Activity, distribution and regulation of phosphofructokinase in salivary gland of rats with streptozotocin-induced diabetes

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Douglas Nesadal Souza**
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ABSTRACT: Although the influence of diabetes on salivary glands is well studied, it still presents conflicting results. In this work, the regulation of the phosphofructokinase-1 enzyme (PFK-1) was studied utilizing the salivary glands of rats. Diabetes was induced by a single intraperitoneal injection of streptozotocin (60 mg/Kg of body weight) in rats (180-200 g). The animals were killed 30 days after the induction of diabetes and the submandibular and parotid salivary glands were used. Hyperglycemia was evaluated by blood sugar determination. The distribution of PFK-1 between the soluble and cytoskeleton fractions, the phosphate content of PFK-1, the content of fructose-2,6-bisphosphate and the activity of the PFK-2 enzyme were determined. The calculated relative glandular weight showed a higher value for the parotid gland in comparison with the control, but not for the submandibular gland. The activity of PFK-1 expressed per gland showed no variation between diabetic and control animals. However, considering the specific activity, the soluble enzyme presented a value 50% higher than that of the control and the cytoskeleton bound form increased by 84% compared to the control. For the parotid gland, no difference in the specific activity between diabetic and control animals was observed. On the other hand, the activity per gland of the soluble enzyme increased in the diabetic animals. The phosphate content of PFK-1 increased in the submandibular and parotid glands of diabetic rats. Both the content of fructose-2,6-bisphosphate and the active form of PFK-2 were reduced in the diabetic glands. In conclusion, the increase in the activity of PFK-1 observed in the salivary glands of rats with streptozotocin-induced diabetes does not seem to be due to its modulator fructose-2,6-bisphosphate.

DESCRIPTORS: Diabetes mellitus; Phosphofructokinase-1; Salivary glands; Parotid gland; Submandibular gland.

RESUMO: Apesar de existirem muitos estudos sobre a influência do diabetes nas glândulas salivares, esses apresentam resultados conflitantes. Neste estudo, a regulação da enzima fosfofrutoquinase-1 (PFK-1) foi estudada utilizando-se glândulas salivares de ratos. O diabetes foi induzido por uma única injeção intraperitoneal de estreptozotocina (60 mg/kg peso corporal) em ratos (180-200 g). Os animais foram sacrificados 30 dias após a indução do diabetes e utilizaram-se as glândulas submandibular e parotídea. A hiperglycemia foi avaliada por determinação da glicemia sanguínea. A distribuição da PFK-1 entre frações solúvel e ligada, concentração de fosfato na PFK-1, concentração de frutose-2,6-bisfosfato e a atividade da enzima PFK-2 foram determinadas. O cálculo do peso glandular relativo mostrou um aumento na glândula parótida de ratos diabéticos comparados ao controle, o que não ocorreu na glândula submandibular. A atividade da PFK-1 expressa por glândula não mostrou variação entre animais diabéticos e controle. Contudo, considerando a atividade específica, a fração solúvel da enzima mostrou aumento de 50% com relação ao controle e a fração ligada ao citoesqueleto um aumento de 84% com relação ao controle. Na glândula parótida não foi observada diferença na atividade específica entre os grupos diabético e controle. Por outro lado, a atividade por glândula da fração solúvel aumentou nos animais diabéticos. A concentração de fosfato da PFK-1 aumentou nas glândulas submandibular e parótida nos animais diabéticos. Tanto a concentração de frutose-2,6-bisfosfato quanto a forma ativa da PFK-2 mostraram redução nas glândulas salivares. Concluindo, o aumento na atividade da PFK-1 observado nas glândulas salivares de ratos com diabetes induzida por estreptozotocina não parece ser modulado pela frutose-2,6-bisfosfato.

DESCRITORES: Diabetes mellitus; Fosfofrutoquinase-1; Glândulas salivares; Glândula parótida; Glândula submandibular.

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INTRODUCTION

Diabetes mellitus is a metabolic disease that affects many organs and systems, including the oral cavity. Some investigations have described the effects of experimental diabetes induced either by streptozotocin or alloxan in the structure and functions of the salivary glands of animals. It has been reported that diabetes decreases norepinephrine content, the density of adrenergic receptor and receptor-adrenal coupling in parotid glands, as well as salivary secretion closely associated with the lowered susceptibility of the muscarinic receptors. Recently it was reported that isolated parotid gland from streptozotocin-induced diabetic rats presented a dose-dependent decrease in amylase release in response to noradrenaline when compared to control parotid gland.

The role of PFK-1 in the regulation of glycolysis has been established for a variety of cell types in various animals. The activity of this enzyme is controlled by multiple positive and negative allosteric factors such as ATP, ADP, AMP, fructose-2,6-bisphosphate (Fru-2,6-P$_2$) and citrate. Fru-2,6-P$_2$ is a potent stimulator of PFK-1 and has been detected in all mammalian tissues. The concentration of Fru-2,6-P$_2$ is the result of a balance between the activity of PFK-2/FBP-2 as kinase and as phosphatase. Similar to other glycolytic enzymes PFK-1 may be covalently modified by phosphorylation/d Dephosphorylation as a form of regulation.

In the submandibular gland, PFK-1 was found to be synergistically regulated by ATP, fructose-6-phosphate and Fru-2,6-P$_2$. In adult rat submandibular gland, Fru-2,6-P$_2$ relieves PFK-1 from inhibition by ATP. In previous publications we have found that PFK-1 in submandibular glands of rats treated with the β-adrenergic agonist isoproterenol has its activity and kinetics properties altered.

Considering that energy is important for the secretory functions of salivary glands, the purpose of the present investigation was to examine, in a short-term experiment, the activity, distribution and regulation of PFK-1 in the submandibular and parotid salivary glands of streptozotocin-induced diabetic rats.

MATERIAL AND METHODS

Animals

Thirty-two male rats of the Wistar strain weighing 180-200 g were housed individually in plastic cages with free access to water and food throughout the experimental period. The animals were randomly divided into control and diabetic groups. Diabetes was induced by a single intraperitoneal injection of streptozotocin (STZ) (Sigma-Aldrich Corporation, St. Louis, USA) dissolved in 0.01 mol/L citrate buffer (Sigma-Aldrich Corporation, St. Louis, USA), pH 4.5 (60 mg/Kg of body weight) in overnight fasted rats. The control animals received only the citrate buffer. The rats were killed 30 days after the induction of diabetes.

All animals were handled in accordance to the guideline of Ethical Principles in Experiments with Animals approved by the “Colégio Brasileiro de Experimentação Animal”.

Tissue preparation

The animals were killed always between 9:00 and 11:00 a.m. to minimize circadian variations. The salivary glands were immediately removed, cleaned from the adherent tissue and frozen between aluminium tongs previously cooled in dry ice and stored at –80°C until analyzed.

Analysis

Blood glucose

It was monitored using the blood from the tail vein through the method of Somogy modified by Nelson (1944). Animals were considered diabetic with blood glucose levels exceeding 19.5 mM.

Determination of the soluble and cytoskeleton bound PFK-1

The soluble and particulated PFK-1 was separated as follows: The frozen tissue was homogenized with 0.15 mol/L sucrose solution (Sigma-Aldrich Corporation, St. Louis, USA) containing 1 mol/L DTT (Sigma-Aldrich Corporation, St. Louis, USA) and 20 mol/L NaF (Sigma-Aldrich Corporation, St. Louis, USA), pH 7.5, in a glass homogenizer with a Teflon pestle. The homogenate was centrifuged at 100 x g for 5 minutes and the supernatant was centrifuged at 27,750 x g for 20 minutes. The enzymatic activity of the supernatant of this centrifugation was considered the soluble enzyme. The enzymatic activity of the sediment was considered the cytoskeleton bound PFK-1, as described elsewhere.

The activity of PFK-1 was assayed spectrophotometrically by monitoring the oxidation of
NADH (Sigma-Aldrich Corporation, St. Louis, USA) at 340 nm in a system coupled to aldolase, triosephosphate isomerase and glycerophosphate dehydrogenase at two pHs: At pH 6.9, the enzyme exhibits typical allosteric kinetics and, at pH 8.2, it presents the maximum activity in a medium (Sigma-Aldrich Corporation, St. Louis, USA) containing 50 mol/L Hepes buffer, 10 mol/L KCl, 6.5 mol/L MgCl₂, 1 mol/L NH₄Cl, 5 mol/L KH₂PO₄, 0.1 mol/L AMP, 0.3 mol/L NADH, 0.5 U/ml aldolase, 0.5 U/ml TIM, 0.1 mol/L Fru-6-P, 0.3 mol/L G-6-P and 1.5 mol/L ATP.

For the determination of the total and active (nonphosphorylated) form of PFK-2, according to the method described by Bartrons et al. (1983), the enzyme fructose-6-phosphotransferase purified from potato tubers was employed. Fructose-2,6-bisphosphate was determined by the ability of this metabolite to activate the enzyme PP₁: fructose-6-phosphate-1-transferase purified from potato tubers.

The phosphate content of PFK-1 purified from both salivary glands was determined based upon the methods described by Hasegawa et al. (1982).

Protein was estimated by the method of Lowry et al. (1951), using the bovine serum albumin as a standard.

Data were submitted to Student’s t test comparing the diabetic and control groups. Differences were accepted as statistically significant at p ≤ 0.05.

**RESULTS**

The diabetic animals lost weight along the experimental period. The calculated relative glandular weight (RGW) of the submandibular gland (RGW = glandular weight x 100/body weight) showed no significant difference between diabetic and control rats (0.067 ± 0.005 and 0.070 ± 0.007, respectively), while for the parotid gland the RGW was statistically higher for the diabetic group (0.060 ± 0.001 and 0.030 ± 0.007 respectively for the diabetic and control groups).

Table 1 shows the data for the submandibular gland. Expressed as mU/gland no difference was observed comparing the glands from the diabetic and control animals for both soluble and particulate enzyme. However, considering the activity per mg of protein (specific activity) it was seen that the soluble enzyme increased by 50% and the bound, by 84% compared to the control, when the activity was determined in conditions to present allosteric properties.

For the parotid gland (Table 2) there was an increase in the activity of the soluble form of PFK-

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**TABLE 1** - Activity of the soluble and citoskeleton bound fractions of PFK-1 of the submandibular salivary glands of rats with streptozotocin-induced diabetes (D) and of control animals (C) determined at pH 6.9 and at pH 8.2.

<table>
<thead>
<tr>
<th>Groups</th>
<th>pH</th>
<th>Soluble</th>
<th>Bound</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>mU/gland</td>
<td>mU/mg protein</td>
</tr>
<tr>
<td>C (8)</td>
<td>6.9</td>
<td>56.0 ± 15.0</td>
<td>7.6 ± 1.9</td>
</tr>
<tr>
<td>D (8)</td>
<td>6.9</td>
<td>59.0 ± 12.0</td>
<td>11.4 ± 3.2*</td>
</tr>
<tr>
<td>C (8)</td>
<td>8.2</td>
<td>124.0 ± 15.0</td>
<td>7.2 ± 2.4</td>
</tr>
<tr>
<td>D (8)</td>
<td>8.2</td>
<td>109.0 ± 19.0</td>
<td>6.9 ± 1.8</td>
</tr>
</tbody>
</table>

Mean ± SD. In parenthesis is the number of rats. The asterisk means statistically significant by Student’s t test comparing the diabetic and control animals (p < 0.05).

**TABLE 2** - Activity of the soluble and cytoskeleton bound fractions of PFK-1 of the parotid salivary gland of rats with streptozotocin-induced diabetes (D) and of control animals (C) determined at pH 6.9 and at pH 8.2.

<table>
<thead>
<tr>
<th>Groups</th>
<th>pH</th>
<th>Soluble</th>
<th>Bound</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>mU/gland</td>
<td>mU/mg protein</td>
</tr>
<tr>
<td>C (8)</td>
<td>6.9</td>
<td>24.0 ± 5.0</td>
<td>6.4 ± 1.6</td>
</tr>
<tr>
<td>D (8)</td>
<td>6.9</td>
<td>33.0 ± 6.0*</td>
<td>5.7 ± 1.1</td>
</tr>
<tr>
<td>C (8)</td>
<td>8.2</td>
<td>156.0 ± 51.0</td>
<td>35.1 ± 9.9</td>
</tr>
<tr>
<td>D (8)</td>
<td>8.2</td>
<td>102.0 ± 39.0*</td>
<td>36.4 ± 8.2</td>
</tr>
</tbody>
</table>

Mean ± SD. In parenthesis is the number of animals. The asterisk means statistically significant by Student’s t test comparing the diabetic and control animals (p < 0.05).
TABLE 3 - The activity of PFK-2 (active form and total) and the content of Fru-2,6-P₂ in submandibular (SM) and parotid (P) glands of rats with streptozotocin-induced diabetes (D) and of control animals (C).

<table>
<thead>
<tr>
<th>Groups</th>
<th>Fru-2,6-P₂ (pmol/g tissue)</th>
<th>PFK-2 (pmol/g/min)</th>
<th>Active</th>
<th>Active/total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Total</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SM</td>
<td>C 87.2 ± 16.8 (8)</td>
<td>27.9 ± 8.3 (8)</td>
<td>13.0 ± 1.7 (8)</td>
<td>0.46</td>
</tr>
<tr>
<td></td>
<td>D 50.2 ± 7.6* (8)</td>
<td>20.0 ± 5.5* (8)</td>
<td>7.2 ± 1.7* (8)</td>
<td>0.36</td>
</tr>
<tr>
<td>P</td>
<td>C 172.5 ± 22.3 (8)</td>
<td>28.2 ± 6.4 (8)</td>
<td>19.3 ± 5.2 (8)</td>
<td>0.68</td>
</tr>
<tr>
<td></td>
<td>D 100.6 ± 22.3* (8)</td>
<td>18.3 ± 2.5* (8)</td>
<td>9.0 ± 2.4* (8)</td>
<td>0.49</td>
</tr>
</tbody>
</table>

Mean ± SD. In parenthesis is the number of animals. The asterisk means statistically significant by Student’s t test comparing the diabetic and control animals. (p < 0.05).

1 determined at pH 6.9 (37%), and a reduction at pH 8.2 (35%) expressed per gland.

The content of Fru-2,6-P₂ was reduced in the submandibular (42.5%) and parotid glands (41.7%) (Table 3). Similarly, the active form of PFK-2 showed reductions of about 44.6% in the submandibular and of about 53.2% in the parotid glands. The total activity also showed a reduction either in submandibular (28.3%) or parotid (35.1%) glands (Table 3).

The phosphate content of PFK-1 was significantly higher in the diabetic group than in the control rats for the submandibular gland (respectively 1.59 and 0.95 mol P/mol PFK-1) and for the parotid gland (respectively 1.07 and 0.74 mol P/mol PFK-1).

DISCUSSION

The results of the calculated RGW showed no variation for the submandibular gland. However, a higher value was found for the parotid gland of the diabetic animals, indicating an enlargement in comparison with the control animals, a fact that has already been reported.

The role of PFK-1 in the regulation of metabolism is well known. The rate of the reaction catalyzed by PFK-1 is important to control the flow of glycolysis. The activity of this enzyme may be controlled by a variety of modulators as well as by phosphorylation/dephosphorylation. Our results for the submandibular gland show an increase in the specific activity of the enzyme determined at pH 6.9 for both forms, soluble (50%) and bound to the cytoskeleton (84%). No differences were observed either for the soluble or particulate enzyme in the activity determined at pH 8.2.

Contrary to the findings for the submandibular gland, the specific activity of the enzyme showed no variation for the parotid gland. However, considering the determination performed at pH 6.9 in which the enzyme presents allosteric properties, the mean activity per gland was higher for the diabetic animals in the soluble fraction and lower than the control animals when determined at pH 8.2.

The diabetic rats consume more food than the control animals (hyperphagia). This increase in consumption may lead to an increase in the mastication and an increase in the consumption of ATP from the glycolytic pathway. PFK-1 is a key enzyme of this metabolic pathway, and the increase in activity may be a consequence of the increase in the glycolytic pathway. The differences in the results of the submandibular and parotid glands may be due to metabolic differences between the two glands. While in the SM gland the metabolism is predominantly anaerobic, the consumption of glucose may be higher than in the P gland, considering that its metabolism is predominantly aerobic and the glycolytic pathway is more efficient. Under allosteric control, the increase in the activity observed in the SM gland shows only an increase in the activation of the PFK-1, but not an increase of the total content and the total activity of PFK-1 in the SM gland. On the other hand, in the P gland, the lack of increase in the specific activity may have led to an increase of the RGW while, under allosteric regulations, the specific activity did not increase, but the total activity did. Without this regulation, we can not explain the decrease of the activity of PFK-1.

Many studies have shown that the induction of diabetes may lead to different results of PFK-1 activity in several tissues. Thus, for the liver, adipocytes, enterocytes, and heart atria, reduced enzymatic activities were reported. The responses of the enzymatic activity to induced diabetes in animals were shown to be non-significant in lung and heart ventricle. On the other hand, in rat peritoneal macrophage cells, PFK-1 activity was increased in cells from diabetic rats compared with those from normal rats.
To examine if the modulator Fru-2,6-P₂ influenced the activity of PFK-1, we have determined the content of this metabolite and the activity of PFK-2. The steady state concentration of Fru-2,6-P₂ is determined by the balance between the activity ratio of the kinase and bisphosphatase of the bifunctional enzyme (PFK-2/FBPase). In liver, Fru-2,6-P₂ is described as a molecule involved in the balance between glycolytic and gluconeogenic pathways, a potent allosteric activator of the glycolytic enzyme PFK-1 and an inhibitor of the gluconeogenic enzyme Fru-1,6-bisphosphatase. Fructose-1,6-bisphosphatase is also present in the submandibular salivary glands of rats, however its activity is very low and does not vary with fasting, suggesting that the gluconeogenic process is not operating in this tissue. Thus, in salivary glands, Fru-2,6-P₂ is probably implicated only in the activation of PFK-1.

The content of the metabolite was reduced by 42.5% for the submandibular gland and by 41.7% for the parotid gland. Reductions in the concentration of Fru-2,6-P₂ were also described in the atria region of the heart, rat small intestine, enterocytes and liver from diabetic animals. However, in macrophage cells, an increased level of this metabolite in diabetes was reported. A reduction in the mean activity of PFK-2 was observed mainly for the active (non-phosphorylated) form in submandibular (44.5%) and in parotid glands (53.4%). It has been pointed out that the reduced activity of PFK-1 observed in some tissues of diabetic animals was due to the reduction in the metabolite Fru-2,6-P₂. On the other hand, in enterocytes isolated from diabetic rats, there was a significant decrease in the level of Fru-2,6-P₂, but not in the activity of PFK-1. In our work both a decrease in Fru-2,6-P₂ and in the activity of PFK-2 in the diabetic animals was not accompanied by a reduction in the activity of PFK-1, suggesting that the diabetic state (decrease of insulin, hyperglicemia) reduces the activity of PFK-2 and the content of Fru-2,6-P₂, but not the activity of PFK-1 in salivary gland of diabetic rats. In contrast, we have found higher specific activity of PFK-1 for the submandibular gland when compared with that of the control animals, and no difference in the parotid gland between experimental and control animals. In view of these results, we are led to conclude that the activation of PFK-1 in the submandibular salivary gland of diabetic rats was not due to the metabolite Fru-2,6-P₂, but to another factor not known so far.

The results of the present study on the state of phosphorylation for the enzyme PFK-1 of the submandibular and parotid salivary glands of control animals are within the values reported in the literature for the liver and muscle. Working with skeletal muscle from mice, Bazaes et al. (1982) reported higher phosphate content and about 30% lower PFK-1 activity in diabetic mice than in control animals. In the present report the values obtained for phosphate content in submandibular salivary glands from diabetic animals were higher than those of the control animals, and the specific activity was also higher than in control animals. In previous papers we have found that the specific activity of PFK-1 in the submandibular gland from rats injected with isoproterenol and killed 12 hours after the injection was higher than in control animals, while the state of phosphorylation of the enzyme was also higher than in control animals. Increasing the state of phosphorylation, we have found a reduction in the activity of PFK-1 in submandibular gland from rats injected with three doses of isoproterenol.

The results of this and of previous investigations have led us to suggest that for the salivary glands, the phosphorylation of PFK-1 has dual activation. Initially, the activity of the enzyme is activated by an increased phosphorylation state. However, a higher state of phosphorylation inhibits the enzyme activity.

CONCLUSION

In conclusion, the increase in the activity of PFK-1 observed in the salivary gland of rats with streptozotocin-induced diabetes was not due to its modulator fructose-2,6-bisphosphate.

ACKNOWLEDGEMENTS

This work was supported by the “Fundação de Amparo à Pesquisa do Estado de São Paulo” (FAPESP 98/07169-9 and 99/03768-8).

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Received for publication on Aug 17, 2005
Sent for alterations on Oct 07, 2005
Accepted for publication on Mar 07, 2006