

## Restoration of skeletal muscle leptin response does not precede the exercise-induced recovery of insulin-stimulated glucose uptake in high-fat-fed rats

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**Ritchie IR, Gulli RA, Stefanyk LE, Harasim E, Chabowski A, Dyck DJ.** Restoration of skeletal muscle leptin response does not precede the exercise-induced recovery of insulin-stimulated glucose uptake in high-fat-fed rats. *Am J Physiol Regul Integr Comp Physiol* 300: R492–R500, 2011. First published November 17, 2010; doi:10.1152/ajpregu.00602.2010.—Leptin administration increases fatty acid (FA) oxidation rates and decreases lipid storage in oxidative skeletal muscle, thereby improving insulin response. We have previously shown high-fat (HF) diets to rapidly induce skeletal muscle leptin resistance, prior to the disruption of normal muscle FA metabolism (increase in FA transport; accumulation of triacylglycerol, diacylglycerol, ceramide) that occurs in advance of impaired insulin signaling and glucose transport. All of this occurs within a 4-wk period. Conversely, exercise can rapidly improve insulin response, in as little as one exercise bout. Thus, if the early development of leptin resistance is a contributor to HF diet-induced insulin resistance (IR) in skeletal muscle, then it is logical to predict that the rapid restoration of insulin response by exercise training would be preceded by the recovery of leptin response. In the current study, we sought to determine 1) whether 1, 2, or 4 wk of exercise training was sufficient to restore leptin response in isolated soleus muscle of rats already consuming a HF diet (60% kcal), and 2) whether this preceded the training-induced corrections in FA metabolism and improved insulin-stimulated glucose transport. In the low-fat (LF)-fed control group, insulin increased glucose transport by 153% and leptin increased AMPK and ACC phosphorylation and the rate of palmitate oxidation (+73%). These responses to insulin and leptin were either severely blunted or absent following 4 wk of HF feeding. Exercise intervention decreased muscle ceramide content (–28%) and restored insulin-stimulated glucose transport to control levels within 1 wk; muscle leptin response (AMPK and ACC phosphorylation, FA oxidation) was also restored, but not until the 2-wk time point. In conclusion, endurance exercise training is able to restore leptin response, but this does not appear to be a necessary precursor for the restoration of insulin response.

leptin resistance; insulin resistance; ceramide; soleus

LEPTIN IS A 16-KDA CYTOKINE produced primarily by adipocytes and is known to regulate food intake and energy metabolism. Disruption of normal leptin signaling in rodent models, which does not express functional leptin (*ob/ob*) or its receptors (*db/db*) is characterized by extreme obesity, hyperglycemia, hyperlipidemia, and insulin resistance (IR) (6, 8, 29). Correction of leptin deficiency by means of injection or implantation reverses these metabolic abnormalities (4, 15). Leptin admin-

istration can also improve insulin-stimulated glucose transport in skeletal muscle from healthy rodents (30). This is likely mediated, at least in part, through changes in fatty acid (FA) metabolism (reduced esterification, increased oxidation) leading to reduced triacylglycerol (TAG) content (18, 21, 27, 30).

Skeletal muscle from obese humans demonstrates an impaired response to leptin, i.e., leptin resistance (18). We have recently shown that high-fat (HF) feeding induces resistance to the adipokines leptin and adiponectin very rapidly (in under 1 wk), potentially contributing to the subsequent increase in lipid accumulation and manifestation of IR in skeletal muscle observed weeks later (11). Interestingly, the impaired insulin response induced by a 3-mo HF diet can be reversed in only 10 days of leptin injections (31). While this reversal is impressive, it is also somewhat paradoxical, given that leptin resistance should also have been present. However, this demonstrates that resistance to leptin in muscle can at least be partially overcome by further increasing circulating leptin in vivo, sufficient to normalize FA metabolism and restore insulin response. It is reasonable to predict, then, that restoring or improving leptin response in skeletal muscle might be an important factor facilitating the restoration of insulin response.

Improved insulin sensitivity is a hallmark outcome of exercise training; importantly, endurance training can restore insulin response in obese, insulin-resistant rodents (10, 16) and humans (2). However, very little work has examined the influence of endurance exercise on skeletal muscle leptin response. We have previously shown that in rats, endurance training concurrent with the feeding of a high-fat (60% kcal) diet over a 4-wk period can partially prevent the development of leptin resistance in skeletal muscle (19). It is possible that an improved response to leptin may be one mechanism by which exercise normalizes muscle FA metabolism and insulin response. To date, no research has examined the ability of exercise to restore skeletal muscle leptin response after leptin resistance has already been induced.

The best identified mediator of leptin signaling is the suppressor of cytokine signaling-3 (SOCS3), which interferes with JAK activity at the leptin receptor (1) and thereby disrupts downstream signaling, including the phosphorylation of AMPK and acetyl-CoA carboxylase (ACC). The expression (mRNA) of SOCS3 is elevated following 4 wk of HF feeding in fed rodents (19); protein content has been less extensively examined, but it has been shown to increase with 12 wk of a HF diet (32). Finally, overexpressing SOCS3 in cultured myotubes derived from lean humans impairs leptin signaling and mimics the leptin resistance observed in myotubes from obese

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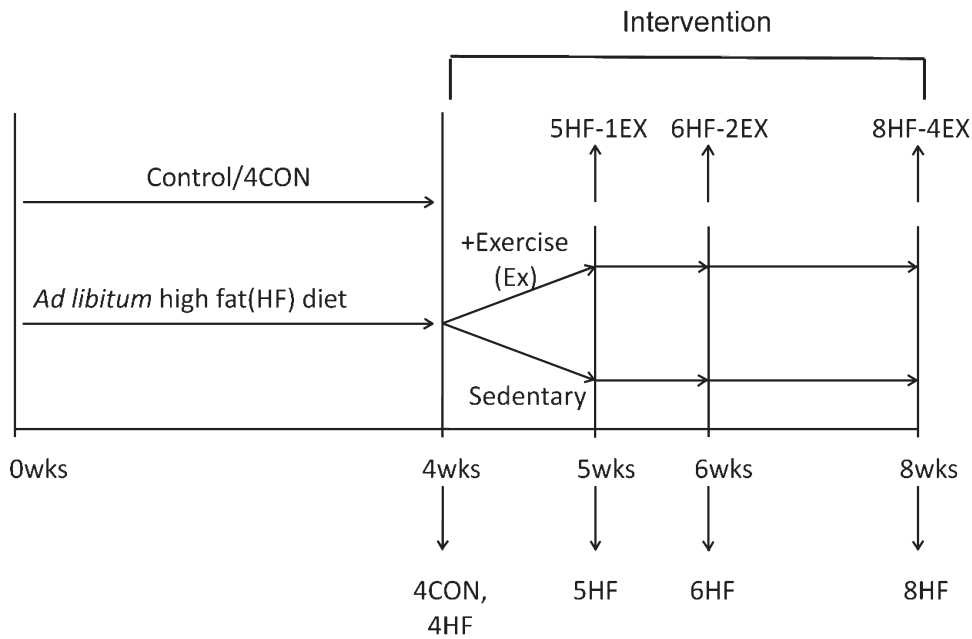


Fig. 1. Schematic of time course. 4CON, control/4 wk of rodent chow; 4HF, 4 wk on a high-fat (HF) diet; 5HF-1EX, 5 wk on a HF diet + 1 wk of exercise (Ex); 6HF, 6 wk on a HF diet; 6HF-2EX, 6 wk on a HF diet + 2 wk Ex; 8HF, 8 wk on a HF diet; 8HF-4EX, 8 wk HF diet + 4 wk Ex.

subjects (17). A deficiency in leptin receptor (ObR) protein content could also account for impaired leptin response. This, however, has not been examined in skeletal muscle from HF-fed rodents.

The objective of the current study was to examine the early time course of changes in leptin and insulin response in muscle induced by endurance training in HF-fed rats. We hypothesized that impaired leptin response (as indicated by a blunted stimulation of FA oxidation and AMPK/ACC phosphorylation) following 4 wk of a HF diet would be associated with an increase in skeletal muscle SOCS3 protein content and a decrease skeletal muscle leptin receptor protein content. Furthermore, we hypothesized that subsequent endurance exercise training would lead to a rapid improvement in leptin response, which would correspond to a reduction in SOCS3 protein content, an increase in leptin receptor content, and restored AMPK/ACC phosphorylation. Finally, we hypothesized that the improved muscle leptin response would precede the reduction of ceramide and DAG, and the restoration of insulin response.

## METHODS

**Animals and diets.** Upon arrival, female Sprague-Dawley rats (145 g; Charles River Laboratories, Quebec, Canada) were housed individually in a reverse 12:12-h light-dark environment and maintained on standard rodent chow. Following a 2–5-day acclimatization period, rats were randomly assigned to 1 of 8 experimental groups ( $n = 12$  per group; Fig. 1). During the first 4 wk, all rats remained sedentary and received ad libitum access to either a LF (CON) diet (Harlan Teklad, Madison, WI) or a diet high in saturated fat (HF; 60% of kilocalories consumed) derived from lard (Research Diets, New Brunswick, NJ; Table 1) with the purpose of inducing muscle insulin and leptin resistance, as we have previously demonstrated (12, 20).

After the initial 4 wk, the animals in the LF group (4CON) were killed as a reference point for the subsequent 4-wk HF diet and exercise intervention period. During the subsequent 4 wk, all rats continued to receive a HF diet, as in the initial 4 wk, and were killed after an additional 1, 2, or 4 wk of HF diet (5HF, 6HF, or 8HF). Half of the rats remained sedentary, while the other half embarked on a

treadmill training program during the remaining 4 wk with the objective of reversing the state of insulin and leptin resistance already created (5HF-1EX, 6HF-2EX, and 8HF-4EX). Thus, the 5HF-1EX designation indicates a total of 5 wk on the HF diet, with the final 1 wk, including exercise training; the absence of “EX” indicates a sedentary condition for the full duration of the study. All surgical and experimental procedures were conducted following a 12-h overnight fast. All procedures were approved, and ethical consent was provided by the Animal Care Committee at the University of Guelph.

**Training protocol.** Two days prior to commencement of training, animals received two acclimatory training sessions (<5 min, 10 m/min, 0% incline). Training took place 5 days a week followed by 2 days of recovery. Animals started at 15 m/min at a 0% incline for 20 min and were rapidly increased to 20 m/min at a 10% incline for 60 min by the end of the first week (the 5th training session). The speed and incline were maintained thereafter, but duration continued to increase to a maximum of 120 min midway through the second week (8th training session), which they maintained for the duration of the study. Animals were allowed a 48-h recovery period following the last training session prior to surgical procedures to avoid any acute effect from the last exercise bout.

**Blood and tissue sampling.** After an overnight fast, animals were anesthetized with an intraperitoneal injection of pentobarbital sodium (6 mg/100 mg body wt). Basal and insulin-stimulated glucose transport, basal and leptin-stimulated FA metabolism, and basal and leptin-stimulated signaling protein activation were assessed in paired, isolated soleus strips (~25 to 30 mg each). Briefly, a 27-gauge needle was used to carefully strip the soleus longitudinally, from tendon to tendon, into three sections, which were incubated under their respective condition. The red gastrocnemius muscle was excised from each

Table 1. *Macronutrient content of experimental diets*

	Control		High Fat	
	g	kcal	g	kcal
Protein	18.6	24	26.2	20
Carbohydrate	44.2	58	26.3	20
Fat	6.2	18	34.9	60
Total		100		100
kcal/g	3.1		5.24	

animal and immediately frozen in liquid N<sub>2</sub> for later Western blot analysis and analysis of whole muscle lipid content. The red gastrocnemius muscle was sampled due to the limited amount of soleus tissue. Following skeletal muscle excision, a terminal blood sample was obtained via cardiac puncture. All blood samples were collected in heparinized tubes and kept on ice. Plasma was separated via centrifugation (5,445 g, 10 min). All plasma samples were stored at -80°C until analyses. Fasting plasma glucose concentrations were determined using a glucometer (Elite XL; Bayer, Toronto, Ontario, Canada). Fasting plasma insulin and leptin concentrations were determined via commercially available RIA kits (Linco, St. Charles, MO) following the specific directions of each kit. Fasting plasma FAs were determined using a Wako assay kit (Wako Chemical, Richmond, VA). All plasma measurements were made in duplicate.

**Basal and insulin-stimulated glucose transport.** Pregassed (95% O<sub>2</sub>-5% CO<sub>2</sub>) Medium 199 containing 0.1% BSA and warmed to 30°C was used as a base for all glucose uptake buffers. Insulin (10 mU/ml) (Humulin R; Eli Lilly, Toronto, Ontario, Canada) was added to all buffers for the insulin-stimulated condition. Immediately following excision, the soleus strips were placed in 20-ml glass vials containing preincubation buffer for 30 min in the presence or absence of insulin (10 mU/ml; maintained in all subsequent buffers). The preincubation buffer consisted of base buffer with 8 mM glucose and 32 mM mannitol. The soleus strips were then washed in two glucose-free buffers, which contained 4 mM pyruvate and 36 mM mannitol, for 10 min each. The soleus muscle was then incubated in base buffer with 4 mM pyruvate, 8 mM 3-O-[<sup>3</sup>H]methyl-D-glucose (0.5 µCi/ml; ARC, St. Louis, MO), and 28 mM [<sup>14</sup>C] mannitol (0.2 µCi/mmol; GE Healthcare, Baie d'Urfe, Quebec, Canada) for 20-min (insulin stimulated) or 40-min (basal) conditions. Soleus muscles were then removed, thoroughly blotted to remove excess buffer, and trimmed of their tendons. Muscles were then weighed and digested for 10 min in 1 ml of 1 M NaOH at 95°C. Two-hundred microliters of muscle digest from each sample was sampled in duplicate and analyzed by liquid scintillation counting, from which glucose transport was calculated.

**FA metabolism.** Pregassed (95% O<sub>2</sub>-5% CO<sub>2</sub>) Medium 199 containing 4% BSA, 5 mM glucose, and 0.5 mM palmitate and warmed to 30°C and used as a base for all buffers. All incubation vessels were capped in 20-ml glass scintillation vials and contained 2 ml of buffer. Immediately following isolation, the soleus strips were placed in glass vials containing preincubation buffer for 30 min. The preincubation buffer consisted only of the base buffer. Following preincubation, muscle strips were carefully transferred to new vials containing incubation buffer for 60 min. The incubation buffer consisted of the base buffer with addition of 0.5 µCi/ml [<sup>1-14</sup>C] palmitate (GE Healthcare). Leptin was added to the buffer in the leptin-stimulated conditions (10 µg/ml; recombinant rat leptin; Peprotech, Rocky Hill, NJ). Following incubation, muscle strips were removed, thoroughly blotted, trimmed of their tendons, weighed, and placed into 5 ml of ice-cold 2:1 chloroform: methanol in 13-ml centrifuge tubes.

One milliliter of incubation buffer from each sample was immediately transferred to a sealed 50-ml Erlenmeyer flask and acidified with 1 ml of 1 M sulfuric acid to liberate <sup>14</sup>CO<sub>2</sub>. <sup>14</sup>CO<sub>2</sub> was captured in 250 µl of 1 M benzethonium hydroxide suspended in a 500-µl tube within the sealed flask. The tube containing benzethonium hydroxide and <sup>14</sup>CO<sub>2</sub> was then counted using a liquid scintillation counter.

Muscle samples were homogenized using a Polytron (PT10-35; Kinematica, Lucerne, Switzerland) and centrifuged for 10 min at 4,500 g. The supernatant was then removed and added to new 13-ml centrifuge tubes containing 2 ml of double-deionized water and placed on a shaker for 10 min to separate the aqueous and lipid-soluble metabolites. The pellets were discarded. Following extraction, the samples were centrifuged for another 10 min at 1,620 g, after which 500 µl of the aqueous phase was sampled in duplicate for measurement of <sup>14</sup>C trapped as β-oxidation intermediates (isotopic fixation) via liquid scintillation counting.

The lipid-soluble phase was removed and transferred to 12 × 75 mm glass tube, and the chloroform was evaporated off under a stream of N<sub>2</sub> gas. The lipids were then resuspended in 100 µl of 2:1 chloroform:methanol containing small amounts of dipalmitin and tripalmitin as markers, of which 50 µl was spotted in individual lanes on dry silica plates. Plates were placed in sealed thin-layer chromatography (TLC) tanks containing 200 ml of 60:40:3 heptane:isopropylether:glacial acetic acid for 45 min. Plates were removed and allowed to dry before being sprayed with dichlorofluorescein dye and visualized with long-wave UV light. The visible TAG and DAG bands for each sample were scraped into individual vials for liquid scintillation counting of <sup>14</sup>C-TAG and DAG.

**Leptin signaling.** Pregassed (95% O<sub>2</sub>-5% CO<sub>2</sub>) Medium 199 containing 4% BSA, 5 mM glucose, and 0.5 mM palmitate, warmed to 30°C, was used as a base for all of the buffers. All incubation vessels were capped in 20-ml glass scintillation vials and contained 2 ml of buffer.

Immediately following isolation, the soleus strips were placed in glass vials containing preincubation buffer for 30 min. The preincubation buffer consisted only of the base buffer. Following preincubation, muscle strips were carefully transferred to new vials containing incubation buffer for 30 min. The incubation buffer consisted of the base buffer alone in the basal condition or with the addition of 10 µg/ml leptin (recombinant rat leptin; Peprotech, Rocky Hill, NJ) in the leptin-stimulated condition. Following incubation, muscle strips were removed, blotted of excess buffer, and immediately frozen in liquid N<sub>2</sub> for later analysis.

**Total muscle lipid content.** Red gastrocnemius (~50 mg) was freeze-dried, powdered, and separated from all visible blood and connective tissue. Lipid species (TAG, DAG, and ceramide) were extracted and separated by TLC, and the individual FA species were measured using gas-liquid chromatography, as described previously (2).

**Western blot analyses.** Muscle samples were homogenized in an ice-cold buffer for the extraction of proteins and preservation of protein phosphorylation states. The buffer contained 50 mM Tris (pH=7.5), 1 mM EDTA, 1 mM EGTA, 50 mM NaF, 5 mM sodium pyrophosphate, 10% (vol/vol) glycerol, 1% (vol/vol) Triton X-100, 2 mg/ml leupeptin, 2 mg/ml aprotinin, 2 mg/ml pepstatin, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride. Muscle homogenates were centrifuged at 20,000 g for 20 min at 4°C, and the supernatant was removed and protein content was determined. Fifty micrograms of whole muscle tissue lysate protein was solubilized in 4× Laemmli's buffer and boiled at 95°C for 10 min, resolved by SDS-PAGE, and wet transferred to PVDF membranes (ObR, SOCS3, GLUT4, pAMPK, and tAMPK for 1 h at 100 V; pACC and tACC for 16 h at 20 V). The membranes were blocked with 2.5–7.5% BSA for 2 h and then incubated with the specific primary antibodies for ObR (all isoforms; Abcam, Cambridge, MA), tAMPK, pAMPK, tACC (Cell Signaling, Danvers, MA), pACC, GLUT4 (Millipore, Billerica, MA), and SOCS3 (Santa Cruz Biotechnology, Santa Cruz, CA). After incubation with the appropriate secondary antibody, the immune complexes were detected by enhanced chemiluminescence and were quantified by densitometry (ChemiGenius 2 Bioimaging system; Syngene, Cambridge, UK). Alpha tubulin (Abcam) was used to ensure consistent protein loading and transferring.

**Calculations and statistics.** All data are reported as means ± SE. Data were analyzed using ANOVA procedures. One-way ANOVA was used to analyze plasma, lipid, and Western blot measurements, where the experimental group was the only independent variable. A randomized block-design two-way ANOVA was used to determine whether the effects of insulin and leptin on glucose transport and FA metabolism were different among the experimental groups. Results from the ANOVA were assessed by a Student-Newman-Keul's post hoc test. Two-tailed paired *t*-tests were used to identify differences in basal and leptin-stimulated AMPK and ACC phosphorylation. Significance was accepted at *P* ≤ 0.05.

Table 2. Fasting plasma measurements and body mass

	4CON	4HF	5HF	5HF-1EX	6HF	6HF-2EX	8HF	8HF-4EX
Free fatty acids, mmol/l	0.30 ± 0.02	0.22 ± 0.03	0.23 ± 0.02	0.28 ± 0.02	0.24 ± 0.02	0.22 ± 0.02	0.26 ± 0.02	0.29 ± 0.04
Glucose, mmol/l	9.6 ± 0.5	10.7 ± 0.5	10.8 ± 0.4	11.1 ± 0.3	11.5 ± 0.3*	10.4 ± 0.3	11.7 ± 0.4*†	9.5 ± 0.5
Insulin, ng/ml	4.4 ± 0.4	3.2 ± 0.4	5.2 ± 0.5	4.8 ± 0.7	5.0 ± 0.5	5.2 ± 0.7	6.9 ± 0.4*†	4.5 ± 0.5
Leptin, ng/ml	1.4 ± 0.2	9 ± 2*	11 ± 1*	11 ± 2*	15 ± 3*†	7 ± 1 ( <i>P</i> = 0.066)	20 ± 2*†	11 ± 2*
Pretrial body mass, g	154 ± 1	154 ± 4	157 ± 5	155 ± 2	152 ± 2	161 ± 4	159 ± 4	152 ± 2
Terminal body mass, g	244 ± 3	282 ± 8	288 ± 5	294 ± 9	304 ± 8	306 ± 9	325 ± 9	336 ± 7

Data are expressed as means ± SE; *n* = 8–12. \*Significantly different from 4CON, *P* ≤ 0.05. †Significantly different from corresponding exercise group. 4CON, 4 wk of rodent chow; 4HF, 4 wk on a high-fat (HF) diet; 5HF, 5 wk on a HF diet; 5HF-1EX, 5 wk on a HF diet +1 wk of exercise (Ex); 6HF, 6 wk on a HF diet; 6HF-2EX, 6 wk on a HF diet +2 wk Ex; 8HF, 8 wk on a HF diet; 8HF-4EX, 8 wk on a HF diet +4 wk Ex.

## RESULTS

**Plasma FAs, glucose, leptin, and insulin.** Fasting plasma FA concentrations did not differ among the groups regardless of treatment (Table 2). Fasting plasma insulin concentration was elevated in the 8HF group above that of the 8HF-4EX and 4CON groups (*P* ≤ 0.05). Otherwise, there were no differences between groups (Table 2). Fasting plasma glucose was elevated in 6HF, 8HF, and 8HF-4EX groups compared with the 4CON group (*P* ≤ 0.05). In the 8HF-4EX group, plasma glucose was significantly lower than the 8HF group (*P* ≤ 0.05). HF feeding significantly increased fasting plasma leptin concentrations in all groups except 6HF-2EX (*P* = 0.66). However, 2 and 4 wk of training significantly reduced plasma leptin compared with their respective sedentary groups (*P* ≤ 0.05; Table 2).

**Skeletal muscle glucose transport.** Insulin stimulation significantly increased glucose transport in all groups at all time points (*P* ≤ 0.05). This effect was significantly diminished following 4, 5, 6, and 8 wk of HF feeding (*P* ≤ 0.05). Exercise training restored insulin-stimulated glucose transport to normal levels within 1 wk (*P* ≤ 0.05). Exercise training significantly increased insulin-stimulated glucose transport compared with their sedentary counterparts at all time points (*P* ≤ 0.05; Fig. 2).

**Skeletal muscle FA metabolism.** There were no differences in rates of FA oxidation under nonstimulated conditions among the groups (Fig. 3A). Leptin stimulation significantly increased FA oxidation in the 4CON group above basal conditions (+73%, *P* ≤ 0.05). This effect was abolished by 4, 5, 6, and 8 wk of HF feeding. Two and four weeks of exercise training, but not 1 wk, partially restored the ability of leptin to stimulate FA oxidation (+27% and +39%, *P* ≤ 0.05).

HF feeding increased the rate of palmitate esterification to TAG compared with the 4CON group (Fig. 3B). Leptin stimulation significantly reduced the rate of FA esterification to TAG in the 4CON group (−24%, *P* ≤ 0.05). This effect was abolished by 4, 5, 6, and 8 wk of HF feeding. Four weeks of exercise training restored the ability of leptin to decrease TAG esterification rates (−23%, *P* ≤ 0.05).

The basal rates of FA esterification to DAG did not differ between groups (Fig. 3C). The rate of FA esterification to DAG was significantly lower in the 8HF-4EX group compared with 8HF group following leptin stimulation (−37%, *P* ≤ 0.05). There were no other significant differences between the groups. The rate of FA esterification to DAG was decreased in response to leptin in the 4CON group (−19%, *P* ≤ 0.05). This effect was lost following 4, 5, 6, and 8 wk of HF feeding. Four,

but not 1 or 2 wk, of exercise training restored the ability of leptin to reduce DAG esterification rates (−23%, *P* ≤ 0.05).

**AMPK and ACC signaling.** There was no significant effect of HF feeding or exercise training on total AMPK and ACC protein content (Fig. 4). Leptin stimulation induced a small increase the pAMPK and pACC when in the 4CON group (+18%, +19%, *P* ≤ 0.05). Leptin stimulation did not increase pAMPK or pACC in any of the HF-fed sedentary groups; this was restored following 2 and 4 wk of exercise training (pAMPK, +20%, +25%, pACC, +15%, +18%, *P* ≤ 0.05).

**Intramuscular lipid content.** HF feeding increased intramuscular TAG content (Fig. 5A) compared with 4CON in all sedentary groups (4HF, +80%; 5HF, +77%; 6HF, +85%; 8HF, +88%; *P* ≤ 0.05). Total TAG content was restored to control concentrations following 1 wk of endurance exercise. Intramuscular ceramide content (Fig. 5B) was elevated following HF feeding in all sedentary groups (4HF +36%; 5HF +27%; 6HF +32%; 8HF +24%; *P* ≤ 0.05). Ceramide content was restored to control levels following 1 wk of endurance exercise. There were no significant differences in intramuscular DAG content (Fig. 5C) between the experimental groups. There were no significant differences in total saturated, poly-

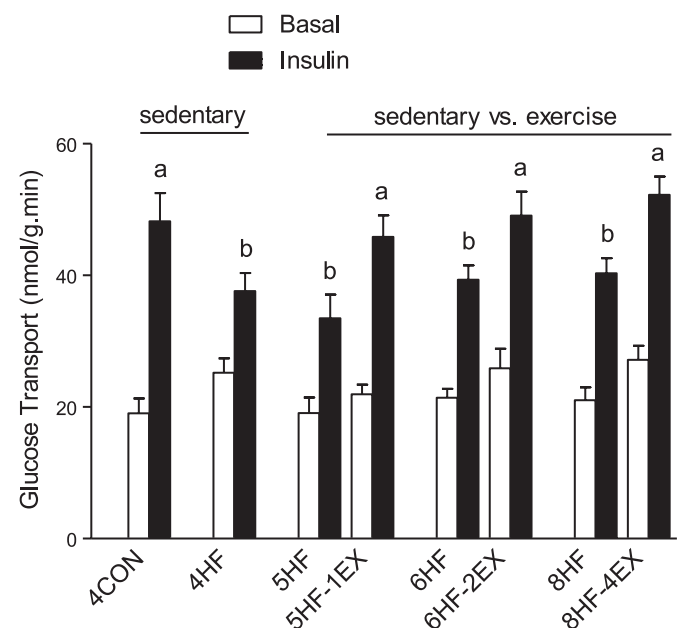


Fig. 2. Basal and insulin-stimulated glucose transport in isolated soleus muscle strips. Data are expressed as means ± SE; *n* = 8–12. <sup>a,b</sup>Bars not sharing a letter are significantly different, *P* ≤ 0.05.

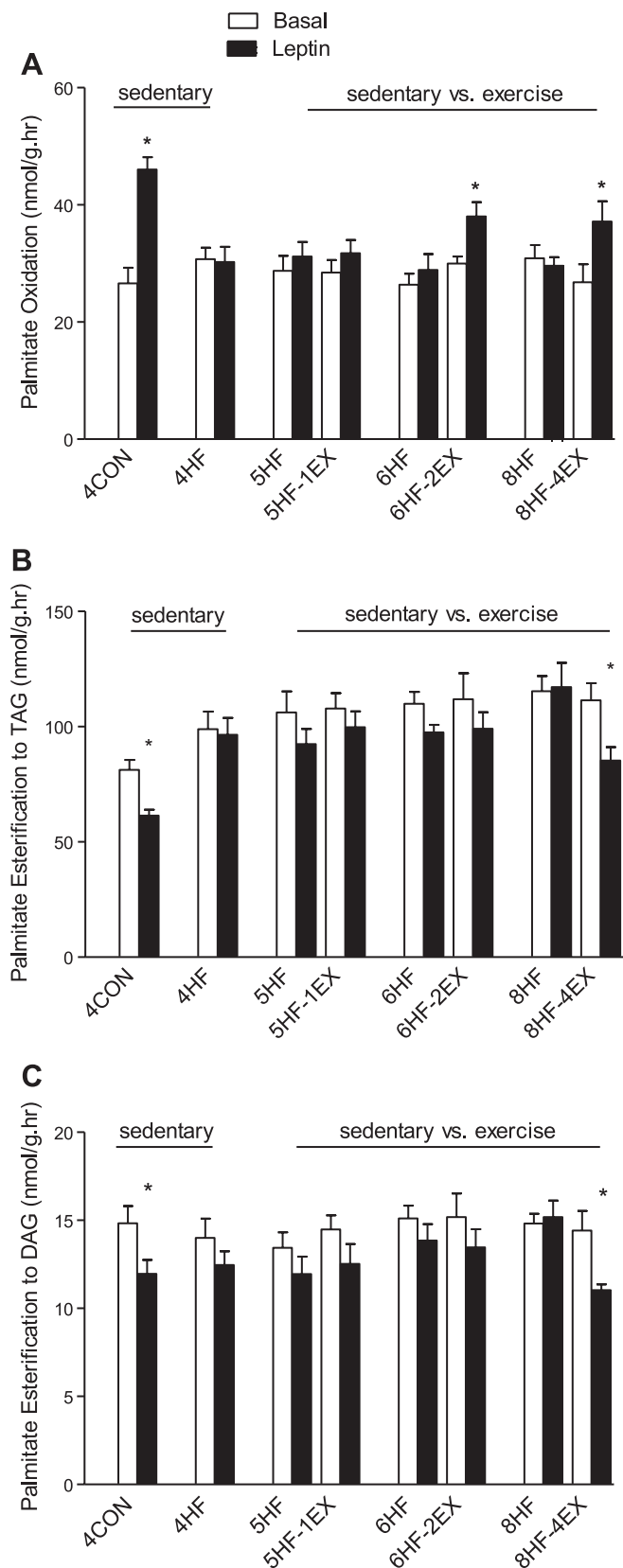


Fig. 3. Skeletal muscle palmitate oxidation (A), esterification to triacylglycerol (TAG; B), and esterification to DAG (C) in isolated soleus muscle strips with or without leptin. Data are expressed as means  $\pm$  SE;  $n = 8-12$ . \*Significantly different from basal condition,  $P \leq 0.05$ .

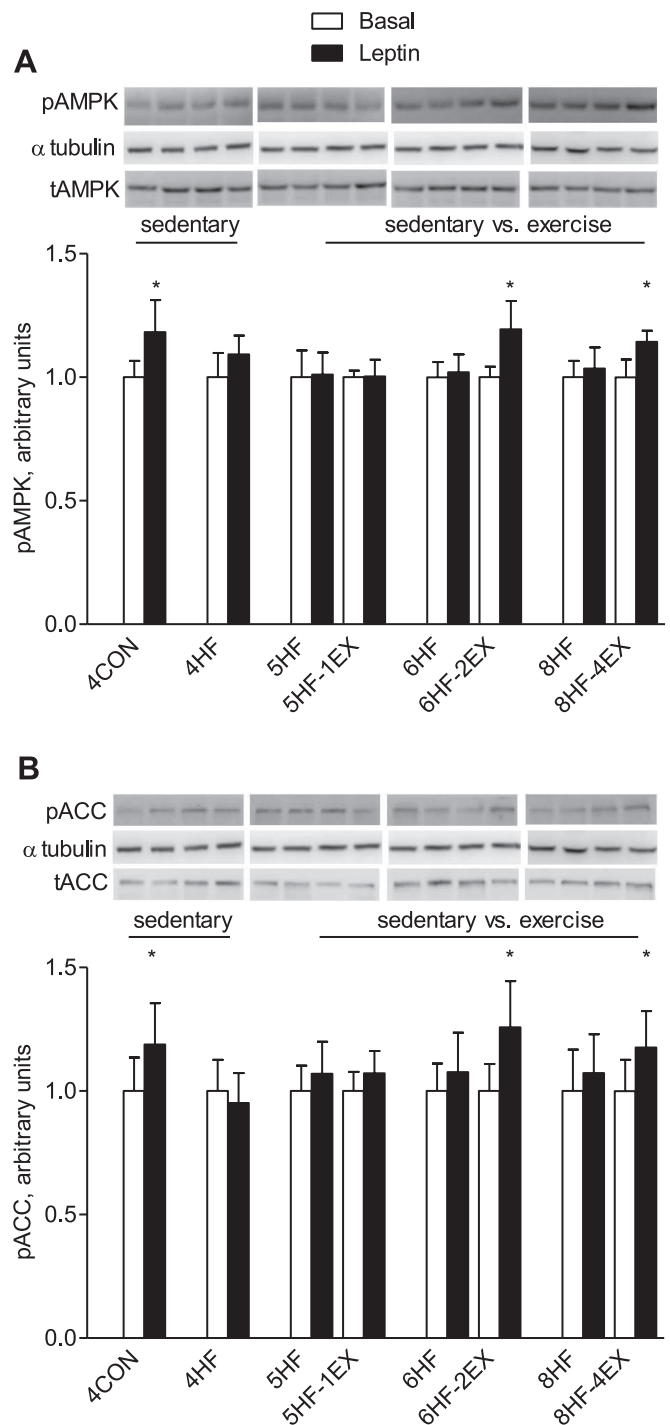


Fig. 4. The effect of acute leptin treatment on total and phosphorylated AMPK (A) and acetyl-CoA carboxylase (ACC; B) in isolated soleus muscle strips. Data are expressed as means  $\pm$  SE;  $n = 8-12$ . \*Significantly different from basal conditions;  $P \leq 0.05$ .

unsaturated, and monounsaturated FA species in the TAG, ceramide, or DAG pools between experimental groups (data not shown).

*Red gastrocnemius GLUT4, SOCS3, and leptin receptor protein expression.* Because of the soleus tissue limitations, Western blot analysis was performed on red gastrocnemius, another highly oxidative muscle. SOCS3 expression increased

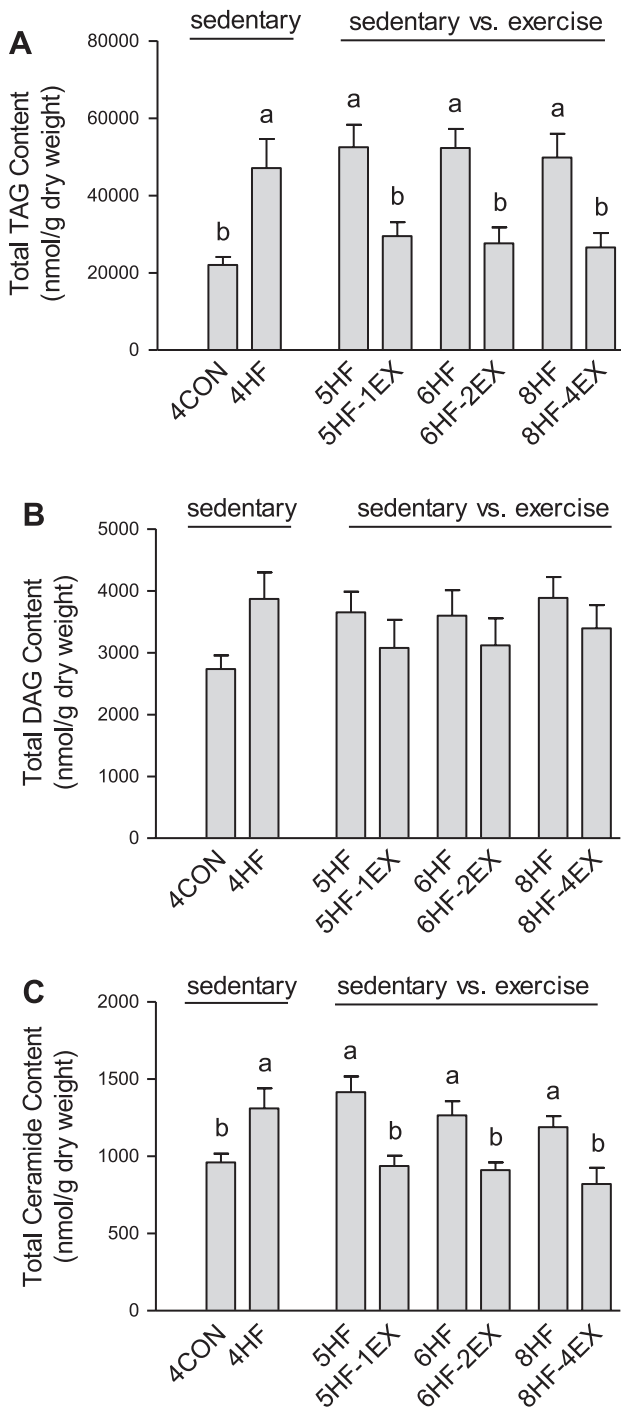


Fig. 5. Total intramuscular TAG (A), DAG (B), and ceramide content (C) measured in red gastrocnemius muscle. Data are expressed as means  $\pm$  SE;  $n = 8-12$ . <sup>a,b</sup>Bars not sharing a letter are significantly different,  $P \leq 0.05$ .

in 5HF, 6HF, and 8HF groups compared with the 4CON group by +26%, +29%, +39%, respectively ( $P \leq 0.05$ ; Fig. 6A). There appeared to be an increase in the 4HF group compared with the 4CON group, but this result was not statistically significant (+17%,  $P = 0.11$ ). The exercise-trained groups were not significantly different from the 4CON group. SOCS3 protein content was significantly different following 4 wk of exercise (8HF-4EX) compared with the respective sedentary group (8HF). While several isoforms of the leptin receptor

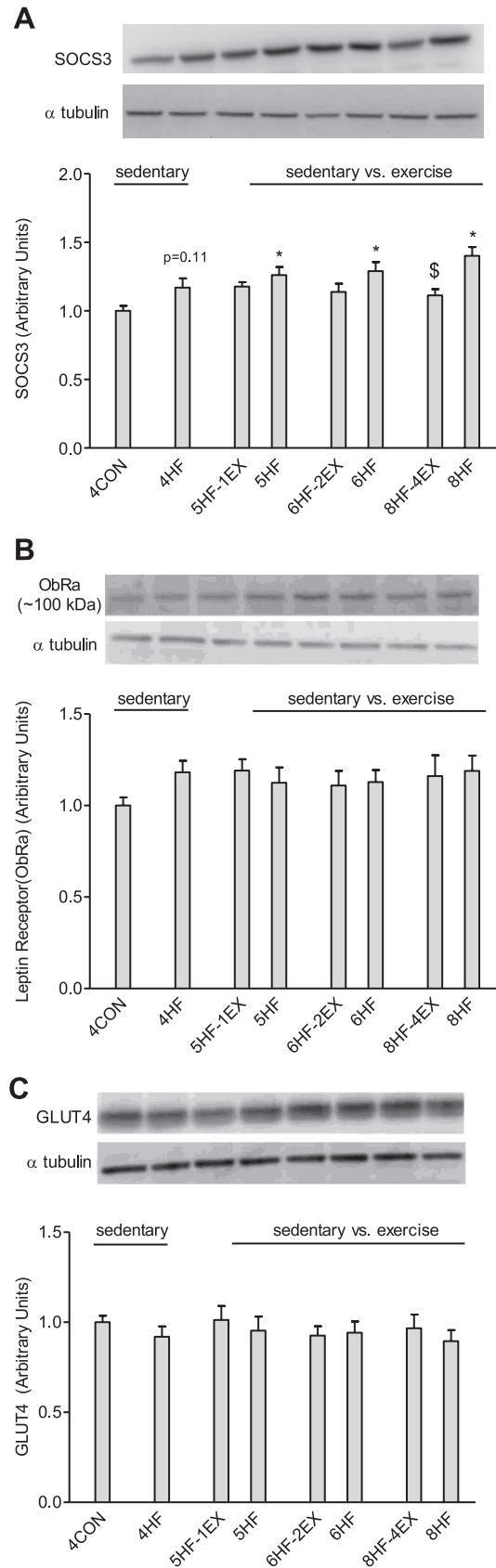


Fig. 6. The effect of HF feeding and exercise training on red gastrocnemius SOCS3 (A), leptin receptor (B), and GLUT4 (C) protein expression. Data are expressed as means  $\pm$  SE,  $n = 8-11$ . \*Significantly different from 4CON group,  $P \leq 0.05$ . \$Significantly different from corresponding HF-fed sedentary group,  $P < 0.005$ .

exist, we were only able to consistently detect the short form of the receptor (ObRa; ~100 kDa). HF feeding had no significant effect on leptin receptor protein content (Fig. 6B). There were no significant differences in whole muscle GLUT4 protein content between any of the groups (Fig. 6C).

## DISCUSSION

Leptin administration improves insulin sensitivity. However, very little work has examined whether improving the responsiveness of skeletal muscle to leptin, without further augmentation of leptin levels, can also improve insulin sensitivity. Therefore, we examined the time course of endurance training-mediated restoration of skeletal muscle insulin and leptin response in soleus muscle from HF-fed rats. The major findings of this study were that skeletal muscle insulin-stimulated glucose transport can be completely restored from a resistant state within 1 wk of endurance exercise training, while maintaining a HF diet; and endurance exercise also restores leptin's ability to stimulate FA oxidation and reduce FA esterification, but importantly, that this occurs after the restoration of insulin response. Interestingly, the loss and restoration of leptin response by HF feeding and exercise training were accompanied by significant increases and decreases in total SOCS3 protein content, but neither were related to changes in total muscle leptin receptor protein content. Overall, our data indicate that exercise training can rapidly reverse a state of leptin and insulin resistance initially evoked in a sedentary, HF-fed state. Contrary to our expectation, an improvement in leptin response did not occur first, and therefore may not be necessary for the observed restoration of insulin-stimulated glucose transport.

*Exercise intervention improves leptin response following HF feeding.* Leptin is well established as a regulator of skeletal muscle FA metabolism, acutely increasing the rate of FA oxidation and diverting FAs from storage (13, 14, 20–23). In vivo, chronic leptin infusion increases FA oxidation and lipolysis, resulting in lower intramuscular TAG content (21, 22). Furthermore, we have published evidence that leptin resistance precedes perturbations in skeletal muscle FA metabolism associated with IR induced by HF feeding (11). Surprisingly, very little work has examined the means by which to treat leptin resistance.

Here, we show that an endurance exercise intervention following HF diet-induced leptin resistance is able to improve leptin response (Fig. 7). The ability of leptin to acutely increase the rate of FA oxidation was restored between 1 and 2 wk of exercise, whereas the ability to acutely reduce the rate of

esterification to TAG or DAG occurred between 2 and 4 wk of exercise. These results are consistent with that of Steinberg et al. (19), who found that exercise could partially prevent the development of leptin resistance in rats when administered in conjunction with the feeding of a HF diet.

The stimulatory effects of leptin are believed to function through the phosphorylation of AMPK and ACC (17, 24) and through a decrease in malonyl CoA, which relieves inhibition on carnitine palmitoyltransferase. Consistent with others (7), we report a loss in the ability of leptin to stimulate the phosphorylation of AMPK and ACC following HF feeding. Concurrent with restoration of leptin's stimulatory effect on FA oxidation, we see a significant increase in the phosphorylation of AMPK and ACC. This suggests that the impairments in leptin signaling may be occurring upstream of AMPK and ACC.

*SOCS3 protein content is regulated by diet and exercise.* SOCS3 is increased in response to chronic leptin stimulation and forms a negative feedback loop with the leptin receptor (1, 5), inhibiting leptin receptor-associated JAK2 proteins, thereby inhibiting further leptin signaling (5). Interestingly, in addition to blocking leptin signal transduction, SOCS3 may also target the leptin receptor for degradation (33). SOCS3 expression (mRNA) is increased in human obesity (17) and following 4 wk of HF feeding in rodents (17, 19, 32), and SOCS3 protein content is elevated following 12 wk of HF feeding (32). In lean human myotubes, overexpression of SOCS3 impairs leptin response similar to that observed in myotubes derived from obese, leptin-resistant subjects (17). It has also been demonstrated that in addition to its inhibition of leptin signaling, SOCS3 can colocalize with IRS-1 and impair insulin signaling (32). Furthermore, it has recently been demonstrated that constitutively overexpressing SOCS3 in mice leads to insulin resistance, but only in conjunction with HF feeding (9). Leptin response was not assessed in this latter study.

This present investigation is the first to examine SOCS3 protein content in skeletal muscle during the early development of insulin and leptin resistance, and the restoration of these responses. Recently, Yaspelkis et al. (32) reported an increase in skeletal muscle SOCS3 protein content following 12 wk of HF feeding. We show a similar increase (~20%) in as little as 5 wk, concurrent with impairments in leptin response. Also, 4 wk of exercise training is sufficient to reduce SOCS3 protein content compared with the HF-fed sedentary controls. None of the exercise-trained groups demonstrated significantly elevated SOCS3 compared with controls. It is tempting to attribute the alterations in leptin response to SOCS3 protein content. How-

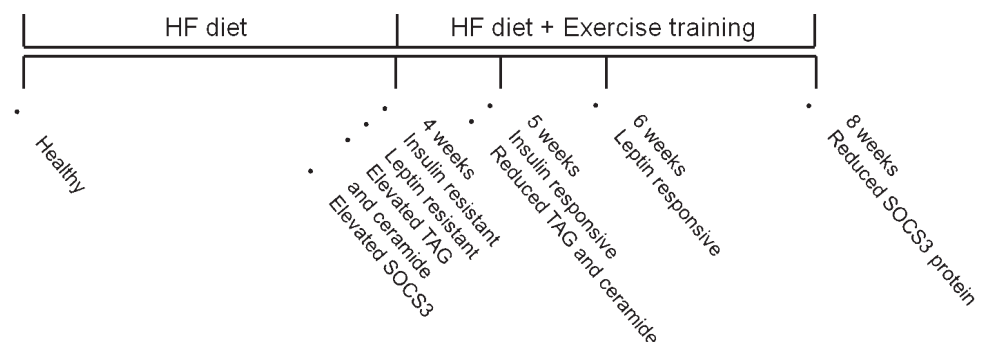


Fig. 7. Summary schematic of exercise training effects on HF-fed-induced insulin and leptin resistance in skeletal muscle.

ever, the increase in SOCS3 with HF feeding did not reach statistical significance (4 wk,  $P = 0.11$ ) until after our first reported impairment in leptin response. Also, although SOCS3 protein content in exercise-trained groups did not significantly differ from the control group, exercise did not significantly decrease SOCS3 protein relative to the HF sedentary condition until the 8-wk time point, 2 wk after the first observation of improved leptin response.

It is possible that it is the cellular location rather than the total amount that is of functional importance. In this regard, it was Yaspelkis et al. (32) who recently reported an increase in SOCS3 colocalization with IRS-1 and the insulin receptor at the plasma membrane in skeletal muscle following HF feeding. Here, the subcellular distribution of SOCS3 in skeletal muscle during states of leptin resistance, and specifically, its interaction with the leptin receptor, was not examined. Therefore, the most important changes during HF feeding and exercise, in terms of altering leptin response, may be the redistribution of SOCS3 from the cytosol to the sarcolemma rather than an increase in SOCS3 protein.

*Endurance exercise reduces intramuscular lipids and restores glucose transport before leptin response.* The major finding of this study was that 2 wk of exercise restored leptin response in leptin-resistant skeletal muscle, but not before maximally insulin-stimulated glucose transport was restored. At all time points, with both HF feeding and exercise training, we demonstrate that intramuscular lipid content and insulin resistance are correlated. Specifically, TAG and ceramide, but not DAG, were significantly increased with HF feeding and reduced within 1 wk of exercise training. Our data support previous work, implicating elevated ceramide with impaired insulin response and reduced ceramide with the restoration of insulin response (3, 26, 28). Ceramides directly activate protein phosphatase 2A, which, in turn, dephosphorylate and deactivate PKB, a central serine kinase in the insulin signaling pathway (25). Collectively, this suggests that an early reduction in ceramide is a potential mechanism for the improvement in insulin response. The primary purpose of this investigation was to determine whether leptin resistance could be reversed by exercise training and, if so, whether this would precede the restoration of insulin-stimulated glucose transport that is known to occur with exercise training (2, 10, 16). As such, identifying the specific impairments within the insulin signaling pathway, as induced by a HF diet was not a priority and has been previously assessed in similar studies (11).

We do not show an acute increase in FA oxidation with leptin stimulation (i.e., restoration) within the 1st wk of training. However, given the rapid (within 3 days) induction of adipokine resistance with HF feeding (11), it is possible that leptin response is improved with exercise, but that HF feeding is able to impair leptin signaling within the 48 h between the last exercise bout and the experimental procedures. It is impossible to know whether this potentially transient improvement in leptin response might have contributed to the observed increase in insulin-stimulated glucose uptake. Nonetheless, at the time of assessment in isolated muscle, an improved insulin-stimulated glucose uptake (after 1 wk of training) was not accompanied by an improved leptin response.

### Perspectives and Significance

Many studies examine the metabolic changes present in the advanced stages of obesity and diabetes. However, it is important to recognize that changes in the response of skeletal muscle to insulin and insulin-sensitizing adipokines, and the causative metabolic disturbances underlying these altered responses can occur very rapidly. This is the first study to examine the early time course of changes in both leptin and insulin response with exercise following HF feeding. Overall, our data indicate that training can rapidly reverse a state of leptin and IR initially evoked in a sedentary, HF-fed state. Contrary to our expectation, an improvement in leptin response did not occur first, and, therefore, may not be necessary for the observed restoration of insulin-stimulated glucose uptake. The impairments and restoration of insulin response were matched closely by significant increases and decreases in TAG and ceramide at all time points. Although this does not demonstrate a cause and effect, it does strongly suggest that ceramide content is an important determinant of insulin response in this case. In the case of exercise interventions, improved leptin signaling does not appear to be necessary for the restoration of insulin response. However, this does not rule out the possibility that improved leptin response may be more relevant to other interventions, such as the administration of pharmaceuticals or lower volume/intensity of endurance exercise. We are also the first to examine SOCS3 protein content during the restoration of leptin response. Although changes in SOCS3 content were generally predictive of altered leptin response in the soleus, the time course of these changes was not an exact match. The factors responsible for these alterations should be the topic of future research, but include examination of the interactions of SOCS3 with the leptin receptor.

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### DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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