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Mononuclear and polymorphonuclear leukocytes show increased fructose-1,6-bisphosphatase activity in patients with type 1 diabetes mellitus

Natalia Sybirna [a], Daria Dziewulska-Szwajkowska [b], Marina Barska [a], Andrzej Dzugaj [b,*]

[a] *Department of Biochemistry, Ivan Franko Lviv National University, Grushevsky Street 4, 79005 Lviv, Ukraine*
[b] *Department of Animal Physiology, Zoological Institute, Wroclaw University, Cybulskiego 30, 50-205 Wroclaw, Poland*

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Abstract

The activity and localization of fructose-1,6-bisphosphatase (FBPase; EC 3.1.3.11) in blood leukocytes of patients with type 1 diabetes mellitus and healthy adults were investigated immunocytochemically. The amount of polymorphonuclear (PMN) and mononuclear (MN) cells with positive FBPase immunocytochemical reaction was 57% and 68%, respectively, in pathological, and 38% and 42%, respectively, in healthy donors. Results of light microscopic investigations were confirmed by measurements of FBPase activity following lysis of PMN and MN cells. The enzyme activity of PMN and MN leukocytes was higher in diabetes mellitus than in healthy adults, by 30% and 127%, respectively. Using immunocytochemistry together with electron microscopy, FBPase was detected not only in the cytoplasm but also in the nucleus of leukocytes of both patients with insulin-dependent diabetes mellitus and healthy donors.

Keywords: Fructose 1,6-bisphosphatase; Diabetes mellitus type 1; Polymorphonuclear cells; Mononuclear cells

1. Introduction

Fructose-1,6-bisphosphatase (FBPase) (EC 3.1.3.11) catalyzes hydrolysis of fructose-1,6-bisphosphate to fructose-6-phosphate and P_i in the presence of divalent cations like magnesium, manganese, zinc or cobalt (Benkovic and De Maine, 1982; Tejwani, 1983). Liver and muscle FBPase isozymes have been found in mammalian tissues (Tillmann et al., 2002). Both isozymes are activated by monovalent cations like potassium or ammonium, inhibited competitively by fructose-2,6-bisphosphate and allosterically by AMP. The basic difference between muscle and liver isozymes concerns their sensitivity to AMP inhibition. $I_{0.5}$ of the muscle isozyme is 50 to 100 times lower than the corresponding values for the liver isozyme (Benkovic and De Maine, 1982; Tejwani, 1983; Skalecki et al., 1999). The liver isozyme has been recognized as the key enzyme of gluconeogenesis, the muscle isozyme as the key enzyme of glyconeogenesis (Tejwani, 1983; Rakus et al., 2004). FBPase activity has been detected in the majority of mammalian cells where either gluconeogenesis or glyconeogenesis occurs. In human tissues the liver isozyme has been found in liver, kidney, lung and monocytes (Dzugaj and Kochman, 1980; Kikawa et al., 1994: Gizak et al., 2001).

Type 1 diabetes mellitus (insulin-dependent diabetes mellitus; IDDM) is a chronic organ-specific autoimmune disease, in which the β cells of pancreatic islets are destroyed by the body's own immune system. The pathogenesis of type-1

Abbreviations: AOF, active oxygen forms; AGE, advanced glycation end-products; BTP, Bis-Tris-Propane; EDTA, ethylenediaminetetraacetic acid; FBPase, D-fructose 1,6-bisphosphate 1-phosphohydrolase (EC 3.1.3.11); IDDM, insulin-dependent diabetes mellitus; NADP, β-nicotinamide adenine dinucleotide phosphate sodium salt; PMN, polymorphonuclear leukocytes; PLO-AOD, Peroxide lipid oxidation–antioxidant defense.

* Corresponding author. Tel.: +48 71 375 9544; fax: +48 71 375 9213.
E-mail address: dzugajan@biol.uni.wroc.pl (A. Dzugaj).

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diabetes mellitus is determined by genetic and environmental factors (Kelly et al., 2001b). IDDM is characterized by hyperglycemia and very low levels of circulating endogenous insulin. The physiological role of liver FBPase in glucose homeostasis as well as in IDDM hyperglycemia has been investigated, but no data on FBPase in white blood cells of diabetic patients are available.

The primary aim of the present paper was to investigate the FBPase activity in leukocytes of patients with IDDM and the subcellular localization of FBPase in these cells. The physiological significance of these findings is discussed.

2. Materials and methods

A group of 12 patients with type 1 diabetes (aged 36 ± 10 years, 4 female and 8 male) was studied. They were all insulin treated, the mean diabetes duration was 12.9 ± 6.1 years, and the mean HbA_{1C} level was 7.5 ± 0.9%. These patients did not receive any medication likely to influence WBC function. A group of 10 volunteers (aged 34.5 ± 10 years, 3 female and 7 male) was analyzed as a control. The volunteers were free of disease and were not taking any medication.

2.1. Chemicals

Gradisol G was purchased from Polfa-Kutno (Poland), Triton X-100 was from BDH Chemicals (England). Anti-rabbit IgG gold-conjugated (10 nm), Biotin-conjugated mouse monoclonal anti-rabbit immunoglobulins, extravidin peroxidase, normal sera, RPMI 1640 medium and other biochemicals were purchased from Sigma (USA). All the reagents were of the highest purity commercially available. Distilled deionized water was used in all the experiments.

2.2. Isolation of leukocytes

Blood samples from patients with type 1 diabetes and healthy volunteers were collected in heparinized tubes. Leukocytes were isolated from heparinized (20 U/ml) whole blood by Gradisol G (a solution of dextran and uropoline) gradient density (1.115 ± 0.002 g/cm^3 at 20 °C), using the Boyum method (Boyum, 1968) with slight modification. Heparinized whole blood was layered onto Gradisol G and centrifuged at 400 × *g* for 25 min at 20 °C. This method allows the simultaneous separation of two highly purified leukocyte fractions: MN, containing 95% lymphocytes and monocytes and PMNs containing 94% PMN. PMN and MN were washed three times with RPMI medium. The purity of isolated PMN and MN was determined by Romanowsky staining. Cell viability was estimated by trypan blue exclusion. The cells were resuspended in 0.4% (w/v) trypan blue in phosphate-buffered saline (PBS). The total number of PMN and MN cells was counted using a Thoma hemacytometer.

2.3. Immunocytochemistry

2.3.1. Light microscopy

Fresh smears were fixed for 5 min in acetone/methanol (50:50). For immunostaining, endogenous peroxidase activity was quenched by incubation in 1% hydrogen peroxide in 100% methanol for 30 min. Smears were washed in Tris-buffered saline (TBS) and blocked with 10% goat serum in TBS for 2 h at room temperature. Primary polyclonal anti-FBPase was diluted 1:600 in TBS and incubated overnight at 4 °C in a humidified chamber. The smears were then washed in TBS (3 × 10 min), followed by biotinylated goat anti-rabbit secondary antibody (Sigma) at 1:4500 in TBS for 120 min at room temperature. After washes in TBS (3 × 10 min), streptavidin horseradish peroxidase (HRP) (Sigma) was added (20 μg/1 ml) and incubated for 1 h. After three washes, the presence of HRP was detected with diaminobenzidine (Sigma). The reaction was stopped by rinsing in water, dehydrated through graded ethanols, and cleared in xylene. In the control experiments primary antibody was omitted, or non-immune rabbit serum was used as the first layer.

Estimation of the positive cytoplasmic and nuclear FBPase immunocytochemical staining was carried out by measuring the intensity of brown pigment in leukocytes. An "intense reaction" corresponds to staining of PMN or MN cytoplasm deep-brown; "medium reaction"—to brown; "slight reaction"—to light-brown; and negative reaction means the absence of color. For quantitative evaluation of immunocytochemical reaction on smears, the percent of positively stained PMN or MN was calculated (Pratt, 2001).

2.3.2. Electron microscopy

Isolated PMN and MN cells were fixed in 1.55% glutaraldehyde in 0.2 M cacodylate buffer pH 7.2 for 2 h at 2 °C and then washed twice in 0.2 M cacodylate buffer pH 7.2. Fixed cells were incubated in $NaJO_4$ for 15 min and washed in deionized water. Then the cells were transferred to NH_4Cl solution for 15 min and washed in deionized water. The samples were dehydrated in a graded series of ethanol solutions and propylene oxide and embedded in Epon 812. Sections were mounted on nickel grids and incubated with 2% normal goat serum, 1% bovine serum albumin (BSA) and 50 mM glycine in Tris-buffered saline (TBS)-Tween before immunostaining. The sections were incubated overnight at 4 °C with polyclonal anti-FBPase (in dilution 1:100–1:600). Then they were washed with TBS and incubated with goat anti-rabbit gold-conjugated antibody (1:200) for 60 min at room temperature. The grids were thoroughly washed with TBS and distilled water to remove unbound antibodies. In control experiments the primary antibody was omitted, or non-immune rabbit serum was used as the first layer. Ultrathin sections were stained with uranyl acetate and examined using a transmission electron microscope Zeiss EM 900.

2.4. FBPase activity determination

FBPase activity was measured in a standard assay mixture which contained: 50 mM Bis-Tris-Propane (BTP), 2 mM $MgCl_2$, 150 mM KCl, 1 mM EDTA, 0.5 mM β-nicotinamide adenine dinucleotide phosphate sodium salt (NADP), 0.2 mM fructose-1,6-bisphosphate, 20 U/ml glucose 6-phosphate dehydrogenase and 10 U/ml glucose 6-phosphate isomerase, pH 7.5, at 37 °C. FBPase was incubated in the assay mixture for 10 min at 37 °C, then fructose-1,6-bisphosphate was added to start the reaction.

Table 1
Total leukocytes (white blood cells) and leukocyte subtypes in blood of patients with type 1 diabetes mellitus and healthy donors

	Healthy donors	Patients with IDDM
White blood cell count (×10^9/L)	5.85 ± 0.95	6.38 ± 1.38
Differential leukocyte count (%)		
Segmented neutrophils	57.6 ± 3.9	48.7 ± 4.3*
Band neutrophils	3.7 ± 1.5	4.3 ± 2.5
Eosinophils	2.5 ± 2.4	3.5 ± 1.8
Basophils	ND	ND
Lymphocytes	25.6 ± 2.2	30.2 ± 3.1*
Monocytes	5.6 ± 2.9	6.2 ± 3.1
Neutropholic myelocytes	ND	ND

*$P < 0.05$ compared to healthy donors. ND, not determined.

Table 2
FBPase activity in leukocytes of patients with type 1 diabetes mellitus and healthy donors

Donors	FBPase activity (mU/10^6 cells)	
	Polymorphonuclear leukocytes	Mononuclear leukocytes
Healthy donors	1.09 ± 0.039	0.98 ± 0.126
Patients with IDDM	1.42 ± 0.142*	2.22 ± 0.689*

*$P < 0.05$ compared to healthy donors.