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Epinephrine modulates cellular distribution of muscle phosphofructokinase

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Abstract

In this paper, we report evidences that cellular distribution of phosphofructokinase can be affected by epinephrine stimulation in rabbit skeletal muscle homogenates. Through co-sedimentation techniques, we observed that in epinephrine-stimulated tissues, approximately 50% of phosphofructokinase activity is co-located in an actin-enriched fraction, against 29% in control. This phenomenon is companied by a 400% increase in specific phosphofructokinase activity in stimulated homogenates. This effect is reproduced by the β-adrenergic agonist isoprenaline. Here we propose that the modulation of cellular distribution of phosphofructokinase may be one of the mechanisms of control of glycolytic flux in mammalian muscle, by β-adrenergic stimulation.
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Keywords: Phosphofructokinase; Epinephrine; Cellular distribution; Muscle; Actin

Introduction

Mammalian muscle phosphofructokinase (ATP:D-fructose-6-phosphate-1-phosphotransferase, PFK-1, EC 2.7.1.11), known as a rate-limiting enzyme on glycolysis, has been described as a link between glycogen phosphorylase activation promoted by epinephrine and the resultant additional substrate flux for Embden–Meyerhoff pathway [1]. It has been reported that muscle phosphofructokinase is activated by epinephrine in both skeletal and heart muscles [1–5]. This activation is described as resultant from changes on intracellular concentrations of several effectors, including fructose 2,6-bisphosphate, a potent activator of phosphofructokinase activity [6], and glucose 1,6-bisphosphate [7,8].

However, some evidences have shown that hormones which can interfere with glycolytic flux on muscle, such as insulin, could do so by mechanisms other than elevation of fructose 2,6-bisphosphate levels [9–11]. It was shown that insulin could affect the intracellular equilibrium between free and actin-associated enzymes [10,11]. Actin-phosphofructokinase association significantly increases enzyme activity and diminishes its K_m for fructose 6-phosphate [12,13], and can be affected by ionic strength [14], calcium concentration [13], and phosphorylation [15,16].

The glycolytic rate can be accelerated by epinephrine stimulation through activation of phosphofructokinase as previously shown with skeletal and heart muscles [1–6]. However, the mechanisms involved on this phenomenon are not completely clear. In this work, we studied the effects of epinephrine and other β-adrenergic effector, isoprenaline, on intracellular distribution of phosphofructokinase activity from rabbit skeletal muscle, and the resulting modulation of enzyme activity.

Materials and methods

Material

ATP and fructose 6-phosphate were purchased from Sigma Chemical (St. Louis, MO, USA). ^{32}Pi was purchased from Instituto de Pesquisas Energéticas e

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Nucleares (São Paulo, Brazil). [γ-^{32}P]ATP was prepared according to [17]. Other reagents were of the highest purity available.

Rabbit skeletal muscle homogenate

Rabbits killed by cervical dislocation were bled by cutting the blood vessels of the neck and the muscles of hind legs and back were quickly removed and cleaned to remove fat and connective tissue. Muscle slices weighting 5 g each were homogenized for 30 s in Polytron (Brinkmann Instruments, Westbury, NY, USA) in the presence of 2 volumes of a solution containing 50 mM Tris–HCl, 30 mM NaF, 4 mM EDTA, and 1 mM dithiothreitol, at pH 7.5 (homogenizing buffer). Homogenized tissues were centrifuged for 5 min at 100*g*, 4 °C, for separation of cellular debris. The resultant supernatant, called total homogenate (TH), represented total enzyme activity.

In order to test the action of effectors, muscle homogenate was incubated for 15 min at 25 °C in saline (0.9% NaCl solution) containing 0.5, 1, 7, 10, 50, or 100 μM epinephrine. In the test with isoprenaline, epinephrine was substituted by the concentration of isoprenaline that presented the highest effects (10 μg/ml). Incubation with saline, in both cases, was used as control.

Tissue fractionation

Tissue fractionation was performed after a modification of the protocol proposed by Lilling and Beitner [18], based on actin-bound enzyme extraction protocols [15,16], as follows.

Total homogenate (TH) was centrifuged for 15 min at 27,000*g* (4 °C). The pellet was resuspended in 200 μl of buffer 1, and called P1. The supernatant (S1) was centrifuged for 30 min at 110,000*g* (4 °C). The supernatant (S2) was collected and the pellet resuspended in 100 μl of homogenizing buffer (P2). All fractions were assayed for phosphofructokinase activity and protein concentration. Protein quantifications were performed as described by Lowry et al. [19].

Radioassay for phosphofructokinase activity

Enzyme activity was measured according to Sola-Penna et al. [20]. The assays were performed at 37 °C in 0.4 ml of reaction medium containing 50 mM Tris–HCl (pH 7.4), 5 mM $MgCl_2$, 5 mM $(NH_4)_2SO_4$, 1 mM fructose 6-P, and 0.1 mM [γ-^{32}P]ATP (4 μCi/μmol). Reaction was started by addition of tissue extracts and quenched after 2 min, when the reaction rate is constant in function of time. Reaction was stopped by addition of 1 ml of activated charcoal suspended in 0.1 N HCl. The suspension was centrifuged at 1500*g* in a refrigerated clinical centrifuge for 15 min and 0.4 ml of the supernatant was counted in a liquid scintillation counter. Blanks for each tube were performed in parallel in the absence of fructose 6-phosphate.

Western blotting

All tissue fractions (30 μg total protein) were subjected to 12% SDS–PAGE, transferred to polyvinylidene difluoride filters (PVDF Hybond-P, Amersham Pharmacia Biotech), blocked with Tween–TBS (20 mM Tris–HCl (pH 7.5), 500 mM NaCl, 0.1% Tween 20) containing 2% bovine serum albumin and subsequently incubated with monoclonal mouse anti-actin (1:200; Santa Cruz Biotechnology). The PVDF filters were then washed three times with Tween–TBS, followed by 1 h incubation with biotin-conjugated anti-mouse IgG (1:1000; Santa Cruz Biotechnology). Subsequently, the filters were incubated with horseradish peroxidase-conjugated streptavidin (1:1000; Caltag Laboratories, Burlingame, CA, USA). Immunoreactive proteins were detected using an enhanced chemiluminescence kit (Amersham Pharmacia Biotech). The bands were analyzed with *Sigma Gel* (Jandel Scientific, San Rafael, CA, USA).

Statistics

Statistical analyses were performed with the use of Statgraphics version 4.0 (Statistical Graphics Corporation/Plus Ware, USA). Results are presented as median (±standard errors). We used paired and unpaired Student's t tests for comparison of perfused and control fractions. Two-tailed p values <0.05 were considered as statistically significant.

Results

Phosphofructokinase activity

The effects of epinephrine on phosphofructokinase activity of rabbit skeletal muscle homogenate were tested. Incubation of muscle homogenates with different concentrations of epinephrine significantly increased phosphofructokinase activity on total homogenate, as presented in Table 1. The presence of the lower concentration tested (0.5 μM) activity was 290% higher than in control. The more pronounced activation (430%) was achieved in the presence of 7 μM epinephrine.

Muscle homogenate incubated with 7 μM epinephrine was fractionated as described in Materials and methods and assayed for phosphofructokinase activity (Fig. 1A). Besides the activation of phosphofructokinase of muscle homogenate, the activities in subcellular fractions did not follow the same pattern. It can be seen that there was a significant net increase in phosphofructokinase activity only in P2 fraction, and that this phenomenon

Table 1
Effect of epinephrine on muscle homogenate phosphofructokinase activity

Epinephrine (μM)	0	0.5	1	7	10	100
Phosphofructokinase activity (U/mg of protein)	0.016 ± 1.3	0.045 ± 12.6*	0.062 ± 12.9*	0.068 ± 5.2*	0.063 ± 14.8*	0.045 ± 7.5*

Phosphofructokinase activity was measured as described in Materials and methods. Values are means ± SE of six independent experiments ($n = 6$).
* Values significantly different from control ($p < 0.05$).

occur in parallel to a proportional decrease in the phosphofructokinase activity in fraction S2. Fig. 1B shows that this phenomenon is reproduced by isoprenaline, another β-adrenergic agonist.

Actin distribution on subcellular fractions

All fractions obtained through differential centrifugation (TH, S1, P1, S2, and P2) were evaluated for their actin content. Fig. 2 shows a representative Western blotting with monoclonal mouse anti-actin antibody for muscle slices treated with saline (control) and 7 μM epinephrine. Densitometry analysis of fractions S2 and P2 revealed that actin content is clearly higher on pelleted fraction (P2) (see Table 2).

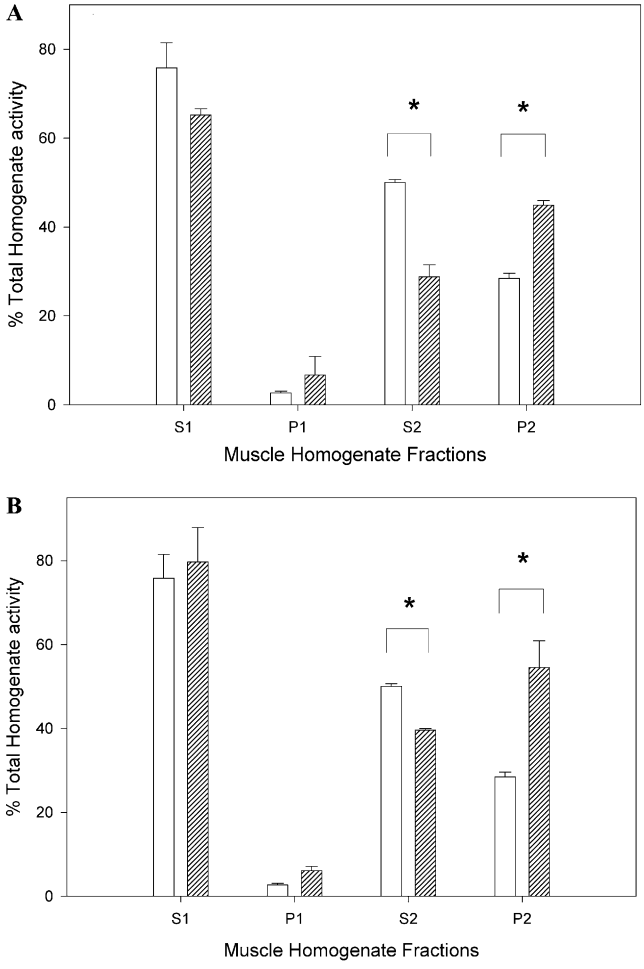

Fig. 1. Effect of β-agonists on distribution of phosphofructokinase activity on S1, P1, S2, and P2 fractions. Phosphofructokinase activity was measured as described in Materials and methods, and presented as percentage of total activity obtained in total homogenate (TH). Values are means ± SE of six independent experiments ($n = 6$). *$p < 0.05$. (A) Empty bars, control; hatched bars, fractions from samples treated with 7 μM epinephrine. (B) Empty bars, control; hatched bars, fractions from samples treated with 40 μM isoprenaline.

Discussion

In this work, we found that epinephrine, as well as isopenaline, are able to affect the distribution of phosphofructokinase activity in rabbit skeletal muscle homogenates. Upon stimulation of muscle homogenate with both β-adrenergic agonists, phosphofructokinase activity increased in an actin-enriched fraction as well as in the total homogenate. Several works suggest an important role of intracellular location of phosphofructokinase (as well as other glycolytic enzymes) on glycolysis regulation [21–23]. Beitner and co-workers [10,11,18,24–26] described the modulation of phosphofructokinase activity in muscle and other cell types by its association to cytoskeleton in response to diverse cellular signals such as insulin and serotonin, intracellular calcium and phospholipase A_2 activity.

The role of epinephrine in skeletal muscle is intimately related to carbohydrate catabolism. It has been described that the main mechanism involved in the regulation of glycolytic flux in muscle is the activation of phosphofructokinase by its allosteric effectors fructose 2,6-bisphosphate [6] and glucose 1,6-bisphosphate [7,8]. However, hormone regulation in muscle tissue may also modulate phosphofructokinase activity by increasing the concentration of glucose 1,6-bisphosphate [9,10,18] and, as stated earlier, by influencing the association of the enzyme to structural elements of the cell [10,11,18]. In this purpose, epinephrine stimuli may possibly affect more than one regulatory mechanism of hexose catabolism and its signal should be more efficient on an intensity and time-related basis. Our data corroborate this fact and suggest that the modulation of the cellular distribution of phosphofructokinase may be one of these mechanisms. Chen-Zion et al. [10] showed that although there are several different mechanisms involved in the regulation of phosphofructokinase, they are even not present or do not have the same intensity at the same time. So, one could expect that the relevance of a modulator at a given time is related to the velocity of

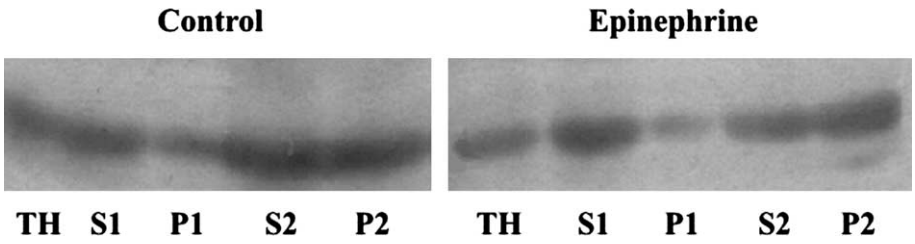

Fig. 2. Cellular distribution of actin on TH, S1, P1, S2, and P2 fractions. Tissue fractionation and Western blotting were performed as described in Materials and methods. Actin was detected using horseradish peroxidase-conjugated streptavidin after separation by SDS–PAGE and transfer to polyvinylidene difluoride filters.

Table 2
Analysis of intensity of actin bands in TH, S1, P1, S2, and P2 fractions obtained by *Western blotting*

	TH	S1	P1	S2	P2
Control	2092	1730	769	1980	2609
(% TH)	100	82.70	36.76	94.65	124.7
Epinephrine	1131	1557	80	610	1515
(% TH)	100	137.67	7.0	53.93	133.95

Band intensity was analyzed on Sigmagel (Jandel Scientific, San Rafael, CA, USA). Values are represented as arbitrary numbers. Numbers in brackets are the percentage of the total homogenate (TH) value.

action of that mechanism. In that matter, we believe in the model previously proposed, in which it is possible that at some point between adrenergic stimulation and the elevation of the concentration of frutose-2,6-bisphosphate in muscle, other factors could be playing a regulatory role on glucose catabolism and skeletal muscle function [10,27]. These other factors may include the modulation of subcellular location of the enzyme showed here.

Association of phosphofructokinase with filamentous actin or microtubules is described to modulates enzyme activity [28–30]. The binding to actin filaments itself diminishes both the inhibition promoted by high levels of ATP and the sensitivity to fructose 2,6-bisphosphate [13,18]. Although the mechanisms involved in this process are not yet completely understood, it seems that the tetrameric configuration of phosphofructokinase (the most active oligomer of enzyme) is stabilized upon binding to F-actin [31].

Some authors have shown that mammalian muscle phosphofructokinase is phosphorylated as a result of cyclic AMP elevation and protein kinase A activation [32–34], despite the fact that its activity is described not to be affected in the process [34,35]. On the other hand, phosphorylation of phosphofructokinase by protein kinase A is said to increase its apparent affinity for F-actin [16].

According to our data, we could observe that treatment of rabbit muscle homogenate with agonists promoted a significant increase in phosphofructokinase activity on a subcellular fraction enriched with F-actin. Since it is well known that the biological effects of catecholamines on skeletal muscle are mediated by elevation of tissue cyclic AMP levels through adenylate cyclase activation [36,37], one can imagine that the stimulation of glycolytic flux promoted by epinephrine may involve phosphorylation of phosphofructokinase by protein kinase A, which would increase phosphofructokinase association to filamentous actin and lastly stimulate net enzyme activity.

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