GLUT4 and Glycogen Synthase Are Key Players in Bed Rest–Induced Insulin Resistance

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To elucidate the molecular mechanisms behind physical inactivity–induced insulin resistance in skeletal muscle, 12 young, healthy male subjects completed 7 days of bed rest with vastus lateralis muscle biopsies obtained before and after. In six of the subjects, muscle biopsies were taken from both legs before and after a 3-h hyperinsulinemic euglycemic clamp performed 3 h after a 45-min, one-legged exercise. Blood samples were obtained from one femoral artery and both femoral veins before and during the clamp. Glucose infusion rate and leg glucose extraction during the clamp were lower after than before bed rest. This bed rest–induced insulin resistance occurred together with reduced muscle GLUT4, hexokinase II, protein kinase B/Akt1, and Akt2 protein level, and a tendency for reduced 3-hydroxyacyl-CoA dehydrogenase activity. The ability of insulin to phosphorylate Akt and activate glycogen synthase (GS) was reduced with normal GS site 3 but abnormal GS site 2+2a phosphorylation after bed rest. Exercise enhanced insulin-stimulated leg glucose extraction both before and after bed rest, which was accompanied by higher GS activity in the prior-exercised leg than the rested leg. The present findings demonstrate that physical inactivity–induced insulin resistance in muscle is associated with lower content/activity of key proteins in glucose transport/phosphorylation and storage. ***Diabetes*** **61:1090–1099, 2012**

Lifestyle-related diseases like type 2 diabetes are rapidly increasing worldwide, and there is strong evidence that physical inactivity contributes to this development (1). The prediabetic and diabetic states are characterized by peripheral insulin resistance (2), and due to the large mass of skeletal muscle, the insulin action in this tissue has major influence on whole-body insulin sensitivity, especially in lean adults (3). Insulin sensitivity in skeletal muscle is to a large extent a dynamic parameter that can be enhanced by exercise training and decreased by physical inactivity (4–7). Thus, research attempting to combat diseases related to insulin resistance has focused on mechanisms by which the sensitivity of the glucose uptake to insulin is regulated.

Type 2 diabetes patients with peripheral insulin resistance have impaired GLUT4 translocation in skeletal muscle (8). Moreover, muscle insulin–resistant individuals and type 2 diabetes patients have impaired insulin-stimulated Akt Thr^{308} and Ser^{473} phosphorylation (9–11), lower insulin-stimulated Akt substrate of 160 kDa (AS160/TBC1D4) phosphorylation on multiple sites (7,9–11), and impaired insulin-stimulated glycogen synthase (GS) activity (9). The impaired GS activity in type 2 diabetes patients is associated with hyperphosphorylation of GS site 2+2a during insulin stimulation, whereas GS site 3a phosphorylation appears to be unaffected (9,12), indicating that GS phosphorylation downstream of Akt is maintained despite lower Akt phosphorylation. Exercise training has been shown to enhance the capacity for glucose transport and glycogen storage in skeletal muscle by increasing GLUT4, hexokinase II (HKII), and TBC1D4 protein content as well as GS activity in both healthy and type 2 diabetic patients, and these adaptations may contribute to the training-induced improvements in insulin sensitivity (13–15). In addition, the improved insulin sensitivity observed after exercise training has been reported to be associated with improved, and in fact normalized, insulin-induced phosphorylation of TBC1D4 in type 2 diabetes (7). In line with the changes observed with training, physical inactivity has been demonstrated to induce insulin resistance (5,16). However, to the best of our knowledge, no study has reported the effects of physical inactivity on key proteins in glucose metabolism in human skeletal muscle.

Muscle insulin sensitivity has been shown to be increased in the period after a single bout of exercise (17). Thus, enhanced insulin-mediated glucose clearance is observed for up to 48 h after a single exercise bout (17–19). It has been shown in rat skeletal muscle that the increased insulin sensitivity to glucose uptake is associated with an increased GLUT4 translocation to the plasma membrane (20). Moreover, multiple studies have documented that insulin-mediated activation of the most proximal insulin signaling elements (e.g., insulin receptor, insulin receptor substrate, phosphatidylinositol-3 kinase, and Akt) is not enhanced after a single bout of exercise (21–23). Interestingly, recent studies have revealed that basal and insulin-induced TBC1D4 phosphorylation are increased in the period after a single bout of exercise (24,25), although unchanged TBC1D4 phosphorylation has also been reported, but in a study not detecting increased glucose uptake after exercise (26). Studies have also reported an increased GS activity 3–4 h after exercise (27), and thus a combination of increased phosphorylation of TBC1D4 (regulating glucose transport)

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and increased GS activity (regulating glycogen synthesis) may explain the increased sensitivity of insulin to stimulate glucose uptake 3–4 h after a single bout of exercise.

Knowing the underlying molecular mechanisms behind physical inactivity–induced insulin resistance and the impact of physical inactivity on exercise-induced insulin sensitivity may provide the basis for developing pharmaceutical agents and interventions that can counteract the development of insulin resistance in skeletal muscle during bed rest after surgery/illness as well as induced by a physically inactive lifestyle. The aim of the current study was to test the hypotheses that *1*) physical inactivity–induced insulin resistance in human skeletal muscle is due to dysregulation of both GS and TBC1D4 and 2) exercise-induced enhancement of insulin sensitivity is lost with physical inactivity.

RESEARCH DESIGN AND METHODS

Subjects. Twelve young, healthy male subjects participated in the study (Table 1). The subjects were physically active, as they all walked or bicycled for >1 h/day. The subjects were given both oral and written information about the experimental protocol and procedures as well as the discomfort they might experience during the experiment. After having received this information, the subjects gave their written consent. The study was performed according to the Declaration of Helsinki and was approved by the Copenhagen and Frederiksberg Ethics Committee in Denmark (H-C-2007-0085).

Bed rest. The subjects were placed in bed for 7 days and were given information about the importance of not moving their legs. All transport of subjects took place in wheelchairs and subjects were told to sit down during showers. The subjects were encouraged to sit in the wheelchair for up to 5 h per day to avoid vascular complications. The daily energy intake during the bed rest came from meals containing 10–20% protein, 50–60% carbohydrates, and 25–35% fat. The meals were served ad libitum.

Physical performance and body composition. Twelve subjects were included in the bed rest part (Table 1). Six of these subjects were chosen randomly to follow a study protocol involving one-legged exercise and a hyperinsulinemic euglycemic clamp. The characteristics of these subjects are given in brackets in Table 1.

Approximately 2 weeks before the bed rest, maximal oxygen uptake ($V_{O_{2max}}$) was determined by an incremental test on a cycle ergometer (Monarch Ergomedic) for all 12 subjects. Body composition and body weight were determined by dual-emission X-ray absorptiometry scanning (GE Healthcare). The $V_{O_{2max}}$ test and the dual-emission X-ray absorptiometry scan were repeated at the end of the bed rest period. In addition, the six subjects participating in the exercise and clamp protocol performed an incremental one-legged knee extensor exercise test to exhaustion on a modified cycle ergometer bike (Monarch Ergomedic) to determine $Watt_{max}$ as previously described (28).

Experimental protocol. The day before the experimental day (both before and after the bed rest period), the subjects consumed a prepackaged, standardized meal and evening snack regulated for body weight (14.3 kcal/kg and 2.9 kcal/kg, respectively). On the experimental day, the subjects arrived to the laboratory in the morning. Catheters were placed in the femoral vein of each leg and in one femoral artery. Three and a half hours after consuming a standardized breakfast (2.9 kcal/kg), the subjects completed 45 min of one-legged knee extensor exercise at 60% of $Watt_{max}$. This relatively low intensity was chosen to ensure that the subjects would be able to complete the exercise bout after the bed rest. Three hours into recovery, a 3-h euglycemic hyperinsulinemic clamp with a constant rate of insulin infusion (50 mU/[min · m^2]) (Actrapid; Novo Nordisk, Bagsvaerd, Denmark) was performed. Arterial blood samples were obtained every 5–10 min for immediate determination of plasma glucose concentration (Radiometer ABL 725 series Acid-Base Analyser). Euglycemia was maintained by subsequent adjustment of the glucose infusion rate (GIR). The duration of 3 h was chosen to approach clamp steady-state conditions at the sampling time points.

Muscle biopsies were obtained from the vastus lateralis of both the previously exercised (Ex leg) and the rested leg (Rest leg) both before (B) and after (I) the clamp using the needle biopsy technique (29) with suction. Incisions for biopsies were made under local anesthesia (lidocaine; AstraZeneca, Södertälje, Sweden) and individual incisions were used for each biopsy. The biopsies were quickly frozen in liquid nitrogen (within 10–20 s) after removing visual blood and connective tissue.

Before as well as at 2, 2.5, and 3 h of the insulin clamp, blood samples were drawn from the three femoral catheters and placed in EDTA-treated tubes. Plasma was collected by centrifugation and stored at −80°C.

Plasma analyses. The femoral arterial and venous plasma glucose concentrations were measured using an acid-base analyzer (Radiometer ABL 725 series Acid-Base Analyser), and arterial insulin concentration was measured by ELISA (K6219; DAKO, Glostrup, Denmark). Glucose extraction across the leg was calculated as the difference between the glucose concentration in the arterial and venous plasma samples. The plasma palmitate concentration was determined by gas chromatography (30)

Muscle samples were freeze dried for >48 h. Visible blood, fat, and connective tissue were removed under a microscope.

Glycogen was measured as glycosyl units after acid hydrolysis using an automatic spectrophotometer as previously described (31,32).

Enzyme activities. The activity of 3-hydroxyacyl-CoA dehydrogenase (HAD) and citrate synthase (CS) was analyzed spectrophotometrically as previously described (32). The GS activity was determined in muscle homogenates as previously described (22,33).

SDS-PAGE and Western blotting. Muscle homogenate, muscle lysate, and protein concentration determination were prepared as previously described (25,34).

The protein or phosphorylation of β-actin, HKII, GLUT4, Akt, GS kinase 3β (GSK-3β), and TBC1D4 in muscle lysates and GS protein and protein phosphorylation in muscle homogenates were measured by SDS-PAGE and Western blotting (25,34). Primary polyclonal antibodies against β-actin (4967; Cell Signaling Technology, Beverly, MA), HKII (2867; Cell Signaling Technology), GLUT4 (ABR-PAI1065; Thermo Scientific, Golden, CO), Akt1 and Akt2 (2967 and 3063, respectively; Cell Signaling Technology), GSK-3α and -3β (9338 and 9315, respectively; Cell Signaling Technology), and TBC1D4 protein (ABCAM, Cambridge, U.K.) as well as against protein phosphorylation of Akt Thr^{308} (05-669; Upstate Biotechnology, Lake Placid, NY) and Ser^{473} (05-802; Millipore, Bedford, MA), GSK-3α Ser^{21} and GSK-3β Ser^{9} (9331 and 9323, respectively; Cell Signaling Technology), TBC1D4 Ser^{588} and Thr^{642} (3028-P2 and 3028-P1, respectively; Symansis, Auckland, N.Z.), and TBC1D4 Ser^{751} (provided by Graham Hardie, Dundee University, Dundee, U.K.) were used. Primary polyclonal antibodies against GS protein (provided by Oluf Pedersen, University of Copenhagen) and phosphospecific antibodies against 2+2a, 1b (provided by Graham Hardie), and 3a (3891; Cell Signaling Technology) were also used. Membranes probed with phosphospecific antibodies were stripped to allow for determination of the level of the corresponding protein as previously described (25).

Statistics. Values presented are means ± SE. Two-way ANOVA with repeated measures was applied to evaluate the effect of exercise and insulin before as well as after bed rest. Also, a two-way ANOVA with repeated measures was

TABLE 1
Subject characteristics before and after bed rest

Subject characteristics	Before bed rest	After bed rest
Age (years)	26 ± 3 (29 ± 5)	—
Height (cm)	181 ± 2 (183 ± 7)	—
Weight (kg)	75.2 ± 3.2 (81.6 ± 4.9)	75.1 ± 3.3 (81.4 ± 5.0)
$V_{O_{2max}}$ (L/min)	3.9 ± 0.2 (4.1 ± 1.0)	3.7 ± 0.2 (3.9 ± 0.4)†
Leg muscle mass (kg)	20.6 ± 1.0 (21.7 ± 1.9)	20.0 ± 0.9 (21.0 ± 1.7)†
Percentage of whole body fat (kg)	18.2 ± 2.1 (21.7 ± 3.3)	18.5 ± 2.1 (21.7 ± 3.1)

Age, height, weight, $V_{O_{2max}}$, leg muscle mass, and percentage of whole body fat before and after 7 days of bed rest. Values are means ± SE; n = 12. Values given in parentheses represent the six subjects randomly chosen for the invasive exercise and clamp study. †Significantly different from before bed rest, $P < 0.05$.

used to evaluate the effect of bed rest and insulin within the rested leg as well as within the exercised leg. The data were log transformed if normality or equal variance tests failed. If significant main effects were found, the Student-Newman-Keuls post hoc test was used to locate differences. Differences were considered significant at $P < 0.05$. A tendency is reported for $0.05 \leq P < 0.1$. Statistical calculations were performed using SigmaStat version 3.1.

RESULTS

Physical characteristics and performance. Total body weight and BMI were unaffected by 7 days of bed rest (Table 1). Leg muscle mass was reduced 3 ± 0.7% ($P < 0.05$), whereas whole body fat did not change significantly with bed rest (Table 1). Seven days of bed rest reduced whole body Vo_{2max} 5 ± 1.4% ($P < 0.05$) (Table 1).

Protein content and enzyme activity. Skeletal muscle HAD activity tended to decrease 8 ± 4.7% ($0.05 \leq P < 0.1$) by bed rest, whereas CS activity did not change significantly (Table 2). The protein content of HKII (66 ± 15%), GLUT4 (74 ± 14%), Akt1 (56 ± 6%), and Akt2 (68 ± 7%) was reduced after bed rest relative to before ($P < 0.05$), whereas the protein level of TBC1D4 and GS and GS total activity were unchanged with bed rest (Fig. 1 and Table 2).

Plasma insulin. The average plasma insulin concentration before exercise, immediately after, and 3 h into recovery was not significantly different before and after bed rest, with an average of 32 ± 15 mmol/L before and 74 ± 20 mmol/L after bed rest. However, during the last hour of the clamp, plasma insulin concentration tended to be higher after (452 ± 66 pmol/L) than before (341 ± 30 pmol/L) bed rest ($0.05 \leq P < 0.1$) (Table 3).

Plasma palmitate. The plasma palmitate concentration was unaffected by bed rest both before and after the insulin clamp. Insulin reduced the plasma palmitate concentration both before and after bed rest ($P < 0.05$).

Plasma glucose and GIR. The arterial plasma glucose concentration during the clamp was similar, 4.9 ± 0.1 mmol/L before and after bed rest. The GIR did not reach a steady state even after 180 min (Fig. 2*A*). At all time points after 50 min, GIR was higher before than after bed rest ($P < 0.05$). During the last hour of the clamp, GIR after bed rest was 75 ± 12% of the level before bed rest ($P < 0.05$) (Fig. 2*A*).

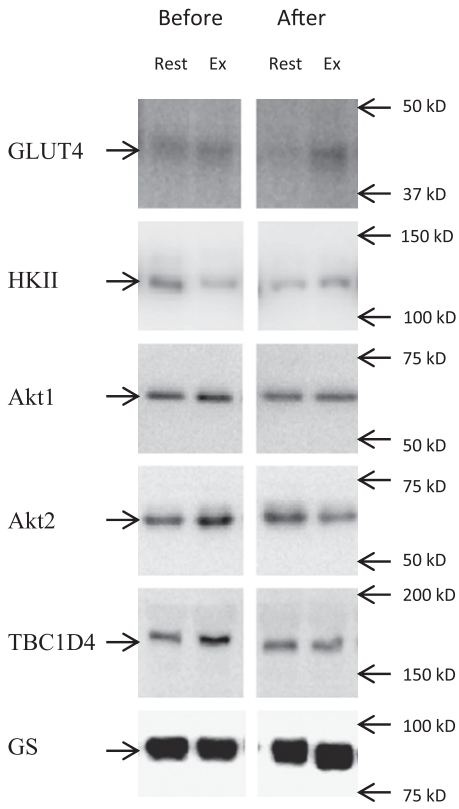

FIG. 1. Representative blots showing GLUT4, HKII, Akt1, Akt2, TBC1D4, and GS protein before and after 7 days of bed rest in rested (Rest) and prior-exercised (Ex) leg for the same subject. Although the average Akt2 protein content of all subjects is reduced with bed rest (Table 2), it may be noted that the abundance of Akt2 is not reduced with bed rest in the subject used for the representative blots. kD, kilodaltons.

TABLE 2
Muscle protein content and activity before and after bed rest

Protein	Before bed rest	After bed rest
GLUT4 protein	1 ± 0.18	0.74 ± 0.14†
HKII protein	1 ± 0.14	0.66 ± 0.15†
Akt1 protein	1 ± 0.13	0.56 ± 0.06†
Akt2 protein	1 ± 0.13	0.68 ± 0.07†
TBC1D4 protein	1 ± 0.22	0.79 ± 0.21
GS protein	1 ± 0.15	0.82 ± 0.21
GS total activity ($nmol \cdot min^{-1} \cdot mg^{-1}$)	30.7 ± 2.3	30.8 ± 1.5
HAD activity ($\mu mol \cdot min^{-1} \cdot kg^{-1}$)	23.5 ± 1.2	21.4 ± 1.4(†)
CS activity ($\mu mol \cdot min^{-1} \cdot kg^{-1}$)	44.9 ± 3.8	41.9 ± 4.2

GLUT4 protein, HKII protein, Akt2 protein, TBC1D4 protein, and GS protein content, as well as GS total activity ($nmol \cdot min^{-1} \cdot mg^{-1}$), HAD activity ($\mu mol \cdot min^{-1} \cdot kg^{-1}$ dry weight), and CS activity ($\mu mol \cdot min^{-1} \cdot kg^{-1}$ dry weight) in vastus lateralis before and after 7 days of bed rest. Target protein content is normalized to β-actin protein content. Values are means ± SE; $n = 12$. †Significantly different from before bed rest, $P < 0.05$. (†)Tendency, $0.05 \leq P < 0.1$.

Glucose extraction. Glucose extraction was evaluated during the last hour as the arterio-venous concentration difference across the leg. It has previously been reported that glucose extraction increased only in the exercising leg during the one-legged exercise (35). Three hours after exercise, glucose extraction across the leg was similar in the exercised and rested leg, reflecting that the acute exercise effect on glucose extraction was fully diminished at 3 h of recovery from exercise (corresponding to pre-insulin) (Fig. 2*B*). This was seen both before and after bed rest. Insulin infusion increased glucose extraction both in the prior-exercised and the rested leg, both before and after bed rest (Fig. 2*B*). During the last hour of the clamp before bed rest, glucose extraction in the rested leg was 11-fold ($P < 0.05$), and the prior-exercised leg sixfold ($P < 0.05$), higher after insulin than at basal. After bed rest, glucose extraction was six- and sevenfold ($P < 0.05$) higher after insulin than at basal in the rested and prior-exercised leg, respectively. The insulin-stimulated glucose extraction in the rested leg after bed rest was 78 ± 10% ($P < 0.05$) of the level before bed rest. Also, insulin-stimulated glucose extraction before bed rest was 1.6 ± 0.2-fold ($P < 0.05$), and after bed rest 1.9 ± 0.2-fold ($P < 0.05$), higher in the prior-exercised than in the rested leg (Fig. 2*B*).

Muscle glycogen. Three hours after exercise, muscle glycogen content was ~20% ($P < 0.05$) lower in the prior-exercised than the rested leg. This was observed both

TABLE 3
Plasma concentrations before and after bed rest

Plasma parameter	Before bed rest		After bed rest	
	B	I	B	I
Glucose (mmol/L)	5.3 ± 0.03	4.9 ± 0.1*	5.3 ± 0.08	5.0 ± 0.07*
Insulin (pmol/L)	32 ± 15	341 ± 30*	74 ± 20	452 ± 66*(†)
Palmitate (μmol/L)	183 ± 11.1	20 ± 1.3*	166 ± 9.8	20 ± 3.3*

Glucose (mmol/L), insulin (pmol/L), and palmitate (μmol/L) concentration in arterial blood plasma before and after 7 days of bed rest. Samples were obtained before (B) and after (I) a 3-h hyperinsulinemic euglycemic clamp performed 3 h after a one-legged exercise bout. Values are means ± SE; $n = 6$. *Significantly different from B, $P < 0.05$. (†)Tends to be significantly different from before bed rest, $0.05 \leq P < 0.1$.

before and after bed rest. There was no change in the muscle glycogen level in response to insulin infusion. Bed rest per se tended to enhance the muscle glycogen content ~20% ($0.05 \leq P < 0.1$) in the rested leg (Fig. 2*C*).

Muscle signaling

Akt phosphorylation. Akt Thr^{308} phosphorylation was similar in the rested and exercised leg at 3 h of recovery both before and after bed rest. Insulin infusion increased Akt Thr^{308} phosphorylation similarly four- to fivefold in both legs before and after bed rest. No effect of prior exercise was observed on insulin-induced Akt phosphorylation. However, bed rest decreased Akt Thr^{308} phosphorylation 20–30% ($P < 0.05$) after the clamp (Fig. 3*A* and *C*). This decrease was observed in both the prior-exercised and rested leg. A similar phosphorylation pattern was observed for Akt Ser^{473} (data not shown). Thus, the insulin resistance induced by bed rest was associated with decreased signaling through Akt, even after accounting for decreased Akt2 protein expression (Table 2).

GSK-3 phosphorylation. The phosphorylation pattern of Akt target sites (GSK-3α Ser^{21} and GSK-3β Ser^{9}) did not reflect the 20–30% lower Akt phosphorylation after bed rest. Phosphorylation of GSK-3β Ser^{9} was however 10–20% ($P < 0.05$) lower in the prior-exercised than the rested leg at 3 h recovery both before and after bed rest, but the ability of insulin to induce phosphorylation was apparently not significantly changed by either bed rest or prior exercise (Fig. 3*B* and *C*). Effects of insulin on GSK-3α Ser^{21} phosphorylation were too small to be detected with the antibody applied (data not shown).

TBC1D4 phosphorylation. Being a direct target of Akt TBC1D4 phosphorylation was evaluated by the use of phosphospecific antibodies toward three phosphorylation sites (Ser^{588}, Thr^{642}, and Ser^{751}). The general phosphorylation pattern revealed by these antibodies was similar (Fig. 4). Thus, both before and after bed rest, TBC1D4 phosphorylation 3 h after exercise was similar in the rested and prior-exercised leg. Insulin stimulation increased the TBC1D4 phosphorylation 1.5- to 3-fold ($P < 0.05$) in both legs before and after bed rest, and there was no change in the insulin-stimulated TBC1D4 phosphorylation with bed rest (Fig. 4).

Glycogen synthase activity and phosphorylation

GS activity. The GS activity was evaluated both as %I-form (not shown) and percent fractional velocity (%FV). The two measures of GS activity revealed the same pattern of regulation. GS %FV activity was enhanced ($P < 0.05$) in the prior-exercised leg relative to the rested leg 3 h after exercise both before ($P < 0.05$) and after bed rest ($0.05 \leq P < 0.1$). Insulin increased GS activity in both legs before and after bed rest and with a similar magnitude ($P < 0.05$). However, in both legs, the level of GS activity tended to be lower after bed rest than before ($0.05 \leq P < 0.1$) (Fig. 5*A*).

GS phosphorylation. GS phosphorylation at site 2+2a was, in general, elevated after bed rest relative to before ($P < 0.05$). Site 2+2a phosphorylation was 30% ($P < 0.05$) lower in the prior-exercised leg than in the rested leg 3 h after exercise only before bed rest (Fig. 5*B* and *C*). Insulin stimulation decreased site 2+2a phosphorylation by 70% ($P < 0.05$) before but not after bed rest.

GS site 3a phosphorylation was reduced 40% ($P < 0.05$) by insulin in the rested leg after bed rest and was reduced 45% in the prior-exercised leg ($P < 0.05$) before and tended to be reduced 35% ($0.05 \leq P < 0.1$) after bed rest in response to insulin. Bed rest resulted in an apparent ($P < 0.05$) 1.2-fold higher GS site 3a phosphorylation in both legs 3 h after exercise (Fig. 5*B* and *D*). GS phosphorylation at site 1b was not different between legs and was not affected by insulin, exercise, or bed rest (data not shown).

DISCUSSION

The main findings of the current study are that bed rest–induced insulin resistance is associated with reduced insulin-stimulated muscular GS activity and Akt signaling as well as decreased protein level of HKII and GLUT4. Thus, this study may indicate that decreased glucose extraction with bed rest occurs as a consequence of both decreased glucose transport/phosphorylation as well as decreased nonoxidative glucose metabolism in skeletal muscle. In addition, the ability of acute exercise to increase insulin-stimulated glucose extraction is well maintained even after 7 days of bed rest. However, acute exercise after bed rest does not fully normalize the ability of skeletal muscle to extract glucose to the level seen when exercise is performed before bed rest.

The observation that 7 days of bed rest resulted in lower insulin-stimulated glucose extraction across the leg is in accordance with previous bed rest studies (5,16). Unfortunately, we are not able to evaluate whether this relates to changes in blood flow. However, based on previous observations, insulin-stimulated blood flow is expected to decrease with bed rest (5,36). Thus, it seems unlikely that the decreased glucose extraction observed after bed rest is a compensation for an increased blood flow. Thus, we are confident that the decreased leg glucose extraction observed reflects decreased leg glucose uptake after bed rest.

The current study provides some indications for the molecular mechanisms behind the observed bed rest–induced insulin resistance. Although it is assumed that the observed

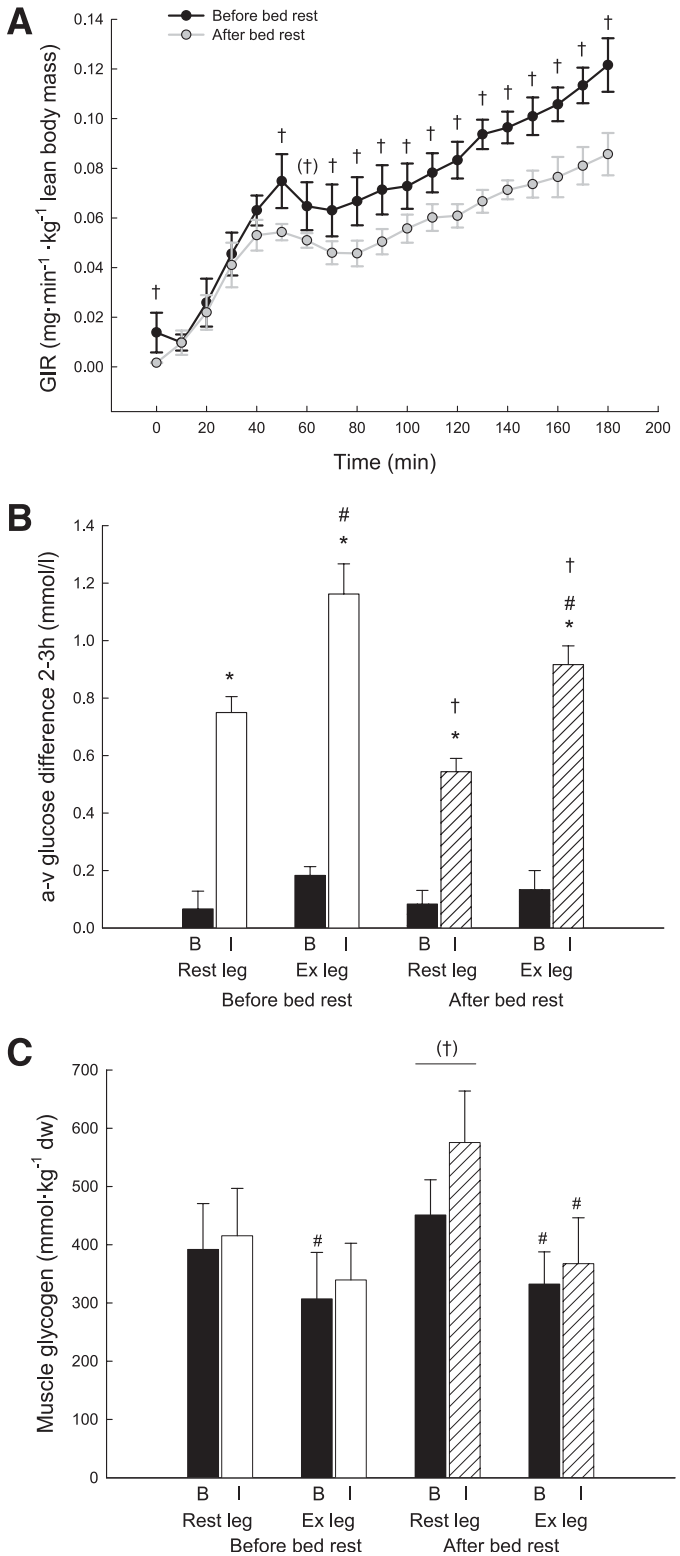

FIG. 2. ***A*: GIR during 3 h of hyperinsulinemic euglycemic clamp initiated 3 h after 45 min of one-legged knee extensor exercise before and after 7 days' bed rest.** ***B*: Arterio-venous (a-v) glucose difference across rested (Rest leg) and prior-exercised (Ex leg) vastus lateralis during 3 h of hyperinsulinemic euglycemic clamp performed 3 h after 45 min of one-legged knee extensor exercise before and after bed rest. Samples were obtained before (B) as well as during (I) the clamp, presented here by the accumulated data during the last hour of the clamp (2–3 h).** ***C*: Muscle glycogen concentration (mmol/kg dry weight [dw]) in rested and prior-exercised leg before (B) and after (I) 3 h of hyperinsulinemic euglycemic clamp performed 3 h after one-legged knee extensor exercise before and after bed rest. Values are means ± SE; $n = 6$. *Significantly different from B, $P < 0.05$. †Significantly different from before bed rest, $P < 0.05$. #Significantly different from Rest leg at given time point, $P < 0.05$. (†)Tendency, $0.05 \leq P < 0.1$.**

molecular changes are due to the physical inactivity in itself, a contribution from a potential positive energy balance cannot be ruled out (37). But the finding that the body weight was maintained by the ad libitum food intake during the bed rest period indicates that the subjects were in energy balance. Central to the observed bed rest–induced insulin resistance is the finding that both basal and insulin-stimulated GS activity was lower after bed rest than before. This impairment is likely one explanatory factor for the decreased leg glucose extraction. The observed lower GLUT4 protein content after bed rest is similar to the previously reported decrease in GLUT4 protein after a period of physical inactivity in trained humans (38) and rats (39). This suggests a similar effect of bed rest and detraining on the regulation of GLUT4 expression. Although our data suggest that physical inactivity–induced insulin resistance is associated with reduced GLUT4 abundance, it is notable that other studies have found no relationship between GLUT4 protein content and insulin action (40). Furthermore, the lower HKII and GLUT4 protein levels after bed rest likely decrease glucose transport and phosphorylation in skeletal muscle. These modifications are likely additional explanatory factors. Although we can only speculate as to the mechanism for the latter observations, it is tempting to suggest that the absence of muscle use during bed rest leads to lack of stimulation of transcriptional/translational processes normally seen in contracting skeletal muscle (41,42).

The current study does not provide insight as to whether translocation of GLUT4 to the plasma membrane is also compromised in bed rest–induced insulin resistance as seen in, for example, insulin-resistant muscle (40). But the 20–30% decreased protein and phosphorylation level of Akt after bed rest, similar to what has recently been observed with reduced levels of physical activity (43), may suggest such a mechanism because impaired Akt phosphorylation is seen in skeletal muscle of type 2 diabetes subjects. A possible upstream regulator responsible for the observed decreased Akt phosphorylation could be phosphatidylinositol-3 kinase, but due to lack of sufficient tissue, this cannot be elucidated further in the current study. However, the impaired phosphorylation on both of the key regulatory sites for Akt activity did not translate to two endogenous substrates of Akt. Thus, in contrast to insulin resistance in type 2 diabetes subjects (7), basal and insulin-induced phosphorylation of TBC1D4 was fully normal on multiple proposed Akt target sites after bed rest. In addition, the present observations do not indicate that reduced TBC1D4 phosphorylation is responsible for muscle insulin resistance after bed rest. However, other mechanisms involved in the GLUT4 recruitment to the plasma membrane, dependent on Akt, may be impaired (e.g., cytoskeleton rearrangements) (44).

Interestingly, the impaired activity and activation of GS after bed rest might be linked to impaired covalent regulation of the enzyme. Thus, whereas GS site 3a dephosphorylation was not compromised after bed rest, the ability of insulin to induce dephosphorylation on site 2+2a was lost. Furthermore, the general level of site 2+2a phosphorylation was increased and potentially contributing to the suppressed GS activity after bed rest. Such a pattern of dysregulation of site 2+2a rather than site 3a is

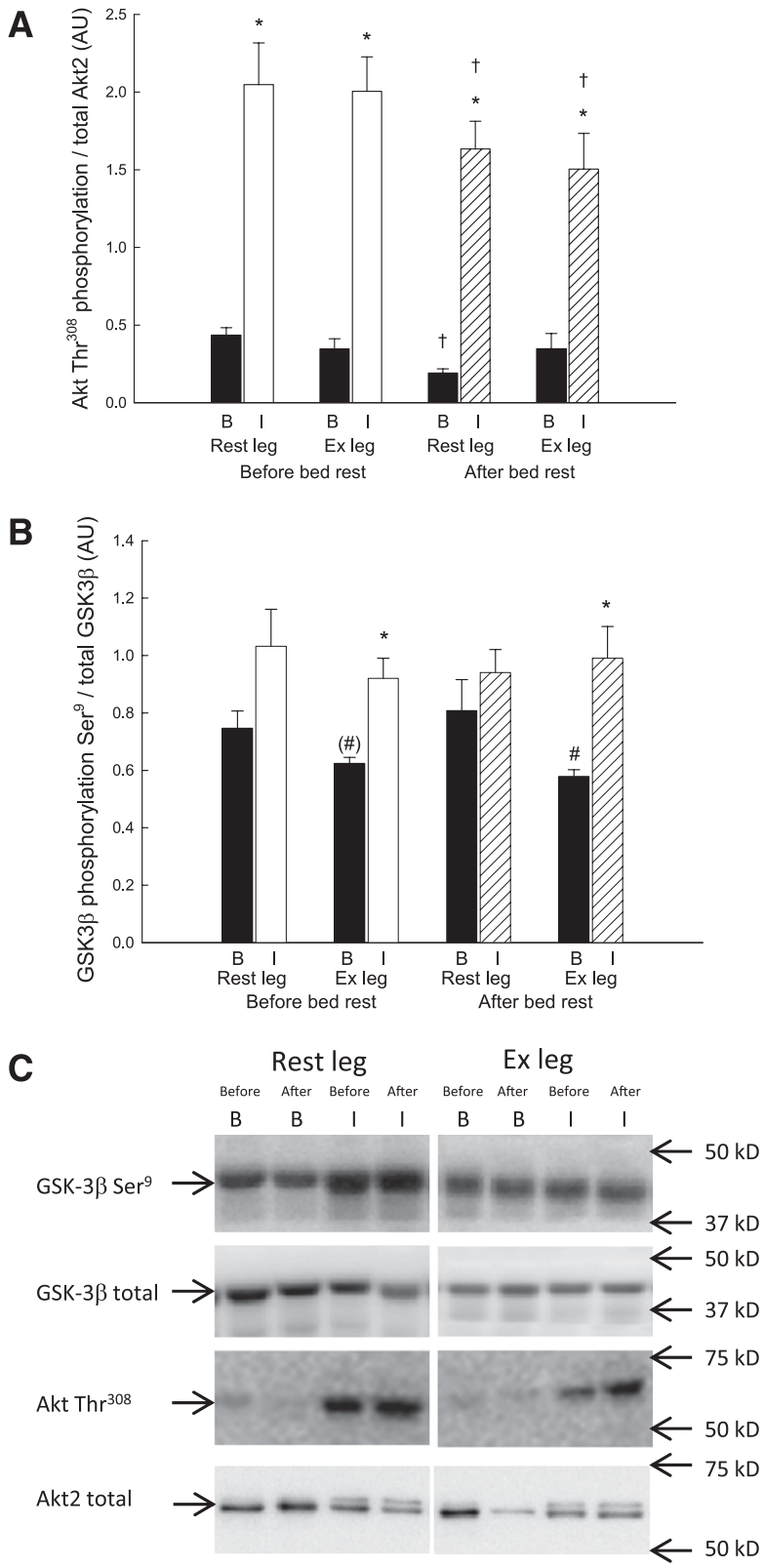

FIG. 3. Akt Thr^{308} phosphorylation (*A*), GSK-3β Ser^{9} phosphorylation (*B*), and representative blots (*C*) in rested (Rest leg) and prior-exercised (Ex leg) vastus lateralis before (B) and after (I) 3-h hyperinsulinemic euglycemic clamp performed 3 h after one-legged knee extensor exercise before and after 7 days of bed rest. AU, arbitrary units; kD, kilodaltons. Values are means ± SE; $n = 6$. *Significantly different from B, $P < 0.05$. †Significantly different from before bed rest, $P < 0.05$. #Significantly different from Rest leg at given time point, $P < 0.05$. (#)Tendency, $0.05 \leq P < 0.1$.

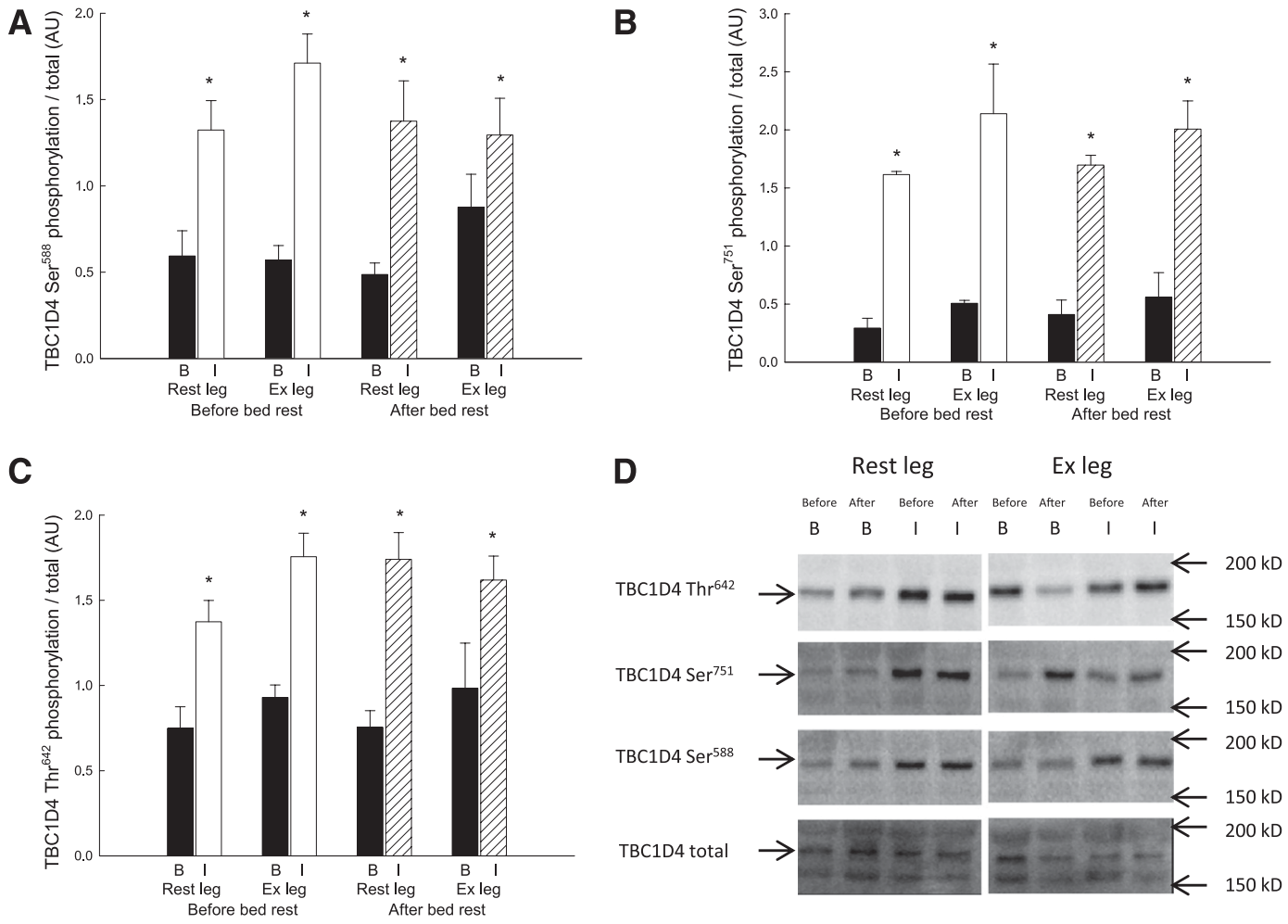

FIG. 4. TBC1D4 Ser^{588} phosphorylation (*A*), TBC1D4 Ser^{751} phosphorylation (*B*), TBC1D4 Thr^{642} phosphorylation (*C*), and representative blots (*D*) in rested (Rest leg) and prior-exercised (Ex leg) vastus lateralis before (B) and after (I) 3-h hyperinsulinemic euglycemic clamp performed 3 h after one-legged knee extensor exercise before and after 7 days of bed rest. AU, arbitrary units; kD, kilodaltons. Values are means ± SE; $n = 6$. *Significantly different from B, $P < 0.05$.

in agreement with observations in muscle insulin resistance related to obesity and type 2 diabetes (9,12). Site 2+2a is apparently not a direct target of either GSK-3 or Akt. Yet it is uncertain whether the impaired Akt signaling indirectly relates to the lack of site 2+2a dephosphorylation seen after bed rest. Site 2+2a is directly targeted by multiple kinases, but so far we have not been able to find indications that these kinases are dysregulated with bed rest. Thus, the fully nonresponsive site 1b phosphorylation indicates that neither calcium calmodulin–dependent kinase nor cAMP-dependent protein kinase is regulated under these conditions (45). Also, AMP-activated protein kinase activity/phosphorylation is not regulated by either insulin or bed rest (data not shown). In addition, both GS site 3 and site 2+2a are dephosphorylated by the multisubstrate phosphatase (PP1) (46), and a dysregulation of PP1 activity would be expected to affect phosphorylation at both sites, and not only site 2+2a as seen in the current study.

Previous studies have suggested that GS site 2+2a phosphorylation is affected by muscle glycogen levels, and that GS site 2+2a phosphorylation is a possible link between the inverse relationship between muscle glycogen and GS activity (47,48). Thus, although mechanistically unresolved, the lower GS activity and higher site 2+2a phosphorylation after bed rest might be related to the higher muscle glycogen level after bed rest. Of notice is, however, that the differences in muscle glycogen concentration eliciting changes in GS activity in previous studies (47–50) are larger than the 20% difference seen in the current study. This makes it less likely that the enhanced glycogen level is the only reason for the differences observed in the current study. The findings that resting muscle glycogen was elevated despite impaired insulin-induced glucose extraction and reduced GS activity after bed rest may at first seem contradictory. However, the impact of the dysregulated GS activation on the glycogen level likely first becomes evident when a significant amount of glycogen has to be synthesized (e.g., in the period after glycogen-depleting exercise).

The finding that insulin-stimulated GS activity in the prior-exercised leg was higher than in the rested leg both before and after bed rest is in line with previous studies (14,22) and indicates that activation of GS may play a role in the exercise-induced enhanced glucose uptake several hours after exercise both before and after the inactivity period. The similar insulin-mediated regulation of GS site 3a and Akt phosphorylation in the rested and prior-exercised leg both before and after bed rest is also in accordance with earlier studies (22,48) and suggests that changes in

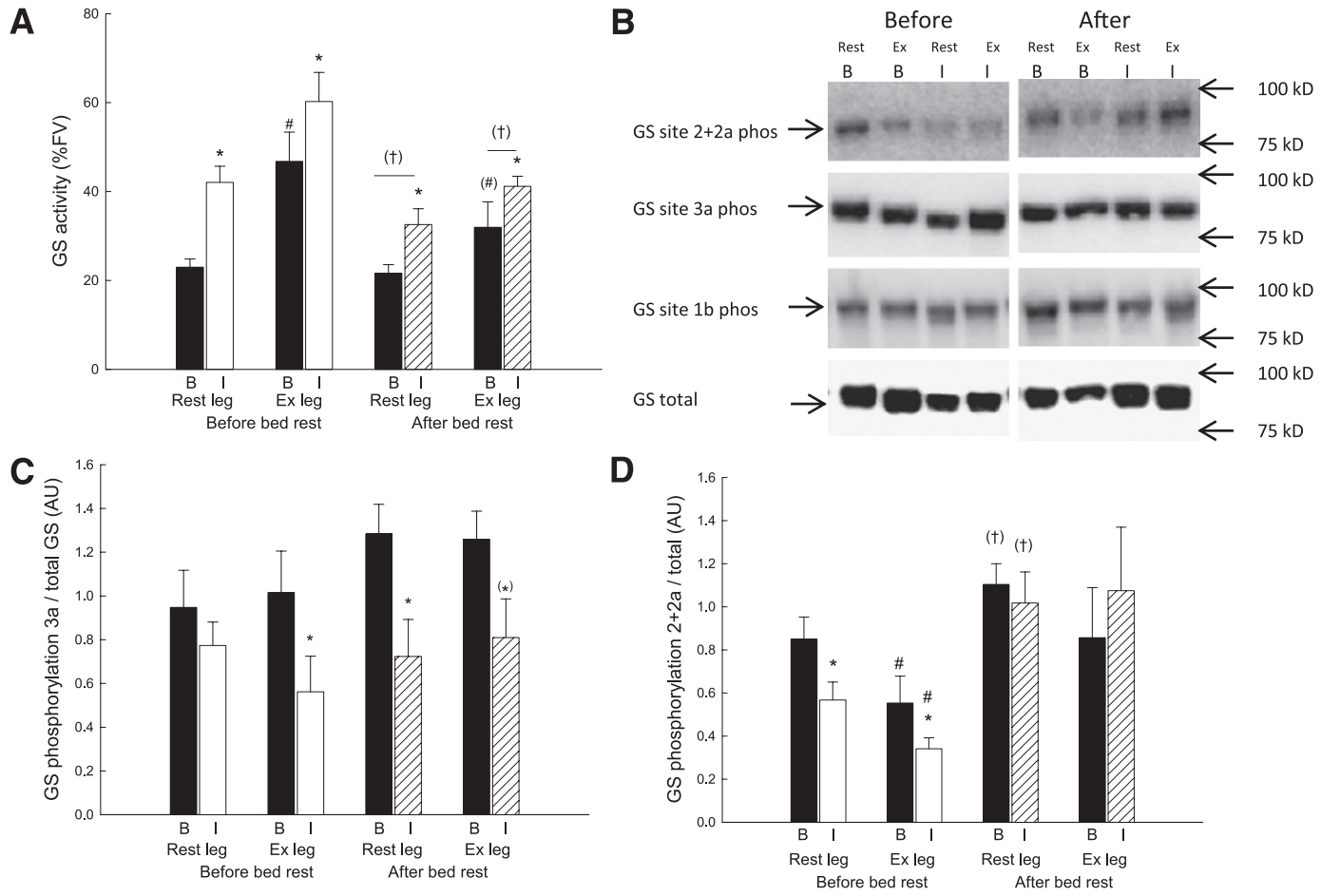

FIG. 5. Glycogen synthase (GS) activity (%FV) (*A*), representative blots (*B*), GS site 3a phosphorylation (*C*), and GS site 2+2a phosphorylation (*D*) in rested (Rest leg) and prior-exercised (Ex leg) vastus lateralis before (B) and after (I) 3-h hyperinsulinemic euglycemic clamp performed 3 h after one-legged knee extensor exercise before and after 7 days of bed rest. AU, arbitrary units; kD, kilodaltons. Values are means ± SE; $n = 6$. *Significantly different from B, $P < 0.05$. †Significantly different from before bed rest, $P < 0.05$. #Significantly different from Rest leg at given time point, $P < 0.05$. Symbols within parentheses indicate tendency, $0.05 \leq P < 0.1$.

GS site 3a and Akt phosphorylation do not contribute to the observed exercise-induced effects on GS activity. Previous findings show that reduced muscle glycogen is associated with dephosphorylation of GS site 2+2a (34), and thus the more marked dephosphorylation of GS site 2+2a could be a link between the reduced muscle glycogen and the increased GS activity in the prior-exercised leg. Previous studies showing that prior exercise increased TBC1D4 phosphorylation both before and after insulin stimulation (25) suggest a role of TBC1D4 in the enhanced glucose clearance in a prior-exercised muscle. However, the current study does not confirm these observations, although it cannot be excluded that a more intense or more prolonged bout of exercise would elicit such changes. Thus, the analyses performed in this study do not bring further insights to the mechanisms behind exercise-induced enhanced insulin sensitivity but do provide the important observation that the mechanisms still seem fully functional in bed rest–induced insulin-resistant muscle.

We are well aware of the limitation in the study by having investigated the effect of acute exercise in only six subjects, leaving us in some cases with near-significant trends. Still we are able to draw some conclusions to potential mechanisms involved in bed rest–induced insulin resistance in skeletal muscle. Thus bed rest–induced insulin resistance in skeletal muscle relates to decreased expression and impaired activation of central players regulating glucose uptake and storage in skeletal muscle, together with a possible impaired insulin-stimulated blood flow. In addition, the similar enhancement of glucose removal by prior exercise before and after bed rest demonstrates that the ability of exercise to increase skeletal muscle insulin sensitivity is maintained after 7 days of physical inactivity.

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R.S.B. designed the study, took part in conducting the human experiments, performed the laboratory analyses, wrote the manuscript, and commented on the manuscript. S.R. designed the study, took part in conducting the human experiments, performed the laboratory analyses, and commented on the manuscript. K.K., C.L., and P.P. designed the study, took part in conducting the human experiments, and commented on the manuscript. N.-J.A.-A., R.K.-M., B.G., G.v.H., and J.A.L.C. took part in conducting the human experiments and commented on the manuscript. J.T.T. provided valuable guidance for specific laboratory analyses and commented on the manuscript. B.S. designed the study and commented on the manuscript. H.P. and J.F.P.W. designed the study, took part in conducting the human experiments, assisted with writing the manuscript, and commented on the manuscript. J.F.P.W. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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