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Chronic leptin treatment enhances insulin-stimulated glucose disposal in skeletal muscle of high-fat fed rodents

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Abstract

The aim of this investigation was to evaluate if chronic leptin administration corrects high fat diet-induced skeletal muscle insulin resistance, in part, by enhancing rates of glucose disposal and if the improvements are accounted for by alterations in components of the insulin-signaling cascade. Sprague–Dawley rats consumed normal (CON) or high fat diets for three months. After the dietary lead in, the high fat diet group was further subdivided into high fat (HF) and high fat, leptin treated (HF-LEP) animals. HF-LEP animals were injected twice daily with leptin (5 mg/100 g body weight) for 10 days, while the CON and HF animals were injected with vehicle. Following the treatment periods, all animals were prepared for and subjected to hind limb perfusion. The high fat diet decreased rates of insulin-stimulated skeletal muscle glucose uptake and glycogen synthesis in the red gastrocnemius (RG), but did not affect glycogen synthase activity, rates of glucose oxidation or nonoxidative disposal of glucose. Of interest, IRS-1-associated PI3-K activity and total GLUT4 protein concentration were reduced in the RG of the high fat-fed animals. Leptin treatment increased rates of insulin-stimulated glucose uptake and glucose oxidation, and normalized rates of glycogen synthesis. Leptin appeared to mediate these effects by normalizing insulin-stimulated PI3-K activation and GLUT4 protein concentration in the RG. Collectively, these data suggest that chronic leptin treatment reverses the effects of a high fat diet thereby allowing the insulin signaling cascade and glucose transport effector system to be fully activated which in turn affects the amount of glucose that is transported across the plasma membrane and made available for glycogen synthesis.
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Keywords: Glycogen synthesis; PI 3-kinase activity; GLUT-4 protein concentration

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Introduction

Leptin, the product of the ob gene (Zhang et al., 1994), has received a great deal of attention since its discovery in 1994 due to the ability of this 16kD protein hormone to reduce visceral adipose deposition (Halaas et al., 1995; Pellymounter et al., 1995) and improve insulin-stimulated glucose disposal in normal muscle (Barzilai et al., 1997; Sivitz et al., 1997; Wang et al., 1999; Yaspelkis et al., 1999). Of interest, we (Yaspelkis et al., 2001) recently demonstrated that chronic leptin administration increases insulin-stimulated glucose uptake and transport in insulin-resistant skeletal muscle and is related to the normalization of the GLUT4 protein concentration (Yaspelkis et al., 2001). However, whether a portion of these improvements might also be attributable to changes in skeletal muscle glucose disposal has not been reported. Therefore, the first aim of this investigation was to determine if chronic leptin administration corrects high fat diet-induced skeletal muscle insulin resistance, in part, by enhancing insulin-stimulated rates of glucose disposal. Leptin administration has been reported to improve IRS-1 associated phosphatidylinositol 3-kinase (PI3-K) activation in liver (Anai et al., 1998), IRS-2 associated PI3-K activation in C_2C_{12} muscle cells (Berti et al., 1997) and enhance insulin-stimulated association of the p85 subunit of PI3-K with IRS-1 in Fao cells (Szanto and Kahn, 2000). However, the effects of chronic leptin treatment on components of the insulin signaling cascade in skeletal muscle from high fat fed rats has not been extensively evaluated. Thus, a secondary aim of this investigation was to assess the effects of high fat diet and chronic leptin treatment on select components of the insulin signaling cascade.

Methods

Experimental animals

Twenty four male Sprague–Dawley rats approximately 6 weeks of age were obtained from ATL (Fremont, CA), and were randomly assigned into one of two groups: Control (CON, n = 8) and High-fat (HF, n = 16) groups. Control animals received a diet composed of normal fat content (17% fat derived calories, Cat# 112386 Dyets Inc., Bethlehem, PA) and High-fat animals ate a high fat content diet (59% fat derived calories, Cat# 112387, Dyets, Inc.). Both the control and high fat diet were provided in powder form. Rats were housed two per cage in a temperature-controlled animal room (21 °C) maintained on a 12-hour light-dark cycle. Animals were provided the respective diets and water ad libitum for 12 weeks. Following the 12-week period, the High-fat group was further subdivided into two groups: High-fat (HF, n = 8) and High-fat Leptin (HF-LEP, n = 8). The leptin group received leptin injections (5 mg/100 g body weight; Amgen, Inc., Thousand Oaks, CA) 2 × / day at 0800 h and 1700 h, while CON and HF animals received PBS injections for a 10 day experimental period. We have previously used this dosing regimen and found that serum leptin levels do not differ between CON and HF animals, but are significantly elevated in HF-LEP animals, but neither leptin nor high fat diet altered blood glucose, insulin or triglyceride concentrations (Yaspelkis et al., 2001). In addition, we did not include a control diet-leptin treated group as we have previously reported that differences in insulin-stimulated skeletal muscle glucose uptake and transport that exist between control diet and control diet-leptin treated animals are due to the effects of chronic leptin treatment, as opposed to weight loss (Yaspelkis et al., 1999).

All experimental procedures were approved by the Institutional Animal Care and Use Committee at California State University Northridge and conformed with the guidelines for the use of laboratory animals published by the U.S. Department of Health and Human Resources.

Hind limb perfusions

Following the experimental periods, all animals were prepared for hind limb perfusions following an ~ 6 h fast. Animals were anesthetized with an intraperitoneal injection of sodium pentobarbital (6.5mg/100g body weight). The surgical technique to prepare the rats for perfusion and perfusion apparatus were similar to those described previously by Ruderman et al. (1971), and modified by Ivy et al. (1989). Just prior to catheterization, the plantaris (Plant) and portions of the red (RG) and white (WG) gastrocnemius were excised from the non-perfused left leg and stored at − 80 °C until analysis. In the non-perfused RG and WG, total IRS-1, Akt, GLUT4 protein concentrations and glycogen content were assessed. In the non-perfused RG, the concentration of the p85, p110α and p110β subunits of PI3-Kinase were assessed. After the skeletal muscle was excised from the non-perfused leg, the right iliac artery was catheterized to the tip of the femoral artery to limit the perfusate flow to the right hind limb. Catheterization of the lower abdominal vena cava to the tip of the iliac vein permitted the collection of effluent perfusate.

Immediately after catheterization, the rats were sacrificed via an intracardiac injection of pentobarbital while the hind limbs were washed out with 20 mL of Krebs–Heinseleit Buffer (KHB). The catheters were then placed in line with a non-recirculating perfusion system, and the hind limb was allowed to stabilize during a 5 min washout period. The perfusate flow rate was set at 5 mL/min during the 5 min stabilization period and subsequent perfusion. The basic perfusate medium consisted of 30% washed time-expired human erythrocytes (Ogden Medical Center, Ogden, UT), KHB (pH 7.4), 4% dialyzed bovine serum albumin (Fraction V; Fisher Scientific, Fair Lawn, NJ), and 0.2 mM pyruvate. The perfusate was continuously gassed with a mixture of 95% O_2 − 5% CO_2 and warmed to 37 °C. Perfusions were performed in the presence of a maximally effective insulin concentration (10 mU/mL) for all experimental groups. Following the stabilization period, the perfusate was changed to one containing 8 mM glucose (0.25 μCi/ml D-[^{14}C(U)] glucose, PerkinElmer Life Sciences, Boston, MA). The [^{14}C] glucose was added to determine glucose uptake, the rates of glucose incorporation into glycogen and glucose oxidation. At the completion of the 25 min perfusion, portions of the RG and WG were removed, blotted on gauze dampened with cold KHB, clamp frozen in liquid N_2 and stored at − 80 °C until analysis of glucose incorporation into glycogen, PI3-K activity, phosphorylated Akt (pAkt) content, and IRS-1 tyrosine phosphorylation (pY-IRS-1).

Determination of glucose uptake and disposal

For determination of glucose uptake, 0.2 ml of perfusate samples were taken from the arterial perfusate and well-mixed venous effluent, deproteinized in 0.8 ml of 10% TCA and centrifuged in a microcentrifuge for 10 min. From the supernatant, 0.3 ml samples were transferred to 7 ml scintillation vials containing 6 ml of Bio-Safe II scintillation counting cocktail (Research Products International, Mount Prospect, IL) and counted for radioactivity. Muscle glucose uptake was calculated from the arteriovenous difference, the perfusate flow rate and the weight of the muscle perfused.

The rate of glucose oxidation was determined from the arteriovenous difference in $^{14}CO_2$ and flow rate. To determine the $^{14}CO_2$ content of the arterial and venous perfusate, duplicate 2.5 ml samples of arterial perfusate were obtained from the perfusate reservoir and 2.5 ml samples of venous effluent samples were obtained by syringe through a rubber adapter on the venous line at 22 min of perfusion. The perfusate samples were immediately injected into 25 ml flasks fitted with serum caps and hanging center wells. The perfusate samples were acidified with 0.5 ml of 1.0 M acetic acid and the released $^{14}CO_2$ trapped on a strip of filter paper located in the center well soaking in 0.4 ml of Solvable (Packard BioScience Company, Meriden, CT). The acidified perfusate was allowed to stand overnight. The filter paper strips and Solvable were then transferred to a scintillation vial containing 6 ml of Bio-Safe II and radioactivity was determined by scintillation spectrophotometry.

Lactate concentration was determined from samples obtained from the arterial reservoir and venous effluent, which was collected on ice during the 25 min perfusion. The samples were deproteinized in 8% perchloric acid and centrifuged. Lactate was determined on the acid extracts according to the method of Hohorst et al. (1965). Lactate accumulation was calculated from the arteriovenous difference, perfusate flow rate and the weight of the muscle perfused.

The rate of [^{14}C]glucose incorporated into glycogen was determined by isolating total muscle glycogen as described by Lo et al. (1970). The muscles were weighed frozen and then placed into test tubes containing 1 ml of 30% KOH saturated with Na_2SO_4 and digested by incubating the tubes for 30 min at 100 °C. After the 30 min incubation, the tubes were cooled to room temperature, 1.2 vol of 95% ethanol was added and the glycogen was allowed to precipitate overnight at −20 °C. The glycogen was pelleted by 30 min of centrifugation (1200 × g at 4 °C) and resuspended in distilled H_2O. A 400 μL aliquot of the suspension was transferred to a scintillation vial containing 6 ml of Bio-Safe II, and radioactivity was determined.

IRS-1, Akt, and GLUT4 protein concentration

Muscles were weighed, and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) run under reducing conditions as described elsewhere (Singh et al., 2003). Antibody binding was visualized using chemiluminescence in accordance to the manufacturer's instructions (West Femto, Pierce Chemical Company, Rockford, IL). Images were captured with a ChemiDoc system (BioRad) and saved to a Macintosh G4 computer. Bands were quantified with Quantity One analysis software (BioRad). The density of the bands was calculated as a percentage of a standard (100 μg protein of a muscle sample) run on each gel.

Plasma membrane GLUT4 protein concentration

Plasma membrane fractions were prepared as described previously (Yaspelkis et al., 2001). Aliquots of the plasma membrane (35 μg of protein) were treated with Laemmli sample buffer and subjected to SDS-PAGE run under reducing conditions on a 10% resolving gel. Resolved proteins were transferred to PVDF membranes as described previously and incubated with affinity labeled polyclonal αGLUT-4 (1:1,000), followed by incubation with HRP-labeled protein A (1:10,000). The bands were visualized and captured as described above. The density of the bands was expressed as a percentage of a heart standard (15 μg of heart protein) run on each gel.

IRS-1 associated PI3-kinase activity

Between 100–150 mg of insulin-stimulated muscle tissue was weighed frozen and homogenized in an ice-cold homogenization buffer (1:10 wt/vol) containing 50 mM Hepes (pH 7.6), 150 mM NaCl, 200 mM sodium pyrophosphate, 20 mM β-glycerophosphate, 20 mM NaF, 2 mM sodium vanadate, 20 mM EDTA (pH 8.0), 1% IGEPAL, 10% glycerol, 2 mM phenylmethylsulfonyl flouride, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 μg/ml leupeptin, and 10 μg/ml aprotinin. Muscles were homogenized using a glass Pyrex homogenizer, then centrifuged at 12,000 rpm at 4 °C for 10 min. The supernatant was extracted, quantified for protein content by the Bradford method (Bradford, 1976) and IRS-1 associated PI3-kinase activity was assessed as described elsewhere (Singh et al., 2003). After a 1 h separation of the phosphoinositides on a TLC plate, the plates were dried, exposed to a storage phosphor screen (Cat# 8700, Eastman Kodak Company, Rochester, NY), the screen was scanned with a phosphor imager (Personal Molecular Imager FX System, BioRad) and the image was imported into a Macintosh G4 computer. Quantification was performed using Quantity One analysis software (BioRad). Kinase activity was calculated as a percentage of an insulin-stimulated muscle standard run on each plate.

IRS-1 tyrosine phosphorylation (pY-IRS-1)

pY-IRS-1 content was determined in aliquots of the RG samples prepared for IRS-1 associated PI3-Kinase activity. Five hundred micrograms of sample protein was immunoprecipitated with 6 μg of αIRS-1 (Cat# 06-248, Upstate Biotechnology) overnight at 4 °C. Protein A-Sepharose beads were prepared as described for PI3-Kinase. After the overnight incubation, a slurry of 200 μL Protein A-Sepharose was added to the αIRS-1 immunoprecipitates for 1.5 h at 4 °C with rotation. The immunocomplex was washed successively with buffer A, buffer B and buffer C (see *IRS-1 associated PI3-kinase activity Methods*). Washing was performed once with each buffer. Sample buffer containing 250 mM Tris HCl (pH 6.8), 10% SDS, 10% β-mercaptoethanol, 40% glycerol, 0.01% Bromophenol Blue) was diluted (1:4) in PI3-K buffer and added to each sample and boiled at 100 °C for 5 minutes. Samples were then centrifuged at 10,000 rpm for 4 minutes. Ten microliters of supernatant was subjected to SDS-PAGE run under reducing conditions on a 7.5% resolving gel. The resolved proteins were then transferred to PVDF membrane as previously described. The membranes were incubated with affinity purified polyclonal αpY (1:1,000; Cat# 06-427, Upstate Biotechnology) overnight followed by 1.5 hours of incubation with goat anti-rabbit IgG conjugated to HRP (1:20,000; sc-2004, Santa Cruz Biotechnology). Antibody binding was visualized and quantified as described above. The density of the bands was calculated as a percentage standard (i.e., an insulin-stimulated muscle sample treated the same as the samples and ran on each gel).

PI 3-kinase subunit (p85α, p110α and p110β) concentration

Portions of the RG from the non-perfused leg were weighed frozen and homogenized in ice-cold PI 3-Kinase homogenization buffer as described in *IRS-1 associated PI3-kinase activity Methods*. Three hundred micrograms of lysate protein were immunoprecipitated overnight at 4 °C with 4 μg of either polyclonal anti-p85α (Cat# 06-248, Upstate Biotechnology), anti-p110α (sc-

7174, Santa Cruz Biotechnology) or anti-p110β (sc-602, Santa Cruz Biotechnology). Immunocomplexes were collected as described above in *pY-IRS-1 Methods*. Twenty microliters of the supernatant was subjected to SDS-PAGE run under reducing conditions on a 7.5% resolving gel. Proteins were transferred to PVDF membranes as previously described and blocked in 5% NFDM for one hour at room temperature. Membranes were then incubated with either polyclonal anti-p85α (1:1,000; Cat# 06-248, Upstate Biotechnology), anti-p110α (1:1,000; sc-7174 Santa Cruz Biotechnology) or anti-p110β polyclonal antibody (1:1,000; sc-602 Santa Cruz Biotechnology) overnight at 4 °C. Membranes were washed twice for 10 minutes using TTBS and incubated with goat anti-rabbit IgG conjugated to HRP (1:20,000, sc-2004, Santa Cruz Biotechnology) for 1.5 hours at room temperature. Antibody binding was visualized and quantified as described above. The density of the bands was calculated as a percentage of a standard muscle sample run on each gel.

Akt phosphorylation

Aliquots of samples that were prepared for IRS-1 associated PI3-Kinase activity were also used for determination of Akt phosphorylation (pAkt) content. One hundred micrograms of sample protein was subjected to SDS-PAGE run under reducing conditions on a 7.5% resolving gel. The resolved proteins were transferred to PVDF membrane as previously described and blocked in 5% NFDM/TTBS. The membranes were incubated with affinity purified polyclonal αpAkt (sc-7985, Santa Cruz Biotechnology) followed by incubation with HRP-labeled Protein A (Amersham Life Sciences). Antibody binding was visualized and quantified as described above. The density of the bands was calculated as percentage of the standard (100 μg protein of an insulin-stimulated muscle sample) run on each gel.

Glycogen concentration

Muscle glycogen concentration was determined as previously described (Yaspelkis et al., 2001).

Glycogen synthase activity

Assessment of glycogen synthase activity in the red gastrocnemius was based on the procedure of Thomas et al. (1968) as modified by Thorburn et al. (1990). Muscle samples (50 mg) were homogenized at 4 °C with an extraction buffer at pH 7.4 (2 mM EDTA, 2 mM 1,4-dithiolthreitol, 20 mM NaF, 50 mM KH_2PO_4, and 50 mM K_2HPO_4. After centrifugation (20,000 g for 20 min), 0.5 mL of the supernatant was added to 2.0 mL of synthase buffer at pH 7.8 (20 mM EDTA, 25 mM NaF, 50 mM Tris-HCl). Glycogen synthase was determined in this diluted extract by measuring the incorporation of radioactive [^{14}C]glucose from uridine 5′-diphosphate glucose (UDPG) into glycogen. To assay glycogen synthase activity, 30 μL of the extract was added to 60 μL of a reaction mixture consisting of 0.03 μCi of uridine diphospho-D-[U-^{14}C]glucose (PerkinElmer, Boston, MA), 1% glycogen, 0.3 mM UDPG and varying concentrations of glucose-6-phosphate (G6P; 0, 5, or 10 mM). After incubating at 30 °C for 30 minutes the reaction was stopped by spotting 75 μL of each sample onto filter paper and washing two times in 66% ethanol (two 20-min periods) before drying in acetone. ^{14}C was then counted in a scintillation spectrophotometer. Protein

Table 1
Glucose uptake, glycogen synthesis, glucose oxidation and lactate accumulation during 25 min perfusion with 10 mU/ml insulin and 8 mM glucose

	CON	HF	HF-LEP
Glucose Uptake, μmol/g/h	22.68 ± 0.47	20.35* ± 0.51	23.87*,† ± 0.44
Glycogen Synthesis, μmol/g/h			
RG	16.02 ± 1.2	13.01* ± 0.2	18.06† ± 0.7
WG	4.16 ± .47	4.71 ± .50	4.63 ± .45
Glucose Oxidation, nmol/g/h	106.8 ± 6.2	102.15 ± 10.0	154.02*,† ± 11.6
Lactate Accumulation, μmol/g/h	4.54 ± 0.32	3.39 ± 0.29	3.95 ± 0.60

Values are means ± SE. CON, control; HF, High-fat diet; HF-LEP, High fat diet-Leptin treated. RG; red gastrocnemius, WG; white gastrocnemius.

* Significantly different from CON ($p < 0.05$).

† Significantly different from HF ($p < 0.05$).

was also assayed in the extract so those units of synthase activity could be expressed as nanomoles of [^{14}C]UDPG incorporated into glycogen per minute per milligram of protein.

Results are expressed in the form of activity ratio and fractional velocity. The activity ratio represents the percentage of enzyme in the I form; the percentage of the enzyme that is active in the absence of glucose 6-phosphate. The fractional velocity was determined by dividing the activity of the enzyme obtained in the presence of a submaximal glucose 6-phosphate concentration (5 mM) by the activity obtained in the presence of saturating levels of glucose 6-phosphate (10 mM) after background (0 mM) was subtracted from each sample. The fractional velocity is a measure of the enzyme's sensitivity to activation by glucose 6-phosphate.

Statistics

A one-way analysis of variance was used on all variables to determine whether significant differences existed between groups. When a significant *F* ratio was obtained, a Fisher's protected least significant difference post hoc test was employed to identify statistically significant differences ($p < 0.05$) among the means.

Table 2
Muscle glycogen concentration and glycogen synthase activity

	CON	HF	HF-LEP
Muscle Glycogen Concentration, μmol/g wet wt			
RG	33.61 ± 0.7	31.41 ± 2.2	29.94 ± 1.7
WG	36.91 ± 1.2	33.44 ± 1.2	33.30 ± 2.2
Glycogen Synthase Activity, %			
Activity ratio	42.1 ± 6.2	35.3 ± 5.6	26.2 ± 1.1
Fractional velocity	66.9 ± 10.1	63.2 ± 6.6	49.1 ± 9.1

Values are means ± SE. CON, control; HF, High-fat diet; HF-LEP, High fat diet-Leptin treated. RG; red gastrocnemius, WG; white gastrocnemius.

Results

Body and epididymal fat pad mass

At Day 0 of the experimental treatment period, the body mass of the CON (496.8 ± 12.1 g), HF (499.7 ± 11.3 g) and HF-LEP (505.8 ± 12.2 g) animals were similar. At the end of the 10 d

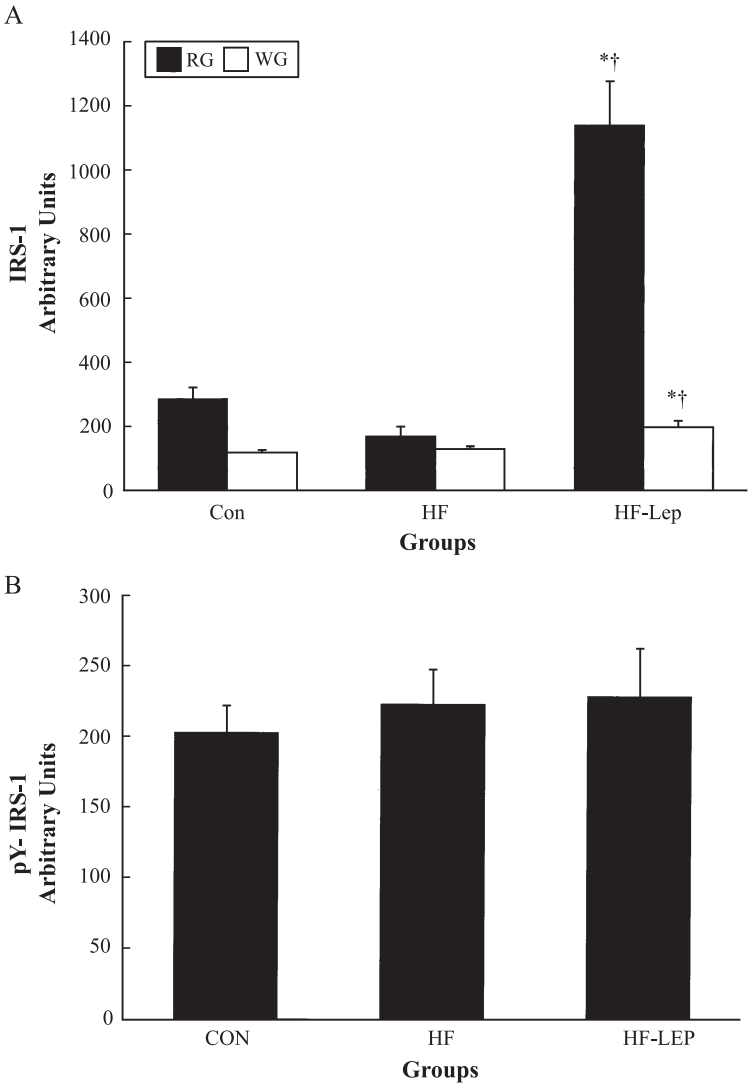

Fig. 1. **A.** Skeletal muscle IRS-1 protein concentration. **B.** Insulin-stimulated IRS-1 tyrosine phosphorylation in red gastrocnemius. Control (CON), High fat diet (HF), and High fat diet-leptin treated (HF-LEP). *, Significantly different from CON ($p < 0.05$). †, Significantly different from HF ($p < 0.05$). RG, red gastrocnemius; WG, white gastrocnemius. Values are means ± SE.

treatment period, the change in body mass of the HF-LEP (− 6.24 ± 1.6 g) animals was significantly different ($p < 0.05$) from that of the CON (6.16 ± 3.6 g) and HF (9.00 ± 3.9 g) animals. However, epididymal fat pad mass was similar among the CON (9.81 ± 1.0 g), HF (11.73 ± 1.0 g) and HF-LEP (11.98 ± 1.0 g) animals.

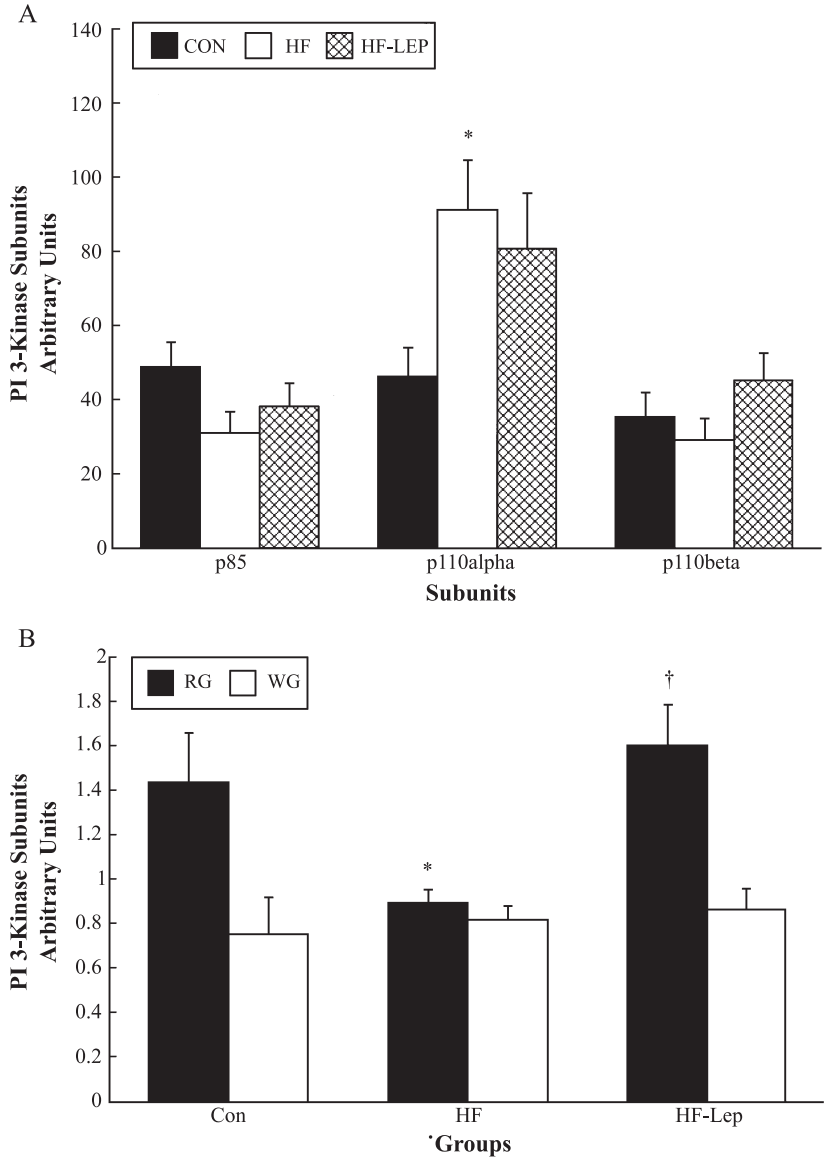

Fig. 2. **A.** PI3-K subunit concentration in red gastrocnemius muscle. **B.** Insulin-stimulated IRS-1-associated PI3-Kinase Activity. Control (CON), High fat diet (HF), and High fat diet-leptin treated (HF-LEP). *, Significantly different from CON ($p < 0.05$); †, significantly different from HF-LEP ($p < 0.05$). RG, red gastrocnemius; WG, white gastrocnemius. Values are means ± SE.

Glucose disposal

Rates of insulin-stimulated glucose uptake were significantly decreased in the hind limb skeletal muscles of the HF animals compared to CON animals (Table 1). Rates of glucose uptake were significantly elevated in the HF-LEP animals compared to both CON and HF animals. While rates of glucose oxidation were similar in the CON and HF animals, HF-LEP animals exhibited rates of glucose oxidation greater than that of CON and HF animals (Table 1). The CON and HF-LEP animals had

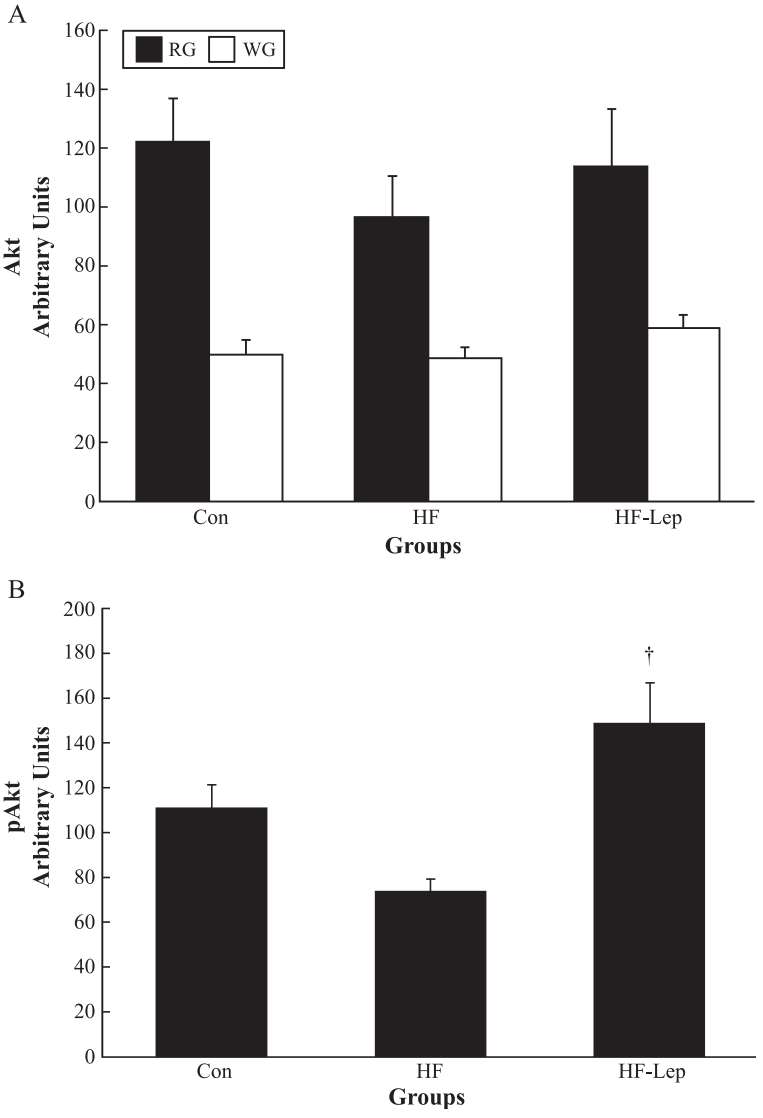

Fig. 3. **A.** Skeletal muscle Akt protein concentration. **B.** Phosphorylated Akt concentration in insulin-stimulated red gastrocnemius. †, significantly different from HF-LEP ($p < 0.05$). RG, red gastrocnemius; WG, white gastrocnemius. Values are means ± SE.

greater rates of glycogen synthesis in the RG compared to the HF animals, but did not exhibit alterations in glycogen synthesis in the WG (Table 1). Lactate accumulation was similar among groups (Table 1).

Differences in muscle glycogen synthesis could not be accounted for by alterations in muscle glycogen concentration or glycogen synthase activity (Table 2).

Insulin signaling cascade

IRS-1 protein content in the RG and WG was not different between the CON and HF groups, but leptin treatment significantly increased IRS-1 protein concentration (Fig. 1A). However, insulin-stimulated IRS-1 tyrosine phosphorylation in the RG was not different among groups (Fig. 1B).

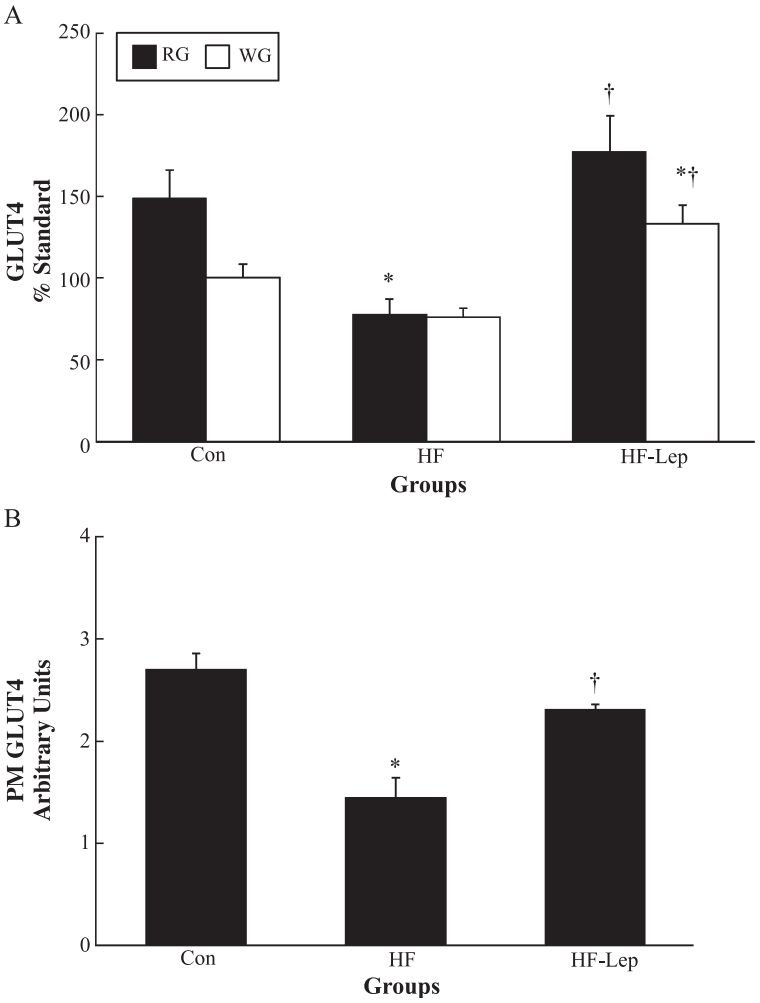

Fig. 4. **A.** Total skeletal muscle GLUT4 protein concentration. **B.** Insulin-stimulated plasma membrane GLUT4 protein concentration. *, Significantly different from CON ($p < 0.05$); †, significantly different from HF-LEP ($p < 0.05$). RG, red gastrocnemius; WG, white gastrocnemius. Values are means ± SE.

The concentration of the PI3-K subunits, p85 and p110β, were not different in the RG among groups (Fig. 2A). Of interest, p110α concentration was significantly greater in the RG of the HF compared to CON and a trend existed ($p = 0.06$) for p110α to also be elevated in the HF-LEP animals in comparison to the CON animals. IRS-1 associated PI 3-K activity was significantly reduced in the RG of the HF group compared to both CON and HF-LEP animals (Fig. 2B). Ten days of leptin treatment normalized IRS-1 associated PI3-K activity in the RG such that no differences existed between CON and HF-LEP groups. PI3-K activity in the WG was similar among groups.

Total Akt protein concentration in the RG and WG was not different among groups (Fig. 3A). Insulin-stimulated Akt phosphorylation in the RG was similar in the CON and HF animals, and in the CON and HF-LEP animals (Fig. 3B). Despite similar levels of protein expression, insulin-stimulated Akt phosphorylation in the RG of the HF group was reduced compared to the HF-LEP group (Fig. 3B).

The high fat diet significantly reduced the total GLUT4 protein concentration in the RG of the HF animals when compared to the CON animals (Fig. 4A). Leptin treatment reversed the effects of the high fat diet as it was found that total GLUT4 protein concentration in the RG was similar between the CON and HF-LEP animals. Although the high fat diet had no effect on the total GLUT4 protein concentration in the WG, leptin treatment was found to elevate the total GLUT4 protein concentration in the WG.

Insulin-stimulated plasma membrane GLUT4 protein concentration from the perfused quadricep was significantly lower in the HF animals compared to the CON animals (Fig. 4B). In contrast, insulin-stimulated plasma membrane GLUT4 protein concentration was normalized in the HF-LEP animals. Plasma membrane fractions were purified in comparison to the crude homogenates as determined by 5′-nucleotidase activity (μmol/min/mg protein) (CON: 127.2 ± 14.2 vs. 526.8 ± 38.9; HF: 119.1 ± 15.7 vs. 541.4 ± 8.7; HF-LEP: 122.4 ± 9.6 vs. 543.9 ± 19.5).

Discussion

We (Yaspelkis et al., 1999) had previously attributed leptin-induced improvements on insulin-stimulated 3-MG transport in normal skeletal muscle to an increased GLUT4 protein concentration, as it has been reported that there is a direct relationship between the total GLUT4 protein concentration and rates of 3-MG transport (Banks et al., 1992; Brozinick et al., 1993; Friedman et al., 1990). Subsequently, we found that chronic leptin treatment normalizes the total GLUT4 protein concentration in the skeletal muscle of high fat-fed insulin-resistant rodents (Yaspelkis et al., 2001). However, alterations in skeletal muscle GLUT4 protein concentration may not fully account for impairments induced by high fat-diet and the reversal of high fat-diet effects by chronic leptin administration. We hypothesized that high fat feeding and leptin treatment may be affecting glucose disposal in skeletal muscle and additionally, modifications in components of the insulin-signaling cascade may contribute to the alterations in skeletal muscle carbohydrate metabolism.

To confirm that the high fat diet induced skeletal muscle insulin resistance, we initially evaluated rates of insulin-stimulated glucose uptake and found hind limb glucose uptake to be reduced in HF animals and normalized in the HF-LEP animals compared to the CON group. This finding is in excellent agreement with previous investigations that have assessed the effects of high fat diet (Barnard et al., 1998; Buettner et al., 2000; Halseth et al., 2000; Hansen et al., 1998; Kraegen et al., 1986; Wilkes et al., 1998; Yaspelkis et al., 2001) and chronic leptin treatment (Singh et al., 2003; Yaspelkis et al., 2001) on

insulin-stimulated glucose uptake and 3-MG transport in rodent skeletal muscle. In an extension to these findings, we observed that while a high fat diet did not affect rates of glucose oxidation and lactate accumulation, the rates of insulin-stimulated glycogen synthesis were impaired in the RG of the HF animals. Furthermore, we found that chronic leptin administration, in addition to normalizing rates of glycogen synthesis in the RG of the HF-LEP animals, substantially increased rates of insulin-stimulated glucose oxidation, but did not alter lactate accumulation. In light of these observations, the question arises whether the differences in glucose uptake contributed to the altered rates of glycogen synthesis and oxidation or vice versa.

A plausible explanation for the reduced rates of glycogen synthesis in the HF animals could be due to decreased activation of glycogen synthase. However, when we evaluated glycogen synthase activity no differences were found among groups and, moreover, muscle glycogen levels were similar among the groups. Thus, under our experimental conditions it does not appear that differences in glycogen synthase activity or muscle glycogen concentration could account for the reduced or normalized rates of glycogen synthesis in the HF and HF-LEP animals, respectively. This raises the possibility that the differences in rates of glycogen synthesis among the experimental animals might be a function of glucose availability and that the ability to transport glucose across the plasma membrane has direct effects on the amount of glucose available for glycogen synthesis as hypothesized by Fisher et al. (Fisher et al., 2002).

Reduced rates of insulin-stimulated glucose transport in the skeletal muscle of high-fat fed rodents have been attributed to a reduced total GLUT4 protein concentration and/or an inability to effectively translocate glucose transporters to the plasma membrane (Hansen et al., 1998; Tremblay et al., 2001; Yaspelkis et al., 2001). Consistent with these observations, we again (Yaspelkis et al., 2001) report that both total and insulin-stimulated plasma membrane GLUT4 protein concentration were reduced in the skeletal muscle of the HF animals and normalized by chronic leptin administration. However, as previously stated, it is unlikely that differences in GLUT4 protein concentration solely account for altered rates of glucose uptake and that the high fat diet and chronic leptin treatment likely affected components of the insulin-signaling cascade as well.

Although the high fat diet did not affect IRS-1 protein concentration, IRS-1 insulin stimulated tyrosine phosphorylation or Akt protein concentrations, it did decrease insulin-stimulated PI3-K activity, which is consistent with the findings of other investigations (Krook et al., 2000; Krook et al., 1998; Song et al., 1999; Tremblay et al., 2001; Zierath et al., 1997). Of interest, we observed the p110α subunit of PI3-K to be elevated in the HF animals and while unknown if this has functional significance, it might represent a compensatory mechanism for reduced PI3-K activity. Activation of Akt, a downstream target of PI3-K, has been reported to be reduced (Krook et al., 1998; Song et al., 1999; Tremblay et al., 2001) or unchanged (Cho et al., 2001; Kim et al., 1999; Kruszynska et al., 2002; Nadler et al., 2001; Singh et al., 2003) in various models of insulin resistance. We have previously reported that a high fat diet does not alter insulin-stimulated (0.5 mU/mL) Akt activation (Singh et al., 2003) and we report here that Akt phosphorylation is not reduced in HF animals even when a maximal insulin concentration (10 mU insulin/mL) is used in the perfusate.

As the high fat diet impaired components of the insulin-signaling cascade, it was possible that the leptin-induced improvements on insulin-stimulated glucose uptake, glycogen synthesis and glucose oxidation may have been a result of alterations in the insulin signaling cascade. Although insulin-stimulated IRS-1 tyrosine phosphorylation was unaffected by leptin treatment, IRS-1 protein concentration was increased, while IRS-1-associated PI3-K activity and Akt activation were normalized in the RG of the HF-LEP animals. However, it is possible that PI3-K activity and Akt activation may have been

normalized due to leptin directly activating PI3-K through leptin activation of IRS-2 which may couple the leptin signaling pathway to the insulin signaling cascade (Kellerer et al., 1997).

Nevertheless, the mechanism by which chronic leptin treatment mediated these effects remains to be fully elucidated. We have previously reported that a high fat diet increases and chronic leptin administration reduces intramuscular triglyceride (IMTG) levels (Singh et al., 2003; Yaspelkis et al., 2001, 2002) and although we did not assess IMTG in the present investigation we suspect that the modifications in IMTG levels would be consistent with our previous observations. Of particular interest, leptin administration has been found to alter skeletal muscle metabolism by shifting lipid storage to fat oxidation (Muoio et al., 1997) and decreasing fatty acid translocase (FAT) and fatty acid binding protein (FABP) (Steinberg et al., 2002), both of which could decrease intramuscular triglyceride content. A reduction in skeletal muscle triglyceride levels has been shown to improve whole body glucose tolerance (Koyama et al., 1997) and it is therefore plausible to suggest that by altering IMTG levels rates of insulin-stimulated skeletal muscle glucose disposal could be enhanced.

Conclusion

A high fat diet decreased rates of insulin-stimulated skeletal muscle glucose uptake and glycogen synthesis, but did not affect rates of glucose oxidation or nonoxidative disposal of glucose. In addition, the high fat diet did not impair glycogen synthase activation. However, IRS-1-associated PI3-K activity and total GLUT4 protein concentration was reduced in the RG of the high fat-fed animals. Leptin treatment increased rates of insulin-stimulated glucose uptake and glucose oxidation, while rates of glycogen synthesis were normalized. It appeared that leptin mediated these effects by normalizing the total skeletal muscle GLUT4 protein concentration and insulin-stimulated PI3-K activation. Collectively, these data suggest that chronic leptin treatment reverses the effects of a high fat diet thereby allowing the insulin signaling cascade and glucose transport effector system to be fully activated which in turn affects the amount of glucose that is transported across the plasma membrane and made available for glycogen synthesis.

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