Determination of Fructose 2,6-Bisphosphate in Human Lymphocytes Is a Potential Laboratory Test in Diabetology

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The conventional micromethod of fructose 2,6-bisphosphate (F-2,6-P_2) determination is optimized for human peripheral blood lymphocytes. The F-2,6-P_2 content in lymphocytes of healthy subjects is 0.8±0.2 pmol/10^6 cells or 6.7±1.1 pmol/mg protein. Preliminary findings show a decrease in the F-2,6-P_2 content in lymphocytes from patients with severe diabetes mellitus.

Key Words: *fructose 2,6-bisphosphate; lymphocytes; peripheral blood*

Experimental streptozotocin diabetes causes substantial changes in the fructose 2,6-bisphosphate (F-2,6-P_2) system [2,10,12,13]. Diabetes enhances cAMP-dependent phosphorylation of bifunctional enzyme, which leads to a decrease in the liver content of F-2,6-P_2. Consequently, the rate of glycolysis drops, while that of glyconeogenesis increases. This may be the major cause of the increase in blood glucose concentration in diabetes mellitus [12].

Recently, it has been shown that the hypoglycemic effect of numerous peroral antidiabetic preparations is related to the inhibition of cAMP-dependent phosphorylation of the bifunctional enzyme and increased liver content of F-2,6-P_2 [13]. It should be remembered that insulin produces a potent effect on the F-2,6-P_2 system, although this effect is indirect [12,13]. Based on these considerations, we think that it is reasonable to examine the F-2,6-P_2 levels in patients with diabetes mellitus of different types and severity treated with various antidiabetic preparations.

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The study was performed on peripheral blood lymphocytes, although their F-2,6-P_2 system slightly different from that of hepatocytes [5]. However, lymphocytes are more available in clinic and have insulin receptors [11]. There is evidence suggesting that in contrast to hepatocytes, protein kinase C but not protein kinase A is involved in the regulation of the activity of the bifunctional enzyme in lymphocytes [5,8]. It was shown that incubation of bifunctional enzyme from lymphocytes in the presence of the catalytic subunit of protein kinase A does not change the enzyme activity [5]. Incubation of B cells with phorbol ester increases the F-2,6-P_2 level and the activity of 6-phosphofructo-2-kinase in these cells [5,8]. As known, phorbol ester imitates the effect of diacylglycerol, a naturally occurring activator of protein kinase C. An increase in the rate of glycolysis and in the F-2,6-P_2 content was observed in lymphocytes incubated with mitogens [5].

A possible activation of protein kinase C in lymphocytes by insulin has been indirectly confirmed by the finding that diacylglycerol and phorbol ester can imitate the inducing effect of insulin on the synthesis of glycerol-3-phosphate dehydrogenase [14].

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Our objective was to optimize the micromethod of the determination of the F-2,6-P_2 content in biological material [15] for human peripheral blood lymphocytes (8 ml of blood for two parallel samples). The main goal was to find out the range within which the F-2,6-P_2 content varies in lymphocytes of healthy subjects.

MATERIALS AND METHODS

Lymphocytes were isolated from human peripheral blood on a Ficoll-Paque gradient [4]. It should be noted that lymphocytes can be counted in an electron counter of blood formed elements prior to the second centrifugation [3]. After counting, cells were pelleted by centrifugation (10 min, 400*g*). The pellet was heated with 0.1 N NaOH (80 μl) for 10 min at 80-85°C, cooled, and pH was adjusted to 7.5-8.0 by adding 60 μl 0.1 N CH_3COOH. The samples were centrifuged for 10 min at 5000*g*, after which the supernatant (100 μl) was collected to determine the F-2,6-P_2 content. The protein content was measured in the remainder after the addition of 100 μl 0.1 N NaOH and mixing [6].

The F-2,6-P_2 content was determined by enzyme assay [15] optimized for human peripheral blood lymphocytes. The method is based on the ability of F-2,6-P_2 to activate pyrophosphate-dependent phosphofructokinase (PP_n-PFK) from potato tubers. The F-2,6-P_2 content was determined by comparing the degree of activation of PP_n-PFK by known concentration of F-2,6-P_2 and by F-2,6-P_2 in the sample (lymphocytes) using a calibration curve. The activity of PP_n-PFK was measured spectrophotometrically at 340 nm by the rate of NADH oxidation in a system coupled with aldolase, triosephosphate isomerase, and glycerol-3-phosphate dehydrogenase. The enzymatic reactions are as follows:

$$\text{fructose 6-phosphate} + H_4P_2O_7 \xrightarrow[\text{(EC 2.7.1.90)}]{PP_n\text{-PFK}} \text{fructose 1,6-bisphosphate} + H_3PO_4;$$

$$\text{fructose 1,6-bisphosphate} \xrightarrow[\text{(EC 4.1.2.13)}]{\text{aldolase}} \text{dihydroxyacetone phosphate} + \text{glyceraldehyde 3-phosphate};$$

$$\text{glyceraldehyde 3-phosphate} \xrightarrow[\text{(EC 5.3.1.1)}]{\text{triosephosphate isomerase}} \text{dihydroxyacetone phosphate};$$

$$\text{2-dihyroacetone phosphate} + 2\ \text{NADH} \xrightarrow[\text{(EC 1.1.1.8)}]{\text{glycerol-3-phosphate dehydrogenase}} 2\ \text{glycerol-3-phosphate} + 2\ \text{NAD}.$$

The following reagents were used: 0.1 M Tris-acetate buffer, pH 8.0, 40 mM $MgCl_2$, 10 mM fructose 6-phosphate containing 17 mM glucose 6-phosphate, 1.5 mM NADH in Tris-acetate buffer, pH 8.0, PP_n-PFK from potato tubers (Sigma) dissolved in Tris-acetate buffer (pH 8.0) containing 50% glycerol (0.01 U is required for one sample), 5 mM sodium pyrophosphate ($H_4P_2O_7$), 10% sodium dodecyl sulfate (SDS), and a coupled enzyme system containing 0.5 U aldolase, 5 U triosephosphate isomerase and 1 U glycerol-3-phosphate dehydrogenase per sample (all from Sigma). The enzyme system can be prepared for several determinations. However, it should be remembered that the crystalline enzyme suspensions in ammonium sulfate require salt elimination, since ammonium sulfate inhibits PP_n-PFK [15].

Protocol of Determination. The reaction mixture contains 200 μl Tris-acetate buffer, 30 μl $MgCl_2$, 60 μl fructose 6-phosphate, 100 μl NADH, 50 μl coupled enzyme system, and 10 μl PP_n-PFK. When several samples are analyzed, 450 μl of the reaction mixture is added to a microcuvette with an optic pathway length of 1 cm. The sample (100 μl) is then added to the cuvette, mixed, and light absorbance is measured at 340 nm (the first point of reference). The reaction is triggered by the addition of 50 μl sodium pyrophosphate followed by thorough mixing. Light absorbance at 340 nm (the second point of references) is measured after a 10-min incubation at 30°C (total volume 600 μl). Providing that the ΔA_{340} value (the difference between the first and second reference points) is known, the F-2,6-P_2 content in the sample is calculated from the calibration curve.

Construction of Calibration Curve. The reaction mixture (0.45 ml), Tris-acetate buffer (pH 8.0), and 1.6×10^{-8} M PP_n-PFK are added to the microcuvette in the amounts given in Table 1. The F-2,6-P_2 content in lymphocytes is calculated from the following for-

TABLE 1. Concentrations of F-2,6-P_2 Used for Construction of Calibration Curve

1.6×10^{-8} M F-2,6-P_2, μl	0.1 M Tris-acetate buffer (pH 8.0), μl	F-2,6-P_2, pmol/sample
0	100	0
25	75	0.4
50	50	0.8
75	25	1.2
100	0	1.6

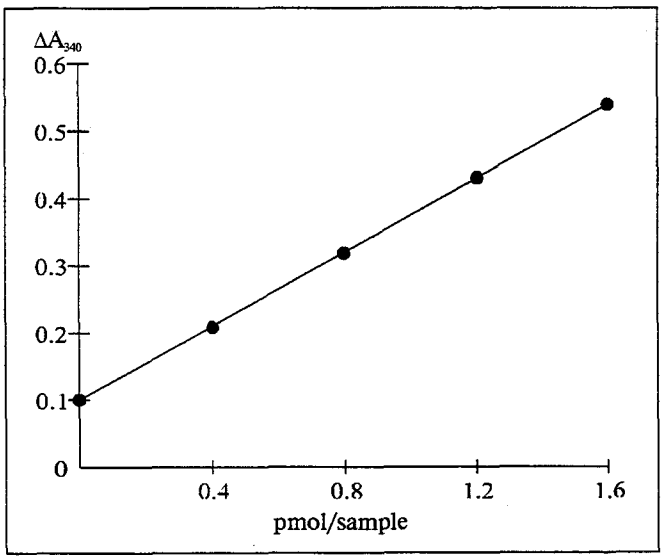

Fig. 1. Calibration curve for determination of the F-2,6-P_2 content in peripheral blood lymphocytes.

mula: $A=B\times1.5/C$, where A is the lymphocyte F-2,6-P_2 content, B is the F-2,6-P_2 concentration calculated from the calibration curve, and C is the total protein content (mg) or cell count (10^6 cells).

RESULTS

Figure 1 illustrates the relationship between changes in light absorbance (ΔA_{340}) and the content of F-2,6-P_2 in the sample. In the original protocol [15], the calibration curve is constructed using the relative activity of PP_n-PFK (v/V, where v is the rate of the enzyme reaction in the sample and V is the maximum reaction rate measured in the presence of 1 μM PP_n-PFK). However, it is more convenient to use the decrease in light absorbance during a 10-min incubation (ΔA_{340}). In this case the dependence is linear up to 1.5 pmol F-2,6-P_2 in the sample. When great numbers of samples are tested, the reaction is stopped by the addition of 1% SDS, after which the NADH content is measured [7]. This approach was used in the present study: after a 10-min incubation 30 μl 10% SDS was added, mixed with the sample, and light absorbance was measured (the second reference point).

It should be noted that the method based on the activation of PP_n-PFK is more sensitive than determination of F-2,6-P_2 concentration by activation of phosphofructokinase-1 [1].

The F-2,6-P_2 content in lymphocytes can be expressed per a definite number cells or per 1 mg protein. In this study, the F-2,6-P_2 content in lymphocytes of healthy volunteers (n=14) varied from 0.6 to 1.2 pmol/10^6 cells. Meanwhile, a wider variation range was reported for this value (0.6-1.6 pmol/10^6 cells). In the available literature we found only two pieces of evidence concerning the F-2,6-P_2 in lymphocytes [8,9]. We obtained a more stable value for the F-2,6-P_2 content in peripheral blood lymphocytes per 1 mg protein (6.7±1.1 pmol/mg protein).

Our preliminary studies showed that the content of F-2,6-P_2 is lowered in the lymphocytes of patients with severe diabetes mellitus.

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