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Influence of streptozotocin-induced diabetes on hexokinase activity of rat salivary glands

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The influence of diabetes on the enzyme hexokinase (HK) was examined in the salivary glands of rats. Diabetes was induced by an intraperitoneal injection of streptozotocin (60 mg/Kg body weight) in overnight fasted rats (180-200 g). The animals were killed 48 hours and 30 days after the induction of diabetes and the submandibular and parotid salivary glands extracted for use. Hyperglycemia was evaluated by determining the blood sugar. The area occupied by each intralobular component, acini, ducts, total parenchyma and stroma was measured, and no differences were observed compared with control. In the soluble fraction of the submandibular gland, no difference in the specific activity of HK was observed, between the diabetic and control animals, however, the activity per gland and per g of tissue showed lower values than control. The specific activity of the bound form was reduced in the diabetic gland. The results obtained for the parotid gland were different from the submandibular. The specific activity of both the soluble and bound forms were increased in the diabetic animals. The DEAE-cellulose column chromatography of the soluble and bound forms of the enzyme from both glands showed a first peak appearing during the washing of the column and two other peaks were eluted by the gradient. Thus, three isoenzymes in the submandibular and parotid salivary glands for the control and diabetic rats have been found.

Key words: Hexokinase, Salivary glands, Diabetic rats, Isoenzymes.

The metabolism of glucose, specially through glycolysis, is of major significance in all living organisms. Phosphorylation of glucose is a fundamental process in mammalian metabolism. After the transport of glucose into cells, the first step in its metabolism is phos-

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phorylation to glucose-6-phosphate, catalyzed by a family of enzymes known as hexokinases (HK). Four isoenzymes of HK have been described in mammalian tissues. HK binds specifically to the outer mitochondrial membrane, and the binding of HK isozymes to mitochondria has been considered a potential mechanism for the regulation of glucose metabolism (6, 33).

It has been reported that insulin induces increased transcription of type II HK but has no effect on expression of the type I HK (25). In skeletal muscle of rats the activity of the enzyme HK decreases in the diabetic animals compared to control rats (11, 12). They have shown that the decreased activity results from a decreased synthesis coupled to increased degradation of the HK II in the diabetic animals. In submandibular salivary glands it was reported that the soluble HK activity represents about 53 % of the activity of this enzyme (19), and that in diabetic rats it decreased by 35 % (21) and 21% (18) compared to the control.

Considering that all functions played by cells of the salivary glands are energy- dependent, and that the regulation of HK play important role in the carbohydrate metabolism, we decided to examine the activity, distribution and isoenzymes of HK in the submandibular and parotid salivary glands of streptozotocin-induced diabetic rats 48 hours and 30 days after the induction

Material and Methods

Animals.– Seventy two male Wistar rats (180-200 g) bred in the animal house from our laboratory were housed individually in plastic cages with free access to water and food. The animals were randomly divided into control and diabetic groups. Diabetes was induced by a single intraperitoneal injection of streptozotocin (STZ) (Sigma Co) (60 mg/Kg body weight) dissolved in 0.01 M citrate buffer, pH 4.5, in overnight fasted rats. The control animals received only citrate buffer. The rats were killed 48 hours and 30 days after the induction of diabetes. Animals care was performed following the guidelines of the "Colégio Brasileiro de Experimentação Animal" (COBEA).

Morphological analysis of salivary glands.– Six rats were randomly divided into three groups: control, 48 hours and 30 days after STZ injection. The animals were anesthetized with chloral hydrate (30 mg/ 100 g of body weight) and perfused with 0.9% NaCl and 4% paraformaldehyde solutions. Both, the parotid and submandibular glands were removed and post-fixed in 4 % paraformaldehyde solution at 4 °C for 6 hours, followed by cryoprotection in 30 % sucrose dissolved in 0.1 M phosphate buffer, pH 7.4 at 4 °C for at least 24 hours. After O.C.T embedding (Baxter), the glands were frozen and cut on a cryostat (Reichert-Jung, Germany). The 7 µm longitudinal sections were placed on gelatin-coated slides (Sigma), heated for 1 hour at 37 °C and kept at –20 °C until utilization. The histological sections were stained with hematoxylin and eosin for the morphometric analysis. The area occupied by each of the main components within the lobules of the glands (acini, ducts, total parenchyma or stroma) was measured using the Image Pro Plus software, version 3.0. At least 24 photographs, each comprising

an area of 18.6 mm^2 , were analyzed for each group.

Tissue preparation and biochemical analysis.– The killing of the animals occurred always between 9:00 and 11:00 a.m. to minimize circadian variations. The salivary glands were removed, trimmed from the adherent tissue and frozen between aluminum tongs previously cooled in dry ice and stored at –80 °C until they were analyzed.

Blood glucose was monitored using the blood from the tail vein by the modified method of Somogy (22). Animals were considered diabetic with blood glucose levels equal or exceeding 19.5 mmol/L.

The distribution of soluble and bound HK between the cytosolic and particulate fractions was determined according to the method described elsewhere (18). Enzyme activities were assayed as described (10). One unit of enzyme activity corresponds to the amount of enzyme that converts 1 µmol of substrate per min; specific activity is expressed in U/mg protein. The multiple isoforms of HK in soluble and bound fractions were separated by DEAE cellulose column chromatography (16) and activity determinated. Protein was assayed by the folin-phenol reagent (20), using bovine serum albumin as standard.

Statistical analysis.– Statistical significance of the morphometric data was determined by analysis of variance with the process of multiple comparison of Tukey. For the data on enzyme activity it was used the Student's *t* test comparing the diabetic and control rats. The level of significance was set at $p < 0.05$.

Results

The diabetic animals lost weight along the experimental period (initial weight 215.1 ± 28.0 g *vs.* final weight 180.6 ± 24.6 g), while the control rats gained weight (initial weight 192.0 ± 27.5 g *vs.* final weight 261.3 ± 45.6 g). It was also seen that although the mean weight of the submandibular gland in diabetic animals was lower than in control rats (121.7 ± 10.2 mg *vs.* 183.1 ± 2.74 mg, $p < 0.05$), the relative glandular weight showed no difference between diabetic and control rats (0.070 ± 0.007 *vs* 0.067 ± 0.005). On the other hand, although the mean weight of the parotid gland did not differ from the control (93.8 ± 10.9 mg *vs.* 115.5 ± 8.2 mg), the calculated relative glandular weight showed higher value than the control (0.030 ± 0.007 *vs.* 0.060 ± 0.001, $p<0.05$).

No significant differences were observed in the morphometric analysis of parotid and submandibular glands between control, 48 hours and 30 days after STZ injection. Table I shows the data obtained from animals killed 48 hours and 30 days after STZ administration. No difference was observed in HK distribution between control and the treated group after 48 hour of administration.

Thirty days after diabetes induction, the results showed that values of submandibular gland HK activity, both in soluble and bound fractions, were lower in diabetic animals than in control rats. Contrarily, the values of soluble and bound HK activity in the parotid gland were increased in diabetic group as compared to the control group.

The DEAE cellulose elution patterns of the soluble and bound forms of HK for both, the submandibular and parotid

Table I. *Activity of the soluble and mitochondrial bound fractions of hexokinase enzyme in the submandibular and parotid salivary glands of STZ-induced diabetic rats and control animals killed 48 hours and 30 days after STZ administration.*
In parenthesis, number of animals. *p < 0.05 *vs.* control group (Student's *t* test).

Groups	48 hours		30 days	
	Control	Diabetics	Control	Diabetics
	Submandibular gland			
Soluble				
mU/gland	177.2 ± 20.3 (6)	193.8 ± 25.0 (7)	134.0 ± 28.0 (8)	79.0 ± 21.0 (8)*
mU/mg prot	15.2 ± 2.2 (6)	17.4 ± 3.4 (7)	17.0± 5.5 (8)	17.4 ± 5.4 (8)
Bound				
mU/gland	2.1 ± 0.4 (6)	2.3 ± 0.5 (7)	12.0 ± 3.0 (8)	8.0 ± 1.0 (8)*
mU/mg prot	78.2 ± 26.1 (6)	94.3 ± 31.2 (7)	91.6 ± 13.7 (8)	23.5 ± 7.6 (8)*
	Parotid gland			
Soluble				
mU/gland	28.8 ± 11.0 (7)	22.1 ± 6.8 (7)	42.0 ± 6.0 (9)	51.0 ± 11.0 (10)*
mU/mg prot	7.5 ± 2.5 (7)	9.1 ± 2.7 (7)	9.2 ± 2.9 (9)	12.3 ± 3.3 (10)*
Bound				
mU/gland	12.7 ± 1.7 (7)	12.2 ± 1.5 (7)	9.0 ± 2.0 (9)	7.0 ± 1.0 (10)
mU/mg prot	16.9 ± 3.8 (7)	19.0 ± 5.0 (7)	8.0 ± 2.2 (9)	14.8 ± 2.0 (10)*

glands, are shown in Fig. 1. In interpreting these results an explanation is necessary. After loading the column with the enzyme and while it was washed, we have saved the washing fractions and determined the absorbance at 280 nm and HK activity. In all experiments with the submandibular gland we obtained three peaks of enzyme activity for soluble and bound fraction in both control and diabetic rats, the first peak (A) belong to the washing fractions, while the other two peaks (B and C) were eluted with a KCl gradient. The same pattern was observed in the parotid gland (Fig. 1B).

Discussion

The results on relative glandular weight (RGW) indicated that no variation was observed for submandibular salivary gland. However the higher RGW value found for parotid suggests that enlargement of this gland had apparently occurred, a fact that has already been reported (4). The mean weight of the parotid gland obtained in this study is lower than that reported elsewhere (1), although, it has been demonstrated (31) that the rat parotid gland suffers a diurnal cycle in gland weight, which is related to the nocturnal feeding habits. The reduction in parotid gland weight is due to the release of secretory material from the gland as the rats eat. Thus, being of nocturnal habits, as the animals are submitted to overnight fasting period, the parotid gland show greater weight. In our work we did not fast the animals, consequently the parotid gland was analyzed after nocturnal feeding habit.

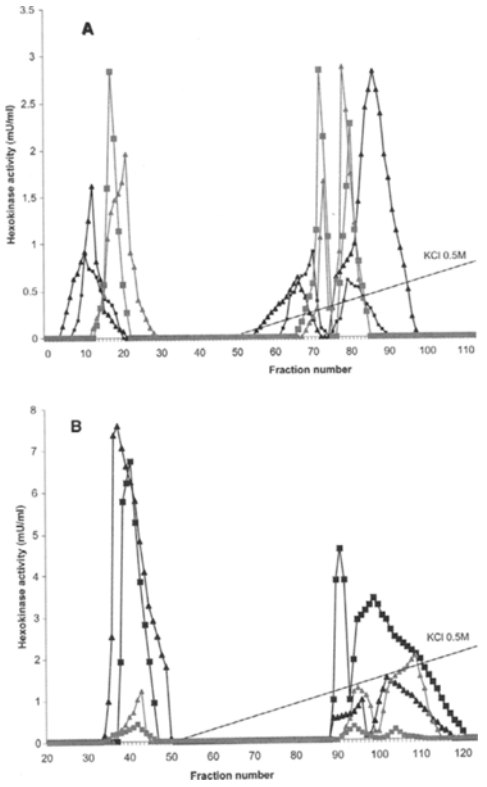

Fig 1. *Chromatographic elution profile of the soluble (black line) and bound (gray line) fraction of hexokinase from the submandibular (A) and parotid (B) glands of STZ-induced diabetic (triangle) and control* rats (square).
The column was developed with linear gradient from 0-0.5 M KCl and fractions of 2 ml were collected and assayed for enzyme activity.

The role of HK in regulating carbohydrate metabolism is well known. HK, the primary enzyme catalyzing the phosphorylation of glucose, is regulated by metabolites (26), its distribution between mitochondria and the soluble compartment (32) and its degree of activation (16).

The data obtained from the rats killed 48 h after STZ administration shows that no differences were observed in the parameters considered between control and experimental.

The specific activity of HK (mU/mg prot) of the soluble fraction of the submandibular gland from rats after 30 days diabetes induction showed no variation, although, the activity per gland disminished about 41 %. There has been reported reductions in the activity of HK of the submandibular gland of diabetic animals of about 35% (21) and 21 % (18). We have also found a disminution in the activity/gland of the bound HK of about 33 %, and of about 74 % in the specific activity.

Different results were obtained for the parotid gland, where an increase in the activity of HK occurred in both, the soluble and bound fractions. The specific activity increased about 34 % and 85 %, respectively, for the soluble and bound forms. The difference found between the submandibular and parotid glands is not a surprise, since both glands presents different metabolic characteristics. In the submandibular gland predominates the anaerobic, while in the parotid gland predominates the aerobic metabolism (23).

Many reports demonstrated that the induction of diabetes results in decreased activities of several enzymes in rat liver, however the HK isoenzymes are reduced most dramatically (14, 17, 30). Notwithstanding the reductions found in liver, it was reported an increase in the activity of HK in macrophages (8), and enterocytes (29) from streptozotocin-induced diabetic rats. In the brain of diabetic rats, administration of insulin caused different responses of mitochondrial and cytosolic HK (24).

It was reported that HK type I is the predominant isoenzyme present in submandibular and parotid salivary glands of rats, although small amounts of HK type II are present (18). Contrary to what these authors have described we have found three peaks of HK activity, which we have called A, B and C. As peaks B and C were eluted by the gradient possibly they are types II and III. The isoenzyme A was present in both glands and in both fractions (soluble and bound) of the control and diabetic rats. Peaks B and C of HK activity of the soluble and bound fractions were also present in the control and diabetic rats. Using ion-exchange chromatography (28) three peaks in human skeletal muscle (peaks S, I and II) in the soluble extract were described. By thermal stability they concluded that peak S was part of peak II. On the other hand, it was demonstrated that thermal inactivation cannot be used as a general and reliable method for distinguishing HK isoenzymes (34). Similarly to what has been found for muscle (28) we have found for the salivary gland a peak of HK activity in the washing fractions. As for as we know, there is no written publication mentioning HK peak activity of the salivary gland in washing fractions and thus we are doing further study to characterize it.

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F. N. NOGUEIRA, M. F. DOS SANTOS y J. NICOLAU. *Influencia de la diabetes sobre la actividad hexoquinasa en glándulas salivales de rata.* J. Physiol. Biochem., **61** (3), 421-428, 2005.

Se estudia la influencia de la diabetes sobre la actividad hexoquinasa (HK) en glándulas salivales de rata. La diabetes se induce con una inyección intraperitoneal de estreptozotocina (60 mg/Kg de peso corporal) en animales mantenidos en ayunas durante una noche (180-200 g). Los animales se sacrificaron 48 h y 30 d tras inducción de la diabetes y se obtuvieron las glándulas submandibulares y las parótidas. La hiperglucemia se valoró determinando el azúcar sanguíneo. El área ocupada por cada componente intralobular, acinos, conductos, parénquima total y estroma no varió entre los animales tratados y control. La actividad específica de la HK (mU/g proteína) en la fracción soluble de la glándula submandibular no varió entre los animales del grupo control y los diabéticos. Sin embargo, la actividad HK referida a g de tejido era menor en diabéticos que en el grupo control. Respecto de la forma ligada, se observó disminución también en la actividad específica en las diabéticas. Los resultados en las glándulas parótidas fueron diferentes que en las submandibulares, ya que se observó incremento de la actividad HK específica, tanto de la forma soluble como de la ligada, en los animales diabéticos. La columna cromatográfica DEAE-celulosa de las formas soluble y ligada de las enzimas de ambas glándulas presentaron la aparición del primer pico durante el lavado de la columna. Otros dos picos fueron eluídos por un gradiente. Por tanto, se han encontrado tres isoenzimas en las glándulas submandibulares y parótidas tanto en los animales control como en los diabéticos.

Palabras clave: Hexoquinasa, Glándulas salivales, Diabetes, Isoenzimas.

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