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The Effect of Insulin and Glucose on Fructose-2,6-P_2 in Hepatocytes

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The effect of insulin and glucose on fructose-2,6-P_2 turnover has been examined using isolated rat hepatocytes. Insulin (>10^{-10}M) increases the fructose-2,6-P_2 level in hepatocytes from fasted rats, or those pretreated with glucagon (<10^{-10}M) but not those from fed rats. Glucose (>10mM) also increases the fructose-2,6-P_2 and hexose-P levels in hepatocytes from starved rats, and its effect appears to be greater than that of insulin. Furthermore, insulin increases fructose-6-P,2-kinase activity and decreases cAMP levels in hepatocytes pretreated with glucagon, and it increases hexose-P concentration in hepatocytes from fasted rats. These results suggest that insulin antagonizes glucagon action by increasing fructose-6-P,2-kinase, decreasing cyclic AMP levels, and increasing hexose-P levels, resulting in increased concentrations of fructose-2,6-P_2.

Fructose-2,6-P_2, a very important activator for hepatic phosphofructokinase, (1-4), has been shown to be under hormonal regulation (2, 5-10). The concentration of this activator decreases rapidly in isolated hepatocytes in response to glucagon (2, 5, 6) or epinephrine (8) and increases in response to glucose concentration (2, 5).

The synthesis of fructose-2,6-P_2 has been shown to be catalyzed by fructose-6-P,2-kinase (11-13) which also appears to be under hormonal regulation (6). For example, administration of glucagon results in the decrease in fructose-2,6-P_2 which corresponds closely with inactivation of fructose-6-P,2-kinase.

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Fructose-6-P,2-kinase can be inactivated in vitro via phosphorylation catalyzed by cAMP-dependent protein kinase (14-16) and activated by dephosphorylation via phosphatase (14).

The effect of insulin on fructose-2,6-P_2, however, has not been extensively investigated although it has been shown that administration of the hormone to a diabetic rat restores fructose-2,6-P_2 levels to normal (17).

In this paper, we examine the effect of insulin on fructose-2,6-P_2 concentration in isolated rat hepatocytes under various conditions. We present evidence that insulin seems to antagonize the effect of glucagon by decreasing cAMP levels, increasing hexose-P levels, and thus increasing the activity of fructose-6-P,2-kinase and the synthesis of fructose-2,6-P_2.

MATERIALS AND METHODS

Purified porcine glucagon (2x crystallized) is obtained from NOVO A.S. (Copenhagen, Denmark), and insulin, from Eli Lilly (Indianapolis, Ind.). Fructose-2,6-P_2 is prepared as described previously (18).

Hepatocytes are prepared from rats which are either fed a standard laboratory diet or starved from 24-48 h. as stated in each experiment, using the procedure of Ishibashi and Cottam (19), based on methods developed by Berry and Friend (20), as well as Ingebretsen and Wagle (21). Hepatocytes (100 mg/ml) are incubated in Krebs-Ringer buffer, pH 7.4, in an atmosphere of 95% O_2, 5% CO_2 for 15 min prior to addition of hormones or glucose. For analysis of fructose-2,6-P_2 levels, an aliquot of cell suspension is rapidly centrifuged and frozen in liquid nitrogen. Cell extracts containing fructose-2,6-P_2 and fructose-6-P,2-kinase are prepared and assayed as described previously (4, 5, 7). A perchloric extract of the cells is prepared using the method of Crow et al. (22), and hexose-P content, determined by the method of Lowry and Passonneau (23).

RESULTS AND DISCUSSION

The Effect of Insulin on Fructose-2,6-P_2 The possible regulation of fructose-2,6-P_2 levels by insulin has been examined using hepatocytes which are either a) glycogen-rich, i.e. prepared from rats fed a normal diet, or b) glycogen-poor, i.e. prepared from rats which are fasted for 24 to 48 h prior to hepatocyte preparation. In order to determine whether glucose is necessary for synthesis of fructose-2,6-P_2, the cells from fed and fasted rats are incubated with insulin in the presence or absence of 11 mM glucose. As shown in Figure 1, insulin (10^{-9}M) increases the concentration of fructose-2,6-P_2 in hepatocytes isolated from a fasted rat, whereas the hormone does not appear to influence the fructose-2,6-P_2 level in cells prepared from a fed rat. The increase in fructose-2,6-P_2 provoked by insulin alone is comparable to the level reached in the pres-

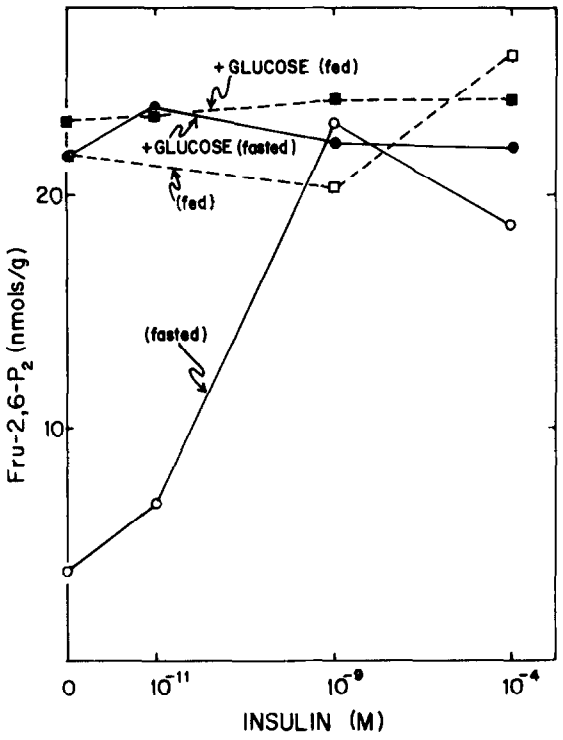

Fig 1 Dose response curves showing fructose-2,6-P_2 concentrations with varying concentrations of insulin in the presence or absence of 11mM glucose, using hepatocytes prepared from fed or 24 to 48 h fasted rats. Experimental details are given in the methods section.

ence of glucose (11 mM) as well as to the level present in hepatocytes isolated from a fed rat.

Insulin Counteracts the Glucagon Effect on Fructose-2,6-P_2 The results which are presented above suggest that insulin appears to regulate fructose-2,6-P_2 metabolism by counteracting the effect of glucagon which is induced during starvation of the animal and which results in decreased levels of fructose-2,6-P_2. Therefore, the ability of insulin to reverse the effect of glucagon on fructose-2,6-P_2 levels is examined using hepatocytes prepared from fed rats and pretreated with glucagon in the presence of 15mM glucose. As shown in Figure 2, within 2 min following administration of insulin (10^{-8}M) to glucagon-pretreated hepatocytes, the fructose-2,6-P_2 level increases approximately 6-fold, and by 10 min, a 9-fold increase has occurred. In order to determine whether insulin is specific for counteracting the effect of glucagon, other hormones which have

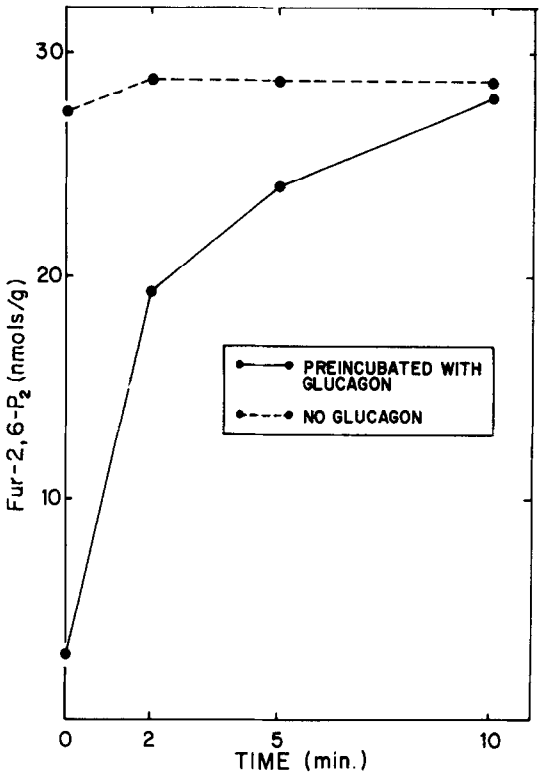

Fig 2 Kinetic curves of fructose-2,6-P_2 concentration in response to insulin, following pretreatment with glucagon or saline. Hepatocytes prepared from a fed rat are incubated 5 min in the presence of 15mM glucose and 10^{-11}M glucagon or saline, at which time 10^{-8}M insulin is added to each incubation. The cells are harvested and extracts prepared and analyzed for fructose-2,6-P_2 content as described in the methods section.

been shown to regulate fructose-2,6-P_2 levels have also been examined. Insulin does not increase fructose-2,6-P_2 levels in cells which have been pretreated with epinephrine, phenylephrine, vasopresin, or angiotensin.

The Effect of Insulin on Fructose-6-P,2-Kinase Since the activity of fructose-6-P,2-kinase, the enzyme responsible for the synthesis of fructose-2,6-P_2, has been shown to closely parallel the concentration of fructose-2,6-P_2 in isolated hepatocytes (6), it is possible that insulin regulates fructose-2,6-P_2 levels by increasing the activity of fructose-6-P,2-kinase. As shown in Table I, administration of low concentrations of glucagon to isolated hepatocytes results in a 50% inactivation in fructose-6-P,2-kinase which can be completely reactivated following administration of 10^{-8}M insulin in the presence or absence of

Table I. Effect of Insulin and Glucose on Fructose-6-P,2-Kinase and Fructose-2,6-P_2

Treatment	Fructose-6-P,2-Kinase (milliunits/g)	Fructose-2,6-P_2 (nmols/g)
Control	0.54	17.8
Glucagon	0.22	3.8
Glucagon, Glucose	0.51	20.1
Glucagon, Insulin	0.51	18.1
Glucagon, Glucose + Insulin	0.51	19.3

Hepatocytes isolated from a fed rat are incubated 15 min in 15mM glucose at which time 10^{-11}M glucagon is added and the incubation continued 10 min; glucose (15mM), insulin (10^{-8}M) or both are added and the incubation continued a period of 10 min. Cells are harvested and extracts prepared and assayed as described in "Methods". These values are from a representative experiment.

exogenous glucose. Similarly, fructose-2,6-P_2 levels decrease after glucagon treatment and increase following addition of insulin to the cells. Under these conditions, addition of insulin (10^{-8}M) decreases the cAMP concentration approximately 70% from 1.4 nmols/gm to 0.4 nmols/gm in less than 10 min.

Since fructose-2,6-P_2 levels appear to be partially regulated by fructose-6-P availability (9), the effect of insulin on hexose-P levels has been examined. As summarized in Table II, insulin, in the presence or absence of glucose, has no effect on hexose-P levels in hepatocytes prepared from fed rats. However, insulin, in the presence or absence of glucose, elevates hexose-P levels 2-fold in the fasted animals. Glucose alone also increases the hexose-P concentration, but no additional increase is observed by both glucose and insulin.

Insulin increases fructose-2,6-P_2 levels in hepatocytes, but only in the cells which are prepared from starved animals that have high concentrations of circulating glucagon or in the cells that are pretreated with low concentra-

Table II. Effect of Insulin and Glucose on Hexose-P levels

Treatment		Hexose-P (μmols/g) Fed	Hexose-P (μmols/g) Fasted	Fructose-2,6-P_2 (nmols/g) Fed	Fructose-2,6-P_2 (nmols/g) Fasted
Control		0.31	0.07	23	4
Glucose		0.34	0.16	24	22
Insulin	10^{-7}M	0.37	0.13	20	19
Insulin	10^{-9}M	0.31	0.16	25	23
Insulin	10^{-7}M + Glucose	0.33	0.14	23	20
Insulin	10^{-9}M + Glucose	0.33	0.21	23	22

Hepatocytes are isolated from fed or fasted animals in the absence of glucose and incubated 15 min without glucose. Glucose (11mM) and/or insulin is added and the incubation continued 10 min. Cells are harvested and assayed for hexose-P as described in "Methods". The values are an average of five experiments.

tions of glucagon (10^{-10}M). Insulin fails to exert any effect on fructose-2,6-P_2 in hepatocytes isolated from fed rats. Thus, the effect of insulin on fructose-2,6-P_2 appears to be the result of counteracting the action of glucagon; insulin does not appear to elicit any direct action by itself. Such an antagonistic action of insulin on the effect of glucagon on glucose production, the formation of cAMP, phosphorylase interconversion, and ion movements has been well established (see a review by Exton and Park, 24). These results suggest that insulin antagonizes the action of glucagon possibly by decreasing cAMP levels, increasing fructose-6-P,2-kinase activity and increasing hexose-P concentration, all of which result in increased fructose-2,6-P_2 levels. Although the above data obtained using our experimental conditions is consistent with the view that insulin action is initiated by decreasing cAMP levels, there is abundant documentation that insulin does not consistently inhibit cAMP accumulation (see a review by Stalmans and Van de Werve, 25). Therefore, another mechanism which does not involve cAMP is possible and cannot be ruled out at present.

The effect of glucose on fructose-2,6-P_2 levels appears to dominate that of insulin. Glucose raises the fructose-2,6-P_2 levels maximally in the absence of insulin, and addition of insulin produces no further increase in glucose-induced fructose-2,6-P_2 level. In contrast, Witters and Avruch (26) using hepatocytes from fed rats, have reported that insulin augments the effect of glucose in affecting a decrease in phosphorylase activity and an increase in glycogen synthase activity. In addition, these authors have reported that the presence of glucose is necessary for insulin action under their experimental conditions. Although insulin does not appear to augment the glucose effect on fructose-2,6-P_2, the presence of glucose appears to be necessary for elevation of fructose-2,6-P_2 levels. Since administration of increasing concentrations of glucose to hepatocytes from starved rats results in elevation of the levels of both fructose-2,6-P_2 and fructose-6-P, the substrate for synthesis of fructose-2,6-P_2, it is possible that the fructose-6-P concentration directly regulates the synthesis of fructose-2,6-P_2 under certain conditions as suggested by Hue et al. (9). Further investigation of the role of glucose and insulin in the turnover of fructose-2,6-P_2 is currently in progress in our laboratory.

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