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Regulation of glycogen synthase activity and phosphorylation by exercise

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Glycogen synthase (GS) catalyses the rate-limiting step of UDP-glucose incorporation into glycogen. Exercise is a potent regulator of GS activity, leading to activation of GS immediately after exercise promoting glycogen repletion by mechanisms independent of insulin. The incorporation of UDP-glucose is energy demanding, and during intense exercise GS is deactivated, diminishing energy utilization but also increasing the potential for glycogen breakdown. An apparent activation of GS is observed during moderate exercise, which could be considered as a potential waste of energy, although the cellular capacity for glycogen breakdown is considerably higher than that for glycogen synthesis. The understanding of this complex regulation of GS activity in response to exercise is just at its beginning. In the present review potential mechanisms by which exercise regulates GS activity are described, factors that may promote GS activation and factors that may deactivate GS are discussed, pointing to the view that GS activity during exercise is the result of the relative strength of these opposing factors.

Exercise: Contraction: Skeletal muscle: Glycogen synthase: Phosphorylation

Glycogen is an essential metabolic substrate during exercise, even at fairly low work intensities. After a glycogen-depleting stimulus, e.g. exercise, the rise in plasma insulin induced by a carbohydrate-rich meal directs a substantial part of the glucose towards repletion of the glycogen stores. This process occurs by insulin-dependent and insulin-independent increases in both glucose uptake and glycogen synthesis. A key enzyme in glycogen synthesis is glycogen synthase (GS), which catalyses the incorporation of UDP-glucose into glycogen. GS activity is regulated allosterically by glucose-6-phosphate (G6P), as well as by reversible phosphorylation and dephosphorylation leading to inactivation and activation of GS respectively. Thus, activation of GS may take place through inactivation of kinases phosphorylating GS and by activation of phosphatases dephosphorylating GS. Several of these upstream enzymes have been identified, although their relative importance in the regulation of GS activity *in vivo* has not been fully clarified. In general, phosphorylation decreases GS activity by causing an increase in the K_m for the substrate UDP-glucose and an increase in the K_a for G6P (Roach & Larner, 1976; Roach *et al*. 1976). GS is subject to phosphorylation at nine or more sites. Two sites, sites 2 and 2a, corresponding to Ser^7 and Ser^{10} on GS from rabbit skeletal muscle, are located near the NH_2 terminus. At least seven sites (sites 3a–c, 4, 5, 1a–b) are located in the COOH-terminal 100 residues of GS. Phosphorylation of sites 2, 2a, 3a and 3b generally decreases the activity more than phosphorylation of the remaining sites that have minor or no effect on GS activity *in vitro* (for review and further references, see Roach, 2002). Notably, initial phosphorylation of site 5 by casein kinase 2 creates a recognition motif for GS kinase 3 (GSK3), which then sequentially phosphorylates sites 4, 3c, 3b and 3a. This phenomenon has been termed hierarchal phosphorylation (see Fig. 1), which has also been demonstrated for the NH_2-terminal sites of GS where modification of site 2 by protein kinase A (PKA), 5′AMP-activated protein kinase (AMPK) or other kinases is a prerequisite for phosphorylation of site 2a by casein kinase 1.

Abbreviations: AICAR, 5′-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside; AMPK, 5′AMP-activated protein kinase; CaMK, Ca^{2+}/calmodulin-dependent kinase; G6P, glucose-6-phosphate; G_M, glycogen-associated PP1 subunit; GS, glycogen synthase; GSK3, GS kinase 3; PhK, glycogen phosphorylase kinase; PKA, protein kinase A; PP1, protein phosphatase 1.

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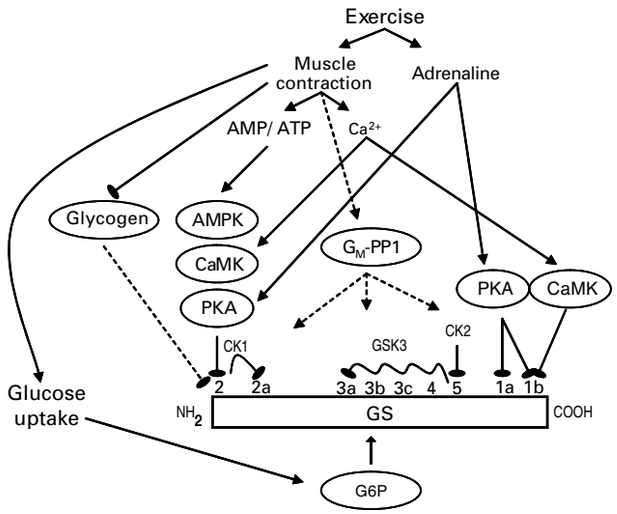

Fig. 1. Regulation of skeletal muscle glycogen synthase (GS) activity and phosphorylation by exercise. Exercise elicits an increase in blood adrenaline levels and muscle contraction leads to changes in intracellular nucleotide and calcium ion levels. Adrenaline activates cAMP-dependent protein kinase (AMPK)/ protein kinase A (PKA), which decreases GS activity by directly phosphorylating site 2 and indirectly phosphorylating site 3 by a mechanism as yet unknown (not indicated). The glycogen-associated protein phosphatase 1 (PP1) subunit G_M, has been shown to be essential in contraction-activation of GS, but the mechanism by which G_M is activated (if at all) by muscle contraction has not been identified. Furthermore, it is not known whether PP1 preferentially dephosphorylates specific sites on GS. Glycogen inhibits GS activity by causing phosphorylation of site 2 on GS, but the mechanism remains elusive. Furthermore, the calcium ion concentration increases in response to muscle contraction and this factor could lead to increased site 2 phosphorylation of GS mediated by calcium ion/calmodulin-dependent kinase (CaMK) or other calcium ion-dependent kinases. Glucose-6-phosphate (G6P) levels often increase during exercise as a result of an increase in glucose uptake and an increase in glycogen breakdown. This change in levels allosterically activates GS. ←, ⊢, Activating and inhibiting factors respectively. -----, Effects that are not completely understood. The GS enzyme is shown in a diagrammatic form with indications of the different phosphorylation sites and the primary kinases thought to act on these sites. Hierarchal phosphorylation is indicated on site 2a and the sites 3–4. Sites 2, 2a, 3a and 3b are the sites thought to be most important in the regulation of GS activity. GSK, GS kinase; CK1 and 2, casein kinases 1 and 2. For further detail and references, see p. 233.

Mechanisms by which exercise regulates glucose synthase activity

Early studies in rodent skeletal muscle have demonstrated that GS activity is increased in response to exercise (Danforth, 1965). However, in response to short-term high-intensity exercise GS activity has actually been observed to be decreased or unchanged during exercise and then rapidly increased after the cessation of exercise (Chasiotis *et al*. 1982; Jiao *et al*. 2001). From a narrow viewpoint, this finding suggests that the energy-consuming process of glycogenesis is diminished only when energy is greatly needed elsewhere, and also that this potential waste of energy is allowed during conditions of more moderate exercise. The hypothesis has been put forward that GS activity is influenced by both stimulatory and inhibitory factors during exercise and the consequent effect of exercise is a result of the relative strength of these opposing signals, which may vary according to the mode, intensity and duration of the exercise. This idea is supported by the observation in rodent skeletal muscle, using phospho-site-specific GS antibodies, that phosphorylation at the NH_2-terminal sites 2 and 2a and the far COOH-terminal sites 1a and 1b is markedly increased during contraction *in vitro* (leading to inactivation of GS), whereas phosphorylation on sites 3a and 3b decreases (leading to activation of GS; JN Nielsen, BF Hansen and JFP Wojtaszewski, unpublished results). There seems to be a consensus that exercise and insulin regulate GS activity differentially (Danforth, 1965; Aschenbach *et al*. 2001; Nielsen *et al*. 2001). This consensus is based on the observations that the regulation of GS activity by exercise is independent of the muscle insulin receptor (Wojtaszewski *et al*. 1999*a*) and phosphatidylinositol 3-kinase, whereas the effect of insulin is not (Marchand-Brustel *et al*. 1995; Wojtaszewski *et al*. 1999*b*). Notably, the stimulatory effects of exercise and insulin are not always additive (Wojtaszewski *et al*. 1999*a*), suggesting that the two stimuli, at least in part, affect GS in the same way. This suggestion is also supported by the observation that both insulin and muscle contraction decrease phosphorylation of GS at sites 3a and 3b (Parker *et al*. 1983; Hojlund *et al*. 2003; Sakamoto *et al*. 2003). The way in which exercise may signal the regulation of the activity and phosphorylation of GS will be discussed, with emphasis on some potential kinases (PKA, AMPK, GSK3), phosphatases (protein phosphatase 1; PP1) and other regulatory elements (G6P, glycogen).

Protein kinase A

As mentioned earlier, GS activity is not always increased, and in some studies even decreased, in response to exercise. Based on *in vitro* observations activation of several kinases could be responsible for the exercise-induced deactivation of GS *in vivo*. A decrease in phosphatase activity during exercise would also be able to decrease the activity of GS. Importantly, an inherent response to exercise is an increase in the plasma adrenaline concentration. The cellular action of adrenaline is thought to be mediated through an increase in cAMP, in turn activating PKA. Although sites 2, 1a and 1b, but not sites 3a and 3b, on GS are phosphorylated by PKA *in vitro*, phosphorylation of sites 3a and 3b is increased *in vivo* with adrenaline infusion (for review, see Roach, 2002). This finding indicates that PKA may exert its effect on GS by direct phosphorylation of sites 2, 1a and 1b and also by indirect mechanisms affecting phosphorylation of sites 3a and 3b (e.g. through PP1 deactivation, as discussed later). A role for adrenaline in exercise-induced inhibition of GS activity is supported by the following observations: (1) adrenaline infusion in resting human subjects induces a marked drop in skeletal muscle GS activity (Chasiotis *et al*. 1983*b*); (2) β-adrenergic blockade (propranolol infusion)

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abolishes the decrease in GS activity induced by short-term isometric or dynamic exercise (Chasiotis *et al*. 1983*a*); (3) in response to electrical stimulation of the sciatic nerve in one leg of the rat, eliciting contractions in one leg while keeping the contra-lateral leg rested, phosphorylation of sites 2, 1a and 1b increases in the rested muscle, pointing to the influence of a systemic factor induced by muscle contraction *in situ* (JN Nielsen, BF Hansen and JFP Wojtaszewski, unpublished results).

5′AMP-activated protein kinase

Based on the view that AMPK is a sensor of cellular energy charge, turning off ATP-consuming anabolic pathways, it could be proposed that AMPK activation may work to decrease the activity of GS during exercise, slowing down the energy-consuming incorporation of UDP-glucose into glycogen. This mechanism was initially indicated by the observations that site 2 on GS is a substrate of AMPK *in vitro* (Carling & Hardie, 1989) and that GS co-immunoprecipitates with AMPK in skeletal muscle (Chen *et al*. 1999). More recently, several observations have been made that strongly support the notion that AMPK indeed phosphorylates GS at site 2 *in vivo*. Pharmacological activation of AMPK by 5′-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) in perfused muscles leads to a decrease in GS activity (Wojtaszewski *et al*. 2002). The change in AMPK activity associated with the α2 AMPK catalytic subunit in response to AICAR is negatively related to the GS activity, and AICAR induces an upward shift in the gel mobility of GS, which is prevented by previous phosphatase treatment (Wojtaszewski *et al*. 2002). Furthermore, GS obtained from AICAR-perfused (and incubated) rat muscles has increased site 2 phosphorylation, in accordance with the original *in vitro* findings (SB Jørgensen, JN Nielsen, DG Hardie, BF Hansen, EA Richter and JFP Wojtaszewski, unpublished results). Finally, in muscles from α2 AMPK knock-out mice AICAR does not induce GS deactivation (SB Jørgensen, B Viollet, DG Hardie, BF Hansen, S Vaulont, EA Richter and JFP Wojtaszewski, unpublished results). Thus, AMPK seems to be a GS site 2 kinase. Whether AMPK activation is important for the observed GS site 2 phosphorylation during exercise is currently under investigation.

Glycogen synthase kinase 3

Apparently, several potential mechanisms exist by which exercise could inhibit GS activity. There still remains the question of how exercise signals to the activation of GS. Although phosphorylation of GS at sites 2 and 2a is markedly increased at the onset of muscle contraction, phosphorylation of sites 3a and 3b is progressively decreased when muscle contraction is sustained for more than a few minutes, leading to an increase in GS activity (JN Nielsen, BF Hansen and JFP Wojtaszewski, unpublished results). This finding raises the possibility that the activity of a kinase with specificity for sites 3a and 3b would decrease in response to exercise. GSK3 consecutively phosphorylates sites 4, 3a, 3b and 3c on GS when site 5 is phosphorylated by casein kinase 2, as discussed earlier. In rodent skeletal muscle GSK3α and β activity and phosphorylation are decreased and increased respectively in response to treadmill exercise, *in situ* contraction and in isolated muscle contracted *in vitro* in the absence of systemic factors (Markuns *et al*. 1999; Sakamoto *et al*. 2002). Thus, GSK3 could be a potential regulator of GS activity during exercise. However, recent data suggest that although GSK3 activity is decreased with muscle contraction, this decrease does not correlate with the increase in GS activity and phosphorylation of sites 3a, 3b, 3c and 4 on GS (Sakamoto *et al*. 2003). It has been demonstrated that both contraction and mechanical stretch of isolated rat muscle increase the phosphorylation of GSK3 (leading to inactivation of GSK3), but only contraction decreases the phosphorylation of GS. Furthermore, the phosphatidylinositol 3-kinase inhibitor wortmannin inhibits the contraction and stretch-induced increase in GSK3 phosphorylation but has no effect on phosphorylation of GS at sites 3a, 3b, 3c and 4 during these stimuli. The absence of a role for GSK3 in exercise-induced activation of GS is also supported by the observation that GSK3 is not deactivated in human skeletal muscle in response to exercise (Wojtaszewski *et al*. 2001). Interestingly, recombinant dual-specificity tyrosine phosphorylated and regulated kinases have recently been shown to phosphorylate muscle GS at site 3 (Skurat & Dietrich, 2003). Whether dual-specificity tyrosine phosphorylated and regulated kinase activity is influenced by exercise in skeletal muscle is presently unknown.

Protein phosphatase 1

It would be tempting to ascribe the decreased phosphorylation of GS at sites 3a and 3b during exercise to the notion that phosphatase activity directed against these sites is increased with exercise. However, an increase in PP1 activity in response to exercise has never been reported. The primary phosphatase acting on GS is the multisubstrate phosphatase PP1. The regulation of PP1 is complex and has recently been the topic of comprehensive reviews (see Brady & Saltiel, 2001). In relation to the regulation of GS activity it should be mentioned that GS, glycogen phosphorylase and phosphorylase kinase are all to a variable extent bound directly to glycogen (Cohen, 1978), but several lines of evidence indicate that these enzymes, as well as PP1, are also bound to PP1-targeting subunits that themselves bind to glycogen and act as molecular scaffolds, thereby having a major impact on the regulation of glycogen metabolism. One such targeting subunit that has received considerable attention is the glycogen-associated PP1 subunit G_M (also termed glycogen-binding regulatory subunit or RGl). Binding of G_M to PP1 enhances dephosphorylation of GS, whereas glycogenolytic agents such as adrenaline cause serine phosphorylation resulting in dissociation and thus inactivation of the G_M–PP1 complex (Hiraga & Cohen, 1986). An essential role for G_M in exercise regulation of GS has recently been indicated by a study in which the G_M gene was knocked out in mice. After either exercise or *in situ* muscle contraction GS activity is increased in the wild type but unchanged in the G_M knock-out mice (Aschenbach *et al*.

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2001). This finding suggests that G_M is essential for the activation of GS after exercise, perhaps through the association with PP1.

Glycogen

GS activity is negatively feedback-regulated by glycogen (Danforth, 1965; Nielsen *et al*. 2001), but the mechanism for this effect is largely unknown. The hypothesis has been put forward that glycogen breakdown is a factor involved in GS activation during exercise. It has been observed that the lower GS activity in muscle high in glycogen compared with GS from muscle low in glycogen is associated with elevated phosphorylation at sites 2 and 2a (JN Nielsen and JFP Wojtaszewski, unpublished results), pointing to the notion that glycogen modulates the ability of kinases and phosphatases to act on GS. Previously, it has been observed that protein phosphatase treatment of GS from muscle high in glycogen does not restore GS activity to the same extent as GS obtained from muscle low in glycogen (Wojtaszewski *et al*. 2002). This finding suggests that events unrelated to phosphorylation also regulate GS activity in response to changes in glycogen content. A potential mechanism could be O-linked N-acetylglucosamine modification, which is a reversible post-translational modification that regulates enzyme activity. Recently, it has been shown in 3T3-adipocytes that exposure to glucosamine induces O-linked N-acetylglucosamine modification of GS, which apparently inactivates the enzyme (Parker *et al*. 2003). If GS is modified by O-linked N-acetylglucosamine in muscle high in glycogen, this may explain the relative resistance to phosphatase treatment (Wojtaszewski *et al*. 2002), because it has been shown that purified GS becomes fully de-phosphorylated in response to phosphatase treatment only after removal of O-linked N-acetylglucosamine (Parker *et al*. 2003). Future studies should address the possibility that the O-linked N-acetylglucosamine modification of GS is changed in response to exercise and/or whether the activity of upstream GS kinases or phosphatases are glycogen sensitive.

Glucose-6-phosphate

As mentioned earlier, G6P is a direct and indirect stimulator of the activity of GS. Although the direct allosteric effect of G6P *in vivo* is not detected when GS activity is measured *in vitro*, it should be considered that G6P may play an important role in regulating GS activity during exercise. Muscle glucose uptake is increased by exercise and this increase often leads to increases in the concentration of G6P levels. Thus, the allosteric activation of GS during exercise could be quite important, as suggested by Bloch *et al*. (1994). GS activity is also increased in contracting skeletal muscles that have been stimulated to contract *in vitro* (incubated) or *in situ* (perfused) in the absence of glucose, keeping G6P levels very low or even unchanged (W Derave, personal communication). This finding suggests that the increase in GS activity induced by exercise is not dependent on an increase in skeletal muscle G6P levels, which is also supported by observations in human muscle cell cultures (Montell *et al*. 1999).

Summary

Exercise regulation of GS is characterized by great complexity, because GS is a substrate of multiple upstream enzymes acting on several phosphorylation sites of GS. Furthermore, exercise activates both stimulatory and inhibitory regulators of GS, and the strength of these regulators may vary over time. Although the field is far from being fully elucidated, the following hypothesis has been put forward, based on the literature and the authors' unpublished data. In brief, during the initial stage of exercise, GS activity is inhibited through the increased phosphorylation of site 2 (rendering site 2a as a substrate for the constitutively-active casein kinase 1) mediated by adrenaline-induced PKA activation, and an increase in AMPK activity. Later in exercise, although the inhibitory signals are undiminished, GS activity is increased through an increase in PP1-mediated dephosphorylation of GS at sites 3a and 3b. Nevertheless, major questions remain unanswered. What is the precise mechanism by which exercise signals to PP1, and do PP1 act site-specifically on GS *in vivo*? What is the role of sites 1a and 1b in the regulation of GS, since phosphorylation of these sites seems to have minor effects on enzyme activity? Finally, it should be considered that O-linked glucosylation might have a role in exercise-regulation of GS activity. Although omitted from the present review, the authors are aware that Ca-sensitive kinases (glycogen phosphorylase kinase, protein kinase C, Ca^{2+}/calmodulin-dependent protein kinase) are also potential GS kinases, which may have additional regulatory roles during exercise, and these kinases are also obvious targets for further research in this area.

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