The Effect of Selected Biologically Active Compounds on the Expression of Fructose-1,6-Bisphosphate (FBPase) in H9C2 Cell Line and Culture Condition

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Abstract

Introduction: Fructose-1,6-bisphosphatase (FBPase) a gluconeogenic enzyme accumulates in the nucleus of proliferating cells, however its physiological role is not elucidated. In heart muscle cells localize on the Z-line, in the nuclei and in the intercalated discs. Accumulation of FBPase on the Z-line is important for proceeding of glyconeogenesis – the synthesis of glycogen from noncarbohydrate precursors like lactate. Recently employing neonatal rat cardiomyocytes in culture we have shown that FBPase localization on the Z-line is regulated in a calcium dependent manner. Aim of the study: In this study, we checked the effect of selected biologically active compound and culture conditions on FBPase localization in H9C2 cell line. Methods: H9C2 cells form embryonic rat heart were used for incubation in the presence of biologically active compounds: insulin, dantrolene and glucose. FBPase localization was examined by immunofluorescence and confocal microscopy. Results: We have found that FBPase in the H9C2 cells is localized in the cytoplasm and, in opposite to the neonatal rat cardiomyocytes in culture, in cell nuclei. Such localization was not affected by any of the stimulation and culture condition. Conclusions: These finding suggest influence on the glyconeogenesis regulation in cells obtained from the heart at different stages of development.

Keywords: H9C2 cell line, FBPase, glyconeogenesis

Introduction

Fructose-1,6-bisphosphatase (FBPase) catalyzes the hydrolysis of fructose-1,6-bisphosphate producing fructose-6-phosphate and orthophosphate. Two isozymes of FBPase [EC 3.1.3.11] have been found in the vertebrate tissues – the muscle and the liver. Both are inhibited by fructose-2,6-bisphosphate and AMP, but the muscle isozyme is 10 to 100 times more sensitive toward the latter [1]. The muscle isozyme of FBPase is indispensable enzyme of glyconeogenesis - the synthesis of glycogen from noncarbohydrate precursors like lactate - and has been hypothesized to regulate this process [2]. In the heart muscle cells the enzyme is localized mainly on the Z-line, in the nuclei and in the intercalated discs [3, 4]. It has been found that FBPase colocalize with aldolase on the Z-line in heart and skeletal muscle cells what is crucial for the proceeding of glyconeogenesis as it has been shown that interaction of FBPase with aldolase desensitizes the former enzyme to AMP inhibition [2]. Gizak et al. suggest that FBPase can be a target of the glycogen synthase kinase-3 (GSK3) pathway. They proved that FBPase localizes in mitochondria and may be a significant relationship between the GSK3 activity, mitochondrial function and cardiac cell survival during high intensity work [5].
Recently we have shown that in permeabilized heart muscle fibres and in neonatal rat cardiomyocytes in culture the localization of FBPase on the Z-line is regulated in a calcium dependent manner [4, 6]. Upon the increase in intracellular calcium concentration FBPase dissociate from the Z-line what result in inhibition of glyconeogensis. Additionally the muscle isozyme is highly sensitive to the inhibition by calcium ions in the opposite to the liver isozyme [4]. Calcium ions in the heart muscle supposedly plays a similar role in the regulation of carbohydrate metabolism as fructose-2,6-bisphosphate in the liver [6].

In this study, we have shown that stimulation and culture conditions leading to changes in intracellular calcium level do not affect intracellular FBPase localization in H9c2 cell line.

The H9c2 cell line is routinely used and treated as a model object in the experiments concerning on the function of heart muscle [7, 8, 9]. The cell line was established from the embryonic rat heart muscle by Kimes & Brandt in 1976. Upon cell fusion leading to the appearance of polinucleated myotubes the H9c2 cells expressed both acetylcholine nicotine receptor and muscle specific isozyme of creatinine kinase. Such findings allows the authors to conclude that the cell line acquire features typical for skeletal muscle [10, 11]. Another group obtained the opposite results. Hescheler and coworkers found the lack of formed myotubes even though the cells were cultured in the condition promoting cell fusion. However, they notice that the cell morphology resemble immature embryonic heart cells despite the lack of T channels, caveoles, junctions, myofibrils. Additionally, electrical and hormonal signal transduction pathways of the H9c2 cells were similar to the adult rat heart muscle cells. Besides that the L-type calcium channel was found present and responsive to the β-adrenergic stimulation in the H9c2 cell [12]. Further research carried out by Mejia-Alvarez suggested that H9c2 cell line represent the stage of differentiation common for skeletal and heart muscle as the L-type calcium channel characteristic for this both kind of muscle was present in the cells [13].

In the current study we examined the changes of subcellular localization of FBPase in H9c2 cell, a cardiac myoblast cell line, with the treatment of insulin, dantrolene, glucagon or ionophore A23187, respectively. According results we suggested that the influence on the glyconeogenesis regulation in cells obtained from the heart at different stages of development, since the responses of H9c2 cell are different from that of rat neonatal cardiomyocytes to variant biologically active compounds.

Materials and Methods

Cell culture media and sera were from Gibco. Glucose free DMEM was purchased from the Laboratory of Chemistry (Institute of Immunology and Experimental Therapy, Polish Academy of Science, Wroclaw, Poland). All other chemicals, anti- α-actinine antibodies and secondary antibodies were from Sigma (Poznan, Poland).
**Cell culture and cells stimulation**

The H9c2 cells derived from embryonic rat heart tissue were used. H9c2 exhibit many of the properties of skeletal muscle. Cell culture was harvested for 4 days (in 5%\(\text{CO}_2\) atmosphere and in 37°C) in the DMEM medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin on the sterile cover slips placed in the round Petri dishes (fi 35mm). The medium was changed every 2 days. The day before stimulation the cells were incubated overnight in the serum free DMEM containing 1% BSA and 1% penicillin/streptomycin (sfDMEM).

To stimulate the H9c2 cells sfDMEM was supplemented with the relevant hormone or other biologically active compounds. Prior to stimulation the cells were once washed in the sfDMEM and next incubated in the present of relevant hormone or biologically active compound: insulin (2h, 100 nM), dantrolene (inhibitor of calcium removal from the intracellular stores, 10 min, 25 \(\mu\text{M}\)). The effect of glucose withdrawal on intracellular FBPase localization was determined by the incubation of H9c2 cells in the glucose free, serum free DMEM supplemented with 1% Bovine Serum Albumin and 1% penicillin/streptomycin (gfsfDMEM). In the further experiments cells were cultured in gfsfDMEM supplemented with calcium ionophore A23187 (10 \(\mu\text{M}, 1h\)) and glucagon (1.7 nM, 1h). Experiments were repeated at least 3 times and in every experiment about 20 cells were analyzed.

**Immunofluorescence staining**

Immediately after stimulation H9c2 cells were once washed with PBS and fixed with 4% paraformaldehyde at room temperature for 10 min. After washing fixed cells were incubated with PBS containing 5% goat serum and next overnight with rabbit antibody against FBPase (1:100) or with mouse antibody against \(\alpha\)-actinin (1:400) at 4°C. After washing cells were incubated with FITC-conjugated anti-rabbit antibodies (1:100) or with TRITC-conjugated anti-mouse antibodies (1:400). Following washing with PBS cells were incubated with RNase (0.6 mg/ml) for 1 h at 37°C and next with propidium iodide (0.01 mg/ml) for 30 min at room temperature. To visualize actin after blocking with goat serum cells were incubated with phalloidin-rhodamine (Sigma, 1:400) for 1 ‘h at room temperature. After washing cell were closed in fluorescent mounting medium (DAKO) and analyzed in confocal microscopy.

**Confocal microscopy and image processing**

The fluorescent confocal FV500 microscope (Olympus) was used to obtain images. Images were taken with 60x oil objective (N.A. 1.4) and each frame was averaged four times to get a good signal to noise ratio. To avoid emission cross-talk separate acquisition for each dye was done. Optical slices of 1 uM are presented. Obtained images were next processed using ImageJ according to standard procedure (http://rsb.info.nih.gov/ij/).
Results

FBPase in the H9c2 cell line is localized in the cytoplasm and cell nuclei (Fig. 1A). The applied hormones and other biologically active compounds affected intracellular localization of FBPase in H9c2 cell line. Dantrolene and the incubation of the cells in glucose-free medium had no effect on the expression of the enzyme (Fig. 1B and C). Glucagon and calcium ionophore A23187 in the gfsfDMEM evoked a decrease of the enzyme localization (Fig. 1D and E). The incubation with insulin (2h, 100 nM) induced increased expression of FBPase in H9c2 cells (Fig. 1F). Either of the intracellular FBPase localization resembles actin patter (Fig 1 and Fig. 2A) as well as there is a lack of characteristic for skeletal and muscle cells striation pattern (Fig. 2B).

Discussion

In the current work we used H9c2 cardiac cells. These cells were proven to express functional receptors for insulin [5]. There is no literature data about the presence of glucagon receptors in H9c2 cell line. In the present study we have revealed that muscle FBPase is present inside the nucleus and cytoplasm of cardiomyocytes. Gizak et al. demonstrated reduced amount of nuclear FBPase in HL-1 cardiomyocytes with GSK3 inhibitors [5]. Other authors presented that FBPase localizes in cell nuclei during S and G2 phase and can interact with histone family members and with several proteins involved in cell cycle regulation and RNA processing [14]. As we demonstrated the addition of the calcium ionophore A23187 or glucagon induced increased distribution of fructose-1,6-bisphosphatase in neonatal cardiomyocytes. In our previous study was also presented that incubation with calcium ionophore A23187 in medium resulted in a dramatic increase of cytosolic resting calcium concentration and the dissociation of FBPase from the Z line in primary cultures of neonatal rat cardiomyocytes (cells derived from 2- to 5-day old rats). There was also proved that glucagon increased the cytosolic calcium concentration [6]. Experiments carried out by Kondomerkos and coworkers showed also the response of neonatal rat heart muscle cell on glucagon stimulation [15]. We found also that incubation with insulin had the stimulatory effect on the nuclear and cytosolic accumulation of FBPase in cardiac myoblasts. Similar effect was proved on primary cell culture [6]. In mammals lactate oxidation in heart muscle cells is an important energy source in the prenatal period of live. Mammals fetuses serum contain 10 mM lactate what tremendously exceeds the level observed in adults during the rest (0.5 mM). Glucose level in fetuses and adults do not differ whereas the level of fatty acid is diminished [16]. Additionally glycogen in the fetus heart muscle cell occupy 30% of the cell volume whereas in adults only 2% [17]. Such high level of glycogen
might cover the energy demand of the heart muscle cell during the experienced hypoxia period by providing the glucose for glycolysis. According obtained results we can conclude that H9c2 cells are more sensitive on insulin inhibitors than primary myocytes, isolated from embryonic rats. It is possible that glyconeogenesis in heart muscle cells of fetuses is constantly active in order to maintain high intracellular glycogen level and the regulatory mechanisms have not been established. Moreover we conclude that different type of the cells sensitivity is caused by their individual metabolism, the level of cell differentiation and morphology. In the case of cardiomyocytes the regulation of FBPase appears more periphrastic and there is a field for further research and more detailed study.

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References


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Figure 1. Rows: A – Control; B – cells incubated in the sfgfDMEM (1h); C – cells incubated in the presence of dantrolene (10 min, 25 μM); D – cells incubated in sfgfDMEM in the presence of A23187 (1h, 10 μM); E – cells incubated in sfgfDMEM in the presence of glucagone (1h, 1.7 nM); F – cells incubated in the presence of insulin (2h, 100nM). PI – propidium iodide, bar – 15 μm.
Figure 2. Localization of actin (A) and alpha-actinin (B) in H9c2 cell line.

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