GLUCAGON REGULATION OF PYRUVATE KINASE IN RELATION TO PHOSPHOENOL PYRUVATE SUBSTRATE CYCLING IN ISOLATED RAT HEPATOCYTES

Michael G Clark
Clinical Biochemistry Unit
School of Medicine
Flinders University of South Australia
Bedford Park SA 5042 AUSTRALIA

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Summary: The rate of flux through pyruvate kinase in isolated rat hepatocytes has been estimated by a new procedure involving direct spectrophotometric measurement of pyruvate production by liver cells suspended in an oxygenated medium containing lactate dehydrogenase and NADH. For the substrates, glucose, dihydroxyacetone, fructose, propionate and galactose only the rate of pyruvate production from glucose and galactose was inhibited by the addition of 1 μM-glucagon. These results imply that glucagon mediates glycolytic flux at a point in the pathway preceding the point of entry of fructose and dihydroxyacetone and not at pyruvate kinase.

Introduction

Liver contains very high activities of pyruvate kinase (PK) (1). Thus for gluconeogenesis to proceed effectively from substrates entering the pathway below phosphoenol pyruvate carboxykinase it is essential that flux through PK be reduced to a minimum. Some evidence exists to indicate long-term (1) and short-term (2,3,4) changes in catalytic activity of PK in relation to changes in dietary and hormonal status. In addition, Rognstad (5) has recently claimed that cyclic AMP induces short-term inhibition of pyruvate kinase flux in the intact liver cell. However, as the latter studies cannot exclude the possibility of indirect effects of cyclic AMP at other sites in the gluconeogenic-glycolytic pathway (e.g. stimulation of flux through FDPase), there still remains no direct evidence that acute hormonal regulation of hepatic gluconeogenesis is mediated by changes in flux rate through pyruvate kinase.

In the present study a new approach has been used to assess the flux rate through PK. The method involves direct spectrophotometric measurement

of pyruvate production by liver cells suspended in an oxygenated medium containing lactate dehydrogenase (LDH) and NADH. By using this method the rate of pyruvate production was determined for several substrates in the presence and absence of glucagon and the potential control of pyruvate kinase flux was assessed.

Methods

Isolated rat liver cells were prepared from 24 hour fasted animals essentially as described by Berry and Friend (6), with the omission of hyaluronidase and substitution of Krebs-Henseleit saline buffer pH 7.4 (7). For the direct spectrophotometric measurement of the rate of pyruvate plus lactate formation incubations were conducted in 3 ml glass cuvettes of 1 cm light path. The cells together with buffer, NADH, NAD^+, dialyzed lactate dehydrogenase, substrates and other additions were maintained at 37°C in a thermostatically regulated cell carriage of a Carl Zeiss PMQ II recording spectrophotometer. To ensure gentle and even mixing of the cell suspension with minimal interruption to spectrophotometric measurement of NADH oxidation an automatic cuvette mixer was used. Each cuvette was in the light path for 15 seconds and was continuously mixed at the rate of 30 strokes/min. Rates of oxidation of NADH were continuously recorded and were measured when linear. This usually occurred after 8 min and continued for up to 20 min. Sufficient oxygen was present in the buffer for approximately 25 min metabolism. Specificity of the method relied upon preparations of rat liver cells that did not leak enzymes. In particular, the system was concluded to be satisfactory if omission of either lactate dehydrogenase or cells from a reaction mixture containing glucose, cells, lactate dehydrogenase, NADH and NAD^+ gave similar and low rates.

Incubations, to which NADH, NAD^+ and LDH had not been added, were conducted as described previously (8). Following a 30 min incubation period at 37°C the reactions were stopped by the addition of 0.5 ml 6% (w/v) $HClO_4$. Precipitated material was removed by centrifugation and the supernatant solution neutralized (methyl orange) with 1M-KOH. Analyses for glucose (9), lactate (10) and pyruvate (11) were conducted on the neutralized perchlorate extract. The rate of pyruvate plus lactate formation is expressed as µmoles of 3-carbon units per min x g^{-1} wet wt. of liver, where 1 g wet wt. of liver is assumed to be equivalent to 10^8 parenchymal cells (12).

To test the extent to which lactate formed extracellularly from the reduction of pyruvate, was utilized, incubations containing 0.1 µmoles L-[U-^{14}] lactate, cells, lactate dehydrogenase, NADH, and NAD^+ were conducted at 37°C for 15 min. [U-^{14}C] Lactate was isolated from the acidified reaction mixture in a manner similar to that described by Rognstad (5).

Results and Discussion

The fundamental assumptions of this study are (i), that all pyruvate formed within the cell is free to move outside and be reduced to lactate and (ii), that lactate once formed outside the cell no longer participates in metabolic events within the cell. Two aspects of the data suggest

that these assumptions are valid. Table 1 shows that the rates of pyruvate plus lactate formation from coupled (i.e. incubation of cells in medium containing a pyruvate trapping system of lactate dehydrogenase and NADH) and free incubations compare favourably. The exception was propionate. For this substrate the rate of pyruvate plus lactate formation in the

TABLE 1

Effect of glucagon on pyruvate plus lactate formation in coupled and free incubations with isolated rat hepatocytes

Isolated liver cells (equivalent to 10 mg wet wt. of liver) from 24 hour-fasted rats were added to cuvettes containing 2 ml of gassed Ca^{2+}-free Krebs Henseleit saline buffer plus 1 µmole NADH, 0.1 µmole NAD^+, 250 units of lactate dehydrogenase, the additions indicated and incubated with constant gentle mixing at 37°C in the cell carriage of a recording spectrophotometer. In these coupled incubations the oxidation of NADH due to pyruvate formation was monitored at 340 nm. For free incubations the reaction mixture contained liver cells (equivalent to 30 mg wet wt. of liver) in 1.5 ml of Ca^{2+}-free Krebs Henseleit saline buffer and the additions indicated. Free incubations were maintained at 37°C for 30 min at which time 0.5 ml 6% (w/v) $HClO_4$ was added. Analyses for lactate and pyruvate were conducted on the neutralized perchlorate extract of the cells and suspending medium. Glucagon (1 µM) was used in these experiments. Mean values ± S.E.M. are given with the no. of experiments shown in parentheses.

	Net rate of pyruvate plus lactate formation (µmoles × min^{-1} × g^{-1} wet wt. of liver)			
	Coupled Incubations		Free Incubations	
	Control	+ Glucagon	Control	+ Glucagon
Final conc. of additions				
5mM-Glucose	0.10±0.02(3)	0.03±0.01(3)	0.08±0.01(5)	0.02±0.005(5)
2.5mM-Galactose	0.08±0.01(3)	0.02±0.01(3)	0.12 (2)	0.01 (2)
2.5mM-Fructose	0.26±0.02(3)	0.26±0.02(3)	0.22±0.02(3)	0.07±0.01 (3)
5mM-Fructose	0.60±0.05(4)	0.60 (2)	0.45 (2)	0.26 (2)
10mM-Fructose	1.24±0.30(3)	1.22 (1)	1.48 (2)	1.22 (2)
5mM-Dihydroxyacetone	0.11±0.02(3)	0.10±0.02(3)	0.15±0.02(3)	0.08±0.01 (3)
5mM-Propionate	0.20±0.03(3)	0.21±0.03(3)	0.04±0.003(3)	0.03±0.003(3)
5mM-Glucose + 1.1mM-$CaCl_2$	0.35 (2)	0.25 (2)		
5mM-Fructose + 1.1mM-$CaCl_2$	0.83 (2)	1.05 (2)		

coupled incubation markedly exceeds that from the free incubation. Secondly the assumption that lactate once formed outside the cell cannot enter into further reactions is supported by the observation that the rate of lactate utilization when added to a coupled incubation is less than 0.005 μmoles x min^{-1} x g^{-1} wet wt. of liver (results not shown).

For the coupled incubations 1 μM-glucagon gave rise to a decreased rate of pyruvate plus lactate formation from glucose and galactose but was without effect on that produced from fructose, dehydroxyacetone, propionate (Table 1) or alanine (results not shown). Providing that all pyruvate produced from each substrate is freely accessible to the extracellular lactate dehydrogenase and NADH these results imply that hormonal control is exercised at a point in the glycolytic pathway prior to the point of entry of fructose, possibly phosphofructokinase (8). For fructose, dihydroxyacetone, and propionate the effects of glucagon on pyruvate plus lactate formation in coupled and free incubations are opposite. In free incubations the hormone gave rise to a marked decrease in the rate from all substrates. It is tempting to speculate that it is the stimulation of FDPase by glucagon with the concomitant inhibition of PFK that produces a marked decrease in pyruvate plus lactate formation in the free incubations and that for the coupled incubations, continual flow of pyruvate out of the cell over-rides the redirecting effect of the glucagon-mediated FDPase-PFK substrate cycle.

The inclusion of Ca^{2+} in an other wise "Ca^{2+}-free" system did not change the pattern of the results. Indeed the measured flux rates through pyruvate kinase were increased by the inclusion of 1.11mM-$CaCl_2$ and rather than a glucagon-mediated inhibition of pyruvate plus lactate formation from fructose the rate was stimulated. Thus the failure to detect a glucagon-mediated inhibition of pyruvate kinase flux does not result from the use of Ca^{2+}-free buffers.

To assess the effect of glucagon on the rate of flux through two

arms of the pyruvate kinase-pyruvate carboxylase-phosphoenol pyruvate carboxykinase substrate cycle, the rates of glucose synthesis and pyruvate plus lactate formation were measured from propionate (Table 2). For the coupled incubation approximately 0.48 parts proceeded to glucose formation and 0.52 parts to pyruvate plus lactate.

The inclusion of 1 μM-glucagon resulted in a 52% increase in the rate of production of glucose with no change in the rate of pyruvate plus lactate formation. Most significantly, glucagon did not act to increase glucose production at the expense of pyruvate plus lactate formation. These results also imply that acute control at pyruvate kinase did not occur. For the free incubation 1 μM-glucagon also resulted in an increased rate of glucose synthesis (Table 2) and indeed the increment in gluconeogenic rate was greater than that seen for the corresponding coupled incubation. However, these results do not indicate whether, for the substrate propionate, glucagon accelerated the rate of glucose synthesis at a site between

TABLE 2

Effect of glucagon on glucose and pyruvate plus lactate formation from propionate.

Incubation conditions were as described for Table 1. Glucagon (1 μM) was used in these experiments. For the coupling incubations the oxidation of NADH due to pyruvate formation was monitored at 340 nm and allowed to proceed for 15 minutes. At this time 0.5 ml 6%(w/v) $HClO_4$ was added. Precipitated material was removed by centrifugation and analyses for glucose conducted on the neutralized (pH 6.5) perchlorate extract.

	Rate (μmoles × min^{-1} × g^{-1} wet wt. of liver)			
	Glucose Formation		Pyruvate plus lactate formation	
	Coupled Incubations	Free Incubations	Coupled Incubations	Free Incubations
Additions				
5mM-Propionate	0.091	0.133	0.20	0.04
5mM-Propionate + Glucagon	0.138	0.212	0.21	0.03

propionate and phosphoenol pyruvate or at a site between phosphoenol pyruvate and glucose.

The occurrence of recycling of pyruvate during gluconeogenesis has been clearly demonstrated by several workers [e.g. for kidney cortex segments (13), perfused rat liver (14) and isolated rat liver cells (5)] and convincing evidence that short-term changes in catalytic activity (2,3,4) and catalytic properties of the enzyme [e.g. increased Km for phosphoenol pyruvate (4)] has been presented. In addition Ljungstrom et al. (15) have demonstrated the phosphorylation of rat liver pyruvate kinase by a cyclic AMP-stimulated protein kinase. Thus the present data contrast with the above evidence by indicating that acute hormonal control by glucagon of pyruvate kinase does not occur concommitant with a glucagon-mediated stimulation of gluconeogenesis in liver cells from fasted rats. It remains to be established whether the glucagon (cyclic AMP)-mediated changes in kinetic constants of pyruvate kinase are related to the acute control of glycolysis and gluconeogenesis elicited by this hormone.

Whereas Rognstad (5) has recently shown that with high concentrations of pyruvate as substrate, cyclic AMP depressed pyruvate kinase flux by about 45%, a cyclic AMP-mediated increase in gluconeogenesis did not accompany the decreased rate of flux through pyruvate kinase. Furthermore, for a cyclic AMP-mediated decrease in pyruvate kinase flux, a more substantial decrease in the proportion of carbon recycling relative to that giving rise to glucose synthesis, would have been expected. For the two experiments shown, this proportion changed from 49.1% and 46.8% 44.3% and 38.4%, respectively. Finally the experimental design employed (5) does not preclude the possibility of indirect effects. For example, a glucagon-mediated change in flux rates of FDPase and PFK has already been shown (8) and is presently shown (e.g. Table 1, free incubations) to indirectly affect the apparent rate of flux through

pyruvate kinase by re-directing carbon from lactate and pyruvate formation to glucose synthesis. A similar situation when either lactate or pyruvate is substrate could occur so that a greater flux rate through reactions beyond phosphoenol pyruvate act to decrease the proportion of phosphoenol pyruvate recycled to pyruvate.

On the basis of the present data it is concluded that hepatic pyruvate kinase is not under acute hormonal control and that whereas independent regulation of pyruvate carboxylase and/or phosphoenol pyruvate carboxykinase may be exerted, the pyruvate carboxylase-phosphoenol pyruvate carboxykinase-pyruvate kinase substrate cycle is not hormonally regulated in a manner totally analogous to that previously described for the FDPase-PFK cycle (8,16).

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References

1. Krebs, H.A. and Eggleston, L.V. (1965) Biochem. J. 94, 3c.
2. Taunton, O.D., Stifel, F.B., Greene, H.L. and Herman, R.H. (1972) Biochem. Biophys. Res. Commun. 48, 1663-1670.
3. Friedrichs, D. (1975) abstract, Post FEBS Symposium No. 38.
4. Cimbala, M. and Blair, J.B. (1975) Fed. Proc. 34, 618.
5. Rognstad, R. (1975) Biochem. Biophys. Res. Commun. 63, 900-905.
6. Berry, M.N and Friend, D.S. (1969) J. Cell. Biol. 43, 506-520.
7. Dawson, R.M.C. (1969) in Data for Biochemical Research. (Dawson, R.M.C, Elliott, D.C., Elliott, W.H. and Jones, K.M., eds) p. 507, Oxford University Press, New York.
8. Clark, M.G., Kneer, N.M., Bosch, A.L. and Lardy, H.A. (1974) J. Biol. Chem. 249, 5695-5703.
9. Werner, W., Rey, H.G. and Wielinger, H. (1970) Z. Anal. Chem. 252, 224-228.
10. Hohorst, H. (1963) In Methods of Enzymatic Analysis (Bergmeyer, H.U. ed.) pp. 226-270, Academic Press, New York.
11. Czok, R. and Eckert, L. (1963) In Methods of Enzymatic Analysis (Bergmeyer, H.U. ed.) pp. 229-233, Academic Press, New York.
12. Zahlten, R.N., Stratman, F.W. and Lardy, H.A. (1973) Proc. Nat. Acad. Sci. U.S. 70, 3213-3218.
13. Rognstad, R. and Katz, J. (1972) J. Biol. Chem. 247, 6047-6054.
14. Friedmann, B., Goodman, E.M., Saunders, M.L., Kostos, V. and Weinhouse, S. (1971) Arch. Biochem. Biophys. 143, 566-578.
15. Ljungstrom, O., Hjelmquist, G. and Engstrom, L. (1974) Biochem. Biophys. Acta 358, 289-298.
16. Kneer, N.M., Bosch, A.L., Clark, M.G. and Lardy, H.A. (1974) Proc. Nat. Acad. Sci. U.S. 71, 4523-4527.