effects of lipids on insulin sensitivity in muscle. In short, there is evidence (1, 13, 47, 48, 57, 59, 62) that increased levels of diacylglycerol (DAG)
and/or ceramide contribute to decreased insulin action, effects that possibly involve activation of the novel protein kinase C-θ (PKCθ) and/or inhibition of Akt (4, 13, 20, 27, 30, 47, 48, 57, 62). However, the effects of leptin on muscle ceramide and DAG levels and insulin signaling pathways are unknown. Furthermore, the effects of leptin on lipid-induced insulin resistance have not been addressed directly, since previous studies have simply demonstrated a correlation between the effects of leptin on insulin action and lipid metabolism, and were performed in models of pre-existing insulin resistance and hyperlipidemia (ob/ob mouse, diet-induced obesity, and lipodystrophy). Thus, the primary goals of the current study were to address these issues. The data demonstrate that leptin-induced improvements in insulin sensitivity are associated with lowered skeletal muscle TG levels, increased skeletal muscle insulin signaling, decreased levels of PKCθ, but not with decreases in skeletal muscle levels of DAG and ceramide. Furthermore, leptin protects against insulin resistance induced by an acute lipid infusion.
MATERIALS AND METHODS

Animal Care and Maintenance. Male Wistar rats weighing 200-280 g were purchased from Charles River Laboratories (Wilmington, MA). Animals were individually housed in environmentally controlled conditions on a constant 12:12 h light-dark cycle, with free access to food and water. All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Pittsburgh, and were in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals.

Study Design. All studies were performed ~10 days after the insertion of indwelling catheters and five days after adenovirus administration in 18-24 h fasted animals, as described in detail below. Studies measuring insulin sensitivity and skeletal muscle lipid levels were performed in separate groups of animals. To allow comparisons between studies and conditions, assessments of insulin sensitivity and lipid levels were always performed subsequent to a saline or lipid infusion, as described below, except in studies assessing skeletal muscle insulin signaling. In three studies, the effects of hyperleptinemia, or hyperlipidemia combined with hyperleptinemia, on insulin sensitivity and lipid levels (triglycerides, diacylglycerol, ceramide) in two skeletal muscles differing in fiber-type composition (soleus muscle, a slow twitch, oxidative muscle composed predominantly of type 1 muscle fibers and superficial vastus, a fast twitch, glycolytic muscle composed predominantly of type 2 muscle fibers) were evaluated. The effects of hyperleptinemia on skeletal muscle insulin signaling were assessed in separate studies, as described below.
**Animal Surgeries.** Catheters (PE-50, Intramedic, Becton Dickinson, Sparks, MD) were placed into the carotid artery (advanced to the aortic arch) and jugular vein (advanced to the right atrium) 5-10 days prior to a study as described previously (14). All subsequent studies were performed in animals that achieved at least 90% of presurgery weight.

**Recombinant Adenovirus Administration.** In studies requiring hyperleptinemia, chronically catheterized rats (~5 days after surgery) received a recombinant adenovirus containing the leptin cDNA (HL, 1 x 10^{12} particles) by tail vein injection as previously described (11). Control animals received a recombinant adenovirus containing the Escherichia coli β-galactosidase cDNA (CONT, 1 x 10^{12} particles). Subsequently, CONT were calorically matched to HL to adjust for leptin-induced decreases in food intake, as previously described (11). Studies (lipid/saline infusions, insulin clamps) described below were performed 5 days after adenovirus administration.

**Lipid and Saline Infusions.** In studies requiring the induction of acute hyperlipidemia, overnight fasted (18-24 h) CONT or HL animals were infused with Liposyn II/heparin (5 mL·kg^{-1}·h^{-1} and 6 U·h^{-1}, respectively, Abbott Laboratories, Chicago, IL) for 6 h. Control animals received a saline infusion (5 mL·kg^{-1}·h^{-1}) for a similar period of time. At the end of the infusions, animals were either sacrificed and tissues (skeletal muscle and blood) isolated for the analysis of lipid levels, or they underwent a hyperinsulinemic clamp as described below.

**Hyperinsulinemic Clamps.** All clamps were performed in 18-24 h fasted HL or CONT animals subsequent to a 6 h saline or lipid infusion as described above. A venous infusion of insulin (Humulin, Eli Lilly, Indianapolis, IN) at 15 mU·kg^{-1}·min^{-1} was begun in conjunction with a variable glucose (30%) infusion to maintain plasma glucose
concentrations (Beckman Glucose II analyzer, Fuller, CA) at euglycemia (~110 mg·dL⁻¹, n=7). The insulin infusion rate used ensured that hepatic glucose output was completely suppressed (44). Arterial blood samples (~30 µL) were taken every 8-10 min throughout the clamp, which lasted 90 min. The glucose infusion rate (GIR) during the final 30 min of the clamp was used as an index of insulin sensitivity (insulin-stimulated whole body glucose disposal), as previously described (2, 7, 14, 20, 34, 40). Total blood volume taken throughout the clamp period did not exceed 5% of total blood volume (approximated from body weight).

**Activation of Insulin Signaling Pathways.** For insulin signaling studies, anaesthetized CONT or HL rats were administered a bolus injection of insulin (10 U·kg⁻¹) via a jugular vein catheter, inserted as described above. Five minutes after administration of the insulin, skeletal muscle was isolated, flash frozen in liquid nitrogen, and stored at -70°C until analysis.

**Skeletal Muscle Analysis.** (i) **DAG and ceramide.** ~30 mg of frozen skeletal muscle was homogenized in 1 mL of ice-cold methanol containing butylated hydroxytoluene (5 µg·mL⁻¹) as an antioxidant. The methanol homogenate was mixed with 2 mL chloroform and lipids extracted using the method of Folch (17). DAGs and ceramides were phosphorylated from a 50 µL aliquot of the lipid extract by the addition of DAG-kinase (5 µL per sample) and 5 µCi ³²P-ATP according to the method of Priess (45). Subsequently, ³²P labeled DAGs and ceramides were separated by thin layer chromatography. After completion of the chromatography, the silica plates were exposed to autoradiograph film. Using the developed film for orientation, DAGs and ceramides were located on the silica plate and scraped into separate scintillation vials. Scintillation
cocktail was added and the product counted for 1 min. DAG and ceramide concentrations were calculated as nMol per mg protein based on DAG and ceramide standards run in conjunction with test samples. This analysis method gives quantitatively similar data to that obtained using high-performance liquid chromatography-fluorescence spectrometry for total ceramide content, and presumably total DAG content (10). Furthermore, the concentration of ceramide and DAG reported in this study are similar to those reported using liquid chromatography tandem mass-spectrometry (62) and gas-liquid chromatography (19). (ii) Triglycerides. A 200 µL aliquot of extracted lipids was air-dried overnight in a glass sample tube. Subsequently, 40 µL of Triton X-114:methanol (2:1 v:v) and 60 µL of tert-butanol were added and the sample vortexed. Samples and standards were assayed spectrophotometrically using a commercially available assay kit (Sigma-Aldrich, St. Louis, MO). (iii) Fatty acid oxidation. Bilateral whole solei were isolated from fasted HL and CONT rats and placed in pregassed 37°C KRH buffer containing 2.4% BSA/0.4 mM ³H-palmitate (1 µCi/mL) for 1 h. Palmitate oxidation was assessed by measuring the quantity of tritiated water released into the medium, as previously described (42, 49). Briefly, at the end of the 1 h incubation, 200 µL of incubation media was transferred to an open 1.5 mL tubes and the tube placed into a 20 mL scintillation vial containing 0.5 mL unlabeled water. The scintillation vial was capped and kept at 50°C for 18 h to allow equilibration of tritiated and unlabeled water. To determine the equilibration coefficient, 200 µL medium containing a known quantity of tritiated water (10 µCi) was incubated similarly to other samples. After the 18 h incubation, 10 mL of scintillation fluid was added to the vials and the samples counted. (iv) PI3-kinase activity. IRS-1 associated PI3-kinase activity was measured as previously
described (26). Briefly, 3 µg of αIRS-1 antibody (Upstate Biotechnology, New York, NY) was incubated with 250 µg of protein for 2 h, followed by the addition of protein A-Sepharose for 1.5 h (Amersham Biosciences). Subsequently, the immune complexes were incubated for 10 min at 22 °C with phosphatidylinositol and $[^{32}\text{P}]$ATP. $^{32}\text{P}$-Containing PI(3)P was separated by TLC and was quantitated by scraping the PI(3)P spot from the TLC plate followed by scintillation counting. (v) Akt, GSK3α, fatty acid transporters, fatty acid binding proteins, ACC, and PKCθ. Protein extracts and/or membrane fractions were prepared from ~100 mg skeletal muscle (gastrocnemius muscle). Total Akt and phosphorylated Akt (Ser$^{473}$), total GSK3α and phosphorylated GSK3α (Ser$^{21}$), phosphorylated ACC (Ser$^{79}$), total FAT/CD36, plasma membrane FABP (FABPpm) and membrane and cytosolic PKCθ levels were determined using standard immunoblot protocols and as described previously (8, 9, 25)

**Statistical analysis.** All data are expressed as mean ± S.E. Statistical significance was determined by unpaired $t$-test using the SPSS (Chicago, IL) statistical software. Significance was assumed at $P < 0.05$. 
RESULTS

*Leptin-induced improvements in insulin sensitivity are associated with lowered skeletal muscle triglycerides, increased ceramides and unaltered diacylglycerol levels.*

Previously, we demonstrated in obese rats (11) that leptin-induced improvements in skeletal muscle insulin sensitivity are correlated with decreased skeletal muscle triglyceride levels. To further address the association of altered skeletal muscle lipid metabolism with leptin-induced improvements in insulin sensitivity we determined the effects of 96 h of hyperleptinemia (HL: 57.8±3.9 ng·mL⁻¹ *versus* CONT: 1.3±0.2 ng·mL⁻¹) in lean rats on the skeletal muscle levels of lipids implicated in decreasing insulin action. In all experiments, CONT were calorically matched to HL during the four days prior to an experiment (HL: 12.8±1.7g/24h *versus* CONT: 12.3±1.1g/24h, an ~40% decrease in daily food intake). There were no statistical differences between HL and CONT in body weight, fasting plasma glucose or plasma insulin concentration on the day of experiments (data not shown). During the clamp insulin was infused at a rate of 15 mU·kg⁻¹·min⁻¹ to suppress hepatic glucose output (44), thus ensuring that the glucose infusion rate represented alterations in insulin-stimulated glucose disposal (2, 7, 20, 34, 40), predominantly in skeletal muscle. As expected, the glucose infusion rate required to maintain euglycemia was increased in HL animals compared to controls (Figure 1). In soleus muscle, a slow twitch, oxidative muscle composed predominantly of type 1 fibers, and superficial vastus, a fast twitch, glycolytic muscle composed predominantly of type 2 muscle fibers, triglyceride content was decreased in HL compared to CONT (Figure 2, Panels A and B). Surprisingly, ceramide levels were increased, while DAG levels were unaltered by hyperleptinemia in both muscles (Figure 2, Panels A and B).
Leptin protects against insulin resistance and alterations in skeletal muscle triglyceride, ceramide, and diacylglycerol levels induced by acute hyperlipidemia. The detrimental effects of acute hyperlipidemia on skeletal muscle insulin sensitivity are well described (7, 12, 24, 33). As such, this model is ideal for directly assessing the capacity of leptin to protect against the effects of lipids on insulin action. As expected, the glucose infusion rates required to maintain euglycemia was decreased in animals receiving a 6 h lipid infusion (LIP) compared to saline-infused (CONT) animals (Figure 3). These changes in insulin sensitivity were associated with increases in soleus muscle DAG and ceramide levels and decreases in TG in LIP compared to CONT (Figure 4, Panel A), in good agreement with the previous study of Yu et al. (62). A similar lipid profile was observed for ceramide and DAG in superficial vastus, but triglycerides were unaltered in this muscle (Figure 4, Panel B). We next determined the effects of leptin on hyperlipidemia-induced insulin resistance. The glucose infusion rate required to maintain euglycemia was not different in HL receiving a lipid infusion (HL-LIP) compared to HL animals receiving a saline infusion (HL-CONT) demonstrating that HL protects against the detrimental effects of lipids on insulin action (Figure 5). Furthermore, soleus DAG, ceramide and triglyceride levels subsequent to a lipid infusion (HL-LIP) were similar to levels observed subsequent to a saline infusion (HL-CONT) (Figure 6, Panel A). A similar pattern was observed in superficial vastus although DAG levels were lower in HL-LIP compared to HL-CONT (Figure 6, Panel B).
Leptin increases insulin-stimulated Akt and GSK3α activity in skeletal muscle. We (11) and others (3, 43, 54, 60, 61) have previously demonstrated that leptin increases insulin-stimulated glucose uptake into skeletal muscle. In the current study we assessed the effects of leptin on the capacity of insulin to activate the PI3-kinase-Akt-GSK3α signaling axis in skeletal muscle. In HL, IRS-1 associated PI3-kinase activity was increased, although the increase did not reach statistical significance (Figure 7, Panel A, \(P=0.06, n=10\)). However, insulin-stimulated activation of Akt was 1.3 ± 0.1 fold greater compared to CONT animals (Figure 7, Panel B, \(P=0.02\)), while phosphorylation of GSK3α, a target of Akt activity was increased 2.1 ± 0.4-fold in HL compared to CONT animals (Figure 3, Panel C, \(P=0.02\)). We conclude from these data that leptin increases responsiveness of the insulin-signaling pathway to insulin in skeletal muscle.

Skeletal muscle fatty acid binding protein/transporter levels and ACC phosphorylation are unchanged, but PKCθ levels are decreased, by hyperleptinemia. We next assessed the effects of leptin on a number of biochemical mechanisms that may play a role in altered lipid metabolism and/or increased insulin sensitivity. Fatty acid entrance into the cell may require plasma membrane fatty acid binding proteins (FABPpm) and fatty acid transporters (FAT/CD36). Furthermore, it has been reported that a chronic leptin infusion decreases FABPpm and FAT/CD36 (56). However, in the current study, no differences in SkM FABPpm and total FAT/CD36 content were observed between HL and CONT animals (Figure 8, Panels A and B).

We next determined the effects of chronic HL on ACC phosphorylation and fatty acid oxidation in SkM. Surprisingly, exogenous palmitate oxidation was not increased in the isolated skeletal muscle (Figure 8, Panel C), nor was ACC phosphorylation increased.
(Figure 8, Panel D) in muscle taken from HL compared to CONT. However, this was most likely due to the measurements being performed ex vivo, since Steinberg et al. reported similar results in the isolated rat muscle (55), and previous studies have demonstrated stimulatory effects of leptin on skeletal muscle fatty acid oxidation in vivo (35, 49).

It has been proposed that increased activity/expression of PKCθ, possibly mediated by DAG, is a contributing mechanism to the development of lipid-induced skeletal muscle insulin resistance (4, 20, 62). Thus, we thought it possible that leptin may decrease PKCθ activity. Subsequent to lipid infusions, both cytosolic and membrane PKCθ levels in skeletal muscle were reduced by ~30% (both P<0.05) in HL compared to CONT (Figure 9), suggesting one potential biochemical mechanism for the protective effects of leptin against lipid-induced insulin resistance.
DISCUSSION

The purpose of the current study was to gain a greater understanding of the effects of leptin on levels of skeletal muscle lipids implicated in the pathogenesis of insulin resistance, to assess the capacity of leptin to protect against the detrimental effects of lipids on insulin action, and to begin to understand the biochemical basis of leptin action on insulin sensitivity. The data demonstrate that leptin-induced improvements in insulin sensitivity are not clearly associated with decreased levels of skeletal muscle lipids implicated in the pathogenesis of insulin resistance, while insulin resistance induced by acute hyperlipidemia is prevented by leptin. Potential biochemical mechanisms mediating these effects include leptin-induced increases in the responsiveness of the skeletal muscle insulin signaling pathway to insulin and reductions in the levels of skeletal muscle PKCθ, a kinase implicated in reducing insulin sensitivity.

The association between leptin effects on lipid metabolism and leptin effects on insulin action suggest that the latter effects may be mediated by decreasing the levels of lipid metabolites such as triglyceride, ceramide and DAG that have been implicated in the pathogenesis of lipid-induced insulin resistance (1, 13, 47, 48, 57, 59, 62). Indeed, leptin has potent lowering effects on plasma and tissue triglyceride levels, but it is thought that triglycerides *per se* are unlikely to play a direct mechanistic role in altering insulin action. Rather, the triglyceride lowering effects of leptin are thought to be a surrogate marker for alterations in other lipid metabolic pathways that may alter insulin action. Thus, it was somewhat surprising in the current study that while leptin increased insulin sensitivity, and the increases were associated with decreases in triglyceride levels in both slow-twitch, oxidative (soleus) and fast-twitch, glycolytic (superficial vastus) muscle fibers,
there were no corresponding decreases in the levels of either ceramide or DAG. Indeed, ceramide increased in both muscle fiber-types examined in hyperleptinemic animals, while DAG was unchanged. Thus, under the conditions examined here (hyperleptinemia in lean animals) the beneficial effects of leptin on insulin sensitivity occur independently of alterations in the levels of these lipid metabolites.

Previous studies in ob/ob and lipodystrophic mice and diet-induced obese rats correlated leptin-induced improvements in insulin sensitivity with improvements in the plasma and tissue triglyceride profile. This led us to ask if leptin could prevent insulin resistance induced by hyperlipidemia, thereby directly addressing the relationship between lipid and leptin effects on insulin action. Importantly, in the presence of hyperleptinemia the detrimental effects of lipids on insulin action were abolished. Arising from this observation is the issue of the mechanism of the protective effects of leptin. One possibility is that leptin acts by preventing the accumulation of DAG and ceramide induced by hyperlipidemia, since neither DAG nor ceramide were increased in lipid-infused compared to saline-infused hyperleptinemic animals, while both metabolites were increased by a lipid infusion in animals with basal leptin concentrations. However, as discussed above, decreases in DAG or ceramide are not correlated with the effects of leptin on insulin action in lean animals. It is possible that leptin effects on other lipid metabolic pathways are involved in leptin-induced increases in insulin sensitivity. Of interest in this regards are the observations that increased fatty acid oxidation in L6 myotubes achieved by overexpression of CPT-1 protects these cells from the detrimental effects of fatty acids on insulin action without altering ceramide or DAG levels (42). Also, in exercise training (18, 46) there is an increase in insulin sensitivity that correlates
with increased skeletal muscle oxidative capacity but not with triglyceride levels, which are paradoxically increased. In the current study we did not observe leptin-induced increases in fatty acid oxidation or ACC phosphorylation, although previous studies suggest that there is a stimulatory effect of leptin on fatty acid oxidation in skeletal muscle (35, 49), possibly mediated by an inactivation of ACC and relief of inhibition of CPT-1. However, it is possible that the model we used i.e. the isolated muscle, explains the current data since previous studies were performed *in vivo* (35, 49) or in isolated muscles receiving electrical stimulation (55). Indeed, in this latter study (55) the authors report that fatty acid oxidation was not increased by leptin in non-stimulated muscles, the conditions that were used in the current study. One conclusion that can be made is that leptin-induced genomic changes that would result in enhanced fatty acid oxidation are not occurring, and that sympathetic innervation may be required to observe leptin-induced increases in fatty acid oxidation.

We demonstrate that the capacity of insulin to activate elements of the insulin-signaling pathway in skeletal muscle is improved by leptin, suggesting one biochemical mechanism underlying the beneficial effects of leptin on insulin action. It is also clear that the increases in the responsiveness of the insulin signaling pathway are most closely associated with decreases in skeletal muscle TG, but are not related to decreases in ceramide or DAG. However it is unknown how decreases in TG would improve insulin signaling. Indeed, it remains a possibility that this association is purely correlative, and that the TG level may reflect increases in the activity of other unknown lipid metabolic pathways or fatty acid oxidation, as discussed above. Alternatively, it is important to consider that other mechanisms that may be unrelated to leptin effects on lipid
metabolism may mediate leptin effects on insulin signaling. An interesting observation was that insulin-stimulated IRS-1 associated PI3-kinase activity was not increased in HL animals, despite increases in Akt and GSK3α phosphorylation. This was somewhat surprising given that Akt is a proximal downstream target of PI3-kinase activity. Furthermore, a recent study suggests that leptin inhibits insulin signaling at the level of IRS-1 (23), demonstrating that there is a potentially complex relationship between leptin-induced increases in insulin sensitivity and leptin effects on insulin signaling.

Several studies have demonstrated a relationship between increased activation of PKCθ and insulin resistance. Thus, in response to a lipid infusion in rodents membrane-associated PKCθ, considered to be the active form of this kinase, is increased, and there is a corresponding decrease in the levels of cytosolic PKCθ (62). Similarly, in obesity levels of skeletal muscle PKCθ are increased (28). Furthermore, PKCθ deficient mice (32) are protected against the effects of acute hyperlipidemia on insulin action. In the current study we demonstrate that both cytosolic and membrane-associated PKCθ are decreased in hyperleptinemic compared to control animals. Thus, a potential mechanism of beneficial effects of leptin on insulin action is to decrease PKCθ. The mechanism by which leptin mediates a reduction in PKCθ is unknown but may be related to effects of leptin on lipid metabolism.

In conclusion, the present study demonstrates that leptin-induced improvements in insulin sensitivity cannot be clearly associated with alterations in skeletal muscle lipid levels. There is an association between leptin effects on insulin action and leptin effects on skeletal muscle triglyceride levels, but this association does not extend to the levels of DAG and ceramide. Under conditions of acute hyperlipidemia, leptin protects against the
deleterious effects of lipids on insulin action. Leptin increases the responsiveness of skeletal muscle to insulin as assessed by activation of Akt and inhibition of GSK3α and decreases the levels of PKCθ, which has been implicated in the pathogenesis of lipid-induced insulin resistance.
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FOOTNOTES

The abbreviations used are: ACC, acetyl Co-A carboxylase; AMPK, 5'-AMP activated protein kinase; CPT-1, carnitine palmitoyltransferase-1; DAG, diacylglycerol; FABPpm, Plasma membrane fatty acid binding protein; FAT/CD36, Fatty acid transporter CD36; GSK3, Glycogen synthase kinase 3; PKC, Protein kinase-C; TG, triglyceride.
FIGURE LEGENDS

Figure 1. Effects of leptin on insulin sensitivity. Rats were chronically cannulated and allowed to recover to ~90% of presurgical body weight as described in "Materials and Methods". Subsequently, rats received a recombinant adenovirus containing the rat leptin cDNA (HL) or β-galactosidase cDNA (CONT). Five days later, all animals were fasted overnight and insulin sensitivity was determined by hyperinsulinemic-euglycemic clamp as described in "Materials and Methods". \( n = \) a minimum of 6 animals/group. Statistical significance is indicated. Results are presented as mean ± S.E.

Figure 2. Effects of leptin on skeletal muscle lipid content. Skeletal muscles differing in fiber-type composition (soleus: type I, slow-twitch, oxidative; superficial vastus: type II, fast-twitch, glycolytic) were isolated from overnight fasted hyperleptinemic (HL) and control (CONT) animals. Subsequently, diacylglycerol (DAG), ceramide, and triglyceride (TG) were measured as described in "Materials and Methods". Panel A and B show lipid content in soleus and superficial vastus muscles, respectively. \( n = \) a minimum of 6 muscles/group. Statistical significance is indicated. Results are presented as mean ± S.E.

Figure 3. Effects of acute hyperlipidemia on insulin sensitivity. Overnight fasted chronically cannulated rats were infused with Liposyn II/heparin (LIP) or saline (CONT) for 6 h. Subsequently, insulin sensitivity was determined by hyperinsulinemic-euglycemic clamp as described in "Materials and Methods". \( n = \) a minimum of 6 animals/group. Statistical significance is indicated. Results are presented as mean ± S.E.
Figure 4. Effects of acute hyperlipidemia on skeletal muscle lipid content. Overnight fasted, chronically cannulated rats were infused with Liposyn II/heparin (LIP) or saline (CONT), and then soleus and superficial vastus muscles were isolated and flash-frozen in liquid nitrogen. Subsequently, diacylglycerol (DAG), ceramide, and triglyceride (TG) were measured as described in "Materials and Methods". Panels A and B show lipid content in soleus and superficial vastus muscles, respectively. n= a minimum of 6 muscles per group. Statistical significance is indicated. Results are presented as mean ± S.E.

Figure 5. Effects of leptin on lipid-induced insulin resistance. Rats were chronically cannulated and allowed to recover to ~90% of presurgical body weight. Subsequently, rats received a recombinant adenovirus containing the rat leptin cDNA (HL). Five days later, all animals were fasted overnight and then were infused with Liposyn II/heparin (HL-LIP) or saline (HL-CONT) for 6 h. Subsequently, insulin sensitivity was determined by hyperinsulinemic-euglycemic clamp as described in "Materials and Methods". n= a minimum of 6 animals/group. Statistical significance is indicated. Results are presented as mean ± S.E.

Figure 6. Effects of lipid infusion on skeletal muscle lipid content in hyperleptinemic animals. Rats were chronically cannulated and allowed to recover to ~90% of presurgical body weight. Subsequently, rats received a recombinant adenovirus containing the rat leptin cDNA (HL). Five days later, all animals were fasted overnight,
and then were infused with Liposyn II/heparin (HL-LIP) or saline (HL-CONT) for 6 h. Soleus and superficial vastus muscles were isolated and flash-frozen in liquid nitrogen. Subsequently, diacylglycerol (DAG), ceramide, and triglyceride (TG) were measured as described in "Materials and Methods". Panel A and B show lipid content in soleus and superficial vastus muscles, respectively. \( n = \) a minimum of 6 animals/group. Statistical significance is indicated. Results are presented as mean ± S.E.

**Figure 7. Effects of leptin on skeletal muscle insulin signaling.** Rats received a recombinant adenovirus containing the rat leptin cDNA (HL) or β-galactosidase cDNA (CONT). Five days later, rats were fasted overnight, anesthetized and an insulin bolus (CONT+ and HL+, 10 U·kg\(^{-1}\)) or saline (CONT- and HL-) administered. Five minutes after administration of insulin, skeletal muscle (gastrocnemius) was harvested and PI3-kinase, Akt and GSK3\(\alpha\) activity assessed. Panel A: IRS-1 associated PI3-kinase activity; Panel B: Akt phosphorylation (Ser\(^{473}\)); Panel C: GSK3\(\alpha\) (Ser\(^{21}\)) phosphorylation. The upper panels show representative autoradiographs for each assay. The lower panels are a quantification of the total data sets as measured by densitometry. \( n = 8-10/\) group. Statistical significance is indicated. Results are presented as mean ± S.E.

**Figure 8. Effect of leptin on skeletal muscle fatty acid binding and transport proteins, ACC phosphorylation, and fatty acid oxidation.** Plasma membrane fatty acid binding protein (FABPpm), fatty acid transporter (FAT/CD36), and phosphorylated ACC (Ser\(^{79}\)) were measured by standard immunoblotting techniques in gastrocnemius muscle from hyperleptinemic (HL) and control animals (CONT). Panel A (upper), Panel
Panel B (upper) and Panel D (upper) shows representative autoradiographs of plasma membrane FABPpm, FAT/CD36, and phospho-ACC, respectively. Panel A (lower), Panel B (lower) and Panel D (lower) is a quantification, as measured by scanning densitometry, of the total data set. Panel C shows oxidation of exogenous fatty acids ([3H]-palmitate) as measured in solei muscles isolated from hyperleptinemic (HL) and control (CONT) animals. n=a minimum of 6 animals/group. Statistical significance is indicated. Results are presented as mean ± S.E.

**Figure 9. Effect of leptin on cytosolic and membrane-associated PKCθ.** Membrane-associated and cytosolic PKCθ content of gastrocnemius muscle were measured from hyperleptinemic (HL) and control animals (CONT). Panel A (upper) and Panel B (upper) shows representative autoradiographs of cytosolic and membrane-associated PKCθ, respectively. Panel A (lower) and Panel B (lower) is a quantification, as measured by scanning densitometry, of the total data set. Panel C is a ratio of total PKCθ divided by the membrane fraction. n= a minimum of 6 muscles per group. Statistical significance is indicated. Results are presented as mean ± S.E.
Figure 1
Figure 2

A. **Soleus**

- DAG (nMol/mg protein)
  - CONT: 2.5 ± 0.5
  - HL: 3.0 ± 0.5
  - P = 0.35

- Ceramide (nMol/mg protein)
  - CONT: 0.5 ± 0.1
  - HL: 1.0 ± 0.2
  - P = 0.001

- TG (μg/mg protein)
  - CONT: 40 ± 5
  - HL: 30 ± 4
  - P = 0.002

B. **Superficial Vastus**

- DAG (nMol/mg protein)
  - CONT: 1.0 ± 0.2
  - HL: 1.5 ± 0.3
  - P = 0.47

- Ceramide (nMol/mg protein)
  - CONT: 0.5 ± 0.1
  - HL: 1.0 ± 0.2
  - P = 0.03

- TG (μg/mg protein)
  - CONT: 25 ± 3
  - HL: 15 ± 2
  - P = 0.001
Figure 3

![Graph showing glucose infusion rate (mg/kg/min) with comparison between CONT and LIP, with P=0.001]

- P=0.001
Figure 4

A. Soleus

B. Superficial Vastus
Figure 5

[Bar graph showing glucose infusion rate with labels HL-CONT and HL-LIP, and a P-value of 0.40]
Figure 6

A. Soleus

B. Superficial Vastus

P-values indicate statistical significance.
Figure 7

A. Insulin PI(3)P
   IP: αRS-1
   P=0.06

B. Insulin Akt-P
   P=0.02

C. Insulin GSK3α-P
   P=0.02
Figure 8

A. 

\[ \text{FABP}_{\text{pm}} \]

\[ \begin{array}{ccc}
\text{CONT} & \text{HL} \\
1.2 & 1.0 & 1.0 & 0.8 & 0.8 & 0.8 & 0.6 & 0.6 & 0.0 & 0.0 & 0.0 \\
\hline
0.0 & 0.2 & 0.4 & 0.6 & 0.8 & 1.0 & 1.2 & 1.4 & 1.6 & 1.8 & 2.0
\end{array} \]

\[ P = 0.18 \]

B. 

\[ \text{FAT/CD36} \]

\[ \begin{array}{ccc}
\text{CONT} & \text{HL} \\
1.2 & 1.0 & 1.0 & 0.8 & 0.8 & 0.8 & 0.6 & 0.6 & 0.0 & 0.0 & 0.0 \\
\hline
0.0 & 0.2 & 0.4 & 0.6 & 0.8 & 1.0 & 1.2 & 1.4 & 1.6 & 1.8 & 2.0
\end{array} \]

\[ P = 0.34 \]

C. 

\[ \text{\mu M palmitate/tissue/h} \]

\[ \begin{array}{ccc}
\text{CONT} & \text{HL} \\
4.5 & 4.0 & 4.0 & 3.5 & 3.5 & 3.5 & 3.0 & 3.0 & 2.5 & 2.5 & 2.0 & 1.5 & 1.5 & 1.0 & 1.0 & 0.5 & 0.5 & 0.0 & 0.0 \\
\hline
0.0 & 0.5 & 1.0 & 1.5 & 2.0 & 2.5 & 3.0 & 3.5 & 4.0 & 4.5 & 5.0 & 5.5 & 6.0 & 6.5 & 7.0 & 7.5 & 8.0 & 8.5 & 9.0 & 9.5 & 10.0
\end{array} \]

\[ P = 0.76 \]

D. 

\[ \text{P-ACC} \]

\[ \begin{array}{ccc}
\text{CONT} & \text{HL} \\
1.4 & 1.2 & 1.2 & 1.0 & 1.0 & 1.0 & 0.8 & 0.8 & 0.6 & 0.6 & 0.4 & 0.4 & 0.2 & 0.2 & 0.0 & 0.0 \\
\hline
0.0 & 0.2 & 0.4 & 0.6 & 0.8 & 1.0 & 1.2 & 1.4 & 1.6 & 1.8 & 2.0 & 2.2 & 2.4 & 2.6 & 2.8 & 3.0 & 3.2 & 3.4 & 3.6 & 3.8 & 4.0
\end{array} \]

\[ P = 0.23 \]
Figure 9

A. Membrane PKC

B. CONT HL

Cytosol
PKC CONT HL

PKC CONT HL

P = 0.01

Cytosolic fraction (AU)

0.0 0.2 0.4 0.6 0.8 1.0 1.2

P = 0.01

Membrane PKC

PKC CONT HL

P = 0.01

Membrane fraction (AU)

0.0 0.2 0.4 0.6 0.8 1.0 1.2

C.

PKC CONT HL

PKC fraction/total (AU)

0.0 0.1 0.2 0.3 0.4 0.5 0.6

CONT HL
REFERENCES


55. Steinberg GR, Bonen A, and Dyck DJ. Fatty acid oxidation and triacylglycerol hydrolysis are enhanced after chronic leptin treatment in rats. *Am J Physiol Endocrinol Metab* 282: E593-600, 2002.