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REGULATION OF L-PYRUVATE KINASE ACTIVITY BY INSULIN AND GLYCOLYTIC INTERMEDIATES

Osamu Miyanaga*, Claudia Evans and G. Larry Cottam

The Department of Biochemistry
The University of Texas Health Science Center at Dallas
5323 Harry Hines Blvd., Dallas, Texas 75235

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Summary. The presence of insulin and various carbohydrates in long term cultures of hepatocytes was studied to examine the mechanism by which insulin and glycolytic metabolites regulate L-pyruvate kinase activity. When hepatocytes were isolated from a control rat and cultured in the presence of insulin, a constant level of enzyme activity (16 EU/mg DNA) was maintained for 12 days. The L-pyruvate kinase activity in hepatocytes from refed rats initially was elevated (45 EU/mg DNA) and decreased to control values by the 5th day in culture. Cells isolated from a fasted rat initially contained a low level of L-pyruvate kinase activity (5 EU/mg DNA) which increased to control values by the 8th day in culture. The enzyme activity was 5 EU/mg DNA when control cells were cultured for 4 days in medium containing either glucose, glycerol or fructose without insulin; 10 EU/mg DNA in medium containing galactose and insulin but without glucose, glycerol or fructose; and 20 EU/mg DNA in medium containing both insulin and either glucose, glycerol or fructose. It is suggested that insulin is essential for the induction and maintainance of L-pyruvate kinase activity, that carbohydrates in the absence of insulin are unable to maintain the enzyme activity in cultures of hepatocytes, and that insulin and glycolytic metabolites may act synergistically to increase the activity of L-pyruvate kinase by increasing the synthesis of the enyzme.

Introduction. Liver (L) pyruvate kinase (ATP: pyruvate phosphotransferase (EC 2.7.1.40) occurs at a functional control point in carbohydrate metabolism (for review, 1,2). During diabetes and fasting the activity of L-pyruvate kinase is low. The administration of insulin to diabetic rats or refeeding a high carbohydrate diet to fasted rats results in a 5 fold increase in the enzyme activity compared to controls (3-5). This induction of the enzyme activity has been correlated with changes in the relative rate of L-pyruvate kinase synthesis (6,7). It has been suggested that insulin may act to increase L-pyruvate kinase mRNA activity by increasing the concentration of specific glycolytic metabolites (8). One method which may permit the study of the induction of specific liver enzymes is the use of hepatocytes maintained in monolayer culture. In the present study, parenchymal cells were

*Permanent address: First Department of Medicine, Faculty of Medicine Kyushu University, Fukuoka, Japan.

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isolated from the livers of control, fasted, and fasted-refed rats. The cells were maintained up to 12 days in culture under well defined conditions. The effects of the presence of insulin and various carbohydrate concentrations in the culture medium upon the level of L-pyruvate kinase activity in the isolated hepatocytes were examined.

Materials and Methods. Male Sprague-Dawley rats (Holtzman Co., Madison, WI) weighing 150 to 250 g were fed ad libitum with standard laboratory chow. When fasted animals were used, food was withdrawn 48 h before the start of the experiment. Animals which were refed were fasted for 48 h and then were given a high carbohydrate diet (6) for 48 h before the start of the experiment.

The L-pyruvate kinase activity was assayed spectrophotometrically by measuring the rate of L-pyruvate formation by coupling with excess lactate dehydrogenase (9). Protein concentrations were measured (10) using bovine serum albumin as a standard. The DNA concentration was measured (11) by using calf thymus DNA (Sigma Chemical Company, St. Louis, Mo.) as a standard. In control rats, a mean value of 16.6 ± 7.4 and 14.1 ± 2.8 units of L-pyruvate kinase activity per mg of DNA were observed in liver homogenates and freshly isolated hepatocytes, respectively (7,12).

Hepatocytes were isolated from rat livers by a slight modification of an existing procedure (13). The cell viability, evaluated by 0.2% Trypan blue exclusion immediately after isolation, routinely exceeded 90%. The cells were immediately suspended in 2.5 ml of Leibovitz L-15 (KC Biological Inc., Lenexa, KS) medium (1×10^6 cells per ml of medium) which contained; 10% heat inactivated fetal bovine serum (Gibco Labs, Grand Island, NY), 20 mM 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid buffer, (pH 7.4), 100 IU of penicillin per ml, 100 µg of streptomycin per ml, 2.5 µg of fungizone per ml, 10^{-6} M insulin and 10^{-6} M dexamethasone. The cells were then innoculated into each 60 mm petri dish (Falcon, Cockeysville, MD) containing a collagen gel layer and cultured as described previously (7,12). At predetermined intervals the hepatocytes were washed and harvested and the L-pyruvate kinase activity assayed (7,12).

Results and Discussion. It has been postulated that the primary action of insulin on glycolysis is on the induction of glucokinase, and that the action of insulin on the induction of L-pyruvate kinase, is not direct but is secondary and may be related to changes in a specific glycolytic metabolite (15-17). A metabolite common to glucose, fructose and glycerol has been suggested as responsible for the induction of the enzyme, and dihydroxyacetone phosphate has been suggested as such a mediator (17). In the studies reported here the individual and combined effects of insulin and various carbohydrates in long term cultures of hepatocytes were investigated to further examine the mechanism(s) by which insulin and glycolytic metabolites regulate L-pyruvate kinase activity.

The optimal glucose concentration required to maintain L-pyruvate kinase activity in cultured hepatocytes was between 10 to 50 mM glucose (Figure 1) and was similar to blood glucose concentrations found *in vivo* (14). Concentrations greater than 100 mM glucose resulted in a

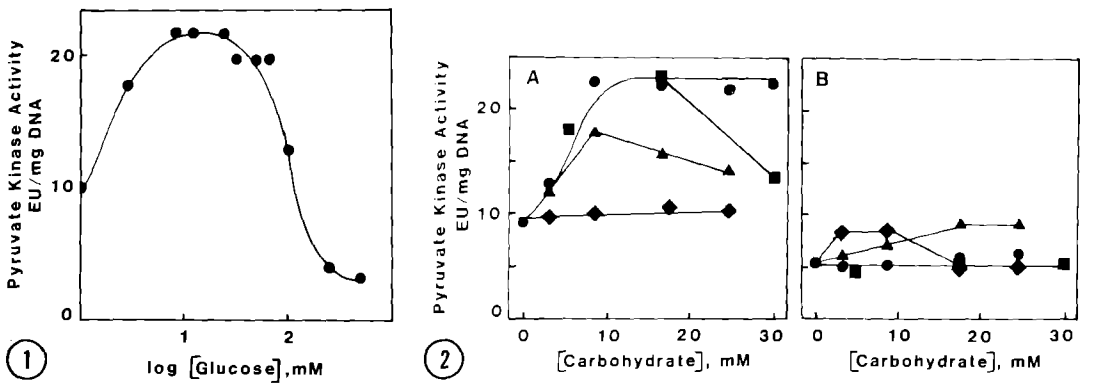

Figure 1. The effect of glucose concentration on L-pyruvate kinase activity in hepatocytes. Hepatocytes were isolated from a control rat, suspended in L-15 medium without hormones and attached to collagen gels at 37°C for 4 h. After 4 h, the medium was replaced with fresh L-15 medium containing 10^{-6} M insulin, 10^{-6} M dexamethasone and the concentrations of glucose indicated. The medium was exchanged every 24 h. The cells were harvested after 4 days and the L-pyruvate kinase activity measured. Each point represents the mean of duplicate plates of cells.

Figure 2. The effect of various carbohydrate concentrations on L-pyruvate kinase activity in hepatocytes. Hepatocytes were isolated from a control rat, suspended in L-15 medium containing 8 mM glucose and 10^{-6} M dexamethasone and attached to collagen gels at 37°C for 4 h. After 4 h the medium was replaced with fresh L-15 medium containing the indicated concentrations of glucose (●), fructose (▲), glycerol (■) or galactose (◆). The medium was exchanged every 24 h. The cells were harvested after 4 days and the L-pyruvate kinase activity measured. Each point represents the mean of duplicate plates of cells. (A) Cells were cultured in the presence of 10^{-6} M insulin, or (B) in the absence of insulin.

significant decrease in the L-pyruvate kinase activity of the hepatocytes.

The L-pyruvate kinase activity of hepatocytes isolated from a control rat increased from 10 to 20 EU/mg DNA during 4 days in culture when the concentration of glucose, fructose or glycerol in the medium was increased (Figure 2A). Maximal enzyme activity was maintained when the medium contained between 8 to 30 mM glucose, 16 mM glycerol or 8 mM fructose. Lebovitz L-15 medium contains 5 mM galactose as a component, and no change in L-pyruvate kinase activity was observed in cultured hepatocytes at higher galactose concentrations.

The increase in the L-pyruvate kinase activity in 4 day cultures of control hepatocytes required insulin regardless of the carbon source in the medium. When hepatocytes were cultured in the absence of insulin (Fig. 2B) a low level of L-pyruvate kinase activity (5 EU/mg DNA) was observed with each of the carbohydrates examined. This observation is in contrast to *in vivo* observations where fructose induced L-pyruvate kinase to normal levels in diabetic rats (15). However, *in vivo* the effects of insulin and dietary fructose cannot be separated since

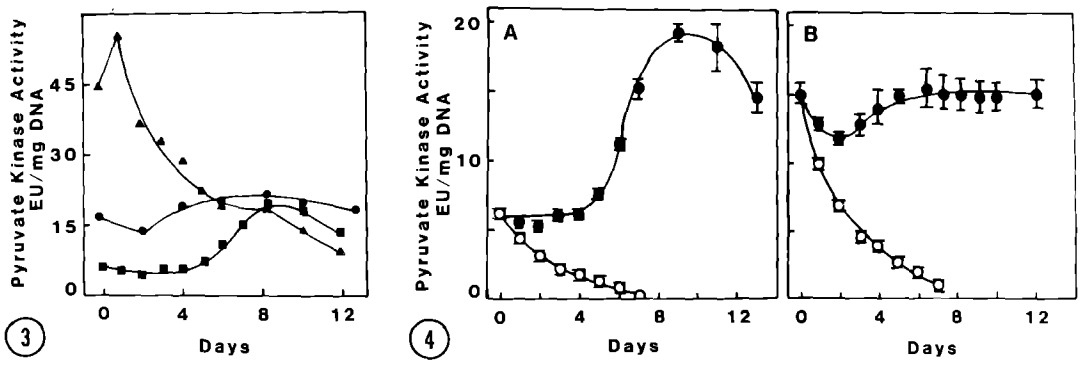

Figure 3. The effect of dietary status of the animals upon the L-pyruvate kinase activity in cultured hepatocytes. Hepatocytes were isolated from control (●), fasted (■), or fasted and refed (▲) rats and suspended in L-15 medium containing 8 mM glucose but without hormones. After 4 h of incubation at 37°C to allow the cells to attach to the collagen gels, the medium was replaced with fresh medium containing 8 mM glucose and 10^{-6} M insulin and 10^{-6} M dexamethasone. This medium was exchanged every 24 h and the cells harvested at the time intervals indicated. The L-pyruvate kinase activity was measured and each point represents the mean from duplicate cultures of cells.

Figure 4. The induction of L-pyruvate kinase activity in hepatocytes isolated from a fasted rat. Hepatocytes were isolated from a fasted (A) or control (B) rat and attached to collagen gels at 37°C for 4 h in medium L-15 containing 8 mM glucose but without hormones. After the 4 h the medium was exchanged with fresh medium containing 8 mM glucose, 10^{-6} M dexamethasone and either with (●) or without (○) 10^{-6} M insulin. The medium was exchanged every 24 h and the cells harvested at the time intervals indicated. The L-pyruvate kinase activity was measured and each point represents the mean and standard deviation from 3 plates.

fructose feeding increases insulin secretion in both diabetic and fasted animals (18).

The L-pyruvate kinase activity of hepatocytes isolated from control, fasted and refed rats were compared as a function of time in culture. Figure 3 shows that when hepatocytes were isolated from a control rat, a constant level of L-pyruvate kinase activity similar to values found *in vivo* (16 EU/mg of DNA) was maintained throughout the culture period. The level of L-pyruvate kinase activity in hepatocytes freshly isolated from refed rats was approximately 3 times greater than in control cells (45 EU/mg of DNA) and decreased to control values by the 5th day in culture. On the other hand, cells isolated from a fasted rat initially contained a low level of L-pyruvate kinase activity (5 EU/mg of DNA) which remained low for 4 days and then increased to control values by the 8th day in culture.

The induction of L-pyruvate kinase activity in hepatocytes isolated from a fasted rat required insulin. Figure 4A shows that when insulin is a component of the medium, a 4 to 5 fold increase in the L-pyruvate kinase activity occurs between the 6th and 8th day in culture of hepatocytes isolated from a fasted rat. The rate of increase in the

enzyme activity was greater when the medium contained 50 mM glucose. No increase in the enzyme activity was observed when fasted cells were cultured in the absence of insulin in medium containing 50 mM fructose. Control cells maintained a constant high level of L-pyruvate kinase activity over the same 12 day period when the medium contained insulin (Figure 4B). When fasted or control cells were maintained in the absence of insulin the L-pyruvate kinase activity steadily declined.

The increase of the L-pyruvate kinase activity in cultured hepatocytes isolated from a fasted rat reflects an increase in the L-pyruvate kinase synthesis, since the increase in the enzyme activity was blocked by the presence of protein synthesis inhibitors. When hepatocytes from a fasted rat were cultured in medium which contained 8 mM glucose, 10^{-6} M insulin and 10^{-6} M dexamethasone the induction in L-pyruvate kinase activity between the 6th and 8th day in culture was blocked by the presence of 0.38 mg/liter of actinomycin D or 10 mg/liter of cycloheximide.

Previously, it was reported that insulin induced liver L-pyruvate kinase synthesis in hepatocytes isolated from a diabetic rat (7). The relative rate of L-pyruvate kinase synthesis in hepatocytes from fasted rats was induced when either insulin or insulin and fructose was added to the culture medium (19,20). In vivo studies indicate that no change in the degradation rate of the enzyme occurred during fasting or refeeding and that the increase in the relative rate of enzyme synthesis upon refeeding fasted rats was related to a synchronous increase in the level of mRNA coding for L-pyruvate kinase (21). Recently, when diabetic rats were fed a high fructose diet, the level of translatable mRNA for L-pyruvate kinase was increased (8). From these experiments and the above results, it is suggested that 1. insulin is essential for the induction of L-pyruvate kinase, 2. in the absence of insulin, glucose, glycerol or fructose are unable to maintain the enzyme activity in cultures of hepatocytes and 3. the increase of L-pyruvate kinase by insulin and glycolytic metabolites is synergistic.

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