

Regulation of Glycogen Synthase Kinase-3 in Human Skeletal Muscle

Effects of Food Intake and Bicycle Exercise

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Studies of skeletal muscle from rodents performed both in vivo and in vitro suggest a regulatory role of glycogen synthase kinase (GSK) 3 in glycogen synthase (GS) activation in response to insulin. Recently, hyperinsulinemic clamp studies in humans support such a role under nearly physiological conditions. In addition, in rats the activation of GS in skeletal muscle during treadmill running is time-related to the deactivation of GSK3. We investigated whether GSK3 was deactivated in human muscle during low- (~50% VO_{2max} for 1.5 h) and high-intensity (~75% VO_{2max} for 1 h) bicycle exercise as well as food intake. We observed a small but significant increase in GSK3 α (10–20%) activity in biopsies obtained from vastus lateralis after both low- and high-intensity exercise, whereas GSK3 β activity was unaffected. Subsequent food intake increased Akt-phosphorylation (~2-fold) and deactivated GSK3 α (~40%), whereas GSK3 β activity was unchanged. GS activity increased in response to both exercise and food intake. We conclude that GSK3 α but not GSK3 β may have a role in the regulation of GS activity in response to meal-associated hyperinsulinemia in humans. However, in contrast to findings in muscle from rats, exercise does not deactivate GSK3 in humans, suggesting a GSK3-independent mechanism in the regulation of GS activity in muscle during physical activity. *Diabetes* 50:265–269, 2001

Glycogen synthase kinase (GSK) 3 is expressed in skeletal muscle and has been identified in two isoforms, α and β . Both isoforms contain potential serine and tyrosine phosphorylation sites. Tyrosine phosphorylation is important for full activation, but the regulatory role for these sites in vivo is still unclear (1–3). Serine phosphorylation on sites 9 and 21 in GSK3 β and GSK3 α , respectively, is highly regulated in response to stimulation (e.g., by insulin) and phosphorylation on these sites leads to deactivation of the enzyme (4–6). In vitro GSK3

phosphorylates two of the four sites on glycogen synthase (GS), which also become dephosphorylated in response to insulin (7). This suggests that insulin activates GS in part through a GSK3-dependent mechanism. Supporting this view are our recent observations that the time course of serine phosphorylation and deactivation of GSK3 and activation of GS is highly identical under both supraphysiological insulin stimulation in the rat (8) and during physiological insulin clamp conditions in humans (9). Interestingly, during physiological conditions in humans only GSK3 α is deactivated by insulin, whereas supraphysiological insulin injection in the rat leads to deactivation of both GSK3 α and GSK3 β (8–10).

In the period after exercise, glycogen resynthesis is a major metabolic challenge for the skeletal muscle. Sustained activation of glucose uptake and activation of GS together with enhanced insulin sensitivity for activation of these processes eventually lead to normalization or even supercompensation of the glycogen stores after exercise, as reviewed by Richter (11). GS, the rate-limiting enzyme for glycogen synthesis, displays enhanced activity ratio when isolated from previously exercised muscle. This suggests that the enzyme becomes dephosphorylated in response to exercise (12), as reviewed by Ivy and Kuo (13). Recently it was reported that treadmill running leads to deactivation of GSK3 α and GSK3 β in rat skeletal muscle concurrent with GS activation (8). This indicates that GSK3 may also regulate GS phosphorylation and activation in response to contractile activity in rat skeletal muscle.

In the current investigation, we compared the effects of food intake and high- as well as low-intensity exercise on the regulation of muscular GSK3 in humans and compared this regulation with that of GS and the upstream kinase Akt.

RESEARCH DESIGN AND METHODS

Subjects. Seven healthy men (25 ± 1 years) gave their informed consent to participate in the study, which was approved by the Copenhagen Ethics Committee. Body weight, height, and BMI were 74 ± 2 kg, 178 ± 2 cm, and 23 ± 1 kg/m², respectively. The subjects participated in regular physical activities twice a week on average and were used to biking on a daily basis for local transportation. One to two weeks before the experiments, maximal pulmonary oxygen consumption was determined during an incremental bicycle ergometer test (55 ± 1 ml · kg⁻¹ · min⁻¹).

Experimental protocol. The subjects randomly underwent two experimental trials separated by 2–3 weeks. Subjects were instructed to eat a controlled diet (carbohydrate ~63%, fat ~22%, and protein ~15%, average 13.5 MJ/day) for 3 days before each experiment and arrived at the laboratory in the morning after an overnight fast. After 45 min of rest, a needle biopsy from the vastus lateralis was obtained under local anesthesia. A venous catheter was inserted in an antecubital vein for blood sampling. The subjects then performed bicycle exercise for 90 min at 50% VO_{2max} (low-intensity trial) or for 55 min at

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%FV, percent of fractional velocity; %I-form, percent of G6P-independent GS activity; G6P, glucose-6-phosphate; GS, glycogen synthase; GSK, GS kinase; PP, protein phosphatase.

75% $\text{Vo}_{2\text{max}}$ plus 5 min at 90% $\text{Vo}_{2\text{max}}$ (high-intensity trial). The total energy expenditure was aimed at being the same and did not differ in the two trials (average 3,800 kJ). Immediately after exercise, another biopsy was obtained from the vastus lateralis. The subjects then rested in the supine or sitting position for 3 h, having free access to water only. Then, a third biopsy was obtained, and the subjects then ingested a carbohydrate-rich meal with an energy content similar to the amount used during the exercise bout plus the expected energy consumption in the 3-h postexercise period. Thus, this meal was identical in the two trials, and consisted of ~75% carbohydrate, ~17% fat, and ~8% protein, which amounted to 4,910 kJ in total. The meal was ingested within 15 min. The subjects then rested in the supine or sitting position for 2 h before drinking a carbohydrate beverage, containing 500 kJ. Another hour elapsed before a final biopsy was obtained from the vastus lateralis. The biopsies before and after exercise were always taken in the same leg, and the biopsies taken 3 and 6 h after exercise were always taken in the contra-lateral leg. Incisions for biopsies were spaced 4–5 cm apart. Biopsies were frozen in liquid nitrogen within 20 s. Before and during the last minute of exercise as well as every following hour, blood samples were obtained for measurements of plasma insulin concentrations.

Analytical procedures. Plasma insulin concentrations were measured as previously described (14). For determination of muscle glycogen content, muscle biopsies were freeze-dried and dissected free of blood, fat, and connective tissue before analysis. Glycogen content was determined as glycosyl units after acid hydrolysis (15). Muscle GS activity was measured in a homogenate by a modification of the method of Thomas (16) as described by Richter et al. (17). GS activity was determined in the presence of 0.02, 0.17, and 8 mmol/l glucose-6-phosphate (G6P) and given either as the percent of G6P-independent GS activity (%I-form) ($100 \times$ activity in the presence of 0.02 mmol/l G6P divided by the activity at 8 mmol/l G6P [saturated]) or as the percent of fractional velocity (%FV) ($100 \times$ activity in the presence of 0.17 mmol/l G6P divided by the activity at 8 mmol/l G6P).

As an indicator of Akt activation, Akt serine⁴⁷³ phosphorylation was measured by Western blotting of muscle lysate using a phosphospecific antibody (New England Biolabs, Beverly, MA) and fluorescent measurements of alkaline phosphatase as a detection system (Promega U.K., Southampton, U.K.), as previously described (14). GSK3 α and GSK3 β were immunoprecipitated from 100 μg of muscle lysate using an anti-GSK3 α (Upstate Biotechnology, Lake Placid, MA) or anti-GSK3 β (Transduction Laboratories, San Diego, CA) antibody. A p81-filter paper assay, with a phospho-GS2-peptide (Upstate Biotechnology) as substrate, was used to measure GSK3 activity, as described previously (8,9).

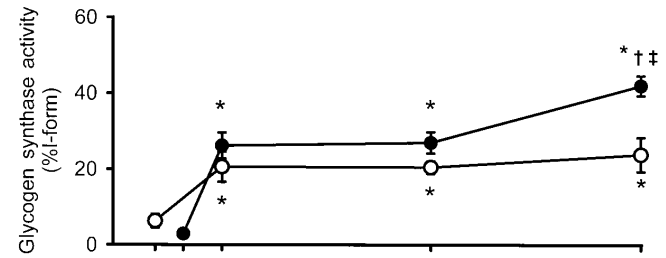
Calculations and statistics. Control samples were added to all immunoblots and activity assays, and assay-to-assay variation was accounted for by expressing the data relative to these samples. Data are expressed as means \pm SE. Statistical evaluation was done by one- or two-way analysis of variance with repeated measures, as appropriate. When analysis of variance revealed significant differences, a post hoc test was used to correct for multiple comparisons (Student-Newman-Keuls test). Differences between groups were considered statistically significant if P was <0.05 .

RESULTS

Exercise and food intake effects on glycogen metabolism.

Figure 1 shows GS activity in the low- and high-intensity exercise trials. Both %I-form and %FV of GS were increased by exercise, and the increase tended to be higher in the high-intensity trial. GS activity was not changed in the first 3 h after exercise. Food intake raised GS activity further only in the high-intensity trial, despite the fact that plasma insulin concentrations were elevated to a similar extent in the two trials (Table 1). The rate of glycogen utilization was

A



B

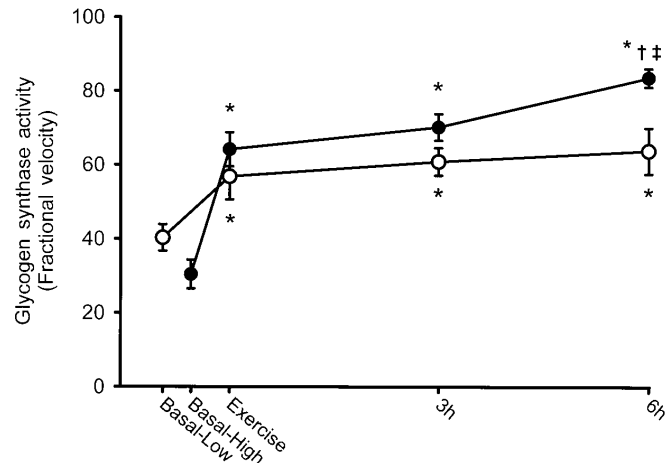


FIG. 1. Muscle GS activity presented as %I-form (A) or as fractional velocity (B) in the low-intensity trial (○) and the high-intensity trial (●). Biopsies were obtained before bicycle exercise (Basal) and immediately after exercise (Exercise) from the same leg. The biopsies obtained 3 (3h) and 6 (6h) h postexercise were taken from the contralateral leg. A carbohydrate-rich meal and a beverage were ingested 3 and 5 h after exercise, respectively. *, †, ‡ Significant differences ($P < 0.05$) from basal, 3 h, and the same measurement in the other trial, respectively. Data are means \pm SE, $n = 7$.

higher during high- compared with low-intensity exercise (4.5 ± 0.7 vs. 1.6 ± 0.3 mmol/kg dry wt/min, $P < 0.05$). In the high-intensity trial, glycogen content decreased further during the first 3 h after exercise, and only in this trial was a significant ($P < 0.05$) glycogen resynthesis (~25%) observed after food intake (Fig. 2).

Bicycle exercise in humans does not lead to deactivation of GSK3. Glycogen-depleting exercise, performed at either a high or low intensity, did not decrease the activity of GSK3 α or GSK3 β (Fig. 3). In fact, exercise increased GSK3 α activity in both trials in all subjects except one, giving rise to

TABLE 1
Plasma insulin concentrations during the low- and high-intensity exercise

| | Basal | Exercise | 1-h post | 2-h post | 3-h post | 4-h post | 5-h post | 6-h post |
|------|---------------|-----------------|---------------|---------------|---------------|---------------|---------------|---------------|
| Low | 6.1 ± 0.8 | $3.4 \pm 0.4^*$ | 4.5 ± 0.4 | 4.7 ± 0.6 | 4.7 ± 0.9 | $90 \pm 12^*$ | $76 \pm 10^*$ | $60 \pm 11^*$ |
| High | 6.0 ± 0.9 | $3.4 \pm 0.5^*$ | 5.4 ± 0.9 | 4.7 ± 0.7 | 5.2 ± 1.0 | $79 \pm 8^*$ | $70 \pm 9^*$ | $61 \pm 12^*$ |

Values ($\mu\text{U/ml}$) are means \pm SE, $n = 7$. *Significant difference ($P < 0.05$) from basal. The statistical analysis of differences between basal and exercise was performed using a paired t test. The value for 4-h post represents measurements obtained 1 h after starting the meal.

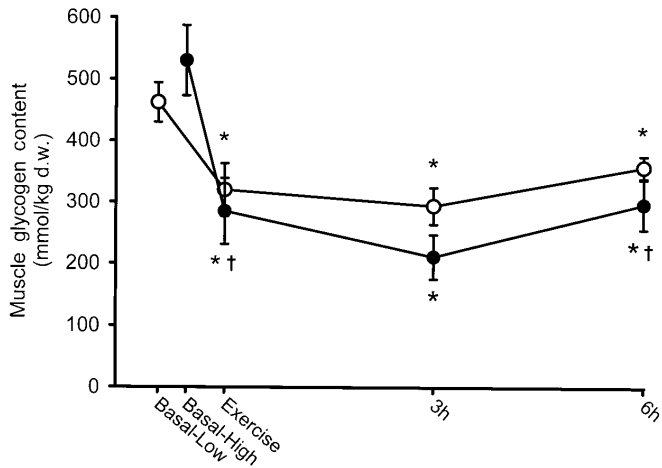
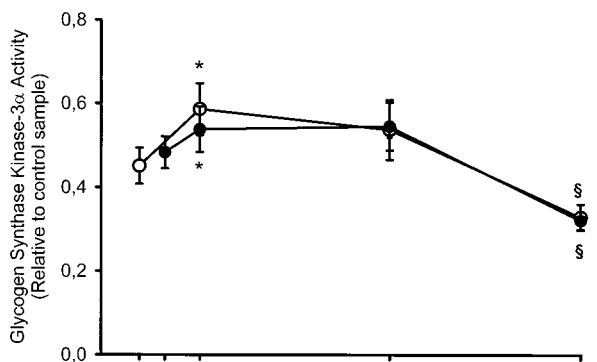


FIG. 2. See Fig. 1 for details. Glycogen content measured in freeze-dried muscle samples dissected free of connective tissue, fat, and blood. *, † Significant differences ($P < 0.05$) from basal and 3 h, respectively. Data are means \pm SE, $n = 6-7$.

a modest but significant ($P < 0.05$) increase in both trials ($\sim 10-20\%$) (Fig. 3A). In accordance with this increase, Akt serine⁴⁷³ phosphorylation decreased during exercise in the majority of experiments (10 of 14) (Fig. 4), perhaps as a result of the significant ($P < 0.05$) insulin-lowering effect of exercise (Table 1). However, overall the decrease in Akt phosphorylation did not reach statistical significance.

A



B

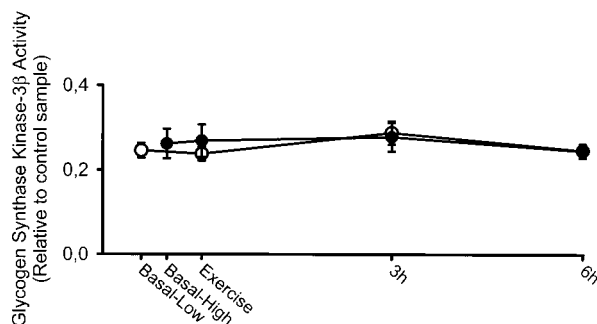


FIG. 3. See Fig. 1 for details. Muscle GSK3 α (A) and GSK3 β (B) activities measured *in vitro* after immunoprecipitation with isoform-specific antibodies. *, § Significant differences ($P < 0.05$) from basal and from all other measurements in the same trial, respectively. Data are means \pm SE, $n = 7$.

Increased plasma insulin levels after food intake lead to phosphorylation of Akt and deactivation of GSK3 α .

Food intake was associated with an increase in plasma insulin concentration, peaking 60 min after food intake in both trials at an average of 85 $\mu\text{U}/\text{ml}$, followed by a slight decrease 2 and 3 h after ingestion (~ 70 and ~ 60 $\mu\text{U}/\text{ml}$, respectively) (Table 1). Plasma insulin concentrations were similar during the two trials at all time points. Serine phosphorylation on site 473 of Akt increased by ~ 2 -fold in response to food intake (Fig. 4). The degree of phosphorylation was independent of the intensity of the prior exercise bout. Likewise, GSK3 α activity decreased significantly by $\sim 40\%$ in response to food intake during both trials, whereas GSK3 β activity was unchanged (Fig. 3).

DISCUSSION

The current results demonstrate that low- and high-intensity physical exercise in humans is not associated with a deactivation of GSK3 in skeletal muscle. In fact, the drop in plasma insulin concentration during exercise may cause the minor, but significant, activation of GSK3 α . Because GS is highly activated during exercise, our data suggest that this activation in response to exercise is not regulated by a GSK3-dependent mechanism in humans. These findings are in line with our recent human data showing an elevated muscle GS activity 4 h after exercise without any concurrent deactivation of GSK3 (9). The lack of deactivation of GSK3 with exercise in the present study contrasts with observations in muscle from treadmill-exercised rats, in which an exercise-induced decrease in both GSK3 α activity and GSK3 β activity was demonstrated (8).

For the first time, we show that a physiological stimulus such as intake of food causes deactivation of muscle GSK3 α in humans. Because deactivation of GSK3 α was recently demonstrated during hyperinsulinemic clamp conditions (9,10), the GSK3 α -deactivation observed in the present study is probably caused by the meal-induced hyperinsulinemia, giving rise to increased Akt phosphorylation (Fig. 4). However, deactivation of GSK3 β is not observed (Fig. 3B). During euglycemic clamp conditions in healthy subjects and patients

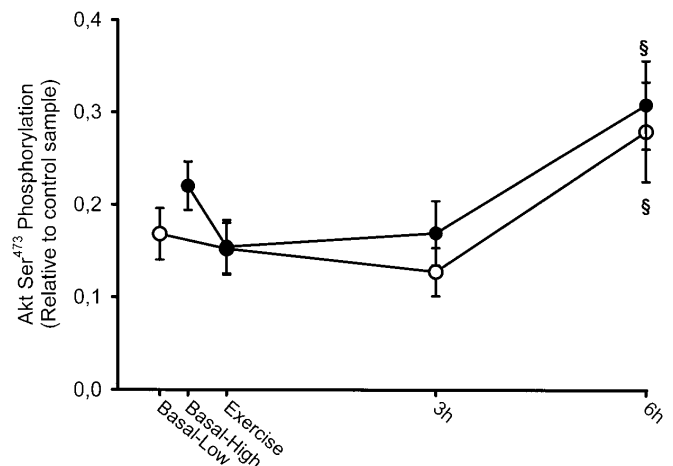


FIG. 4. See Fig. 1 for details. Muscle Akt serine⁴⁷³ phosphorylation measured by phosphospecific immunoblotting of muscle lysates. § Significant differences ($P < 0.05$) from all other measurements in the same trial. Data are means \pm SE, $n = 7$.

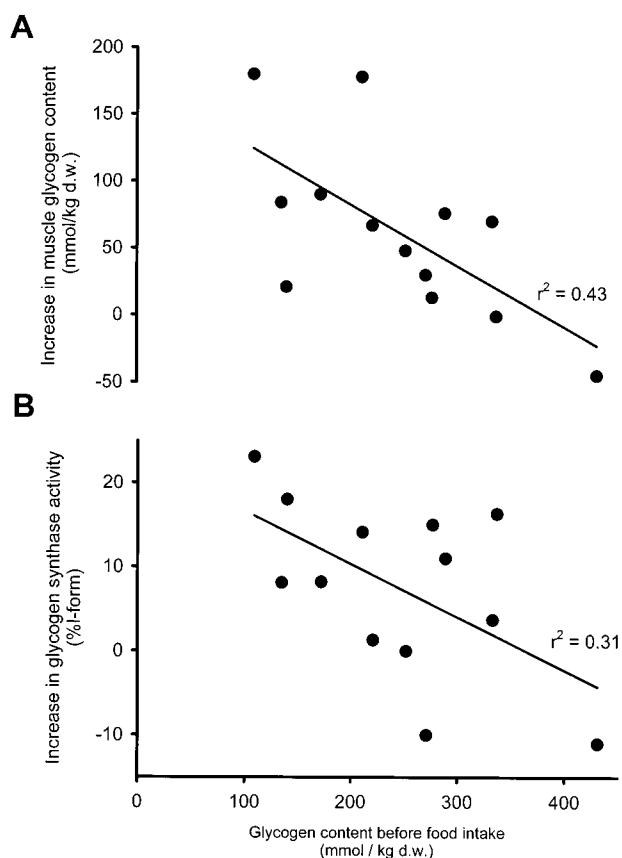


FIG. 5. Linear regressions between muscle glycogen level before food intake (3 h postexercise). **A:** The increase in glycogen content after a meal ($\Delta = 6$ h minus 3 h). **B:** The increase in GS activity after a meal ($\Delta = 6$ h minus 3 h). A statistical significant correlation is present in both cases. $P < 0.01$ (A) and $P < 0.05$ (B).

with type 2 diabetes, deactivation of GSK3 β was also not observed ([10] and unpublished observation). In contrast, a marked deactivation of GSK3 β was observed in rat skeletal muscle after injections of very large doses of insulin (10 U/kg body wt) (8). The differences between the findings in humans and in rats may relate to the species differences or possibly to the level of hyperinsulinemia achieved, which was clearly supraphysiological in the rats.

Another possibility is that insulin injections in rats result in hypoglycemia giving rise to epinephrine secretion (18). β -adrenergic stimulation of primary adipocytes decreased GSK3 activity immunoprecipitable with an antibody recognizing both α and β isoform of GSK3 (19). If β -adrenergic stimulation by epinephrine in response to hypoglycemia in the insulin-injected rats also deactivates GSK3 in muscle, this could be the indirect mechanism by which supraphysiological insulin concentrations cause deactivation of GSK3 β . However, if so, we would expect similar effects of epinephrine during exercise in humans because plasma epinephrine levels, especially during the high-intensity exercise trial, are known to be elevated four- to sixfold compared with rest (20). Thus, more studies are needed to clarify the role of epinephrine in the regulation of GSK3 activity in skeletal muscle.

Hyperinsulinemia as a result of food intake activates GS to a greater extent after high-intensity exercise compared with low-intensity exercise, and is accompanied by a significant

glycogen resynthesis in the period after food intake. Interestingly, this enhanced insulin action is not mediated through changes in insulin signaling, as both Akt and GSK3 were affected similarly in the two trials. Recently, we reported that enhanced insulin action in exercised versus rested human skeletal muscle was also not a consequence of enhanced intracellular insulin signaling (9,14). In fact, in those studies, a very strong correlation existed between the degree of glycogen breakdown during the exercise bout and the subsequent rate of muscle glucose uptake after insulin stimulation ($r^2 = 0.52$, $n = 14$, $P < 0.01$ [unpublished observations]). In the present study, a significant glycogen resynthesis occurred in response to food intake in the high-intensity trial, in which a lower glycogen content was also evident after exercise. In fact, good negative correlations exist between the glycogen level before food intake and the following: 1) the increase in glycogen content after food intake ($r^2 = 0.43$, $n = 13$, $P < 0.01$) and 2) the increase in GS activity after food intake (e.g., for %I-form $r^2 = 0.31$, $n = 13$, $P < 0.05$) (Fig. 5). These observations are in line with other recent observations indicating that muscle glycogen content is important for the ability of both insulin and contractions to stimulate muscle glucose uptake in rats (21–23).

In contrast to the present study in humans, exercise in rats leads to deactivation of GSK3 α and GSK3 β in muscle (8). The explanation of this difference is not easily found, but it cannot be excluded that even more severe exercise than in the present study, leading to more severe glycogen depletion, could cause deactivation of GSK3 also in human muscle. Nevertheless, our findings that two rather strenuous exercise bouts consuming close to 4 MJ, leading to marked glycogen depletion and GS activation, do not lead to GSK3 deactivation suggests that GSK3 deactivation during exercise is not physiologically important for activation of GS in human skeletal muscle. A putative alternative mechanism for dephosphorylation of GS in response to exercise could be an activation of protein phosphatases (PPs), especially PP1 (24). PP1 is targeted to glycogen through binding to regulatory binding proteins. For example, protein targeting to glycogen (PTG) has been shown to form complexes to the catalytic subunit of PP1 and to glycogen. In addition, there may be an association with other enzymes involved in glycogen metabolism (25). Thus, regulation of the binding properties of these binding proteins may be important for the regulation of GS activity in response to exercise. In fact, a very recent but preliminary report indicates that in the absence of the regulatory glycogen binding subunit G_M (or R_{GL}) of PP1, a marked reduction in GS activation in response to contractile activity is observed in mouse skeletal muscle (26). To our knowledge, only one study has measured muscular PP1 activity in response to exercise. Thus, in humans PP1 activity actually decreases during maximal isometric muscle contractions and returns to pre-exercise levels early in the recovery period (27). Whereas these findings do not offer any explanation for the postexercise-increased GS activity after isometric contractions, they do not exclude a regulatory role of PP1 during more dynamic glycogen-depleting exercise as in the present study.

In conclusion, food intake is associated with muscular deactivation of GSK3 α , supporting a physiological role for GSK3 α in the regulation of GS activity in human skeletal muscle. However, the regulation of GS in response to food

intake (elevated plasma insulin) is not solely dependent on GSK3 α deactivation because muscle glycogen content clearly has a signaling-independent influence on the effects of food intake on insulin action. Finally, in contrast to rodents, moderate and intense exercise in humans does not lead to GSK3 deactivation in skeletal muscle, excluding the kinase as an important regulator of GS activity during physical activity in humans.

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