Inhibitor-1 Is Not Required for the Activation of Glycogen Synthase by Insulin in Skeletal Muscle*

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Glycogen synthase is an excellent in vitro substrate for protein phosphatase-1 (PP1), which is potently inhibited by the phosphorylated forms of DARPP-32 (dopamine- and cAMP-regulated phosphoprotein, $M_r = 32,000$) and Inhibitor-1. To test the hypothesis that the activation of glycogen synthase by insulin is due to a decrease in the inhibition of PP1 by the phosphatase inhibitors, we have investigated the effects of insulin on glycogen synthase in skeletal muscles from wild-type mice and mice lacking Inhibitor-1 and DARPP-32 as a result of targeted disruption of the genes encoding the two proteins. Insulin increased glycogen synthase activity and the synthesis of glycogen to the same extent in wild-type and knock out mice, indicating that neither Inhibitor-1 nor DARPP-32 is required for the full stimulatory effects of insulin on glycogen synthase and glycogen synthesis in skeletal muscle.

Insulin lowers blood glucose by inhibiting hepatic glucose output and by increasing the uptake of glucose by various target tissues. In lean individuals, skeletal muscle represents the largest mass of insulin-sensitive tissue, and the majority of the glucose taken up in response to insulin following a meal is converted to muscle glycogen (1, 2). Thus, the stimulation of glycogen deposition in skeletal muscle is of particular importance in the maintenance of glucose homeostasis. Insulin promotes glycogen synthesis both by increasing glucose entry into muscle fibers and by increasing conversion of the intracellular glucose into glycogen. This response involves activation of glucose transport and glycogen synthase.

Glycogen synthase catalyzes the reaction in which glucose from UDP-glucose is incorporated into glycogen. The enzyme is subject to complex control by both allosteric and covalent mechanisms (3). Skeletal muscle glycogen synthase may be phosphorylated in 10 or more sites, which are clustered in regions near the NH$_2$ and COOH termini (4). Insulin activates the enzyme by promoting dephosphorylation of sites in both regions (5, 6). In general, phosphorylation decreases glycogen synthase activity (3). However, the allosteric effector, glucose-6-phosphate (G6P), is able to activate fully even highly phosphorylated forms of the enzyme. When provided with sufficient substrate, nonphosphorylated glycogen synthase is fully active even in the absence of G6P (7). Consequently, the activation of glycogen synthase may be monitored by the increase in the activity ratio (G6P/G6P).

Despite several decades of investigation, the mechanism by which insulin activates glycogen synthase is still not clear. The multisite dephosphorylation of glycogen synthase is suggestive of phosphatase activation. Indeed, insulin has been shown to increase the activity of PP1 (8), an enzyme that is capable of dephosphorylating both the NH$_2$ and COOH-terminal sites in glycogen synthase. The action of PP1 in cells is dependent on its subcellular localization, which is determined by different regulatory/targeting subunits. R$_{65}$ (also known as the G subunit or G$_M$) and PTG are two glycogen-binding subunits that target PP1 to glycogen particles in skeletal muscle (9, 10). These glycogen-bound forms of PP1 are believed to be responsible for the dephosphorylation of glycogen synthase, which is also bound to glycogen. PP1 activity is controlled by two related heat-stable proteins, Inhibitor-1 (11, 12) and DARPP-32 (13). Inhibitor-1 (I-1) is expressed in a wide variety of tissues, including skeletal muscle (12). DARPP-32 is expressed in certain neurons (14), kidney, and adipocytes (15). The nonphosphorylated forms of I-1 and DARPP-32 are essentially devoid of PP1 inhibitory activity, but both proteins become potent inhibitors of PP1 after phosphorylation by cAMP-dependent protein kinase (13).

It was recently proposed that the activation of glycogen synthase by insulin in 3T3-L1 adipocytes is mediated by activation of PP1 via a mechanism involving decreased susceptibility of the PTG-bound form of the phosphatase to inhibition by DARPP-32 (16, 17). If this mechanism were to apply to skeletal muscle, the most important site of insulin-stimulated glycogen deposition, then the effect of insulin would presumably depend on I-1, as skeletal muscle does not express DARPP-32 (15). To investigate this possibility, we have compared the effects of insulin on glycogen synthase and glycogen synthesis in muscles from wild-type mice and mice lacking I-1 and DARPP-32 as a result of targeted-disruption of the genes encoding the two proteins.

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1 The abbreviations used are: G6P, glucose 6-phosphate; DARPP-32, dopamine- and cAMP-regulated phosphoprotein, $M_r = 32,000$; EDL, extensor digitorum longus; G1P, glucose 1-phosphate; MAP, mitogen-activated protein; PP1, type 1 protein phosphatase; PP1G, a form of PP1 containing a catalytic subunit bound to R$_{65}$; PTG, a PP1-targeting subunit; R$_{G1L}$, muscle glycogen-binding regulatory subunit of PP1G.
EXPERIMENTAL PROCEDURES

Mouse Lines—Mice lacking I-1 were generated by targeted disruption of the I-1 gene. The preparation of these animals and additional phenotypic characterization will be described in detail elsewhere. Briefly, the I-1 gene was disrupted in the E14 embryonic stem cell line (18) by using a targeting vector containing 4.5 kilobases (5′) and 5.5 kilobases (3′) of I-1 locus genomic DNA flanking a neomycin resistance gene (PGK-neo). Homologous recombination at the embryonic locus resulted in replacement of a 400-bp genomic fragment with PGK-neo. The deleted genomic fragment contains the I-1 exon encoding the initiation of translation. Correctly targeted clones were identified by the shift of a genomic restriction fragment from 10.1 to 11.5 kilobases, as determined by Southern (DNA) blot analysis. After C57BL/6J blastocyst transfer and embryo transfer, chimoric offspring were crossed to C57BL/6J females, and those mice carrying the mutation were further backcrossed to C57BL/6J for five generations. Male I-1 knockout mice and wild-type littermates 5–8 months of age were selected from the offspring of heterozygous breeding pairs. To generate mice lacking both I-1 and DARPP-32, homozygous I-1 knockout mice were bred with homozygous DARPP-32 knockout mice (19). The F-1 offspring were intercrossed to generate wild-type and double knockout lines (20/9Ia-C57BL/6J, backcrossed to C57BL/6J for five generations). Male mice from these lines were age-matched and studied at 4–8 months. For all mice used, genotype was determined by Southern blot or polymerase chain reaction analysis of tail DNA. To confirm the presence/absence of the protein(s), skeletal muscle extracts were subjected to SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes, which were then probed with polyclonal antibodies to I-1 (20) and glycogen synthase (21), or monoclonal ascites to DARPP-32 (22).

Incubation of Muscles in Vitro—Media used for muscle incubations were continuously gassed by bubbling with a 19:1 mixture of O2:CO2. Hemidiaphragm and extensor digitorum (EDL) muscles were incubated at 30 °C in Dulbecco’s modified Eagle’s medium (30 ml/muscle) for 30 min to remove endogenous hormones. The muscles were then incubated without or with either 250 milliunits/ml insulin (Humulin, Eli Lilly Co.) or 10 μM epinephrine at 37 °C in Krebs-Henseleit buffer (118 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl2, 1.2 mM MgSO4, 20 mM NaHCO3) containing 5 mM glucose. To terminate the incubations, the muscles were blotted on tissue paper and immediately frozen in liquid nitrogen. The frozen tissues were manually ground with a porcelain mortar and pestle that had been chilled in liquid nitrogen.

Measurements of Glycogen Synthase Activities—Samples (30 μl) were added to solutions (60 μl) containing 10 mM rabbit liver glycogen, 20 mM EDTA, 25 mM KF, 10 mM UDP-[1-14C]glucose (100,000 cpm, Amersham Pharmacia Biotech), 50 mM Tris-HCl (pH 7.8 at 30 °C) and incubated without or with 10 mM G6P at 30 °C for 20 min. The glycogen synthase activity ratio was determined by dividing the activity measured without added G6P by the activity measured in the presence of 10 mM G6P (total activity). Phosphorylase activity was measured in the direction of glycogen synthase from [U-14C]G1P by using the method of Gilboe et al. (24). Samples (30 μl) of extracts were added to solutions (60 μl) containing 10 mg/ml rabbit liver glycogen, 20 mM KF, 100 mM [U-14C]G1P (50,000 cpm, NEN Life Science Products), and incubated without or with 5 mM 5′-AMP at 30 °C for 20 min. The activity ratio was determined by dividing the activity measured without 5′-AMP by the activity measured in the presence of 5′-AMP.

Measurements of [U-14C]Glucose Incorporation into Glycogen—The amount of glucose incorporated into glycogen was measured as described previously (25). Following the incubation to remove endogenous hormones, muscles were incubated in medium containing 5 mM [U-14C]glucose (ICN, Irvine, CA). Samples of muscles were weighed, then dissolved by heating for 45 min at 100 °C in 30% KOH (1 ml/100 mg of tissue). Ethanol was added to a final concentration of 70%, and the glycogen was allowed to precipitate at −20 °C. After 8 h, the samples were centrifuged at 2000 × g for 20 min to pellet the glycogen. The glycogen pellets were washed four times with 66% ethanol before the amount of 14C-labeled glycogen was determined by liquid scintillation counting.

RESULTS AND DISCUSSION

FIG. 1. Immunoblot analysis of diaphragm muscle from wild-type and Inhibitor-1 knockout mice incubated without (+) or with (+) 250 milliunits/ml insulin for 20 min at 37 °C. Muscle extracts (30 μg) were separated by SDS-polyacrylamide gel electrophoresis. The proteins were transferred to polyvinylidene difluoride membranes and probed with anti-I-1, anti-DARPP-32, or anti-glycogen synthase antibodies, as indicated. Rat brain extracts (25 μg) were included in the DARPP-32 blot as a control (BE). The positions of the standard marker proteins, phosphorylase B (97 kDa), bovine serum albumin (66 kDa), and carbonic anhydrase (29 kDa) are indicated on the right.

FIG. 2. Effect of insulin on glycogen synthase activity in Inhibitor-1 knockout mice. Glycogen synthase activity was measured in hemidiaphragms from wild-type and I-1 knockout mice treated without (CONTROL) or with (INSULIN) 250 milliunits/ml insulin for 20 min at 37 °C. Glycogen synthase activity was measured in the presence and absence of G6P. Total activities (+G6P) were as follows (in μmol/min/g protein): wild-type littermate 89.95 ± 21.03, plus insulin 83.0 ± 13.2; Inhibitor-1 knockout 64.57 ± 12.03, plus insulin 76.46 ± 21.62. The results are mean values ± S.E. for muscles obtained from five experiments performed on different days (*, p < 0.05, control versus insulin-stimulated).

Activation of Glycogen Synthase in Skeletal Muscle

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versus insulin-stimulated).

Type littermate mice were incubated at 37 °C for 30 min without (control) or with (INSULIN) 20 milliunits/ml insulin in medium containing [14C]glucose. The amount of [14C]glycogen synthesized (in nmol/min/g wet weight) was determined by liquid scintillation counting. The results are mean values ± S.E. from five experiments performed on different days (*, p < 0.05, control versus insulin-stimulated).

The stimulation of glycogen synthesis by insulin involves multiple steps. To determine whether I-1 was essential for the overall process of glycogen synthesis, we assessed the rates of [U-14C]glucose incorporation into glycogen in diaphragm muscles from wild-type and I-1 knockout mice (Fig. 3). Basal rates of 14C-labeled glycogen synthesis were not significantly different in muscles from wild-type and I-1 knockout animals. Insulin increased 14C-labeled glycogen accumulation by approximately 9-fold in muscles from wild-type mice. Insulin was equally effective in increasing 14C-labeled glycogen synthesis in muscles from the I-1 knockout animals. Thus, I-1 is not an essential component of the signal transduction pathway leading to the stimulation of glycogen synthesis.

Regulation of Glycogen Synthase in I-1/DARPP-32 Knockout Mice—A potential problem in interpreting results from knockout animals is that the absence of a gene product during development may result in compensatory expression of another functionally related protein. For this reason, we considered the possibility that expression of DARPP-32 might be induced in the I-1 knockout animals. This did not appear to be the case, as
DARPP-32 was not detected in muscles from the I-1 knockout animals (Fig. 1, middle blot). Nevertheless, to be certain that the activation of glycogen synthase did not depend on either I-1 or DARPP-32, experiments were performed using muscles from animals that lacked both I-1 and DARPP-32. The activation of glycogen synthase in muscles from the double knockout animals was indistinguishable from that observed in muscles from the wild-type control animals (Fig. 4). The effect of insulin on incorporation of glucose into glycogen was assessed in EDL muscles from the same animals (Fig. 5). Basal rates of [U-14C]glucose into glycogen were almost identical in muscles from wild-type and double knockout mice. Insulin increased [U-14C]glucose into glycogen by approximately 2-fold in muscles from both groups of animals. The stimulation of [14C]glycogen synthesis by insulin was lower in the EDL muscles than in diaphragm (Fig. 3). This difference is most likely due to the different fiber type composition of the two muscles. Diaphragm is composed primarily of oxidative fiber types, which are more responsive to insulin than fast-glycolytic fibers, which comprise approximately half of the fibers in the EDL (26).

The possibility that insulin might control glycogen metabolism by decreasing the phosphorylation of I-1 in skeletal muscle has been investigated previously. Khatra et al. (27) observed no effect of insulin on I-1 phosphorylation in perfused rat hindlimb. In contrast, Foulkes et al. (28) reported that insulin decreased the phosphorylation of I-1 in hindlimb muscles in a perfused hemi-limb model. Later, it was found that I-1 phosphorylation was elevated in the hemi-limb model due to circulating epinephrine and that the effect of insulin to decrease I-1 phosphorylation was dependent on the presence of the β-adrenergic agonist (29). Unfortunately, we were unable to use the I-1 knockout mice to investigate the role of I-1 in the inactivation of glycogen synthase in response to epinephrine because epinephrine was without effect on the glycogen synthase activity in either the wild-type or I-1 knockout muscles. Epinephrine did activate phosphorylase and the response was essentially identical in muscles from the wild-type and I-1 knockout mice (Fig. 6). It is not clear why glycogen synthase was not inactivated by epinephrine in the mouse muscles. Nevertheless, it is well established that insulin activates glycogen synthase in isolated muscles incubated without epinephrine (30, 31), and that the effect of insulin is not inhibited by the β-adrenergic receptor antagonist, propranolol (29, 32). Therefore, previous findings indicate that the direct activation of glycogen synthase by insulin in skeletal muscle does not depend on decreased phosphorylation of I-1.

The concept that targeting subunits might mediate insulin action on glycogen synthase arose from investigations of PP1ζ, a form of PP1 that is targeted to glycogen by RGL (33). In vitro studies demonstrated that phosphorylation of RGL in site 1 increased glycogen synthase phosphatase activity (34). Subsequently, injecting rabbits with insulin was reported to increase site 1 phosphorylation (35). Identification of an insulin-activated kinase that phosphorylated this site as p90RSK (36) apparently linked the activation of glycogen synthase to the MAP kinase signaling pathway, as p90RSK was known to be activated by MAP kinase. However, findings with activators and inhibitors of MAP kinase and p90RSK indicated that activation of these two kinases were neither necessary nor sufficient for the activation of glycogen synthase (21, 37–40). The recent finding that insulin is able to activate glycogen synthase in muscles of mice lacking RGL provides yet another reason to question the role of PP1ζ in the activation of glycogen synthase by insulin (41). Thus, the PTG-bound form of PP1 is becoming a more attractive candidate for a target of insulin action. Although our results would be consistent with a role of this form of the phosphatase in the activation of glycogen synthase by insulin, our findings demonstrate that neither I-1 nor DARPP-32 is required for the full stimulatory effects of insulin on glycogen synthase and glycogen synthase in skeletal muscle. An important implication is that the model proposed for the activation of glycogen synthase in 3T3-L1 adipocytes involving decreased inhibition of PTG-bound form of PP1 by DARPP-32 (42) cannot explain the activation of glycogen synthase in skeletal muscle, the most important site of insulin-stimulated glycogen deposition.

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