Effect of Hyperglycemia on the Hexose Monophosphate Shunt in Islets of Langerhans

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SUMMARY

The activities of glucose-6-P dehydrogenase, 6-P-gluconate dehydrogenase, ribulose-5-P epimerase, ribulose-5-P isomerase, transketolase and transaldolase were measured in frozen dried specimens of the islets of Langerhans and exocrine cells of the pancreas. Activities of all five enzymes were comparable in the two cell types. The effect of hyperglycemia on the concentration of 6-P-gluconate was also studied. A ten-fold increase in blood glucose concentration resulted in a thirteen-fold increase in 6-P-gluconate levels in islet tissue but no change in the exocrine portion of the pancreas. The rate of increase of 6-P-gluconate in the islets during hyperglycemia was slower than the rate of increase of either glucose or glucose-6-P. The rise of 6-P-gluconate appears to be too slow a process to serve as a trigger for the insulin release mechanism. DIABETES *17*:475-80, August, 1968.

It has been demonstrated that hyperglycemia causes a marked increase of the glucose-6-P levels in the islets of Langerhans.[1] It is not unreasonable to suppose that this unusual response is related to the well-known release of insulin from the pancreas by hyperglycemia. Since glucose-6-P stands at a crossing for several pathways of glucose metabolism, a change in glucose-6-P level might influence the flux through any or all of these pathways. This paper is concerned with the question of whether or not, in the islets of Langerhans, the flux through the pentose-P pathway is stimulated by increased availability of the first metabolite of that pathway, glucose-6-P.

MATERIAL AND METHODS

Animal experiments. Details of the procedure have been described.[1] Adult, obese, hyperglycemic mice weighing 30 to 60 gm. (Bar Harbor, Maine, Jackson Memorial Laboratory) were used. The animals were anesthetized with ether or intraperitoneal injections of phenobarbital (140 to 200 mg./kg.). The blood glucose levels were altered by various means. Levels ranging from 4 to 10 mM were obtained by fasting the animals overnight. Levels ranging from 25 to 100 mM and relatively stable from sixty to ninety minutes were produced by injection of glucose under the skin of the back. To achieve more rapid increases in blood levels, glucose was injected into the femoral vein over a period of thirty seconds. In this manner the sugar level of the blood could be raised to approximately 50 mM within less than sixty seconds, and levels then remained practically constant for as long as five minutes. Blood was taken from the tail or from the abdominal cavity immediately after the removal of the pancreas. The whole pancreas was cut out rapidly with scissors and dropped into Freon-12 (CCl_2F_2) chilled to the freezing point (—150°) with liquid nitrogen. Removal and freezing of the pancreas was usually completed within less than five seconds.

Sections 20 to 30 μm. thick were cut at —20° in a cryostat and dried from the frozen state.[2] As described[1] islets of Langerhans were dissected from the dry sections and weighed, together with specimens of comparable size (0.1 to 1 μg.) from the exocrine portion of the pancreas.

Analytical methods

All measurements were made with TPN or DPN dependent enzymatic-fluorometric assays. Glucose in blood was determined with a direct fluorometric method.[3] The analysis of enzymes in samples of islets and acini was carried out by methods based on the native fluorescence of pyridine nucleotides or the fluorescence induced by strong alkali.[4] The analysis of 6-P-gluconate (given below) was made with an enzymatic cycling for TPNH.[5]

Source of reagents: All enzymes, except ribulose-5-P epimerase, ribulose-5-P isomerase and transketolase, were obtained from Boehringer and Sons, Mannheim, West Germany. Ribulose-5-P epimerase and ribulose-5-P isomerase were prepared from calf spleen according to a

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modification of the procedure described by Ashwell and Hickman.[8] Transketolase was prepared from bakers' yeast according to a modification of the method described by Srere et al.[10] The details of the modified purification procedures are the subject of a separate report. All biochemical cofactors and substrates of the assays except for erythrose-4-P (Calbiochem) were bought from Sigma Chemical Company, St. Louis, Missouri.

Analysis of enzymes. Dry tissue samples were placed on the bottom of small glass tubes with an inner diameter of 3 mm. and ice cold reagent of the composition described in the accompanying table (table 1) was added. More information regarding the assay principles and preparation of the auxiliary enzymes will be presented in a separate publication.[6] After suspension of the tissue in the assay medium the tubes were kept at 0° in ice water until starting of the incubation at a desired temperature.

Analysis of 6-P-gluconate. This assay was carried out with an oil well method.[9] Dry specimens were placed on the bottom of small holes drilled into a Teflon block (3 mm. inner diameter, 4 mm. deep). Then 0.08 μl. of 0.03 N HCl was added to the sample and the resulting tissue suspension was quickly covered with a mixture of 40 per cent light mineral oil and 60 per cent hexadecane. Reagent blanks and standards of 6-P-gluconate (five duplicates ranging from 10^{-14} to 10^{-13} moles) were also added to the Teflon wells. The samples were then heated for twenty minutes at 75°.

TABLE 1

Assay conditions for pentose-P cycle enzymes. The assay volume was in all instances 10 μl. Times and temperatures for incubation were as follows: transketolase and transaldolase one hour at 25°, ribulose-5-P isomerase thirty minutes at 25°, ribulose-5-P epimerase twenty minutes at 25°, and glucose-6-P as well as 6-P-gluconate dehydrogenase, thirty minutes at 38°. All DPN dependent reactions were terminated by addition of 1.0 μl. of 5.0 N HCl to destroy excess DPNH. The total sample of about 11 μl. was transferred to 1 ml. of 6 N NaOH and heated for fifteen minutes at 60° to induce fluorescence. All TPN dependent assays were terminated by transfer of the total sample to 1 ml. of 0.05 M Na_2CO_3-0.005 M $NaHCO_3$ and the native fluorescence was read directly.

Enzyme	Buffer	Substrates	Cofactors	Auxiliary enzymes	Other additions
Glucose-6-P dehydrogenase*	2-amino-2 methyl 1,3 propanediol 0.1 M pH 9.0	glucose-6-P, 2 mM	TPN^+ 1 mM	none	EDTA 0.5 mM bovine serum albumin 0.05%
6-P-gluconate dehydrogenase*	tris, 0.1 M pH 8.2	6-P-gluconate 1 mM	TPN^+ 0.2 mM	none	EDTA 0.3 mM bovine serum albumin 0.05%
Ribulose-5-P epimerase	imidazole 0.05 M, pH 7.6	ribose-5-P 2.5 mM	DPNH 0.1 mM Thiamine-PP 0.02 mM	α-glycero-P DH 15 μg./ml., triose-P-isomerase 12.5 μg./ml., ribulose-5-P isomerase 4.5 mmoles/1./hr. transketolase 6.0 mmoles/1./hr.	nicotinamide 20 mM sodium amytal 2 mM bovine serum albumin 0.01% $MgCl_2$-2 mM
Ribulose-5-P isomerase	same	ribose-5-P 5 mM	DPNH 0.2 mM Thiamine-PP 0.02 mM	same, except ribulose-5-P isomerase replaced by epimerase 4.2 mmoles/1./hr.	same
Transketolase	same	xylulose-5-P 0.2 mM,† ribose-5-P 1 mM	DPNH 0.1 mM Thiamine-PP 0.02 mM	α-glycero-P DH 15 μg./ml. triose-P-isomerase 12.5 μg./ml.	same
Transaldolase	same	erythrose-4-P 0.1 mM, fructose-6-P 0.2 mM‡	same	same	same, except $MgCl_2$ omitted

*Assay conditions according to McDougal et al.[7]

†Xylulose-5-P added in an equilibrium mixture of pentose phosphates generated by treating of ribulose-5-P with ribulose-5-P epimerase according to Ashwell and Hickman[8]

‡Fructose-6-P added in an equilibrium mixture of hexose phosphate generated by treating 50 mM G-6-P with phosphohexoseisomerase for thirty minutes at 25° followed by heating the mixture at 100° for three minutes to destroy phosphohexoseisomerase.

After cooling to 22°, 0.08 μl. was added of a reagent with the following composition: 0.3 M Tris-HCl, pH 8.0, 0.2 per cent bovine serum albumin, 0.5 mM dithiothreitol, 0.03 mM TPN^+ and 10 μg./ml. of 6-P-gluconate dehydrogenase (prepared from yeast). The Teflon rack was incubated for forty minutes at 22° and 0.8 μl. of 0.3 N NaOH was then added to the wells. A heating step followed (twenty minutes at 75°). The TPNH generated during the enzymatic oxidation of 6-P-gluconate was then measured by enzymatic cycling.[5] For this purpose, to the Teflon wells cooled on ice was added 7 μl. of ice-cold cycling reagent A (0.2 M Tris-HCl), pH 8.0, 5 mM α-ketoglutarate, 1 mM glucose-6-P, 5 mM NH_4+-acetate, 0.3 mM ADP, 50 μg./ml. glucose-6-P dehydrogenase and 250 μg./ml. glutamic dehydrogenase). When low levels of 6-P-gluconate (20 μmoles/kg. dry weight or less) or small pieces of tissue (0.3 μg. or less) were to be analyzed, only 3 μl. of a cycling reagent (cycling reagent B) was added, which contained twice the enzyme concentrations (100 μg./ml. glucose-6-P dehydrogenase and 500 μg./ml. glutamic dehydrogenase). After cycling (1 to 1.5 hrs. at 37°) the Teflon rack was heated for ten minutes in an oven of 100°.

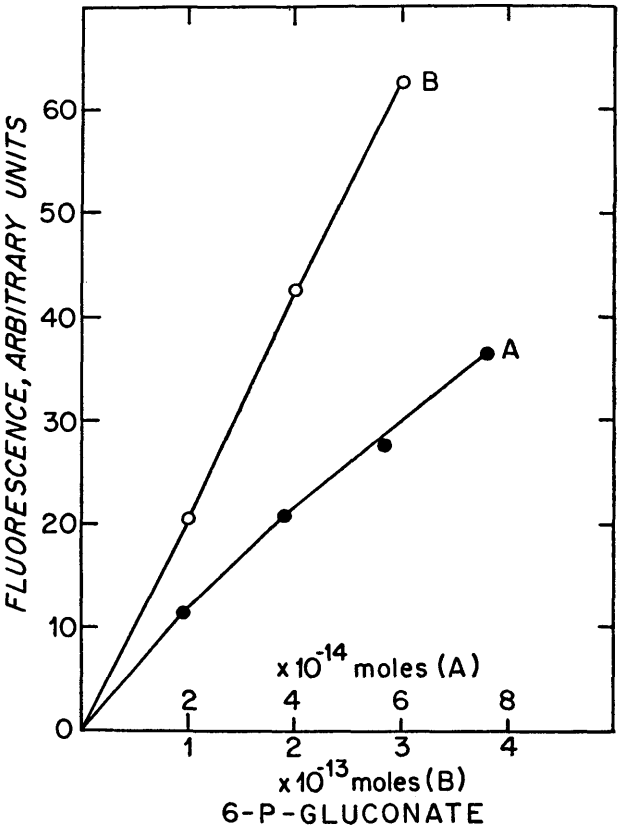
The indicator step for 6-P-gluconate was performed in fluorometer tubes (75 × 10 mm.) in 1 ml. of the following reagent (0.05 M Tris-HCl, pH 8.0, 0.05 mM TPN^+, 10 μg./ml. of 6-P-gluconate dehydrogenase from yeast). This reaction was completed after incubation at 22° for forty five minutes. The native fluorescence of the TPNH generated was finally measured.[5]

Comments on the 6-P-gluconate assay. With the described assay the sum of 6-P-gluconate and its lactone are measured. The half-time of the assay was about two minutes, as determined directly in the fluorometer in a test volume of 1 ml. Glucose-6-P, when added at ten times the 6-P-gluconate concentration did not interfere with the assay. Dithiothreitol was included in the reagent to prevent oxidation of TPNH by oxidized glutathione from the tissue and glutathione reductase, which frequently is a contaminant of commercial 6-P-gluconate dehydrogenase. The heat treatment of the acid tissue suspension destroys enzymes present in the tissue, which might interfere. Acidification eliminates endogenous TPNH. The purpose of the alkaline heat treatment following completion of the enzymatic oxidation of 6-P-gluconate to ribulose-5-P and CO_2 is to destroy excess TPN^+.

RESULTS AND DISCUSSION

Validation of 6-P-gluconate assay

To demonstrate the validity of the oil well method for 6-P-gluconate two standard curves are given which cover a thirty-fold range of sensitivity from 10^{-14} to 3×10^{-13} moles of the compound (figure 1). With authentic 6-P-gluconate, the standard deviation of the method was 12 per cent or less, permitting the estimation of mean values to within about 5 per cent with four or five samples. The precision of the procedure is comparable with similar methods published earlier.[9] The reproducibility achieved when specimens of islets or acini were analyzed was not as good. The standard error amounted to 15 per cent of the mean value, when four or five samples were analyzed (table 2). This is in all likelihood an indication of biological variability. The precision obtained in the analysis of



STANDARD CURVES OF OIL WELL TECHNICS FOR 6-P-GLUCONATE

FIG. 1. In experiment A, 3 μl. of cycling reagent A (see Methods) were used and cycling proceeded for 1.5 hrs. In experiment B, 7 μl. of cycling reagent B were used and cycling time was 1 hr. Amplification was 13,000-fold in case A and 6,000-fold in case B. Each point is the mean of two to three determinations.

TABLE 2

Reproducibility of 6-P-gluconate measurements in islets of Langerhans and pancreatic acinar tissue. The results of the analysis of individual samples from two animals are shown. The means and standard errors of 6-P-gluconate levels are also recorded. The ratio of the fluorescence delta and fluorescence blank indicate the degree of sensitivity of the assays. The control animal (Experiment 1) had a blood glucose of 4.1 mM, the hyperglycemic animal (Experiment 2) of 45 mM. In the analysis of Experiment 1, cycling Reagent I and of Experiment 2 cycling Reagent II were used.

Weight of sample (μg. dry tissue)	Ratio of fluorescence delta to fluorescence blank	6-P-gluconate (μmoles/kg. dry tissue)
	Islets of Langerhans	
Experiment 1		
0.38	0.88	36.4
0.40	0.61	20.6
0.33	0.69	30.2
0.31	0.93	40.5
0.26	0.35	26.8
		31±4
Experiment 2		
0.32	4.6	935
0.33	1.8	354
0.22	2.8	825
0.31	2.9	605
0.33	3.2	645
		675±114
	Acinar tissue	
Experiment 1		
1.33	2.2	28.2
1.00	1.3	24.0
0.94	2.3	42.7
0.87	1.3	27.7
		30.8±4.7
Experiment 2		
1.16	1.9	104
1.08	1.4	82
1.58	1.4	55
0.86	0.8	57
		71±12

enzymes in tissue specimens is indicated by the standard errors of table 3.

Pattern of enzymes of the pentose-P cycle in islets of Langerhans and acini

The in vitro activities of the enzymes of the pentose-P pathway are essentially the same in islets and acinar tissue (table 3). It is doubtful that the slight differences observed are of metabolic significance. These observations are in agreement with work of other investigators who demonstrated that the in vitro activities of glucose-6-P dehydrogenase and 6-P-gluconate dehydrogenase were similar in islets and acinar tissue.[11] The relatively constant proportions of the five enzymes in islets and acinar tissue support the concept that the capacity to catabolize glucose via the pentose-P-cycle is the same for both types of cells. Activities in pancreatic cells are comparable to activities demonstrated in brain.[6] Two other pertinent observations indicated similarities regarding the role of the pentose-P-shunt in endocrine and exocrine cells of the pancreas. Both types of cells contain comparable amounts of TPN[1] and respond with a similar increase of the activities of the two dehydrogenases of the shunt to chronic treatment with glucose.[12]

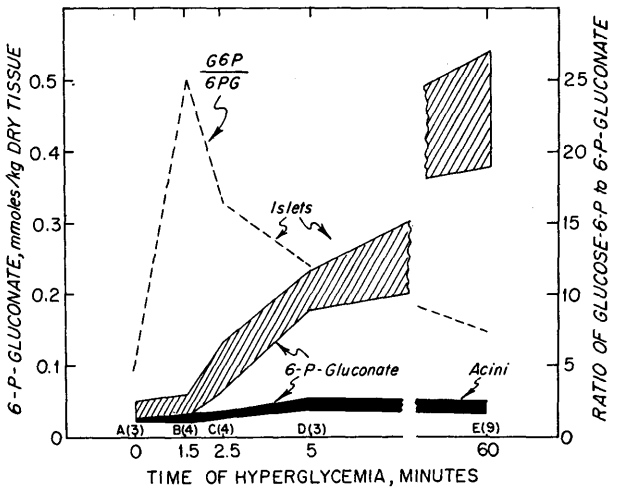
Effect of hyperglycemia on levels of 6-P-gluconate in islet and acinar cells

A peculiarity of the hexose monophosphate shunt in β-cells became apparent, when the 6-P-gluconate levels of islets of Langerhans and acinar tissue were compared at various stages of hyperglycemia (figure 2). Within one hour, hyperglycemia of about 50 mM caused an average thirteen-fold increase of the 6-P-gluconate concentration in β-cells whereas the level of the intermediate in acinar cells was little affected. The rise of

TABLE 3

Pattern of enzymes of the pentose-P-cycle in endocrine and exocrine portions of the pancreas. Each value represents the mean and standard error of usually five determinations carried out in an individual animal. The proportions of activities are recorded in reference to the activity of glucose-6-P DH.

Enzyme	Activities		Activities relative to glucose-6-P dehydrogenase	
	Islets	Acini	Islets	Acini
	moles/kg. dry tissue/hr.			
Glucose-6-P dehydrogenase	1.35±0.07	0.93±0.07	1	1
6-P-gluconate dehydrogenase	0.43±0.06	0.25±0.06	0.32	0.27
Ribulose-5-P epimerase	2.00±0.06	1.97±0.22	1.48	2.12
	1.85±0.13	1.88±0.21	1.37	2.00
Ribulose-5-P isomerase	0.75±0.09	0.60±0.09	0.55	0.65
	0.56±0.07	0.31±0.03	0.41	0.34
Transketolase	0.11±0.02	0.18±0.02	0.08	0.19
	0.16±0.03	0.13±0.01	0.12	0.14
Transaldolase	0.22±0.03	0.31±0.02	0.16	0.33
	0.23±0.01	0.27±0.04	0.17	0.29



EFFECT OF HYPERGLYCEMIA ON 6-P-GLUCONATE LEVEL IN ISLETS OF LANGERHANS AND EXOCRINE PANCREAS

FIG. 2. Animals were anesthetized with phenobarbital and divided into five groups: (A) fasted for 8 hrs. (B) not fasted, sampled 90 sec. after intravenous injection of 0.2 ml. of 2 M glucose. (C) like B, but sampled 150 sec. after the glucose load. (D) like B, but sampled 300 sec. after the glucose load. (E) not fasted, sampled 1 hr. after 1 ml. of 2 M glucose injected subcutaneously. The average blood sugar was in group A about 5 mM, in groups B-E, about 50 mM. Usually five determinations were made to obtain the mean level for each individual pancreas. The mean of the levels of the different organs is represented. The number of animals is given in parentheses. The width of the bands represents two standard errors. The glucose-6-P data for calculation of the ratio of glucose-6-P/6-P-gluconate were taken from Matschinsky and Ellerman[1] and were obtained from the same groups of animals.

6-P-gluconate in the β-cells during hyperglycemia was markedly slower than the rate of accumulation of glucose and glucose-6-P.[1]* Five minutes after an intravenous glucose load the concentration of 6-P-gluconate in islet cells had increased approximately five-fold to about 50 per cent of the peak level, whereas under comparable conditions glucose-6-P levels at that time had nearly reached their maximum.[1] This delay of 6-P-gluconate accumulation is clearly expressed by the ratio of glucose-6-P to 6-P-gluconate computed for different time intervals after glucose injection. The ratio of 4.5, found at control conditions, is slightly lower than observed in brain.[13] Ninety seconds after glucose injection, the value had reached a maximum of 25 and declined then, slowly reaching a value of 7.5 after one hour.

*Since in the assay no distinction is made between 6-P-gluconate and its lactone, there exists the theoretical possibility that either molecule may rise faster than their sum.

Evaluation of the present data is made difficult by the possible presence of regulatory steps in the pentose-P pathway, which do not obey simple mass action laws. There is some indirect evidence that glucose-6-P dehydrogenase may be influenced allosterically by ATP and TPNH[14] and P_i[15]. The variation in the ratio of glucose-6-P/6-P-gluconate during hyperglycemia observed in the present investigation similarly indicates some control of the oxidative limb of the hexose monophosphate shunt. Altogether, however, data obtained in the present study indicate that in the islets of the pancreas the levels of the intermediates of the pentose-P cycle rise and fall with the blood sugar level similarly as observed for other phosphorylated metabolites of glucose.

The delayed rise of 6-P-gluconate and probably also of the pentose-P intermediates of the shunt appears to be too slow a process to trigger the release mechanism of insulin, assuming the β-cells of obese hyperglycemic mice respond as rapidly as the β-cells of the rat to glucose with the release of insulin.[16] On the other hand, it has been observed that hyperglycemia enhances RNA synthesis in pancreatic islets.[17] An activation of the pentose-P cycle as indicated here and also suggested by tracer studies[18,19] could well be the primary cause for this stimulation of RNA synthesis by providing high levels of precursors for RNA. In addition to the well-known, almost instantaneous, effect on the insulin release mechanism, glucose could then by this means turn on RNA synthesis to initiate a restoration of the hormone supply.

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