Defective Insulin Response of Phosphorylase Phosphatase in Insulin-resistant Humans

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Abstract

Insulin-stimulated glycogen synthase activity in human muscle is reduced in insulin-resistant subjects. Insulin regulation of human muscle glycogen synthase may require activation of a type-1 protein phosphatase (PP-1). We investigated the change of phosphorylase phosphatase and glycogen synthase activities in muscle biopsies obtained during a 2-h hyperinsulinemic euglycemic clamp in 12 insulin-sensitive (group S) and 8 insulin-resistant (group R) subjects.

Fasting phosphorylase phosphatase activity was lower in group R than in group S, and did not increase significantly with insulin infusion in group R until 20 min. In group S, phosphorylase phosphatase was significantly stimulated by 10 min, remaining significantly higher than in group R at all time points. The insulin-mediated changes in phosphatase activities were not decreased by 3 nM okadaic acid but were completely inhibited by 1 μM okadaic acid, thereby verifying that insulin-stimulated phosphorylase phosphatase is accounted for by a PP-1. Subcellular fractionation demonstrated reduced fasting PP-1 activities in both the glycogen and cytosolic fractions of muscle obtained from subjects in group R compared to those in group S.

These results suggest that insulin activation of PP-1 could contribute to the stimulation of glycogen synthase by this hormone in human muscle. Lower fasting PP-1 activity in cytosol and glycogen fractions plus lower insulin-stimulated PP-1 activity could explain, in part, reduced insulin-stimulated glycogen synthase in skeletal muscle of insulin-resistant subjects. (*J. Clin. Invest.* 1992. 89:610–617.) Key words: glucose disposal • glycogen synthase • okadaic acid • skeletal muscle • type-1 protein phosphatase

Introduction

Glucose storage via glycogen synthesis in skeletal muscle is a major determinant of insulin-mediated glucose disposal rate (1–3). A rate-limiting enzyme in glycogen synthesis is glycogen synthase, which is regulated by both reversible phosphorylation and by allosteric modifications (4–9). Phosphorylation (inactivation) of glycogen synthase is catalyzed by several protein kinases (9–12), whereas the reverse reaction (activation) is catalyzed by protein phosphatases (13, 14). Insulin administration results in a rapid increase in glycogen synthase activity in both animal and human tissues (1, 3, 15–21). Our recent data demonstrate that transient activation of glycogen synthase phosphatase is associated with insulin-mediated glycogen synthase activation in insulin-sensitive humans (21). This response was defective in insulin-resistant subjects. Based on studies of muscle glycogen synthase phosphatase in animals (13), the human enzyme should be a type-1 phosphatase (PP-1),[1] which is conveniently assayed using phosphorylase *a* as a substrate. The present study demonstrates that the abnormalities in skeletal muscle glycogen synthase phosphatase activity from insulin-resistant subjects can also be detected using phosphorylase *a* as a substrate. Based on sensitivity to inhibition by okadaic acid, the phosphatase that accounts for this abnormal activity has been identified as a PP-1. The mechanisms and regulatory proteins involved in control of PP-1 activity in the glycogen-microsomal subcellular fraction appear to be different from those in the cytosolic fraction of skeletal muscle (22). In order to further define the cause of this abnormal PP-1 activity, the subcellular location of this reduced activity was studied in muscle from insulin-resistant subjects.

Methods

Subjects. 12 insulin-sensitive subjects (group S: four Pima Indians and eight Caucasians) and 8 insulin-resistant subjects (group R: six Pima Indians and two Caucasians) participated in this study. In vivo insulin-mediated glucose disposal rates (M) were measured by using the hyperinsulinemic euglycemic clamp (see below). Subjects with an M < 8 mg/kg fat-free mass · min were considered to be insulin resistant. Subject characteristics are listed in Table I. Body weight ($P < 0.01$), percent body fat ($P < 0.001$), and fasting plasma insulin ($P < 0.001$) were significantly higher in group R than in group S.

Informed consent was obtained and fitness for the study was determined by medical history, physical examination, electrocardiography, and routine blood biochemical and hematological testing. None of the subjects was taking medication. After consuming a weight-maintaining diet (20% protein, 50% carbohydrate, and 30% fat) for at least 2 d, each subject had a 75-g oral glucose tolerance test. None of the subjects had diabetes mellitus but four in group R had impaired glucose tolerance according to the criteria established by the National Diabetes Data Group (23). Body fat as a percentage of body weight was estimated by underwater weighing with simultaneous measurement of residual lung volume (24).

Hyperinsulinemic euglycemic clamp. After at least 3 d on a weight maintenance diet and an overnight fast, a hyperinsulinemic euglycemic clamp was performed as previously described (21). The clamp was

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1. *Abbreviations used in this paper:* M, insulin-mediated glucose disposal rate; PP-1, type-1 protein phosphatase.

Table I. Patient Characteristics

No.	Sex	Age	Race	Body weight	Body fat	Glucose disposal rate	Fasting plasma	
							Glucose	Insulin
		yr		*kg*	%	*fat-free mass mg/kg·min*	*mg/dl*	*μU/ml*
Group S								
1	M	26	P	65	22	14.2	93	3
2	M	21	P	154	39	12.4	81	11
3	M	32	P	64	17	18.7	96	2
4	M	25	P	72	24	12.2	91	5
5	F	28	C	56	20	9.4	101	4
6	M	26	C	66	8	14.7	91	2
7	M	22	C	74	8	10.3	92	6
8	M	27	C	106	25	13.2	87	1
9	M	34	C	75	11	11.6	104	4
10	M	35	C	99	20	11.8	97	4
11	M	34	C	63	17	12.4	106	4
12	M	23	C	84	15	11.7	92	14
Mean±SE		28±2		82±8	19±3	12.7±0.7	98±2	5±1
Group R								
1	M	38	P	190	41	6.4	98	35
2	M	43	P	148	42	5.3	100	40
3	M	42	P	86	30	7.5	99	10
4	F	24	P	117	38	6.9	108	38
5	M	33	P	113	34	5.9	97	19
6	M	27	P	119	38	5.4	96	33
7	M	39	C	174	42	6.2	115	8
8	M	24	C	122	39	7.0	83	39
Mean±SE		34±8		134±13*	38±2‡	6.3±0.3‡	100±4	28±5‡

Four patients in group R (nos. 2, 5, 6, and 7) are impaired glucose tolerant. Abbreviations for race: P, Pima Indians; C, Caucasians. Symbols: *t* test, * $P < 0.01$; ‡ $P < 0.001$ between groups.

initiated by a primed continuous high-dose insulin infusion (600 mU/min·m^2) which was continued for 120 min while the steady-state plasma glucose was maintained at ~ 100 mg/dl. The plasma insulin concentrations were determined at 0, 10, 20, 40, 60, 80, and 120 min. The plasma glucose concentrations were determined before the start of insulin infusion and every 2.5 or 5 min through the end of the clamp. The M (mg/kg fat-free mass·min) was determined during the period from 80 to 120 min. The basal and steady-state plasma insulin concentrations (Table II) were higher in group R than in group S ($P < 0.001$ at 0 min, $P < 0.05$ at 120 min).

Plasma glucose and insulin concentrations were measured by the glucose oxidase method using a glucose analyzer (Beckman Instruments, Inc., Fullerton, CA) and by radioimmunoassay using a radioassay analyzer (Concept 4, ICN, Horsham, PA), respectively.

Muscle biopsy. Before the start of insulin infusion and at the indicated times, percutaneous muscle biopsies were taken from the quadriceps femoris muscle using a Bergstöm needle (Depuy, Phoenix, AZ) as previously described (21). Specimens (100–200 mg) were frozen in liquid nitrogen within 15 s and stored at −70°C. To reduce the assay variability between specimens the biopsies were lyophilized; dissected free of blood, fat, and connective tissue; and powdered. The powder was thoroughly mixed and stored at −70°C before analysis.

Enzyme assay. Glycogen synthase activity, determined by a modified method (21) of Guinovart et al. (25) and Thomas et al. (26) was

Table II. Plasma Glucose and Insulin Concentrations during Clamp

Time (*min*)	0	10	20	40	60	80	100	120
Plasma glucose (*mg/dl*)*								
Group S	94±2	99±1	92±3	98±3	105±3	103±2	102±1	101±1
Group R	101±3	105±3	106±3*	103±2	100±1	101±2	101±1	100±2
Plasma insulin (*μU/ml*)‡								
Group S	7±2	1,472±146	1,839±223	2,146±221	2,159±176	2,343±188	—	2,513±209
Group R	28±5§	1,844±146	2,081±218	2,573±334	2,963±390	3,160±407	—	3,584±458‖

* Mean±SE of samples drawn at 2.5–5-min intervals using the 10- or 20-min interval before indicated times. ‡ Mean±SE of samples drawn at indicated times. Symbols: *t* test, § $P < 0.05$, ‖ $P < 0.001$ between groups.

expressed as units per gram tissue dry weight. 1 U equals 1 μmol of [^{14}C]glucose incorporated into glycogen per minute at 30°C. The active form of glycogen synthase and total glycogen synthase were assayed at 0.17 and 7.2 mM glucose-6-phosphate, respectively. The ratio of active form to total glycogen synthase was used as fractional glycogen synthase activity. Glycogen synthase phosphatase activity was assayed by using rabbit glycogen synthase D as a substrate as described by Miller (27) with some modification (21). Phosphatase activity was calculated as the increase in the active form of glycogen synthase per minute and expressed as the change in glycogen synthase units per gram tissue dry weight. Interassay variations of glycogen synthase and glycogen synthase phosphatase were 5% and 10%, respectively.

Phosphorylase phosphatase activity was determined using [^{32}P]-phosphorylase *a* as a substrate as described by Nimmo and Cohen (28). Dry muscle was homogenized in solution A (18 μl/mg dry weight, unless otherwise indicated) containing 50 mM Tris-HCl, 1 mM EDTA, 50 mM 2-mercaptoethanol, pH 7, at 4°C using a Potter-Elvehjem tissue grinder (Radnoti Glass Technology Inc., Monrovia, CA). The homogenates were centrifuged at 10,000 *g* for 10 min at 4°C. The resulting postmitochondrial supernatant was used for the time course study of phosphorylase phosphatase during insulin infusion. The postmitochondrial supernatant was centrifuged at 200,000 *g* for 45 min to separate the pellet containing glycogen and microsomes from the cytosol. The pellet was resuspended in the original volume of solution A for assay of phosphorylase phosphatase activity. This fraction will be referred to as the glycogen fraction. ^{32}P-labeled phosphorylase *a* was prepared by phosphorylation of rabbit muscle phosphorylase *b* (P-6635, Sigma Chemical Co., St. Louis, MO) with phosphorylase kinase (P-2014, Sigma Chemical Co.) and [^{32}P]ATP (sp act 50–80 cpm/pmol, New England Nuclear, Boston, MA) as described in Antoniw et al. (29). The phosphorylase phosphatase activity was assayed using 40 μl of extract preincubated for 5 min at 25°C and mixed with 25 μl of [^{32}P]phosphorylase *a* (2 mg/ml in solution A plus 0.25 M NaCl) which had been preincubated 2 min at 25°C. The reaction at 25°C was terminated after 5 min by adding 100 μl of ice-cold 17.5% trichloroacetic acid and 100 μl of 6 mg/ml bovine serum albumin. Denatured protein was removed by centrifugation at 10,000 *g*, for 4 min and radioactivity was determined in 200 μl of the supernatant. Phosphorylase phosphatase activity is expressed as nanomoles of ^{32}P hydrolyzed per gram dry weight muscle per minute. In order to estimate the relative contributions of type 1 and type 2A protein phosphatases to the total phosphorylase phosphatase activity, the extracts were incubated with either 3 nM or 1 μM okadaic acid (a generous gift from P. Cohen, University of Dundee, Scotland) to inactivate type 2A and types 1 plus 2A phosphatases, respectively (30, 31). Nonspecific dephosphorylation of phosphorylase *a* was < 5% of the activity with extract and was subtracted from each activity. The extent of dephosphorylation of [^{32}P]phosphorylase *a* was < 30% of the total giving a linear response of activity with time during the 10-min incubation. Interassay variation of phosphorylase phosphatase activity was 10% and intraassay variation using the same extract was 3%.

Preliminary studies demonstrated that the use of wet and lyophilized muscle gave similar results for activities of glycogen synthase, synthase phosphatase and phosphorylase phosphatase when expressed per gram dry weight (data now shown). The average dry muscle weight was 23% (22–24%) of wet weight. The soluble protein content of postmitochondrial supernatant was 50–60% of dry weight in both groups and was not changed by insulin infusion. Protein determination was carried out by the Bradford method (32).

Statistics. Statistical significance of the differences was analyzed with Student's paired or nonpaired *t* test. Data were expressed as mean±SE unless otherwise indicated.

Results

Effect of homogenate concentration on phosphorylase phosphatase activity. The influence of homogenate concentration on the apparent phosphorylase phosphatase activities was determined in the postmitochondrial supernatant, glycogen, and cytosolic fractions as shown in Fig. 1. The extract prepared with 18 μl of buffer/mg dry muscle was diluted serially. The concentration at which linearity of the assay was lost was different in the three fractions. Compared to the postmitochondrial supernatant and cytosol, linearity was maintained at higher homogenate concentrations in the glycogen fraction. In the most concentrated extracts, specific activity in the glycogen fraction was higher than in the postmitochondrial supernatant or the cytosol. Phosphorylase phosphatase activities in fasting and insulin-stimulated muscle postmitochondrial supernatant were determined using four different dilutions of the homogenate (Fig. 2). Significant insulin activation of phosphorylase phosphatase was observed only when it was assayed at the highest (18 μl/mg, $P < 0.01$) concentration. Because the insulin effect depended on homogenate concentration, subsequent experiments used muscle powder homogenized with 18 μl of buffer/mg dry weight.

Phosphorylase phosphatase and glycogen synthase activities during insulin infusion. Changes of total and fractional glycogen synthase and phosphorylase phosphatase activities during insulin infusion are shown for the postmitochondrial supernatant in Fig. 3. In group S, total glycogen synthase activity was increased by 10% at 120 min (Fig. 3 *A*, $P < 0.01$). Differences of both basal and insulin-stimulated total glycogen synthase activities between groups were not significant. In group S, fractional glycogen synthase activity was significantly increased by 10 min (Fig. 3 *B*, $P < 0.001$). In group R, the increase of fractional glycogen synthase was slower and values were lower than in group S ($P < 0.05$ at 10 min, $P < 0.01$ at 120 min).

In group S, insulin infusion resulted in a significant increase of phosphorylase phosphatase activity by 10 min reaching a maximum increase of 19% at 20 min (Fig. 3 *C*). In group R, phosphorylase phosphatase activity did not increase at 10 min, but increased significantly by 17% at 20 min ($P < 0.01$). Both basal and insulin-stimulated phosphorylase phosphatase activities were lower in group R compared to group S ($P < 0.01$ at all time points). Results for insulin action on phosphorylase phosphatase are shown by race in Table III.

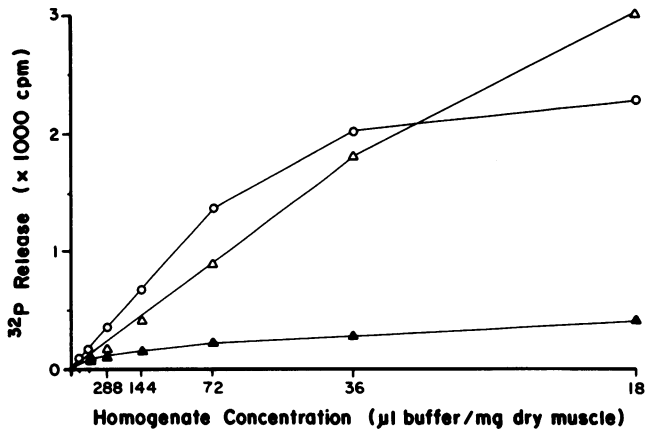

Figure 1. Effect of homogenate concentration on phosphorylase phosphatase activity in postmitochondrial supernatant (○), glycogen fraction (△), and cytosol (▲). Data are means of duplicate determinations using a pool of fasting muscle from five subjects.

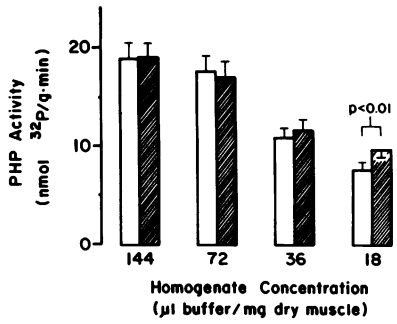

Figure 2. The influence of homogenate concentration on measured insulin activation of phosphorylase phosphatase (*PHP*) in the postmitochondrial supernatant fraction. Phosphorylase phosphatase activities in the postmitochondrial supernatant fraction prepared from fasting (*open bars*) and insulin-stimulated muscles (*shaded bars*) from five subjects were determined using four different homogenate dilutions. Significant insulin effect was observed only at 18 μl of buffer/mg dry muscle.

Subcellular distribution of phosphorylase phosphatase activity and its sensitivity to okadaic acid. Phosphorylase phosphatase activities in the three subcellular fractions prepared from fasting muscle were compared in the two groups. As previously shown in Fig. 1, the relative subcellular distribution of apparent activities is partially dependent upon the concentration of the homogenate used to prepare the subcellular fractions. The apparent proportion of phosphorylase phosphatase activity is similar in both groups (Table IV). Also in both groups, activity in the glycogen fraction obtained from the postmitochondrial supernatant exceeded the activity of the intact postmitochondrial supernatant. In all fractions, phosphorylase phosphatase activities in group R are ~ 25% lower than in group S ($P < 0.01$ in postmitochondrial supernatant and glycogen fraction, $P < 0.05$ in cytosol fraction).

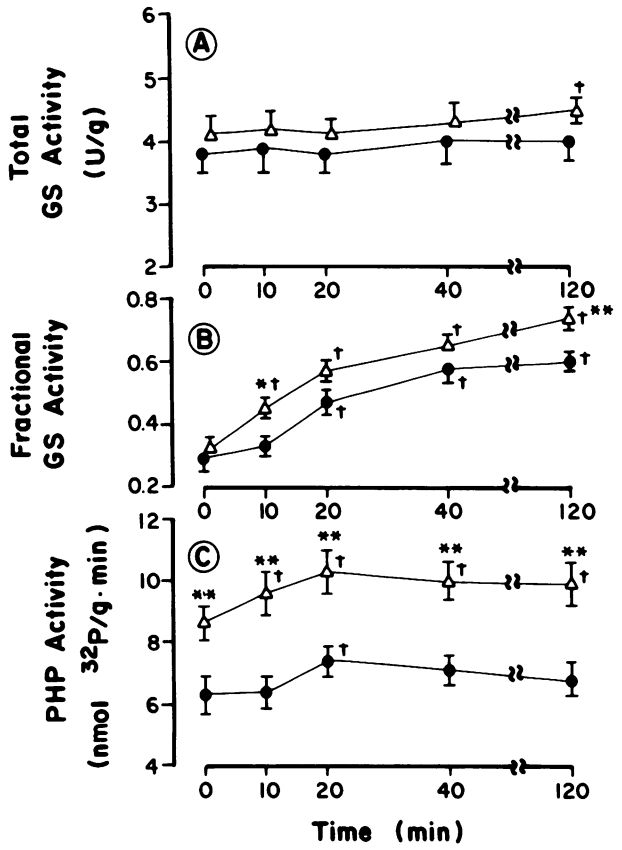

Figure 3. Change of (*A*) total glycogen synthase (*GS*) activity, (*B*) glycogen synthase fractional activity, and (*C*) phosphorylase phosphatase (*PHP*) activity in 12 subjects from group S (△) and 8 subjects from group R (●) during insulin infusion. Symbols: *t* test, $^{*}P < 0.05$; $^{**}P < 0.01$ between groups. Paired *t* test, $^{\dagger}P < 0.01$, vs. 0 min.

Table III. Effect of Insulin on Phosphorylase Phosphatase Activity by Race

	Caucasian		Pima Indian	
Insulin infusion	Group S (n = 8)	Group R (n = 2)	Group S (n = 4)	Group R (n = 6)
min	*nmol ^{32}P/g·min*			
0	8.4±0.4	6.9±0.6	9.5±1.6	6.1±0.7
10	9.2±0.4	7.0±0.0*	10.5±1.7	6.2±0.7*
20	10.3±0.5	7.4±0.1*	10.4±1.8	7.4±0.6
40	10.0±0.5	7.3±0.1*	10.2±1.6	7.1±0.6
120	9.9±0.6	7.6±0.3	9.8±1.6	6.5±0.5

Symbol: *t* test, * $P < 0.05$ between groups of the same race.

Phosphorylase phosphatase activities were also determined in subcellular fractions of fasting and insulin-stimulated muscles. Significant insulin activation of phosphorylase phosphatase was observed in the postmitochondrial supernatant and glycogen fractions, but not in cytosol (Fig. 4). After treatment with 1 μM okadaic acid, no significant fasting or insulin-stimulated phosphorylase phosphatase activity could be measured in either group S (Fig. 4) or group R subjects (data not shown). Insulin stimulation of phosphorylase phosphatase activity could be observed when assayed in the presence of 3 nM okadaic acid, suggesting that insulin stimulates primarily glycogen-microsome–associated type 1 but not type 2A protein phosphatase in human muscle postmitochondrial supernatant.

Comparison of phosphatase activity on glycogen synthase and phosphorylase after insulin infusion. Phosphorylase phosphatase and glycogen synthase phosphatase activities after insulin infusion were compared in three subjects. Activation of phosphorylase phosphatase peaked at 20 min and persisted throughout the insulin infusion (Fig. 5 *A*). Activation of glycogen synthase phosphatase is transient (Fig. 5 *B*) and peaked at 10 min in two subjects and 20 min in one subject (individual data not shown). Similar to the results shown above for phosphorylase phosphatase activity, insulin-stimulated glycogen

Table IV. Subcellular Distribution of Phosphorylase Phosphatase Activity

	Postmitochondrial supernatant	Glycogen	Cytosol
Group S (n = 4)	10.4±0.5	17.2±0.5 (165%)	3.2±0.1 (31%)
Group R (n = 4)	7.5±0.4*	13.1±0.7 (175%)*	2.4±0.4 (32%)‡

Phosphorylase phosphatase activities in postmitochondrial supernatant, glycogen fraction, and cytosol were determined by using fasting muscle (homogenized with 18 μl buffer/mg dry muscle) from four subjects in each group. Percentages in parentheses compare activities in subcellular fractions to the postmitochondrial supernatant. Symbols: *t* test, * $P < 0.01$, ‡ $P < 0.05$ between groups.

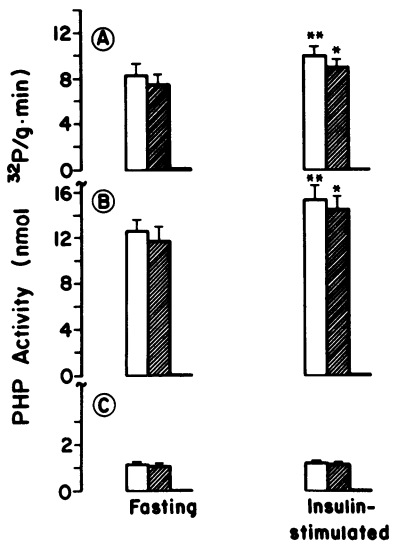

Figure 4. Insulin effect on phosphorylase phosphatase (*PHP*) activity in three subcellular fractions. Phosphorylase phosphatase activities in (*A*) postmitochondrial supernatent, (*B*) glycogen fraction, and (*C*) cytosol were determined using fasting and insulin-stimulated muscles from five subjects in group S. Activities were assayed with zero (*open bars*), 3 nM (*shaded bars*) and 1 μM (*closed bar*) okadaic acid, as described in the Methods section. Paired *t* test, $*P < 0.05$; $**P < 0.01$ between fasting and insulin-stimulated muscle.

synthase phosphatase activity was observed in the presence of 3 nM but not with 1 μM okadaic acid, suggesting that insulin stimulation of glycogen synthase phosphatase is also mediated by activation of PP-1 (data not shown).

Discussion

Insulin-resistant subjects with low insulin-stimulated glucose disposal rates have low insulin-stimulated skeletal muscle glycogen synthase activity (1, 3). The data presented here also demonstrate that glycogen synthase activation by insulin is low in group R compared with group S and indicate that insulin-resistant subjects have a defect in PP-1 activity.

Glycogen synthase activity is regulated by both noncovalent allosteric interactions and by reversible phosphorylation (4–10). Phosphorylation states of several specific sites in the glycogen synthase molecule alter its activity (10, 11). It is well known that insulin is a potent activator of glycogen synthase in liver, fat, and muscle in animals. Stimulation of dephosphorylation by activation of a protein phosphatase (13, 14, 18, 20, 21) has been proposed as a mechanism for insulin activation of glycogen synthase. We have previously reported the transient activation of skeletal muscle glycogen synthase phosphatase by insulin in humans (21). Insulin resistance was associated with a low fasting glycogen synthase phosphatase activity and a defect of its response to insulin. The previous study (21), however, did not characterize this abnormal phosphatase activity either in terms of its subcellular location or its sensitivity to okadaic acid. Here we further characterize the abnormal phosphatase activity by using phosphorylase *a* as a substrate for the reaction. [^{32}P]phosphorylase *a* is a preferred substrate for measuring PP-1 based on its availability in pure form, single site for dephosphorylation, and the relative simplicity of the phosphatase assay (29).

Using concentrated homogenates (18 μl of buffer/mg dry muscle), the glycogen fraction separated from the cytosol has higher phosphorylase phosphatase activity than the original postmitochondrial supernatant (Fig. 4). This observation suggests the presence of inhibitors of PP-1 primarily in the cytosolic fraction. Insulin activation of phosphatase is also concentration dependent (Fig. 2). Significant insulin stimulation of phosphorylase phosphatase in human muscle is only observed using concentrated homogenates (18 μl of buffer/mg dry muscle). These results may be explained by the interaction of a type-1 phosphatase catalytic subunit with insulin-sensitive regulatory proteins as discussed below.

The time courses for phosphorylase phosphatase and glycogen synthase phosphatase stimulation in response to insulin are different. In insulin-sensitive subjects, glycogen synthase phosphatase activity returned to fasting levels after peaking at 10 min (21). In order to verify this difference, three subjects were compared for phosphorylase phosphatase and glycogen synthase phosphatase activities during insulin infusion. Phosphorylase phosphatase activation in these subjects reached a plateau by 20 min which persisted until the end of insulin infusion (Fig. 5 *A*). Consistent with our previous observation (21), glycogen synthase phosphatase peaked at 10 min in two subjects, and at 20 min in one subject, and then decreased to basal levels (Fig. 5 *B*). The results suggest a feedback regulation developing after 20 min of insulin infusion which specifically inhibits PP-1 action on glycogen synthase but not on phosphorylase *a*. This regulatory mechanism would not be limited to an in vivo modification of the substrate (glycogen synthase) in that the mechanism is also capable of altering phosphatase action on the rabbit muscle glycogen synthase D added to the in vitro assay mixture. Apparent independent regulation of liver PP-1 activity on phosphorylase and synthase has previously been reported. Alloxan diabetic rats had decreased glycogen synthase phosphatase activity but phosphorylase phosphatase activity was not decreased (33). The different timecourse results in Fig. 5 could be explained by distinct catalytic or regulatory subunits responsible for specific interaction with phosphorylase and syn-

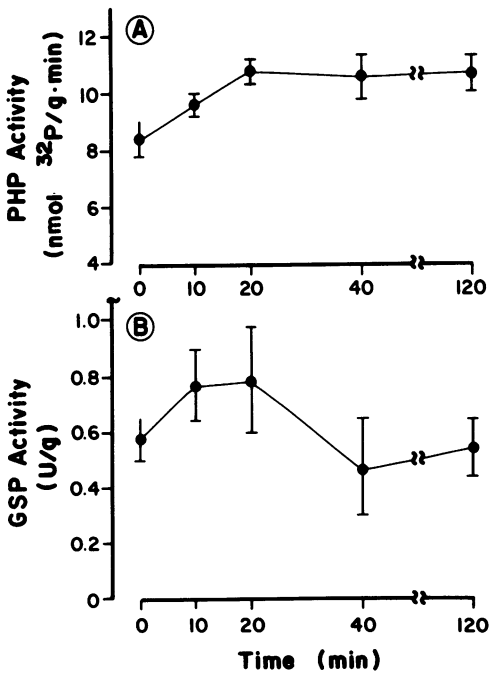

Figure 5. Comparison of (*A*) phosphorylase phosphatase (*PHP*), and (*B*) glycogen synthase phosphatase (*GSP*) activities during insulin infusion in three insulin-sensitive subjects.

thase. Similar okadaic acid inhibition results for assays with either substrate suggest that distinct catalytic subunits would probably be isoforms of PP-1. Isoforms have been identified in tissues from rat (34) and *Drosphila* (35). Increasing glycogen can inhibit glycogen synthase phosphatase (15). Increases in muscle glycogen content during 40 min of insulin infusion, however, are < 5% and unlikely to cause such a marked inhibition of glycogen synthase phosphatase. The action of PP-1 on the two substrates may only appear to be different considering that use of synthase as a substrate required a measure of enzyme activity subject to modification by kinases and metabolites which would not necessarily alter measures of the direct dephosphorylation of phosphorylase *a*.

The phosphorylase phosphatase assay was used to compare muscle PP-1 activities in group S and group R after insulin administration. Fasting phosphorylase phosphatase activity in the postmitochondrial supernatant was lower by 27% in group R compared to group S (Fig. 3 *C*). Response of phosphorylase phosphatase to insulin was slower in group R compared to group S. The percent stimulation above fasting at 20 min was similar in both groups, but insulin-stimulated phosphorylase phosphatase activity remained lower in group R over the entire 2 h. These results demonstrate that in spite of a different time course for insulin stimulated activity (Fig. 5), PP-1 assayed with either phosphorylase (Fig. 3) or synthase (21) as substrate showed reduced fasting and insulin-stimulated activities for insulin-resistant subjects. The results demonstrate that studies of abnormal PP-1 in insulin-resistant subjects can be pursued using phosphorylase *a* as a substrate. Although the abnormality in initial insulin stimulation of PP-1 is detected by both assays, they do not apparently measure the result of identical regulatory mechanisms at least beyond the first 10–20 min of insulin infusion.

In order to study specific mechanisms for altered phosphorylase phosphatase activity in insulin-resistant humans, it is first necessary to define which characterized phosphatase is responsible for the abnormal enzyme activity. In rabbit skeletal muscle extracts, PP-1 accounts for 85–90% of the phosphorylase phosphatase activity (36, 37). Okadaic acid at 1–2 nM has been previously shown to inhibit protein phosphatase 2A leaving PP-1 unaffected (30, 31). Raising the okadaic acid to 1 μM completely inhibits both type-1 and 2A protein phosphatases but not types 2B and 2C. Based on the okadaic acid inhibition of phosphatase activity observed here (Fig. 4), human muscle phosphorylase phosphatase and its insulin-stimulated activity are primarily attributed to PP-1. In addition, PP-1 is the phosphatase activity which is low in the fasting state and fails to respond normally to insulin in group R. Insulin stimulation of PP-1 in only the glycogen fraction (Fig. 4) suggests that this insulin action primarily utilizes regulators of PP-1 unique to the glycogen-microsomal fraction. The G-subunit of PP-1 has been described as a glycogen bound regulator of this enzyme and is discussed below.

In the fasted state, insulin-resistant subjects have reduced PP-1 activity but glycogen synthase activity is not significantly reduced. Another apparent discrepancy between these two enzymes is the continued increase in synthase activity beyond 20 min of insulin infusion which is accompanied by a plateau (phosphorylase as substrate) or a decrease (glycogen synthase as substrate) in phosphatase activity. These observations suggest (21), that the observed insulin-mediated changes in glycogen synthase activity are the result of a coordinate regulation by multiple enzymes including changes in PP-1 activity. Insulin stimulation of glycogen synthase kinase 3 activity has been reported (38) and would be expected either to inhibit glycogen synthase by direct phosphorylation (39) or to stimulate it secondary to the phosphorylation of inhibitor 2, which has been suggested as a mechanism for the activation of PP-1 (40). Regulation of glycogen synthase kinase 3 in human muscle has not been studied. Phosphorylation of glycogen synthase by casein kinase II does not directly alter synthase activity, but it may contribute to glycogen synthase regulation by enhancing glycogen synthase kinase 3 phosphorylation of both glycogen synthase (41) and inhibitor 2 (42). Elevated casein kinase II activity has recently been reported in muscle from both fasting and insulin-stimulated subjects with insulin resistance (43). It is not yet clear what role these two kinases, both with potential for activation or inactivation of glycogen synthase, have in explaining the abnormal glycogen synthase activity of insulin resistance.

Elevated cAMP-dependent protein kinase activity has also been reported in muscle from insulin-resistant subjects (44). In addition, inhibition of this kinase activity by insulin is reduced in insulin-resistant subjects. This elevated kinase activity in insulin-resistant subjects could contribute to reduced glycogen synthase activity as a result of the deactivation of PP-1. cAMP-dependent protein kinase has been reported to phosphorylate both the G-subunit (22) and inhibitor 1 (45). Phosphorylation of the G-subunit has been reported to release the catalytic subunit of PP-1 from glycogen where it can be inactivated by the phosphorylated form of inhibitor 1 (46). Recently, an insulin-stimulated kinase, which activates PP-1 bound to the G-subunit, has been reported (47). The activities of glycogen synthase and protein phosphatase reported here, therefore, are likely to be, in part, secondary to regulation of kinase action on the G-subunit or inhibitors 1 and 2.

The postmitochondrial supernatant was fractionated into glycogen-microsomal and cytosolic fractions from group R subjects in order to determine if phosphatase regulators unique to either of these subcellular fractions were responsible for low PP-1 activity in insulin-resistant subjects. The results in Table III indicate a similar reduction of PP-1 activity in both glycogen and cytosolic fractions of fasting group R subjects. These results could be explained by (*a*) an abnormal structure for or a reduced concentration of the PP-1 catalytic subunit available to both glycogen and cytosolic fractions, or (*b*) abnormally high inhibition of PP-1 by regulatory peptides in the cytosol such as inhibitors 1 or 2 plus an abnormal regulation of the G-subunit in the glycogen fraction. We have recently reported an increase in the concentration of PP-1 catalytic subunit in the postmitochondrial supernatant from insulin-resistant compared to sensitive subjects (48). The second explanation seems unlikely because trypsinization of homogenates from insulin-resistant subjects was recently reported to produce the expected stimulation (49, 50) of PP-1 activity but did not normalize this activity compared to control subjects (48). Trypsinization removes the effects of inhibitors 1 and 2 on PP-1 (14). This suggests that inhibitors 1 and 2 are not primary to the mechanism of abnormal PP-1 activity in insulin resistance. Abnormal G-subunit regulation of liver PP-1 activity has been reported for alloxan diabetic rats (33). A primary role for the G-subunit in causing insulin resistance seems unlikely in human muscle considering

the similar magnitude of the defect in glycogen and cytosol fractions (Table IV). Although further studies are needed to clarify the mechanism for abnormal PP-1 activity in insulin-resistant subjects, abnormal intrinsic activity of the PP-1 catalytic subunit currently appear to be the most likely explanation.

In summary, fasting and insulin-stimulated phosphorylase phosphatase activity in human skeletal muscle is primarily mediated by a type-1 phosphatase. Both fasting and insulin-stimulated phosphorylase phosphatase activities are reduced in the postmitochondrial supernatant of muscle from subjects with insulin resistance. Low fasting phosphatase activity in the glycogen and cytosolic fractions, and the reduced activity after insulin administration are likely to contribute to the reduced insulin-stimulated glycogen synthase activity associated with reduced insulin-stimulated glucose disposal rates in insulin-resistant subjects.

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