ACCUMULATION OF CITRATE IN PANCREATIC ISLETS OF OBESE HYPERGLYCEMIC MICE

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Received November 4, 1968

In several publications dealing with the mechanism of insulin release, the citrate molecule has been implicated as an essential element in the secretory process (1-3). However, the actual levels of citrate in islets have never been measured. Accordingly, in order to further the study of the role of citrate in the islets of Langerhans, a method for the measurement of citrate in microscopic samples was developed. Using this method, determinations have been made in the cells from islet and acinar portions of the pancreas of obese hyperglycemic mice and of lean control animals of the same strain. The effect of citrate on P-fructokinase of islets was also studied *in vitro*. It was found that the citrate levels in pancreatic islets of obese hyperglycemic animals were twice as high as in the islets of the lean controls. It was demonstrated that P-fructokinase of islets is subject to inhibition by citrate *in vitro*.

Methods

Frozen-dried specimens of islets of Langerhans and acini from obese and lean mice (C 57 BL/6 J-ob strain) were sampled as described (4). Blood was collected from the abdominal cavity immediately after the pancreas was taken out. The blood was heparinized and plasma separated by centrifugation. Citrate was measured enzymatically using a fluorometric version of the assay described by

Gruber and Moellering (5). Plasma was analyzed directly, without deproteinizing, by adding 20 µl to 1 ml of reagent with the following composition: 0.1 Tris-HCl buffer, pH 7.8, 0.5 mM $MgCl_2$, 10 µM NADH, and 3 µg/ml of crystalline malic dehydrogenase. After 10 min at 25°, the fluorescence was recorded and 10 to 20 µg of citrate lyase (Boehringer) were added in 2 µl of 0.05 M Tris-HCl buffer, pH 7.5, which contained 1 mM EDTA and 0.05% bovine serum albumin. The reaction was completed within 10 min and the fluorescence decrease was measured. Appropriate blanks and standards (20 µl of 0.2 to 0.4 mM citrate) were carried through the procedure. For analysis of citrate in whole pancreas about 50 mg of tissue were extracted with 0.5 ml of cold 0.5 N $HClO_4$. Of the extract, neutralized with K_2CO_3, 50 µl were added to 1 ml of citrate reagent and the assay was performed as for plasma, except that the NADH in the reagent was reduced to 5 µM and the standards to 0.1 mM. For the analysis of citrate in microscopic samples, the necessary sensitivity was gained by working in small volumes under oil and by using a catalytic assay for NAD^+ (6-8). To 0.1 µg of frozen-dried tissue placed in the well of a Teflon rack was added 0.04 µl of 0.05 M NaOH. The droplet was covered with oil and the sample heated for 20 min at 70° as described (7). After cooling, 0.04 µl was added of a reagent which contained 0.3 M Tris-HCl buffer, pH 7.8, 1 mM $MgCl_2$, 30 µM NADH, 0.05 % bovine serum albumin, 50 mM ascorbic acid, 6 µg/ml of malic dehydrogenase and 40 µg/ml of citrate lyase. (The serum albumin had been thoroughly dialyzed against dilute Tris-HCl, pH 8.0 to remove citrate.) After 15 min at 25°, 0.12 µl of 0.5 N HCl was added and 10 min later the Teflon rack was cooled on ice (7). For the catalytic determination of NAD^+, to each well was added 10 µl of the following reagent (8): 0.1 M phosphate buffer, pH 7.4, 10 mM sodium arsenate, 10 mM EDTA, 0.02 % bovine serum albumin, 5 mM

ammonium-acetate, 3 mM glyceraldehyde-3-P, 5 mM α-ketoglutaric acid, 0.3 mM ADP, 100 µg/ml of NAD-free glyceraldehyde-P-dehydrogenase prepared from yeast (freed by centrifuging of excess ammonium-sulfate) and 500 µg/ml of glutamic dehydrogenase in glycerol. 75 µl of 0.3 M Na_2CO_3 was added to neutralize glyceraldehyde-3-P (stored at pH 3) and α-ketoglutaric acid. To achieve an amplification of approximately 5000-fold, samples were incubated for 1 hr at 37°. To stop the cycling reaction and destroy α-ketoglutarate, 0.6 µl of 3 % H_2O_2 was added and the rack heated for 15 min at 70°. The contents of the droplet from each well was transferred to 200 µl of the following reagent: 0.2 M hydrazine-HCl buffer, pH 9.5, 0.3 mM NAD^+, 0.02 % bovine serum albumin, 0.3 mM ADP and 30 µg/ml of glutamic dehydrogenase in glycerol. After 30-40 min at 37° 1 ml of water was added to the tubes and the fluorescence of NADH recorded in the fluorometer. Appropriate blanks and standards (10^{-13} to 3×10^{-12} moles citrate) were carried along. Pancreatic islets from obese animals are larger than from controls, and therefore the frozen-dried samples were larger (about 0.5 µg). Since, in addition, citrate levels are higher, all volumes were increased 10-fold and the cycling step was performed in 10 x 75 mm glass tubes. Glyceraldehyde-P dehydrogenase and glutamic dehydrogenase concentrations were reduced to approximately one-fifth of those used with normal islets to give amplification of about 1000-fold. Glucose in plasma was determined according to (9).

P-fructokinase and the effect of citrate on this enzyme was studied in the following manner: to 1 to 2 µg of frozen-dried islet tissue was added 100 µl of 50 mM imidazole-HCl buffer, pH 7.1 containing 150 mM potassium acetate, 0.01 % bovine serum albumin, 5 mM $MgCl_2$, 7.5 mM sodium arsenate, 0.1 mM NAD^+, 1 mM mercaptoethanol, 1 mM EDTA, 0.13 mM fructose-6-P, 0.2 mM ATP,

45 µg/ml of aldolase, 15 µg/ml of triose phosphate isomerase, and 45 µg/ml of glyceraldehyde-P-dehydrogenase. NADH formation was followed directly in the fluorometer. Fructose-1,6-P_2 served as standard.

Results and Discussion

With the oil well technique the final readings were linear with citrate concentrations from 10^{-13} to 3 x 10^{-12} moles (Fig. 1). The overall blank of the more sensitive method (A) was equivalent to 3 x 10^{-13} moles of citrate and of the less sensitive method (B) to 10^{-12} moles of citrate. The degree of precision was acceptable (standard deviation less than $\pm$ 10 %). Recovery of citrate added to tissue was 87 %. Pyruvate, a possible source of error, did not interfere at 10 µM concentration.

Levels of citrate in the pancreas of fed obese animals were twice as high as in the controls (Table 1). This was true for the pancreas as a whole on a wet weight basis, as well as for acinar tissue on a dry weight basis. The difference in the acinar tissue was less pronounced after brief starvation. The citrate levels in islets were also elevated in the obese mice but were 20 to 40 % higher than in acinar tissue in all 4 groups of animals. The citrate concentrations in plasma likewise appeared to be somewhat higher in obese hyperglycemic animals. The fed groups had comparable plasma sugar levels. Only after overnight fasting, were the levels lower in the lean animals.

The finding of higher citrate levels in pancreatic islets of the pathologic animals deserves special attention, because of the major role attributed to the citrate molecule in the regulation of glycolysis (10-12). It has been demonstrated that citrate added *in vitro* leads to increased levels of glucose-6-P in islets and enhanced insulin release by glucose (3). It is, therefore, con-

TABLE I

CITRATE LEVELS IN PANCREAS AND PLASMA

Half of the animals were used for the determination of citrate in microscopic samples of islets and acini. The other half were used for plasma and whole pancreas analyses. The average weight of the controls was 22 g, the average weight of the obese animals was 58 g. The mean of results, the standard errors of the means and the number of animals studied are given. $^{a}p \leq 0.1$, $^{b}p \leq 0.05$, $^{c}p \leq 0.01$ (all compared to controls given the same treatment).

Nutritional State	Plasma glucose mmoles/liter	Plasma citrate mmoles/liter	Citrate in Pancreas: Average Pancreas mmoles/Kg. wet tissue	Citrate in Pancreas: Islets mmoles/Kg. dry tissue	Citrate in Pancreas: Acini mmoles/Kg. dry tissue
			Lean animals		
fed	9.6 ± 0.9(5)	0.18 ± 0.01(5)	0.65 ± 0.06(5)	3.73 ± 0.2(6)	2.97 ± 0.3(6)
fasted 10 hrs.	6.9 ± 0.5(5)	0.29 ± 0.03(5)	0.54 ± 0.04(5)	4.82 ± 0.3(5)	3.94 ± 0.2(5)
			Obese animals		
fed	10.8 ± 1.5(5)	0.23 ± 0.02(5)^{a}	1.00 ± 0.02(5)^{a}	7.75 ± 0.7(5)^{c}	6.22 ± 0.3(5)^{b}
fasted 10 hrs.	11.0 ± 1.2(5)^{b}	0.38 ± 0.01(5)^{b}	1.00 ± 0.02(5)^{c}	7.00 ± 0.7(7)^{b}	4.90 ± 0.6(5)

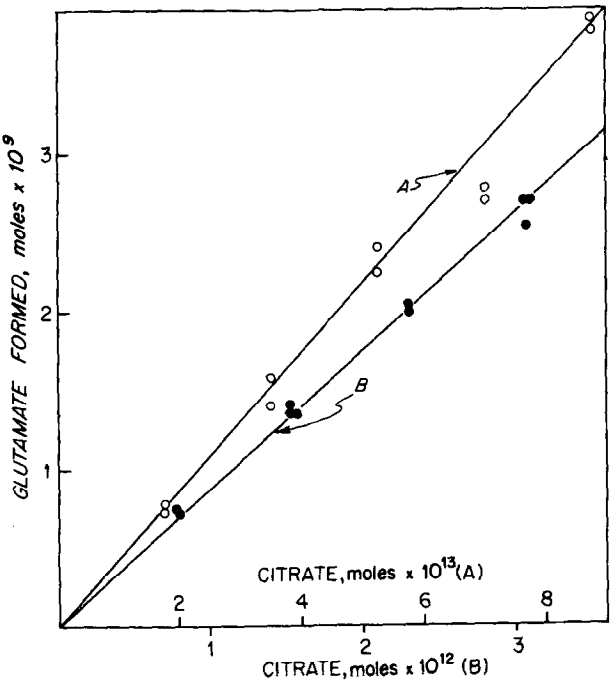

Fig. 1. Yield of product in the catalytic assay for citrate. Assay A is that used for 0.1 μg samples of normal islets; Assay B is that used for 0.5 μg samples of islets from obese mice (see Methods). It is seen that the amplification at the cycling step was approximately 4500-fold for Assay A and nearly 1000-fold for Assay B.

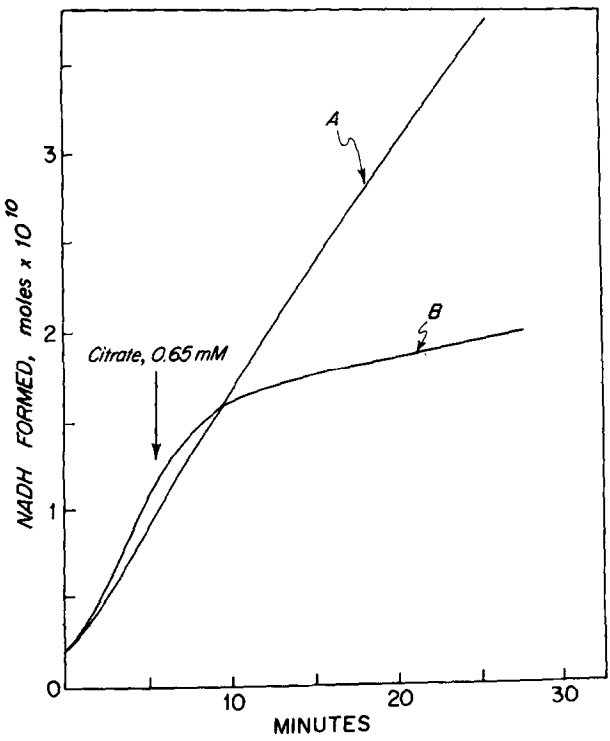

Fig. 2. Effect of citrate on P-fructokinase from pancreatic islets of obese mice. The amount of tissue in Assay A was 1.57 μg and in Assay B 1.67 μg. The arrow indicates when citrate was added to Assay B.

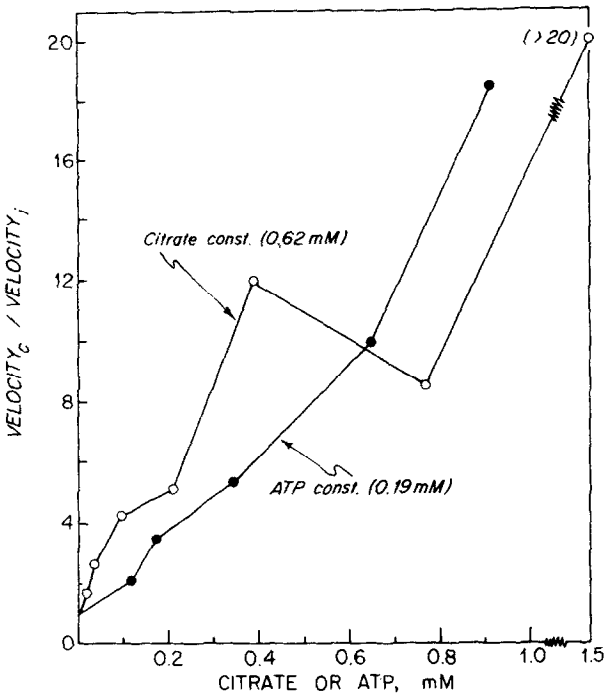

Fig. 3. Inhibition of islet P-fructokinase by citrate and ATP. With ATP at an essentially non-inhibitory level (0.19 mM) citrate was varied as shown. Conversely, citrate was held constant at a level of 0.62 mM and ATP was varied. The basic composition of the reagent is given in Methods. The basal activity with 0.19 mM ATP and no citrate was 267 ± 30 mmoles of fructose-1,6-P_2 per kg dry weight per hour (average of 6 samples ± standard error). Velocity$_c$ and velocity$_i$ refer respectively to control velocity (0.19 mM ATP, no citrate) and inhibited velocity.

ceivable that the elevated citrate in the islets of the obese animals is, at least in part, responsible for the known increased plasma levels of insulin (13, 14). If so, the effect is probably indirect and might be attributed to inhibition of P-fructokinase by citrate. This possibility is supported by *in vitro* studies (Figs. 2, 3) which show that P-fructokinase from islets of Langerhans can indeed be inhibited in the same manner as the enzyme from other sources (10-12). Whether this interpretation of the increased insulin levels in the obese mice is valid and, if so, whether it has further implications for the understanding of diabetes, can only be decided by further studies. Thus, it will be necessary to determine the effect of citrate accumulation on glucose-6-P and fructose diphosphate levels in β-cells and also to thoroughly investigate the effect of citrate on the rate of insulin secretion.

Acknowledgement

These studies were supported by research grant AM-1059 from the National Institutes of Health.

References

1.) Maske, H., Diabetes *6*, 335 (1957).
2.) R-Candela, J. L., "The Structure and Metabolism of Pancreatic Islets", ed. S. E. Brolin, B. Hellman, H. Knutson, Macmillan New York (1964), 349.
3.) Montague, W. and Taylor, K. W., Nature *217*, 853 (1968).
4.) Matschinsky, F. M. and Ellerman, J. E., J. Biol. Chem. *243*, 2730 (1968).
5.) Gruber, W. and Moellering, H., Biochem. J. *346*, 85 (1966).
6.) Lowry, O. H., Harvey Lectures *58*, 1 (1963).
7.) Matschinsky, F. M., Passonneau, J. V., and Lowry, O. H., J. Histochem. Cytochem. *16*, 29 (1968).
8.) Matschinsky, F. M., Rutherford, C., and Guerra, L., Proc. 2nd. Internat. Congr. Histochem. Cytochem., New York (1968).
9.) Lowry, O. H., Passonneau, J. V., Hasselberger, F. X., and Schulz, D. W., J. Biol. Chem. *239*, 18 (1964).
10.) Passonneau, J. V. and Lowry, O. H., Biochem. Biophys. Res. Communs. *13*, 372 (1963).
11.) Parmeggiani, A. and Bowman, R. H., Biochem. Biophys. Res. Communs. *12*, 268 (1963).
12.) Garland, P. B., Randle, P. J., and Newsholme, E. A., Nature London *200*, 169 (1963).
13.) Stauffacher, W., Lambert, A. E., Vecchio, D., and Renold, A.E. Diabetologica *3*, 230 (1967).
14.) Westman, S., Diabetologica *4*, 141 (1968).