

# Dysregulation of muscle glycogen synthase in recovery from exercise in type 2 diabetes

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## Abstract

**Aims/hypothesis** Insulin and exercise stimulate skeletal muscle glycogen synthase (GS) activity by dephosphorylation and changes in kinetic properties. The aim of this study was to investigate the effects of insulin, exercise and post-exercise insulin stimulation on GS phosphorylation, activity and substrate affinity in obesity and type 2 diabetes.

**Methods** Obese men with type 2 diabetes ( $n=13$ ) and weight-matched controls ( $n=14$ ) underwent euglycaemic–hyperinsulinaemic clamps in the rested state and 3 h after 60 min of cycling (70% maximal pulmonary oxygen uptake [ $\dot{V}O_{2max}$ ]). Biopsies from vastus lateralis muscle were obtained before and after clamps, and before and immediately after exercise.

**Results** Insulin-stimulated glucose uptake was lower in diabetic patients vs obese controls with or without prior exercise. Post exercise, glucose partitioning shifted away from

oxidation and towards storage in both groups. Insulin and, more potently, exercise increased GS activity (fractional velocity [FV]) and substrate affinity in both groups. Both stimuli caused dephosphorylation of GS at sites 3a+3b, with exercise additionally decreasing phosphorylation at sites 2+2a. In both groups, changes in GS activity, substrate affinity and dephosphorylation at sites 3a+3b by exercise were sustained 3 h post exercise and further enhanced by insulin. Post exercise, reduced GS activity and substrate affinity as well as increased phosphorylation at sites 2+2a were found in diabetic patients vs obese controls.

**Conclusions/interpretation** Exercise-induced activation of muscle GS in obesity and type 2 diabetes involves dephosphorylation of GS at sites 3a+3b and 2+2a and enhanced substrate affinity, which is likely to facilitate glucose partitioning towards storage. Lower GS activity and increased phosphorylation at sites 2+2a in type 2 diabetes in the recovery period imply an impaired response to exercise.

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**Keywords** Exercise · Glycogen synthase · Insulin resistance · Skeletal muscle · Type 2 diabetes

## Abbreviations

AMPK	AMP-activated protein kinase
FV <sub>(0.1/1.67)</sub>	Fractional velocity (at 0.1/1.67 mmol/l UDP-glucose)
G6P	Glucose 6-phosphate
GDR	Glucose disposal rate
GS	Glycogen synthase
GSK3	Glycogen synthase kinase 3
I-form	Glycogen synthase activity independent of G6P
NOGD	Non-oxidative glucose disposal
RM ANOVA	Repeated measures ANOVA

UDP-glucose Uridine diphosphate-glucose  
 $\dot{V}O_{2\max}$  Maximal pulmonary oxygen uptake

## Introduction

In type 2 diabetes, insulin-mediated glucose uptake is reduced by about 40% compared with weight-matched control individuals, with the defect largely accounted for by reduced glycogen synthesis in skeletal muscle [1–3]. In accordance, insulin activation of glycogen synthase (GS), the key enzyme in the regulation of glycogen synthesis, has consistently been found defective in muscle of patients with type 2 diabetes and obese insulin-resistant individuals [3–7].

Exercise plays a fundamental role in the prevention and treatment of type 2 diabetes [8, 9]. Beneficial effects of a single bout of moderate-intensity exercise include an immediate increase in muscle glucose uptake [10]. This is followed by a more prolonged (<48 h) increase in insulin sensitivity to stimulate glucose uptake in both healthy, insulin-resistant and type 2 diabetic individuals [11–13]. Both basal and insulin-stimulated muscle GS activity is elevated in recovery from exercise, indicating that GS indirectly mediates glucose uptake after exercise [7, 14] and facilitates glycogen replenishment. In line, correlation between insulin-stimulated glucose storage and GS activity at recovery from exercise has been reported [12]. Interestingly, glycogen-lowering exercise also increases GS activity per se [15]. This regulation does not seem to be impaired in insulin-resistant muscle [16, 17], indicating differential regulation of GS by insulin and exercise.

The activity of GS is regulated both by its allosteric activator glucose 6-phosphate (G6P) and covalently by phosphorylation of at least nine serine residues, of which sites 2, 2a, 3a and 3b are considered the most important [18, 19]. Dephosphorylation promotes activation of GS [20]. Insulin stimulation induces GS dephosphorylation at sites 3a+3b as well as sites 2+2a in muscle of healthy individuals [6, 21, 22]. Impaired insulin activation of GS in insulin-resistant conditions such as obesity and type 2 diabetes is associated with dysregulation of GS dephosphorylation in skeletal muscle, in particular at sites 2+2a [4–6]. Also, the activation of GS during endurance exercise is associated with dephosphorylation of sites 3a and 3b but seemingly unchanged phosphorylation at sites 2 and 2a [15, 16]. Interestingly, glycogen content is tightly associated with GS activity, and glycogen depletion by exercise is associated with decreased phosphorylation of sites 2+2a [15]. GS affinity for its substrate uridine diphosphate-glucose (UDP-glucose) is regulated by phosphorylation, and assessment of both substrate affinity and traditional measurements of GS activity may provide more comprehensive insights to GS function [23]. A recent study of GS kinetic properties and phosphorylation in skeletal muscle indicates that the response to exercise and exercise recovery is

preserved in obesity and type 2 diabetes [16]. However, that study neither compared the effect of acute exercise with that of insulin nor investigated the effect of insulin in recovery from exercise.

The present study was undertaken to investigate whether dysregulation of GS by insulin in type 2 diabetes is influenced by prior exercise. In a well-matched cohort of type 2 diabetic patients and obese control individuals, we investigated GS activity, phosphorylation and kinetic properties in response to acute moderate-intensity exercise and physiological insulin stimulation. In addition, these properties of GS were investigated in the insulin-stimulated state during recovery from exercise.

## Methods

**Participants** Thirteen obese male patients with type 2 diabetes and 14 obese healthy male control individuals, matched for age, BMI and physical activity level, participated in the study (Table 1). Medication details and eligibility criteria are given in the electronic supplementary material (ESM) **Methods**. Informed consent was obtained from all participants before participation. The study was approved by the local ethics committee and was performed in accordance with the Helsinki Declaration.

**Study design** One week prior to the first trial, participants underwent blood screening tests, including plasma glucose, HbA<sub>1c</sub> and lipids, ECG and exercise tests [24] to determine maximal pulmonary oxygen uptake (see ESM **Methods**). Subsequently, participants underwent two experimental tests, a baseline ('baseline day') and a post-exercise ('exercise day') euglycaemic–hyperinsulinaemic clamp, separated by 4–8 weeks (ESM Fig. 1). All medications were withdrawn 1 week prior to both test days (see ESM **Methods**), and individuals were instructed to refrain from strenuous exercise for 48 h before each test.

**Baseline euglycaemic–hyperinsulinaemic clamp** After an overnight fast, participants underwent a euglycaemic–hyperinsulinaemic clamp with tracer glucose (2 h basal period and 4 h insulin infusion, 40 mU m<sup>-2</sup> min<sup>-1</sup>) combined with indirect calorimetry (see ESM **Methods**) to assess total glucose disposal rates (GDR), rates of glucose and lipid oxidation, and non-oxidative glucose disposal (NOGD) as described previously [4, 6, 25]. Muscle biopsies were obtained from vastus lateralis muscle before and after insulin (see ESM **Methods**).

At 4–8 weeks after the baseline clamp, the participants returned to the clinic after an overnight fast. They were rested for 30 min in the supine position, and two incisions (4–5 cm apart) were made in the same leg. A first biopsy was obtained

**Table 1** Clinical and metabolic characteristics of participants at study entry

Characteristic	Controls	Type 2 diabetes
<i>n</i>	14	13
Age (years)	55±2	55±2
Height (m)	1.79±0.02	1.80±0.02
BMI (kg/m <sup>2</sup> )	29.0±0.9	29.7±1.0
Fat free mass (kg)	69.1±2.4	68.3±2.0
Fat mass (kg)	24.5±1.9	28.1±2.4
Fasting plasma glucose (mmol/l)	5.6±0.1	10.0±0.7***
HbA <sub>1c</sub> (%)	5.5±0.1	7.0±0.2***
HbA <sub>1c</sub> (mmol/mol)	37±4.0	53±7.9***
Plasma cholesterol (mmol/l)	5.7±0.3	4.4±0.4*
Plasma LDL-cholesterol (mmol/l)	3.8±0.3	2.0±0.2***
Plasma HDL-cholesterol (mmol/l)	1.3±0.1	1.2±0.1
Plasma triacylglycerol (mmol/l)	1.5±0.2	3.2±1.5
IPAQ score	5,248±952	5,558±943
$\dot{V}O_{2max}$ (l/min)	3.50±0.17	3.22±0.23
$W_{max}$ (W)	236±12	196±20
Diabetes duration (years)	-	3.5±1.2

Data are means ± SEM

\* $p < 0.05$  and \*\*\* $p < 0.001$  vs controls

IPAQ score, metabolic equivalent minutes according to the short form of the International Physical Activity Questionnaire;  $W_{max}$ , maximal workload capacity

from vastus lateralis muscle through the lower incision (pre-exercise biopsy). The participants then exercised on a cycle ergometer for 60 min at an intensity (~70% maximal pulmonary oxygen uptake [ $\dot{V}O_{2max}$ ]), which has been shown to increase insulin sensitivity immediately after and up to 48 h afterwards [11]. On completion of exercise, a second muscle biopsy was obtained through the upper incision (exercise biopsy). Participants then rested in bed for 1 h, after which a post-exercise euglycaemic–hyperinsulinaemic clamp study identical to the previous clamp was started. Muscle biopsies (4–5 cm apart) were then taken from the other leg before (3 h into recovery) and after insulin stimulation (7 h into recovery including 4 h insulin infusion). Muscle homogenates were prepared as described in ESM [Methods](#).

**GS activity and  $K_m$  value** All GS activity measurements were performed in 96 well microtitre plates as previously described [6]. The fractional velocity (FV) of GS was determined in the presence of 0.1 mmol/l (FV<sub>0.1</sub>) and 1.67 mmol/l (FV<sub>1.67</sub>) UDP-glucose. The FV was calculated as:  $100 \times$  activity in the presence of 0.17 mmol/l G6P/activity at 8 mmol/l G6P. The activity of GS independent of G6P (I-form) was determined in the presence of 1.67 mmol/l UDP and calculated as:  $100 \times$  activity in the presence of 0.02 mmol/l G6P/activity at 8 mmol/l G6P. The affinity of GS for UDP-

glucose (expressed as the  $K_m$ , i.e. an inverse measure of enzyme affinity for substrate) was analysed in the presence of 0.17 mmol/l G6P and the following concentrations of UDP-glucose: 0.1, 0.3, 0.7 and 1.67 mmol/l. Data were analysed as Eadie–Hofstee plots, with  $K_m$  calculated as the reciprocal to the slope.

**Glycogen content** Muscle glycogen content was measured in muscle homogenates (150 µg protein) as glycosyl units after acid hydrolysis determined by a fluorometric method [26].

**SDS-PAGE and western blotting** Protein levels and phosphorylation patterns were measured using SDS-PAGE with self-cast Tris-HCl gels (7.5%). Proteins were transferred by semidry transfer to polyvinylidene difluoride (PVDF) membranes (Immobilion Transfer Membrane; Millipore, Billerica, MA, USA). The membranes were blocked by incubation in 3% skimmed milk (15 min, room temperature). After primary (overnight, 4°C) and secondary antibody treatment (45 min, room temperature), membranes were incubated in a chemiluminescent substrate (Immobilion Western Chemilum HRP Substrate; Millipore). The primary antibodies used were as previously described [6].

**Statistics** Statistical analyses were performed by SigmaStat version 3.5 (Systat Software, San Jose, CA, USA). Baseline characteristics of diabetic and control individuals were compared via Student's unpaired *t* tests. Separate two-way repeated measures analysis of variance (two-way RM ANOVA) was performed to evaluate the effect of insulin ('baseline day', 'exercise day') and exercise/recovery (pre-exercise, exercise and 3 h recovery data on the exercise day). Significant main effects or interactions were further analysed by the Student–Newman–Keul post-hoc test. The linear dependence between different groups of data was assessed by Spearman's rank correlation coefficient. Data are presented as means ± SEM. Significance was accepted at  $p < 0.05$ .

## Results

**Euglycaemic–hyperinsulinaemic clamps** In the insulin-stimulated period of both clamps, euglycaemia (~5.5 mmol/l) and hyperinsulinaemia (~400 pmol/l) were achieved in each group (Table 2). During the baseline clamp, insulin-stimulated GDR, glucose oxidation and NOGD were lower in type 2 diabetic patients vs weight-matched obese controls (Table 2). Reduced NOGD accounted for ~70% of the reduction in insulin-stimulated GDR in diabetic patients. Similar differences between the groups were seen during the post-exercise clamp. When comparing the baseline day with the exercise day, neither basal nor insulin-stimulated GDR was altered by prior exercise in either group (Table 2). However, prior

**Table 2** Metabolic characteristics during clamps

Characteristic	Baseline		Post exercise	
	Controls	Type 2 diabetes	Controls	Type 2 diabetes
Plasma glucose, basal (mmol/l)	5.6±0.1	9.5±0.8 <sup>‡‡‡</sup>	5.5±0.1	8.2±0.4 <sup>‡‡‡</sup>
Plasma glucose, clamp (mmol/l)	5.5±0.1	5.4±0.1 <sup>*</sup>	5.4±0.1	5.4±0.1 <sup>*</sup>
Serum insulin, basal (pmol/l)	40±5	56±12	44±7	58±10
Serum insulin, clamp (pmol/l)	427±14	407±19	395±15 <sup>§§</sup>	395±24
GDR, basal (mg m <sup>-2</sup> min <sup>-1</sup> )	80±2	90±4	87±4	93±3
GDR, clamp (mg m <sup>-2</sup> min <sup>-1</sup> )	349±35 <sup>*</sup>	242±35 <sup>‡‡‡</sup>	333±28 <sup>*</sup>	245±28 <sup>‡‡‡</sup>
Glucose oxidation, basal (mg m <sup>-2</sup> min <sup>-1</sup> )	47±7	38±6 <sup>†</sup>	29±7 <sup>¶</sup>	30±6 <sup>¶</sup>
Glucose oxidation, clamp (mg m <sup>-2</sup> min <sup>-1</sup> )	113±9 <sup>*</sup>	80±11 <sup>††</sup>	95±8 <sup>*¶</sup>	65±8 <sup>‡‡‡¶</sup>
NOGD, basal (mg m <sup>-2</sup> min <sup>-1</sup> )	33±7	52±6	58±8	63±5 <sup>¶</sup>
NOGD, clamp (mg m <sup>-2</sup> min <sup>-1</sup> )	236±30 <sup>*</sup>	162±27 <sup>‡‡</sup>	238±28 <sup>*</sup>	180±23 <sup>*¶</sup>
Ra basal (mg m <sup>-2</sup> min <sup>-1</sup> )	80±3	85±3	89±4	90±4
Ra clamp (mg m <sup>-2</sup> min <sup>-1</sup> )	41±6 <sup>*</sup>	34±7 <sup>*</sup>	31±5 <sup>*</sup>	34±6 <sup>*</sup>
Lipid oxidation, basal (mg m <sup>-2</sup> min <sup>-1</sup> )	29±2	36±2 <sup>†</sup>	39±3 <sup>§§</sup>	39±2
Lipid oxidation, clamp (mg m <sup>-2</sup> min <sup>-1</sup> )	9±3 <sup>*</sup>	19±4 <sup>††</sup>	14±2 <sup>*</sup>	22±3 <sup>*</sup>
Plasma lactate, basal (mmol/l)	1.0±0.1	1.1±0.1	0.8±0.1 <sup>¶</sup>	1.1±0.1 <sup>‡</sup>
Plasma lactate, clamp (mmol/l)	1.2±0.1	1.1±0.1	1.0±0.1 <sup>¶</sup>	0.9±0.1

Data are means ± SEM

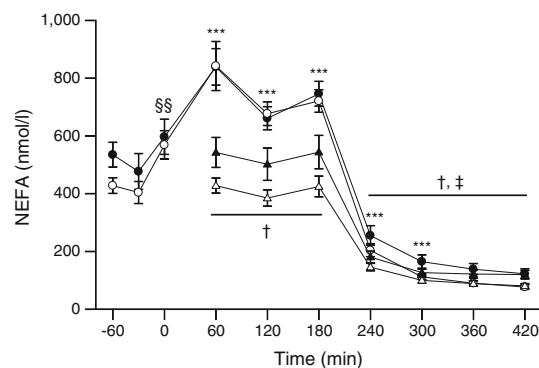
<sup>\*</sup>*p*<0.001 main effect of insulin; <sup>†</sup>*p*<0.05 main effect of diabetes; <sup>‡</sup>*p*<0.05, <sup>‡‡</sup>*p*<0.01 and <sup>‡‡‡</sup>*p*<0.001 vs controls; <sup>§§</sup>*p*<0.01 vs baseline; and <sup>¶</sup>*p*<0.05 main effect of exercise

Ra, endogenous glucose production rate

exercise did lower basal and insulin-stimulated glucose oxidation in both groups. In line, prior exercise increased basal and insulin-stimulated NOGD in diabetic patients. In obese controls previous exercise did not significantly alter NOGD when evaluated by two-way RM ANOVA. However, when evaluated by Student's paired *t* test, basal NOGD was increased after exercise (*p*<0.01). In response to exercise, plasma NEFA at the beginning of the basal clamp period increased from 543±52 to 838±63 and from 428±26 to 842±85 nmol/l in diabetic patients and obese controls, respectively (*p*<0.001), and remained significantly elevated during the recovery period (*p*<0.001) as well as 1 and 2 h into the following insulin-stimulated period (Fig. 1). During the last 2 h of the insulin-stimulated period, plasma NEFA were higher in diabetic patients vs obese controls (*p*<0.05) both with and without prior exercise (Fig. 1).

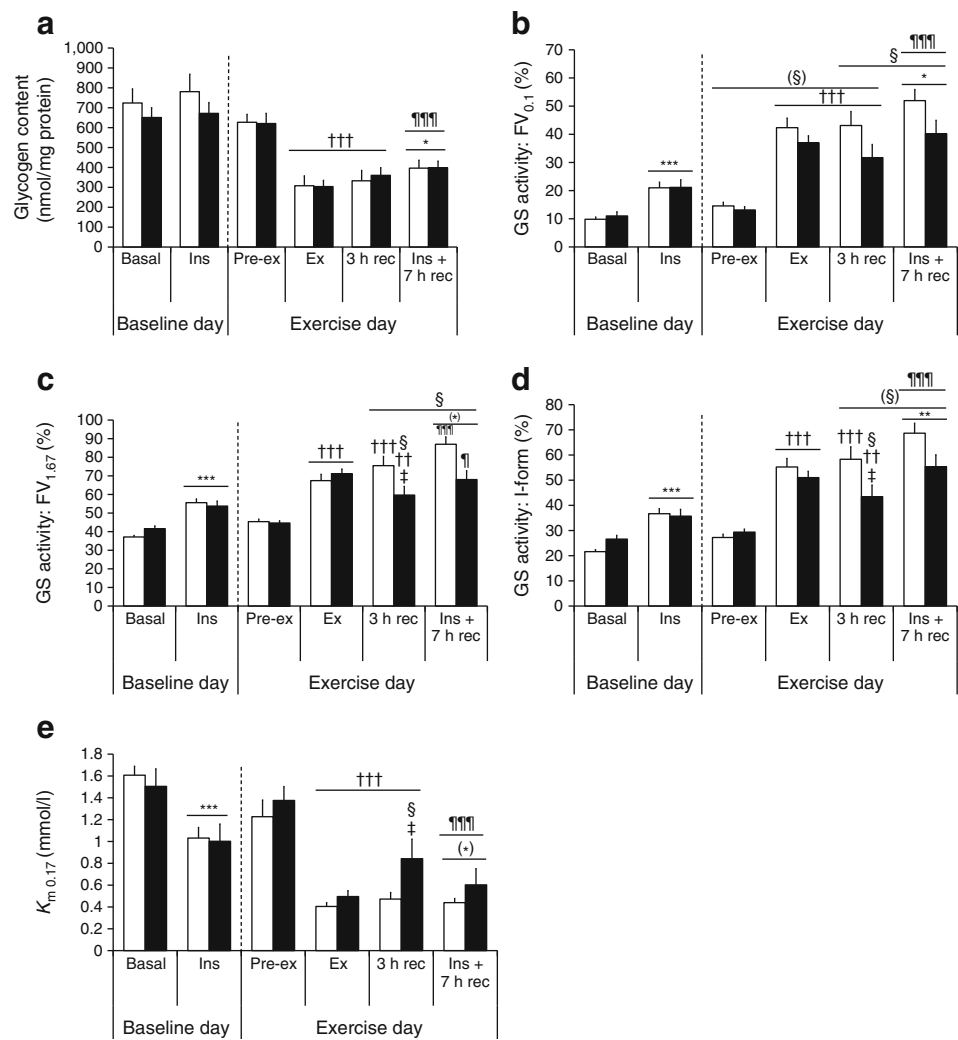
**Glycogen content** On the baseline day, muscle glycogen content was similar between groups (Fig. 2a). On the exercise day, exercise robustly decreased muscle glycogen content by ~50% (*p*<0.001) in both groups. Glycogen content did not change during the 3 h recovery period. After subsequent insulin stimulation, glycogen content increased equally in the groups (*p*<0.05). However, glycogen levels were not restored to pre-exercise levels (Fig. 2a).

**GS protein content** No differences in total GS protein content or GS total activities, either measured in the presence of 1.67 mmol/l (high) or 0.1 mmol/l (low) UDP-glucose, were found at any time or between groups (data not shown).



**Fig. 1** Plasma NEFA levels in patients with type 2 diabetes (black circles/triangles) and controls (white circles/triangles) on: (1) the baseline day (triangles) during the basal (60–180 min) and insulin-stimulated (180–420 min) periods; and on (2) the exercise day (circles) during exercise (–60–0 min) and during the basal and insulin-stimulated periods post exercise. Insulin was infused at a rate of 40 mU m<sup>-2</sup> min<sup>-1</sup> for 4 h. Exercise consisted of 60 min cycling at 70%  $\dot{V}O_{2max}$ . Data are means ± SEM. <sup>\*\*\*</sup>*p*<0.001 vs baseline day (main effect day); <sup>†</sup>*p*<0.05 type 2 diabetes vs controls on baseline day (main effect group); <sup>‡</sup>*p*<0.05 type 2 diabetes vs controls on exercise day (main effect group); and <sup>§§</sup>*p*<0.01 vs NEFA levels at –60 min (main effect time)

**Fig. 2** Muscle glycogen content (a); GS FV assayed with 0.1 mmol/l (b) and 1.67 mmol/l UDP-glucose (c); GS I-form activity (d) and  $K_m$  value assayed with 0.17 mmol/l of G6P (e) in skeletal muscle biopsies obtained from patients with type 2 diabetes (black bars) and obese controls (white bars) on the baseline day before and after insulin and on the exercise day before and after exercise and before and after insulin. Data are means  $\pm$  SEM. (\*) $p$ <0.10, \* $p$ <0.05, \*\* $p$ <0.01 and \*\*\* $p$ <0.001 vs before insulin; †† $p$ <0.01 and ††† $p$ <0.001 vs before exercise; † $p$ <0.05 vs after exercise; (§) $p$ <0.10 and (§§) $p$ <0.05 vs controls; ¶ $p$ <0.05 and ¶¶¶ $p$ <0.001 vs after insulin. Basal, before insulin; Ins, after insulin; Pre-ex, before exercise; Ex, after exercise; 3 h rec, before insulin (3 h into recovery on exercise day); Ins+7 h rec, after insulin (7 h into recovery on exercise day)



Average total GS activity ( $n=102$ ) was  $18.8 \pm 0.6 \text{ nmol min}^{-1} \text{ mg}^{-1}$  at high and  $6.5 \pm 0.1 \text{ nmol min}^{-1} \text{ mg}^{-1}$  at low UDP-glucose concentration.

**GS activity** On the baseline day, insulin stimulation increased GS activity measured as FV<sub>0.1</sub>, FV<sub>1.67</sub> and I-form similarly in each group (Fig. 2b–d). Exercise robustly increased these measures of GS activity with no differences between groups. Activation of GS by exercise per se was markedly higher than the effect of insulin per se ( $p < 0.001$ ). At 3 h into recovery, these measures of GS activity remained elevated in obese controls, but decreased in diabetic patients, so that the GS FV<sub>1.67</sub>, and I-form activities were lower in diabetic patients vs obese controls. Subsequent insulin stimulation increased all measures of GS activity in both groups, but GS FV<sub>0.1</sub> and FV<sub>1.67</sub> activities were still lower in the diabetic patients vs obese controls. The activities of GS FV<sub>0.1</sub>, FV<sub>1.67</sub> and I-form were higher in the post-exercise clamp vs the baseline clamp in both groups (Fig. 2b–d).

**GS affinity for UDP-glucose** On the baseline day, basal muscle GS affinity for UDP-glucose (assessed with a physiological [0.17 mmol/l] concentration of G6P) was similar between groups ( $K_m \sim 1.5 \text{ mmol}$ ) (Fig. 2e). Insulin increased GS affinity for UDP-glucose as  $K_m$  decreased to  $\sim 1.0 \text{ mmol/l}$  in both groups ( $p < 0.001$ ). Exercise also increased GS substrate affinity ( $p < 0.001$ ) and in fact more potently than insulin per se to  $K_m$  values around 0.4–0.5 mmol/l in both groups. After 3 h of recovery,  $K_m$  remained decreased compared with pre-exercise in both groups.

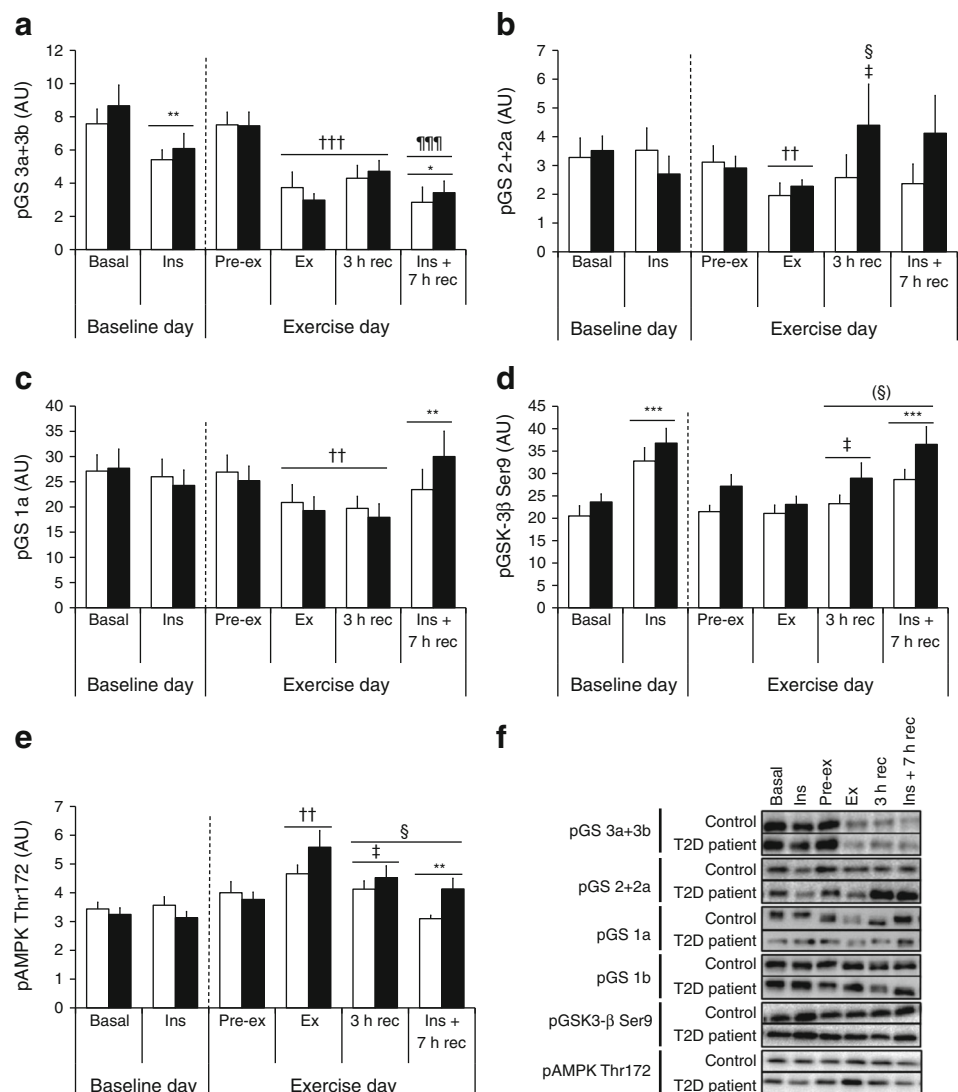
Comparison of  $K_m$  values for the first three time points of the exercise day using two-way RM ANOVA showed significant effects of exercise ( $p < 0.001$ ) and group ( $p < 0.05$ ), with no significant interaction between group and exercise ( $p = 0.43$ ). However, comparing only the exercise values with the 3 h recovery values demonstrated that  $K_m$  was higher in diabetic patients vs obese controls 3 h into recovery, and actually increased during recovery in the diabetic patients. Post exercise, insulin tended to decrease  $K_m$  further ( $p = 0.06$  for main effect) with no significant differences between groups. These

observations on GS substrate affinity ( $K_m$ ) are in line with the GS activity pattern described above. Finally, insulin-stimulated  $K_m$  values were markedly lower in the post-exercise state than during the baseline condition in both groups ( $p < 0.001$ ).

**GS phosphorylation** Dephosphorylation of GS at sites 2+2a and 3a+3b is an important mechanism by which GS is activated [18–20, 27]. On the baseline day, insulin decreased GS sites 3a+3b phosphorylation in both groups ( $p = 0.008$ ) (Fig. 3a). GS sites 3a+3b phosphorylation was also robustly decreased in response to exercise ( $p < 0.001$ ), and remained decreased 3 h into recovery in both groups ( $p < 0.001$ ). Post-exercise insulin stimulation further decreased GS sites 3a+3b phosphorylation vs 3 h into recovery ( $p = 0.012$ ) causing reduced GS sites 3a+3b phosphorylation compared with the baseline insulin-stimulated condition in both groups ( $p = 0.001$ ). No differences in GS sites 3a+3b phosphorylation were seen between diabetic patients and controls.

GS phosphorylation at sites 2+2a was similar between the groups on the baseline day and was not influenced by insulin (Fig. 3b). Exercise caused significant dephosphorylation of GS sites 2+2a ( $p < 0.01$  vs pre-exercise), with no difference between groups. After 3 h of recovery, a significant (115%) increase in GS phosphorylation at sites 2+2a vs the level at exercise was observed in the diabetic patients ( $p = 0.02$ ). On the exercise day, GS phosphorylation at sites 2+2a was again not affected by insulin in either group. GS site 1a phosphorylation was similar between groups at all time points (Fig. 3c). Whereas insulin did not affect this phosphorylation site on the baseline day, exercise caused significant GS site 1a dephosphorylation ( $p < 0.01$ ), and it remained decreased 3 h into recovery. Post-exercise insulin stimulation increased GS site 1a phosphorylation ( $p < 0.01$  vs recovery), with a tendency for this effect to be confined to the diabetic group ( $p = 0.09$  for interaction between group and insulin). GS site 1b phosphorylation was similar in diabetic patients and obese controls at

**Fig. 3** Phosphorylation of GS at sites 3a+3b (a), sites 2+2a (b) and site 1a (c), and Ser9 phosphorylation of GSK3- $\beta$  (d) and Thr172 phosphorylation of AMPK (e) in skeletal muscle biopsies obtained from patients with type 2 diabetes (black bars) and controls (white bars) on the baseline day and the exercise day (see Fig. 2). Representative immunoblots are shown in (f). Quantified data in the bar graphs are given in arbitrary units. Data are means  $\pm$  SEM. \* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.001$  vs before insulin; †† $p < 0.01$  and ††† $p < 0.001$  vs before exercise; ‡ $p < 0.05$  vs after exercise; (§) $p < 0.10$  and (§§) $p < 0.05$  vs controls and †††† $p < 0.001$  vs after insulin. AU, arbitrary units; Basal, before insulin; Ins, after insulin; Pre-ex, before exercise; Ex, after exercise; 3 h rec, before insulin (3 h into recovery on exercise day); Ins+7 h rec, after insulin (7 h into recovery on exercise day); T2D, type 2 diabetes



all time points and was unaffected by insulin or exercise (ESM Fig. 2).

Glycogen synthase kinase 3 (GSK3) and AMP-activated protein kinase (AMPK) are major upstream regulators of GS [27, 28]. Insulin stimulation inhibited GSK-3 $\beta$  activity by increasing Ser9 phosphorylation on both experimental days ( $p < 0.001$ ). No effect of acute exercise was seen on GSK-3 $\beta$  Ser9 phosphorylation (Fig. 3d). GSK-3 $\beta$  Ser9 phosphorylation was slightly higher 3 h into recovery vs exercise, and tended to be increased in the diabetic group in the recovery period compared with obese controls ( $p = 0.099$ ). AMPK phosphorylation at Thr172 (reflecting kinase activation) was increased modestly by exercise in both groups ( $p = 0.002$ ) (Fig. 3e). AMPK phosphorylation decreased in recovery from exercise ( $p = 0.029$ ) and was further decreased by insulin ( $p = 0.009$ ) on the exercise day. Interestingly, AMPK phosphorylation was elevated in the post-exercise period in type 2 diabetic patients compared with obese controls ( $p = 0.03$ ). Total protein levels of AMPK ( $\alpha 2$  subunit) and GSK-3 $\beta$  were similar between groups and unaffected by interventions except for a small increase in GSK-3 $\beta$  protein by insulin on the baseline day ( $p < 0.001$ ) (data not shown).

**Correlation analysis** To explore possible mechanisms underlying the regulation of GS activity, phosphorylation and substrate affinity ( $K_m$ ) by insulin, exercise and insulin post exercise, we examined the relationship between these measures as well as glucose metabolism and glycogen content in the study cohort.

On the baseline day, insulin-stimulated GDR and NOGD correlated positively with GS  $FV_{0.1}$ ,  $FV_{1.67}$  and I-form ( $r = 0.41$  to  $0.66$ , all  $p < 0.05$ ). No significant associations between these variables were seen on the exercise day.

On the baseline day, insulin-stimulated GS  $FV_{0.1}$ ,  $FV_{1.67}$  and GS I-form activities correlated inversely with GS phosphorylation at sites 3a+3b and 2+2a ( $r = -0.48$  to  $-0.61$ , all  $p < 0.01$ ).  $K_m$  correlated with GS phosphorylation at sites 2+2a ( $r = 0.50$ ,  $p < 0.01$ ) and tended to correlate with GS phosphorylation at sites 3a+3b ( $r = 0.37$ ,  $p = 0.06$ ).

On the exercise day, GS  $FV_{0.1}$ ,  $FV_{1.67}$  and I-form activities after exercise correlated inversely with GS sites 3a+3b phosphorylation ( $r = -0.55$  to  $-0.68$ , all  $p < 0.01$ ) and at sites 2+2a ( $r = -0.41$  to  $-0.56$ , all  $p < 0.05$ ).  $K_m$  correlated with GS sites 3a+3b phosphorylation ( $r = 0.42$ ,  $p = 0.03$ ) and tended to correlate with GS phosphorylation at sites 2+2a ( $r = 0.35$ ,  $p = 0.08$ ). During recovery, GS  $FV_{0.1}$ ,  $FV_{1.67}$  and I-form activities all showed an inverse correlation with GS phosphorylation at sites 3a+3b and 2+2a ( $r = -0.64$  to  $-0.83$ , all  $p < 0.001$ ), whereas  $K_m$  correlated with GS phosphorylation at both sites 3a+3b and sites 2+2a ( $r = 0.61$  to  $0.71$ ,  $p < 0.001$ ).

On the exercise day, the changes ( $\Delta$  values) in muscle glycogen content in response to exercise correlated inversely with the changes in GS  $FV_{0.1}$ ,  $FV_{1.67}$  and I-form activities ( $r =$

$-0.43$  to  $0.56$ , all  $p < 0.05$ ), and positively with the changes in  $K_m$  ( $r = 0.53$ ,  $p < 0.01$ ) and with the changes in the corresponding phosphorylation of GS at sites 3a+3b ( $r = 0.56$ ,  $p < 0.01$ ) and sites 2+2a ( $r = 0.41$ ,  $p = 0.03$ ).

## Discussion

In the present study, we demonstrate that moderate-intensity exercise reduces muscle glycogen, increases muscle GS activity and substrate affinity, and dephosphorylates GS at sites 3a+3b and 2+2a to a similar degree in type 2 diabetic patients and well-matched controls. These effects may explain the observed increase in glucose partitioning toward storage and away from oxidation. Importantly, we show that, 3 h into exercise recovery and during insulin stimulation post exercise, GS activity and substrate affinity in muscle are reduced in diabetic patients compared with controls concomitant with increased phosphorylation of GS at sites 2+2a. This dysregulation of muscle GS in type 2 diabetes implies some degree of impaired response to recovery from exercise.

Our findings on the effects of exercise on GS activity, substrate affinity and sites 3a+3b phosphorylation are in accordance with a recent study that reported similar changes in these variables and with comparable efficacy in lean, obese and type 2 diabetic individuals [16]. In contrast to our results, Jensen et al could not demonstrate an effect of acute exercise on GS phosphorylation at sites 2+2a or differences in measures of GS activity and phosphorylation 3.5 h into recovery between the diabetic and control groups [16]. However, in that study the post-exercise muscle biopsies were only available from four diabetic participants, which may explain the inability to detect such changes. This, together with differences in sex composition and a lower baseline  $\dot{V}O_{2max}$ , may explain these discordant findings. Mutational studies [18, 19] as well as analysis on muscle biopsies from humans [15, 21, 22, 29] have found phosphorylation of GS at sites 2+2a and 3a+3b to be the most important for GS activity. In accordance, we demonstrate an inverse correlation between both insulin- and exercise-induced measures of GS activity and GS phosphorylation at these sites. This also suggests that the increased phosphorylation at GS sites 2+2a caused the reduced GS activities in our diabetic cohort in the recovery period.

Glycogen is a proposed regulator of GS activity [30] and GS phosphorylation at sites 2+2a [28]. Consistent with this, the exercise-associated decrease in muscle glycogen content has been shown to be a factor involved in GS activation [30–32]. Recently, a positive correlation between muscle glycogen levels and GS phosphorylation at sites 2+2a, 3a, and 3a+3b was reported in pooled data from healthy young volunteers before, during and after glycogen-depleting exercise [15]. We extend these findings to a cohort of obese individuals with or without type 2 diabetes, reporting a positive

correlation between changes in muscle glycogen content during moderate-intensity exercise and changes in phosphorylation of GS at sites 2+2a and 3a+3b. In skeletal muscle of both normal and insulin-resistant rats, contraction-induced glycogen utilisation is associated with dephosphorylation of GS on sites 2+2a [33, 34]. It is, therefore, interesting that GS phosphorylation at sites 2+2a increased 3 h into recovery in our patients with type 2 diabetes despite a sustained depletion of glycogen. This suggests that in type 2 diabetes, recovery from exercise is associated with activation of kinases and/or inhibition of phosphatases acting on muscle GS. Our data suggest that increased activation of the stress-activated kinase AMPK during exercise recovery might be associated with enhanced GS site 2+2a phosphorylation in the type 2 diabetic patients compared with obese controls. The pattern of GSK-3 $\beta$  regulation does not support a direct role for this kinase in the differential GS regulation during the post-exercise period between the groups.

Several studies have found insulin sensitivity, as assessed by the euglycaemic–hyperinsulinaemic clamp technique, to be increased for a prolonged period after a single bout of moderate- to strenuous-intensity cycle exercise [11, 12]. An enhanced post-exercise insulin sensitivity has been demonstrated both for glucose-tolerant individuals and for various insulin-resistant populations, including patients with type 2 diabetes, with the change mainly accounted for by an increase in NOGD [11–13]. As glycogen synthesis is the primary pathway of NOGD [1], these changes favour glycogen replenishment after exercise [13, 17, 35–37].

It was thus somewhat surprising that we did not find an effect of prior exercise on insulin-stimulated GDR in either diabetic or obese control individuals. Indeed, the observed exercise-induced increase in plasma NEFA, which was sustained at least 3 h into recovery in both groups as shown previously [38], is a physiological response that might explain the lack of increase in whole-body insulin-stimulated glucose metabolism by both changes in substrate preferences and perhaps signalling mechanisms [39–41]. Moreover, NEFA levels remained elevated during the post-exercise clamp in the diabetic individuals. This indicates that insulin resistance at the level of adipose tissue is not ameliorated in response to exercise. Nevertheless, prior exercise did in fact direct basal and insulin-stimulated glucose uptake toward storage and away from oxidation within both groups, in accordance with previous observations [42].

Glucose storage has consistently been reported to correlate with GS activity [6, 7, 12]. In accordance with the effect of exercise on intracellular glucose metabolism, we observed increased basal and insulin-stimulated values for GS activity and substrate affinity after exercise in both diabetic and obese individuals. As noted by Jensen et al [16], with a GS  $K_m$  value (in the mmol/l range) far above the physiological concentration of UDP-glucose (estimated to  $\sim$ 0.03 mmol/l in resting muscle), an increase in substrate affinity would be likely to

increase the rate of glucose storage into glycogen after exercise. Correspondingly, we demonstrate a reduction in the GS  $K_m$  at a physiological concentration of G6P (0.17 mmol/l) from above 1 mmol/l in the rested state to  $<$ 0.5 mmol/l after exercise in both study groups.

Previously, we have found GS hyperphosphorylation or lack of dephosphorylation on sites 2+2a together with impaired GS activation by insulin in muscle of patients with type 2 diabetes compared with lean and obese controls in some [4, 5], but not all studies [6]. However, in the present study, we could not demonstrate these differences between patients with type 2 diabetes and weight-matched obese controls in the baseline rested state. In line with previous studies of insulin-resistant conditions including individuals with obesity, PCOS and type 2 diabetes [4–6, 25, 29], we did, however, observe a lack of insulin-induced dephosphorylation of GS at sites 2+2a in both type 2 diabetes and obesity. These phosphosites have previously been found to be responsive to insulin stimulation in a number of healthy lean participant populations [4, 25, 43].

Although impaired insulin activation of muscle GS represents one of the more consistent defects found in insulin-resistant cohorts with obesity, polycystic ovary syndrome (PCOS) and type 2 diabetes [4, 6, 7, 25], not all studies have been able to detect a difference between type 2 diabetic and weight-matched obese individuals [6, 17, 44]. In the present study, the insulin-stimulated GDR in the type 2 diabetic patients was 51–77% higher than values reported in our previous studies [6, 44]. This could be explained by the higher cardiorespiratory fitness ( $\dot{V}O_{2max}$ ) in this diabetic cohort compared with cohorts in previous studies [5, 45], and may have contributed to higher insulin-stimulated GS activity in the rested state. Nevertheless, our data also demonstrate that changes in GS activity, substrate affinity and phosphorylation cannot explain all the differences in whole-body insulin sensitivity between type 2 diabetic patients and weight-matched controls, indicating a role for other factors, e.g. those involved in glucose delivery (capillary recruitment) or glucose transport (Glut4 recruitment) and phosphorylation (hexokinase II activation).

In summary, the present study demonstrates that a single bout of exercise stimulates muscle GS activity with similar efficiency in type 2 diabetic patients and weight-matched controls. This effect involves GS dephosphorylation and enhanced substrate affinity. In both groups these exercise-induced effects on GS were sustained 3 h into recovery. Thus, the subsequent enhanced insulin-stimulated responses may contribute to increased glucose partitioning towards storage and away from oxidation in the period after exercise. Interestingly, compared with obese controls, increased phosphorylation of GS at sites 2+2a in concert with impaired GS activation was observed in the diabetic group during recovery from exercise. Further studies are needed to establish to what extent these post-exercise defects in the regulation of GS leave type 2



diabetic patients refractory to post-exercise recovery. Although not fully illuminated, our data suggest that elevated AMPK activity in the post-exercise recovery period might be part of the mechanisms involved.

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**Contribution statement** AJTP, KH and JFPW were responsible for the conception and design of the study. AJTP conducted the in vivo experiments and wrote the first version of the manuscript. JRH and MF conducted the laboratory experiments. AJTP, JRH MF, JMK and KH contributed to analysis of data. All contributed to the interpretation of the results, revised the manuscript and approved the final version. KH and JFPW are responsible for the integrity of the work as a whole.

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