INHIBITORY EFFECTS OF Zn²⁺ ON MUSCLE GLYCOLYSIS AND THEIR REVERSAL BY HISTIDINE

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Summary Of a number of divalent cations investigated, Zn²⁺ strongly inhibited lactate production from glucose 6-phosphate in rat muscle cytosol fraction. The I₅₀ value for lactate production influenced by Zn²⁺ was 10 µM and was increased to 200 µM and 18 µM by the addition of 10 mM histidine and 10 mM carnosine, respectively. The inhibitory effect of 50 µM Zn²⁺ on lactate production was completely reversed by the addition of 1.2 mM histidine and the apparent Kₘ was found to be 0.34 mM.

The inhibitory site for Zn²⁺ was investigated by the estimation of glycolytic intermediates. It occurred at point between fructose 6-phosphate and fructose 1,6-bisphosphate. Purified rat muscle phosphofructokinase was inhibited by Zn²⁺. The I₅₀ values for Zn²⁺ were calculated to be 12, 5.5 and 1.5 µM in the presence of 3, 0.3 and 0.06 mM fructose 6-phosphate, respectively. The addition of histidine removed the inhibitory effect of Zn²⁺ on the glycolytic key enzyme. These studies indicate that in rat skeletal muscle the inhibitory effect of Zn²⁺ on lactate production might result from the inhibition of phosphofructokinase and that histidine could remove the effect.

Keywords zinc, glycolysis, histidine, carnosine, glycolytic intermediates, phosphofructokinase, lactate.

It is becoming increasingly clear that the effect of a metabolite in the regulation of glycolysis has to be considered in terms of its function as a reactant. Imidazole activated the lactate production of ascites tumor (1) and stimulated

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³ Abbreviations: G-6-P, glucose 6-phosphate; F-6-P, fructose 6-phosphate; FBP, fructose 1,6-bisphosphate; DHAP, dihydroxyacetone phosphate; GAP, glyceraldehyde 3-phosphate; 1,3-BPG, 1,3-bisphosphoglycerate; 3-PG, 3-phosphoglycerate; 2-PG, 2-phosphoglycerate; PEP, phosphoenolpyruvate.
glycolysis of rat skeletal muscle (2), diaphragm (3) and renal cortex (4). Also, histidine activated both jejunal and hepatic pyruvate kinase activity (5). However, it has not been clarified if the activation of glycolysis by imidazole depends on the stimulation of pyruvate kinase.

Zinc is an essential component of several dehydrogenases involved in carbohydrate metabolism (6). The addition of $10^{-4}$ M Zn(OH)$_2$, however, inhibited the formation of lactate by smooth muscle extracts (7). On the other hand, histidine has the role of a chelating agent (8, 9) and a buffer at neutral pH (9).

The work described in the present report concerns the effect of Zn$^{2+}$ and histidine on the production of lactate from glucose 6-phosphate by rat skeletal muscle. In the course of the studies we found that histidine could restore the phosphofructokinase activity inhibited by Zn$^{2+}$.

MATERIALS AND METHODS

**Chemicals.** All chemicals used were of analytical grade and purchased from Nakarai Chemicals, Ltd., if not otherwise stated. All nucleotides and auxiliary enzymes were obtained from Boehringer Mannheim GmbH. L-Carnosine was purchased from Sigma Chemicals. Sepharose CL-6B was purchased from Pharmacia and Cibacron blue F3G-A was kindly provided by Dr. H. Bosshard of Ciba-Geigy, Basel, Switzerland. Cibacron blue F3G-A-Sepharose CL-6B was prepared from Sepharose CL-6B and Cibacron F3G-A according to Finderknecht et al. (10).

**Animals.** Male Sprague-Dawley rats, weighing 200 to 250 g, were housed in individual screen-bottom cages in a room maintained at 23 ± 1°C with 50% humidity and light regulation (12 hr light per day). The animals were given commercial food pellets (CLEA Japan, Inc.) and water ad libitum. After the sacrifice of the rats, hind leg muscle was quickly removed and was provided as a sample for analysis of glycolysis.

**Lactate production by rat skeletal muscle.** Hind leg muscle was homogenized with a glass homogenizer in 5 vol. of 90 mM potassium phosphate (pH 7.0), containing 180 mM KCl. After the homogenate was stood for 1 hr at 4°C, the precipitate was removed by centrifugation at 12,000 g for 30 min and at 105,000 g for 1 hr. The supernatant solution was desalted by passing it through a Sephadex G-25 column equilibrated with 20 mM potassium phosphate, pH 7.0, containing 280 mM KCl and 5 mM 2-mercaptoethanol. Protein fraction was pooled and was used as the source of glycolytic enzymes in these studies. Protein concentration was measured by the biuret method with albumin as standard (11). Glycolysis was measured as the rate of lactate production from glucose 6-phosphate. Incubation was carried out in a final volume of 2.0 ml containing 10 mM triethanolamine-HCl (pH 7.4), 10 mM potassium phosphate (pH 7.4), 5 mM MgCl$_2$, 30 mM KCl, 0.5 mM 2-mercaptoethanol, 0.5 mM ATP, 20 mM glucose 6-phosphate, 0.4 mM NAD and 2.3 mg of the above enzyme protein. The incubations were performed at 37°C for
10 min using air as the gas phase, and were terminated by addition of ice-cold trichloroacetic acid to a final concentration of 7%. Glycolytic intermediates including lactate were estimated in neutralized aliquots of the medium by enzymatic methods (12, 13).

**Enzyme purification and enzyme assay.** Rat skeletal muscle phosphofructokinase was purified by the modification method of Ramadoss et al. (14). Cibacron blue F3G-A-Sepharose CL-6B was used instead of ATP-Sepharose in the process of purification. The activity of phosphofructokinase was analyzed with the following test system: 2 mL of 0.1 M triethanolamine-HCl buffer, pH 7.4 including 5 mM MgCl₂, 0.3 mM NADH, 0.5 mM ATP, 3 mM fructose 6-phosphate, 1 unit aldolase, 5 units triosephosphate isomerase and 0.5 unit glycerol 3-phosphate dehydrogenase.

The above purified enzyme was homogeneous by gel electrophoresis in the presence or absence of sodium dodecylsulfate. Rat skeletal muscle phosphofructokinase was prepared with specific activity of 140 μmol × min⁻¹ × mg⁻¹ under standard assay conditions in the presence of 1 mM AMP at 25°C.

**Quantitative Zn²⁺ analysis.** Atomic absorption spectroscopy was employed to measure the concentration of the stock Zn²⁺ solution and the sample solution. Measurements were made with a Hitachi 208 atomic absorption spectrophotometer at 213.8 nm. Glassware used in preparation and storage of the samples was soaked in aqua regia and then rinsed well with doubly distilled deionized water.

**RESULTS**

**Effect of buffers on lactate production by rat skeletal muscle**

Of many buffers investigated, only potassium phosphate showed excellent lactate production and substrate utilization with linearity for 60 min (Fig. 1). The lactate production diminished when potassium phosphate was replaced by triethanolamine, Tris, L-histidine, L-carnosine or imidazole of the same pH. The increasing molarity of L-histidine, L-carnosine or imidazole to 500 mM did not affect the lactate production. Also, no increasing lactate production was found by the addition of KCl to imidazole buffer until 100 mM. From these findings, 10 mM potassium phosphate was added to the standard reaction mixture for lactate production.

**Removal of inhibitory effect of divalent cations on lactate production by histidine or carnosine**

A number of divalent cations inhibited rat muscle lactate production and Zn²⁺ was found to be an effective inhibitor of enzyme activity at low concentration (Table 1) (Fig. 2). The I₅₀ value was increased 20- and 1.8-fold by the addition of 10 mM histidine and carnosine, respectively. However, the extent of inhibition by Cu²⁺ or Ca²⁺ was at the same level in the presence or absence of 10 mM histidine.
Fig. 1. Effect of buffers on utilization of glucose 6-phosphate and production of lactate by rat skeletal muscle. In each experiment, lactate production was determined on the addition of each of the following buffers at a concentration of 20 mM: potassium phosphate, triethanolamine, Tris, L-histidine, L-carnosine and imidazole. Other conditions were described in MATERIALS AND METHODS. ●, potassium phosphate; ▲, triethanolamine or Tris; ■, L-histidine; ×, L-carnosine or imidazole.

Table 1. Inhibition by divalent cations of muscle lactate production.

<table>
<thead>
<tr>
<th></th>
<th>None</th>
<th>Histidine (10 mM)</th>
<th>Carnosine (10 mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZnCl₂</td>
<td>10 µM</td>
<td>200 µM</td>
<td>18 µM</td>
</tr>
<tr>
<td>NiCl₂</td>
<td>160</td>
<td>1,000</td>
<td>660</td>
</tr>
<tr>
<td>CuSO₄</td>
<td>540</td>
<td>680</td>
<td>620</td>
</tr>
<tr>
<td>CoCl₂</td>
<td>1,000</td>
<td>1,000</td>
<td>1,000</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>50,000</td>
<td>46,000</td>
<td>53,000</td>
</tr>
</tbody>
</table>

The values shown are apparent I₅₀ and were calculated from six to ten divalent cation concentrations. The lactate production was estimated as described in MATERIALS AND METHODS.

as well as 10 mM carnosine. D-Histidine also protected the inhibitory effect of Zn²⁺ on lactate production as well as L-histidine. The lactate production inhibited by 50 µM Zn²⁺ was completely reversed by the addition of 1.2 mM histidine, with an apparent Kₘ of 0.34 mM (Fig. 3).

Zn²⁺ content of rat skeletal muscle

The Zn²⁺ content of the supernatant fraction after centrifugation at 105,000 g for 1 hr as described in MATERIALS AND METHODS was estimated, revealing 130 ± 26 (n = 5) nmoles/g wet tissue. After the supernatant fraction was filtered with
Fig. 2. Inhibition of rat muscle lactate production by Zn\(^{2+}\). ○, control; □, 10 mM L-histidine; ■, 1 mM L-histidine; ×, 0.1 mM L-histidine; ▲, 10 mM L-carnosine. Other conditions were the same as the standard assay conditions.

Fig. 3. Reactivation of inhibited lactate production by histidine. The lactate production was assayed in the presence of 50 μM ZnCl\(_2\). Other conditions were the same as the standard assay conditions. The dotted line shows the lactate production in the absence of ZnCl\(_2\).

Amicon Centriflo Membrane Cones CF 25, the filtrate was found to contain 8.7 ± 1.3 (n=5) nmoles Zn\(^{2+}\)/g wet tissue.

Effect of Zn\(^{2+}\) on muscle glycolytic intermediates and removal of its effect by histidine

Table 2 summarizes the results of experiments performed to determine the site of action of Zn\(^{2+}\) in inhibiting lactate production. Lactate production was measured from a variety of different substrates in the glycolytic pathway. When 20 μmoles of glucose 6-phosphate was added to the incubation mixture, the sum of glycolytic intermediates including glucose 6-phosphate was approximately 90%. The concentrations of glycolytic intermediates measured after incubation with 10 μM Zn\(^{2+}\) in the presence or absence of histidine were expressed as a percentage of the concentrations of control and were shown in the form of a crossover plot.
Table 2. Effect of Zn$^{2+}$ on muscle glycolytic intermediates in the presence or absence of histidine.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Control</th>
<th>10 $\mu$M Zn$^{2+}$</th>
<th>10 $\mu$M Zn$^{2+}$ and 1 mM histidine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>× 10$^{-9}$ mol</td>
<td>× 10$^{-9}$ mol</td>
<td>× 10$^{-9}$ mol</td>
</tr>
<tr>
<td>G-6-P</td>
<td>6,016 ± 37</td>
<td>8,060 ± 717*</td>
<td>5,444 ± 445</td>
</tr>
<tr>
<td>F-6-P</td>
<td>3,205 ± 432</td>
<td>4,924 ± 516*</td>
<td>2,798 ± 31</td>
</tr>
<tr>
<td>FBP</td>
<td>1,803 ± 131</td>
<td>1,212 ± 320*</td>
<td>2,179 ± 175</td>
</tr>
<tr>
<td>DHAP</td>
<td>372 ± 50</td>
<td>275 ± 20</td>
<td>399 ± 35</td>
</tr>
<tr>
<td>GAP</td>
<td>302 ± 77</td>
<td>195 ± 42</td>
<td>337 ± 74</td>
</tr>
<tr>
<td>1,3-BPG</td>
<td>464 ± 105</td>
<td>427 ± 96</td>
<td>545 ± 79</td>
</tr>
<tr>
<td>3-PG</td>
<td>5,035 ± 1,102</td>
<td>3,218 ± 598</td>
<td>4,648 ± 714</td>
</tr>
<tr>
<td>2-PG</td>
<td>459 ± 102</td>
<td>388 ± 106</td>
<td>503 ± 116</td>
</tr>
<tr>
<td>PEP</td>
<td>1,035 ± 231</td>
<td>923 ± 43</td>
<td>988 ± 237</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>93 ± 14</td>
<td>77 ± 11</td>
<td>105 ± 5</td>
</tr>
<tr>
<td>Lactate</td>
<td>5,489 ± 483</td>
<td>2,572 ± 255**</td>
<td>6,045 ± 509</td>
</tr>
</tbody>
</table>

Total 24,273 ± 1,735 (17,986) 22,271 ± 1,206 (18,469) 23,991 ± 2,308 (17,574)

Substrate concentrations are 20 $\mu$molar. Values are mean ± S.E. of three samples. Two moles of 1,3-BPG, 3-PG, 2-PG, PEP, pyruvate and lactate were stoichiometrically synthesized from one mole of G-6-P. The correct values are shown in parentheses. * $p<0.05$, ** $p<0.01$ compared to control.

Fig. 4. Crossover plot demonstrating the effect of ZnCl$_2$ on muscle lactate production in the presence or absence of L-histidine. Incubation conditions are shown in Table 2. ●, 10 $\mu$M ZnCl$_2$; ○, 10 $\mu$M ZnCl$_2$ and 1 mM L-histidine.

(Fig. 4). The result indicated that crossover occurred at a point between fructose 6-phosphate and fructose 1,6-bisphosphate. A reduction in fructose 1,6-bisphosphate levels could represent the low level of other glycolytic intermediates including lactate. These results suggest that the inhibition of muscle glycolysis caused by...
Zn$^{2+}$ occurs at the level of the key glycolytic enzyme: phosphofructokinase. The high concentration of fructose 6-phosphate in the presence of 10 $\mu$M Zn$^{2+}$ was reduced to control level by the addition of 1 mM histidine. Also, the decreased level of other glycolytic intermediates recovered to control level by the addition of histidine (Table 2) (Fig. 4).

**Effect of Zn$^{2+}$ on purified muscle phosphofructokinase and removal of its effect by histidine**

From the crossover point, Zn$^{2+}$ should inhibit phosphofructokinase activity. In the presence or absence of Zn$^{2+}$, the velocity profiles of muscle phosphofructokinase as a function of fructose 6-phosphate concentration are shown in Fig. 5. Addition of 10 $\mu$M Zn$^{2+}$ to the assay medium reduced the maximum velocity to approximately 67% of that obtained in the absence of Zn$^{2+}$, with a concomitant increase in the $K_{1/2}$ value from 0.047 to 0.32 mM. The Hill coefficient was increased from 1.0 to 1.3 by the addition of 10 $\mu$M Zn$^{2+}$. In the presence of 5 mM Mg$^{2+}$, the purified muscle phosphofructokinase activity was inhibited by Zn$^{2+}$ and the inhibition was found to correspond to a progressive increase in lower substrate concentration of fructose 6-phosphate (Figs. 5 and 6). The $I_{50}$ values for Zn$^{2+}$ were calculated to be 12, 5.5 and 1.5 $\mu$M in the presence of 3, 0.3 and 0.06 mM fructose 6-phosphate, respectively. The inhibitory effect of Zn$^{2+}$ on the enzyme activity was relieved by the addition of histidine (Fig. 6). The addition of histidine in the absence of Zn$^{2+}$ had no effect on the enzyme activity. In the presence of 3 mM fructose 6-phosphate, the phosphofructokinase activity inhibited by 10 $\mu$M Zn$^{2+}$

![Fig. 5. The effect of increasing concentration of fructose 6-phosphate on purified rat muscle phosphofructokinase. Phosphofructokinase was dialyzed overnight at about 4°C against 50 mM potassium phosphate, pH 7.0, including 1 mM fructose 6-phosphate and 5 mM 2-mercaptoethanol. Auxiliary enzymes were also dialyzed against 0.1 M triethanolamine, pH 7.4. The enzyme activity was measured by the method described in MATERIALS AND METHODS. ●, control; ○, 10 $\mu$M Zn$^{2+}$.](image)
DISCUSSION

Glycolysis is activated or inhibited by ions, amino acids, nucleotides, hormones or carbohydrates. Imidazole has also been shown to enhance the production of lactate from glucose 6-phosphate (3, 4) and glucose 1-phosphate (2). Our results, however, showed that imidazole did not activate the lactate production in rat skeletal muscle (Fig. 1). This discrepancy might be caused by removing the low molecular weight substances from the enzyme source with Sephadex G-25. In particular, divalent cations including Zn$^{2+}$ might exist in the fractions and they could inhibit lactate production. Also, phosphate is the most effective buffer for glycolysis as shown in ascites tumor (15), erythrocyte (16) and muscle (Fig. 1).

Of many divalent cations, Zn$^{2+}$ inhibited glycolysis of rat skeletal muscle at low concentrations (Table 1) (Fig. 2). Zinc is an indispensable mineral element for animals, distributed in the tissues of several species (17). Recently, it has been proposed that metallothionein acts as the regulator of Zn$^{2+}$ metabolism (18, 19). In normal animal muscle, the level of metallothionein is much lower than in other tissues (20, 21). Therefore, the molarity of Zn$^{2+}$ in muscle low molecular fraction (8.7 $\mu$M) is sufficient to reveal the toxicity to glycolysis (Fig. 2). The addition of 1 mM histidine completely overcame the inhibition of glycolysis by Zn$^{2+}$, and 0.1 mM histidine and 10 mM carnosine modulated the inhibition. The contents of histidine, anserine ($\beta$-alanyl-$N^\alpha$-methylhistidine) and carnosine ($\beta$-alanylhistidine) in rat skeletal muscle were 0.2, 2 and 6 mM, respectively (22). These histidine...
compounds could remove the inhibitory effect of Zn\(^{2+}\) on lactate production.

The crossover data suggest that Zn\(^{2+}\) acts at the level of the rate-limiting glycolytic enzyme, phosphofructokinase. Phosphofructokinase catalyses the principal rate-limiting reaction of the glycolytic pathway (23). Recently, substrate cycling of fructose 6-phosphate through reactions catalyzed by phosphofructokinase and fructose 1,6-bisphosphatase has been found in muscle. The enzyme activity of purified rat muscle phosphofructokinase was inhibited by Zn\(^{2+}\) and the addition of histidine also removed the inhibitory effect (Figs. 5 and 6). From these findings, the inhibitory effect of Zn\(^{2+}\) on lactate production might result from the inhibition of phosphofructokinase. We found Zn\(^{2+}\) is also a potent inhibitor of muscle pyruvate kinase (Tamaki et al., in preparation). The addition of 10 nM Zn\(^{2+}\) in overall production of lactate from glucose 6-phosphate did not reduce the concentration of phosphoenolpyruvate (Table 2). However, it does not lessen the probability that Zn\(^{2+}\) affects glycolysis and gluconeogenesis in other tissues by the reaction of pyruvate kinase, pyruvate carboxylase and phosphoenolpyruvate carboxykinase.

Our observations suggest that physiological concentrations of Zn\(^{2+}\) could inhibit carbohydrate metabolism and that chelating agents including histidine might relieve the inhibition.

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REFERENCES


