Enhanced insulin action on glucose transport and insulin signaling in 7-day unweighted rat soleus muscle

Matthew P. O’Keefe, Felipe R. Perez, Julie A. Sloniger, Marc E. Tischler, and Erik J. Henriksen

Muscle Metabolism Laboratory, Department of Physiology, and Department of Biochemistry and Molecular Biophysics, University of Arizona College of Medicine, Tucson, Arizona 85721-0093

Submitted 18 December 2003; accepted in final form 27 February 2004

Hindlimb suspension (HS) of the rat has been used as a terrestrial model of simulated weightlessness by inducing muscle unweighting and elicits several adaptive responses in skeletal muscle contractile function and metabolism (see Refs. 1 and 36 for comprehensive reviews). A hallmark of prolonged (>3 days) unweighting of the soleus muscle is the development of significant atrophy and growth failure (36). Moreover, soleus unweighting induces dynamic alterations in insulin action on glucose transport activity and metabolism. Whereas unweighting initially (within 24 h) causes a condition of insulin resistance of glucose transport and metabolism (19, 32), with more prolonged unweighting (>3 days of HS), a condition of enhanced insulin action on carbohydrate metabolism in the soleus muscle develops (3, 20, 21, 27, 35). The increased insulin action on glucose transport in unweighted soleus muscle is associated with both increased insulin binding (3, 21) and enhanced protein expression of the GLUT-4 glucose transport isoform (16, 17). However, further information on the potential intracellular mechanisms underlying this adaptive response to prolonged soleus unweighting is not available. Moreover, no study to date has evaluated the response of whole body insulin sensitivity to prolonged HS.

Skeletal muscle glucose transport is acutely regulated by insulin through the coordinated actions of several intracellular proteins (see Refs. 33 and 39 for reviews). Briefly, insulin activation of tyrosine kinase in the β-subunit of the insulin receptor leads to tyrosine phosphorylation of insulin receptor substrate (IRS)-1. Phosphorylated IRS-1 then interacts with the p85 regulatory subunit of phosphatidylinositol 3-kinase (PI3-kinase), thereby activating the p110 catalytic subunit of this enzyme. The lipid moieties produced by PI3-kinase subsequently activate phosphoinositide-dependent kinases. One target of phosphoinositide-dependent kinases is the serine/threonine protein kinase Akt (also known as protein kinase B). The specific role of Akt in the glucose transport process in skeletal muscle remains controversial (12, 26, 28, 37). However, it is clear that the activation of these steps causes translocation of vesicles containing the glucose transporter protein isoform GLUT-4 to the plasma membrane, where glucose transport takes place via a facilitated diffusion process.

In addition, the functionality of these insulin signaling factors can be modulated by several serine/threonine kinases, including the stress-activated kinase p38 MAPK. Whereas most evidence indicates that p38 MAPK can phosphorylate and negatively regulate specific elements of the insulin signaling cascade, such as IRS-1 (10), other investigators have provided evidence that p38 MAPK may be involved in regulation of insulin-dependent glucose transport (11). Short-term soleus unweighting is associated with an enhancement of p38 MAPK protein expression and activation (32) and with the development of insulin resistance (19, 32). It is presently unknown whether prolonged skeletal muscle unweighting induced by HS can alter the regulation of the protein expression and/or functionality of the aforementioned insulin signaling factors or the response of p38 MAPK.

In this context, the present investigation was designed to test the overall hypothesis that the enhanced insulin action on glucose transport in unweighted rat soleus muscle is due to...
increased protein expression and functionality of insulin signaling elements. Therefore, the specific purposes of this study were 1) to determine the whole body response of insulin sensitivity to 3 and 7 days of HS of juvenile, female Sprague-Dawley rats; 2) to assess the adaptive responses to soleus unweighting of the protein expression and functionality of specific insulin signaling factors, including the insulin receptor, IRS-1, PI3-kinase, and Akt; and 3) to determine whether soleus unweighting affects the protein expression and activation of the stress-activated p38 MAPK.

MATERIALS AND METHODS

Animals and HS. Female Sprague-Dawley rats (100–110 g, Harlan, Indianapolis, IN) were received shortly before use. Animals were lightly anesthetized by a low-dose intraperitoneal injection of pentobarbital sodium (Nembutal; 10 mg/100 g body wt), tail casted, and suspended in a 30° head-down position so that their hindlimbs were elevated above the floor of the cage, while their forelimbs were available for locomotion and feeding (25). Tail casts consisted of Hexcelite orthopedic tape (Kirschner Medical, Timonium, MD) and 6382 RTS elastomer (Factor II, Lakeside, AZ). Vaseline was smeared on the underside of the animals so that urine rolled off rather than saturated the undersides hair. All procedures were approved by the University of Arizona Animal Care and Use Committee.

Insulin tolerance tests. After an overnight fast, at 0800, animals underwent an insulin tolerance test (ITT). An initial blood sample (0.25 ml) from a tail vein was obtained. An insulin load of 0.3 U/kg body wt was then administered by intraperitoneal injection. Blood samples were taken and centrifuged at 13,000 g to obtain plasma 30, 60, 90, and 120 min after the insulin administration. Plasma was used for determination of glucose concentration by using a Glucose Analyzer 2 (Beckman, Brea, CA). After the last blood draw, the animals received 1.25 ml of 0.9% saline subcutaneously to compensate for plasma volume loss.

Assessment of intramuscular triglycerides. GLUT-4, and glucose enzymes. Clamp-frozen soleus and extensor digitorum longus (EDL) muscles were pooled from each animal, and a portion (~20 mg) was used for determination of intramuscular triglycerides (IMTG) by using the chloroform-methanol extraction described by Folch et al. (8), followed by the processing method of Frayn and Maycock (9), as modified by Denton and Randle (6). Glycerol was ultimately assayed spectrophotometrically by using a commercially available kit (Sigma Chemical, St. Louis, MO).

The remaining piece (~30 mg) of soleus and EDL muscle was homogenized in 30 volumes of ice-cold 20 mM HEPES acid buffer (pH 7.4), containing 1 mM EDTA and 250 mM sucrose. The homogenate was divided into aliquots for analysis of total protein [bicinchoninic acid (BCA) assay; Sigma], total GLUT-4 protein (14), hexokinase (38), and citrate synthase (34).

Muscle glucose transport activity. After an overnight fast, at 0800, a separate group of animals was weighed and deeply anesthetized with an intraperitoneal injection of pentobarbital sodium (50 mg/kg body wt). Soleus and EDL muscles were dissected and prepared into two strips (~25–30 mg) and incubated in the unmounted state. Each muscle was initially incubated for 30 min at 37°C in 3 ml of oxygenated (95% O2-5% CO2) Krebs-Henseleit buffer (KHB) supplemented with 8 mM glucose, 32 mM mannitol, and 0.1% BSA (radioimmunoassay grade, Sigma Chemical) in the presence or absence of a maximally effective concentration of insulin (2 mU/ml; Humulin, Eli Lilly, Indianapolis, IN). After the initial incubation period, the muscles were rinsed for 10 min at 37°C in 3 ml oxygenated KHB containing 8 mM mannitol and 0.1% BSA, in the presence or absence of insulin. Thereafter, the muscles were transferred to 2 ml KHB, containing 1 mM 2-deoxy-[1,2-3H]glucose (2-DG) (300 μCi/ mmol; Sigma), 39 mM [U-14C]mannitol (0.8 μCi/mmol; ICN Radiochemicals, Irvine, CA), and 0.1% BSA, in the presence or absence of insulin. At the end of this final 20-min 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 of 0.5 M NaOH. After the muscles were completely solubilized, 5 ml of scintillation cocktail were added, and the specific intracellular accumulation of 2-DG was determined as described previously (15). This method for assessing glucose transport activity in isolated muscle has been validated (13).

Expression of insulin signaling factors. Soleus muscle was homogenized in 8 vol of 50 mM HEPES (pH 7.5) containing 150 mM NaCl, 20 mM sodium pyrophosphate, 20 mM α-glycerophosphate, 10 mM NaF, 2 mM Na3VO4, 2 mM EDTA (pH 8.0), 1% Triton X-100, 10% glycerol, 1 mM MgCl2, 1 mM CaCl2, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 2 mM phenylmethylsulfonylfluoride. Thereafter, homogenates underwent end-over-end rotation for 20 min at 4°C, followed by a 20-min centrifugation at 13,000 g. The homogenate was then analyzed for total protein (BCA assay, Sigma). Samples were then diluted to equal protein concentrations and combined with sodium lauryl sulfate (SDS) sample buffer [125 mM Tris (pH 6.8), 4% SDS, 20% glycerol, 10% β-mercaptoethanol, and 0.0025% bromophenol blue]. This mixture was boiled for 5 min. After boiling, 200 μg of protein were loaded on either 7.5 or 12% Tris-HCl Ready Gels (Bio-Rad Laboratories, Hercules, CA) for SDS-PAGE. After electrophoresis, samples were transferred to nitrocellulose paper for Western blotting.

For analysis of Akt1/2 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 h at room temperature. It was then washed three times with TBS/T. The nitrocellulose was then incubated overnight at 4°C with anti-Akt antibody (recognizing both the Akt1 and Akt2 isoforms) (Cell Signaling Technology, Beverly, MA) diluted 1:1,000 in TBS/T containing 5% BSA. On completion, the nitrocellulose was washed three times with TBS/T and then incubated for 1 h with goat anti-rabbit IgG horseradish peroxidase (HRP)-conjugated secondary antibody (Chemicon International, Temecula, CA) diluted 1:1,000 in TBS/T containing 5% nonfat dry milk. The nitrocellulose was then washed three 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 h at room temperature in PBS 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 two times with water. Thereafter, the nitrocellulose was incubated for 1.5 h 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 min and then four additional times with water. For analysis of IR-β and p38 MAPK, the nitrocellulose was blocked for 1 h 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 (Santa Cruz Biotechnology, Santa Cruz, CA) diluted in TBST-MLK. The nitrocellulose was washed three times with TBS/T.

Downloaded from http://jap.physiology.org/ by IP 10.220.32.246 on August 29, 2017
above for Akt expression analysis. Analysis of phosphorylated Akt used antiphospho-Akt (Ser473) antibody (Cell Signaling) for the primary antibody.

Measurement of tyrosine-phosphorylated IRS-1, IR-β, and IRS-1 associated with p85 was performed via immunoprecipitation and immunoblotting. Each soleus muscle was homogenized in 1 ml of 50 mM HEPES (pH 7.4) containing 150 mM NaCl, 1% Triton X-100, 2 μg/ml aprotonin, 0.2 mM leupeptin, 2 mM phenylmethylsulfonyl fluoride, 0.1 mM antipain, and 0.5 U/ml aprotinin. Thereafter, homogenates were incubated for 20 min at 4°C followed by 20-min centrifugation at 13,000 g. The homogenate was then analyzed for total protein (BCA assay; Sigma). Samples were then diluted to 1 mg/ml for analysis of phosphorylated IR-β, 0.5 ml of homogenate were immunoprecipitated with 10 μl of recombinant agaroose-conjugated anti-phosphotyrosine antibody (4G10, Upstate Biotechnology) and gently rocked overnight at 4°C. The solution was then placed in a microcuf¬rifuge for ~5 s. The supernatant was removed, and the beads were washed three times with PBS. The beads were then exposed to SDS sample buffer and boiled for 5 min. For analysis of phosphorylated IRS-1 and IRS-1 associated with p85, 1 ml of diluted homogenate was combined with 20 μl of agaroose-conjugated anti-IRS-1 antibody (Upstate Biotechnology) and gently rocked overnight at 4°C. The solution was then placed in a microcentrifuge for ~5 s. The supernatant was removed, and the beads were washed three times with PBS. The beads were then exposed to SDS-PAGE sample buffer and boiled for 5 min. Thereafter, 40 μl of sample were loaded on a 7.5% Tris-HCl Ready Gels for SDS-PAGE. After electrophoresis, samples were transferred to nitrocellulose paper for Western blotting.

Immunoblotting for detection of phosphorylated IR-β and IRS-1 associated p85 was conducted as described above for detection of protein expression of IR-β and p85. For analysis of phosphorylated IRS-1, the nitrocellulose was blocked for 1 h 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 three times with TBST. The nitrocellulose was then incubated for 1 h 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 by using enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ) and exposure to Kodak X-Omat AR Film (Sigma). Densitometric analysis was carried out by using a Bio-Rad GS-800 Calibrated Densitometer.

Statistical analysis. All data are presented as means ± SE. The significance of differences between multiple groups was assessed by a factorial ANOVA with a post hoc Fisher’s paired least significant difference test (StatView version 5.0, SAS Institute, Cary, NC). A P value of <0.05 was considered to be statistically significant.

RESULTS

Body weights, muscle weights, and muscle glucose metabolism enzymes. After 3 days of HS, the final body weight of the suspended animals (107 ± 1 g) was significantly (P < 0.05) less than that of the weight-bearing control animals (115 ± 2 g). This difference in final body weight was even more pronounced after 7 days of HS (118 ± 3 vs. 131 ± 6 g). The relative muscle wet weights for the soleus were 16 and 46% less in the 3- and 7-day HS animals, respectively, compared with the weight-bearing soleus value (Table 1). Moreover, the total protein concentration in the 3- and 7-day HS soleus was decreased by 11 and 13%, respectively. The relative weight and total protein concentration of the EDL were not altered by HS. Total GLUT-4 protein (62%) and the activities of total hexokinase (93%) and citrate synthase (34%) were increased in the 7-day HS soleus. In the EDL, only the activity of total hexokinase (53%) was significantly increased after 7 days of HS.

Whole body insulin sensitivity. Fasting plasma glucose values were not different among the three groups (control, 109 ± 4 mg/dl; 3-day HS, 113 ± 5 mg/dl; and 7-day HS, 104 ± 4 mg/dl), and fasting plasma insulin was only marginally greater in the 7-day HS group (12.9 ± 1.0 μU/ml) compared with the control (10.6 ± 1.0 μU/ml) and 3-day HS (10.5 ± 1.0 μU/ml) groups. The relative response of plasma glucose during an ITT was used as an index of whole body insulin sensitivity (Fig. 1). Whereas the plasma glucose response during the ITT in the 3-day HS animals did not differ from that of the weight-bearing control animals, the glucose values at the 30-, 60-, 90-, and 120-min time points in the 7-day HS animals were all significantly lower compared with the corresponding weight-bearing control values. Moreover, the integrated decrease in plasma glucose induced by the insulin load over the 120-min ITT was 25% greater in the 7-day HS group than in the control group.

Muscle glucose transport activity. Glucose transport activity, assessed by 2-DG uptake, in the absence of insulin was not altered in the 3- and 7-day unweighted soleus compared with control (Fig. 2A). However, the rate of 2-DG uptake in the presence of insulin and the increase in 2-DG uptake above basal due to insulin were significantly enhanced compared with control in the 3-day HS soleus (55 and 72%, respectively) and in the 7-day HS soleus (118 and 158%, respectively). In contrast, HS had no effect on either basal or insulin-stimulated glucose transport.
Glucose transport activity in the EDL muscle of the 3- and 7-day animals (Fig. 2B).

**IMTG.** Because of the inverse relationship between whole body and skeletal muscle insulin action and the triglyceride concentration in skeletal muscle (18, 23, 30, 31), we assessed IMTG levels in soleus and EDL muscles of the HS animals (Fig. 3). The IMTG level in the soleus (a type I muscle) was more than twofold greater than in the EDL (a type II muscle). Moreover, compared with the weight-bearing control group, the IMTG concentration was decreased by 45% in both the 3-day HS soleus and the 7-day HS soleus, whereas the IMTG concentration in the EDL was not affected by HS.

**Protein expression and functionality of insulin signaling factors.** Because HS affected insulin action on glucose transport only in the soleus muscle, the determination of the protein expression and functionality of insulin signaling factors were restricted to this muscle type. There were no changes in the protein expression or insulin-stimulated tyrosine phosphorylation state of IR-β in the unweighted soleus (Fig. 4). In contrast, the protein expression of IRS-1 in unweighted soleus was significantly less than the control value after 3 days (66% of control) and 7 days (36% of control) of HS (Fig. 5). Interestingly, the protein expression of another IRS isoform in muscle, IRS-2, was also reduced in the 3-day HS (69% of control) and 7-day HS (63% of control) soleus (data not shown). Despite this substantial decline in IRS-1 protein level, the absolute amount of insulin-stimulated IRS-1 tyrosine phosphorylation was not compromised in the unweighted soleus (Fig. 5, center). Consequently, the tyrosine phosphorylation of IRS-1 per unit IRS-1 protein was enhanced by 62 and 184% in the 3- and 7-day HS soleus, respectively (Fig. 5, right).

Soleus unweighting did not alter the protein expression of either the p85 subunit of PI3-kinase (Fig. 6) or Akt1/2 (Fig. 7). The unweighting-induced increase in insulin action on the relative IRS-1 tyrosine phosphorylation was accompanied by a 47% enhancement of insulin action on IRS-1-associated p85 in the 7-day HS soleus (Fig. 6). Interestingly, the Ser473 phosphorylation of Akt in the absence of insulin was reduced by 57% in the 7-day HS soleus. Nevertheless, the phosphorylation state of Akt in the presence of insulin did not differ between the HS and weight-bearing control groups (Fig. 7).

**Protein expression and activation of p38 MAPK.** After 3-day HS, there was not a significant change in the protein expression and activation, as reflected by the phosphorylation state, of the glucose transport activity in the EDL muscle of the 3- and 7-day animals (Fig. 2B).

Fig. 1. Glucose responses during an insulin tolerance test in weight-bearing and 3- or 7-day hindlimb-suspended (HS) animals. Left: glucose concentrations following the insulin load. Right: integrated decreases in glucose concentration over the 120-min test period. Values are means ± SE for 21 weight-bearing animals and 7–9 HS animals. *P < 0.05 vs. weight-bearing control.

Fig. 2. Effect of 3- or 7-day HS on glucose transport activity in rat soleus (A) and extensor digitorum longus (EDL) (B) muscles. Bars represent the rates of 2-deoxyglucose uptake in the absence of insulin (–), in the presence of insulin (+), and the increase above basal due to insulin (Δ) in isolated muscle strips. Values are means ± SE for 5–6 animals per group. Within all groups, the insulin stimulation above basal was significant (P < 0.05). *P < 0.05 vs. weight-bearing control group.
Fig. 3. Effect of 3- or 7-day HS on intramuscular triglyceride concentration in soleus and EDL muscles. Values are means ± SE for 5–9 animals per group. *P < 0.05 vs. weight-bearing control group.

Fig. 4. Effect of 3- or 7-day HS on insulin receptor β-subunit (IR-β) protein expression and insulin-stimulated IR-β tyrosine phosphorylation in soleus muscle. Representative bands from the immunoblots are shown above each bar. Values are means ± SE for 6–8 animals per group. IR-β tyrosine phosphorylation in the absence of insulin was undetectable. *P < 0.05 vs. weight-bearing control group.

Fig. 5. Effect of 3- or 7-day HS on insulin (INS) receptor substrate-1 (IRS-1) protein expression, insulin-stimulated IRS-1 phosphotyrosine, and the relative insulin-stimulated IRS-1 tyrosine phosphorylation per unit IRS-1 protein in soleus muscle. IRS-1 tyrosine phosphorylation in the absence of insulin was undetectable. Representative bands from the immunoblots are shown above each bar. Values are means ± SE for 11–15 weight-bearing (WB) animals and 5–8 HS animals per group. *P < 0.05 vs. weight-bearing control group.
stress-activated serine/threonine protein kinase, p38 MAPK (Fig. 8). However, the protein expression of p38 MAPK was decreased by 32% in the 7-day HS soleus, and the p38 MAPK activation was 18% less than in the weight-bearing control group and 45% less than in the 3-day unweighted group.

Effect of PI3-kinase inhibition on glucose transport in unweighted soleus muscle. The PI3-kinase inhibitor wortmannin (1 μM) was used to assess the dependence of the increase of insulin-stimulated glucose transport activity in unweighted soleus muscle on intact activity of PI3-kinase (Fig. 9). In the absence of insulin, wortmannin had no significant effect on glucose transport activity in any group. In contrast, wortmannin completely prevented insulin-mediated glucose transport in weight-bearing control soleus muscles. This inhibition by wortmannin of insulin-mediated glucose transport was incomplete in the 3-day (92% inhibition, P < 0.05) and 7-day (89% inhibition, P = 0.09) unweighted soleus.

DISCUSSION

In the present investigation, we have made the novel observation that the increases in insulin action on glucose transport activity in the 7-day unweighted soleus muscle of juvenile, female rats (Fig. 2A) are associated with enhanced functionality of specific proteins in the insulin signaling cascade. The most critical adaptive responses to soleus unweighting were the enhanced relative tyrosine phosphorylation of IRS-1 (Fig. 5), despite a marked decline in IRS-1 protein expression (Fig. 5), and the increased association of IRS-1 with the p85 subunit of PI3-kinase (Fig. 6), a surrogate index of PI3-kinase activation. Interestingly, no significant enhancement of insulin action was observed for IRS-tyrosine phosphorylation (Fig. 4) or Akt serine phosphorylation (Fig. 7), implying that these steps are not involved in the upregulation of glucose transport in the unweighted soleus muscle. Moreover, the enhanced insulin response of the soleus from HS animals appears to be a local, unweighting-induced phenomenon, as insulin action on the EDL, which is not involved in the weight-bearing process, was not altered by HS (Fig. 2B), which is in agreement with most previous studies (36).

Another important adaptive response to soleus unweighting was the increase in total GLUT-4 protein expression (Table 1), confirming previous investigations (16, 17). Again, this adap-

Fig. 6. Effect of 3- or 7-day HS on protein expression of the p85 regulatory subunit of phosphatidylinositol-3-kinase and insulin-stimulated IRS-1 associated with p85 in soleus muscle. IRS-1 associated with p85 in the absence of insulin was undetectable. Representative bands from the immunoblots are shown above each bar. Values are means ± SE for 14 weight-bearing animals and 4–6 HS animals per group. *P < 0.05 vs. weight-bearing control group.

Fig. 7. Effect of 3- or 7-day HS on Akt protein expression and insulin-stimulated Akt Ser473 phosphorylation in soleus muscle in the absence (−) or presence (+) of insulin. Values are means ± SE for 5–8 animals per group. Within all groups, the insulin stimulation above basal was significant (P < 0.05). *P < 0.05 vs. weight-bearing control group.
tive response was restricted to the unweighted soleus and was not observed in the EDL (Table 1). It is noteworthy that parallel increases in total hexokinase and citrate synthase also occur in unweighted soleus muscle (Table 1; Ref. 16), ensuring that the capacities for glucose phosphorylation and mitochondrial oxidation are not limiting for intracellular glucose catabolism in the face of an enhanced capacity for insulin-stimulated glucose transport. The present data support the concept that the enhanced activation of PI3-kinase is involved in the recruitment of GLUT-4 protein from an enlarged cellular pool to the plasma membrane, thereby allowing for a greater rate of insulin-stimulated glucose transport. However, the fact that the PI3-kinase inhibitor wortmannin was not able to completely suppress insulin-stimulated glucose transport activity in the unweighted soleus (Fig. 9) indicates that a small but important contribution to this enhanced glucose transport response must come from a PI3-kinase-independent mechanism. The identification of this PI3-kinase-independent mechanism should be the subject of future investigations.

Seven days of HS were also associated with an increase in whole body insulin sensitivity, as reflected by the substantially larger decline in plasma glucose during the 2-h ITT (Fig. 1). The enhanced disappearance of plasma glucose during the ITT was likely due to increased skeletal muscle glucose disposal, as the large bolus of insulin provided (0.3 U/kg) would suppress hepatic glucose production completely and would thereby eliminate the potential confounding influence of the altered liver glucose output. Although insulin action on glucose uptake was specifically enhanced in the unweighted soleus, this muscle represents only a very small fraction of total muscle mass, and it is clear that other muscles in the hindlimb, which experience increased insulin responsiveness during prolonged suspension (35), must also contribute to this increased whole body response to insulin. In addition, the lesser body weight of the 7-day suspended animals (Table 1), likely due to a lesser fat mass, may have contributed to the increased whole body insulin sensitivity.

The IMTG concentration in the unweighted soleus was substantially less than in the weight-bearing control muscle (Fig. 3). In light of the well-established inverse relationship between IMTG and whole body and skeletal muscle insulin action (18, 23, 30, 31), it is possible that the lesser availability of these lipids or their degradation products, which normally have a negative effect on insulin signaling and glucose trans-
port (5), contributed to the enhanced insulin signaling at the IRS-1 and PI3-kinase steps and allowed for the increased glucose transport activity in the unweighted soleus muscle. The exact mechanism for the decline in IMTG in the unweighted soleus is unknown, although we have shown that this decrease in IMTG is an early event during unweighting of the soleus, occurring within the first 24 h of HS (32), and may be caused by increased lipolysis associated with an increase in stress hormones, such as glucocorticoids (24).

An important finding of this study was the decrease in p38 MAPK protein expression and activation in the 7-day HS soleus (Fig. 8). Contradictory information on the exact role of this stress-activated serine/threonine kinase in the regulation of glucose transport exists in the literature. Whereas recent studies provide evidence that p38 MAPK acts as a negative modulator of insulin signaling and glucose transport (10, 22, 29), other investigations indicate that activation of p38 MAPK by insulin is necessary for stimulation of GLUT-4 activity and, therefore, for full activation of glucose transport (see Ref. 11 for a recent review). Our findings support the concept that p38 MAPK elicits a negative effect on insulin-stimulated glucose transport activity in rat skeletal muscle, as the decrease in p38 MAPK protein and activation seen in the 7-day HS soleus (Fig. 8) was associated with a further enhancement of insulin-stimulated glucose transport activity (Fig. 2A). Furthermore, in the 1-day unweighted soleus, in which a transient state of insulin resistance is observed (32), there is an increase in p38 MAPK protein expression and activation (32).

The mechanism for these dynamic alterations in p38 MAPK protein expression during the course of soleus unweighting is unclear. Childs et al. (4) have recently suggested that muscle activity may be an important factor in this event. They report an enhancement of p38 MAPK protein in soleus from hind-limb-immobilized rats, in which electromyographic (EMG) activity is markedly decreased (7). Our laboratory has also shown an increase in p38 MAPK protein expression in 1-day unweighted soleus (32), in which EMG activity is completely absent (2). Interestingly, the decrease in p38 MAPK protein toward control in the 3-day HS soleus and the further decrease below control levels in the 7-day HS soleus (Fig. 8) parallel exactly the time course for the spontaneous recovery of EMG activity in unweighted soleus (2). How muscle activity may regulate the protein level and activation state of the p38 MAPK is presently unknown.

Whereas the changes in the relevant insulin signaling elements and associated factors that regulate glucose transport were manifest in the 7-day unweighted soleus, there were some inconsistencies in these adaptive responses in 3-day HS soleus. This may be related to the fact that the 3-day time point is in a period of transition from a condition of whole body and skeletal muscle insulin resistance (1-day HS) (19, 32) to one of markedly enhanced insulin action (7-day HS or longer) (present study; Refs. 3, 20, 21, 27, 35). The time courses for increases in the functionality of factors directly involved in insulin signaling (p85; Fig. 6), for alterations in factors that can modulate insulin signaling (p38 MAPK; Fig. 8), and for enhancement of GLUT-4 protein expression (Table 1) may be variable over this transitional period. Changes in these variables may not yet be statistically significant at the 3-day time point but could nevertheless be physiologically relevant.

In summary, the present results indicate that the increased insulin action on glucose transport in the 7-day unweighted soleus of juvenile female rats is associated with increased insulin signaling through IRS-1 and PI3-kinase and with decreased protein expression and activation of the stress-activated p38 MAPK. However, because of the incomplete inhibitory effect of the PI3-kinase inhibitor wortmannin on insulin-stimulated glucose transport in the unweighted soleus, PI3-kinase-independent mechanisms must also play a small role in this adaptive response to HS. Further investigation is needed to identify these PI3-kinase-independent factors.

ACKNOWLEDGMENTS
The authors thank Cody Diehl and Tyson Kinnick for excellent technical assistance.

GRANTS
This study was supported in part by National Aeronautics and Space Administration Grant NAG2–1187 and by Minority Access to Research Careers National Institute of General Medical Sciences Grant GM08718.

REFERENCES


32. O’Keefe MP, Perez FR, Kinnick TR, Tischler ME, and Henriksen EJ. Development of whole-body and skeletal muscle insulin resistance after one day of hindlimb suspension. Metabolism In press.


