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Original Communication

Na^+, K^+-ATPase response to neurotensin is altered by Streptozotocin administration

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ABSTRACT

Neurotensin is a basic tridecapeptide which can act as a neuromodulator or a neurotransmitter and binds to a group of receptors. Neurotensin is able to inhibit Na^+, K^+-ATPase activity, an effect blocked by the presence of antagonist SR48692, suggesting the involvement of high affinity neurotensin (NTS1) receptor. Diverse evidences suggest a relationship between neurotensinergic system and glycemia levels. For this reason, potential Na^+, K^+-ATPase regulation by neurotensin in brain membranes obtained from rats turned hyperglycaemic was explored. As a model to produce diabetes mellitus, rats were administered with Streptozotocin (STZ), a specific toxic agent to the pancreatic beta cells. Our findings indicated that Na^+, K^+-ATPase activity in synaptosomal membranes isolated from diabetic rats failed to respond to neurotensin. The treatment decreased the affinity of NTS1 receptor for neurotensin and the expression of Na^+, K^+-ATPase alpha3 subunit in cerebral cortex. The results led us to conclude that STZ administration alters the response of Na^+, K^+-ATPase to neurotensin. Such effect seems to involve a decrease in enzyme alpha3 isoform expression and NTS1 receptor affinity for neurotensin.

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KEYWORDS: Streptozotocin, neurotensin, Na^+, K^+-ATPase activity, alpha 3 isoform, NTS1

ABBREVIATIONS

High affinity neurotensin receptor, NTS1 receptor; low affinity neurotensin receptor, NTS2 receptor; Streptozotocin, STZ; phenylmethyl-sulfonylfluoride, PSMF; sodium dodecyl sulphate, SDS; polyacrylamide gel electrophoresis, PAGE; bovine serum albumin, BSA; National Institutes of Health, NIH

INTRODUCTION

Streptozotocin (STZ) is an antimicrobial substance which has been used as a chemotherapeutic alkylating agent. STZ administration exerts a specific toxic effect to the pancreatic beta cells, which undergo destruction by necrosis [1]. For this reason, STZ is commonly used as a model to study diabetes mellitus.

Neurotensin is a basic tridecapeptide which can act as a neuromodulator or a neurotransmitter and binds to a group of receptors [2, 3]. Two of them, NTS1 and NTS2, are seven transmembrane domain receptors coupled to G proteins, which bind neurotensin with high and low affinity, respectively [3]. Whereas, a third receptor termed NTS3 is an intracellular receptor [4]. The peptide exerts a wide spectrum of actions at both peripheral [5] and central [6, 7] nervous systems.

Diverse evidences suggest a relationship between neurotensinergic system and glycemia levels. Neurotensin induces hyperglucagonemia and hyperglycaemia in rats [8, 9] and dogs [10]. Peptide hyperglycemic effect seems to be due to the stimulation of hepatic glycogenolysis. Neurotensin activates hepatic glycogen phosphorylase and reduces hepatic glycogen content. Phosphorylase activation does not occur *in vitro* and the possible involvement *in vivo* of cathecolamines (or other intermediary substances) has been suggested [11]. The hyperglycemic effect might be mediated by a decrease in insulin and/or an increase in glucagon [8].

Sodium and potassium transport across the membrane depends on an ouabain-sensitive Na^+, K^+-ATPase activity [12]. Synaptosomal membrane Na^+, K^+-ATPase activity is inhibited *in vitro* by neurotensin, suggesting a regulatory action of the peptide on this enzyme activity [13, 14, 15].

Potential Na^+, K^+-ATPase regulation by neurotensin in brain membranes obtained from rats which had been subjected to a hyperglycaemic condition was explored. Neurotensin effect on the activity of synaptosomal membrane Na^+, K^+-ATPase, the expression of this enzyme α3 subunit as well as [^{3}H]-neurotensin binding to membranes after STZ administration were tested.

MATERIALS AND METHODS

Animals and drugs

Adult male Wistar rats weighing 250 g were used. All studies described were conducted in accordance with the Guide for Care and Use of Laboratory Animals provided by the National Institutes of Health (USA). Animals had access to a standard commercial diet and water *ad libitum* and were kept in a room maintained at 25 °C ± 2 °C with a 12 h light/dark cycle. STZ, disodium ATP (grade I, prepared by phosphorylation of adenosine), ouabain, and neurotensin acetate were from Sigma Chemical Co., St. Louis, MO, USA. Peptide solutions in redistilled water were freshly prepared for each experiment. Mouse monoclonal IgG1 anti-Na^+, K^+-ATPase α3 subunit [XVIF9-6-10] was from Affinity Bioreagents Inc. Golden, CO, USA. Secondary antibody anti-mouse IgG, HRP linked whole antibody (from sheep), ECL Western blotting detection reagent and Hyperfilm ECL were from GE Healthcare, U.K. Accutrend glucose test strips were from Roche Diagnostics (Mannheim, Germany). OptiPhase "Hisafe" 3 was from Amersham Biosciences, UK and [^{3}H]-neurotensin, specific activity 3.33 TBq/mmol, was from New England Nuclear, Du Pont, Boston, MA, USA. Levocabastine clorhidrate was kindly provided by Jansen-Cilag, Argentina. Reagents were analytical grade.

STZ administration

Lots of 6 rats were used for each experiment. One half of the rats received (day 0) a single i.p. injection of STZ at a dose of 60 mg/kg body weight to induce diabetes mellitus. The drug was dissolved immediately before use in cold 0.01 M citrate buffer, pH = 4.5 (vehicle). To serve as controls, the other half of the rats was injected i.p. in parallel with the vehicle. Blood glucose levels were estimated using glucose test strips and animals were considered as diabetic if their blood glucose values were above 250 mg/dl, which occurred at day 4 after STZ administration in all STZ-injected rats. It was observed that STZ treated rats progressively lost weight, while control animals gained weight, in agreement with previous results [16].

Preparation of synaptosomal membrane fractions

Synaptosomal membranes from rat cerebral cortex were isolated by differential and sucrose gradient centrifugation as previously described [17]. In each experiment, lots of four or eight rats were processed. Half of the animals received STZ and the other half received the vehicle. Cerebral cortices were homogenized and subjected to differential centrifugation to separate the crude mitochondrial pellet, which was resuspended in redistilled water for the osmotic shock. A pellet containing the synaptosomal membranes was separated by centrifugation (20,000 x *g* for 30 min), then resuspended in 0.32 M sucrose, layered on top of a sucrose gradient (0.8; 0.9; 1.0, and 1.2 M) and ultracentrifuged at 50,000 x *g* for two hours. The fraction at the level of 1.0 M sucrose was collected and spun down (100,000 x *g* for 30 min) to obtain the synaptosomal membrane fraction. Pellets were stored at -70 °C and, prior to enzyme assay, resuspended by brief homogenization in

redistilled water, stored frozen and used for three weeks without appreciable change in enzyme activities.

Enzyme assays

ATPase activity was measured as described by Albers *et al*. [18]. Total ATPase activity was assayed in a medium containing 100 mM NaCl, 20 mM KCl, 3 mM $MgCl_2$, 0.20 M Tris-HCl buffer (pH 7.4) and 4 mM ATP. Mg^{2+}-ATPase activity was determined in a similar medium with no added Na^+ and K^+ and containing 1 mM ouabain. The difference between activities was taken to correspond to Na^+, K^+-ATPase. Before performing ATPase assay, samples of synaptosomal membrane fractions were preincubated with 0.20 M Tris-HCl buffer (pH = 7.4) or 1.0 x 10^{-8} M to 1.0 x 10^{-6} M neurotensin at 37 ºC for 10 min; incubation volume (µl) was 35:5 for buffer and membranes. Aliquots of preincubated fractions (3 µl) were distributed in two series of microtubes containing the respective medium (40 µl) for the assay of total- and Mg^{2+}-ATPase activities, and incubated at 37 ºC for 30 min. The reaction was stopped with 30% trichloroacetic acid solution. ATPase activity was monitored by colorimetric determination of orthophosphate released [19]. In all enzyme assays, tubes containing enzyme preparations and assay media maintained at 0 ºC throughout the incubation period were used as blanks.

Membrane fractions for western blot assay

For Western blot analyses, cerebral cortex was harvested from each rat and processed separately; tissues were homogenized in a Teflon glass Potter Elvehjem type at 10% w/v in ice-cold 0.32 M sucrose (pH 7.4) containing protease inhibitor cocktail (1 mg/ml aprotinine, leupeptine, pepstatine and PSMF) and 0.5 mM EDTA. Samples were then centrifuged at 1,000 x *g* for 10 min at 4 ºC to remove nuclei and cell debris. Supernatants were centrifuged at 10,000 x *g* for 20 min to obtain the sediment containing the crude membranes which was stored at -20 ºC until use. Pellets were resuspended in 0.32 M sucrose (pH 7.4) containing protease inhibitor cocktail and 0.5 mM EDTA, and Western blotting was performed according to the method described by Kalluri *et al*. [20].

Western blot analysis

The content of protein in membranes was determined before Western blot analysis to ensure equal loading in lanes. Membrane sample aliquots containing 80 µg protein were mixed with Laemmli buffer for sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). Samples were denatured by boiling for 5 min in Laemmli sample buffer containing 5% of β-mercaptoethanol [21] and proteins were separated by the use of 7.5% SDS-PAGE under a reducing condition. Polypeptides were transferred to a nitrocellulose membrane. The membrane was washed with a blocking solution containing 50 mM Tris base, 150 mM sodium chloride, 0.15% (v/v) Tween 20 and 5% low fat milk (TBS-T). The blot was then incubated with anti-Na^+, K^+-ATPase α3 antibody at the concentration of 1 µg per ml. The blot was washed several times with TBS-T containing 0.15% Tween 20 and was then incubated with the secondary antibody peroxidase-conjugated antimouse IgG, diluted by 1:5000. The immunoreactive bands were visualized using ECL and hyperfilm. Relative changes in polypeptide levels were evaluated by measuring the intensity of the immunoreactive bands using Image J 1.38 X (NIH) software (2007).

[^{3}H]-Neurotensin binding

Lots of 6 rats were used for each experiment. Half of the animals received STZ and the other half received the vehicle as indicated. Rat cerebral cortices were homogenized in 10 volumes of buffer A (5.0 mM Tris-HCl, 1 mM EDTA, pH 7.4 at 4 °C). The homogenate was centrifuged at 40,000 x *g* for 20 min at 4 °C, the supernatant removed, and the pellet resuspended in buffer A (10.0 mg/ml) and recentrifuged a total of three times. The final pellet was then resuspended in buffer B (50 mM Tris-HCl containing 0.2% BSA, 0.1 nM 1,10-phenanthroline, pH 7.4).

All incubations were performed at 25 °C in buffer B with 0.06 to 10 nM [^{3}H]-neurotensin, 1.0 µM levocabastine (a NTS2 receptor antagonist), with or without 1.0 µM unlabeled neurotensin to determine nonspecific binding. The final reaction volume was 500 µl. The final reaction volume was 500 µl. Twenty minutes after incubation the reaction was terminated by the addition of ice-cold buffer C (50 mM Tris-HCl, pH 7.4) followed

by rapid filtration under reduced pressure through Whatman GF/B glass fibre filters presoaked in ice-cold buffer C containing 0.3% poly-ethylenimine. Filters were then rinsed three times with 5 ml of ice-cold buffer C. Filters were transferred to vials and after addition of 10 ml OptiPhase "Hisafe"3, radioactivity was quantified in a Beckman Coulter-LS 6500 scintillation counter with 64% efficiency. Specific binding was calculated by subtracting binding found in the presence of 1.0 µM unlabelled neurotensin. Non-specific binding accounted for less than 10% of total membrane-bound radioactivity [22].

The whole experiment (administration of drugs, tissue harvesting, membrane preparation and binding assay in triplicate) was carried out in at least three different occasions.

Protein measurement

Protein content in membrane fractions was determined by the method of Lowry *et al*. [23], using bovine serum albumin as standard.

Statistical analysis

Data for Na^+, K^+-ATPase activity were expressed as µmol released inorganic phosphate per mg protein per hour (mean ± SD).

Data for [^{3}H]-neurotensin binding were expressed in nmolar and fmol per mg protein for Kd and Bmax, respectively. Values are presented as means ± SD from three assays carried out with three membrane preparations obtained in different occasions.

In each Western blot assay, paired samples (treated and control) were developed and quantified. For each gel, the comparison between bands was done as follows: the data for optical density were expressed as the ratio between treated versus control values. To calculate the ratios, data were normalized by assigning a value of 1.00 to optical density for the control. The ratio indicates how much a given subunit is expressed in the treated in comparison with the control. Data are presented as mean values (± SD) from *n* experiments; *n* indicates the number of gels processed with different membrane samples isolated from at least two or three animals processed in different occasions.

Statistical significance of differences was determined by one way analysis of variance (ANOVA) followed by Bonferroni Comparison test or by one-sample Student's *t*-test. A probability level indicative of $p < 0.05$ was considered significant.

For saturation assays, non-linear regression of the data were processed using the program Graph Pad Prism 4.0 (2003). Scatchard transformation of the data was employed to show whether more than one receptor population was operative.

RESULTS

ATPase activities were assayed in cerebral cortex synaptosomal membranes isolated from rats injected with STZ and the vehicle. Basal Na^+, K^+-ATPase activity in control and STZ membranes was roughly 29 and 27 µmol Pi. mg. $prot^{-1}$. h^{-1}, respectively. The difference between values was not statistically significant (Fig. 1A). In control membranes, the addition of 1.0 x 10^{-8} M-1.0 x 10^{-6} M neurotensin dose-dependently decreased Na^+, K^+-ATPase activity, leading to 36% inhibition with the latter concentration. The effect of STZ administration was tested on Na^+, K^+-ATPase response to neurotensin, to observe that the peptide at 1.0 x 10^{-8} M and 1.0 x 10^{-7} M concentration failed to modify this enzyme activity. At variance, with 1.0 x 10^{-6} M neurotensin a trend to enhance Na^+, K^+-ATPase activity was recorded (Fig. 1A).

Basal synaptosomal membrane Mg^{2+}-ATPase activity was roughly 14 and 13 µmol Pi. mg. $prot^{-1}$. h^{-1} for control and STZ membranes, respectively. These values remained unaltered in all conditions tested (Fig. 1B).

Previous studies indicate that neurotensin is able to inhibit Na^+, K^+-ATPase activity and that this effect is blocked by the presence of antagonist SR48692, suggesting the involvement of NTS1 receptor [13]. Therefore, it was of interest to analyze whether the lack of neurotensin effect on Na^+, K^+-ATPase after STZ was due to an alteration of peptide binding to its receptor. Preliminary assays of [^{3}H]-neurotensin binding to membranes isolated from STZ rats differed from that recorded in membranes isolated from vehicle treated rats (data not shown). In order to

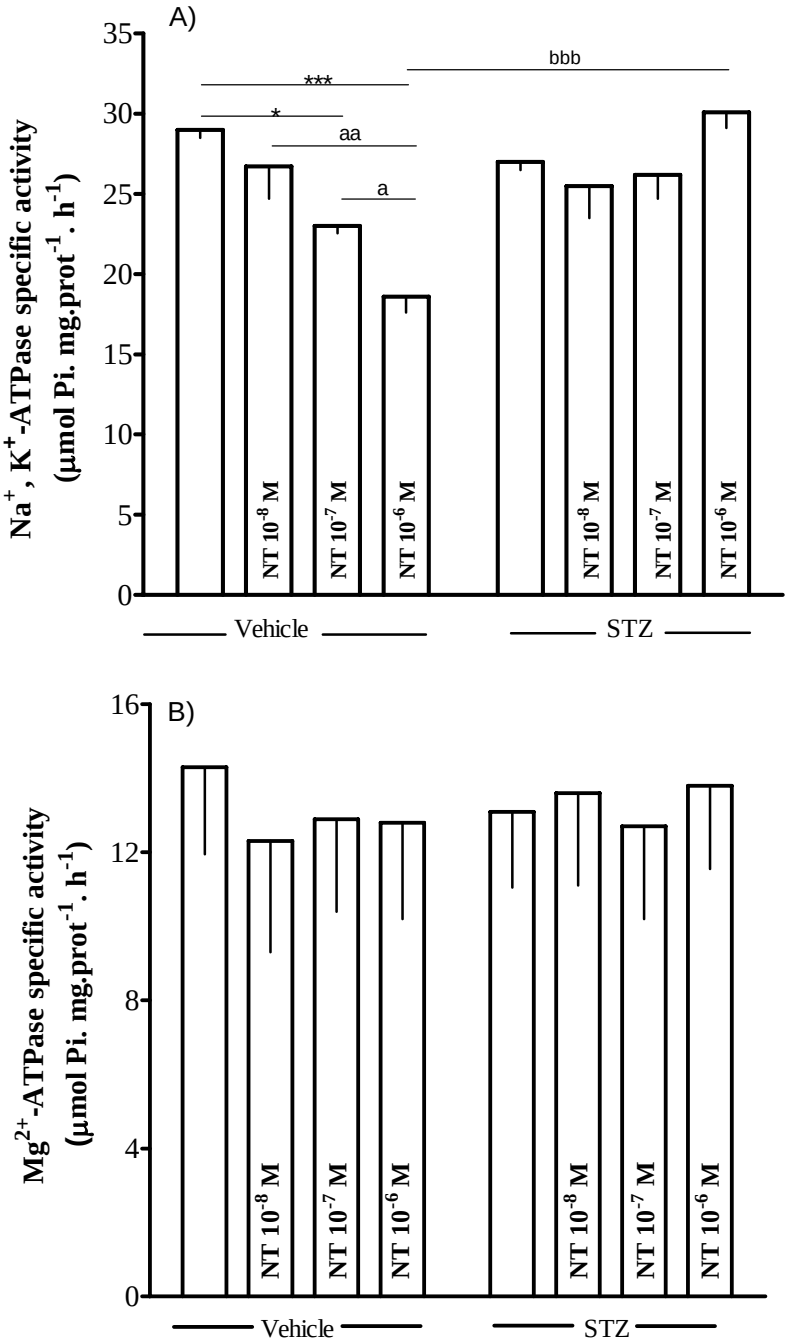

Fig. 1. Neurotensin effect on ATPase activities in rat cerebral cortex after streptozotocin (STZ) administration. Rats received i.p. a single dose of STZ or vehicle and four days later, were decapitated, tissues harvested and subjected to subcellular fractionation to obtain synaptosomal membranes. Membrane samples were preincubated in the absence or presence of 3.5 x 10^{-8} M-3.5 x 10^{-6} M neurotensin (NT) and assayed for ATPase activities. Results are expressed as specific enzyme activity. SD of four experiments performed per triplicate is indicated within the bars. (A) Na^+, K^+- ATPase activity; (B) Mg^{2+}-ATPase activity. $^*p < 0.05$; $^{***}p < 0.001$ with respect to the values recorded without neurotensin, by one way ANOVA followed by Bonferroni Comparison test. $^{aa}p < 0.01$; $^{a}p < 0.05$ between neurotensin concentrations in vehicle treated rats, by one way ANOVA followed by Bonferroni Comparison test. $^{bbb}p < 0.001$ with respect to the values recorded with 10^{-6} M neurotensin in vehicle and STZ treated rats, by one way ANOVA followed by Bonferroni Comparison test.

determine whether binding changes were due to modifications in affinity and/or site number, [^{3}H]-neurotensin binding was studied at variable ligand concentrations. Saturation values were attained with 10 nM ligand in membranes obtained either from rats injected with vehicle or with STZ.

Scatchard analysis of [^{3}H]-neurotensin binding data recorded at equilibrium were carried out. After STZ treatment a significant increase of 47% for Kd value was observed whereas no change in receptor site number was recorded. Hill number was close to unity and remained unaltered by STZ treatment (Fig. 2).

Na^+, K^+-ATPase α3 isoform expression was assayed in cerebral cortex membranes isolated from control and diabetic STZ rats. Western blot analyses indicated that α3 isoform expression was 17% lower in STZ treated rats *versus* vehicle treated animals (Fig. 3).

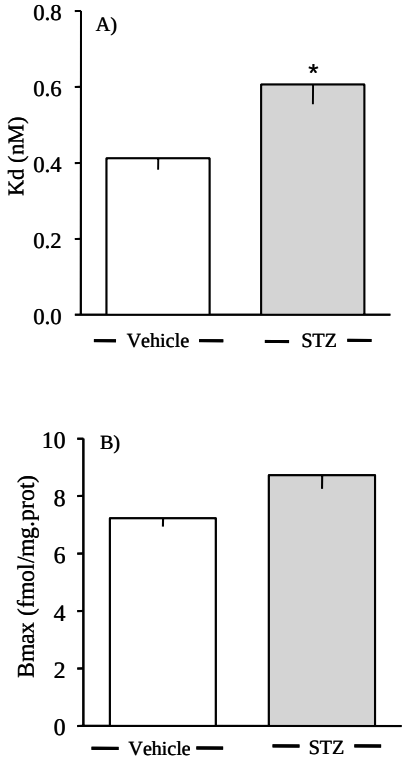

Fig. 2. Kinetic constants for [^{3}H]-neurotensin binding to rat cerebral cortex membranes after STZ administration. Rats received i.p. a single dose of STZ or vehicle and four days later, were decapitated, tissues harvested and subjected to subcellular fractionation to obtain membrane fractions for binding assays. Results presented are mean values (± SD) from four assays carried out in membranes obtained in at least three different occasions. **p* < 0.05, STZ treatment *versus* vehicle treatment, by Student´s *t*-test.

DISCUSSION

In previous work, the ability of neurotensin to inhibit synaptosomal membrane Na^+, K^+-ATPase activity was described, an effect which involves NTS1 receptor. Present findings indicated that neuronal Na^+, K^+-ATPase activity in membranes isolated from diabetic STZ rats failed to respond to neurotensin. The treatment decreased the

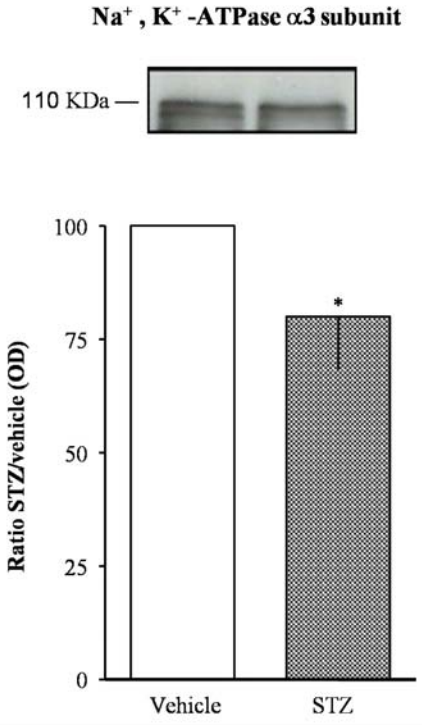

Fig. 3. Western blot analysis of Na^+, K^+-ATPase α3 subunit in rat cerebral cortex membranes after STZ administration. Rats received i.p. a single dose of STZ or vehicle and four days later, were decapitated, tissues harvested and subjected to subcellular fractionation to obtain membrane fractions for Western blot. Results shown are immunoblots from a single representative experiment. C, control; STZ, treated with STZ. The histogram shows the results expressed as the ratio between treated *versus* control values for optical density (OD). Data presented are mean values (± SD) of 9 assays carried out in membrane preparations obtained in at least 4 different occasions. **p* < 0.05, STZ *versus* vehicle, by one-sample Student's *t*-test.

affinity of NTS1 receptor for neurotensin and the expression of Na^+, K^+-ATPase alpha3 subunit in cerebral cortex.

Na^+, K^+-ATPase (sodium- and potassium-activated adenosine 5'-triphosphatase), discovered by Skou [24], also called Na^+ pump or Na^+, K^+-pump, is an ubiquitous membrane transport protein in mammalian cells. It regulates K^+ entry with Na^+ exit from the neuron, and therefore is responsible for Na^+/K^+ equilibrium maintenance through neuronal membranes. This mechanism is essential in the normal cell cycle, cell volume regulation, osmotic balance, nervous system differentiation as well as in the maintenance and restoration of the resting membrane potential in excitable cells [25]. Neurotensin is able to inhibit synaptosomal membrane Na^+, K^+-ATPase activity, an effect which most likely involves high affinity neurotensin (NTS1) receptor because it is entirely blocked by antagonist SR 48692 [13, 14, 15].

Several studies focussed on the potential association of diabetic state with Na^+, K^+-ATPase activity changes. The brain cortex is more susceptible to the hyperglycemic insult than other areas of the brain [26]. Therefore the present study was carried out in cerebral cortex.

After STZ administration, hyperglycemia is enhanced. Four phases were described between 4 days and 8 weeks, when the permanent diabetic hyperglycemic phase occurs [27]. The hyperglycemia induces CNS damage, most likely due to alteration of synaptic membrane integrity because of free radicals overproduction [28].

In the diabetic state induced by STZ in rats, statistically significant decreases (15% to 37%) in enzyme activity are observed at 2 to 8 weeks after drug administration [29, 30, 31]. The reduction of Na^+, K^+-ATPase activity at 8 weeks after STZ administration is accompanied by changes in membrane fluidity [32]. The present study was carried out after a short exposure time to STZ (4-day STZ treatment), when only a trend (-7%) to decrease Na^+, K^+-ATPase activity was observed. However, at that time, the abrogation of Na^+, K^+-ATPase response to neurotensin was recorded, indicating that this change occurs at a stage which is previous to enzyme activity decrease.

Present findings indicated that neuronal Na^+, K^+-ATPase activity in membranes isolated from diabetic STZ rats was not inhibited by neurotensin. In order to know whether this effect is attributable to an alteration of NTS1 receptor, [^{3}H]-neurotensin binding to cerebral cortex membranes isolated from STZ rats was assayed. An increase of 47% in Kd value was observed whereas no change in receptor site number was recorded. Therefore, a reduction in receptor affinity for the peptide may offer an explanation for the lack of inhibitory effect of neurotensin on Na^+, K^+-ATPase activity after STZ administration.

Insulin is one of the many hormones which regulate Na^+, K^+-ATPase activity, and changes in this enzyme have been implicated in diverse diabetic complications, including impaired nerve conduction [33]. Insulin stimulates the activity of membrane-bound ATPase isolated from rat brain [34], an effect which depends on the experimental assay conditions and the integrity of some cell membranes [35]. Maximal insulin effect occurs at relatively high hormone concentrations, most likely involving the high affinity enzyme subunit [36].

At 4 weeks after treatment with STZ, 20% decreases of both alpha3 subunit expression and Na^+, K^+-ATPase activity are observed [29]. At an early stage (4 days) after STZ administration, a decrease in the expression of Na^+, K^+-ATPase alpha3 subunit was recorded whereas enzyme activity was only slightly diminished (present results). These findings suggest that STZ may alter first *per se* subunit expression. At longer periods, the lower enzyme activity may be a consequence of the hyperglycemic diabetic state.

In summary, at an early stage after STZ administration, though synaptosomal membrane Na^+, K^+-ATPase activity was only slightly diminished, an alteration of this enzyme properties seem to occur, because it became unable to respond to neurotensin. This effect is attributable to decrease of $\alpha 3$ Na^+, K^+-ATPase subunit expression and/or to the decrease of NTS1 receptor affinity to peptide neurotensin.

CONCLUSION

STZ administration to rats alters the response of Na^+, K^+-ATPase to neurotensin. Such effect seems to involve a decrease in enzyme alpha3 subunit expression and NTS1 receptor affinity for neurotensin.

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