Development of Whole-Body and Skeletal Muscle Insulin Resistance After One Day of Hindlimb Suspension

Matthew P. O'Keefe, Felipe R. Perez, Tyson R. Kinnick, Marc E. Tischler, and Erik J. Henriksen

Hindlimb suspension (HS) of rats is a model of simulated weightlessness and induces dynamic alterations in insulin action. In the present study, the effect of acute (1-day) HS on whole-body glucose tolerance and insulin action on skeletal muscle glucose transport was assessed in juvenile, female Sprague-Dawley rats. Compared to weight-bearing control rats, 1-day HS animals displayed significantly decreased glucose tolerance and diminished whole-body insulin sensitivity. Glucose transport activity in the 1-day unweighted soleus muscle was significantly decreased ($P < .05$) compared to weight-bearing control muscles both in the absence and presence of insulin (2 mU/mL). Insulin-mediated glucose transport activity in the extensor digitorum longus (EDL) muscles also tended ($P = .09$) to be lower. There was no change in the protein expression of insulin receptor β-subunit (IR-β), insulin receptor substrate-1 (IRS-1), IRS-2, the p85 subunit of phosphatidylinositol-3 kinase (PI3-kinase), Akt, and glucose transporter protein 4 (GLUT-4). The activities of these proteins were also unchanged, as insulin-stimulated IR-β tyrosine phosphorylation, IRS-1 tyrosine phosphorylation, IRS-1-associated p85, and Akt serine phosphorylation were similar to controls. However, basal Akt phosphorylation was significantly depressed ($P < .05$) in the 1-day HS soleus. In addition, the protein expression and basal phosphorylation of the stress-activated p38 mitogen-activated protein kinase (p38 MAPK) were significantly elevated ($P < .05$) in the 1-day unweighted soleus. These results indicate that the development of insulin resistance in the 1-day unweighted soleus is not due to impaired functionality of elements involved in the IR/IRS-1/PI3-kinase/Akt signaling pathway. However, activation of p38 MAPK may play a role in this response.

UNWEIGHTING of the rat soleus muscle by hindlimb suspension (HS) has been used as an earth-based model of simulated weightlessness and induces numerous functional and metabolic adaptations.[1,2] Unweighting results in rapid muscle atrophy.[1] The development of significant muscle atrophy and growth failure with prolonged unweighting is accompanied by an enhancement of insulin action on glucose transport activity in the soleus.[3-7]

The metabolic consequences of acute (24 hours or less) HS is less well characterized. One day of soleus unweighting causes a decrease in both basal and insulin-stimulated glucose transport activity,[8] with corresponding decreases in glycogen synthesis and glucose oxidation,[8] indicating the development of an insulin-resistant state. However, the potential underlying cellular mechanism for this acute response to soleus unweighting has not been elucidated. Moreover, no study to date has evaluated the whole-body response of insulin sensitivity and glucose metabolism to an acute episode of HS.

Glucose transport activity in muscle is acutely regulated by insulin through the activation of a series of intracellular proteins.[9,10] Insulin binding enhances the tyrosine kinase activity of the insulin receptor β-subunits, and the activated insulin receptor then phosphorylates insulin receptor substrates (primarily IRS-1 in skeletal muscle) at conserved pYXXM sequences. Tyrosine-phosphorylated IRS-1 can then dock with the SH2 domains of the p85 regulatory subunit of phosphotidylinositol-3-kinase (PI3-kinase), thereby activating the p110 catalytic subunit of this enzyme. PI3-kinase catalyzes the production of phosphoinositide moieties, which can subsequently activate 3-phosphoinositide-dependent kinases (PDK). A downstream target of PDK is Akt, a serine/threonine kinase. Several lines of investigation support a role of Akt in the regulation of glucose transport,[11-13] although other studies indicate that Akt is not involved in this process.[14] The activation of these steps ultimately results in the translocation of the glucose transporter protein 4 (GLUT-4) isoform to the sarcolemmal membrane, where glucose transport takes place via a facilitative diffusion process. In addition, the stress-activated serine/threonine kinase, p38 mitogen-activated protein kinase (p38 MAPK) may negatively interact with upstream aspects of the insulin signaling cascade, particularly IRS-1, inducing insulin resistance.[15] Whether the regulation of these insulin signaling factors is affected by acute HS is currently not known.

In the context of the foregoing information, the purposes of the present investigation were (1) to determine the whole-body response of glucose tolerance and insulin sensitivity to 1 day of HS of juvenile, female Sprague-Dawley rats, and (2) to assess whether the previously observed insulin resistance of the acutely unweighted soleus[8] from these animals is associated with an impairment of the protein expression and functionality of specific insulin signaling factors, including the insulin receptor, IRS-1, PI3-kinase, and Akt, or with activation of the stress-activated p38 MAPK.

MATERIALS AND METHODS

Animals and Hindlimb Suspension

Female Sprague-Dawley rats (100 to 110 g; Harlan, Indianapolis, IN) were received shortly prior to use. Animals were lightly anesthetized by a low-dose intraperitoneal injection of pentobarbitol sodium (Nembutal; 10 mg/100 g body weight), tail-casted, and suspended in a

From the Muscle Metabolism Laboratory, Department of Physiology, and the Department of Biochemistry and Molecular Biophysics, University of Arizona College of Medicine, Tucson, AZ.

Submitted December 12, 2003; accepted February 28, 2004.

Supported in part by Grant No. NAG2-1187 from the National Aeronautics and Space Administration to M.E.T. and E.J.H., and by a fellowship from Minority Access to Research Careers Grant No. GM08718 from the National Institutes of Health to F.R.P.

Address reprint requests to Erik J. Henriksen, PhD, Department of Physiology, Ina E. Gittings Building #93, University of Arizona, Tucson, AZ 85721-0093.

0026-0495/04/5309-0046$30.00/0

doi:10.1016/j.metabol.2004.02.025

30-degree head-down position so that their hindlimbs were elevated above the floor of the cage, while their forelimbs were available for locomotion and feeding.[16] Tail casts consisted of Hexcelite orthopedic tape (Kirschner Medical, Timonium, MD) and 6382 RTS elastomer (Factor II, Lakeside, AZ). Vaseline was smeared on the belly of the animals so that urine rolled off rather than saturating the underside hair. All animals were provided with rat chow and water ad libitum until 8 PM of the evening before the experiments. All procedures were approved by the University of Arizona Animal Care and Use Committee.

Oral Glucose Tolerance Tests

Following an overnight fast, at 8 AM, animals underwent an oral glucose tolerance test (OGTT). An initial blood sample (0.25 mL) from a tail vein was obtained. A glucose load of 1 g/kg body weight was administered via gavage. Fifteen, 30, 60, and 90 minutes after glucose administration, blood samples were taken. Each blood sample was separated into 2 microcentrifuge tubes and centrifuged at 13,000 $\times$ *g* until plasma separation. One plasma sample was used for determination of glucose concentration using a Glucose Analyzer 2 (Beckman, Brea, CA). The second plasma sample was used for determination of insulin using a rat insulin radioimmunoassay (RIA) kit (Linco, St Charles, MO). Following the last blood draw, the animals received 1.25 mL of 0.9% saline to compensate for plasma volume loss.

Muscle Glucose Transport Activity

Following an overnight fast, at 8 AM, a separate group of animals was weighed and deeply anesthetized with an intraperitoneal injection of pentobarbital sodium (50 mg/kg body weight). Soleus and extensor digitorum longus (EDL) muscles were dissected and prepared into 2 strips (~25 to 30 mg) and incubated in the unmounted state. Each muscle was initially incubated for 30 minutes at 37°C in 3 mL of oxygenated (95% O_2–5% CO_2) Krebs-Henseleit buffer (KHB) supplemented with 8 mmol/L glucose, 32 mmol/L mannitol, and 0.1% bocine serum albumin (BSA; radioimmunoassay grade, Sigma Chemical, St Louis, MO) in the presence or absence of a maximally effective concentration of insulin (2 mU/mL; Humulin R, Eli Lilly, Indianapolis, IN). Following the initial incubation period, the muscles were rinsed for 10 minutes at 37°C in 3 mL oxygenated KHB containing 40 mmol/L mannitol, 0.1% BSA, in the presence or absence of insulin. Thereafter, the muscles were transferred to 2 mL KHB, containing 1 mmol/L 2-deoxy-[1,2-^{3}H] glucose (2-DG; 300 mCi/mmol; Sigma), 39 mmol/L [U-^{14}C] mannitol (0.8 mCi/mmol; ICN Radiochemicals, Irvine, CA), 0.1% BSA, in the presence or absence of insulin. At the end of this final 20-minute incubation period at 37°C, the muscles were removed, trimmed of excess fat and connective tissue, quickly frozen between aluminum blocks cooled in liquid nitrogen, and weighed. The frozen muscles were dissolved in 0.5 mL 0.5N NaOH. After the muscles were completely solubilized, 5 mL of scintillation cocktail was added, and the specific intracellular accumulation of 2-DG was determined as described previously.[17] This method for assessing glucose transport activity in isolated muscle has been validated.[18]

Assessment of Intramuscular Triglycerides, GLUT-4, and Glucose Enzymes

Clamp-frozen soleus and EDL muscles were pooled from each animal and a portion (~20 mg) was used for determination of intramuscular triglycerides (IMTG) using the chloroform-methanol extraction described by Folch et al.[19] followed by the processing method of Frayn and Maycock,[20] as modified by Denton and Randle.[21] Glycerol was ultimately assayed spectrophotometrically using a commercially available kit (Sigma Chemical).

The remaining piece (~30 mg) of soleus and EDL muscle was homogenized in 30 vol of ice-cold 20 mmol/L N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic (HEPES) acid buffer (pH 7.4) containing 1 mmol/L ethylenediaminetetraacetic acid (EDTA) and 250 mmol/L sucrose. The homogenate was divided into aliquots for analysis of total protein (bicinchoninic acid [BCA] assay, Sigma), GLUT-4 protein,[22] total hexokinase,[23] and citrate synthase.[24]

Expression of Insulin Signaling Factors

Soleus muscle was homogenized in 8 vol of 50 mmol/L HEPES (pH 7.5) containing 150 mmol/L NaCl, 20 mmol/L Na pyrophosphate, 20 mmol/L α-glycerophosphate, 10 mmol/L NaF, 2 mmol/L Na_3VO_4, 2 mmol/L EDTA (pH 8.0), 1% Triton X-100, 10% glycerol, 1 mmol/L $MgCl_2$, 1 mmol/L $CaCl_2$, 10 μg/mL aprotinin, 10 μg/mL leupeptin, and 2 mmol/L phenylmethylsulfonylfluoride (PMSF). Thereafter, homogenates underwent end-over-end rotation for 20 minutes at 4°C followed by a 20-minute centrifugation at 13,000 $\times$ *g*. The homogenate was then analyzed for total protein (BCA assay). Samples were then diluted to equal protein concentrations and combined with sodium dodecyl sulfate (SDS) sample buffer (125 mmol/L Tris [pH 6.8], 4% SDS, 20% glycerol, 10% β-mercaptoethanol, and 0.0025% bromophenol blue). This mixture was boiled for 5 minutes. Following boiling, 200 μg of protein was loaded on either 7.5% or 12% Tris-HCl Ready Gels (Bio-Rad Laboratories, Hercules, CA) for SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Following electrophoresis, samples were transferred to nitrocellulose paper for Western blotting.

For analysis of Akt expression, the nitrocellulose paper was blocked in Tris-buffered saline (TBS) containing 0.1% Tween-20 (TBS/T) and 5% nonfat dry milk for 1 hour at room temperature. It was then washed 3 times with TBS/T. The nitrocellulose was then incubated overnight at 4°C at with anti-Akt antibody (Cell Signaling Technology, Beverly, MA) diluted 1:1,000 in TBS/T containing 5% BSA. Upon completion, the nitrocellulose was washed 3 times with TBS/T and then incubated for 1 hour with goat anti-rabbit IgG horseradish peroxidase conjugated secondary antibody (HRP; Chemicon International, Temecula, CA) diluted 1:1,000 in TBS/T containing 5% nonfat dry milk. The nitrocellulose was then washed 3 times with TBS/T.

For analysis of IRS-1, IRS-2, and the p85 subunit of PI3-kinase, the nitrocellulose paper was blocked for 1 hour at room temperature in phosphate-buffered saline containing 3% nonfat dry milk (PBS-MLK). The nitrocellulose was then incubated overnight at 4°C with either anti–IRS-1, anti–IRS-2, or anti-p85 antibody (Upstate Biotechnology, Lake Placid, NY) diluted to 2 μg/mL in PBS-MLK. The nitrocellulose was then washed 2 times with water. Thereafter, the nitrocellulose was incubated for 1.5 hours at room temperature with HRP diluted 1:1,000 in PBS-MLK. The nitrocellulose was then washed with water twice, once with PBS containing 0.05% Tween-20 for 5 minutes, and then 4 additional times with water.

For analysis of IR-β and p38 MAPK, the nitrocellulose was blocked for 1 hour at room temperature with TBS/T containing 5% nonfat dry milk (TBST-MLK). The nitrocellulose was then incubated overnight at 4°C with 2 μg/mL of anti–IR-β-HRP or anti-p38 MAPK antibody (Santa Cruz Biotechnology, Santa Cruz, CA) diluted in TBST-MLK. The nitrocellulose was washed 3 times with TBS/T. For analysis of p38 MAPK, the nitrocellulose was incubated at room temperature for 1 hour in HRP diluted 1:1,000 in TBST-MLK. Following this the nitrocellulose was washed 3 times with TBS/T.

Determination of Activation State of Insulin Signaling Factors

Muscles were incubated for 30 minutes in 3 mL of oxygenated KHB containing 8 mmol/L glucose, 32 mmol/L mannitol, and 0.1% BSA, and maintained at 37°C. One soleus strip from each animal was incubated in the absence of insulin, while a second soleus strip was incubated in the presence of a maximally effective concentration of insulin (5 mU/mL). Following incubation in insulin, the middle two

thirds of each soleus strip was excised, blotted, frozen using aluminum clamps dipped in liquid nitrogen, and then weighed. Analysis of phosphorylated Akt and p38 MAPK was conducted in an identical manner described above for Akt expression analysis. Analysis of phosphorylated Akt used anti-phospho Akt (ser^{473}) and anti-phospho p38 MAPK (thr^{180}/tyr^{182}) antibody (Cell Signaling Technology) for the primary antibody.

Measurement of tyrosine-phosphorylated IRS-1, IR-β, and IRS-1 associated with p85 was performed via immunoprecipitation. Each soleus muscles was homogenized in 1 mL of 50 mmol/L HEPES (pH 7.4) containing 150 mmol/L NaCl, 1% Triton X-100, 2 μg/mL aprotinin, 0.2 mmol/L leupeptin, 2 mmol/L PMSF, 0.1 mmol/L antipain, and 0.5 U/mL α-macroglobulin. Thereafter, homogenates were incubated for 20 minutes at 4°C followed by 20-minute centrifugation at 13,000 $\times$ *g*. The homogenate was then analyzed for total protein (BCA assay). Samples were then diluted to 1 mg/mL.

For analysis of phosphorylated IR-β, 0.5 mL of homogenate was immunoprecipitated with 10 μL of recombinant agarose-conjugated anti-phosphotyrosine antibody (4G10, Upstate Biotechnology) and gently rocked overnight at 4°C. The solution was then placed in a microcentrifuge for approximately 5 seconds. The supernatant was removed and the beads washed 3 times with PBS. The beads were then exposed to SDS sample buffer and boiled for 5 minutes.

For analysis of phosphorylated IRS-1 and IRS-1 associated with p85, 1 mL of diluted homogenate was combined with 20 μL of agarose-conjugated anti–IRS-1 antibody (Upstate Biotechnology) and gently rocked overnight at 4°C. The solution was then placed in a microcentrifuge for approximately 5 seconds. The supernatant was removed and the beads washed 3 times with PBS. The beads were then exposed to SDS-PAGE sample buffer and boiled for 5 minutes. Thereafter, 40 μL of sample were loaded on a 7.5% Tris-HCl Ready Gels (Bio-Rad) for SDS-PAGE. Following electrophoresis, samples were transferred to nitrocellulose paper for Western blotting.

Analysis of phosphorylated IR-β–and IRS-1–associated p85 was conducted as described above for detection of expression of IR-β and p85. For analysis of phosphorylated IRS-1, the nitrocellulose was blocked for 1 hour at room temperature with TBST-MLK. The nitrocellulose was then incubated overnight at 4°C with 2 μg/mL of anti-phosphotyrosine antibody (PY99, Santa Cruz Biotechnology) diluted in TBST-MLK. The nitrocellulose was washed 3 times with TBS/T. The nitrocellulose was then incubated for 1 hour at room temperature in goat anti-mouse IgG-HRP conjugated antibody (Santa Cruz Biotechnology) diluted 1:1,000 in TBST-MLK. Bands of interest were visualized using enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ) and exposure to Kodak X-Omat AR Film (Eastman Kodak, Rochester, NY). Densitometric analysis was performed using a GS-800 Calibrated Densitometer (Bio-Rad).

Statistical Analysis

All data are presented as means $\pm$ SE. Differences between 2 groups were determined by an unpaired Student's *t* test. The significance of differences between multiple groups was assessed by a factorial analysis of variance (ANOVA) with a post hoc Fisher's protected least significant difference (PLSD) test (StatView version 5.0, SAS Institute, Cary, NC). A *P* value of less than .05 was considered to be statistically significant.

RESULTS

Animal Weights, Muscle Weights, GLUT-4 Protein, and Glucose Enzymes

After 1 day of HS, there was no difference in body weight between the suspended animals (107 $\pm$ 3 g; n = 20) and the weight-bearing controls (106 $\pm$ 4 g; n = 31). In addition, the relative muscle wet weights for the soleus (39.3 $\pm$ 0.8 mg/100 g body weight *v* 40.3 $\pm$ 0.8) and EDL (43.2 $\pm$ 0.8 *v* 42.7 $\pm$ 0.7) were not different between the HS and control groups. Finally, no differences between groups were found for total GLUT-4 protein or for the activities of total hexokinase and citrate synthase (data not shown).

Oral Glucose Tolerance and Whole-Body Insulin Sensitivity

The fasting plasma glucose concentration in the 1-day HS animals was 27% greater ($P < .05$) than that of the weight-bearing control animals (Fig 1). The plasma glucose concentration continued to be significantly elevated in the 1-day HS animals at the 60- and 90-minute time points of the OGTT, and the glucose area under the curve (AUC) was 13% greater in the HS group compared to the weight-bearing controls (Fig 2). Fasting plasma insulin was elevated by 23% in the 1-day HS animals. Moreover, the insulin response during the OGTT was significantly greater in the 1-day HS animals compared to the control animals at the 15-, 30-, and 90-minute time points, and the insulin AUC was 35% greater in the HS group. The glucose-insulin index, the product of the glucose AUC and the insulin AUC and inversely related to peripheral insulin sensitivity,[25] was 58% greater in the 1-day HS group compared to the value in the corresponding weight-bearing animals, indicating the development of whole-body insulin resistance due to acute HS.

Muscle Glucose Transport Activity

Glucose transport activity, as assessed by 2-deoxyglucose (2-DG) uptake, was determined in isolated soleus and EDL muscles in the absence and presence of a maximally effective insulin concentration (2 mU/mL) (Fig 3). In the 1-day unweighted soleus, basal glucose transport was diminished by 30%, insulin-stimulated glucose transport was decreased by 19%, and the increase above basal due to insulin was 16% less than in the weight-bearing control soleus. While basal and insulin-stimulated glucose transport activities was not significantly diminished in the EDL of the 1-day HS animals, there was a tendency ($P = .09$) for the increase above basal due to insulin to be 18% less in EDL from the suspended animals compared to EDL from weight-bearing controls.

Intramuscular Triglycerides

Because of the well-established inverse relationship between whole-body and skeletal muscle insulin action and the lipid concentration in human and rodent skeletal muscle,[26-29] IMTG levels were determined in soleus and EDL muscles (Fig 4). The IMTG level in the 1-day unweighted soleus was reduced to 35% ($P < .05$) of the weight-bearing control value, whereas no difference in IMTG concentration was observed in the EDL between HS and weight-bearing groups.

Insulin Signaling in Soleus Muscle

Because the insulin resistance of glucose transport was more pronounced in the 1-day unweighted soleus, assessment of the protein expression and functionality of insulin signaling factors (Figs 5 and 6) was restricted to this muscle type. In the 1-day unweighted soleus, neither the protein expression nor the ty-

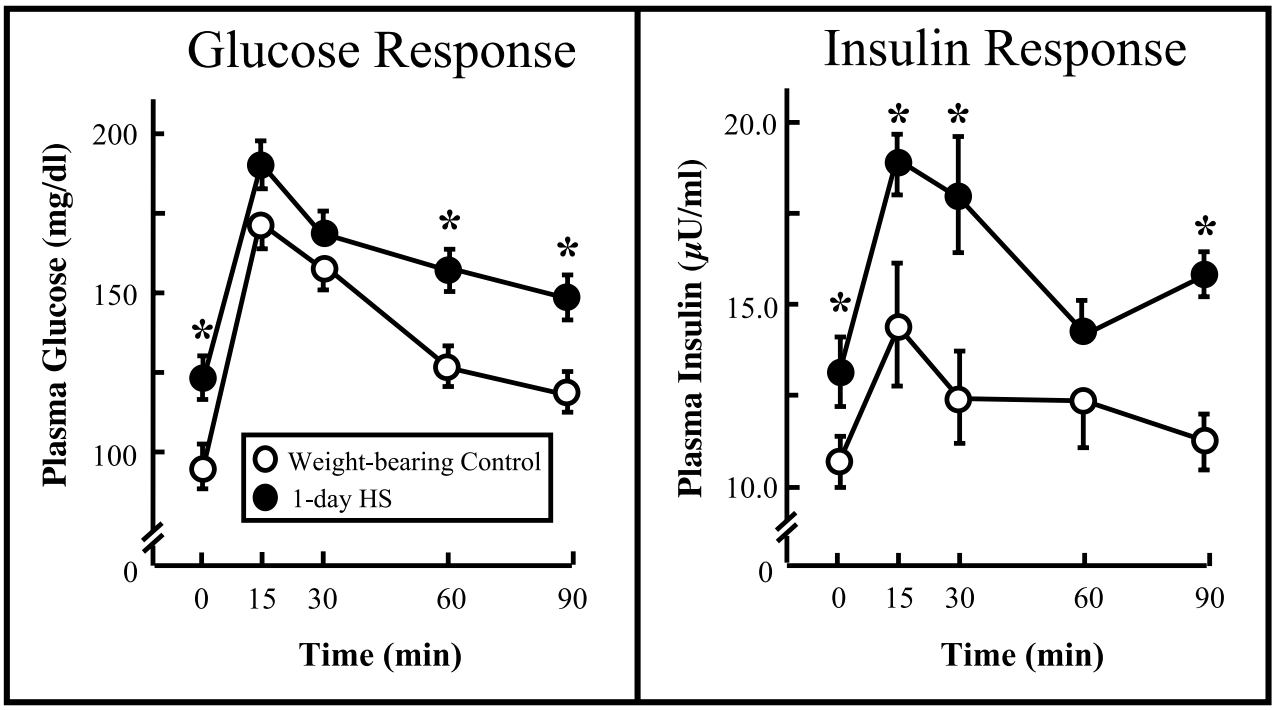

Fig 1. Glucose and insulin responses during an OGTT in weight-bearing and 1-day HS rats. Values are means ± SE for 10 weight-bearing control animals and 12 suspended animals. *$P < .05$ *v* weight-bearing control group.

rosine phosphorylation status of IR-β was different from that of the weight-bearing control soleus (Fig 5). However, there was a tendency for a 21% decrease ($P < .1$) in IRS-1 protein expression in the 1-day HS soleus (Fig 5). Despite this protein deficit, insulin-stimulated tyrosine phosphorylation of IRS-1 was normal (98% of control) in the unweighted soleus compared to weight-bearing control. IRS-2 protein expression was not affected in the 1-day HS soleus (data not shown). Likewise, the protein expression of the p85 regulatory subunit of PI3-kinase and the insulin-stimulated association of IRS-1 with p85

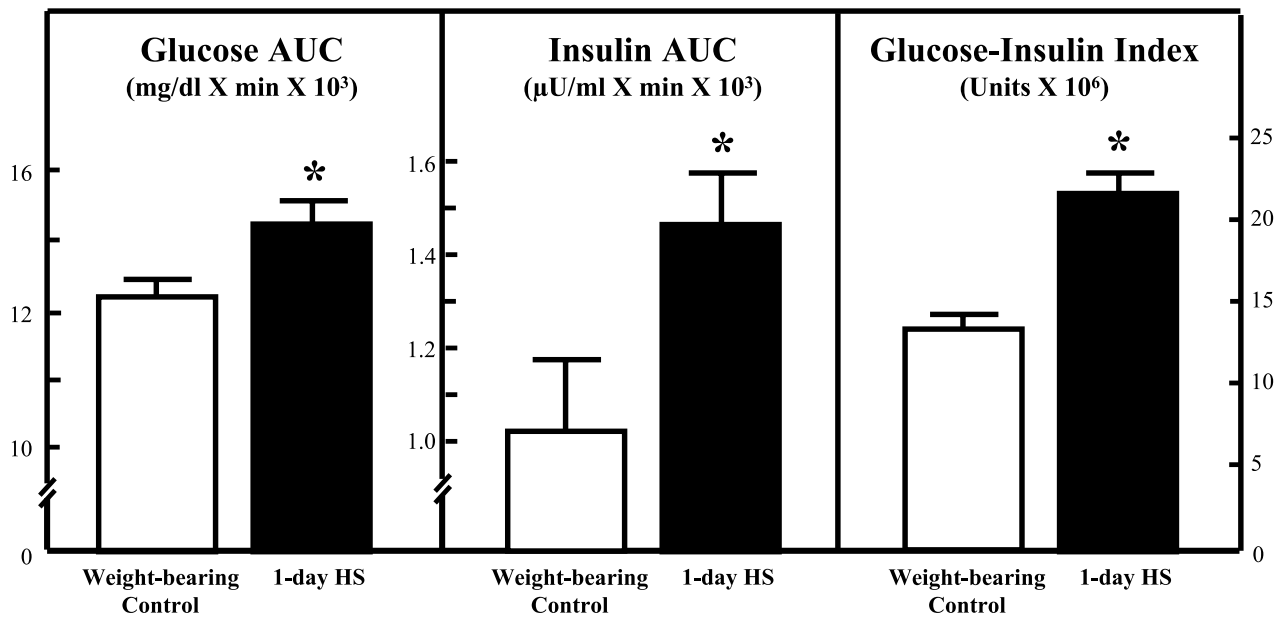

Fig 2. Areas under the curves for glucose and insulin during the OGTTs and the glucose-insulin index in weight-bearing and 1-day HS rats. Data were calculated from Fig 1. *$P < .05$ *v* weight-bearing control group.

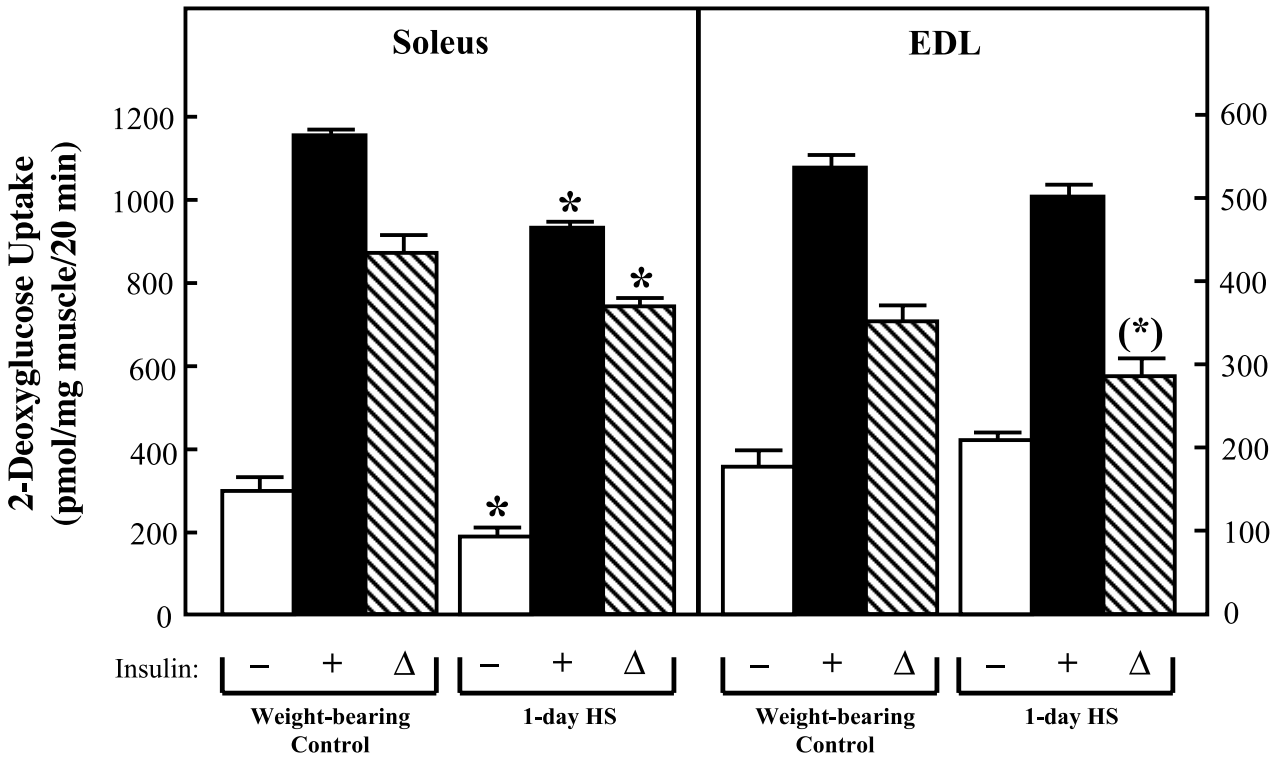

Fig 3. Effect of 1-day HS on glucose transport activity in rat skeletal muscle. Bars represent the rates of 2-DG uptake in the absence of insulin, in the presence of insulin, and the increase above basal due to insulin (Δ) in isolated soleus and EDL muscles. Values are means ± SE for 5 weight-bearing control animals and 11 suspended animals. *$P < .05$ *v* weight-bearing control group. (*)$P < .1$ *v* weight-bearing control group.

were not altered in the soleus muscle by 1 day of HS (Fig 5). The protein expression of the downstream insulin signaling factor Akt in soleus muscle was not different between 1-day HS and weight-bearing control groups (Fig 6). Interestingly, the ser^{473} phosphorylation of Akt in the absence of insulin was reduced by 27% ($P < .05$) in the 1-day HS soleus. Nevertheless, in the presence of insulin, the phosphorylation state of Akt was not different between HS and control groups.

The potential effect of acute unweighting on the stress-activated serine/threonine kinase, p38 MAPK, was investigated (Fig 7). The protein expression of p38 MAPK was increased by 19% ($P < .05$) in the 1-day HS soleus. Moreover, the basal phosphorylation state of the p38 MAPK was 81% greater ($P < .05$) in the 1-day unweighted soleus compared to the weight-bearing control muscle. While some investigators have reported the effect of insulin to enhance p38 MAPK phosphorylation,[30] in our hands the acute effect of insulin (5 to 30 minutes) on this parameter in rat soleus muscle is quite small (maximal 10% to 20% increase) and inconsistent (V. Saengsirisuwan, M.P. O'Keefe, E.J. Henriksen, unpublished observation).

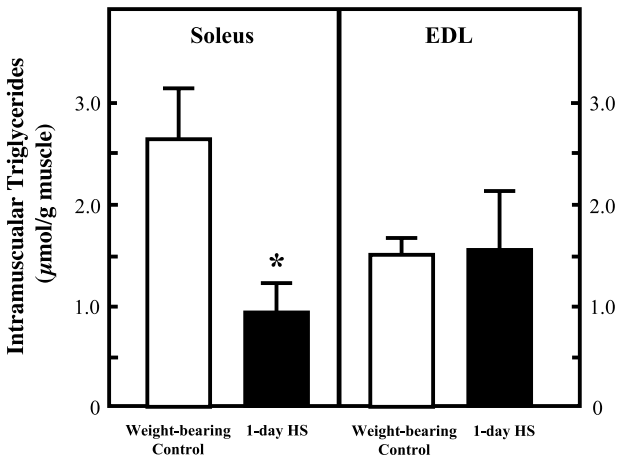

Fig 4. Effect of 1-day HS on intramuscular triglyceride concentration in soleus and EDL muscles. Values are means ± SE for 5 weight-bearing control animals and 5 suspended animals. *$P < .05$ *v* weight-bearing control group.

DISCUSSION

The results of the present investigation clearly indicate that an acute (1 day) episode of simulated weightlessness using the HS model induces an insulin-resistant state in juvenile, female rats, in both the whole animal and skeletal muscle. Insulin resistance at the systemic level was evidenced by increases in fasting plasma glucose and insulin (Fig 1), by exaggerated glucose and insulin responses to an oral glucose load (Figs 1 and 2), and a marked increase in the glucose-insulin index derived from the OGTT results (Fig 2). Insulin resistance at the skeletal muscle level was demonstrated in the type I soleus from HS animals (Fig 3), with decreases in both the basal rate

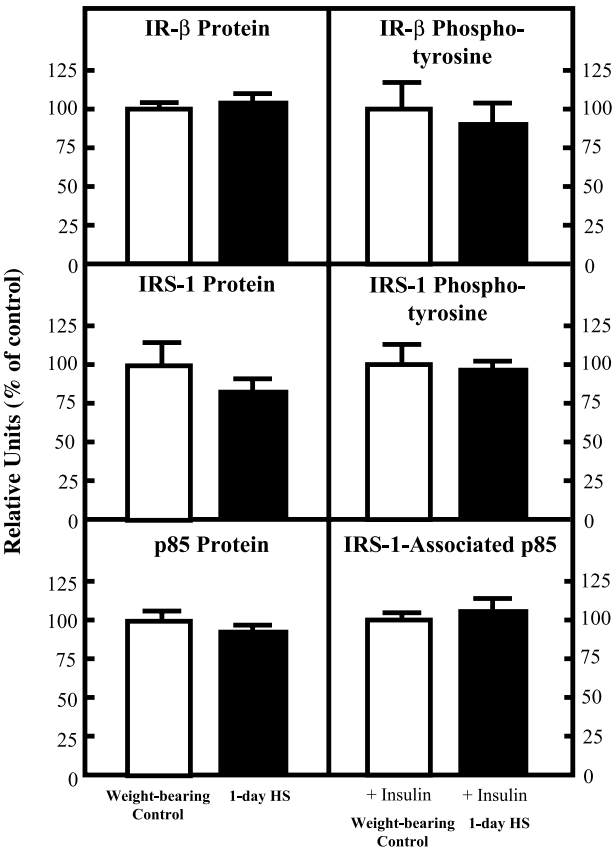

Fig 5. Effect of 1-day HS on protein expression and insulin stimulation of IR-β and IRS-1 and on protein expression of the p85 regulatory subunit of PI3-kinase and insulin stimulation of IRS-1 associated with p85 in soleus muscle. Values are means ± SE for 5 or 6 weight-bearing control animals and 8 suspended animals. Tyrosine phosphorylation IR-β and IRS-1 and IRS-1 associated with p85 in the absence of insulin was undetectable.

of glucose transport activity and in the ability of insulin to increase glucose transport activity above basal. Although HS did not affect basal glucose transport in type II EDL muscles, there was a tendency for a reduction in insulin-mediated glucose transport activity in this flexor muscle.

Taken together, these data support the concept that a systemic factor could be responsible for the induction of impaired insulin action in skeletal muscle of the HS animals. One candidate for this systemic factor is the glucocorticoid hormone corticosterone. Plasma levels of corticosterone are elevated after just 1 day of HS in juvenile, female rats,[31] and chronic systemic exposure of skeletal muscle to glucocorticoid hormones induces impairments in insulin action on glucose transport activity,[32,33] possibly due to reductions in IRS-1 protein expression[32,34] and GLUT-4 translocation to the plasma membrane.[33] Although the duration and magnitude of the elevation of corticosterone in these 1-day HS animals[31] was much less than in these other studies (5 days or more),[32-34] we also observed a 21% decrease in IRS-1 protein in the soleus muscle (Fig 5). It is clear that further investigation is required to delineate the potential role of glucocorticoids in the induction of whole-body and skeletal muscle insulin resistance of the 1-day HS rats.

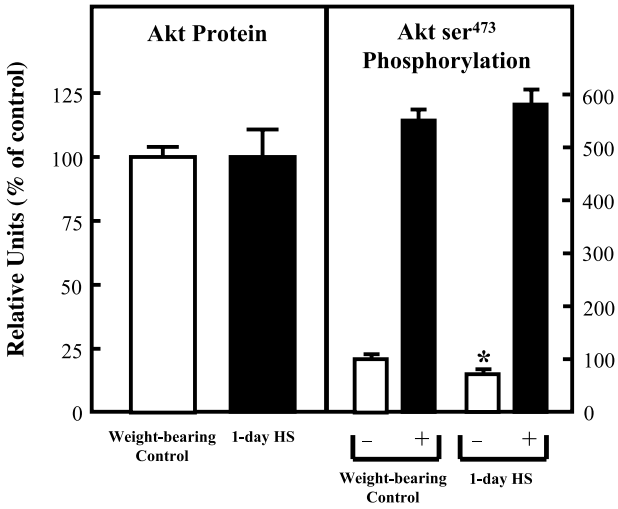

Fig 6. Effect of 1-day HS on Akt protein expression and insulin-stimulated Akt ser^{473} phosphorylation in soleus muscle. Values are means ± SE for 7 weight-bearing control animals and 8 suspended animals. *$P < .05$ *v* weight-bearing control group.

The present data do not support alterations in insulin signaling through IR, IRS-1, PI3-kinase, and Akt as the mechanism underlying the impaired insulin action on glucose transport activity in the 1-day unweighted soleus muscle. The protein expression of IR-β and Akt was not changed by unweighting, and the reductions in IRS-1 (21%) and p85 (10%) protein levels were modest. Moreover, the functionality of all of these insulin signaling factors was normal. Only the 27% decrease in basal Akt phosphorylation (Fig 6) could be associated with the decrease in basal glucose transport activity in the 1-day HS soleus (Fig 3). These results indicate that the site of the impairment of insulin action in the 1-day HS soleus lies distal to the signaling factors investigated in the present study.

As previously noted, there exists an inverse relationship

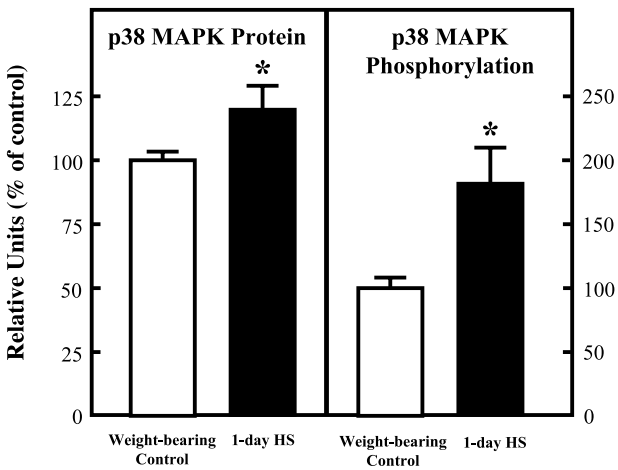

Fig 7. Effect of 1-day HS on p38 MAPK protein expression and p38 MAPK thr^{180}/tyr^{182} phosphorylation in soleus muscle. Values are means ± SE for 5 weight-bearing control animals and 11 suspended animals. *$P < .05$ *v* weight-bearing control group.

between insulin sensitivity and the myocellular lipid concentration.[26-30] As the IMTG concentration of the soleus was significantly decreased and that of the EDL was unchanged following 1-day of HS (Fig 4), it is unlikely that these lipids or their degradation products caused the development of skeletal muscle insulin resistance in these animals. While the elucidation of the mechanism for the reduction in IMTG in the 1-day HS soleus was not a focus of the present study, one could speculate that it resulted from both an enhanced rate of lipolysis (possibly due to increased levels of lipolytic hormones, such as glucocorticoids, catecholamines, and growth hormone) and a reduction in glucose transport activity (Fig 3), thereby limiting glucose carbons available for incorporation into triglycerides.

An intriguing finding in this study was the increase in p38 MAPK protein expression and basal phosphorylation in the 1-day HS soleus (Fig 7). The role of p38 MAPK in insulin signaling remains controversial. Some investigations support a role of this stress-activated serine/threonine protein kinase as a negative modulator of insulin signaling and glucose transport in skeletal muscle.[15,35,36] However, others provide evidence indicating that activation of p38 MAPK by insulin is necessary for stimulation of GLUT-4 activity and therefore for full activation of glucose transport.[30] The findings of the present investigation are consistent with the concept that p38 MAPK elicits a negative effect on insulin-stimulated glucose transport activity in rat skeletal muscle, as the elevation of p38 MAPK protein and phosphorylation observed in the 1-day HS soleus (Fig 7) were associated with a reduction in glucose transport activity (Fig 3). Moreover, insulin stimulation of unweighted soleus muscle does not cause an increase in p38 MAPK phosphorylation (M.P. O'Keefe, E.J. Henriksen, unpublished observation). Interestingly, a more prolonged period (7 days) of soleus unweighting is associated with both a reduction in p38 MAPK protein expression and activation and an enhancement of insulin signaling and insulin-stimulated glucose transport activity.[37]

The mechanism for the enhanced protein expression and phosphorylation state of p38 MAPK in the 1-day unweighted soleus remains unclear. A similar enhancement of p38 MAPK protein expression and phosphorylation was recently reported in soleus muscle of chronically hindlimb-immobilized rats.[38] The authors speculated that the substantial reduction in electromyographic (EMG) activity (5% to 15% of control) in immobilized soleus[39] may be linked to the activation of this enzyme. In this context, it is noteworthy that 1-day of HS is also associated with a nearly complete absence of soleus EMG activity.[40] How this apparent link between muscle electrical activity and p38 MAPK protein expression and functionality is mediated at the cellular and molecular level should be addressed in future investigations.

It is important to note that the insulin-resistant state induced by acute HS is only transient. With more prolonged periods of HS (3 days or longer), the systemic and skeletal muscle insulin resistance disappears and a state of enhanced insulin sensitivity subsequently develops.[3,4,37] Interestingly, despite a continued decrease in IRS-1 protein expression in 3-day and 7-day unweighted soleus, insulin signaling through PI3-kinase and insulin stimulation of glucose transport activity are enhanced,[37] possibly due to recovery of EMG activity at these time points.[40] The duration of HS is clearly an important factor in determining the metabolic response of the unweighted soleus muscle.

In summary, an acute (1 day) period of HS in juvenile, female rats induced insulin resistance at the systemic and skeletal muscle levels. The insulin resistance of glucose transport activity in the 1-day unweighted soleus was not due to impaired functionality of elements involved in the IR/IRS-1/PI3-kinase/Akt signaling pathway. However, p38 MAPK may be an important factor in the development of this insulin-resistant state, as both the protein expression and the phosphorylation state of this serine/threonine protein kinase were upregulated in the 1-day unweighted soleus.

ACKNOWLEDGMENT

The authors wish to thank Julie Sloniger and Cody Diehl for excellent technical assistance.

REFERENCES

1. Thomason DB, Booth FW: Atrophy of the soleus muscle by hindlimb unweighting. J Appl Physiol 68:1-12, 1990

2. Adams GR, Caiozzo VJ, Baldwin KM: Skeletal muscle unweighting: Spaceflight and ground-based models. J Appl Physiol 95:2185-2201, 2003

3. Henriksen EJ, Tischler ME, Johnson DG: Increased response to insulin of glucose metabolism in the six-day unloaded rat soleus muscle. J Biol Chem 261:10707-10712, 1986

4. Henriksen EJ, Tischler ME: Time course of the response of carbohydrate metabolism to unloading of the soleus. Metabolism 37: 201-208, 1988

5. Bonen A, Elder GCB, Tan MH: Hindlimb suspension increases insulin binding and glucose metabolism. J Appl Physiol 65:1833-1839, 1988

6. Stump CR, Balon TW, Tipton CM: Effects of insulin and exercise on rat hindlimb muscles after simulated microgravity. J Appl Physiol 73:2044-2053, 1992

7. Koebel DA, Fell RD, Steffen JM: Increased glucose and 2-deoxy-D-glucose uptake in skeletal muscle of suspended rats. Aviat Space Environ Med 64:1016-1022, 1993

8. Henriksen EJ, Tischler ME: Glucose uptake in rat soleus: Effect of acute unloading and subsequent reloading. J Appl Physiol 64:1428-1432, 1988

9. Shepherd PR, Kahn BB: Glucose transporters and insulin action. N Engl J Med 341:248-257, 1999

10. Zierath JR, Krook A, Wallberg-Henriksson H: Insulin action and insulin resistance in human skeletal muscle. Diabetologia 43:821-835, 2000

11. Kohn AD, Summers SA, Birnbaum MJ, et al: Expression of a constitutively active Akt Ser/Thr kinase in 3T3-L1 adipocytes stimulated glucose uptake and glucose transporter 4 translocation. J Biol Chem 271:31372-31378, 1996

12. Hajduch E, Alessi DR, Hemmings BA, et al: Constitutive activation of protein kinase Bα by membrane targeting promotes glucose and system A amino acid transport, protein synthesis, and inactivation of glycogen synthase kinase 3 in L6 muscle cells. Diabetes 47:1006-1013, 1998

13. Ueki K, Yamamoto-Honda R, Kaburagi Y, et al: Potential role of protein kinase B in insulin-induced glucose transport, glycogen synthesis, and protein synthesis. J Biol Chem 273:5315-5322, 1998

14. Kitamura T, Ogawa W, Sakaue H, et al: Requirement for activation of the serine-threonine kinase Akt (protein kinase B) in insulin

stimulation of protein synthesis but not of glucose transport. Mol Cell Biol 18:3708-3717, 1998

15. Fujishiro M, Gotoh Y, Katagiri H, et al: Three mitogen-activated protein kinases inhibit insulin signaling by different mechanisms in 3T3-L1 adipocytes. Mol Endocrinol 17:487-497, 2003

16. Jaspers SR, Tischler ME: Atrophy and growth failure of rat hindlimb muscles in tail-cast suspension. J Appl Physiol 57:1472-1479, 1984

17. Henriksen EJ, Jacob S: Effects of captopril on glucose transport activity in skeletal muscle of obese Zucker rats. Metabolism 44:267-272, 1995

18. Hansen PA, Gulve EA, Holloszy JO: Suitability of 2-deoxyglucose for in vitro measurement of glucose transport activity in skeletal muscle. J Appl Physiol 76:979-985, 1994

19. Folch J, Lees M, Sloane Stanley JH: A simple method for the isolation and purification of total lipides from animal tissues. J Biol Chem 226:497-509, 1957

20. Frayn KN, Maycock PF: Skeletal muscle triacylglycerol in the rat: Methods for sampling and measurement, and studies of biological variability. J Lipid Res 21:139-144, 1980

21. Denton RM, Randle PJ: Concentration of glycerides and phospholipids in rat heart and gastrocnemius muscles: Effects of alloxan-diabetes and perfusion. Biochem J 104:416-422, 1967

22. Henriksen EJ, Halseth AE: Early alterations in soleus GLUT-4, glucose transport, and glycogen in voluntary running rats. J Appl Physiol 76:1862-1867, 1994

23. Uyeda K, Racker E: Regulatory mechanisms in carbohydrate metabolism. VII. Hexokinase and phosphofructokinase. J Biol Chem 240:4682-4688, 1965

24. Srere PA: Citrate synthase. Methods Enzymol 13:3-10, 1969

25. Cortez MY, Torgan CE, Brozinick JT, et al: Insulin resistance of obese Zucker rats exercise trained at two different intensities. Am J Physiol Endocrinol Metab 261:E613-E619, 1991

26. Koyama K, Chen G, Lee Y, et al: Tissue triglycerides, insulin resistance, and insulin production: Implications for hyperinsulinemia of obesity. Am J Physiol Endocrinol Metab 273:E708-E713, 1997

27. Oakes ND, Cooney GJ, Camilleri S, et al: Mechanisms of liver and muscle insulin resistance induced by chronic high-fat feeding. Diabetes 46:1768-1774, 1997

28. Jacob S, Machann J, Rett K, et al: Associated of increased intramyocellular lipid content with insulin resistance in lean nondiabetic offspring of type 2 diabetic subjects. Diabetes 48:1113-1119, 1999

29. Henriksen EJ, Teachey MK, Taylor ZC, et al: Isomer-specific actions of conjugated linoleic acid on muscle glucose transport in the obese Zucker rat. Am J Physiol Endocrinol Metab 285:E98-E105, 2003

30. Furtado LM, Poon V, Klip A: GLUT4 activation: Some thoughts on possible mechanisms. Acta Physiol Scand 178:287-296, 2003

31. Jaspers SR, Henriksen E, Jacob S, et al: Metabolism of branched-chain amino acids in leg muscles from tail-cast suspended intact and adrenalectomized rats. Metabolism 38:109-114, 1989

32. Giorgino F, Almahfouz A, Goodyear LJ, et al: Glucocorticoid regulation of insulin receptor and substrate IRS-1 tyrosine phosphorylation in rat skeletal muscle in vivo. J Clin Invest 91:2020-2030, 1993

33. Dimitriadis G, Leighton B, Parry-Billings M, et al: Effects of glucocorticoid excess on the sensitivity of glucose transport and metabolism to insulin in rat skeletal muscle. Biochem J 321:707-712, 1997

34. Saad MJ, Folli F, Kahn JA, et al: Modulation of insulin receptor, insulin receptor substrate-1, and phosphatidylinositol 3-kinase in liver and muscle of dexamethasone-treated rats. J Clin Invest 92:2065-2072, 1993

35. Koistinen HA, Chibalin AV, Zierath JR: Aberrant p38 mitogen-activated protein kinase signalling in skeletal muscle from type 2 diabetic patients. Diabetologia 46:1324-1328, 2003

36. Ho R, Alcazar O, Fujii N, Hirshman M, et al: p38γMAPK regulation of glucose uptake in L6 myotubes and mouse skeletal muscle. Diabetes 52:A279, 2003 (suppl 1, abstr)

37. O'Keefe MP, Perez FR, Sloniger JA, et al: Enhanced insulin action on glucose transport and insulin signaling in 7-day unweighted rat soleus muscle. J Appl Physiol (in press)

38. Childs TE, Spangenburg EE, Vyas DR, et al: Temporal alterations in protein signaling cascades during recovery from muscle atrophy. Am J Physiol Cell Physiol 285:C391-C398, 2003

39. Fischbach GD, Robbins N: Changes in contractile properties of disused soleus muscles. J Physiol 201:305-320, 1969

40. Alford EK, Roy RR, Hodgson JA, et al: Electromyography of rat soleus, medial gastrocnemius, and tibialis anterior during hind limb suspension. Exp Neurol 96:635-649, 1987