FBPase is in the nuclei of cardiomyocytes

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Abstract Intracellular localization of FBPase in the cardiac muscle of the pig was studied by immunohistochemistry. In contrast to data from skeletal muscle [Gizak, A., Rakus, D. and Dzugaj, A. (2003) Histol. Histopathol. 18, 135–142], in cardiomyocytes FBPase was present not only in the cytoplasm, but surprisingly, also in the nucleus. Results of the microscopic investigation were confirmed by immunoblotting, measurement of FBPase activity in isolated cardiomyocyte nuclei and by determination of the nuclear FBPase $I_{0.5}$ toward adenosine monophosphate (AMP), which was the same as for the purified enzyme.

Key words: Fructose-1,6-bisphosphatase; Cardiomyocyte; Nucleus

1. Introduction

Fructose-1,6-bisphosphatase (D-fructose-1,6-bisphosphate 1-phosphohydrolase, EC 3.1.3.11, FBPase) catalyzes the hydrolysis of fructose-1,6-bisphosphate to fructose-6-phosphate and inorganic phosphate, in the presence of divalent metal ions like magnesium, manganese, cobalt or zinc [1,2]. Vertebrate FBPases are inhibited competitively by fructose-2,6-bisphosphate, and allosterically by adenosine monophosphate (AMP) [3,4].

Liver and muscle isozymes have been found in vertebrate tissues [2,5,6]. The liver FBPase is recognized as a regulatory enzyme of gluconeogenesis. The muscle isozyme participates in the glycogen synthesis from lactate and in the regulation of glycolysis [7–9]. The basic difference between the liver and muscle isozymes concerns their sensitivity to AMP inhibition. In the case of the muscle enzyme, the $I_{0.5}$ value for AMP is about 0.1 μM, and it is 10–100 times lower than the same value determined for the liver isozyme [2,10–12].

Tissue distribution of FBPase isozymes has been investigated. The liver isozyme has been found primarily in gluconeogenic tissue like liver, kidney, and lung. In skeletal muscle tissue only the muscle isozyme is expressed, but in other tissues simultaneous expression of the two isozymes has been observed [6].

Not many papers are available on cellular and subcellular FBPase isozymes localization. Schmoll et al. [13] have found FBPase to be an astrocyte-specific enzyme. Gizak et al. [14] have located FBPase in pneumocyte II. Saez et al. [15] have reported that in hepatic and renal cells FBPase is located in the perinuclear area. Recently Gizak et al. have located FBPase on both sides of the Z-line of skeletal muscle [16]. The primary aim of the present paper is to locate FBPase in subcellular structures of cardiomyocytes. Evidence for localization of FBPase in the nuclei of cardiomyocytes is presented, and physiological meaning of this finding is discussed.

2. Materials and methods

Antibody diluent and 3,3′-diaminobenzidine (DAB) chromogen were purchased from Dako (USA), Paraformaldehyde, glutaraldehyde, Coomassie brilliant blue R-250 were from Fluka (Switzerland); polyester wax was from EMS (USA). Anti-rabbit IgG gold conjugated (10 nm) was from ICN (USA). Biotin conjugated mouse monoclonal anti-rabbit immunoglobulins, extravidin-horseradish peroxidase, normal sera, nitrocellulose membranes and other reagents were from Sigma (USA).

All the reagents were of the highest purity commercially available.

2.1. Antibody production and Western blot

Antiserum against the muscle FBPase was raised in a rabbit by intracutaneous injection of the electrophoretically pure enzyme (isolated from fresh rabbit skeletal muscle according to [17]) and Freund's complete adjuvant. Immunoserum was partially purified using acetone powder. Reactivity of the anti-FBPase serum was estimated using the double diffusion method [18]. Immunodiffusion resulted in a strong reaction between the antiserum and the pure enzyme. To check specificity of the antibodies immunoblotting was performed as described by Towbin et al. [19].

2.2. Immunohistochemistry

2.2.1. Light microscopy. Fresh porcine heart was provided by a local slaughterhouse. Tissue was fixed in Bouin's fluid and embedded in polyester wax. Immunostaining of 5 μm sections was carried out as described previously [16] and involved incubation of the sections with anti-muscle FBPase antibody, biotinylated secondary antibody, and extravidin-biotinylated peroxidase complex.

In control reactions the primary antibody was omitted, or normal rabbit serum was used as a first layer.

2.2.2. Electron microscopy. Small fragments of porcine heart muscle tissue were fixed in 4% paraformaldehyde and 0.25% glutaraldehyde in phosphate buffer, and embedded in epon. Sections were mounted on nickel grids. Before immunostaining, sections were incubated with 2% normal goat serum, 1% bovine serum albumin (BSA) and 50 mM glycine in Tris-buffered saline (TBS)-Tween. Then they were incubated with polyclonal antibodies against muscle FBPase (in dilution 1:100–1:1000) overnight at 4°C, washed with TBS and incubated with goat anti-rabbit antibodies gold conjugated (1:200) for 1 h at room temperature (RT). The grids were thoroughly washed with TBS and distilled water to remove unbound antibodies. Before microscopic examination the sections were stained with uranyl acetate.

In controls the primary antibody was omitted, or non-immune rabbit serum was used as a first layer.

2.3. Heart muscle fractionation

5 g of porcine ventricles were placed in nine volumes of buffer A (10 mM HEPES, 1 mM dithiothreitol (DTT), 0.1 mM ethyleneglycol-

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bis-(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 0.1 mM ethylenediamine tetraacetic acid (EDTA), 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 mM $MgCl_2$, 0.25 M sucrose; pH 7.4, at 4°C) and homogenized. The homogenate was filtered through two and four layers of cheesecloth and fractionated by differential centrifugation method [20].

Each pellet was dissolved in a small amount of buffer and centrifuged again for maximal removal of cytosol. Fractions were pre-incubated with 0.5% Triton for 15 min at RT, and FBPase activity was measured.

2.4. Isolation of cardiomyocyte nuclei

Cardiomyocyte nuclei were isolated using a non-enzymatic extraction technique as described by Boheler et al. [21]. All steps were performed at 4°C or on ice.

5 g of porcine ventricles were placed in nine volumes of buffer A and homogenized for 15 s.

The homogenate was filtered as described above and centrifuged at 1000×*g* for 10 min. The pellet was resuspended in 15 volumes of buffer, filtered through Millipore filter (pore size 41 μm) and centrifuged as before. The pellet was resuspended in 10 ml of buffer with 0.5% Triton X-100 to further lyse the cells, centrifuged as before, and finally resuspended in 2 volumes of buffer containing 10 mM HEPES, 1 mM DTT, 0.1 mM EGTA, 0.1 mM EDTA, 1 mM PMSF, 2 mM $MgCl_2$, 2.2 M sucrose; pH 7.4, at 4°C. Nuclei resuspended in the above-mentioned buffer were layered on two ice-cold layers of sucrose (2.7 M and 2.4 M sucrose) and centrifuged in TLS-55 Beckman rotor at 36 000 rpm for 2 h. After centrifugation, cardiomyocyte nuclei could be isolated at the interface of the 2.4/2.7 M sucrose.

Then the nuclei were suspended in appropriate volume of buffer with 0.25 M sucrose to achieve 2.2 M final concentration of sucrose, and layered again on sucrose gradient and centrifuged as described above. These steps were performed to ensure high purity of cardiomyocyte nuclei.

To determine the number of isolated nuclei, Thom-Zeiss cell was used.

FBPase activity was assayed in 50 mM Bis Tris Propane (BTP), 2 mM $MgCl_2$, 150 mM KCl, 1 mM EDTA, 0.2 mM nicotinamide adenine dinucleotide phosphate (NADP), 5 units/ml glucose-6-phosphate dehydrogenase, 2 units/ml glucose-6-phosphate isomerase, 50 μM fructose-1,6-bisphosphate, pH 7.5; the assay was carried out at 37°C. The substrate was used to start the reaction.

For measuring the inhibition of heart muscle cytosolic and nuclear FBPase by AMP, concentration of AMP was determined using 15 400 M^{-1} cm^{-1} as the molar absorption coefficient at 259 nm. $I_{0.5}$ was calculated with the use of GraFit program [22].

3. Results

3.1. Immunolocalization of FBPase in the pig heart

Examination of pig heart muscle tissue with light microscopy revealed strong positive reaction in the nuclei of the cardiomyocytes and, much weaker, in the cytoplasm (Fig. 1A). No labeling occurred in the absence of the primary antibody, or when the normal serum was used instead of the primary antibody (Fig. 1B).

Table 1
Subcellular distribution of FBPase from pig heart

Fraction	FBPase activity (U/g tissue)		% Activity
	A	B[a]	
Homogenate	0.29	0.53	100
Nuclear fraction	0	0.014	2.6
Mitochondrial fraction	0	0	0
Microsomal fraction	0	0.001	0.18
Cardiomyocyte nuclei	0	0.006	1.14

[a]Fractions pre-incubated with Triton X-100.

To check specificity of the interaction between the antiserum and its antigen, Western blot was performed. Results showed one band of 37 kDa in the homogenate of the pig heart, corresponding to purified muscle FBPase, indicating that antibodies against rabbit muscle FBPase react with FBPase, but do not react with other proteins from pig heart crude extract (Fig. 2).

Analysis of ultrathin sections of heart muscle tissue under the electron microscope and gold conjugated secondary antibody has corroborated the results obtained from light microscopy. Additionally, in nuclei, positive reaction was restricted to the heterochromatin area (Fig. 3A). In cytoplasm, weak staining was observed on the Z- and M-lines (Fig. 3B). No labeling occurred in the absence of the primary antibody, or when the normal serum was used as a first layer (Fig. 3C, D). The same results were obtained from rat heart tissue analysis (data not shown).

3.2. Pig heart fractionation and isolation of cardiomyocyte nuclei

Activity of FBPase from seven porcine hearts was investigated in the course of this study. Fractions obtained through differential centrifugation enabled a preliminary estimation of subcellular distribution of FBPase.

FBPase activity was clearly detectable in the initial homogenate, but not in particulate subcellular fractions (Table 1). Pre-incubation with Triton X-100, however, resulted in a 1.8-fold increase of FBPase activity in the homogenate and in the appearance of activity in the nuclear fraction, which constituted 2.6% of the homogenate activity. Very low level of FBPase activity was also found in the microsomal fraction.

Control reaction indicated that Triton had no effect on FBPase activity determination.

The enzyme activity found in the final high-speed supernatant (data not shown) was similar to that of the initial step of

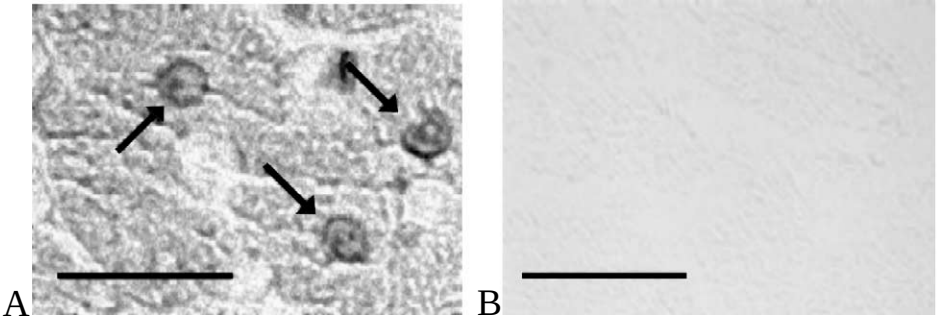

Fig. 1. A: FBPase in pig heart muscle. Arrows point to cardiomyocyte nuclei. B: Control reaction. Bar = 20 μm.

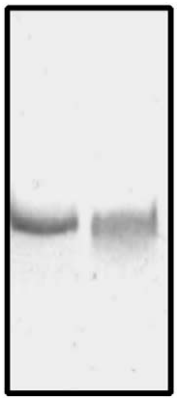

Fig. 2. Western blot of 25 μl of pig heart homogenate (left lane) and purified muscle FBPase (right lane).

fractionation, indicating that no activity was lost during the experiment.

It is well known that non-cardiomyocyte nuclei constitute about 70% of all cardiac nuclei [23]. A non-enzymatic extraction technique [21] was used to isolate a pure fraction of cardiomyocyte nuclei from the total pool. Importantly, this technique ensured that no cytosol remained in the fraction. No residual contamination by myofilaments or membranes was observed.

The isolated fraction of myocyte nuclei was then analyzed for FBPase by both immunoblotting and activity measurement. No detectable activity was found in the fraction not pre-treated with Triton. After incubation with Triton the activity of FBPase detected in myocyte nuclei constituted 1.14% of the activity in the homogenate (Table 1).

Western blot analysis of the nuclei resulted in a thin single band that migrated identically to purified muscle FBPase (Fig. 4).

To determine the size of cardiomyocytes from paraffin-embedded material, ocular micrometry was used; in addition, a morphometric study of cardiomyocyte nuclei was performed, and nuclear volume was calculated. Assuming that mean nuclear volume was 307 μm^3, concentration of FBPase in the nucleus was about 0.61 μM comparing with 0.127 μM in the whole cardiomyocyte.

3.3. Inhibition by AMP

Al-Robaiy and Eschrich [16] have observed that in rat heart muscle there is not only high expression of muscle FBPase isozyme, but also low expression of liver FBPase mRNA. This encouraged the speculation that in pig cardiomyocytes the muscle FBPase isozyme is expressed in cytosol, while the liver isozyme in the nuclei.

Because the most characteristic kinetic property of the muscle isozyme is its high sensitivity towards AMP ($I_{0.5} = 0.1$ μM AMP, while for the liver isozyme it is 10–100 times higher), $I_{0.5}$ was determined for FBPase from pig cardiomyocyte cytosol and nuclei.

Results of the experiment (Fig. 5) indicate that FBPase from both fractions was inhibited in the same manner, with $I_{0.5}$ values characteristic for the muscle isozyme ($I_{0.5} = 0.067$ μM for cytosolic and 0.058 μM for nuclear fraction).

4. Discussion

Presence of FBPase in the nuclei of cardiomyocytes was detected by light and electron microscopy and confirmed by Western blot. Determining the $I_{0.5}$ for FBPase for AMP from the heart homogenate and from the purified nuclear fraction (which was the same as for the purified enzyme, in the range

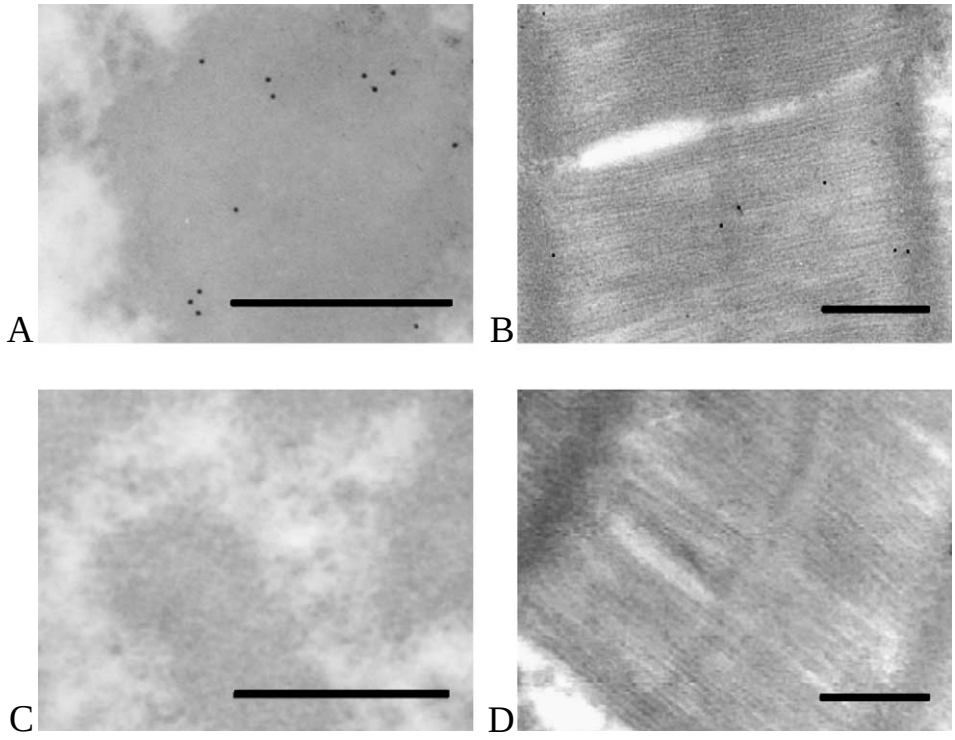

Fig. 3. Localization of FBPase in cardiomyocyte nucleus (A) and cytoplasm (B). C, D: Control reactions. Bar = 0.5 μm.

of experimental error) proved FBPase's presence. Although FBPase activity in nuclei consists of ca. 1% of the total enzyme activity, its concentration therein is four to five times higher than in the cytosol. Thus, non-covalent binding of FBPase with nuclear structures may be expected. Detection of FBPase activity only in nuclei pre-treated with Triton supports the above hypothesis.

The presence of FBPase in the nuclei of cardiomyocytes raises a question concerning its physiological role. Several enzymes of carbohydrate metabolism have so far been found in cell nuclei: glucokinase [24], aldolase [25], glyceraldehyde-3-phosphate dehydrogenase (GAPDH), lactate dehydrogenase (LDH), phosphoglycerate kinase (PGK) (for review see: [26]), and glycogen synthase [27], and their physiological roles have been discussed. Unlike glycogen synthase, whose function is supposedly the same in the cytosol and the nucleus, the other enzymes' physiological roles seem to vary from cytosol to the nucleus. It has been hypothesized that PGK may participate in DNA synthesis and cell cycle progression [28]. GAPDH recognizes the sequence and structural features of the RNA and is involved in transcription [29]; LDH is a recognized stabilizing nuclear factor, and it participates in DNA reparation [28].

One of the signals directing protein to the nucleus is phosphorylation. There is no evidence on in vivo phosphorylation of liver FBPase. On the other hand, Rakus et al. [12] have found that rabbit muscle isozyme is phosphorylated. Phosphorylated FBPase has higher affinity to aldolase, which increases a muscle cell's gluconeogenic capacity [12]. In the liver, stimulation of gluconeogenesis goes via phosphorylation of fructose-2,6-bisphosphatase/6-phosphate-2-fructokinase (FBPase2/PFK2). The muscle isozyme of FBPase2/PFK2 cannot be phosphorylated, therefore, different regulation of gluconeogenesis may be expected. The activation of muscle gluconeogenesis may proceed via phosphorylation of FBPase. That might be the signal inducing the movement of the enzyme to the nucleus where it could induce the expression of its own gene, thus increasing the cell's gluconeogenic capacity, although a possibility that FBPase influences the expression of other genes cannot be ruled out.

A great number of proteins entering the nucleus possess a characteristic nuclear localization sequence (NLS) in their primary structure. One of such sequences is the KKKGK motif [30]. It is present in all known muscle (but not liver – except human liver) FBPase sequences (human, rabbit, rat and mouse), and it could potentially act as an NLS. Although the primary structure of pig muscle FBPase has not yet been determined, all muscle FBPases are characterized by a very high degree of homology (99%) and identity (95%). Therefore the same NLS in pig muscle FBPase might be expected.

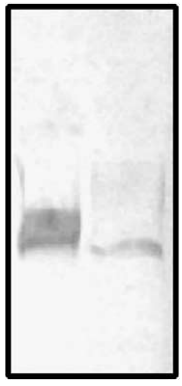

Fig. 4. Western blot analysis of a fraction of cardiomyocyte nuclei (right lane) and purified muscle FBPase (left lane).

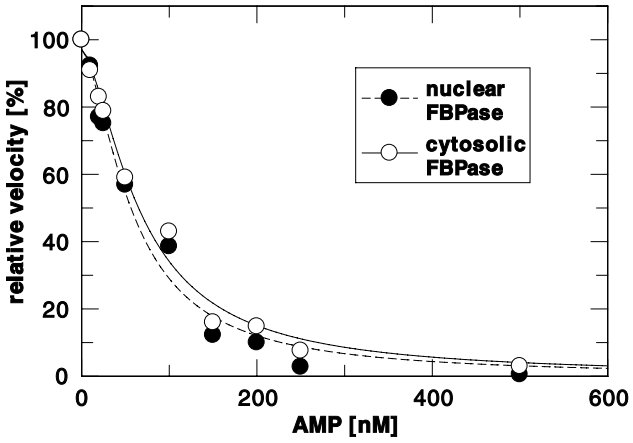

Fig. 5. Inhibition of pig heart muscle FBPase by AMP.

Our paper is the first unequivocal report on localization of muscle FBPase in the nuclei of cardiomyocytes.

In skeletal muscle, FBPase has been located on both sides of the Z-line [16]. Heart muscle, like skeletal muscle, is considered a striated muscle. Nonetheless, differences in metabolic pathways and their regulation between these two types of muscle are well documented; therefore, different subcellular localization and different physiological role of FBPase in skeletal and heart muscle may be expected.

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