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Lactate downregulates the glycolytic enzymes hexokinase and phosphofructokinase in diverse tissues from mice

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ABSTRACT

We examined the effects of lactate on the enzymatic activity of hexokinase (HK), phosphofructokinase (PFK) and pyruvate kinase (PK) in various mouse tissues. Our results showed that lactate inhibited PFK activity in all the analyzed tissues. This inhibitory effect was observed in skeletal muscle even in the presence of insulin. Lactate directly inhibited the phosphorylation of PFK tyrosine residues in skeletal muscle, an important mechanism of the enzyme activation. Moreover, lactate indirectly inhibited HK activity, which resulted from its cellular redistribution, here attributed to alterations of HK structure. PK activity was not affected by lactate. The activity of HK and PFK is directly related to glucose metabolism. Thus, it is conceivable that lactate exposure can induce inhibition of glucose consumption in tissues.

1. Introduction

In the last century, lactate had been considered as the end product of glycolytic flux with no major metabolic functions other than inducing metabolic acidosis and tissue damage [1,2]. However, in recent years, lactate has been studied based on its ability to serve as an energy source and a cell-signaling and tissue-repairing molecule [1,3]. Chronic hyperlactatemia has been described as an independent risk factor for diabetes development, with lactate being an important factor for maintaining insulin resistance [4,5]. To date, only a few metabolic explanations have been provided for this effect of lactate. Depré et al. reported decreased tissue glucose consumption in the presence of lactate [6]. Lombardi et al. proposed that hyperlactatemia could decrease the GLUT-4 level and glucose uptake by skeletal muscle [7], a rate-limiting step of glucose metabolism in skeletal muscle. In addition, Choi et al. demonstrated that lactate could induce insulin resistance in skeletal muscle by inhibiting glycolytic flux through suppressing insulin signaling [8]. However, they did not specify which step(s) of the glycolytic flux was (were) inhibited. In a previous study, we demonstrated that lactate could inhibit 6-phosphofructo-1-kinase (PFK, phosphofructokinase), a regulatory enzyme of glycolytic flux, by dissociating the active enzyme tetramers into the less active dimers [9].

This study aimed to contribute to, and to expand the knowledge regarding, the action mechanism of lactate in skeletal muscle, liver, kidney and heart. Our results demonstrate that lactate can inhibit both hexokinase (HK) and PFK, but not pyruvate kinase (PK) in a variety of tissues, supporting the hypothesis of negative regulation of glucose consumption by glycolytic flux downregulation.

2. Materials and methods

2.1. Materials

ATP, fructose-6-phosphate, fructose-2,6-biphosphate (F2,6BP), hexokinase, insulin and glucose were obtained from Sigma Chemical Co. (St. Louis, MO, USA). ^{32}Pi was obtained from the Instituto de Pesquisas Energéticas e Nucleares (São Paulo, Brazil). [γ-^{32}P]ATP was prepared according to Maia et al. [10].

2.2. Mouse tissue homogenates

All mouse Experiments were performed according to the animal experimental protocols. Male Swiss mice (20–25 g) fed *ad libitum*

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were sacrificed by cervical dislocation. The heart, liver, kidney and quadriceps were promptly removed, stripped of fat and connective tissue and incubated in the homogenization buffer consisting of 50 mM Tris–HCl (pH 7.4), 250 mM sucrose, 20 mM KF, 0.2 mM β-mercaptoethanol and 0.5 mM PMSF (1:3). The tissues were then treated with or without 5 or 10 mM lactate, depending on the requirements of the experiments.

2.3. Tissue fractionation

Tissue fractionation was performed according to a modification of the Lilling and Beitner protocol [11] proposed by Alves and Sola-Penna [12]. The pH of all tissues homogenate and fractions were controlled before and after the addition of lactate to assure that the pH was controlled.

2.4. Enzymatic activity assays

HK and PFK enzymatic activities were assessed by the radiometric method described by Sola-Penna et al. [13] with the modifications proposed by Zancan and Sola-Penna [14,15]. This assay was performed at 37 °C in a 0.4-ml reaction system containing 50 mM Tris–HCl (pH 7.4), 5 mM $MgCl_2$ and [γ-^{32}P]ATP (4 μCi/μmol). PK was evaluated in a basic medium containing 50 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 5 mM phospho(enol)pyruvate (PEP), 5 mM ADP and 120 mM KCl. The reaction was initiated by the addition of enzyme preparation. Aliquots were withdrawn 2, 4, 6, 8 and 10 min after the reaction was initiated and the reaction was stopped by the addition of 0.1 N HCl. The medium was neutralized with NaOH and the ATP content was evaluated using the commercial kit ATPlite 1 step (PerkinElmer, MA, USA). Blanks were performed in parallel in the absence of PEP.

2.5. Intrinsic fluorescence spectroscopy

Intrinsic fluorescence analysis was perfumed on a spectrofluorimeter (Jasco ®) in media consisting of 100 mM Tris–HCl (pH 7.4), 5 mM $(NH_4)_2SO_4$ and purified hexokinase (5 μg/ml) in the presence or absence of 5 or 10 mM lactate. Respective spectra were subtracted for background and interference correction. The excitation wavelength used was 280 nm, and fluorescence emission was scanned from 300 to 400 nm (0.5 nm intervals at a rate of 100 nm/min). The center of mass of the intrinsic fluorescence spectrum was calculated according to Leite et al. [9] using the SigmaPlot 10.0 (Systat) software.

2.6. Immunoprecipitation and Western blotting

Immunoprecipitation and western blotting were performed as previously described [16], using a polyclonal anti-PFK antibody produced in our laboratory according to Meira et al. [17] and a monoclonal anti-phosphotyrosine antibody (clone pT-154, Sigma Chemical Co, MO, USA). Briefly, homogenates from tissues from mice were treated with or without 10 mM lactate in the presence or absence of 100 nM insulin for 3 h. Subsequently, the treated homogenates were subjected to analysis.

2.7. Statistical analysis

Results are expressed as the mean ± standard error of the mean (S.E.M.). Statistical analysis was performed using the SigmaPlot software (v.10.0, Systat Inc., CA, USA) integrated with SigmaStat (v. 3.2, Systat Inc. CA, USA). We used the Student *t*-test to compare different groups, and $P < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. Effects of lactate on PFK activity

We assessed the ability of lactate to modulate PFK and found that lactate inhibited PFK activity in the analyzed tissues under several conditions (Fig. 1). Acute exposure to 10 mM lactate exerted no effect on PFK activity in skeletal and cardiac muscle. However, we observed a 20% and 33% reduction in PFK activity in the liver and kidney, respectively. Unlike the acute exposure to 10 mM lactate, lactate preincubation for three hours inhibited PFK activity in skeletal and cardiac muscle. However, in the liver and kidney, lactate preincubation did not cause a further decrease in PFK activity. To evaluate if the ability of lactate to inhibit PFK activity remains in the presence of hormones that stimulate glycolytic flux, isolated mouse tissues were incubated in a buffer containing 4.5 mM glucose and stimulated with 100 nM insulin (Fig. 1). We observed that after insulin exposure, the ability of lactate to inhibit PFK was lost, independent of the analyzed tissue type and time frame. The only exception was found in skeletal muscle, where we observed a 52% reduction in PFK activity in the tissue preincubated with 10 mM lactate, even in the presence of 100 nM insulin (Fig. 1). Insulin can promote an increase in intracellular synthesis of F2,6BP, a known positive allosteric factor of PFK [18,19]. Therefore, it is possible that the increased F2,6BP synthesis triggered by insulin could counterbalance the inhibitory effect of lactate on PFK activity.

To evaluate whether F2,6BP is capable of reversing the inhibitory effect of lactate on PFK activity, isolated mouse tissues were preincubated with or without 10 mM lactate for three hours and subsequently treated with 100 nM F2,6BP (Fig. 2). As expected, PFK was activated when tissues were treated with 100 nM F2,6BP. As shown in Fig. 2, PFK activity in skeletal muscle, heart, liver and kidney was increased by approximately 148%, 156%, 132% and 134%, respectively. However, the stimulatory effect of F2,6BPF2, 6BP on PFK activity was decreased by preincubating the tissues with lactate for three hours. Under these conditions, PFK activity in skeletal muscle, heart, liver and kidney was approximately 70%, 121%, 104% and 96%, respectively, of the levels observed in control samples. Only in skeletal muscle was the PFK activity significantly lower than that in the control samples.

It has been shown that PFK is regulated by different protein kinases capable of phosphorylating its serine, threonine and/or tyrosine residues [20–22]. Therefore, we proceeded to examine if lactate is capable of regulating the phosphorylation levels of these residues in PFK. For this purpose, skeletal and cardiac muscle, as well as liver and kidney tissues, was incubated for three hours with 10 mM lactate or 100 mM insulin (positive control) for evaluating the PFK phosphorylation levels. As expected, insulin stimulation led to an increase in PFK phosphorylation in all the analyzed tissues (Fig. 3). The representative western blot in Fig. 3 showed that previous exposure of the tissues to 10 mM lactate for three hours caused a 40% decrease in PFK tyrosine phosphorylation level only in skeletal muscle. Phosphorylation of serine and threonine residues, however, was not altered under these conditions independent of the analyzed tissue type (data not shown). These data indicate that lactate can inhibit PFK activity through both a direct effect independent of the insulin signaling cascade (by inhibiting PFK phosphorylation) and an antagonistic effect to the insulin signaling cascade (by counteracting the effects of F2,6BP).

3.2. Effects of lactate on hexokinase/glucokinase activity

Another possible action mechanism of glycolytic flux inhibition by lactate is its ability to modulate hexokinase, another enzyme involved in controlling the flux. To evaluate the role of lactate in

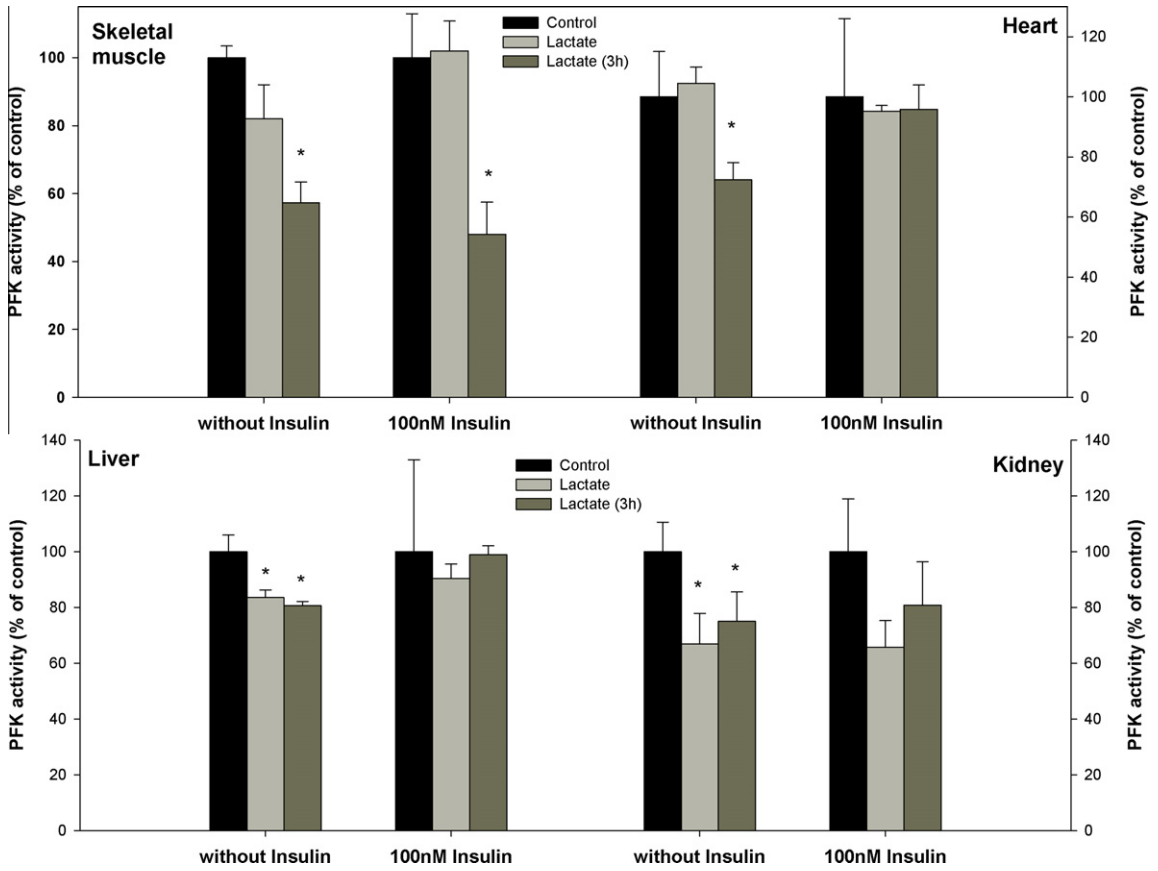

Fig. 1. Effect of lactate on PFK activity in mouse tissue homogenates. Tissues were isolated and preincubated for 3 h in the presence or absence of 10 mM lactate and/or 100 nM insulin. After incubation, the tissues were homogenized, and PFK activity was measured. Pre-incubation with lactate affected the PFK activity in all the analyzed tissues, with the renal and hepatic tissues being sensitive to lactate independent of the exposure time. Only in skeletal muscle was PFK activity modulated by lactate in the presence of insulin. Values are presented as the mean ± S.E.M. of at least five independent experiments. $^{*}P < 0.05$ indicates a statistically significant difference between the treated sample and the respective control in the absence of lactate (black bars).

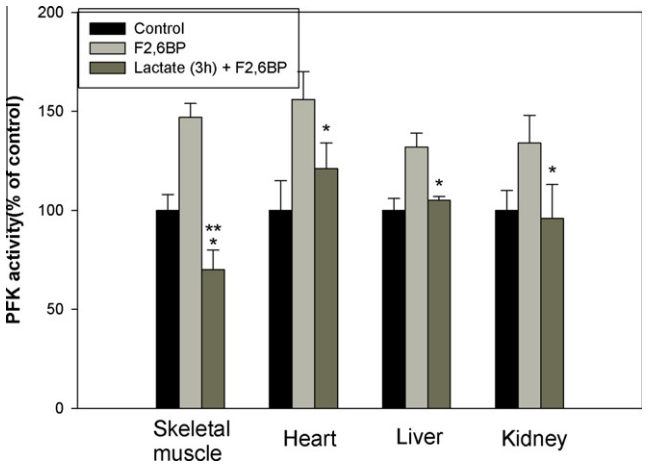

Fig. 2. Effect of lactate on PFK stimulation by F2,6BP (F26BP). Tissues were isolated and preincubated in the presence or absence of 10 mM lactate for three hours. After incubation, the tissues were homogenized and stimulated with 100 nM F26BP, and PFK activity was measured. Preincubation with lactate led to a decrease in the stimulatory effect of F26BP on PFK activity in all the analyzed tissues. Values are presented as the mean ± S.E.M. of at least five independent experiments. $^{*}P < 0.05$ indicates a statistically significant difference between the group stimulated with F26BP and the group stimulated with F26BP pre-treated with lactate. $^{**}P < 0.05$ indicates a statistically significant difference between the control group (black bars) and the group pre-treated with lactate and F26BP.

modulating hexokinase activity, mouse tissues were extracted and submitted to different treatments as indicated in Fig. 4. Lactate did not modulate hexokinase activity in the heart or kidney (data not shown). Similar to what we observed for PFK activity, acute exposure to 10 mM lactate inhibited hexokinase activity in skeletal muscle (Fig. 4A). Exposure of skeletal muscle to 100 nM insulin increased hexokinase activity by five folds. However, we found that the stimulatory effect on hexokinase activity was completely abolished by simultaneous treatment with 100 nM insulin and 10 mM lactate for three hours: the activity levels were similar to those observed in controls (Fig. 4A).

Another hexokinase isoform, hexokinase IV or glucokinase, is present in the liver. To evaluate the modulation of glucokinase by lactate, mouse livers were preincubated with 100 nM insulin and 10 mM lactate for 3 h. We observed that lactate inhibited glucokinase activity independent of the glucose concentration used (Fig. 4B). These results suggest that lactate can affect glucose metabolism in both skeletal muscle and hepatic tissue by at least two distinct mechanisms, i.e., PFK inhibition and hexokinase (glucokinase) inhibition. The inhibition of glucokinase, even in the presence of insulin, suggests that lactate, in addition to being a substrate for gluconeogenesis, can also act as a positive modulator of this metabolic pathway. We suggest that lactate is not only a substrate for gluconeogenesis but also capable of inhibiting both PFK (Fig. 1) and glucokinase (Fig. 4), two of the most important enzymes regulating hepatic glucose consumption.

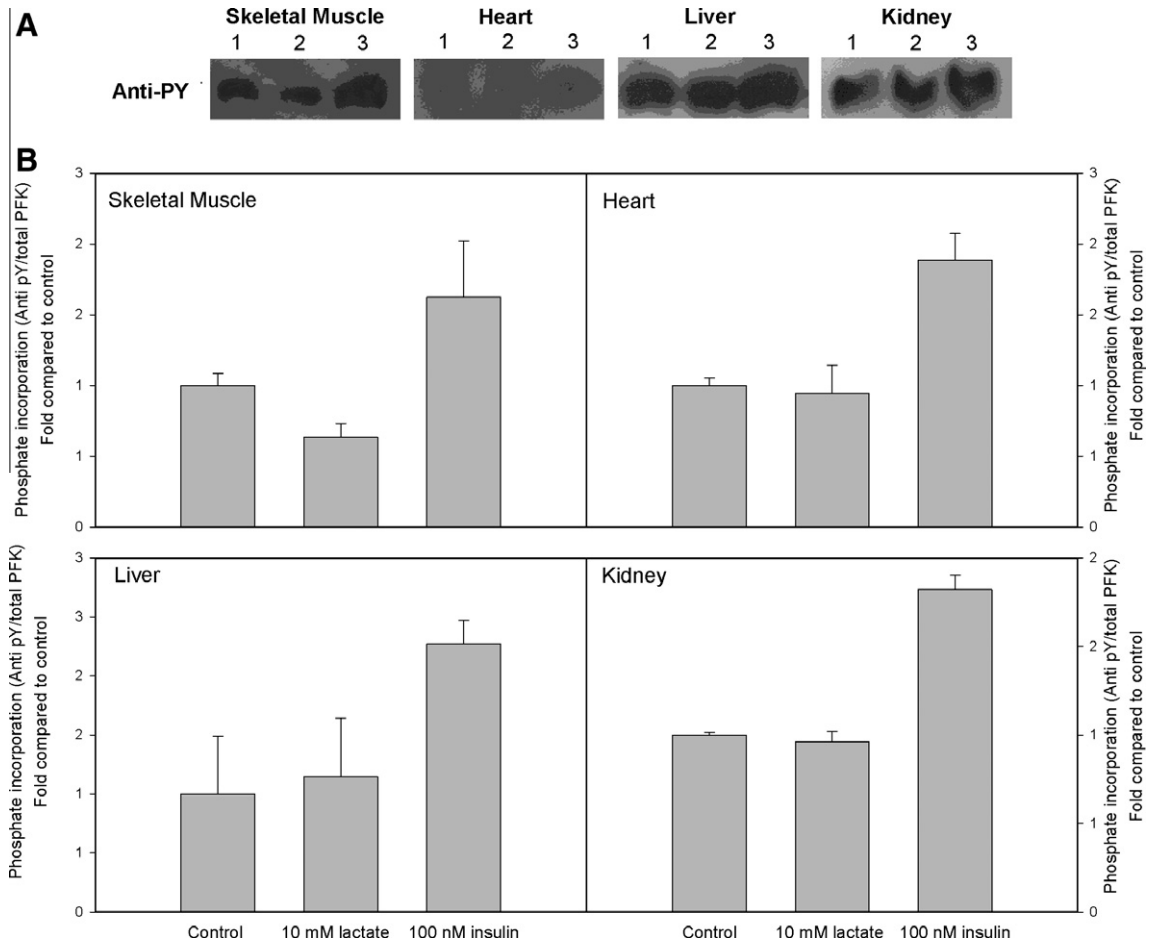

Fig. 3. Effect of lactate on phosphorylation level of PFK tyrosine residues**.** Tissues were isolated and preincubated for 3 h in the presence or absence of 10 mM lactate and/or 100 nM insulin for analyzing the phosphorylation levels of PFK tyrosine residues. (A) Western blot of different tissues blotted with an anti-phosphotyrosine antibody (1: control; 2: 10 mM lactate; 3: 100 nM insulin). (B) Phosphate incorporation in tyrosine residues normalized to total PFK. Exposure to 10 mM lactate for 3 h decreased the overall phosphorylation level of PFK tyrosine residues in skeletal muscle. Insulin treatment promotes an increase in the phosphorylation level of PFK tyrosine residues in all the analyzed tissues.

To examine the mechanism by which lactate inhibits hexokinase and glucokinase activity, we evaluated its ability to modulate the intracellular sublocalization of these enzymes. The binding of hexokinase I to mitochondria renders the enzyme less susceptible to the inhibition by its product [6]. In order to evaluate the cellular distribution of hexokinase in the presence of 10 mM of lactate, mouse tissues were isolated and subjected to differential centrifugation. We analyzed the enzymatic activity in two fractions, total homogenate (TH) and the mitochondria and nuclear compartment-enriched fraction (P1). As shown in Fig. 4C, lactate inhibited hexokinase activity in TH from skeletal muscle and liver, which was likely associated with cellular redistribution of hexokinase because we observed significant hexokinase inhibition in the P1 fraction from both tissues (Fig. 4C). Lactate did not modulate the activity or the cellular distribution of hexokinase in the heart and kidney (data not shown).

We next evaluated the ability of lactate to inhibit the hexokinase activity stimulated by increasing concentrations of the P1 fraction. For this purpose, the activity of purified hexokinase was determined in the presence or absence of 10 mM lactate in the P1 fraction from skeletal muscle, heart, liver and kidney. Fig. 5 showed the activity of purified hexokinase in the presence of the mitochondria-enriched P1 fraction from skeletal muscle. We noted that even the P1 fraction at the lowest analyzed concentration (1 μg/ml) was sufficient to saturate the activity of the purified hexokinase. The same activation pattern was observed in all the analyzed tissues (data not shown). We suggest that binding of the enzyme to mitochondria and the subsequent auto-activation caused the 4.5-fold increase in activity.

We further evaluated whether the hexokinase inhibition by lactate is direct, similar to that of PFK inhibition [9]. For this purpose, we performed an assay using purified hexokinase in the presence of different concentrations of glucose in the presence or absence of 10 mM lactate (Fig. 6A). The data showed that lactate did not modulate the activity of the purified hexokinase because it did not alter its affinity for glucose or ATP (data not shown). Together with the data shown in Figs. 4 and 5, these results suggest that lactate inhibits hexokinase activity by dissociating the enzyme from mitochondria or nuclei, rather than by a direct inhibition. Even though lactate does not directly inhibit hexokinase, it could indirectly inhibit hexokinase activity by mobilizing it to the soluble fraction. It is possible that lactate could cause enzyme dissociation through altering the structure of hexokinase. To test this hypothesis, we measured the center of mass of the intrinsic fluorescence spectrum of purified hexokinase in the presence or absence of 10 mM lactate (Fig. 6B). This technique is commonly used to evaluate the transitions between the intermediate states of proteins in response, for instance, to ligands [23] or different enzymatic concentrations [24,25]. As shown in Fig. 6B, lactate could indeed alter the structure of hexokinase. This structural alteration was not

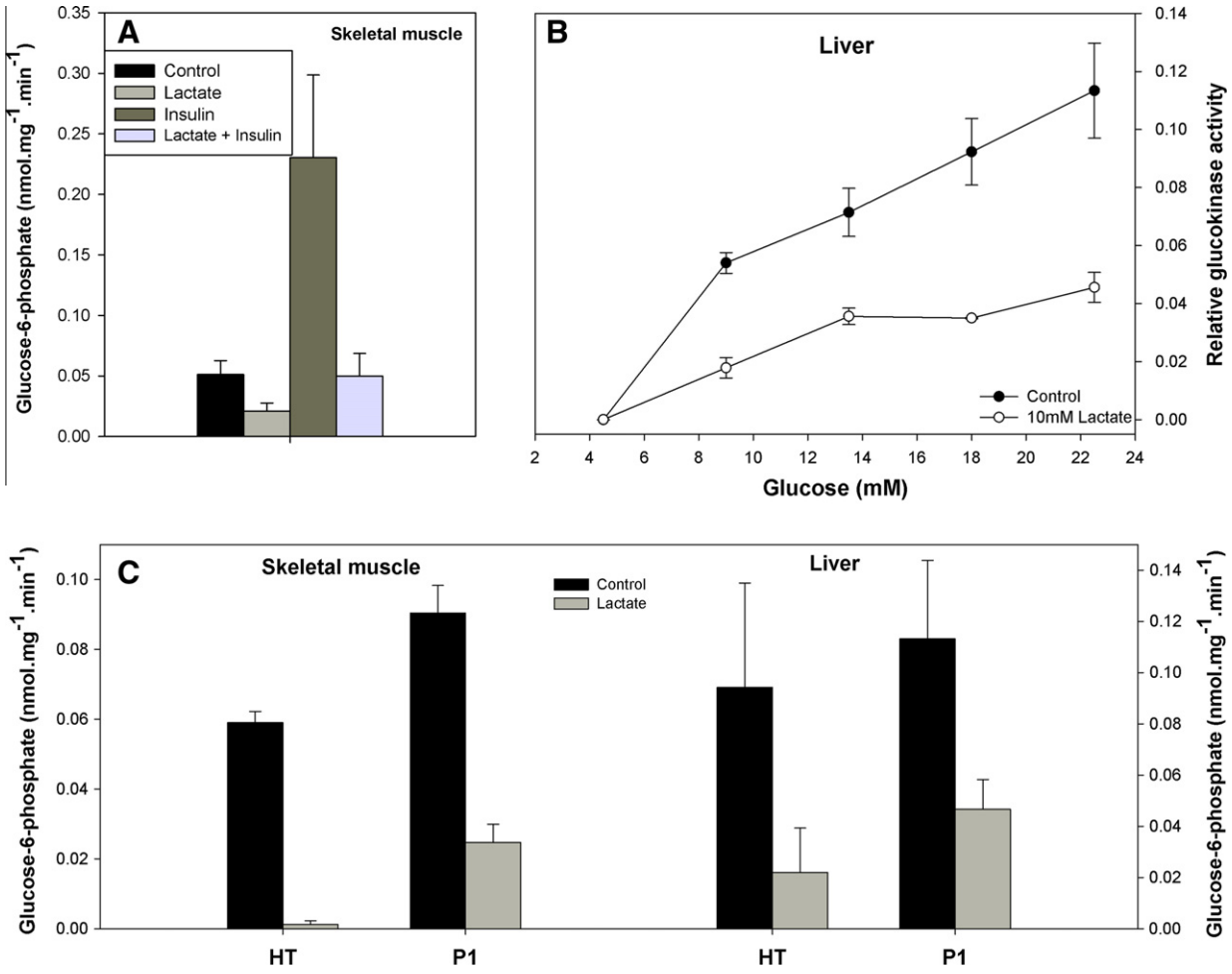

Fig. 4. Effect of lactate on the activity and cellular distribution of hexokinase and glucokinase. HK or glucokinase activity was assessed as described in Section 2 in skeletal muscle (A) and liver (B), respectively. Panel C shows the results after fractionation. Values are presented as the mean ± S.E.M. of six independent assays.

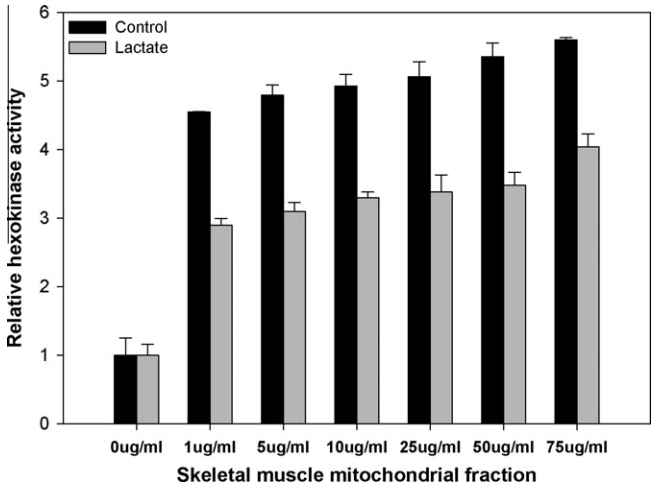

Fig. 5. Effect of lactate on the association of purified hexokinase to mitochondria in skeletal muscle. HK activity was assessed as described in Section 2. Values are presented as the mean ± S.E.M. of three independent assays.

caused by protein denaturation because the enzymatic activity was preserved under these experimental conditions (Fig. 6A). Although these results do not provide a definitive explanation, they suggest that lactate can alter the structure of hexokinase and that this modification may lead to the dissociation of the enzyme from mitochondria and nuclei, which, in turn, causes its inhibition.

The effects of lactate on PK activity was also evaluated. For this purpose, we used an alternative method for the assay of PF activity, instead of the classical coupled enzyme linked assay. This was necessary since the classical method uses lactate dehydrogenase to evaluate the pyruvate formed through its reduction measuring the coupled oxidation of NADH to NAD^+. However, the presence of lactate in the reaction medium would displace the equilibrium of this reaction in the opposite direction, interfering with the measurements. To solve this issue, we analyzed the ATP formed using a commercial kit. The method was efficient but lactate did not promote any effect on PK activity under all tested conditions (data not shown).

Our results support the hypothesis that lactate is not a mere byproduct of glycolysis without relevant metabolic roles. In fact, several groups have shown that lactate metabolism is highly versatile and associated with several pathological conditions [26–31]. The inhibitory effects of lactate on the enzymes known to be important in regulating glycolytic flux, such as hexokinase and PFK, in tissues, such as skeletal muscle and liver, can have a inhibitory effect on carbohydrate metabolism [14,15,17]. Because skeletal muscle is one of the most important tissues in glucose metabolism, glycolytic flux inhibition caused by lactate in this tissue, even in the presence of insulin, can have a systemic impact on hexose metabolism in the whole body [32]. We suggest that the inhibitory effect of insulin is in part due to (I) antagonistic effects

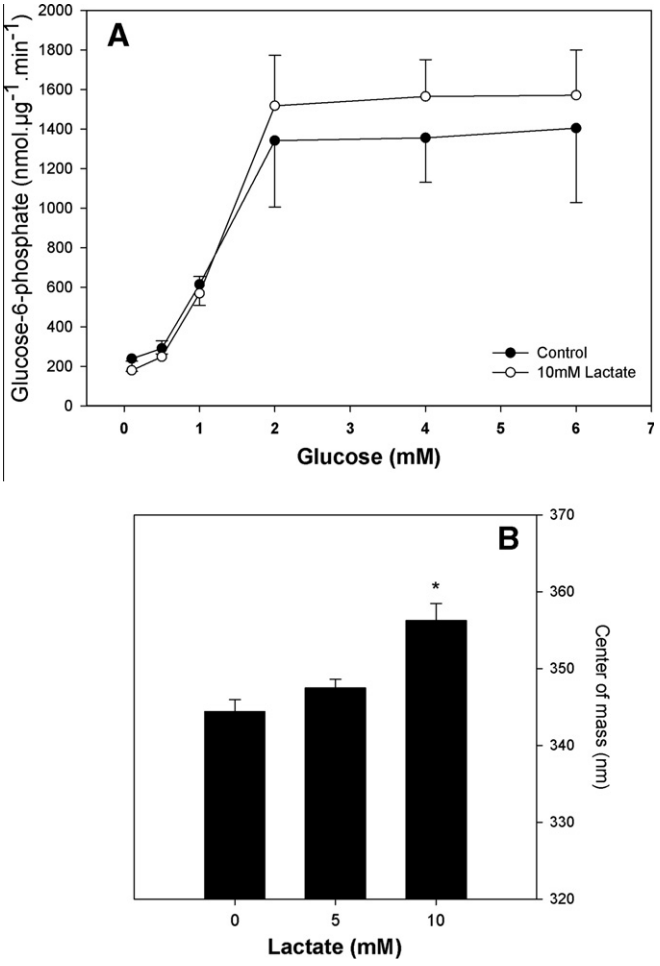

Fig. 6. Effects of lactate on the activity and structure of purified hexokinase. (A) HK activity (A) and intrinsic fluorescence spectra (B) were performed as described in Section 2. Values are presented as the mean ± S.E.M. of three independent experiments. $^{*}P < 0.05$ indicates a statistically significant difference between the experimental group and the control group without lactate.

of lactate on PFK activity when stimulated with fructose-2,6-biphosphate; (II) decreased phosphorylation level of PFK tyrosine residues and (III) PFK dimerization that inhibits its activity [9]. Furthermore, hexokinase inhibition occurs mainly through regulating the association of the enzyme to cellular structures such as mitochondrial and nuclear membranes. There is no direct modulation of hexokinase I by lactate. The modulation of the association with mitochondrial and nuclear membranes is dependent on the alteration of the enzyme structure without protein denaturation, consistent with lactate-induced insulin resistance because the ability of insulin to increase glucose uptake depends on both GLUT4 externalization and increased mobilization of hexokinase to mitochondrial membrane [33], an effect antagonized by lactate. Therefore, we suggest that lactate acts as an antagonist of insulin regarding hexokinase activity and contributes to the decreased glucose consumption by skeletal muscle, a common characteristic under pathological conditions with insulin resistance. In the liver, lactate modulates the major enzymes of the gluconeogenesis pathway, thereby contributing to the hyperglycemia characteristic of diabetes. Finally, our results are in agreement with the theory supported by several groups that lactate should be recognized as an extremely versatile molecule, not simply the end product of fermentation glycolysis.

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