Activation of rat heart phosphofructokinase-2 by insulin in vivo

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Fifteen minutes after the intravenous injection of overnight starved, anaesthetized rats with insulin, the concentration of fructose 2,6-bisphosphate was increased more than 2-fold in the hearts of these animals. Insulin injection also caused a 2–3-fold increase in the V_{max} of phosphofructokinase-2 with no detectable change in K_m values. The effect persisted after precipitation of the enzyme with polyethylene glycol or after gel filtration through Sephadex G-25.

Fructose 2,6-bisphosphate *Phosphofructokinase-2* *Heart* *Insulin* *Glycolysis*

1. INTRODUCTION

Insulin stimulates glycolysis in a variety of mammalian tissues including skeletal muscle, heart and white adipose tissue. Fru-2,6-P_2, which is the most potent positive effector of PFK-1, is a likely candidate for the regulation of glycolysis. This is the case in the liver where the control of glycolysis may be brought about by changes in the concentration of Fru-2,6-P_2, at least under certain conditions [1,2]. Fru-2,6-P_2 is also present in heart, skeletal muscle and adipose tissue [3], and was found to be decreased in the hearts of alloxan diabetic rats [4]. In perfused rat hindlimb muscles, insulin caused a 2-fold increase in Fru-2,6-P_2 and a stimulation of glycolysis [3]. In white adipose tissue, Fru-2,6-P_2 may be important in the regulation of glycolysis by the concentration of its substrates. However, in this tissue, the concentration of Fru-2,6-P_2 decreased when glycolysis was stimulated by insulin (unpublished). Here we report an increase in the concentration of Fru-2,6-P_2 in the hearts of anaesthetized rats after insulin administration. This effect is explained by an increase in the tissue concentration of hexose 6-phosphates and an activation of PFK-2, the enzyme which catalyzes Fru-2,6-P_2 synthesis.

Abbreviations: PFK-1, phosphofructokinase-1 (E.C. 2.7.1.11); PFK-2, phosphofructokinase-2 (E.C. 2.7.1.-); Fru-2,6-P_2, fructose 2,6-bisphosphate

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2. MATERIALS AND METHODS

Enzymes and biochemical reagents were from Sigma or Boehringer.

2.1. *Experimental procedure*

Male Wistar rats (150–200 g, starved overnight) were anaesthetized (nembutal 60 mg/kg, intraperitoneally) and injected intravenously with 0.15 M NaCl or insulin (10 units/kg). After 15 min, the hearts were rapidly excised and freeze-clamped [5]. Blood was collected from the vena cava for determination of plasma glucose concentration.

2.2. *Measurement of metabolites*

The freeze-clamped hearts were powdered under liquid N_2 and homogenized with 1.5 vol. of 5% $HClO_4$, in a salt/ice bath, using an Ultra-Turrax tissue disintegrator (4 × 15 s). The denaturated proteins were removed by centrifugation and the

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supernatants were neutralized with saturated K_2CO_3. These neutralized extracts were assayed for glucose 6-phosphate, fructose 6-phosphate, triose phosphates, fructose 1,6-bisphosphate and *sn*-glycerol 3-phosphate as in [6]. For the measurement of Fru-2,6-P_2 heart extracts were prepared and assayed as in [7].

2.3. *Measurement of PFK-2 activity*

Frozen hearts were homogenized at 0°C, with an Ultra-Turrax (4 × 15 s), in 5 vols of 20 mM Hepes/30 mM KCl/20 mM KF/5 mM EDTA/1 mM dithioerythritol/0.1 mM PMSF/0.1 mM fructose 6-phosphate/0.3 mM glucose 6-phosphate, pH 7.5 (extraction medium). After centrifugation 40 000 × *g* for 20 min) the supernatant was assayed for PFK-2 activity. The precipitation of PFK-2 by polyethylene glycol was performed by mixing 1 vol. of the 40 000 × *g* supernatant with 1 vol. of a solution of 40% polyethylene glycol (M_r 6000) in the extraction medium. The mixture was stirred for 30 min at 0–4°C, the precipitate was collected by centrifugation (2 min in an Eppendorf microfuge) and resuspended in 1 vol. extraction medium. This procedure precipitated 65–70% of the lactate dehydrogenase activity present in the homogenate. PFK-2 activity present in 25 μl of these extracts was assayed at 30°C in a final volume of 0.2 ml containing 50 mM Hepes/100 mM KCl/20 mM KF/5 mM potassium phosphate/1 mM dithioerythritol, pH 7.1 and the concentrations of fructose 6-phosphate and ATPMg indicated in the figures. Glucose 6-phosphate was also added to give a glucose 6-phosphate/fructose 6-phosphate ratio equal to 3. The reactions were stopped by the addition of 0.2 ml of 50 mM NaOH, heated for 10 min at 80°C and Fru-2,6-P_2 was measured as in [7]. The assays were linear for up to 10 min. Blank reactions were incubated in the absence of ATPMg for 10 min to correct for Fru-2,6-P_2 present in the homogenate. The activity is expressed as pmol Fru-2,6-P_2 formed/min per unit lactate dehydrogenase, assayed as in [8].

2.4. *Purification of rat heart PFK-2*

Rat hearts (30 g) were homogenized in 4 vols (v/w) of 20 mM Hepes/50 mM KCl/5 mM $MgCl_2$/2 mM EDTA/1 mM dithioerythritol/0.1 mM fructose 6-phosphate/0.3 mM glucose 6-phosphate, pH 7.5, at 4°C using a Waring blendor (4 × 15 s). The supernatant (40 000 × *g* for 30 min) was fractionated twice with polyethylene glycol (M_r 6000). The protein precipitated in the 6–15% fraction was resuspended in the homogenization medium and applied to a column of DEAE-cellulose. PFK-2 was eluted (0.25 M KCl) with a linear gradient of CL^- (0.05–0.5 M KCl) and further purified on Blue Sepharose. The enzyme was eluted (0.7 M KCl) with a linear gradient of Cl^- (0.1–1 M KCl) and had a specific activity of 0.5 nmol Fru-2,6-P_2 formed/min per mg protein. The enzyme was purified more than 700-fold with a yield of 20%.

2.5. *Statistical methods*

The statistical significance of the results was tested on unpaired samples using a 2-sided Student's *t*-test. For kinetic studies on purified PFK-2, the hyperbolic curves were fitted to the experimental data with a computer program presented in [9].

3. RESULTS AND DISCUSSION

The intravenous injection of insulin to overnight starved rats resulted in a more than 2-fold increase in the concentration of Fru-2,6-P_2 in the heart (table 1). PFK-1 from heart, like the enzyme from other mammalian tissues, is sensitive to Fru-2,6-P_2

Table 1

The effect of insulin on metabolite concentrations in rat heart in vivo

Metabolite	Metabolite concentration (nmol/g wet wt)	
	Control	Insulin
Glc-6-P (4)	191 ± 22	254 ± 20*
Fru-6-P (4)	39 ± 4	55 ± 6*
Fru-2,6-P_2 (7)	0.7 ± 0.1	1.5 ± 0.3*
Fru-1,6-P_2 (4)	32 ± 6	34 ± 6
Triose phosphates (4)	28 ± 13	12 ± 5
sn-Glycerol-3-P (4)	12 ± 2	13 ± 1
Plasma glucose (11)	5.3 ± 0.2	2.3 ± 0.1*

Values shown are the means ± SE for the number of experiments shown in parentheses. Triose phosphates are the sum of glyceraldehyde 3-phosphate and dihydroxyacetone phosphate. * Statistically significant effect of insulin ($P < 0.05$). The plasma glucose concentrations are expressed as μmol/ml

[10] and the extreme concentrations of Fru-2,6-P_2 measured in hearts were between 0.6 (control) and 3.4 (insulin) nmol/g, i.e., a concentration range within which PFK-1 is sensitive to the stimulator. Therefore, such an increase in Fru-2,6-P_2 should lead to a stimulation of PFK-1 activity and so participate in the concerted mechanism by which insulin stimulates glycolysis in the heart.

Table 1 also shows that insulin treatment caused a 30 and 40% increase in glucose 6-phosphate and fructose 6-phosphate concentration, respectively. This increase might result from the well known stimulation of glucose transport by insulin [11]. The concentrations of fructose 1,6-bisphosphate and triose phosphates were, however, unaltered after insulin, indicating that these metabolites are readily used by the enzymes further down the glycolytic pathway and that the reactions catalyzed by these enzymes are not rate-limiting.

The saturation curves of PFK-2 purified from rat hearts were hyperbolic with respect to its substrates and the K_m values for fructose 6-phosphate and ATPMg were 0.11 and 0.81 mM, respectively (fig.1). Therefore, the increase in Fru-2,6-P_2 brought about by insulin cannot be explained solely by the observed rise in fructose 6-phosphate (table 1). Accordingly the effect of insulin on PFK-2 activity was investigated.

As shown in fig.2, the activity of PFK-2 measured in a crude homogenate was increased 2–3-fold; this resulted mainly from an increase in V_{max} with no clear cut difference in K_m for the substrates. The effect of insulin on PFK-2 activity persisted when the enzyme was precipitated by polyethylene glycol (fig.3a,b) or after filtration of

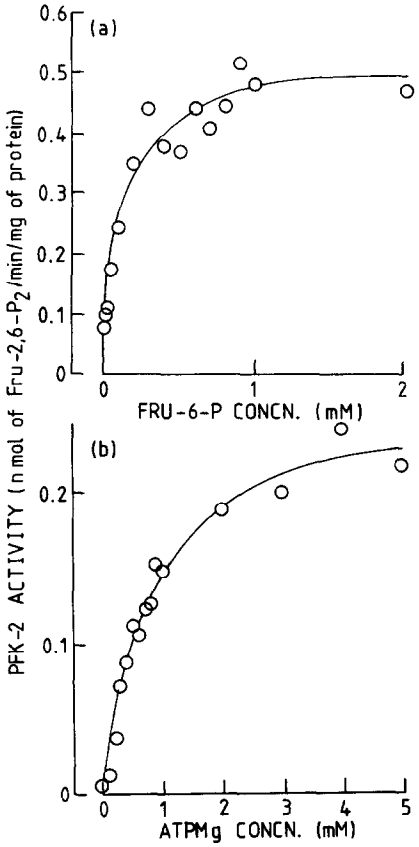

Fig.1. The kinetics of purified PFK-2 from rat heart. The saturation curves of purified PFK-2 were measured for fructose 6-phosphate (a) and ATPMg (b). In (a) the concentration of ATPMg was 5 mM and in (b) the concentration of fructose 6-phosphate was 0.11 mM. Both curves were fitted to a hyperbola (correlation coefficients 0.98 and 0.99).

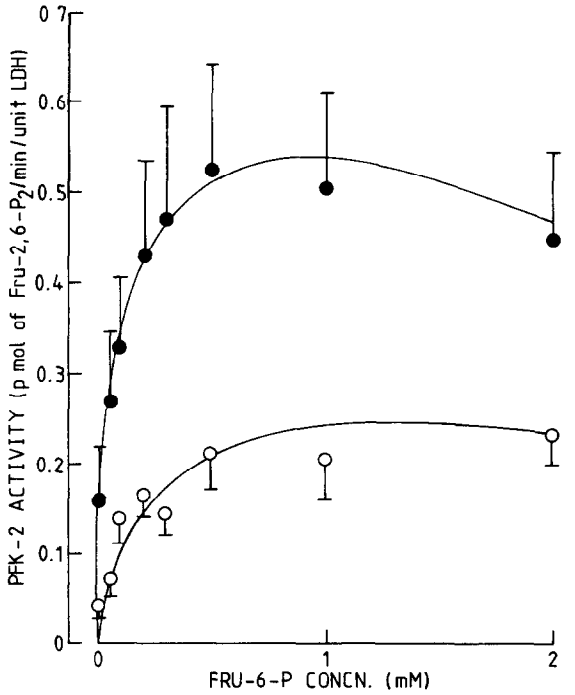

Fig.2. Effect of insulin on PFK-2 activity measured in crude homogenates of rat heart. The saturation curves of PFK-2 for fructose 6-phosphate in homogenates prepared from the hearts of 4 control and 5 insulin-treated rats were measured at 5 mM ATPMg. Closed symbols = insulin. The results show the means ± SE.

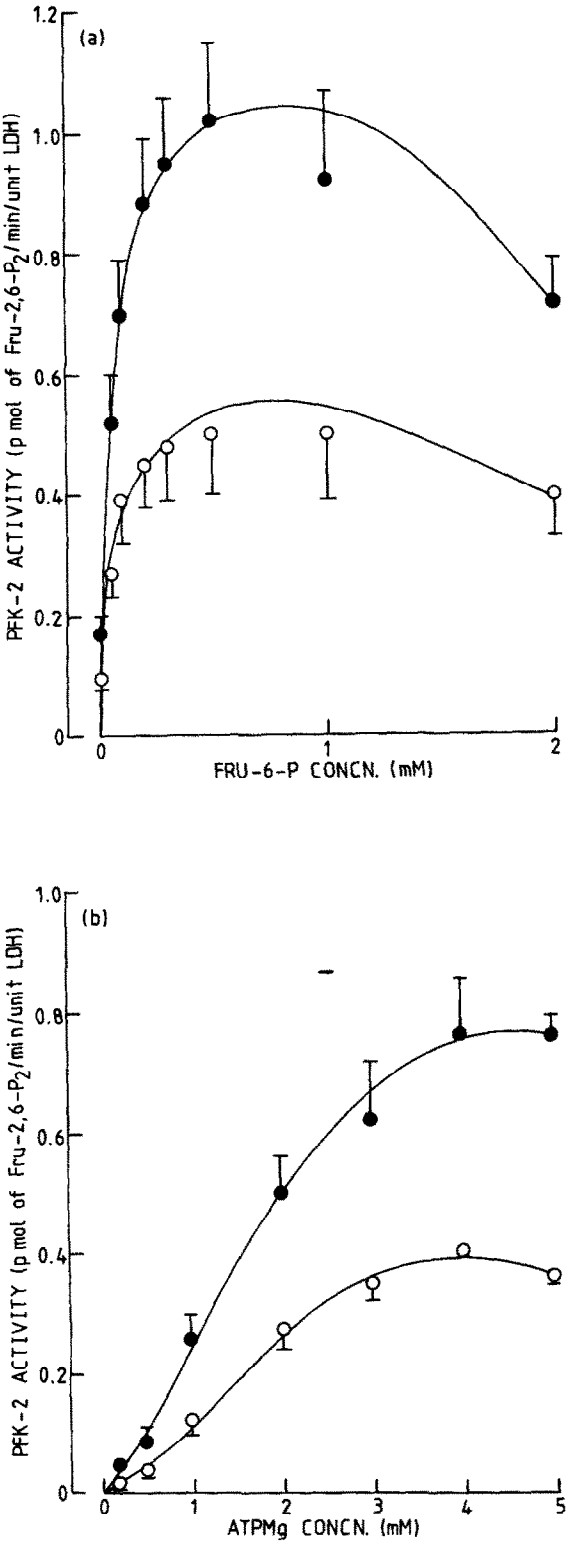

Fig.3. Effect of insulin on PFK-2 activity in 20% polyethylene glycol fractions of rat heart homogenates. PFK-2 activity was measured at the indicated concentrations of fructose 6-phosphate with 5 mM ATPMg (a), and the indicated concentrations of ATPMg with 0.11 mM fructose 6-phosphate (b). Closed symbols represent PFK-2 activities of polyethylene glycol fractions prepared from the hearts of insulin-treated rats. The results show the means ± SE.

the crude homogenates through Sephadex G-25 (not shown). The persistence of the insulin effect after separation of the enzyme from small molecular mass regulators would suggest that some stable change, such as covalent modification, is involved.

Fig.3a also shows that the polyethylene glycol treatment in itself doubled the V_{max} of PFK-2 when compared with the crude homogenate. This effect cannot wholly be explained by the incomplete precipitation of lactate dehydrogenase by polyethylene glycol and may suggest the presence of inhibitory factor(s) in the crude homogenate or a stimulation of the enzyme by polyethylene glycol. The latter possibility is probably true since filtration through Sephadex G-25 did not result in a stimulation of enzyme activity (not shown). It is also apparent from fig.3a that the enzyme precipitated by polyethylene glycol was inhibited by an excess of fructose 6-phosphate. Such an inhibition was not observed with the purified enzyme and may therefore result from the stimulation of phosphatases by polyethylene glycol. The saturation curve for ATPMg of the polyethylene glycol precipitated enzyme was slightly sigmoid with an apparent K_m(fig.3b) higher than that of the purified enzyme (fig.1b). This may result from the co-precipitation of ATPases by polyethylene glycol.

4. GENERAL CONCLUSIONS

The intravenous injection of insulin causes a rapid activation of PFK-2 in rat hearts. This activation, which may involve covalent modification of the enzyme, is probably responsible for the increase in Fru-2,6-P_2 which, in turn, may stimulate PFK-1 and glycolysis. This effect of insulin is the

first rapid hormonal effect on PFK-2 which is observed in extra-hepatic tissues.

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