Regulation of Glucose Synthesis in Hormone-Sensitive Isolated Rat Hepatocytes

(glucagon/insulin/pyruvate/pyruvate dehydrogenase/calcium ionophore)

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ABSTRACT A simplified procedure was developed for isolation of intact, hormone-sensitive liver cells in a high and reproducible yield. These cells produce glucose from various precursors at rates comparable to those achieved in isolated perfused liver. Glucagon enhanced glucose synthesis from pyruvate, dihydroxyacetone, fructose, or xylitol more effectively at low than at high substrate concentration. At high pyruvate concentrations (>2 mM), glucagon or adenosine 3'5-cyclic monophosphate (0.1 mM) exerts a curious inhibition of gluconeogenesis that can be reverted to stimulation on addition of ethanol. It is suggested that glucagon and cyclic AMP inhibit pyruvate dehydrogenase and thus limit the supply of reducing equivalents needed for glucose formation. Supporting evidence for hormonal control of pyruvate dehydrogenase in isolated liver cells is provided by the fact that glucagon decreases and insulin increases decarboxylation of [1-14C]pyruvate. Calcium salts (1.3 mM) enhance glucose formation from pyruvate but greatly enhance the inhibition exerted by the divalent cationophore, A23187. Inhibition by glucagon of glucose synthesis from pyruvate is additive with the effects of A23187 + Ca++. However, with dihydroxyacetone as substrate, glucagon partially reverses the inhibition exerted by A23187 + Ca++. The results are consistent with glucagon effecting an inhibition of pyruvate dehydrogenase and a stimulation of hexosediphosphatase activities.

The use of isolated perfused livers has dominated investigations of gluconeogenesis despite the complexity of equipment required, the limited number of experiments possible in a given time, and the variability between livers from similarly-treated animals of uniform breeding. More recently, the introduction by Berry and Friend (1) and Ingebreten and Wagle (2) of improved techniques for isolation of hepatocytes has led many investigators to adopt these cells for the study of liver metabolism. These cells synthesize glucose from various substrates at rates comparable to those occurring in isolated, perfused livers (1-3). Hepatocytes exposed to glucagon increase their content of adenosine 3'5-cyclic monophosphate (4, 5) but increase their rate of gluconeogenesis only slightly and after a long lag-period (5). A fast action of glucagon on gluconeogenesis and glycoenolysis in isolated hepatocytes was recorded by Garrison and Haynes (6), but no detailed report has appeared. Thus the relatively small number of publications dealing with hormonal regulation of metabolic processes in isolated hepatocytes seemed to reflect the difficulty of preserving cell membranes and their hormone-receptor sites during enzymic digestion of connective tissues and intercellular cementing substances.

We therefore investigated conditions required for preparation of glucagon-responsive hepatocytes and report here the simplified procedure that was developed. Some previously unrecognized effects of glucagon on gluconeogenesis from pyruvate are described.

MATERIALS AND METHODS

Male rats (250-300 g, Sprague-Dawley, Madison, Wisc.) were fasted 24 hr before experimentation. Crystalline insulin and glucagon (gifts from Dr. W. Bromer, Eli Lilly Co., Indianapolis) were prepared as fresh solutions in calcium-free Krebs-Henseleit bicarbonate buffer (7), containing 1.5% gelatin (Difco Laboratories). We shall call this solution "Buffer K-H." Standard solutions of substrates such as fumarate (Boehringer Mannheim Corp.), glucose (Matheson, Coleman and Bell), and glutamic acid (Mann Research Lab.), which were cofactors in the Modified (Ca++ free) Krebs improved Ringer Buffer 1 Buffer (7) ("Buffer K-R") used for perfusion, were kept frozen at -25°. The fourth component of the perfusate buffer, pyruvate (Boehringer Mannheim Corp.), was always prepared immediately before use. Other substrates used for incubations were dihydroxyacetone, xylitol, dL-β-hydroxybutyrate (Sigma), and fructose (Nutritional Chemical Corp.). Collagenase (CLS 9 LC, 144 U/mg and CLS OIE, 133 U/mg) was from Worthington Biochemical Corp. Hyamine hydroxide and glass scintillation vials for incubations and counting were obtained from Research Products International Corp. The ionophore, A23187 (a gift from Dr. Robert Hamill, Eli Lilly Co., Indianapolis), was dissolved in dimethylformamide (1 µg/µl). [1-14C]pyruvate (Amersham/Searle) (13.1 Ci/mol) was freshly diluted before the experiment with nonlabeled Na-pyruvate.

Hepatocytes Were Isolated by modifications of the methods of Berry and Friend (1), and Ingebreten and Wagle (2). Isolated liver was perfused at 37° for 15 min with calcium-free Buffer K-R without added collagenase. The first 50 ml of perfusate were discarded to eliminate erythrocytes and serum proteins. The final volume of the recirculating medium was 100 ml. The medium was gassed by bubbling 95% O2-5% CO2 directly into the main reservoir at a rate of 2-3 liters/min. After this preliminary period, 30 mg of collagenase in 2 ml of 0.9% NaCl solution was added and the perfusion was continued at a flow rate of at least 100 ml/min for 10-15 min. When the liver was extensively swollen, the major blood vessels and adventitious tissues were trimmed off and the organ was minced with a scissors in 20 ml of calcium-free Buffer K-R containing 1.5% gelatin. During all manipulations 95% O2-5% CO2 was bubbled into the cell suspensions at
frequent intervals. The crude suspension was filtered through a layer of cheesecloth by applying a slight stirring motion with a Teflon pestle. The cell suspension from one liver (total volume about 40 ml) was washed four times with 30 ml of calcium-free Buffer K-H containing 1.5% gelatin, by sedimenting the cells in a centrifuge at 50 X g for 75-90 sec. The cells were suspended in this same medium and counted in a Neubauer hemocytometer. The volume was adjusted to obtain 6.5 X 10^6 cells per ml. The number of cells originating from 1 g of liver wet weight was determined by measuring the dry weight of liver tissue, connective tissue, and isolated cells, and the intra- and extracellular water space. We estimated that 1 g of wet liver weight contains 9.8 ± 1.5 (SEM) X 10^6 parenchymal cells (n = 5). The cells recovered after the washing procedure were about 70% of the total in the cheesecloth filtrate. The cells were >90% microscopically intact and >95% excluded trypan blue.

Incubations were performed on a Dubnoff shaker (90 cycles/min) at 37° in 2.5 X 5-cm glass vials containing 1.5 ml of Buffer K-H (+ 1.5% gelatin) and 3.25 X 10^6 cells. The vials containing buffer and substrate were gassed for 5 min with 95% O2-5% CO2, the cells were added, the vials capped, and incubation was begun. Reactions were stopped by addition of perchloric acid to a final concentration of 0.33 N, and the mixture was centrifuged for 15 min at 1700 X g.

Glucose was determined on aliquots of the supernatant solution with glucose oxidase (Boehringer Mannheim Corp.).

\[ ^{14}\text{CO}_2 \text{ Collection.} \] After hepatocytes were incubated with [\(^{14}\text{C}\)]pyruvate (0.17 Ci/mol), CO\(_2\) was collected in hyamine injected into a plastic cup suspended from a rubber cap on the vial. CO\(_2\) was displaced from the medium by injecting 0.2 ml of 4 N perchloric acid through the rubber cap and continuing the incubation for an hour. The cups were then placed in scintillation vials containing 10 ml of toluene-PPO(2,5-diphenyloxazole)-POPOP (1,4-bis[2-(4-methyl-5-phenyl-oxazolyl)]-benzene) and counted in a LS250 Beckman scintillation counter.

RESULTS

Isolation and Incubation of Liver Cells. Hormone-sensitive rat-liver cells were successfully isolated in a high and reproducible yield. Continual nourishment was provided during the recirculating perfusion by the substrates supplied in the perfusate (Buffer K-R1). Hank's solution and the incubation after perfusion, with enzymes (1) were eliminated. Several other modifications were recognized as important for obtaining hormone sensitivity, sufficient digestion, and high yield: (a) flow rate through the liver during perfusion should be above 100 ml/min for an 8- to 10-g rat liver; (b) 95% O\(_2\)-5% CO\(_2\) should be bubbled directly into the hemoglobin-free perfusate of the central reservoir; and (c) 10-15 min of preliminary perfusion in the absence of collagenase improves digestion (2). Calcium-free solutions were used throughout the isolation procedure for the following reasons: (a) calcium present in the perfusate led to rapid digestion (8) but induced heavy aggregation and destruction of cells (This detrimental effect of calcium appears only in the presence of collagenase and not when calcium-containing solutions are used for the washing procedure after enzymatic digestion.); (b) calcium in the washing and incubation medium did not essentially alter.
hormone sensitivity, and influenced glucoenic rate (see Fig. 8B) only slightly. Albumin (Pentex, fraction V, fatty acid-free) was eliminated from the washing steps and incubation since it seemed to inhibit gluconeogenesis. The inclusion of 1.5% gelatin in Buffer K-H provides the desired viscosity and protection of the cells.

Gluconeogenesis from Various Substrates. Isolated liver cells produced glucose from various precursors (10 mM) at rates varying from 3 μmol/min per g with fructose to 0.5 μmol with pyruvate. The rates of glucose formation were in the order fructose > dihydroxyacetone > xylitol > pyruvate. Glucagon enhanced glucose synthesis from each of these substrates but to different extents and with differing optimal conditions.

Gluconeogenesis from Fructose, Dihydroxyacetone, or Xylitol. The effect of glucagon on gluconeogenesis from fructose and dihydroxyacetone in isolated perfused livers has been controversial. Ross et al. (9) and Exton and Park, (10, 11), using high concentrations of substrates, found this hormone to be without significant effect whereas Veneziale (12, 13), using 2-4 mM substrate, found glucagon to enhance the initial rates of glucose formation and Blair et al. (14) found glucagon to increase the total yield of glucose from dihydroxyacetone and xylitol.

With isolated hepatocytes, glucagon (3 μg/ml) enhanced glucose formation from moderate concentrations of fructose, dihydroxyacetone, and xylitol (Figs. 1 and 2). Stimulation by glucagon is about 100% with 5 mM dihydroxyacetone and decreases to 80% at 10 mM. Glucagon did not enhance the initial rate of gluconeogenesis from xylitol or 10 mM fructose (not shown) but more nearly maintained the initial rate when substrate became limiting.

In other experiments, negligible quantities of glucose were formed from added fructose 1,6-diphosphate, which indicates that the integrity of the plasma membranes is retained in these hepatocytes.

Gluconeogenesis from Pyruvate. Rates of glucose formation from varying concentrations of pyruvate are shown in Fig. 3. Glucagon influenced glucose synthesis in an unexpected manner. At low concentrations (1-2 mM) of pyruvate, glucagon effects were not apparent initially but the initial rates of gluconeogenesis were better maintained in the presence of glucagon than in its absence. Above 2 mM pyruvate, glucagon inhibited gluconeogenesis (Fig. 3C and D). The effects of 3 μg of glucagon per ml are mimicked by 0.1 mM adenosine 3',5'-cyclic phosphate (Fig. 4), which indicates that the effects of glucagon may be mediated through the cyclic nucleotide.

Pyruvate, Ethanol, and dl-β-Hydroxybutyrate. We assumed that the rate of gluconeogenesis from pyruvate at high concentrations was probably limited by a general deficiency of reducing equivalents in mitochondria and cytosol. The effect of glucagon on glucose formation from 10 mM pyruvate in the presence of 5 mM ethanol or 20 mM dl-β-hydroxybutyrate (Fig. 5) was examined. Ethanol increased gluconeogenesis from pyruvate by about 60% at 40 min, and glucagon...
in the presence of ethanol stimulated gluconeogenesis above the value with ethanol alone. Thus under these conditions ethanol permitted the expression of hormonal stimulation. Addition of dl-β-hydroxybutyrate (Fig. 5) to the incubation medium in the presence of 10 mM pyruvate also increased glucose formation for the first 20 min, but thereafter the rate was not different from the control. Glucagon in the presence of dl-β-hydroxybutyrate displayed no further stimulation.

Thus, supplying reducing equivalents directly to the cytosolic NAD by alcohol dehydrogenase gave the greater enhancement of gluconeogenesis and permitted glucagon stimulation. β-Hydroxybutyrate, which supplies reducing equivalents to the mitochondria and indirectly to the cytosol by malate, completely overcame the inhibitory effect of glucagon but did not permit glucagon to stimulate gluconeogenesis.

**Glucagon, Insulin, and Pyruvate Decarboxylation.** Pyruvate decarboxylation must be considered the essential step for generation of reducing equivalents for gluconeogenesis from pyruvate when this substrate is at high (5–10 mM) concentrations. At low pyruvate concentrations, there is a smaller sink for reducing equivalents and small amounts of endogenous substrates may provide some electrons for generating NADH. If glucagon were to inhibit pyruvate dehydrogenase, a decrease of gluconeogenic rates would be expected. Fig. 6 shows the effects of glucagon and insulin on gluconeogenesis from 5 mM pyruvate (A) and on decarboxylation of [1-14C]-pyruvate (B) under identical conditions. Insulin exerted no significant effect on glucose synthesis either in the presence or absence of glucagon. However, insulin increases and glucagon decreases decarboxylation of [1-14C]-pyruvate (Fig. 6B). These striking effects indicate that glucagon as well as insulin imposes a control upon pyruvate dehydrogenase in rat liver.

**Calcium and Ionophore A23187.** Since pyruvate dehydrogenase was implicated in the hormonal control of gluconeogenesis from pyruvate (Fig. 6) and because glucagon initiated ion movement in rat liver (15), the effect of calcium and the ionophore A23187 (16) was studied in isolated liver cells (Figs. 7 and 8). The presence of 1.3 mM CaCl₂ led to a small increase in gluconeogenic rate and amplified slightly the inhibition induced by glucagon (Fig. 7B). We observed that A23187 at 1.3 μg/ml induced almost precisely the same degree of inhibition of glucose formation from pyruvate in calcium-free medium (Fig. 7C) as did glucagon (Fig. 7A). This effect was also obtained by using only 0.7 μg of the ionophore per ml (data not shown). When calcium ions were added in the presence of A23187 a striking inhibition of glucose formation from pyruvate was obtained (Fig. 7D). Under these conditions glucagon further additively decreased glucose synthesis to a very low rate. To compare these effects of A23187 and calcium ions a second substrate, dihydroxyacetone, was chosen which does not involve pyruvate dehydrogenase in its gluconeogenic pathway (Fig. 8A–D). Calcium in the medium did not significantly influence the course of glucose formation either in the presence or absence of glucagon (Fig. 8B). A23187 alone or in the presence of calcium ions had only small effects on the course of glucose formation (Fig. 8C). When glucagon was added in the presence of A23187 with or without calcium, glucose formation was stimulated during the first 20 min but ceased thereafter.

**DISCUSSION**

**Substrate Concentration and Hormonal Stimulation.** In isolated hepatocytes the degree of glucagon stimulation of glucose formation from pyruvate, dihydroxyacetone, fructose, or xylitol depends on substrate concentration (Figs. 1–3). This may in part explain Exton and Park’s (10) lack of a glucagon response in livers perfused with 30 mM fructose or 40 mM dihydroxyacetone, whereas Veneziale (12, 13) found glucagon to stimulate gluconeogenesis from 2 mM fructose and 4 mM dihydroxyacetone or glyceroldehyde. In this laboratory, glucagon enhanced glucose formation from

**Fig. 7.** Influence of glucagon (3 μg/ml), A23187, (1.3 μg/ml), and CaCl₂ (1.3 mM) on gluconeogenesis from 10 mM pyruvate. ○, Control; A, A23187; ○, glucagon; A, A23187. (B) Pyruvate + CaCl₂; ○, glucagon. (D) Pyruvate + CaCl₂ + A23187; A, glucagon.

**Fig. 8.** Effect of glucagon (3 μg/ml), A23187 (1.3 μg/ml), and CaCl₂ (1.3 mM) on gluconeogenesis from 5 mM dihydroxyacetone. Solid symbols, control; open circles, glucagon; open triangles, CaCl₂. (A) Dihydroxyacetone; (B) dihydroxyacetone + CaCl₂; (C) dihydroxyacetone + A23187; (D) dihydroxyacetone + A23187 + glucagon.
dihydroxyacetone (14), xylitol (14), and d-xylulose (Cook, D. E., Gillillan, C. and Lardy, H. A., unpublished results) at concentrations up to 10 mM. With all substrates, glucagon strikingly inhibited lactate formation from the added substrate. This might have been mediated by an inhibition of glycolytic enzymes or indirectly by an activation of a gluconeogenic enzyme such as hexosediphosphatase. If the affinity of hexosediphosphatase for its substrate were enhanced through an influence of glucagon, an increased flux could be expected, especially at low concentration of fructose diphosphate. Blair et al. (14) described, in the presence of dihydroxyacetone, a drastic fall of hepatic fructose 1,6-diphosphate concentration after glucagon which could be the result of increased flux of sugar phosphates through hexosediphosphatase towards glucose. Xylitol enters the gluconeogenic pathway through d-glyceraldehyde 3-phosphate as well as through fructose 6-phosphate (17). Assuming that fructose diphosphatase is one of the hormonally regulated enzymes, any stimulation at this step would affect only one part of the overall intermediate supply for glucose synthesis. The glucagon-induced increment of glucose formation from xylitol would therefore be expected to be rather small in comparison to that from fructose or dihydroxyacetone as a precursor. This is, in fact, what we observed at xylitol concentrations as low as 2.5 mM.

Glucagon, Pyruvate, and Pyruvate Dehydrogenase. High concentrations of pyruvate would lead to the following metabolic changes in rat liver (10): increase of NAD/NADH ratio in cytosol because pyruvate is reduced to lactate by this system; the consequent relative deficiency of reducing equivalents in the cytosol causes the triosephosphate dehydrogenase step to become rate limiting for gluconeogenesis (18); and activation of pyruvate dehydrogenase in mitochondria (19) with increased pyruvate oxidation. In isolated hepatocytes, oxidizable metabolites are probably largely removed by diffusion during washing. Pyruvate oxidation would then be essential for generating reducing equivalents for transport of oxalacetate as malate into the cytosol where these reducing equivalents are used at the triosephosphate dehydrogenase step. Addition of glucagon under these circumstances led to a paradoxical inhibition of glucose formation (Figs. 3, 4, and 6). The simplest interpretation of this effect would be to assume a hormonally induced inhibition of pyruvate dehydrogenase which would prevent alleviation of the relative deficiency of NADH in the hepatocytes. Support for the role of pyruvate dehydrogenase in the glucagon-induced inhibition of glucose synthesis is the finding that glucagon inhibits decarboxylation of [1-14C]pyruvate (Fig. 6B). Addition of ethanol stimulated gluconeogenesis from pyruvate at 10 mM and permitted glucagon to induce a further increase above the ethanol-stimulated glucose formation (Fig. 5). In isolated liver perfusion (20) and in vivo (21), addition of ethanol leads to a rapid decrease of the NAD/NADH ratios in cytosol and mitochondria. Thus, the reducing equivalents supplied by ethanol to the mitochondria and cytosol will facilitate reduction of oxalacetate to malate and of diphosphoglycerate to triosephosphate. A glucagon-induced stimulation at the fructose diphosphatase level could now result in an enhanced flux of intermediates to glucose.

Pyruvate dehydrogenase, Calcium, and Ionophore, A23187. Glucagon induced a rapid, transient release of calcium and potassium ions from isolated perfused rat liver (15). This ion movement preceded the hormonal effect on gluconeogenesis. However, a wide variation in the calcium concentration of the perfusate did not significantly influence basic rates of glucose formation or hormonal effects in isolated perfused liver (22). This result agrees with our finding that calcium in the external medium did not cause great quantitative changes in glucose formation or hormone action (Fig. 8).

Calcium ions were observed to stimulate partially purified pyruvate dehydrogenase (23) by activation of pyruvate dehydrogenase phosphatase (24, 25). Insulin administered in vivo was reported to activate liver pyruvate dehydrogenase (26), but glucagon and insulin had no effect on the enzyme activity in isolated perfused liver (19). The decrease and increase of [1-14C]pyruvate decarboxylation observed in the presence of glucagon or insulin, respectively, indicates that the liver pyruvate dehydrogenase is under hormonal control (Fig. 6B). The effect of glucagon could be mediated by phosphorylation of the pyruvate dehydrogenase through its intrinsic protein kinase. A simultaneous release of intracellular calcium as observed in the isolated perfused liver (15) could result in inactivation of the pyruvate dehydrogenase phosphatase and favor a shift of the phosphorylation-dephosphorylation balance toward an inhibited, phosphorylated pyruvate dehydrogenase. Part of the stimulatory effect of insulin on pyruvate decarboxylation (Fig. 6B) could be mediated by increased calcium uptake with subsequent activation of pyruvate dehydrogenase phosphatase as has been discussed by Martin et al. (27) for the adipose tissue enzyme.

That ion movement can participate in regulation of pyruvate dehydrogenase is also indicated by the glucagon-like inhibitory effect of the ionophore A23187 on gluconeogenesis from pyruvate (Fig. 7C). A23187 can, under specific conditions, increase calcium and potassium efflux from mitochondria and calcium influx with potassium release in rat erythrocytes (16, 28). Our experiments suggest that in the absence of added calcium, A23187 may induce ion movements similar to those brought about by glucagon, leading to inactivation of pyruvate dehydrogenase phosphatase through calcium depletion (29). In the presence of calcium in the medium, A23187 induced a striking inhibition of glucose formation from pyruvate, and glucagon enhanced this inhibitory effect (Fig. 7D). Presumably, A23187 in the presence of added calcium leads to uncontrolled calcium influx with a striking inhibition of gluconeogenesis because of adverse effects on mitochondrial phosphorylation (16). The actions of A23187 and glucagon are quite different when dihydroxyacetone is the substrate (Fig. 8). When glucagon and A23187 are both present there is a stimulation of gluconeogenesis in the first 20 min followed by an abrupt cessation of glucose formation.

These findings suggest roles for glucagon and calcium in regulating pyruvate dehydrogenase in the mitochondria. Glucagon also affects gluconeogenesis at other sites—presumably cytosolic enzymes—as is evidenced in experiments with dihydroxyacetone or xylitol as substrates for hepatocytes.

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