Biomedicine & Pharmacotherapy 103 (2018) 228–233

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

journal homepage: www.elsevier.com/locate/biopha

Insulin specifically regulates expression of liver and muscle phosphofructokinase isoforms

Priscila Ausina[a], Daniel Da Silva[a], David Majerowicz[b], Patricia Zancan[c], Mauro Sola-Penna[a,*]

[a] Laboratório de Enzimologia e Controle do Metabolismo (LabECoM), Departamento de Biotecnologia Farmacêutica, Faculdade de Farmácia, Universidade Federal do Rio de Janeiro, Rio de Janeiro, 21941-903, RJ, Brazil
[b] Laboratório de Alvos Moleculares (LAM), Departamento de Biotecnologia Farmacêutica, Faculdade de Farmácia, Universidade Federal do Rio de Janeiro, Rio de Janeiro, 21941-903, RJ, Brazil
[c] Laboratório de Oncobiologia Molecular (LabOMol), Departamento de Biotecnologia Farmacêutica, Faculdade de Farmácia, Universidade Federal do Rio de Janeiro, Rio de Janeiro, 21941-903, RJ, Brazil

ARTICLE INFO

Keywords:
Glycolysis
Phosphofructokinase
Isoform
Expression
Regulation
Insulin

ABSTRACT

Phosphofructokinase (PFK) is a key regulatory enzyme of glycolysis, being considered the pacemaker of this pathway. In mammals, this enzyme exists as three different isoforms, PFKM, PFKL and PFKP, presenting different regulatory and catalytic properties. The expression of these isoforms is tissue-specific and vary according to the cell differentiation and signalization. Although it is known that the expression of the different PFK isoforms directly affects cell function, the information regarding the regulation of PFK isoforms expression is scarce. In the present work, we evaluate the role of insulin signalization on the expression of three PFK isoforms on skeletal muscle, liver, and epididymal white adipose tissue (eWAT) of mice. For this, Swiss mice were treated with streptozotocin (STZ) to disrupt pancreatic ß-cells and, thus, insulin production. Control group were treated with citrate buffer (STZ vehicle). These groups were then treated with insulin or saline twice a day for ten consecutive days when animals were euthanized and tissues used for the evaluation of PFK isoforms expression by quantitative PCR (qPCR). Our results revealed that the lack of insulin significantly impacted the expression of PFKL, presenting mild effects on PFKM and no effects on PFKP. The decrease of PFKL and PFKM mRNA levels observed on the group treated with STZ was reversed by the treatment with insulin. In conclusion, insulin, the most known regulator of glucose consumption, specifically regulates the expression of PFKL and PFKM, which impact the regulation of glycolysis in the cell.

1. Introduction

Glycolysis is the major glucose-consuming pathway, responsible for energy and metabolite production in most living organisms [1–3]. It is a multi-regulated pathway, presenting three rate-liming steps catalyzed by hexokinase (HK), phosphofructokinase (PFK) and pyruvate kinase (PK), the first, third and tenth sequenced enzymes in the pathway, respectively [4–6]. Each of these enzymes presents several isoforms with different catalytic properties. The expression of the isoforms is tissue-specific and can be modulated accordingly to extracellular and intracellular signals [1,7–9]. During the last years, other non-glycolytic properties have been attributed to some of the isoforms of the three enzymes, such as the anti-apoptotic effects of HK isoform II, the transcriptional co-activator properties of PK isoform M2 and, more recently, the expression regulatory properties of PFK isoform P [7–11]. All of these works revealed that the regulation of the expression of these isoforms might be related not only to the control of glycolysis but also to general cell physiology.

Among the three rate-liming glycolytic enzymes, PFK plays a central role, being considered the pacemaker enzyme of the pathway [1,12]. Three isoforms of the enzymes are expressed and were named accordingly to their primary site of expression: PFKM (primarily expressed in skeletal muscle), PFKL (in the liver) and PFKP (from platelets). Although there is high homology in the amino acid sequences (> 65%), these enzymes are encoded by individual genes on different chromosomes, and their differences are mostly present in their allosteric regulatory sites, conferring unique allosteric regulation for each isoform [1]. Moreover, PFK monomers associate into homo- and heterodimers and tetramers. PFK tetramers are the smaller catalytically active conformation of the enzyme, while PFKP dimers have been reported as the conformation responsible for the non-glycolytic properties of this PFK isoform [1,10,12]. On the other hand, PFKM dimers can bind to two

* Corresponding author.
E-mail address: msolapenna@me.com (M. Sola-Penna).

https://doi.org/10.1016/j.biopha.2018.04.033
Received 22 February 2018; Received in revised form 23 March 2018; Accepted 5 April 2018

Ca^{2+}/calmodulin molecules (one at each PFKM monomer) to form a fully active and not susceptible to allosteric inhibition configuration of the enzyme [1,13–15]. The equilibrium between PFK dimers and tetramers is modulated by the enzyme's substrates (ATP and fructose 6-phosphate) as well as its allosteric regulators, such as fructose 2,6-bisphosphate, ATP (> 1 mM), fructose 1,6-bisphosphate, citrate, lactate and others [1,13–23]. Therefore, the expression levels of each PFK isoform is a determinant of the PFK catalytic and non-catalytic properties in each tissue.

The differential expression of PFK isoforms directly affects cell physiology and function. For instance, the glycolytic efficiency, *i.e.*, the ratio between lactate produced per glucose consumed, varies according to the proportion of PFK isoforms present [24]. Moreover, stabilization of PFKP expression promotes tumorigenesis in glial cell lineages, an effect that is promoted by AKT signalization [25], suggesting the participation of insulin signaling in the differential expression of PFK isoforms. Conversely, it is described that impairment of insulin signalization, either in type 1 or type 2 diabetes mellitus (T1DM and T2DM, respectively), decreases the expression of PFK, corroborating its regulation by insulin [26,27]. However, there is no reported information for the role of insulin on the differential expression of PFKM, PFKL, and PFKP in mammalian tissues. In the present work, Swiss mice were treated with streptozotocin (STZ) to impair insulin production then treated for ten consecutive days with 2 U/Kg insulin twice a day (saline was used as the control). The expression of the three PFK isoforms was evaluated and showed a specific insulin-regulated expression of the PFK isoforms.

2. Material and methods

2.1. Animal protocol

Forty Swiss male mice at the age of 8 weeks were initially divided into two groups of 20 animals each. One group received an intraperitoneal (i.p.) injection of STZ (200 mg/Kg) diluted in fresh 100 mM citrate buffer (pH 4.5) and thus was named "STZ". The other group received an i.p. injection of the same citrate buffer and was named as "control". After ten days, all animals from the STZ group presented a fasting glycemia higher than 300 mg/dl and were considered with type-1 diabetes mellitus. Thirteen days after the initial STZ injections, both groups were further randomly subdivided into two groups to be treated for ten consecutive days with two daily i.p. injections (12/12 h) of insulin (2 U/Kg; Humulin R, Lilly, IN, USA) or saline. The first day of insulin or saline treatment was defined as "day 1″ and the last day of this treatment as "day 10". Therefore, a total of four experimental groups with 10 animals each were set as follows: 1) "Control" (those that were initially injected with citrate buffer and were further treated with saline for ten days); 2) "Control + Insulin" (initially treated with citrate buffer and further treated with insulin for ten days); 3) "STZ" (initially treated with STZ and then with saline); and 4) "STZ + Insulin" (treated with STZ and then with insulin). At the day 10, animals were anesthetized with isoflurane and euthanized by cardiac puncture followed by cervical dislocation. The blood, the liver, gastrocnemius muscle and epididymal white adipose tissue (eWAT) were collected for further analyses. The animal protocol was previously approved by the Ethics Committee for the Use of Laboratory Animals, of the Health Sciences Center of the Federal University of Rio de Janeiro (CEUA-CCS/UFRJ, protocol FAR03).

2.2. Biochemical parameters

The collected blood was centrifuged (2000 × *g* for 5 min) and serum was used for all further measurements. Glycemia was analyzed using an Accu-Chek® Active apparatus (Roche Diagnostics GmbH, Mannheim, Germany). Insulinemia was analyzed using Rat/Mouse Insulin ELISA kit (Millipore, Burlington, MA, USA). Triacylglycerol (TAG), alanine aminotransferase (ALT), high-density lipoprotein (HDL) and total cholesterol were analyzed using the appropriate commercial kits from Doles Reagents (Panamá, GO, Brazil).

2.3. PFK activity

PFK activity was evaluated by the method developed by Sola-Penna and co-workers [28] with the modifications introduced by Zancan and Sola-Penna [29,30]. For this, 100 mg of each tissue was extracted in the extraction buffer containing 50 mM Tris-HCl (pH 7.4), 2 mM EDTA, 2 mM EGTA, 0.1% phenylmethanesulfonyl fluoride (PMSF, Sigma-Aldrich Co., St. Louis, MO, USA), 0.1% Triton X-100 (Sigma-Aldrich Co., St. Louis, MO, USA). For the enzyme activity assays, 50 µg of extracted protein was used.

2.4. RNA extraction and cDNA synthesis

Approximately 100 mg of each tissue were homogenized in 500 µl of TRIzol Reagent (Thermo Fisher Scientific, Waltham, MA, USA) and total RNA was extracted following the manufacturer's protocol. At the end of extraction, the RNA was quantified and analyzed using a Picodrop spectrophotometer (Picodrop Limited, Hinxton, UK). The A260/A280 ratios of all samples were between 1.8 and 2.0. The RNA integrity was analyzed by 1% agarose gel electrophoresis. Immediately after extraction, 1 µg of RNA was treated for 30 min at 37 °C with 1 U of DNAse 1 (Sigma-Aldrich Co., St. Louis, MO, USA) in a final volume of 10 µl. After stopping the reaction by incubation at 70 °C for 10 min, cDNA was synthesized using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's protocol, in a final volume of 20 µl.

2.5. Quantitative PCR (qPCR)

qPCR was performed in a LineGene 9600 equipment (Hangzhou Bioer Technology Co. Ltd., Hangzhou, China), using GoTaq® qPCR Master Mix (Promega, Fitchburg, WI, USA). Reactions were conducted in 10 µl containing 6 pmol of the primers (forward and reverse; Table 1) and 0.2 µl of the cDNA template, using Axygen PCR-96-FLT-C plates (Corning, NY, USA). For amplification, the following program was used: 10 min at 95 °C, 40 cycles of 15 s at 95 °C and 1 min at 60 °Cm and a dissociation curve. Beta-actin (ß-actin) was used as endogenous control and did not present significant alteration in its expression after the treatments [31]. Primers were designed using the Primer-Blast tool [32]. The optimization of all qPCR conditions was previously performed following international standards [33] and used in the current work.

Table 1
Primers used for qPCR. All the primers were optimized for 600 nM use in the experiments.

Gene	Protein	Primer sequence		Amplicon length (bp)	Efficiency (%)
		Forward	Reverse		
Actb	ß-actin	TGGATCGGTGGCTCCATCCTGG	GCAGCTCAGTAACAGTCCGCCTAGA	131	96.0
PFKM	PFK isoform M	GGAGTGCGTGCAGGTGACCAAA	ATCACGGCCACTGTGTGCAACC	171	99.3
PFKL	PFK isoform L	CATGAATGCAGCTGTGCGCTCC	CCAGCCCACTTCTTGCACCTGA	118	102.6
PFKP	PFK isoform P	ACAGACACGTGCGACCGCAT	AGTGCACGACGTTGGACTGCA	187	98.5