Regulation of glycogen synthesis in rat skeletal muscle after glycogen-depleting contractile activity: effects of adrenaline on glycogen synthesis and activation of glycogen synthase and glycogen phosphorylase

Jesper FRANCH*, Rune ASLESEN†‡ and Jørgen JENSEN†§[1]

*Institute of Sport Sciences and Clinical Biomechanics, Odense University, Faculty of Health Sciences, Campusvej 55, DK-5230, Odense M, Denmark, †Department of Anatomy, Institute of Basic Medical Sciences, University of Oslo, P.O. Box 1105 Blindern, N-0317 Oslo, Norway, ‡Norwegian University of Sport and Physical Education, P.O. Box 4014, Ullevål, Hageby, N-0806 Oslo, Norway, and §Department of Physiology, National Institute of Occupational Health, P.O. Box 8149 Dep., N-0033 Oslo, Norway

We investigated the effects of insulin and adrenaline on the rate of glycogen synthesis in skeletal muscles after electrical stimulation *in vitro*. The contractile activity decreased the glycogen concentration by 62%. After contractile activity, the glycogen stores were fully replenished at a constant and high rate for 3 h when 10 m-i.u./ml insulin was present. In the absence of insulin, only 65% of the initial glycogen stores was replenished. Adrenaline decreased insulin-stimulated glycogen synthesis. Surprisingly, adrenaline did not inhibit glycogen synthesis stimulated by glycogen-depleting contractile activity. In agreement with this, the fractional activity of glycogen synthase was high when adrenaline was present after exercise, whereas adrenaline decreased the fractional activity of glycogen synthase to a low level during stimulation with insulin. Furthermore, adrenaline activated glycogen phosphorylase almost completely during stimulation with insulin, whereas a much lower activation of glycogen phosphorylase was observed after contractile activity. Thus adrenaline does not inhibit contraction-stimulated glycogen synthesis.

Key words: electrical stimulation, glucose 6-phosphate, insulin.

INTRODUCTION

The two most important physiological stimuli of glycogen synthesis in skeletal muscles are insulin and glycogen-depleting exercise [1,2]. Insulin-stimulated glycogen synthesis in skeletal muscles is particularly important for the regulation of blood glucose. After carbohydrate ingestion, the major part of the insulin-stimulated glucose uptake occurs in skeletal muscle, where the glucose is incorporated primarily into glycogen [3,4]. However, glycogen also has an important role as an energy substrate during exercise: the size of the glycogen stores is a limiting factor in muscular endurance [5]. During exercise, glycogen is broken down; after exercise, glycogen synthesis is stimulated in the muscles, where the glycogen concentration is decreased [6–9].

Glycogen synthesis has been described as occurring in two phases after severe glycogen-depleting exercise. Glycogen is synthesized initially at a high rate, but the rate declines as the glycogen concentration increases [6,8,10–12]. The initial rapid phase occurs even in the absence of insulin [6,9,11]. During the next slow phase, contraction-stimulated glycogen synthesis is low; however, in this period the effect of insulin is increased [7,8]. Glycogen synthesis after exercise therefore seems to be stimulated via two mechanisms: contraction stimulates glycogen synthesis itself and increases the effect of insulin.

The intracellular mechanisms of contraction-stimulated glycogen synthesis and of increased insulin sensitivity are not fully understood, but the signalling pathway for contraction differs from that of insulin [2,13,14]. Because insulin and contraction stimulate glucose uptake via different signalling pathways, there is an additive effect of contraction and insulin on glucose uptake and glycogen synthesis [15–19]. Furthermore, the prolonged increase in insulin action after contraction makes the distinction between the rapid and slow phase in glycogen synthesis less evident when a high concentration of insulin is present [20]. It is not known whether the glycogen stores can be fully replenished at a high rate in the presence of a maximally stimulating insulin concentration. The first purpose of the present study was to investigate the rate of glycogen synthesis during replenishment of the glycogen stores after glycogen-depleting contractile activity in the presence of a maximally stimulating insulin concentration.

The signalling pathway for contraction-stimulated glucose uptake and glycogen synthesis has aroused great interest, because muscle contraction can stimulate glucose transport and the translocation of GLUT4 even in insulin-resistant muscles [18,21]. Adrenaline is a potent inhibitor of insulin-stimulated glucose uptake and glycogen synthesis [19,22–25]. However, it is still uncertain whether adrenaline inhibits glycogen synthesis stimulated after contraction. We have recently reported that adrenaline is a much less powerful inhibitor of glucose phosphorylation during contraction than during stimulation with insulin [19]. Furthermore, adrenaline inhibits the insulin-stimulated activation of glycogen synthase [26–28], but adrenaline seems to inhibit only in part the activation of glycogen synthase after exercise [26,29]. The second purpose of the present study was to investigate the effect of adrenaline on glycogen synthesis and glycogen-metabolizing enzymes after glycogen-depleting exercise.

MATERIALS AND METHODS

Muscle preparation

Male Wistar rats were obtained from Møllegård Breeding Centre (Lille Skensved, Denmark) and housed for at least 1 week in our animal facilities. The rats were kept at a 12 h light:12 h dark cycle and fed with rat chow and water *ad libitum*, as described

[1] To whom correspondence should be addressed at the National Institute of Occupational Health (e-mail jorgen.jensen @stami.no).

previously [30]. The experiments and procedures were approved by the local responsible veterinarian and conducted in conformity with laws and regulations controlling experiments and procedures for animals in Norway. Each animal's body weight was 110–150 g on the day of the experiment.

The rats were anaesthetized with 0.15 ml of pentobarbital (50 mg/ml) injected intraperitoneally. The epitrochlearis muscles were dissected out and mounted on contraction apparatuses at their resting length, as described previously [19]. The muscles were preincubated for 30 min in 3 ml of a modified Krebs–Henseleit buffer (KHB) containing 116 mM NaCl, 4.6 mM KCl, 1.16 mM KH_2PO_4, 25.4 mM $NaHCO_3$, 2.5 mM $CaCl_2$, 1.16 mM $MgSO_4$, 0.1 % BSA, 5 mM Hepes, 5.5 mM D-glucose and 2 mM pyruvate, pH 7.4. All incubations were performed at 30 °C and the buffers were gassed continuously with O_2/CO_2 (19:1). After preincubation the muscles were incubated in 3 ml of fresh KHB and either stimulated with impulse trains of 200 ms (10 V square wave pulses 0.2 ms in duration) delivered at a rate of one train every 1 s for 30 min or kept resting for another 30 min. The pulse trains were generated by a Pulsar 6-bp stimulator (Frederic Haer) and amplified by equipment built at the Institute of Basic Medical Sciences (University of Oslo, Oslo, Norway).

For measurements of glycogen synthesis, the muscles were transferred to KHB containing 0.2 μCi/ml D-[U-^{14}C]glucose (298 mCi/mmol; NEN). Glycogen synthesis was measured after contraction (or rest) with or without 10 m-i.u./ml insulin in the incubation buffer. Adrenaline (1 μM) was added to some muscles during glycogen synthesis. In these experiments, 0.5 % ascorbic acid was added in all groups. See the Figure legends and Tables for further information.

Immediately after incubation, the muscles were removed from the contraction apparatuses, blotted on filter paper and frozen in liquid nitrogen before being stored at −80 °C. The muscles were freeze-dried and weighed before analysis.

Glycogen synthesis

Analysis of glycogen synthesis from the incorporation of [^{14}C]glucose into glycogen was performed as described by Cuendet et al. [31]. In brief, muscles were dissolved in 500 μl of 1 M KOH at 70 °C for 20 min before 100 μl of saturated Na_2SO_4 and 100 μl glycogen (25 mg/ml) were added and mixed. Ethanol (1.5 ml) was added, and the glycogen was precipitated overnight at −20 °C. After centrifugation (3000 ***g*** for 20 min at 4 °C) the supernatant was discarded; the precipitate was dissolved in 500 μl of distilled water for 20 min at 70 °C and the glycogen was reprecipitated with ethanol. The new precipitate was dissolved in 300 μl of distilled water; 250 μl was added to 3 ml of scintillation solution (Hionic-Fluor; Packard) and counted for radioactivity (Tri-Carb 460C; Packard).

Glycogen and metabolites

The glycogen in the muscle samples was hydrolysed to glucose in 500 μl of 1 M HCl at 100 °C for 2.5 h and the glucose units were measured fluorometrically by the method of Lowry and Passonneau [32]. Muscle metabolites were extracted in 0.6 M $HClO_4$ for 30 min at 0 °C as described earlier [19]. The concentration of glucose 6-phosphate was measured fluorimetrically by the method of Lowry and Passonneau [32].

Glycogen phosphorylase

Freeze-dried muscle samples were weighed and homogenized twice for 15 s (Polytron PT 1200) in 1/400 vol. of buffer [50 mM Mes/100 mM NaF/5 mM EDTA/1 mM 2-mercaptoethanol (pH 6.1)]. The homogenate was centrifuged at 4 °C for 10 min at 3000 ***g*** and the supernatant was frozen at −80 °C. Glycogen phosphorylase activity was measured by the method of Gilboe et al. [33]. In brief, 50 μl of supernatant was added to 50 μl of assay buffer containing 50 mM Mes, 0.2 mM NaF, 27 mM 2-mercaptoethanol, 5 mM EDTA, 46 ± 1 mM glucose 1-phosphate, 0.0005 mCi/ml [^{14}C]glucose 1-phosphate (223.9 Ci/mmol; NEN) and 10 mg/ml glycogen. Total phosphorylase activity was measured in the presence of 3 mM AMP in the assay buffer, and phosphorylase *a* activity was measured in the absence of AMP; the percentage of phosphorylase in the *a* form was calculated from these values. After 30 min of reaction at 25 °C, 70 μl of the homogenate/assay buffer was spotted on a Whatman filter (1 cm × 2.5 cm). The filters were dropped into cold (4 °C) 66 % (v/v) ethanol (approx. 10 ml per filter) for 30 min to precipitate glycogen; in addition, they were washed twice at room temperature for 20 min each in 66 % (v/v) ethanol (approx. 7 ml per filter) to remove [^{14}C]glucose 1-phosphate. After being dried, the filters were transferred to scintillation vials; 3 ml of scintillation solution (Ultima Gold XR, Packard) was added to each and the mixtures were counted for radioactivity (Tri-Carb 1900 TR; Packard).

Glycogen synthase activity

Freeze-dried muscle samples were homogenized twice for 15 s (Polytron PT 1200) in 1/400 vol. of buffer [50 mM Tris/HCl/100 mM NaF/10 mM EDTA (pH 7.8)]. The homogenate was centrifuged at 4 °C for 30 min at 3000 ***g*** and the supernatant was frozen at −80 °C. A modification of the filter-paper method [34] was used for measurements of glycogen synthase activity. In brief, 20 μl of homogenate was added to 40 μl of assay buffer containing 25 mM Tris/HCl, 50 mM NaF, 5 mM EDTA, 2.5 mM UDP-glucose, 0.0005 mCi/ml [^{14}C]UDP-glucose (287.4 Ci/mmol; NEN), 20 mg/ml glycogen and various concentrations of glucose 6-phosphate. The reaction was performed at 37 °C; after 8 min, 50 μl of the homogenate/assay buffer was spotted on a Whatman filter paper (1 cm × 2.5 cm). The procedure described for glycogen phosphorylase was used for the precipitation of glycogen, for the removal of [^{14}C]UDP-glucose and for counting radioactivity. Maximal glycogen synthase activity was determined at saturating concentration of glucose 6-phosphate (8 mM). In addition, glycogen synthase activity was measured at three different concentrations of glucose 6-phosphate (0, 0.17 and 0.5 mM) and fractional activities were calculated.

Statistical analysis

Results are presented as means ± S.E.M. Analysis of variance was performed to establish differences between groups; least-significant-difference testing was performed *post hoc*. $P < 0.05$ was considered as significant.

RESULTS

In non-contracted epitrochlearis muscles the glycogen concentration was 152.2 ± 7.7 mmol/kg dry weight ($n = 13$). Electrical stimulation for 30 min decreased the glycogen concentration to 58.2 ± 4.0 mmol/kg dry weight ($P < 0.0001$; $n = 14$). Subsequent incubation of the muscles in buffer containing 10 m-i.u./ml insulin brought the glycogen concentration back to normal within 3 h (162.5 ± 9.8 mmol/kg dry weight; $n = 12$). In the absence of insulin, the glycogen concentration was increased only to 99.5 ± 4.9 mmol/kg dry weight after 3 h of incubation.

The rate of glycogen synthesis, calculated from the incorporation of [^{14}C]glucose into glycogen, was negligible in non-

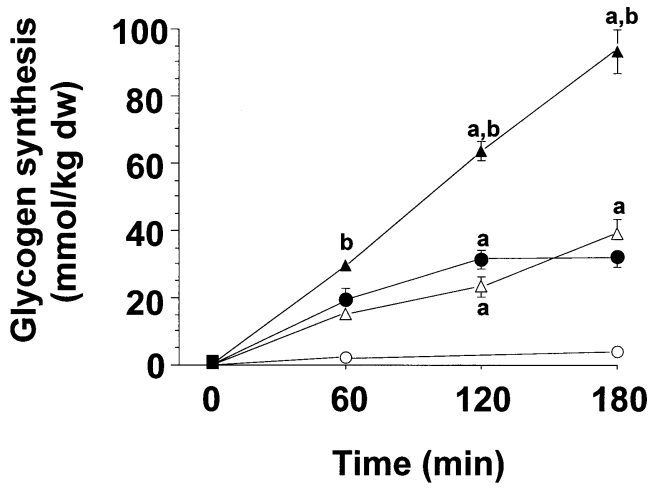

Figure 1 Effect of insulin on glycogen synthesis in epitrochlearis muscle after contractile activity or rest

Muscles were stimulated electrically for 30 min (▲, ●) or rested (△, ○) before glycogen synthesis was measured without (●, ○) or with (△, ▲) insulin. Results are means ± S.E.M.; $n = 8$–14. The letters labelling the points have the following meanings: a, significantly different from previous value; b, significantly different from contraction and from insulin. Abbreviation: dw, dry weight.)

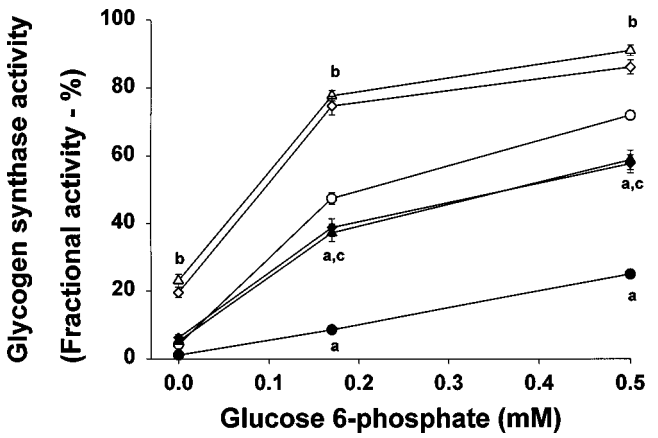

Figure 2 Effect of adrenaline on the fractional activity of glycogen synthase after contraction and during stimulation with insulin

Muscles were stimulated electrically for 30 min and then incubated for 10 min in the absence (△, ▲) or presence (◇, ◆) of 10 m-i.u./ml insulin without (△, ◇) or with (▲, ◆) 1 μM adrenaline. Other muscles were kept rested for 30 min (○, ●) and then incubated with 10 m-i.u./ml insulin in the absence (○) or presence (●) of 1 μM adrenaline. Total glycogen synthase activity was measured (8 mM glucose 6-phosphate) and the fractional activity was calculated at three different concentrations of glucose 6-phosphate. Results are means ± S.E.M.; $n = 6$ or 7. The letters labelling the points have the following meanings: a, significant effect of adrenaline; b, significantly higher than all other groups; c, significantly lower than insulin.

Table 1 Effect of adrenaline on glycogen synthesis after contractile activity and during stimulation with insulin

Muscles were stimulated electrically or kept rested for 30 min and then incubated for 1 h with or without insulin (10 m-i.u./ml) in the absence or presence of 1 μM adrenaline. Glycogen synthesis was calculated from the incorporation of [^{14}C]glucose. Results are means ± S.E.M. The number of muscles in each group is given in parentheses. Abbreviation: n.d., not determined. *Significant difference between muscles incubated with and without adrenaline; †significant difference from contraction in the presence of insulin.

	Glycogen synthesis (mmol/h per kg)	
Condition	Without adrenaline	With adrenaline
Control	2.2 ± 0.3 (10)	n.d.
Insulin	16.2 ± 1.4† (13)	5.9 ± 0.4* (9)
Contraction	19.9 ± 1.7† (14)	19.0 ± 1.5 (9)
Contraction in presence of insulin	30.1 ± 2.4 (12)	21.7 ± 1.4* (8)

contracted muscles in the absence of insulin (Figure 1). After contraction, glycogen synthesis was 19.9 ± 1.7 mmol/h per kg dry weight during the first hour; this decreased to 12 mmol/h per kg dry weight in the second hour. Although the glycogen concentration was only 65 % of the initial value, no further glycogen synthesis was observed during the third hour after contraction when insulin was absent (Figure 1).

The rate of maximal insulin-stimulated glycogen synthesis in the non-contracted muscles was similar to that of glycogen synthesis in muscles after contraction when insulin was absent (16.2 ± 1.4 compared with 19.9 ± 1.7 mmol/h per kg dry weight; Figure 1). Contractile activity and insulin had an additive effect on glycogen synthesis during the first hour after contractions (Figure 1). Furthermore, in the presence of insulin, the rate of glycogen synthesis was constant during the 3 h during which the glycogen stores were fully replenished, although contraction did not itself stimulate glycogen synthesis during the third hour.

Adrenaline decreased the insulin-stimulated glycogen synthesis by 74 % (Table 1). Contraction-stimulated glycogen synthesis, in contrast, was not influenced by adrenaline. In the absence of adrenaline, contraction and insulin had an additive effect on glycogen synthesis; however, when adrenaline was present, glycogen synthesis after contraction was similar whether or not insulin was present (Table 1).

Adrenaline decreased the fractional activity of glycogen synthase to low levels in insulin-stimulated non-contracted muscles (Figure 2). In non-contracted muscles, adrenaline decreased the fractional activity of glycogen synthase to similar low levels when insulin was absent (results not shown). The fractional activity of glycogen synthase was much higher after contraction than during stimulation with insulin; insulin did not increase the fractional activity of glycogen synthase further after contractile activity (Figure 2). After contraction, adrenaline did not decrease glycogen synthase activation to a low level similar to that in non-contracted muscles. In contracted muscles, the fractional activity of glycogen synthase was almost as high when adrenaline was present as in insulin-stimulated non-contracted muscles incubated without adrenaline (Figure 2).

The percentage of glycogen phosphorylase in the *a* form was lower after contraction than during stimulation with insulin in both the presence and absence of adrenaline (Table 2). After contraction, only 38 % of phosphorylase was in the *a* form when adrenaline was present, whereas adrenaline increased the percentage of glycogen phosphorylase *a* to 80 % in non-contracted insulin-stimulated muscles. Adrenaline caused similar activations of glycogen phosphorylase in non-contracted muscles in the absence and the presence of insulin (results not shown).

The concentration of glucose 6-phosphate was increased during stimulation with adrenaline in rested insulin-stimulated muscles (Table 2). After contraction, adrenaline did not increase the glucose 6-phosphate concentration significantly. Furthermore, the concentration of glucose 6-phosphate during adrenaline

Table 2 Effects of adrenaline on glycogen phosphorylase activation and glucose 6-phosphate concentration in epitrochlearis muscles after contractile activity or during stimulation with insulin

Muscles were stimulated electrically for 30 min and then incubated for 10 min in the absence or presence of 1 μM adrenaline. Other muscles were kept rested for 30 min and then incubated with 10 m-i.u./ml insulin in the presence or absence of 1 μM adrenaline. Results are means $\pm$ S.E.M.; $n = 7$ for each group. The percentage of phosphorylase in the *a* form was 16.1% ($n = 5$) and the glucose 6-phosphate concentration was 0.38 mmol/kg dry weight ($n = 6$) in non-contracted muscles incubated without any hormones. *Significant difference between muscles incubated with and without adrenaline; †significant difference from muscles stimulated electrically.

	Phosphorylase in *a* form (%)		Glucose 6-phosphate (mmol/kg dry weight)	
Condition	Without adrenaline	With adrenaline	Without adrenaline	With adrenaline
Insulin	17.4 $\pm$ 1.0†	80.1 $\pm$ 3.4*†	0.33 $\pm$ 0.08	2.45 $\pm$ 0.29*†
Contraction	8.9 $\pm$ 1.1	37.9 $\pm$ 3.7*	0.73 $\pm$ 0.28	1.37 $\pm$ 0.35

stimulation was lower after contraction than during stimulation with insulin (Table 2).

DISCUSSION

Effect of insulin on glycogen synthesis after contraction

A new finding in the present study is that the rate of glycogen synthesis was constant until the glycogen store was fully replenished after exercise when a maximal stimulating concentration of insulin was present. In the absence of insulin, the rate of glycogen synthesis clearly shows the two phases as described *in vivo* after severe glycogen-depleting exercise [6,10,12], and only approx. 65% of the initial glycogen concentration was synthesized. It has been reported that most of the glycogen was synthesized at a fairly constant high rate when the insulin concentration was clamped at 100 μ-i.u./ml [20]; the amount of glycogen synthesized at a high rate therefore seems to depend on the insulin concentration.

The fact that the rate of glycogen synthesis remained unchanged for the 3 h after contraction when insulin was present, although contraction itself did not stimulate glycogen synthesis during the third hour, shows that the effect of insulin was increased during this period. The intracellular mechanism for increased insulin action is unknown but decreased glycogen concentration is believed to be an important determining factor [30,35,36]. The present study indicates that the mechanism for increased insulin action differs from that of contraction-stimulated glycogen synthesis. However, because the increased insulin action develops as the contraction-stimulated glycogen synthesis declines, a possible link between the two effects might exist.

Effect of adrenaline on glycogen synthesis

As expected on the basis of other studies, adrenaline decreased insulin-stimulated glycogen synthesis [22–25]. To our surprise, however, adrenaline did not inhibit glycogen synthesis after contraction. To the best of our knowledge this is the first study in which the effect of adrenaline on glycogen synthesis after contraction has been studied.

The reason for the failure of adrenaline to inhibit glycogen synthesis after contraction is not obvious. Contraction and insulin stimulate glucose uptake through different pathways [13,14], which raises the possibility of different actions of adrenaline. For glycogen synthesis, glucose has to be transported across the cell membrane, phosphorylated to glucose 6-phosphate and incorporated into glycogen. Adrenaline does not influence the transport of glucose across the cell membrane, either during stimulation with insulin or during contractile activity [19,37]. During stimulation with insulin, adrenaline is a powerful inhibitor of glucose phosphorylation and glycogen synthesis [19,24,37]. In contrast, we have found recently that adrenaline is a much less powerful inhibitor of glucose phosphorylation during contractile activity than during stimulation with insulin [19]. The present study extends this finding by showing that even glycogen synthesis occurs in the presence of adrenaline after contractile activity.

Glycogen synthase activity, which incorporates the glycosyl units into glycogen particles, is a rate-limiting step for glycogen synthesis. Glycogen synthase is regulated by phosphorylation and dephosphorylation by different kinases and phosphatases in a hierarchical manner [27,28,38,39]. In the present study, adrenaline reduced insulin-stimulated fractional activity of glycogen synthase and glycogen synthesis to very low levels in agreement with other studies [26,29].

Adrenaline caused similar decreases in the fractional activity of glycogen synthase after contraction and during stimulation with insulin. However, because the the fractional activity of glycogen synthase was much higher after contraction than during stimulation with insulin, it was almost as high when adrenaline was present after contraction as it was in insulin-stimulated muscles without adrenaline. It has previously been reported that the ability of adrenaline to inhibit the fractional activity of glycogen synthase to some extent is overcome by contraction [26,29]. The reason for the high activation of glycogen synthase after contraction even in the presence of adrenaline is not obvious. Glycogen concentration participates in the regulation of glycogen synthase activity [26], and it is possible that the low concentration of glycogen after contractile activity influences the effect of adrenaline. Glycogen synthase was, however, activated much more after contraction than during stimulation with insulin, suggesting different dephosphorylations in the two situations. Because glycogen synthase is phosphorylated in a hierarchical manner, it is possible that the effect of adrenaline differs in the two situations [27,40].

Adrenaline normally activates glycogen phosphorylase and stimulates glycogen breakdown in skeletal muscles [41,42]. The high activation of glycogen phosphorylase when adrenaline was present during stimulation with insulin is consistent with the low rate of glycogen synthesis that we observed. After contraction the activation of glycogen phosphorylase and the concentration of glucose 6-phosphate were decreased during adrenaline stimulation. Contractile activity has been reported to decrease the activation of glycogen phosphorylase in the absence of adrenaline [43,44] as well as in its presence [7,29,45,46]. The decreased β-adrenergic activation of glycogen phosphorylase after glycogen-

depleting contractions corroborates the inability to inhibit glycogen synthesis.

The glycogen synthesis observed in skeletal muscles after glycogen-depleting exercise in the presence of adrenaline might be an important physiological mechanism. Skeletal-muscle glycogen is an extremely important energy substrate in 'fight or flight' situations and therefore for survival. During prolonged stress and elevated concentration of adrenaline, it is important that glycogen can be replenished during repeated periods with muscular activity. The glycogen synthesis observed after glycogen-depleting exercise even in the presence of adrenaline shows that this is possible. Furthermore, because insulin-stimulated glycogen synthesis is inhibited, this mechanism also directs the glucose to the glycogen-depleted muscles, where glycogen synthesis is important for managing the next 'fight or flight' situation.

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