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THE EFFECT OF DEXAMETHASONE ON PYRUVATE KINASE ACTIVITY IN PRIMARY CULTURES OF HEPATOCYTES

OSAMU MIYANAGA *, CLAUDIA EVANS and G. LARRY COTTAM **

Department of Biochemistry, University of Texas Health Science Center at Dallas, 5323 Harry Hines Blvd., Dallas, TX 75235 (U.S.A.)

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Pyruvate kinase activity in primary cultures of hepatocytes isolated from a normal rat was maintained at a constant level similar to that found in vivo (14.0 ± 2.8 units per mg of DNA) for over 6 days when both dexamethasone and insulin were included in the medium. Yet the pyruvate kinase activity decreased 50% when the cells were cultured for 2 days and 4 days, respectively, in the presence of either dexamethasone or insulin alone. A brief, 10 min incubation of hepatocytes in the presence of dexamethasone was sufficient to maintain the enzyme activity of cells subsequently cultured for 4 days in the presence of insulin. The optimal dexamethasone concentration was 1 μM. Three other glucocorticoids were able to maintain the pyruvate kinase activity in cells cultured in medium containing insulin. The presence of the protein synthesis inhibitors, actinomycin D or cyclohexamide in cells cultured in the presence of dexamethasone and insulin resulted in a 25% decrease in the pyruvate kinase activity. Therefore, it is suggested that the synergistic effect of glucocorticoids and insulin to maintain pyruvate kinase activity in primary cultures of hepatocytes is dependent upon the ability of these cells to maintain protein synthesis.

Introduction

The synthesis of the L-form of pyruvate kinase (ATP: pyruvate phosphotransferase, EC 2.7.1.40) in the rat liver undergoes induction depending upon the dietary and hormonal status of the animal [1–8]. Insulin, for example, causes an induction of the synthesis of the enzyme 3–4-fold over basal levels upon treatment of diabetic rats with insulin for three days [9]. Likewise, in vitro when hepatocytes from diabetic rats are maintained in medium containing 1 μM insulin, the relative rate of pyruvate kinase synthesis is increased 3-fold within 3 days [9]. Similar alterations in pyruvate kinase synthesis stimulated by dietary manipulation were accompanied by parallel changes in the translatable mRNA coding for the enzyme [6]. Glucagon, on the other hand, whose action is likely mediated through cAMP [10,11], stimulates the phosphorylation of pyruvate kinase [10–12] by a cAMP-dependent protein kinase which increases the $K_{0.5}$ of the enzyme for phospho*enol*pyruvate [11,13–15]. Glucocorticoids also affect the activity of liver pyruvate kinase; however, the data are not completely consistent. The administration of glucocorticoids to rabbits results in a rapid decrease in the activity of hepatic pyruvate kinase but no equivalent decrease in the enzyme concentration determined immunologically [16,17]. On the other hand, the administration of glucocorticoids to rat hepatocytes maintained in primary culture decreases the phosphorylation of the type-L pyruvate

* Permanent address: First Department of Medicine, Faculty of Medicine, Kyushu University, Fukuoka, Japan.
** To whom correspondence should be addressed.

kinase by cAMP dependent protein kinases in cell-free extracts [18].

Although the influence of adrenal glucocorticoids on gluconeogenesis is well established and involves the modulation of the sensitivity of the liver to glucagon and other hormones, the mechanism of its action is poorly understood (for review, see Ref. 19). Little is known about the influence of glucocorticoids on key regulatory glycolytic enzymes. In the present study, rat liver parenchymal cells have been isolated from normal rats and cultured up to 14 days under well defined conditions to investigate the effect of glucocorticoids on liver pyruvate kinase.

Experimental Procedures

Male Sprague-Dawley rats (150–250 g, Holtzman Co., Madison, WI) given food and water ad libitum were used throughout these studies unless otherwise noted.

Assay of pyruvate kinase activity

The pyruvate kinase activity was assayed by the spectrophotometric procedure as described previously [20]. The assays were carried out at 30°C and the reaction mixture contained 100 mM Tris-HCl (pH 7.4), 100 mM KCl, 4 mM $MgCl_2$, 0.15 mM NADH, 5 units of lactate dehydrogenase, 2 mM ADP, 1 mM fructose 1,6-bisphosphate, and 1 mM phospho*enol*pyruvate in a total volume of 1 ml. 1 unit of pyruvate kinase activity will catalyze the formation of 1 μmol of pyruvate per min under the conditions defined. Protein concentrations were measured [21] using bovine serum albumin as a standard. The DNA concentration was measured by using calf thymus DNA (Sigma Chemical Company, St. Louis, MO) as a standard. A mean value of 16.6 ± 7.4 and 14.1 ± 2.8 units of pyruvate kinase activity per mg of DNA were observed in liver homogenates and freshly isolated hepatocytes, respectively, from control rats [9].

Preparation of collagen gels

Rat tail collagen was purified and solubilized [23,24] before it was centrifuged for 24 h at 17000 rpm. This results in a clear solution of collagen which was stored at 4°C. Collagen gel layers were prepared by pipetting 1.7 ml of the collagen solution and 0.4 ml of a solution containing 10-times concentrated Eagle's minimal essential medium (Gibco Lab., Grand Island, NY) and 0.38 M sodium hydroxide (2 : 1, v/v) into each 60 mm petri dish (Falcon Cockeysville, MD) and mixed well before the gel was allowed to form [23].

Isolation and incubation of rat liver parenchymal cells

Hepatocytes were isolated from livers of rats (150–250 g) as described [25,26]. Rat livers were isolated and flushed to remove blood, then perfused for 10 min at 37°C by recirculation of 100 ml of oxygenated calcium-free Hanks' solution containing 10 mM glucose. 50 mg of collagenase (Millipore Corporation, Bedford, MS) were added to the perfusion medium and the perfusion was continued for 10 min before addition of $CaCl_2$ to a final concentration of 5 mM [27]. 10 min after the addition of calcium the perfusion was stopped, the liver was minced and the cells were filtered through a mylon mesh. The cell suspension was centrifuged for 1 min at $50 \times g$ to collect the parenchymal cells which were washed an additional 4 times using Krebs-Ringer bicarbonate buffer (pH 7.4), containing 10 mM glucose. The cell viability, evaluated by 0.2% Trypan blue exclusion immediately after the isolation, routinely exceeded 90%. The cells were immediately suspended in 2.5 ml of medium A ($1 \cdot 10^6$ cells per ml of medium) and then were innoculated into each petri dish containing the collagen gel. Medium A was Leibovitz L-15 (KC Biological Inc., Lenexa, KS) which contains; 10% heat inactivated fetal bovine serum (Gibco Labs, Grand Island, NY), 20 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid buffer, (pH 7.4), 1.5 mg/ml glucose, 100 IU/ml penicillin, 100 μg/ml streptomycin and 2.5 μg/ml fungizone. All incubations were carried out at 37°C in a humidified atmosphere. After the initial 4 h incubation period for the attachment of the cells to the collagen layer, the medium was changed to remove the non-attached cells and then 2 ml fresh medium A containing the hormones were added and the incubation continued. Thereafter, the culture medium was removed and replaced with fresh culture medium at 24 h intervals.

To assay pyruvate kinase in the cultured

hepatocytes, the medium was removed and the attached cells were washed three times with ice-cold Krebs-Ringer phosphate buffer (pH 7.4). The cells were then detached from the collagen gels by incubation for 20 min at 37°C in 2 ml Krebs-Ringer phosphate buffer (pH 7.4), containing 1 mg/ml collagenase. The detached cells were then transferred to a centrifuge tube and collected by centrifugation for 5 min at $50 \times g$. The cells were washed three times with Krebs-Ringer phosphate buffer (pH 7.4) and collected by centrifugation for 1 min at $50 \times g$. The final cell pellet was suspended in 1 ml, 0.1 M Tris-HCl buffer (pH 7.4), 0.1 M KF, 15 mM EDTA, 0.2 mM dithiothreitol and sonicated for 30 s at 1.5 on a sonicator (Model W 375 for Heat Systems-Ultrasonics, Inc., Plainview, N.Y.).

Results

The influence of dexamethasone on pyruvate kinase in hepatocytes

The pyruvate kinase activity in hepatocytes attached in the presence of 1 μM dexamethasone and cultured in medium A containing both 1 μM dexamethasone and 1 μM insulin was maintained at a constant level of 14.1 ± 2.8 units per mg of DNA during 12–14 days in culture [9]. The effect of dexamethasone alone on pyruvate kinase activity in primary cultures of hepatocytes maintained in medium A up to 24 h is illustrated in Fig. 1. The hepatocytes were attached to the collagen matrix for 4 h in the absence of hormones then either 1 μM dexamethasone or 1 μM dexamethasone and 1 μM insulin was added to the medium. Exposure of hepatocytes in primary culture to 1 μM dexamethasone up to 12 h does not significantly alter the total liver pyruvate kinase activity. At 24 h, however, cells cultured in the presence of only dexamethasone have less total pyruvate kinase activity than cells cultured in the presence of both dexamethasone and insulin. The total liver pyruvate kinase activity (EU/mg of DNA) decreased markedly in cells attached in the absence of hormones then maintained in culture for longer periods in either no hormones, dexamethasone or insulin alone (Fig. 2). Similar results were observed when the enzyme activity was expressed as EU/mg of protein. However, when 1 μM dexamethasone and 1 μM insulin were present in the medium during the remaining culture period, the pyruvate kinase activity was maintained at an elevated level. The pyruvate kinase activity in hepatocytes, therefore, was only maintained at a high level when both dexamethasone and insulin were present during culture.

The combined effect of dexamethasone and in-

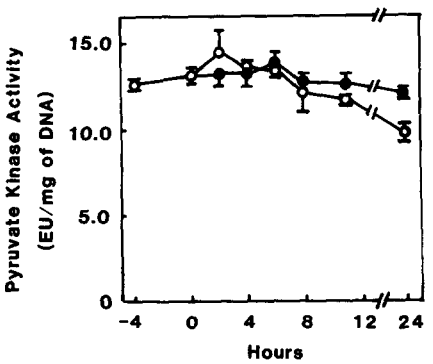

Fig. 1. The short-term effect of dexamethasone on pyruvate kinase activity in hepatocytes. Hepatocytes were isolated from a normal rat, attached to collagen gels at 37°C in the absence of hormones (−4 to 0 h), and were subsequently maintained for 24 h (from 0 hour) in medium A containing 1 μM dexamethasone (○) or 1 μM dexamethasone and 1 μM insulin (●). The points represent the mean and standard deviation of pyruvate kinase activity from three plates.

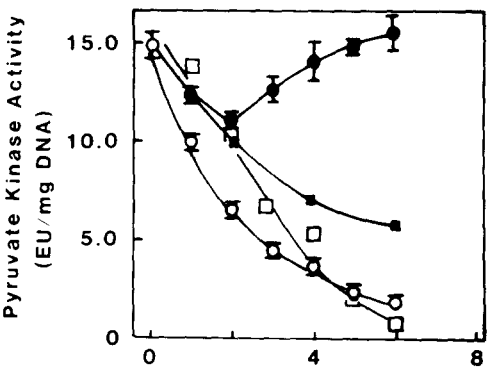

Fig. 2. The long-term effect of dexamethasone on pyruvate kinase activity in hepatocytes. Hepatocytes were isolated from a normal rat, attached to collagen gels by incubation at 37°C for 4 h in medium containing no hormones and subsequently maintained in medium A containing 1 μM dexamethasone (○), 1 μM insulin (■), 1 μM dexamethasone and 1 μM insulin (●), or no hormone (□). The values are the mean and standard deviation of pyruvate kinase activity from at least three plates. Values on x-axis represent days.

TABLE I

THE COMBINED EFFECT OF DEXAMETHASONE AND INSULIN UPON PYRUVATE KINASE ACTIVITY IN HEPATOCYTES

Hepatocytes were isolated from a normal rat and maintained in medium A at 37°C. Cells were attached to collagen gels and maintained in culture in the presence or absence of hormones as noted. Following the 4 h attachment period the medium was replaced with fresh medium containing the hormones as indicated. When hormones were present their final concentration was 1 μM. The cells were harvested after 4 days of incubation and the liver pyruvate kinase activity and DNA content assayed. The attachment period was the first 4 h of incubation. The culture period was the incubation time following the 4 h attachment period. The pyruvate kinase activity in hepatocytes freshly isolated from normal rats was 14.1 ± 2.8 units per mg DNA. The mean and standard deviation were calculated from at least 3 cultures.

Attachment period		Culture period		Pyruvate kinase activity (EU/mg DNA)
Dexamethasone	Insulin	Dexamethasone	Insulin	
−	−	−	−	5.4 ± 0.2
−	−	+	−	3.8 ± 0.1
−	−	−	+	6.2 ± 0.3
−	−	+	+	14.1 ± 0.9
+	−	+	−	5.8 ± 0.8
+	−	+	+	11.6 ± 1.7
−	+	+	−	9.2 ± 4.0
+	−	−	+	12.9 ± 1.2
+	+	−	−	6.4 ± 0.6

sulin on pyruvate kinase activity in hepatocytes is demonstrated in Table I. Hepatocytes were isolated from a normal rat and attached to collagen gels and maintained in medium A at 37°C for 4 days. The ability of various combinations of dexamethasone and insulin during the attachment and subsequent culture period to maintain pyruvate kinase in hepatocytes was examined. Maximal pyruvate kinase activity in primary cultures of hepatocytes was obtained when cells were cultured in the presence of insulin, for example: (1) when cells were attached in absence of hormones and cultured in the presence of dexamethasone and insulin and (2) when dexamethasone was present during attachment and insulin was present during culture. However, when insulin was present only during attachment and dexamethasone was present during culture, a moderate amount of pyruvate kinase activity was observed in hepatocytes after 4 days in culture.

The presence of dexamethasone and insulin is also required to maintain the percentages of pyruvate kinase activity immunoprecipitable by anti-liver pyruvate kinase goat antisera. In hepatocytes freshly isolated from a normal rat approx. 90% of the pyruvate kinase activity was precipitated by anti-liver pyruvate kinase antisera and 85% after 10 days in culture [9]. When cells were attached without hormones and maintained in the absence of dexamethasone but the presence of insulin, within 6 days fibroblast growth was obvious and would overgrow the hepatocytes resulting in an altered isozyme pattern. After 10 days the level of immunoprecipitable liver pyruvate kinase in these cultures had decreased to 18% of the total pyruvate kinase activity. In contrast cells attached in the presence of dexamethasone and maintained in culture medium containing either insulin or dexamethasone and insulin the percentage of immunoprecipitable pyruvate kinase remained between 71 and 85% of the total pyruvate kinase activity.

The effect of the time of exposure of cells to 1 μM dexamethasone during attachment to the collagen matrix was investigated (Fig. 3). The cells were subsequently cultured in medium containing 1 μM insulin but no dexamethasone. The cells were maintained for 4 days then harvested and assayed for liver pyruvate kinase activity. As illustrated in Fig. 3, a 10 min exposure of hepatocytes to dexamethasone during the attachment phase was sufficient to maintain maximal liver

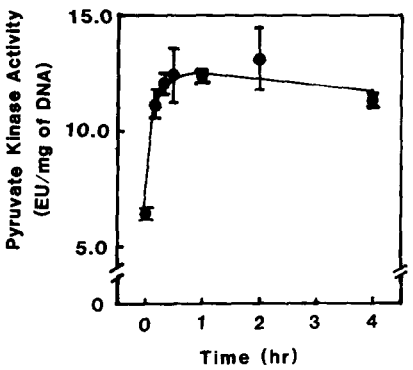

Fig. 3. Maintenance of pyruvate kinase activity in hepatocytes when exposed to dexamethasone for increasing time intervals during attachment. Hepatocytes were isolated from a normal rat and attached to collagen gels at 37°C for 4 h with 1 μM dexamethasone present for the time intervals noted. Following the attachment period the cells were maintained in medium containing 1 μM insulin for four days. The points represent the mean and standard deviation of pyruvate kinase activity from three plates.

pyruvate kinase activity throughout the subsequent 4 days in culture.

The dose response of pyruvate kinase activity to dexamethasone concentration is shown in Fig. 4. Hepatocytes were isolated from a normal rat, attached to collagen gels in the absence of hormones and maintained in the presence of medium A containing 1 μM insulin and various concentrations of dexamethasone. The cells were maintained in this manner for 4 days then harvested and the pyruvate kinase activity was determined. A concentration of dexamethasone of 1.0 μM consistently resulted in the maximal maintenance of the liver pyruvate kinase activity. Half maximal effects were observed at $\simeq 10^{-8}$ M dexamethasone.

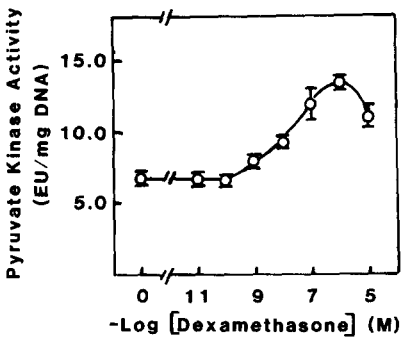

Fig. 4. The effect of dexamethasone concentration on pyruvate kinase activity in hepatocytes. Hepatocytes were isolated from a normal rat and attached to collagen gels at 37°C for 4 h in the absence of hormones. After 4 h the medium was replaced with fresh medium A containing 1 μM insulin and the concentrations of dexamethasone as indicated. Each point represents the mean and standard deviation of pyruvate kinase activity from three plates 4 days later.

The ability of other glucocorticoids to maintain the level of pyruvate kinase activity in hepatocytes was also investigated. Cells were isolated from a normal rat and attached to collagen gels for 4 h in medium A containing no hormone additions. The medium was then replaced with fresh medium A which contained 1 μM insulin and 1 μM of various glucocorticoids. The hepatocytes were cultured for 4 days, harvested, and the total liver pyruvate kinase activity was measured. All glucocorticoids tested resulted in a similar ability to maintain the level of pyruvate kinase activity in cultured hepatocytes (Table II).

The ability of insulin and glucocorticoids to maintain pyruvate kinase activity in hepatocytes in culture may require protein synthesis. The pyruvate kinase activity of cells cultured in the presence of both insulin and dexamethasone and in the presence of either actinomycin D or cycloheximide

TABLE II

THE EFFECT OF VARIOUS STEROID HORMONES ON THE MAINTENANCE OF PYRUVATE KINASE ACTIVITY IN HEPATOCYTES

Hepatocytes were isolated from a normal rat, attached to collagen gels in the absence of hormones, and maintained at 37°C in medium A containing 1 μM insulin and 1 μM of the steroid hormone indicated. After 4 days in culture the cells were harvested and assayed for pyruvate kinase activity. The steroids were dissolved in ethanol and the final ethanol concentration in the culture medium was 0.05%. The values are the mean ± S.D. from at least three plates.

Glucocorticoid	Pyruvate kinase activity (EU/mg of DNA)
None	5.9 ± 1.0
Dexamethasone	16.1 ± 1.3
Hydrocortisone	11.9 ± 0.2
Prednisolone	13.5 ± 1.9
Triamcinolone	13.1 ± 0.9

for two days was 25% lower than the corresponding enzyme activity of cells cultured in the absence of protein synthesis inhibitors. This observation suggests that the synergistic ability of these hormones, insulin and dexamethasone, to maintain the activity of pyruvate kinase in primary cultures of hepatocytes requires active protein synthesis.

Discussion

Pyruvate kinase is a key enzyme in carbohydrate metabolism in liver and its activity fluctuates during dietary [1–4,9] or hormonal stress [5–12,28]. Glucocorticoids have been reported to depress liver pyruvate kinase activity in vivo [1,16,17]. However, Blair and Kletzien [18], report no difference in pyruvate kinase activity in hepatocytes maintained in culture for 48 h and in the presence of dexamethasone for 12 h before harvesting. In the studies reported here the effect of glucocorticoids upon pyruvate kinase activity in long term cultures of hepatocytes was investigated. The presence of both dexamethasone and insulin were required to maintain pyruvate kinase activity at a constant high level (similar to values measured in vivo) throughout 1 to 2 weeks in culture. When cells were cultured in the presence of either hormone alone a significant decrease in the pyruvate kinase activity resulted with apparent half-lives of between 2 and 4 days.

The permissive effects of glucocorticoids on the stimulation of gluconeogenesis in the liver by glucagon and epinephrine is well documented [19]. The mechanism by which glucocorticoids exert their action is not clear, since these steroids are not required for cAMP generation or for protein kinase activation [29]. Consequently, it has been proposed that glucocorticoids are involved in the maintenance of the normal intracellular ionic environment [30]. Cortisol and insulin are reported to act in antagonism [2]. However, apparently under some circumstances glucocorticoids and insulin act cooperatively. It has been suggested that the early gluconeogenic effect of glucocorticoids on hepatic glycogen synthesis is insulin dependent [31,32], while other evidence supports an antagonistic relationship between glucocorticoids and insulin in muscle tissue [33].

In the present experiments the glucocorticoid, dexamethasone, and insulin acted cooperatively in maintaining the pyruvate kinase activity in primary cultures of hepatocytes. Exposure of hepatocytes to dexamethasone during their attachment to collagen gels was sufficient to maintain maximal pyruvate kinase activity if following attachment, insulin was incorporated into the culture medium. Likewise, exposure of hepatocytes to insulin during the attachment period was sufficient to maintain maximal liver pyruvate kinase activity if following attachment dexamethasone was incorporated into the culture medium. In addition, when cells were attached in the absence of hormones, maximal liver pyruvate kinase activity was maintained only when hepatocytes were cultured in the presence of both insulin and dexamethasone.

When dexamethasone was included in the hepatocyte culture medium fibroblasts were not evident. Dexamethasone supplementation has been reported to increase longevity and maintain the polygonal epithelial morphology [34]. The optimal concentration of dexamethasone in the culture medium was 1 μM which resulted in maintenance of the pyruvate kinase activity. There was a decrease in the enzyme activity when 10 μM dexamethasone was used in the culture medium. Bonney et al. [35] have reported decreased activity of tyrosine aminotransferase in liver parenchymal cells cultured in 10 μM dexamethasone. Hepatocytes have been reported to rapidly metabolize cortisol, 66% within 30 min [36]. A rapid incorporation or metabolism of dexamethasone could explain the observation that the presence of dexamethasone for only 10 min during attachment was sufficient to maintain the pyruvate kinase activity throughout 4 days in culture. The ability of dexamethasone to maintain liver pyruvate kinase activity in hepatocytes constant for over a week in culture [9] seems to be a general glucocorticoid effect, since three other glucocorticoids yielded similar results. In contrast, in primary cultures of hepatocytes maintained in 1 μM insulin and 1 μM dexamethasone, the glucokinase activity decreased from 180 to 4 enzyme units/mg of DNA, and hexokinase activity increased from 2 to 6 enzyme units/mg of DNA over 4 days in culture [37]. Under similar culture conditions, the tyrosine

aminotransferase of liver parenchymal cells was induced to 3–4-fold over basal levels within 24 h [35]. The addition of 60 μg of cyclohexamide per ml of culture medium completely prevented the induction of tyrosine aminotransferase by dexamethasone [35]. The observation that the presence of either actinomycin D or cyclohexamide decreased the pyruvate kinase activity in hepatocytes cultured in both dexamethasone and insulin for two days, suggests that the action of these hormones represents the ability of these cells to maintain protein synthesis.

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