Defective Insulin Response of Cyclic Adenosine Monophosphate-dependent Protein Kinase in Insulin-resistant Humans

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Abstract

Insulin-stimulated glycogen synthase activity in human muscle correlates with insulin-mediated glucose disposal and is reduced in insulin-resistant subjects. Inhibition of the cyclic AMP-dependent protein kinase (A-kinase) is considered as a possible mechanism of insulin action for glycogen synthase activation. In this study, we investigated the time course of insulin action on human muscle A-kinase activity during a 2-h insulin infusion in 13 insulin-sensitive (group S) and 7 insulin-resistant subjects (group R). Muscle biopsies were obtained from quadriceps femoris muscle at times 0, 10, 20, 40, and 120 min.

Insulin infusion resulted in significant inhibition of A-kinase activity at 20 and/or 40 min using 0.2, 0.6, and 1.0 μM cyclic AMP in group S. A-kinase activities both before and after insulin administration were lower in group S than in group R using 0.6 μM cyclic AMP. The decrease in apparent affinity for cyclic AMP during insulin infusion was larger for group S compared with group R. Glycogen synthase activity increased significantly after insulin infusion in both groups and was higher in group S compared with group R.

The data suggest that a defective response of A-kinase to insulin in insulin-resistant subjects could contribute to their reduced insulin stimulation of skeletal muscle glycogen synthase. (*J. Clin. Invest.* 1991. 87:673–679.) Key words: glycogen synthase • protein phosphorylation • muscle

Introduction

Glucose storage via glycogen synthesis in skeletal muscle is a major determinant of insulin-mediated glucose disposal (1–3). A rate-limiting enzyme in glycogen synthesis is glycogen synthase which is regulated by both covalent phosphorylation-dephosphorylation and allosteric modifications (4–8). Phosphorylation (inactivation) of glycogen synthase is catalyzed by several different protein kinases acting on different phosphorylation sites (9–11), while the reverse reaction (activation) is catalyzed by protein phosphatases (12, 13).

Insulin administration results in a rapid increase in the percentage of synthase in the I (G6P-independent) form and stimulates glycogen synthesis in both animal and human tissues (1, 3,

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14–20). However, the mechanism by which insulin stimulates a conversion of synthase D to I is as yet unclear in human skeletal muscle. It could result from a decrease in protein kinase activity and/or an increase in synthase phosphatase activity (5–7, 11, 12, 20–22). cAMP-dependent protein kinase (A-kinase)[1] is regulated hormonally and plays a central role in the regulation of glycogen metabolism (23, 24). Previous data have demonstrated that insulin stimulation of glycogen synthase is associated with a decrease in endogenous protein phosphorylation or inhibition of A-kinase in animals (11, 21, 25, 26). Our recent data also demonstrated an insulin-mediated inhibition of A-kinase activity in human skeletal muscle after a hyperinsulinemic clamp (22). However, the possibility that abnormal insulin regulation of A-kinase could contribute to abnormal insulin activation of glycogen synthase in insulin-resistant man has not been studied.

Here, we characterize the A-kinase assay using Kemptide as a substrate and investigate the time course for insulin regulation of A-kinase and glycogen synthase during a hyperinsulinemic, euglycemic clamp. Results are compared for human skeletal muscle from insulin-sensitive and -resistant subjects.

Methods

Subjects. 13 insulin-sensitive subjects (group S, 8 Caucasians and 5 Pima Indians) and 7 insulin-resistant Pima Indians (group R) participated in this study. These subjects were selected to have an insulin-mediated glucose disposal rate in mg/kg fat free mass · min (M), which was significantly lower in group R than in group S ($P < 0.001$). Subjects with M values < 8 were considered to be insulin-resistant. Pima Indians and Caucasians in group S had similar activity and insulin response for both glycogen synthase and A-kinase. Sex, age, body weight, percent body fat, M, fasting plasma glucose, and insulin levels 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 any 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 3 subjects in group R had impaired glucose tolerance according to the criteria established by the National Diabetes Data Group (27). After at least 3 d on a weight maintenance diet, a hyperinsulinemic, euglycemic clamp was performed. Body fat was estimated by underwater weighing with simultaneous measurement of residual lung volume (28).

Table I. Patient Characteristics

No.	Sex	Age	Race	Body weight	Body fat	M	FPG	FIRI
		yr		kg	%	mg/kg fat free mass per min	mg/dl	μU/ml
Group S								
1	m	27	C	68	15	11.6	90	7
2	m	34	C	75	11	11.6	100	5
3	f	28	C	56	20	9.3	89	4
4	m	21	C	82	18	13.4	88	4
5	m	22	C	74	8	10.3	89	5
6	m	23	C	84	15	11.7	88	14
7	m	21	C	66	11	13.5	77	3
8	m	35	C	99	20	11.8	90	4
9	m	26	P	65	22	14.2	77	3
10	m	27	P	97	33	9.2	86	6
11	m	27	P	84	24	8.7	92	13
12	m	32	P	64	17	18.7	95	3
13	f	32	P	94	42	9.2	105	23
Mean±SE		27±1		78±4	20±3	11.8±0.8	90±8	7±2
Group R								
1	f	37	P	111	47	7.5	99	47
2	m	42	P	86	30	7.5	90	10
3	m	43	P	148	42	5.3	106	45
4	m	33	P	113	34	5.9	103	19
5	f	19	P	123	42	5.5	88	37
6	m	26	P	114	36	6.7	92	30
7	m	38	P	190	41	6.4	92	40
Mean±SE		34±4		126*±14	39‡±2	6.4‡±0.4	96±3	33‡±6

m, male; *f*, female; *C*, Caucasian; *P*, Pima Indian; *M*, insulin-mediated glucose disposal rate; *FPG*, fasting plasma glucose; *FIRI*, fasting plasma insulin. *t* test: * $P < 0.01$, ‡ $P < 0.001$ between groups.

Hyperinsulinemic, euglycemic clamp. After an overnight fast, a hyperinsulinemic, euglycemic clamp was performed as previously described (20). The clamp was initiated by a primed-continuous high dose insulin infusion (600 mU/min per m²) for 120 min. After the start of insulin infusion, a variable infusion of 20% glucose was given as necessary to maintain the plasma glucose concentration at 100 mg/dl for all subjects.

The plasma insulin concentration was determined before the start of insulin infusion and at 10, 20, 40, 60, 80, and 120 min during the clamp. The plasma glucose was determined before the start of insulin infusion and every 2.5 or 5 min through the end of the clamp. Insulin-stimulated glucose disposal rate (mg/kg fat-free mass per min) was determined during the period from 80 to 120 min. The steady-state plasma insulin and glucose were ~ 3,000 μU/ml and 100 mg/dl, respec-

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

Muscle biopsy. Before the start of insulin infusion (time = 0) and at the indicated time points after insulin infusion (Figs. 4–7), percutaneous muscle biopsies were taken from the quadriceps femoris muscle using Bergström needle (Depuy, Phoenix, AZ) as previously described (20). Specimens (80–120 mg) were frozen in liquid nitrogen within 15 s and stored at −70°C. These biopsies were lyophilized, dissected free of blood, fat, and all visible connective tissue, and powdered. The powder was thoroughly mixed and stored at −70°C before analysis.

Enzyme assay. Glycogen synthase activity was determined after

Table II. Plasma Glucose and Insulin Concentrations During Insulin Infusion

Time (min)	0	10	20	40	60	80	100	120
Plasma glucose (*mg/dl*)								
Group S	98±1	102±2	96±3	96±3	101±3	102±2	101±1	99±2
Group R	99±1	103±2	101±3	102±1	99±1	99±1	100±1	98±2
Plasma insulin (*μU/ml*)								
Group S	7±2	1672±130	2076±187	2407±204	2586±203	2820±220	—	3061±283
Group R	29±4*	1710±123	1908±170	2336±255	2623±306	2784±300	—	3252±364

Glucose values are the mean±SE of samples drawn at 2.5- to 5-min intervals using 10- or 20-min intervals before indicated times. Insulin values are the mean±SE of samples drawn at indicated times. *t* test: * $P < 0.001$ between groups.

modification of the method of Guinovart (29) and Thomas (30) as previously described (20). Briefly, dry muscle powder was homogenized in a 30% glycerol, 10 mM EDTA, 50 mM potassium fluoride (KF), pH 7 solution (200 μl/mg dry weight), using a Potter-Elvehjem tissue grinder (Radnoti Glass Technology, Inc., Monrovia, CA) at 4°C. The homogenate was centrifuged at 10,000 g for 20 min at 4°C. The supernatant was diluted with a buffer containing 50 mM Tris, 20 mM EDTA, 130 mM KF, pH 7.8, and used for glycogen synthase assay. Glycogen synthase activity was assayed at 0.17 and 7.2 mM glucose-6-phosphate (G6P) (total glycogen synthase activity). Fractional activity is the ratio of synthase activity measured at 0.17 and 7.2 mM G6P. The activities are expressed as units per gram dry weight (U/g). One unit equals 1 μmol [^{14}C]glucose incorporated into glycogen per minute at 30°C. G6P and uridine diphosphate glucose (UDPG) were purchased from Sigma Chemical Co. (St. Louis, MO) and ^{14}C-UDPG from New England Nuclear (Boston, MA). The glycogen synthase assay was linear with time over the range of activity measured and interassay variation was 5%.

Human muscle A-kinase was determined using Kemptide (31) as a substrate and methods from previous publications (11, 22, 32). Dry muscle powder was homogenized in a buffer (54 μl/mg dry weight unless otherwise indicated) containing 10 mM Tris-HCl, 10 mM EDTA, 100 mM KF, pH 7.8, using a Potter-Elvehjem tissue grinder. The homogenate was centrifuged at 12,800 g for 10 min at 4°C and the supernatant was assayed for protein kinase activity in the presence or absence of cAMP. The extract (18 μl) was incubated with 102 μl of reaction mixture giving a final concentration of 200 μM Kemptide (K1127; Sigma Chemical Co.), 0.3 mM gamma-[^{32}P]ATP (sp act = 50–80 cpm/pmol, NEN Research Products, Boston, MA), 50 mM 2-[N-morpholino]-ethanesulfonic acid, 8 mM MgCl$_2$, pH 6.5, in the absence or presence of 0.2, 0.6, 1, and 100 μM cAMP at 30°C. The final concentration of 15 mM KF in the assay should prevent significant hydrolysis of phosphorylated Kemptide by endogenous type-1-protein phosphatase (33). At 0 and 3 min, 30-μl aliquots were spotted on 2 × 2 cm phosphocellulose papers (Whatman P81; Whatman International, Limited, Maidstone, England). The papers were washed twice with 150 mM H$_3$PO$_4$ and rinsed with 95% ethanol. Nonspecific phosphorylation of Kemptide without extract during incubation was < 0.01% of cAMP-independent activity. Protein kinase activity was expressed as ^{32}P-incorporation into Kemptide per minute per gram muscle dry weight (nmol ^{32}P/min · g).

^{32}P-incorporation into Kemptide using basal or insulin-stimulated samples with or without cAMP was linear up to 5 min. Comparable linearity for subjects from groups S and R (Fig. 1) demonstrated the absence of significant phosphodiesterase activity after insulin infusion and during the 3-min incubation period of the enzyme assay. This was verified by testing the decrease in exogenous (0.3 μM) cAMP added to homogenates in the A-kinase assay system at 30°C. The mean 3-min decrease in cAMP concentration from four biopsies (two obtained before and two after insulin stimulation) was 7.4±1.4%. Similar results were obtained from group S and group R subjects (data not shown). The linear increase of activity with time also indicated the absence of significant changes in free catalytic subunit concentration during the 30°C incubation. The interassay variations of cAMP-dependent and -independent activities were 5–10 and 10–15%, respectively. cAMP-independent activities were ~ 10% of maximum activity and were subtracted from activities assayed with cAMP to obtain cAMP-dependent kinase activities shown in Figs. 1, 2, 3, 5 B, and 6. The apparent activation constant (K_a) (inversely proportional to apparent affinity) for cAMP activation of protein kinase was determined from individual dose-response curves at 0, 0.2, 0.6, 1.0, and 100 μM cAMP. K_a was determined graphically as the concentration of cAMP required for one-half maximal stimulation of kinase activity between 0 and 100 μM cAMP. Activities of glycogen synthase and A-kinase were 105±5 and 89±3 percent of values obtained on frozen nonlyophilized samples from the same subjects.

Muscle cAMP concentrations were determined in 14 subjects (10 in group S, 4 in group R) using cAMP RIA kit (TRK 432; Amersham
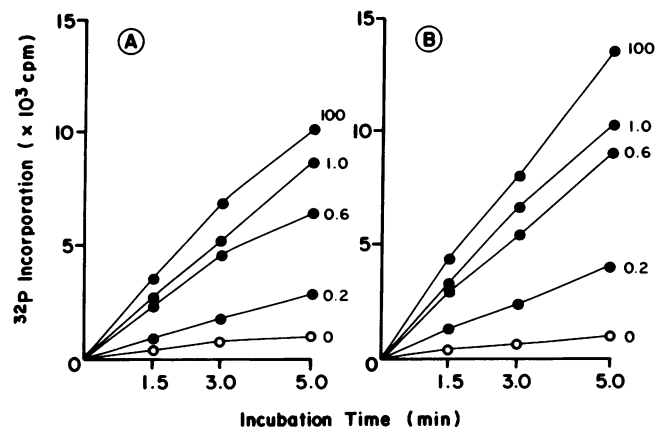

Figure 1. Protein kinase activity with respect to time. Activity was measured at the indicated μM cAMP concentrations. cAMP-dependent activity (●) has been corrected for cAMP-independent activity (○). All results are from a single biopsy after 20 min of insulin infusion. Each point is the mean of duplicate determinations. (A) Group S subject; (B) group R subject.

Corp., United Kingdom) after 0.5 M perchloric acid extraction. Interassay variation was 5%. The average (range) of dry muscle weight was 23% (22–24%) of wet weight.

Statistics. Data generated from repeated biopsies before and during 120 min of insulin infusion were analyzed by repeated measures analysis of variance (RMAV) of one grouping factor (i.e., insulin-sensitive versus -resistant) and five "within factors" (time). Significance (P < 0.05) was also analyzed with Student's paired or nonpaired t test. All data were expressed as mean±SE unless otherwise indicated.

Results

A-kinase in human muscle. The relationship between homogenate protein concentration and kinase activity was determined using fasting samples (Fig. 2). The relationship between A-kinase activity and homogenate concentration was not linear and peaked between 36 and 108 μl/mg muscle (2.1 to 0.6 mg protein/ml in assay). With the increase in homogenate concentration from 108 to 12 μl/mg, the dose-response curve shifted to the right and the K_a for cAMP increased from 0.33 to 0.62 μM (Fig. 3). Based on these observations, muscle powder homogenized with 54 μl buffer/mg dry weight was used for analysis of protein kinase activity unless otherwise indicated.

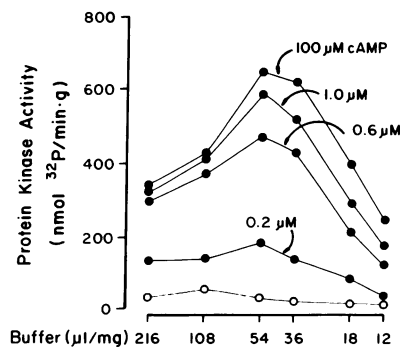*Glycogen synthase activity during insulin infusion.* Glyco-

Figure 2. Relationship between homogenate concentration and cAMP-independent (○) or -dependent (●) kinase activity normalized per gram dry muscle. The highest A-kinase activity was obtained using extract homogenized with 54 μl buffer/mg dry weight (1.3 mg protein/ml in assay). All results are from a single fasting muscle biopsy. Each point is the mean of duplicate determinations.
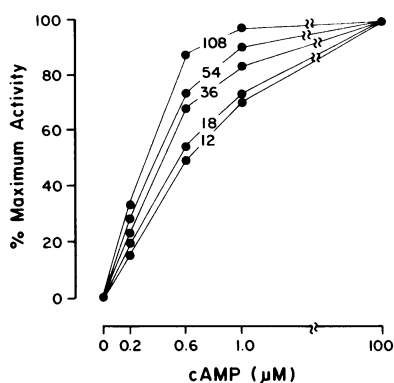
Figure 3. Dose-response curves using five different homogenate concentrations. Numbers (12–108 μl buffer/mg dry weight) indicate homogenate concentration. Activities were corrected for cAMP-independent activities before conversion to percent maximum activity. All results are from a single fasting human muscle biopsy. Each point is the mean of duplicate determinations.

gen synthase total activity and its fractional activity during insulin infusion are shown in Fig. 4. Using RMAV, there was no significant change of total activity with time or difference in total activity between the two groups (Fig. 4 A). Before insulin infusion, there was no significant difference in glycogen synthase fractional activity between groups (Fig. 4 B). Fractional activity at 10 and 120 min were, however, significantly higher (t test) in group S compared with group R ($P < 0.05$). Fractional activity was also significantly higher by RMAV for group S during the time course for insulin infusion ($P < 0.05$). In addition, fractional activity increased significantly with time (RMAV), but this change was not significantly different between groups. Caucasian and Pima subjects in group S had similar changes in fractional activity after insulin infusion (Table III).

Insulin-mediated change of kinase activity and cAMP. There was no significant change of cAMP-independent Kemptide kinase and maximum (100 μM cAMP) dependent Kemptide kinase activity in either group during the insulin infusion (Fig. 5 A and 5 B). Although there was no significant difference in maximum activity between the two groups by RMAV, the mean maximum activities at each time point in group R were about 10% higher than in group S (Fig. 5 B).

In group S but not in group R, only 0.2 and 0.6 μM cAMP-dependent kinase activities decreased significantly ($P < 0.05$ by RMAV) during insulin infusion (Fig. 6). The activities tested between 0 and 120 minutes by RMAV were significantly higher in group R compared with group S at 0.2, 0.6, and 1.0 μM cAMP ($P < 0.005$, 0.001, and 0.05). The greatest difference in A-kinase activity between the two groups appears to occur after 20 min of insulin infusion where at 0.6 μM cAMP the S group has only 70% of the activity measured in the R group.

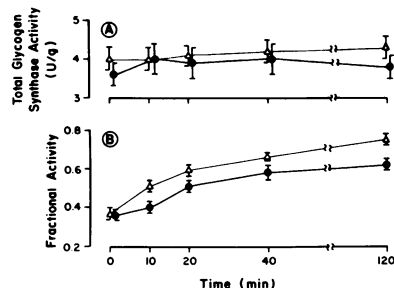The K_a of A-kinase for cAMP is shown in Fig. 7. The K_a

Table III. Insulin-mediated Change in Glycogen Synthase Fractional Activity by Race

Insulin infusion	Group S		Group R
	Caucasian	Pima Indian	Pima Indian
min			
10	0.13±0.01*	0.15±0.01*	0.04±0.01
20	0.22±0.04	0.22±0.02	0.14±0.02
40	0.28±0.04	0.31±0.03	0.22±0.04
120	0.40±0.02*	0.36±0.02	0.26±0.02

t test: * $P < 0.05$ vs. group R.

increased with time in both groups but was significantly higher ($P < 0.001$ by RMAV) in group S compared with group R. The change in K_a with insulin infusion was significantly different comparing the two groups ($P < 0.05$). In group R, K_a did not increase significantly (paired *t* test) until 40 min ($P < 0.05$) compared with the significant rise for K_a in group S at all time points ($P < 0.05$ at 10 and 120 min, $P < 0.001$ at 20 and 40 min). There was no significant relationship between body fat and K_a in group S after insulin infusion. The A-kinase K_a values were similar for Pima and Caucasian subjects in group S (Table IV). When these studies were repeated on 6 subjects in group S and 5 subjects in group R using 18 μl/mg tissue by weight in the homogenate, no significant changes in K_a were observed after insulin infusion (data not shown).

Muscle cAMP concentrations were measured over the time course for insulin infusion. There were no significant changes with time of insulin infusion or between groups (data not shown).

Discussion

We have reported a positive correlation between insulin-stimulated glucose disposal rate and insulin-stimulated skeletal muscle glycogen synthase activity in vivo in man and suggested the regulation of glycogen synthase may contribute to the reduced glucose disposal associated with insulin resistance in Pima Indians (1, 3). A similar conclusion was reached in an in vivo study of normal glucose tolerant Caucasians (34).

Glycogen synthase data in this study confirmed the association of impaired activation of insulin-stimulated glycogen synthase with insulin resistance in man. We assayed the active form of glycogen synthase under physiological concentrations (0.17 mM) of G6P in an effort to more closely approximate in

Figure 4. Total glycogen synthase activity (A) and glycogen synthase fractional activity (B) in group S ($\triangle$) and group R ($\bullet$) during insulin infusion.
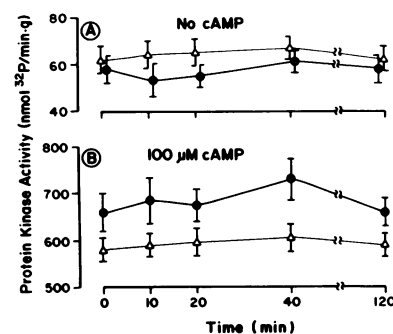

Figure 5. cAMP-independent kinase activity (A) and maximum (100 μM cAMP) dependent kinase activity (B) during insulin infusion. Symbols are the same as in Fig. 4.
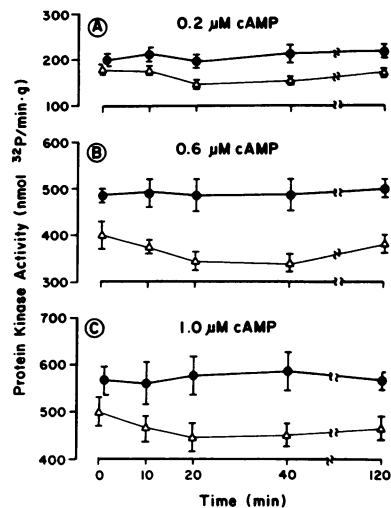
Figure 6. cAMP-dependent kinase activities during insulin infusion assayed in the presence of 0.2 (A), 0.6 (B), and 1 μM (C) cAMP. Symbols are the same as in Fig. 4.

Table IV. Effect of Insulin on A-kinase K_a by Race

Insulin infusion	Group S		Group R
	Caucasian	Pima Indian	Pima Indian
min			
0	0.41±0.02	0.41±0.03	0.38±0.02
10	0.45±0.01[‡]	0.43±0.03	0.38±0.02
20	0.52±0.02[‡]	0.49±0.02[‡]	0.39±0.02
40	0.54±0.02[‡]	0.49±0.03	0.43±0.03
120	0.45±0.02[‡]	0.44±0.02[*]	0.37±0.02

t test: * $P < 0.05$, ‡ $P < 0.01$ vs. group R.

vivo activity in skeletal muscle. We used fractional activity as a marker of glycogen synthase activation. After insulin infusion, the fractional activity was significantly higher for group S. The 120-min activity was 21% higher in group S. Using RMAV, an increase of total glycogen synthase activity could not be verified in either group. We have previously reported that increased glucose flux into human muscle at constant insulin concentration fails to stimulate glycogen synthase activity (35). This study demonstrates that muscle glycogen synthase is not regulated by changes in glucose transport. The reduced stimulation of glycogen synthase activity in insulin-resistant subjects is also not a function of increased muscle glycogen (4). Fasting muscle glycogen was either similar (1) or reduced (36) in two studies of insulin-resistant subjects. Insulin-resistant subjects also have a significantly smaller increase in muscle glycogen following insulin infusion (36, 37).

Glycogen synthase activity is regulated by both noncovalent, allosteric interaction, and covalent phosphorylation/dephosphorylation (5–8). Phosphorylation states of several specific sites in the glycogen synthase molecule alter its activity (9, 10). It is well known that insulin is a potent activator of glycogen synthase in liver, fat, and muscle, but the sequence of events leading to insulin-stimulated glycogenesis is not clear in humans. Inhibition of phosphorylation by inactivation of protein kinases (10, 11, 21, 22, 25) and/or stimulation of dephosphorylation by protein phosphatase activation (12, 17, 19, 20) have been demonstrated as mechanisms of insulin action.

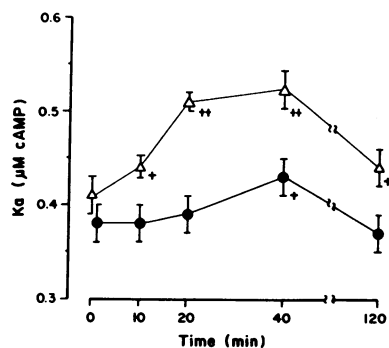Recent literature has demonstrated inhibition of the A-ki-

Figure 7. K_a for cAMP during insulin infusion. Each point represents the mean±SE of K_a values determined from individual dose-response plots for each subject in groups S and R. Symbols are the same as in Fig. 4. Paired t test: + $P < 0.05$, ++ P < 0.001 versus 0 min.

nase in insulin-treated animals (11, 21, 26). This kinase phosphorylates glycogen synthase on sites 1a, 1b, and 2, and to a lesser extent sites on 3 and 4 (10, 38, 39), resulting in inactivation of glycogen synthase. In addition, A-kinase phosphorylates and activates inhibitor 1 which inhibits protein phosphatase type 1 (40, 41). A-kinase also phosphorylates the G-subunit of protein phosphatase type 1, which promotes translocation of the phosphatase from glycogen particles to the cytosol and may lead to phosphatase inactivation by inhibitor-1 (42). Therefore, inhibition of A-kinase in response to insulin could contribute to the activation of glycogen synthase. In human muscle, Okubo et al. (22) used histone type 2A as a substrate and demonstrated insulin inhibition of skeletal muscle A-kinase activity at the end of a 200-min hyperinsulinemic clamp. However, the time course for insulin inhibition of A-kinase was not determined. In addition, the kinase activity was not characterized in subjects with reduced insulin-mediated glucose disposal. The purpose of this study is to further characterize the assay of A-kinase using human skeletal muscle and to investigate the time course of A-kinase activity during insulin infusion in insulin-sensitive and -resistant subjects.

Preliminary data (not shown) demonstrated that both cAMP-dependent and -independent kinase activities using Kemptide were ~ 4–6 times higher than activities using histone type 2A as a substrate. The relationship between A-kinase activity and extract protein concentration is nonlinear (Fig. 2). Similar observations using rat muscle were reported by Oron et al. (26) and Walkenbach et al. (11). Dilution increases the apparent affinity for cAMP (Fig. 3) at the same time that maximum A-kinase activity (100 μM cAMP in Fig. 2) is increasing. These two parameters of A-kinase diverge, however, above 54 μl/mg dry weight where maximum activity decreases but apparent affinity continues to increase. These biphasic effects of homogenate concentration on enzyme activity may reflect dilution of a family of mediators (activators and inhibitors of A-kinase) which have been previously reported in rat skeletal muscle (43, 44).

Muscle cAMP concentrations in this study averaged 3.1±0.6 nmol/g dry weight (0.72±0.14 μmol/kg wet weight) and are similar to previously reported values in resting human muscle (45). Intracellular cAMP concentration was estimated as ~ 0.90±0.18 μM. Because intracellular fluid is diluted ~ 83 times in the assay, endogenous cAMP concentration during the reaction would be estimated at 0.01 μM. Effects of endogenous cAMP on activation of A-kinase during the assay would be negligible.

The activity measured in the absence of cAMP in Fig. 5 is the result of free catalytic subunit from A-kinase as well as other kinase activities which can phosphorylate Kemptide. Comparison of this activity with the maximum cAMP-dependent activity at 100 μM cAMP suggests that, at most, the A-kinase holenzyme is $\sim$ 10% dissociated with no significant change produced by 2 h of insulin infusion. This low activity and presumed low level of cAMP binding (46) is similar in both groups and is ideal for an in vitro estimation of the response of the holenzyme to cAMP in the two groups. The assumption that this low ratio represents a high degree of association between regulatory and catalytic subunits in vivo, however, may be incorrect. It is also possible that during homogenization and assay, this low ratio resulted from a rapid reassociation of catalytic subunit with the regulatory subunit which may have been largely unbound in vivo. Skeletal muscle in rat has been reported to contain two types of regulatory subunit, which have been characterized in adipocytes and heart muscle as having differing stabilities toward subunit reassociation during homogenization (47). Although homogenization conditions have been reported which minimize these changes in subunit association and enzyme activity (48, 49), no completely satisfactory system has been reported (50). It is therefore possible that even though no activity differences were observed between groups in the absence of cAMP (Fig. 5 A), the in vivo concentrations of free catalytic subunit could be different between groups and could change with insulin infusion.

Fig. 6 shows that group S has significant changes of A-kinase activities with time of insulin infusion using physiological concentration of cAMP. Differences of the kinase activity between the two groups were most remarkable at 0.6 μM cAMP (Fig. 6 B). The data in Fig. 7 demonstrates a significant difference in K_a for cAMP after insulin infusion in the two groups. In group S, reduced affinity for cAMP during 120 min of insulin infusion, without a change in maximum activity, appears to explain the reduced activity of A-kinase measured at physiologic cAMP concentrations. Several authors have demonstrated that insulin-mediated inhibition of A-kinase is associated with reduced cAMP binding to the regulatory subunit of A-kinase (51, 52). On the other hand, in group R, K_a increased significantly at 40 min with a concomitant increase in maximum activity (Fig. 5). The net result was no change of A-kinase activity at 40 min. The physiological significance of these changes in K_a and maximum activities is not clear. An increase in the homogenate concentration to 18 μl/mg tissue dry weight removed the insulin-mediated increase in K_a in group S subjects. Although the mechanism involved here is not known, a similar result has been reported using rat diaphragm muscle (11). The increase in K_a (apparent decrease in enzyme affinity for cAMP) is a transient phenomena for both groups. In contrast is the continuous increase in glycogen synthase activity observed over the 120-min insulin infusion. These different temporal profiles emphasize that mechanisms in addition to the change in A-kinase K_a exist for insulin activation of glycogen synthase. A similar conclusion was reached in studies of insulin stimulation of glycogen synthase phosphatase in human muscle (20). Here phosphatase stimulation peaked at 10 min while glycogen synthase activity continued to increase for 120 min. Together these studies on the time course for insulin regulation of human muscle kinase and phosphatase activities suggest that stimulation of glycogen synthase during euglycemic insulin infusion in man is a composite of continuous

change in both kinase and phosphatase activities. Insulin stimulation of glycogen synthase kinase-3 has recently been reported (53). Although stimulation of this enzyme could directly inactivate glycogen synthase (54), this kinase also phosphorylates inhibitor-2 which results in activation of the type-1 protein phosphatase complex or Fc $\cdot$ M (55). Insulin regulation of glycogen synthase kinase-3 may also, therefore, contribute to the dephosphorylation and activation of glycogen synthase observed here.

Insulin has been shown to produce compounds that mediate its action (56–58). A low molecular weight peptide mediator has been purified and identified as an A-kinase inhibitor (59). The mediator binds to A-kinase holoenzyme which leads to lower cAMP binding and reduced release of active catalytic subunit (57). In this study, a significant increase of the K_a for cAMP was observed during insulin infusion in insulin-sensitive subjects. The fact that a shift to lower apparent affinity for cAMP is a result of both increased homogenate concentration (Fig. 3) and of insulin action (Fig. 7) raises the possibility that the same regulator being concentrated in the first experiment is being increased by insulin in the second experiment. The reduced insulin-mediated inhibition of A-kinase activities in the R group at subsaturating (0.6 μM) cAMP concentration (Fig. 6) may be caused by the increased apparent affinity for cAMP which is secondary to reduced insulin mediator concentrations. It is also possible that the observed low values for K_a in group R are the result of a different composition of types I and II regulatory subunit which bind the cAMP with different affinities (47).

This is the first report that demonstrates reduced insulin action to inhibit A-kinase activities in skeletal muscle from insulin-resistant man. The defective response of A-kinase to insulin may in part explain the diminished insulin-stimulated glycogen synthase activation in subjects with reduced insulin-mediated glucose disposal.

Acknowledgments

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