PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 156, 223-228 (1977)

Control of Rat Adipose Tissue Hexokinase Isoenzymes by Sugars during *in Vitro* Incubation[1] (39911)

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Hexokinase (ATP:D-hexose-6-phosphotransferase, EC 2.7.1.1) in mammalian tissue exists as four isoenzymes with distinct kinetic properties and tissue distribution (1–3). In rats, large amounts of hexokinase-II(HK-II) have been found in insulin-sensitive tissues. This isoenzyme is adaptive in adipose tissue; it is decreased in fasting, aging, diabetes, and glucocorticoid excess (3–8), all of which are associated with decreased glucose utilization in adipocytes. Correction of insulin deficiency (4–6) or of starvation (5–7) restores HK-II levels in the adipocytes. *In vitro* rates of insulin-stimulated glucose utilization by adipocytes after a variety of previous *in vivo* physiological manipulations are closely correlated to the activity of this isoenzyme (8, 9). Thus, it is of great interest to elucidate the control mechanisms for adipose tissue HK isoenzymes and their relation to glucose utilization and insulin sensitivity.

In order to separate the factors which control the enzyme from concomitant physiological counter-regulatory influences, we have studied the activity of HK isoenzymes after 18-hr *in vitro* incubation of pieces of epididymal fat pads. Studies in this laboratory and others have shown that incubation of adipose tissue with glucose increases the activity of HK-II and that insulin enhances this effect (5, 6, 10, 11). However, it has not been clear which characteristics of the glucose molecule are responsible for the regulation of HK-II, and whether insulin acts directly or through increased glucose utilization. In this study, glucose analogs and other substrates have been used in an attempt to characterize the separate roles of hormone and substrate in the control of HK isoenzyme activity.

Materials and methods. Male Wistar rats, 175–200 g, were maintained on laboratory chow until the day before sacrifice. They were fasted overnight and decapitated the next afternoon. The epididymal fat pads were rapidly excised distal to the main blood vessel, under sterile conditions, and placed in sterile saline in a petri dish. Pads from at least 5 animals were used for each experiment. They were cut into approximately 30-mg pieces and pooled. Four to six fat pad pieces were incubated in each flask, and five or six replicate flasks for each treatment were used during each experiment. Each flask was incubated and assayed separately.

Incubations were performed in TC 199 medium with Hank's salts and without glucose (specially made by Gibco, Grand Island, N.Y.), containing 0.3 mg of *N*-2-droxyethylpiperazine-*N* - 2 - ethanesulfonic acid (HEPES, Sigma, St. Louis, Mo.), 10 mg of bovine serum albumin, 5000 U of penicillin G, and 50 μg of streptomycin/ml, as modified from Smith (12). Each 25-ml polyethylene Ehrlenmeyer flask (Nalgene, Rochester, N.Y.) contained 10 ml of medium and less than 1 additional ml of insulin and substrate combined. Final concentrations were 300 μU of insulin and 3 mg of substrate/ml. Tissue was incubated at 37° in a metabolic shaker for 18 hr in room air. After incubation, the tissues were washed in saline, blotted, weighed, homogenized in 0.25–0.4 ml of HK extraction mixture (7), and centrifuged at 800*g* for 5 min. HK isoenzymes in the aqueous suspension were assayed as previously described (7).

[1] Supported in part by NIH Grant No. AM 17200 and American Heart Association Grant-in-Aid No. 73 933.

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ISSN 0037-9727

Results were expressed as milliunits of HK per gram wet weight. Absolute values of HK isoenzymes varied considerably when expressed in this fashion, despite attempts to maintain uniform animal size. These differences were caused by: (a) great variability of fat pad weight, and presumable adipocyte size, between batches of animals; and (b) declining HK-II activity per milligram of protein or per cell with increasing cell size in this weight range (7, 8). It can be seen that both increased cell size and decreased HK-II per cell tend to decrease HK-II per gram. In contrast to the differences in activity between batches, there was much less variability for any treatment group within a given tissue batch, and the effects of a given treatment relative to control tissue (incubated without added substrate) were uniform from batch to batch. Preliminary studies indicated very little change in fat cell size during the incubation, so that tissue weight is an appropriate denominator for enzyme activity.

Results. The presence of glucose and/or insulin in the incubation medium has a major effect on HK activity (Fig. 1). Tissue incubated in medium containing glucose has higher HK-I than tissue incubated without glucose; in contrast, there is no effect of insulin on HK-I. Glucose and insulin independently maintain higher HK-II activity than control tissue, and their effects are additive. The changes in HK-II were large and were seen consistently, whereas those in HK-I were not present in every experiment.

In order to characterize the properties of the glucose molecule which maintain the higher HK-II activities, a series of experiments was performed in which glucose was replaced by another sugar in the tissue culture medium (Table I). In each experiment, a set of control flasks was incubated with no added sugar, and the remainder of the flasks was incubated with glucose or with an alternate sugar. Insulin was either absent or present in all treatment groups in a given experiment, so that the effects seen were due only to changes in substrate. Fructose and mannose, both of which are substrates of HK and potential energy sources in adipose tissue, showed higher levels of HK-II

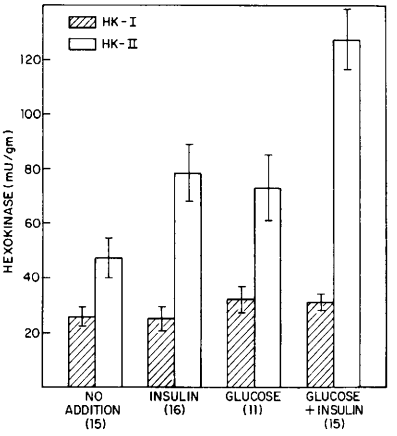

FIG. 1. Activity of hexokinase isoenzymes after 18-hr incubations in the presence of glucose (3 mg/ml) or insulin (300 μU/ml). The number of experiments, each with five or six replicates for each condition, is in parentheses. Values are means ± SEM of the treatment means from the individual experiments. By paired *t* test, *P* values were: HK-I insulin effect, NS; HK-I glucose effect, <0.01 without or with insulin; HK-II insulin effect, <0.05 without glucose, <0.001 with glucose; HK-II glucose effect, <0.01 without insulin, <0.001 with insulin.

than did control tissue. The effect of mannose was similar to that of glucose, both with and without insulin in the incubation medium, whereas the effect of fructose was less than that of glucose in the presence of insulin. 2-Deoxyglucose (2DG) and xylose, nonmetabolizable sugars which compete with glucose for the active site of the enzyme (13, 14), both diminished HK-II below the activity in control incubations. 2DG, which is a substrate of the enzyme, was the more-potent inhibitor. Maximal inhibition of hexokinase-II activity was seen with 2DG concentrations as low as 0.03 mg/ml during the 18-hr incubation (not shown here). 3-*O*-Methylglucose (3*O*MG) and galactose (13) were studied as hexoses which do not interact with the active site of HK. Neither sugar altered HK-II activity in the presence of insulin. However, there was a small but consistent elevation of HK-II above the level in control tissue when 33*O*MG was present in the medium in the absence of insulin.

Since the effects of the metabolizable sugars might have been caused by their role as energy sources, we studied a group of

compounds which can be used for energy by the adipocyte, but are not substrates of HK (Table II). None of these substrates caused an increase in HK-II above control incubations; alanine and glycerol were associated with significant decreases in the enzyme in the absence of insulin, and acetate reduced HK-II when insulin was present. In addition, in order to see whether the inhibition of HK-II activity during incubation with 2DG or xylose might be reversed by provision of additional energy, we added acetate to incubations with these inhibitors (Table III). There was no increase in HK-II from acetate in the incubations with xylose, and only a small increase in incubations with 2DG. In the latter case, enzyme levels were still considerably below

TABLE II. EFFECT OF ALTERNATE ENERGY SOURCES (3 mg/ml) ON HK-II ACTIVITY AFTER 18-hr INCUBATION ABSENCE OF INSULIN.[a]

	−Insulin			+Insulin		
	No. of experi-	Hexokinase-II		No. of experi-	Hexokinase-II	
Substrate	ments	% of control	% of glucose	ments	% of control	% of glucose
Mannose	2	188 ± 17**	108 ± 10	2	184 ± 31*	104 ± 15
Fructose	2	151 ± 15*	102 ± 10	2	195 ± 27**	72 ± 6**
Galactose	1	101 ± 19	–	1	64 ± 14	–
3-*O*-Methylglucose	4	138 ± 11**	–	3	71 ± 12	–
Xylose	1	74 ± 11	–	2	48 ± 7***	–
2-Deoxyglucose	1	20 ± 7**	–	1	11 ± 4***	–

[a] There were five or six replicates in each experiment. Values are expressed as means ± SEM of percentage of simultaneous control incubations (without added substrate) or of incubations with glucose.
* $P < 0.05$ (by t test).
** $P < 0.01$ (by t test).
*** $P < 0.001$ (by t test).

TABLE II. EFFECT OF ALTERNATE ENERGY SOURCES (3 mg/ml) ON HK-II ACTIVITY AFTER 18-hr INCUBATION IN THE PRESENCE OR ABSENCE OF INSULIN.[a]

	−Insulin		+Insulin	
Substrate	No. of experiments	Hexokinase-II (% of control)	No. of experiments	Hexokinase-II (% of control)
Glycerol	1	36 ± 11*	1	113 ± 18
Lactate	3	137 ± 20	1	83 ± 27
Alanine	2	45 ± 8**	1	82 ± 18
Acetate	3	110 ± 22	3	70 ± 9*

[a] There were five or six replicates in each experiment. Values are expressed as means ± SEM of percentage of simultaneous control incubations (without added substrate).
* $P < 0.05$ (by t test).
** $P < 0.01$ (by t test).

TABLE III. EFFECT OF ACETATE (3 mg/ml) ON THE INHIBITION OF HEXOKINASE BY XYLOSE OR 2-DEOXYGLUCOSE AFTER 18-hr INCUBATION.[a]

Substrate	Hexokinase-I (mU/g)	P vs control	Hexokinase-II (mU/g)	P vs control
None	11 ± 3	–	49 ± 7	–
Xylose (3 mg/ml)	11 ± 2	NS	32 ± 4	<0.05
Xylose + acetate	12 ± 2	NS	29 ± 4	<0.05
None	13 ± 2	–	57 ± 10	–
2-Deoxyglucose (3 mg/ml)	5 ± 1	<0.01	7 ± 2	<0.001
2-Deoxyglucose + acetate	9 ± 2	NS	17 ± 3*	<0.001

[a] There were two experiments with each substrate and five or six replicates in each experiment. Values are means ± SEM.
* $P < 0.01$ vs 2-deoxyglucose.

those in control tissue. Thus, these studies make it unlikely that provision of energy sources can maintain increased levels of HK-II, although acetate partially reverses the inhibition caused by 2DG, probably by regenerating ATP.

Since glucose-6-phosphate (G6P) is a product of the hexokinase reaction, and would be expected to increase after incubation with glucose, fructose, mannose, or insulin, we studied the direct effect of this intermediate on HK-II (Fig. 2). Tissue incubated in the presence of G6P had higher levels of both isoenzymes than did control tissue. The addition of insulin did not further increase HK-II. The activity measured was not due to contamination of the assay with G6P from the medium, since: (a) The rate of generation of NADPH in the assay was linear for 30 min, whereas over 90% of added G6P is oxidized in the first 10 min of the enzyme assay; and (b) any exogenous G6P would increase NADPH production in both heated and unheated aliquots, and thus apparent HK-II activity, the difference in activity between these aliquots, would not be altered.

Discussion. The incubation system used in these studies is an excellent one for the study of adipose tissue HK *in vitro*, because it maintains viability for up to 30 weeks (12) and permits the activity of HK-II to increase appropriately in the presence of glucose and insulin (i.e., conditions simulating the carbohydrate-fed state) (10). Even in the absence of glucose, it contains acetate and large quantities of amino acids, both of which can be used as energy sources, as well as many cofactors.

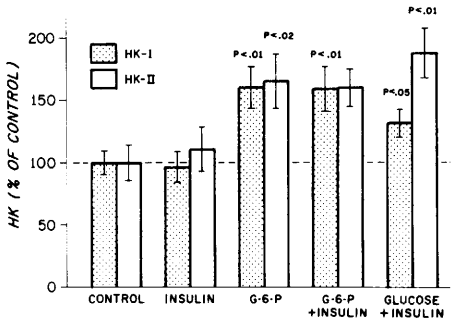

FIG. 2. Activity of adipose tissue HK isoenzymes after 18-hr incubation with G6P (3 mg/ml) in the presence and absence of insulin (300 μU/ml). Values are means ± SEM of 10 replicates in two experiments with identical protocols, expressed as the percentage of the mean of simultaneous control incubations without added energy source or insulin. *P* values are vs the control tissue incubated with the same amount of insulin as the experimental tissue.

Using this medium, we have shown that glucose, mannose, and fructose, all substrates of hexokinase and of the glycolytic system, increase HK-II activity above the levels seen after incubation in the absence of added substrate, and that xylose and 2DG, both of which interact with the active site as competitive inhibitors of glucose phosphorylation (13, 14), but are not glycolytic substrates, decrease HK-II. However, these interactions with the enzyme are not consistent among substrates which alter HK-II. 3*O*MG, which is not a substrate for HK and does not alter the rate of glucose phosphorylation by the enzyme, increases HK-II in the absence of insulin. Alanine, glycerol, and acetate decrease HK-II under some conditions. While it is possible that 3*O*MG might interact with another glucose receptor, it is unlikely that the other substrates could be similarly involved. The role of alanine is more surprising, because lactate, which also enters the metabolic pathway at the level of pyruvate, has no effect.

We have not determined whether control of cellular HK activity is due to regulation of synthesis, activation, or degradation of the enzyme. Indeed, different substrates might act through different mechanisms. It has been demonstrated that the effect of glucose and insulin on adipose tissue HK involves induction of protein synthesis (6, 11). Under the conditions in this study, incubation with glucose and insulin causes an increase in activity over preincubation levels, which suggests production of new enzyme or activation of preexisting protein. However, under most conditions, enzyme levels are lower than before incubation, and, even in the presence of glucose and insulin, it is quite possible that regulation of enzyme degradation could play a major role in control of enzyme levels. Ligands, such as glucose or G6P, protect the enzyme from *in vitro* inactivation by heat, acid, or

proteolytic enzymes (14, 15), and might decrease enzyme catabolism *in vivo*. Binding of the enzyme to mitochondria might also be involved in control of total HK if the rate of degradation is different for bound and free HK. Binding is decreased *in vitro* by G6P, ATP, and ADP, and increased by phosphate (16).

We have suggested that G6P may mediate the effects of substrates on HK-II. This would be paradoxical, because G6P inhibits HK activity in homogenates (14). However, all of the sugars which are associated with increased HK-II, except 3*O*MG, would also be expected to increase G6P production. Insulin in the presence of glucose also increases G6P (17), and fasting and glucocorticoids, which decrease HK-II (5–8, 10, 11), cause decreased G6P (18, 19). Thus a role of G6P is consistent with previous *in vivo* data. The higher levels of HK-II after incubation with G6P are remarkable in view of the relatively small amount of phosphorylated sugar which would be expected to enter the cell. It is unlikely that the effects seen during incubation with G6P were due to contamination of the preparation with glucose or to hydrolysis of the G6P by membrane phosphatases, because the magnitude of the response and lack of insulin effect are inconsistent with the earlier results seen with low concentrations of glucose (10).

There are some discrepancies between our results and those of previous workers. Thus, Hansen *et al.* (11) found no effect of insulin in the absence of an energy source, and found a significant stimulation of HK by alanine and pyruvate, which was ascribed to their role as energy sources. We found an effect of insulin in the absence of added energy sources, but no stimulation by alanine, lactate, or acetate. Indeed, in the present study, alanine decreased HK-II activity in the absence of insulin and acetate lowered activity in the presence of insulin. The major difference between the experimental conditions in these two studies is that Hansen *et al.* used a minimal essential medium, whereas our medium, as discussed above, provides additional substrate in the form of acetate and amino acids. In our system, glucose in the absence of insulin was able to maintain total HK activity (10), but, in their system, total HK and cell protein decreased during the incubation. Thus, the tissue in the studies of Hansen *et al.* was undergoing net catabolism, and the added energy sources might have merely slowed the rate of catabolism. Borrebaek (6), who used 0.2% casein hydrolysate in his medium, also found stimulation of HK by insulin in the absence of an added energy source.

The altered HK activities after incubation of adipose tissue with sugars provide a means to determine the physiological role of this adaptive enzyme. We are currently using these preparations to study the effect of altered HK-II on glucose metabolism, to see whether this enzyme has any role as a modulator of insulin action.

Summary. Substrate control of HK-II activity was studied in rat adipose tissue incubated *in vitro* for 18 hr in a modified TC-199 medium. In addition to glucose, the metabolizable substrates of the enzyme, fructose and mannose, maintained higher levels of HK-II than did control incubations. 2DG and xylose, both nonmetabolizable sugars which compete with glucose for binding to HK, decreased enzyme activity. Hexoses which do not bind to the active site of the enzyme, 3*O*MG and galactose, have minimal effect on enzyme activity. Nonhexose energy sources did not maintain high HK-II activity. G6P, the product of glucose phosphorylation by HK, increased HK-II despite its minimal transport into the cell. It is suggested that G6P may mediate the effects of sugars and insulin on HK-II.

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Received May 23, 1977. P.S.E.B.M. 1977, Vol. 156.