THE HORMONAL CONTROL OF GLYCOGEN METABOLISM: DEPHOSPHORYLATION OF PROTEIN PHOSPHATASE INHIBITOR-1 IN VIVO IN RESPONSE TO INSULIN

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1. Introduction

Over the past few years evidence has accumulated that protein phosphatase-1 is of central importance in the regulation of glycogen metabolism in mammalian skeletal muscle. This enzyme catalyses the dephosphorylation of glycogen phosphorylase, phosphorylase kinase and glycogen synthase, and therefore carries out the reactions which inhibit glycogenolysis or activate glycogen synthesis (reviewed [1,2]).

Protein phosphatase-1 has been shown to be inhibited in vitro by two heat-stable proteins termed inhibitor-1 and inhibitor-2, one of which (inhibitor-1) is only an inhibitor after it has been phosphorylated by cyclic AMP-dependent protein kinase [3,4].

Inhibitor-1 and inhibitor-2 have been purified to homogeneity in this laboratory [5,6] and characterized extensively [1,7–9]. Inhibitor-1 is phosphorylated by cyclic AMP-dependent protein kinase in vitro at a similar rate to phosphorylase kinase and glycogen synthase [7]. It inhibits the phosphorylase phosphatase, phosphorylase kinase phosphatase and glycogen synthase phosphatase activities of protein phosphatase-1 in a reversible non-competitive manner, and half-maximal inhibition is observed at 3–4 nM [8]. The concentration of inhibitor-1 in rabbit skeletal muscle is 1.8 μM [6], which is higher than the concentration of protein phosphatase-1 [1].

The properties of inhibitor-1 determined in vitro are consistent with the idea that this protein is important in the regulation of protein phosphatase-1 in vivo. It has been demonstrated that inhibitor-1 is phosphorylated in rabbit skeletal muscle in vivo, and that its degree of phosphorylation increases markedly in response to adrenaline [10]. Here we demonstrate that insulin decreases the phosphorylation of inhibitor-1 in rat skeletal muscle. The implications of this finding for the control of glycogen metabolism and mechanism of action of insulin are discussed.

2. Methods

2.1. *Rat hemicorpus perfusions*

Male rats of the Sprague-Dawley strain (Charles River Breeding Labs.) were maintained on Purina Lab. chow and water ad libitum. Rats of 200–300 g were heparinized (10 mg/kg) and anaesthetized with Nembutal (50 mg/kg) in preparation for hemicorpus perfusion [11]. The perfusion medium consisted of Krebs-Henseleit bicarbonate buffer containing 3% (w/v) bovine serum albumin (Miles, fraction V) and 30% (v/v) washed bovine erythrocytes. The perfusate was gassed with humidified O_2–CO_2 (95 : 5) and maintained at 37°C. Following an initial 50 ml washout, 100 ml perfusate were recirculated at 14 ml/min flow-rate. After 60 min perfusion, the medium was supplemented with isoproterenol (1×10^{-7} M), insulin (3.3×10^{-9} M), isoproterenol plus insulin, or saline (control). Tissue samples were obtained 25 min after the additions by rapidly freezing a portion of hind-limb muscle (3–4 g) in clamps cooled in liquid nitrogen. Frozen muscle was prepared for analysis by pulverisation in a percussion instrument at liquid nitrogen temperature and stored at −70°C.

2.2. *State of phosphorylation of inhibitor-1*

The frozen muscle powders were extracted with 4 vol. 1.5% trichloroacetic acid–4 mM EDTA and centrifuged for 45 min at 6000 × *g*. The supernatants

were decanted and treated with 100% trichloroacetic acid to give 15% (w/v) final conc. After standing at 4°C for 3 h, the suspensions were centrifuged at 20 000 × *g* for 10 min. The precipitates were resuspended in 0.5 M Tris–HCl (pH 8.0) titrated to pH 7.4 with ammonium hydroxide, and the volume was adjusted to 2/7th of the original weight of the tissue. The solutions were lyophilized and stored at −20°C until analysis.

The lyophilized powders were taken up in water, dialysed against 5 mM Tris–HCl (pH 7.0) and heated for 5 min at 100°C. The suspensions were cooled to 0°C, centrifuged for 2 min at 10 000 × *g* and the supernatants lyophilized. The freeze dried material was redissolved in 0.1 ml water and the state of phosphorylation of inhibitor-1 was then measured as in [10].

3. Results

3.1. *Phosphorylation of inhibitor-1 in vivo*

It has been shown in [10] that inhibitor-1 is not precipitated or inactivated when rabbit skeletal muscle is homogenized with 1.5% trichloroacetic acid. The same result was obtained here using rat skeletal muscle. Extraction of the tissue in this manner is therefore a very effective method of destroying the interconverting enzymes which would otherwise alter the state of phosphorylation of inhibitor-1.

The percentage phosphorylation of inhibitor-1 was determined by making the following three measurements:

(i) Inhibitor activity was measured without any pretreatment;
(ii) The total inhibitor activity was measured after maximal phosphorylation by the catalytic subunit of cyclic AMP-dependent protein kinase;
(iii) The inhibitor activity was measured after maximal dephosphorylation by protein phosphatase-1.

As described for rabbit skeletal muscle, the inhibitor activity was completely destroyed by incubation with protein phosphatase, since inhibitor-2 is eliminated during the initial centrifugation of the 1.5% trichloroacetic acid homogenate [10].

The results of the experiments carried out with the perfused hind-limb system are summarized in table 1. In the absence of both insulin and adrenaline, the phosphorylation of inhibitor-1 averaged 52 ± 12%. The degree of phosphorylation was reduced to 28 ± 11% when insulin was included in the perfusate. The β-adrenergic agonist isoproterenol raised the phosphorylation to 73 ± 11%, and this hormone effectively blocked the reduction in phosphorylation that occurred in response to insulin.

4. Discussion

These results demonstrated that physiological concentrations of insulin lower the state of phosphorylation and the activity of inhibitor-1 in rat skeletal muscle in vivo. Substantial decreases in the activity of inhibitor-1 were recorded in every experiment (table 1). The results suggest that the dephosphorylation of

Table 1
Influence of insulin and isoproterenol on the state of phosphorylation of inhibitor-1 in rat skeletal muscle

Expt	Control	Phosphorylation of inhibitor-1 (%)		
		Insulin	Insulin + isoproterenol	Isoproterenol
1	56	17	55	73
2	66	33	56	71
3	60	20	64	89
4	–	29	–	80
5	65	49	71	72
6	43	36	54	55
7	40	26	–	–
8	36	17	–	–
Av. (±SD)	52 ± 12	28 ± 11	60 ± 7	73 ± 11

Experiments were carried out as in section 2

inhibitor-1 by insulin is likely to be important in the stimulation of glycogen synthesis by this hormone.

We reported in [10] that the degree of phosphorylation of inhibitor-1 in normal fed rabbits was 31 ± 7%, and that it increased to 70 ± 12% following an intravenous administration of adrenaline. Since the levels of circulating insulin would be expected to be moderately high in the normal fed state, the values of 28 ± 11% and 60 ± 7% for the insulin and insulin + isoproterenol experiments obtained in the present work with rat skeletal muscle are in good agreement with that data.

Four interconvertible proteins which are regulated by cystosolic protein kinases and protein phosphatases have been shown to become dephosphorylated in vivo in response to insulin, namely inhibitor-1 and glycogen synthase [12] in skeletal muscle, and hydroxymethylglutaryl-CoA (HMG-CoA) reductase and HMG-CoA reductase kinase in hepatocytes [13]. These observations suggest that a protein kinase becomes inhibited or a protein phosphatase becomes activated in response to insulin, and raises the possibility that many of the intracellular actions of this hormone take place through the same general mechanism.

Glycogen synthase was the first enzyme which was shown to become dephosphorylated in response to insulin [12] and consequently this system has been the most intensively studied. However the regulation of this enzyme has turned out to be unexpectedly complex, since it can be phosphorylated and inactivated in vitro by at least three protein kinases (cyclic AMP-dependent protein kinase, phosphorylase kinase and glycogen synthase kinase-3) which phosphorylate 6 different serine residues [2,14,15]. These findings have raised the question of which glycogen synthase kinase might be under the control of insulin. The recent finding that insulin activates glycogen synthase in the soleus muscle of ICR/IAn mice which lack phosphorylase kinase [16], appears to eliminate this enzyme as the primary target for insulin action. Furthermore as phosphorylase kinase is completely dependent on Ca^{2+} for activity and is the only Ca^{2+}-dependent protein involved in glycogen metabolism [1], the possibility that insulin stimulates glycogen synthesis by decreasing the $[Ca^{2+}]$ in the muscle cytoplasm appears to be excluded.

The present finding that inhibitor-1 is dephosphorylated in response to insulin, simplifies the situation considerably and narrows down the search for the target protein for insulin action. Inhibitor-1 is not phosphorylated by either phosphorylase kinase or glycogen synthase kinase-3 [14] and no enzyme other than cyclic AMP-dependent protein kinase has been shown to phosphorylate this protein in vitro [5,8]. Insulin must therefore either inactivate cyclic AMP-dependent protein kinase or activate a protein phosphatase which dephosphorylates inhibitor-1 in vivo.

Incubation of rat hemidiaphragms with insulin does not cause any detectable decrease in the concentration of cyclic AMP under conditions where glycogen synthase is activated [17,18]. Similarly, no alteration in the concentration of cyclic AMP is observed in the rat hemicorpus system used here, when insulin is included in the perfusate (L.S.J., unpublished). If insulin inhibits the cyclic AMP-dependent protein kinase, it must therefore do so by a mechanism unrelated to decreases in the concentration of cyclic AMP.

A remarkable property of the protein phosphatase-1–inhibitor-1 system is that inhibitor-1 does not inhibit its own dephosphorylation, even at concentrations 500-fold higher than those which inhibit the dephosphorylation of other substrates. Consequently, inhibitor-1 is dephosphorylated by protein phosphatase-1 with kinetic constants which are very similar to those observed for other substrates of the enzyme [1,8]. This property means that there is no need to invoke the existence of a separate phosphatase for the dephosphorylation of inhibitor-1, and the state of phosphorylation of inhibitor-1 may simply be a reflection of the relative activities of cyclic AMP-dependent protein kinase and protein phosphatase-1 [1].

The existence of other inhibitor-1 phosphatases is not however excluded, and protein phosphatase-2 has also been shown to dephosphorylate inhibitor-1 in vitro [8]. Protein phosphatase-2 is an enzyme which dephosphorylates the α-subunit of phosphorylase kinase 40-fold faster than the β-subunit, in contrast to protein phosphatase-1 which dephosphorylates the β-subunit 50-fold faster than the α-subunit [1,19]. Protein phosphatase-2 has lower phosphorylase phosphatase and glycogen synthase phosphatase activity than protein phosphatase-1, and its activity is unaffected by inhibitor-1 and inhibitor-2 [19,20].

A low molecular weight substance in skeletal muscle has been partially purified whose concentration increased in response to insulin, and which inhibited cyclic AMP-dependent protein kinase in vitro [21,22]. This fraction did not inhibit phosphorylase kinase or

two other cyclic AMP-independent glycogen synthase kinases tested [22]. However the same fraction activated a crude preparation of glycogen synthase phosphatase 2-fold [22]. These results are consistent with the conclusion drawn here; namely, that the primary site of insulin action is restricted to either cyclic AMP-dependent protein kinase and/or protein phosphatase-1.

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