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EFFECT OF EXPERIMENTAL DIABETES ON GLYCOLYTIC INTERMEDIATES AND REGULATION OF PHOSPHOFRUCTOKINASE IN RAT LENS

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SUMMARY: The pattern of glycolytic intermediates in the lens of alloxan-diabetic rats was indicative of regulation at phosphofructokinase. The changes in metabolites influencing phosphofructokinase activity in the diabetic, relative to the normal, rat lens were: glucose 6-phosphate, 182%; fructose 6-phosphate, 107%; fructose diphosphate, 57%. There was also a marked decrease in phosphoenolpyruvate, pyruvate, lactate and ATP but no significant change in other triose phosphates or cyclic AMP. The results are considered in relation to the early changes in $[Ca^{2+}]$ known to occur in lens in diabetes and to the coordinating effect of fructose diphosphate on flux through the glycolytic route.

There is a significant decline in the total ATP of the lens in diabetes [1,2], seen as early as one week after induction of diabetes with alloxan [1]. There is evidence that approximately 70% of the lens ATP is generated via the glycolytic pathway, the remaining 30% arising from mitochondrial systems [3,4]; thus, a change in the flux of glucose through key regulatory enzymes of glycolysis in lens could be anticipated in diabetes. The evidence for such changes and the site(s) and mechanism of regulation of the glycolytic route are not entirely clear. On the one hand, there is evidence for a decrease in the activity of a number of glycolytic enzymes, including phosphofructokinase and pyruvate kinase, in extracts of alloxan-diabetic rat lens and human cataractous lenses [5-7], while on the other, it has been reported that diabetes results in only minor changes in glucose uptake and lactate formation in the intact lens [1,8]. Further, there is the apparent anomaly that hexokinase, an enzyme widely held to exert

Abbreviations: PFK, phosphofructokinase (ATP: D-fructose 6-phosphate 1-phosphotransferase, EC 2.7.1.11); cyclic AMP, adenosine 3'5'-monophosphate.

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a 'pacemaker' role in the regulation of glycolysis in lens [4,9-11], increase two-fold in activity four weeks after induction of diabetes [12].

The examination of the metabolite profile of a tissue before and after perturbation by hormonal factors has proved to be a powerful tool in the determination of sites of regulation [13,14]. The present study reports on changes in the metabolite profile of glycolytic intermediates in the lens in diabetes in parallel with measurement of the activities of enzymes of the glycolytic pathway.

METHODS

Adult male albino rats of the Wistar strain were treated with alloxan-monohydrate (19 mg/100 g body weight) by subcutaneous injection into rats previously starved for 24 hours; thereafter insulin was given (2 units protamine zinc insulin daily for one week) and standard laboratory cube diet and water allowed ad lib; the animals were used six weeks later for measurement of enzyme activities in dialysed lens homogenates and for metabolite determination in rapidly frozen rat lenses. Rat lens homogenates for enzyme determinations were prepared as previously described [11], the homogenizing medium contained: 0.25M sucrose, 20 mM triethanolamine buffer pH 7.4, 0.1 mM dithiothreitol and defatted bovine serum albumin 0.5% final concentration. PFK was assayed using the following system: 50 mM Tris buffer pH 8.0; 4 mM $MgCl_2$; 0.34 mM NADH; 2.0 mM fructose 6-phosphate; 1.3 mM ATP; 0.4 units aldolase; glycerol 3-phosphate dehydrogenase/triose isomerase mixture with 0.9 and 2.7 units respectively; the reaction was initiated by addition of lens extract. The ancillary enzymes and substrates were obtained from Boehringer Corporation, London. Other enzymes of the glycolytic pathway were measured as previously described [15,16]. Metabolites were measured in neutralised perchloric acid extracts of rapidly frozen lens tissue, groups of eight lenses were pooled to make a single extract. The metabolites were estimated by procedures given in Bergmeyer [16], cyclic AMP was determined by the competitive binding protein method of Tovey et al. [17]. The results are given as means ±SEM.

RESULTS AND DISCUSSION

Metabolite content of normal and diabetic rat lens

These results are shown in Fig. 1 in the form of a 'crossover plot'. The metabolite profile showed a marked increase in glucose 6-phosphate content in accord with the increased hexokinase activity observed in diabetic rat lens [12]. There was a significant decrease in fructose diphosphate content, indicative of an inhibition of PFK in the diabetic group; in addition, there was evidence for a further regulatory site at enolase and/or pyruvate kinase as shown by the marked decrease in phosphoenolpyruvate and

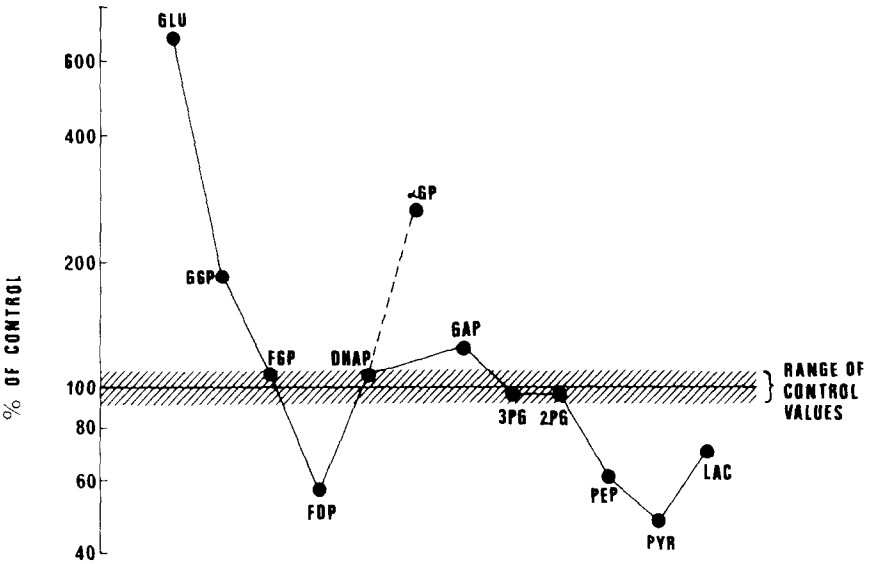

Fig. 1. Crossover plot of glycolytic intermediates in diabetic rat lens as a percentage of those in normal rat lens.

The intermediates of the glycolytic pathway in diabetic-rat lens are shown as a percentage of the normal control group, calculated on the basis of nmol/g lens. Each point is the mean of not less than eight separate extracts, each of which contained lenses pooled from 4 rats. The results are shown on a logarithmic scale. The abbreviations used together with the normal lens values and asterisks indicating significant differences between normal and diabetic groups (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$) are: GLU, glucose 0.614±0.56***; G6P, glucose 6-phosphate 0.060±0.006***; F6P, fructose 6-phosphate 0.027±0.006; FDP, fructose diphosphate 0.054±0.004***; DHAP, dihydroxyacetone phosphate 0.051±0.006; αGP, glycerol 3-phosphate 0.330±0.063***, GAP, glyceraldehyde 3-phosphate 0.048±0.004; 3PG, 3-phosphoglycerate 0.077±0.007; 2PG, 2-phosphoglycerate 0.040±0.006; PEP, phosphoenolpyruvate 0.061±0.008*; PYR, pyruvate 0.118±0.010***; LAC, lactate 8.69±0.98*.

pyruvate content of diabetic rat lens: these metabolite shifts were not matched by corresponding changes in the activities of enzymes measured under optimal conditions *in vitro*. The decrease in lens pyruvate and lactate is consistent with an overall decrease in the glycolytic flux in diabetic rat lens. A highly significant increase in the lens content of glycerol 3-phosphate was seen in diabetes, this increased more than two-fold and may be indicative of a more reduced state of the NAD^+/NADH couple in lens; it may be noted that the pyruvate/lactate quotient also moves towards a more reduced state.

The intermediates of the sorbitol pathway, glucose, sorbitol and fructose were 680%, 1780% and 500% of the control value of 100%

respectively in the alloxan-diabetic group, even at this early stage of diabetes.

The comparison of the activities of glycolytic enzymes in lens extracts with the regulatory points shown in the metabolite profile led to the conclusion that PFK, enolase and pyruvate kinase may be subject to regulation by effector molecules or by covalent modification in the diabetic rat lens since, of the sequence of enzymes measured in vitro, aldolase alone showed a significant decline falling to 67% of the control value (1.02±0.09 and0.63±0.08 units/g lens for 6 control and 8 diabetic values respectively, $P < 0.05$).

The properties of lens PFK and pyruvate kinase have been examined in a number of laboratories and it has been shown that PFK is subject to regulation by citrate and ATP, the latter being reversed by high concentration of cyclic AMP [10,11] and that pyruvate kinase is a variant of M type and is subject to regulation by fructose diphosphate [6,18]. The effect of alloxan diabetes on the lens content of a number of intermediates involved in the regulation of PFK and pyruvate kinase is shown in Table 1. Significant decreases were observed for ATP and fructosediphosphate, citrate was too low to measure.

Table 1. The effect of alloxan diabetes on the lens content of metabolites and effector molecules involved in regulation of phosphofructokinase and pyruvate kinase.

	Normal	Alloxan Diabetic	%	P
	µmol/g lens			
ATP	1.20±0.06(11)	0.85±0.07(9)	70	<0.01
ADP	0.151±0.004(7)	0.149±0.018(7)	95	NS
AMP	0.041±0.004(7)	0.038±0.002(6)	85	NS
Fructose 6-P	0.027±0.006(10)	0.029±0.004(8)	107	NS
Fructose 1,6-P	0.054±0.004(11)	0.031±0.002(9)	57	< 0.001
P-enolpyruvate	0.061±0.008(10)	0.037±0.006(9)	61	< 0.05
Alanine	2.21±0.22(5)	2.04±0.39(6)	98	NS
	nmol/g lens			
Cyclic AMP	0.130±0.011(10)	0.102±0.009(10)	79	0.06

The values given are the means ±SEM; the figures in parentheses are the number of separate extracts measured, each of which contained lenses pooled from 4 rats.

Regulatory role of fructose diphosphate. The decline in the lens fructose diphosphate content could be of particular significance in the coordination and overall regulation of glycolytic flux in the diabetic rat lens since this intermediate is an activator of both PFK and pyruvate kinase [6,10,19]. It has been shown by Cheng et al. [6] that a 10 min pre-incubation of rat lens with fructose diphosphate activated the lens pyruvate kinase; the plots of enzyme activity v phosphoenolpyruvate concentration changed from semi-sigmoidal to Michaelis-Menten type kinetics following this treatment. It may be noted that lens epithelium pyruvate kinase has a Km for phosphoenolpyruvate of 1.67 mM without prior treatment with fructose diphosphate, after preincubation with this compound the Km fell to 0.065 mM, a figure close to the present recorded value for the phosphoenolpyruvate content of normal rat lens of 0.061 mM (Table 1). This excellent agreement between the normal lens content of phosphoenolpyruvate and the apparent Km of the activated pyruvate kinase further emphasises the possible importance, and synergistic effects, of the decline in lens fructose diphosphate and phosphoenolpyruvate in diabetes. However, it must be remembered that the present data are for the whole lens content and not the concentration in a particular site, such as the lens epithelium.

Regulation of phosphofructokinase. While the decline in lens fructose diphosphate provides one mechanism for the coordinated regulation of glycolysis in diabetes, this change must be secondary to a more immediate regulation of PFK. Regulation of lens PFK by covalent modification is an attractive hypothesis, particularly in the light of recent studies by Kagimoto and Uyeda [20] and Castano et al. [21] who have shown that glucagon-stimulated phosphorylation of liver PFK, while not affecting the maximum catalytic activity of the enzyme, did render the enzyme significantly more sensitive to ATP inhibition at subsaturating concentrations of substrate. This mechanism is similar to the cyclic AMP-dependent inactivation of pyruvate kinase at subsaturating concentrations of phosphoenol-pyruvate [22].

Preliminary experiments were undertaken to determine whether similar mechanisms operated in the regulation of lens PFK of diabetic animals where the hormonal balance swings towards glucagon. In the presence of high substrate concentrations, 2.0 mM fructose 6-phosphate and 1.4 mM ATP, no significant difference was found in

the PFK activity of diabetic rat lens relative to control (1.02± 0.06(8) and 0.94±0.06(8) units/g lens respectively), however, with subsaturating concentrations of substrate (0.4-0.6 mM fructose 6-phosphate and 0.4-0.8 mM ATP) a consistent depression of 20-25% in the activity of the PFK in diabetic rat lens relative to the control was observed. No significant change in the whole lens cyclic AMP was found in the present experiments (Table 1), however these results do not preclude significant changes in specific regions of the lens. A knowledge of the epithelial concentration of cyclic AMP would be of particular importance in relation to the regulation of PFK and pyruvate kinase which are concentrated in this region of the lens [6].

A well established early change known to occur in the lens in diabetes is the accumulation of calcium [23]; 3- to 20-fold increases have been reported in different types of cataractous lenses [24,25]. The normal rat lens calcium content is reported as 1.2 mM [24] but the association of Ca^{2+} with lens proteins will markedly affect the free concentration of this cation [23,25]. The effect of Ca^{2+} on the activity of normal rat lens PFK and enolase was studied; with concentrations of 1.8 mM Ca^{2+} there was 1 40% inhibition of PFK and a 60% inhibition of enolase, with 3.6 mM Ca^{2+} inhibitions of 70% and 90% respectively were observed. An inhibition of PFK and enolase by Ca^{2+} would lead, in turn, to a depression in fructose diphosphate and phosphoenolpyruvate which would depress pyruvate kinase activity and ATP generation. There is the additional possibility of Ca^{2+} effects mediated via the adenylate cyclase system (see [26]).

The metabolite profile presented in Fig. 1 points to a more marked inhibition of the lower segment of the glycolytic pathway relative to reactions leading to formation of triose phosphates. Such an imbalance would be consistent, not only with depressed ATP formation and glycolytic flux, but also with the more reduced state of the NAD^+/NADH couple and with the diversion of triose phosphate into glycerol 3-phosphate in lens in diabetes.

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