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Prolyl, cystyl and pyroglutamyl peptidase activities in the hippocampus and hypothalamus of streptozotocin-induced diabetic rats

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ABSTRACT

Prolyl, cystyl and pyroglutamyl peptidases are emerging targets for diabetes and cognitive deficit therapies. The present study is focused on the influence of diabetes mellitus induced by streptozotocin on levels of representative hydrolytic activities of these enzymes in the rat hypothalamus and hippocampus. Streptozotocin-diabetic rats presented about 348 mg glucose/dL blood, and a slightly increased hematocrit and plasma osmolality. The activities of soluble and membrane-bound dipeptidyl-peptidase IV, and soluble cystyl aminopeptidase did not differ between diabetic and control rats in both brain areas. Hippocampal soluble prolyl oligopeptidase presented similar activities between diabetic and controls. Increased activities in diabetics were observed for soluble prolyl oligopeptidase (1.78-fold) and membrane-bound cystyl aminopeptidase (2.55-fold) in the hypothalamus, and for membrane-bound cystyl aminopeptidase (5.14-fold) in the hippocampus. In both brain areas, the activities of membrane-bound and soluble pyroglutamyl aminopeptidase were slightly lower (<0.7-fold) in diabetics. All modifications (except hematocrit) observed in streptozotocin-treated rats were mitigated by the administration of insulin. Glucose and/or insulin were shown to alter *in vitro* the hypothalamic activities of soluble pyroglutamyl aminopeptidase and prolyl oligopeptidase, as well as membrane-bound cystyl aminopeptidase. These data provide the first evidence that diabetes mellitus generates direct and indirect effects on the activity levels of brain peptidases. The implied regional control of regulatory peptide activity by these peptidases suggests novel potential approaches to understand certain disruptions on mediator and modulatory functions in diabetes mellitus.

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Abbreviations: ADAM, A disintegrin and metalloproteinase; APM, aminopeptidase M; AVP, vasopressin; BSA, Bovine serum albumin; CAP, cystinyl or cystyl aminopeptidase; CSF, cerebrospinal fluid; DPPIV, dipeptidyl-peptidase IV; DTT, DL-dithiothreitol; EDTA, ethylenediamine tetraacetic-acid; GHRH, growth hormone-releasing hormone; GLP-1, glucagon-like peptide-1; HC, hippocampus; HT, hypothalamus; IRAP, insulin-regulated aminopeptidase; LAP, leucyl aminopeptidase; LDH, lactate dehydrogenase; LHRH, luteinizing hormone releasing hormone; M, solubilized membrane-bound form; NADH, nicotinamide adenine dinucleotide reduced form disodium salt; NPY, neuropeptide Y; OT, oxytocin; PAP, pyroglutamyl aminopeptidase; PMSF, phenyl methyl sulphonyl fluoride; POP, prolyl oligopeptidase; S, soluble form; STZ, streptozotocin; TRH, thyrotrophin-releasing hormone

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1. Introduction

Certain prolyl, cystyl and pyroglutamyl peptidases, which have been implicated in diabetes, and cognitive deficits [9,25,60], overlap their broad hydrolytic activities on vasopressin (AVP), oxytocin (OT), neuropeptide Y (NPY) and pyroglutamyl peptides such as thyrotrophin-releasing hormone (TRH), luteinizing hormone releasing hormone (LHRH), neurotensin and bombesin [8,18,44]. There is a body of evidences that the hydrolytic activities of these peptidases can contribute to the turnover of these peptides in the hypothalamus and hippocampus [3,18,25,39,44,55–57,67]. In this turn, these peptides have their balance affected by diabetes mellitus in areas of the central nervous system (CNS) that regulate energy homeostasis and memory, such as the hypothalamus and hippocampus [17,21,40,41,48].

AVP and OT neurons in the hypothalamic paraventricular and supraoptic nuclei of streptozotocin (STZ)-diabetic rats have been found to be hypertrophied with concurrent ultrastructural changes [21] and upregulated AVP and OT immunoexpression [41], which may be indicative of hyperactivity and degeneration. Suppressed gonadotropins have also been reported in adverse metabolic conditions, such as undernutrition and diabetes mellitus [17]. The desensitization of TRH neuronal responses to subsequent changes in glucose concentrations *in vitro* has also been observed in the STZ-diabetic rats [40]. Reduced neurotensin concentrations have been found in the hypothalamus of the Aston diabetic mice, which might contribute to hyperphagia and decreased energy expenditure, two major defects that contribute to diabetes in the Aston syndrome [66]. In the fasted and the STZ-induced diabetic male rat, hypothalamic growth hormone-releasing hormone (GHRH) mRNA levels, and pulsatile growth hormone release are decreased. These changes are believed to be due to a rise in the hypothalamic NPY that inhibits GHRH expression [48].

Prolyl oligopeptidase (POP) (EC 3.4.21.26) is involved in the catabolism of AVP, TRH, LHRH [8], OT and neurotensin [60]. Dipeptidyl-peptidase IV (DPPIV) (the canonical EC 3.4.14.5, identical to the cell membrane serine protease CD26) cleaves N-terminal dipeptides from polypeptides when the second residue is a proline or an alanine, such as substance P [4] and the most important mediators of energy homeostasis, i.e. NPY [26] and glucagon-like peptide-1 (GLP-1) [22], as well generates the form of peptide YY, PYY 3-36, which selectively binds to Y2 receptors and might also bind to other NPY receptors depending on several factors, including range of concentration [29]. Cystyl aminopeptidase (CAP) activity has been attributed to leucyl aminopeptidase (LAP/oxytocinase) (EC 3.4.11.1) [62] and is also referred to as insulin-regulated aminopeptidase (IRAP) and vesicle protein of 165 kDa (vp165) [61], which is able to degrade AVP, OT, LHRH, Met-enkephalin and dynorphin A (1–8) [44]. LAP/IRAP has also been identified as the specific high-affinity binding site for the hexapeptide angiotensin IV [15]. The pyroglutamyl aminopeptidase (PAP) type I (EC 3.4.19.3) displays a broad pyroglutamyl substrate specificity, which includes the cleavage of TRH, LHRH, neurotensin and bombesin [18].

The degradation or processing of AVP, OT, NPY and pyroglutamyl peptides by brain peptidases might alter the functionality of these peptides. At this time, very little is known about brain peptidases with this hydrolytic ability in animal models of diabetes mellitus. In the present work, we tested the hypothesis that streptozotocin (STZ) model of diabetes mellitus affects the levels of soluble (S) and solubilized membrane-bound (M) forms of DPPIV activity, CAP(LAP/IRAP) activity, and the broad-spectrum pyroglutamyl peptidase activity PAP-I, as well as the S form of POP activity in the hippocampus and hypothalamus of rats. We have also attempted to distinguish any direct effect of insulin and/or glucose *in vitro* on the levels of these peptidase activities in hippocampal and hypothalamic slices.

2. Materials and methods

2.1. Materials

Commercially available Bio-Rad protein assay kit (Hercules, USA), H-Cys-4-methoxy-β-naphthylamide, amastatin, bestatin, diprotin A (Bachem, USA) and insulin (Humulin®, Lilly, Brazil). Ala-β-naphthylamide, H-Gly-Pro-4-methoxy-β-naphthylamide and Z-Gly-Pro-β-naphthylamide were from Peninsula, USA. Bovine serum albumin, D-L-dithiothreitol, ethylenediaminetetraacetic-acid, 4-methoxy-β-naphthylamine, β-naphthylamine, nicotinamide adenine dinucleotide reduced form, puromycin, phenyl methyl sulphonyl fluoride; L-Pro-β-naphthylamide, L-pyroGlu-β-naphthylamide, propylene glycol, sodium pyruvate solution, streptozotocin, Triton X-100 and Z-Pro-Pro were from Sigma, USA. All other reagents of analytical grade were from Merck, Brazil.

2.2. Animals and treatments

Male healthy Wistar rats were maintained in a restricted-access room in a isolated and ventilated container (Alesco Ind. Com. Ltd., Brazil), with a controlled temperature of 25 °C, relative humidity of 65.3 ± 0.9% and 12 h light:12 h dark photoperiod (lights on at 6:00 h a.m.), and were housed in cages (inside length × width × height 56 cm × 35 cm × 19 cm), with a maximum of four rats per cage, with food and tap water *ad libitum*.

To induce diabetes, STZ diluted in citrate buffer pH 4.5 was intraperitoneally (ip) administered (50 mg/kg BM) in a maximum volume of 0.5 mL between 3:00 and 4:00 h of light period to weaned 21–25 days old rats that had been fasted for 18 h. The normal control group received only 0.5 mL citrate buffer. After 30 h, STZ-injected animals with blood glucose levels higher than 200 mg/dL, and those injected only with citrate buffer, which presented blood glucose levels between 60 and 90 mg/dL, were selected. Selected STZ-diabetic animals were then divided into two groups, one receiving subcutaneous (sc) injection of insulin (4 IU/kg BM) in a maximum volume of 0.3 mL NaCl 0.9% once a day between 7:00 and 8:00 h of light period during 60–75 days (STZ + I), and other receiving only 0.3 mL saline 0.9% under the same scheme of injection (STZ). This regimen of insulin administration was previously reported to promote successful recovery of normal glycemia [43]. Selected normoglycemic citrate-injected animals received 0.3 mL NaCl 0.9% once a day in the same scheme of injection (controls). After this period of 60–75 days, animals

were weighed and blood glucose was measured. Animal care and procedures used here were in accordance with guidelines of the Brazilian Council Directive (COBEA, Brazil) and were approved by the Ethics Committee of the Instituto Butantan (141/2003).

2.3. Blood and brain collections

Animals were anesthetized with Equithensin (42.5 g cloral hydrate/L, 9.72 g Nembutal/L, 22.74 g $MgSO_4$/L, and 39.6% of propylene glycol) (intraperitoneal injection of 2.5 mL/kg BM) at 4:00–6:30 h during the light phase. As the procedure followed, individual 1.0-mL blood samples were quickly obtained from the left cardiac ventricle of each rat, with a heparinized syringe and immediately centrifuged at 3000 × *g*, at 5 °C, for 20 min (microcentrifuge H-240, Hsiangtai Machinery Ind. Co. Ltd., Taiwan) to obtain plasma. Cardiac perfusion was then performed with 0.9% NaCl plus 50 mM phosphate buffer, pH 7.4, over a period of 15 min at a flow rate of 12–15 mL/min, with the circulatory circuit opened by an incision in the right atrium in order to ensure the elimination of blood. Immediately after the perfusion, the brain was quickly removed, frozen in dry ice and stored at −80 °C for a maximum period of 10 days until the use for measurements of enzyme activities and protein concentration. The brain of these animals was used for all measurements, except for *in vitro* incubation. For this last procedure, another group of healthy nonhandled anesthetized animals were submitted to cardiac perfusion (2–3 min) under the same scheme described above, but adopting the clamping of the descendent aorta. Immediately after the perfusion, the brain was quickly removed and the hippocampus and hypothalamus were dissected to obtain slices for using in the *in vitro* incubation procedure.

2.4. In vitro incubation of hippocampal and hypothalamic slices

The middorsal parts along the saggital plane of the hippocampus and hypothalamus from 11 to 14 weeks old rats were sectioned into 0.5 mm-thick slices, which were incubated for 30 min at 37 °C in artificial cerebrospinal fluid, Krebs-phosphate (124 mM NaCl; 5 mM KCl; 1.2 mM $MgSO_4$; 1.2 mM K_2PO_4; 20 mM Na_2HPO_4; 10 mM glucose; 2 mM $CaCl_2$; pH 7,2) without (CSF control) or with insulin (4 IU/mL) (CSF + I), and with additional 10 mM glucose without (CSF + G) or with insulin (4 IU/mL) (CSF + G + I). The ratios of incubation volume (mL) to slice wet weight (g) were about 30 or 80, respectively for the hippocampus or hypothalamus.

2.5. Blood glucose

Glucose was measured by Accu-Chek Advantage® kit (Roche S/A, Brazil) in the second drop of whole blood drawn from 18-h fasted individuals, using tail lancet.

2.6. Hematocrit

Microhematocrit values were obtained by centrifugation (microcentrifuge H-240, Hsiangtai Machinery Ind. Co. Ltd., Taiwan) immediately after blood sampling.

2.7. Osmolality

Osmolality was determined in 10 μL samples of fresh plasma (Osmette II cryoscopic osmometer, Precision Systems Inc., USA).

2.8. Obtention of soluble (S) and solubilized membrane-bound (M) fractions from selected brain structures

Selected structures (hippocampus and hypothalamus) were surgically dissected by manual slicing. The approximate wet weights of the dissected hippocampus and hypothalamus were respectively 70 and 25 mg. The obtention of S and M fractions followed the methodology described by Ramirez et al. [51]. Briefly, these dissected samples were homogenized in 10 mM Tris–HCl buffer, pH 7.4, for 3 min at 800 rpm (pestle mixer Tecnal TE-099, Brazil) and ultracentrifuged (HIMAC CP60E, Hitachi, Japan) at 100,000 × *g* for 35 min. The ratio of buffer volume (mL) to wet weight (g) was 50. The resulting supernatants were S fractions. To avoid contamination with the S fraction, the resulting pellet was washed three times with 10 mM Tris–HCl buffer, pH 7.4. The pellet was then homogenized for 3 min at 800 rpm in 10 mM Tris–HCl buffer, pH 7.4, plus 0.1% Triton X-100 and ultracentrifuged at 100,000 × *g* for 35 min. The resulting supernatants thus obtained were M fractions. All steps were carried out at 4 °C. The loss of enzyme activity of about 15% in both areas studied was estimated by time-dependent degradation curve of S and M fractions incubated at 4 °C until 90 min.

2.9. Fractionation markers

As markers for the fractionation procedure, lactate dehydrogenase (LDH) and aminopeptidase M (APM) activities were assayed in the S and M fractions from control animals. LDH was determined at 340 nm with a spectrophotometer (Power Wave X®, Bio-Tek, USA) by the method of Bergmeyer and Brent [10]. Samples of S (3.5–4.5 μg protein), or M (0.7–1.0 μg protein) from the hippocampus and hypothalamus were incubated in 100 mM phosphate buffer, pH 7.4, containing 200 mM NaCl and 1.6 mM sodium pyruvate solution plus 0.2 mM nicotinamide adenine dinucleotide, reduced form (NADH) disodium salt to a final volume of 300 μL. Values of LDH activity were obtained by the results of subtraction of the absorbance read at 0 time from that read at 10 min of incubation at 37 °C, and extrapolated by comparison with a standard curve of NADH. LDH activity was expressed as mmol NADH oxidized min^{-1} mg $protein^{-1}$. APM activity was determined fluorometrically using the methodology adapted from Gillespie et al. [28], and identically expressed as described in the item 2.11 for the other peptidases. Briefly, samples of S (10–15 μg protein) and M (30–45 μg protein), from the hippocampus and hypothalamus, were incubated in 0.05 M phosphate buffer pH 7.4, containing BSA 0.1 mg/mL, 20 μM puromycin and 0.125 mM Ala-β-naphthylamide to a final volume of 300 μL.

2.10. Protein

Protein was spectrophotometrically measured at 630 nm in triplicate by the Bio-Rad protein assay [14]. Protein content was extrapolated by comparison with a standard curve of

bovine serum albumin (BSA) diluted in 10 mM Tris–HCl buffer, pH 7.4, containing or not 0.1% Triton X-100.

2.11. *Fluorometric peptidase assay on soluble and solubilized membrane-bound fractions of hippocampal and hypothalamic homogenates*

Undiluted supernatants containing S and those containing M were used for the quantification of peptidase activities on the basis of the amount of 4-methoxy-β-naphthylamine (for DPPIV and CAP) or β-naphthylamine (for all other peptidases) released as a result of the enzymatic activity of hippocampal and hypothalamic S (60–75 or 120–150 μg protein, respectively for DPPIV and POP, or CAP and PAP-I) or M (30–50 or 60–100 μg protein, respectively for DPPIV and POP, or CAP and PAP-I), incubated for 30 min in 96-well flat botton microplates (Corning Inc., USA) with each respective prewarmed substrate solution diluted to 0.125 mM for CAP, PAP-I and POP, or to 0.2 mM for DPPIV, in corresponding 0.05 M buffers containing BSA 0.1 mg/mL in a final volume of 300 μL.

β-Naphthylamine or 4-methoxy-β-naphthylamine were estimated fluorometrically (FL600FA Microplate Fluorescence/Absorbance Reader, Bio-Tek, USA) at 460/40 nm emission wavelength and 360/40 nm excitation wavelength in triplicate samples. The value for incubates at zero time (blank) was subtracted and the relative fluorescence was converted to picomoles of β-naphthylamine or 4-methoxy-β-naphthylamine by comparison with a respective standard curve. Peptidase activity was expressed as picomoles substrate hydrolyzed min^{-1} mg $protein^{-1}$. Assays were linear with respect to time of hydrolysis and protein content. Peptidase activity was measured as previously described [42], using H-Gly-Pro-4-methoxy-β-naphthylamide in Tris–HCl buffer, pH 8.3, for DPPIV; H-Cys-4-methoxy-β-naphthylamide in Tris–maleate, pH 5.9, for CAP; L-pyroGlu-β-naphthylamide in phosphate buffer, pH 7.4, containing 2 mM DL-dithiothreitol (DTT) and 2 mM ethylenediaminetetraacetic-acid (EDTA), for PAP-I; Z-Gly-Pro-β-naphthylamide in phosphate buffer, pH 7.4, with 2 mM DTT, for POP.

All peptidase activities were measured in control and STZ-diabetic groups, while only those changed in STZ-diabetic group relative to the controls were measured in animals concomitantly treated with insulin and STZ (STZ + I) and in the slices after *in vitro* incubation.

2.12. *Inhibition studies*

The soluble or membrane-bound fractions obtained from 5 animals were individually pre-incubated at 37 °C during 10 min with preconized concentrations of each potential inhibitors amastatin (10^{-6} M), bestatin (10^{-5} M), puromycin (2×10^{-3} M), diprotin A (10^{-4} M) [38], Z-Pro-Pro (Z-prolinal) (5×10^{-2} M) [8], phenyl methyl sulphonyl fluoride (PMSF) (10^{-3} M) [37] and Zn^{2+} (10^{-3} M) [19] before the addition of fluorogenic substrate. The enzyme activity was then measured as described above. The results (means ± S.E.M) were recorded as the percentage of residual activity relative to control reactions, which run simultaneously in the absence of any inhibitor.

2.13. *Statistical analysis*

Data were analyzed statistically using GraphPad Prism® and Instat® softwares. Regression analysis was performed to obtain standard curves. Student's *t*-test was performed to compare the values of body mass, glycemia, hematocrit, plasma osmolality or peptidase activities in the same brain structures between control group and STZ-treated with (STZ-I group) or without (STZ group) insulin injection. Student's *t*-test was also performed to compare the values of peptidase activities between tissues incubated *in vitro* in control CSF and CSF-containing additional glucose with (CSF + G + I) or without (CSF + G) insulin, as well the values of LDH between S and M fractions and also the peptidase activities in the presence or absence of potential inhibitors. Differences were considered statistically significant at a minimum level of $P < 0.05$.

3. Results

Table 1 shows the decrease of body mass (about 34%) with increased glycemia (about 370%), hematocrit (about 16%) and osmolality (about 8%) in STZ-diabetic rats compared to controls. As also shown in Table 1, these changes (except hematocrit) were mitigated by concomitant treatment of STZ animals with insulin.

Table 2 shows higher levels of LDH activity in S than in M fractions, as well the detection of APM only in M fraction, which reflect the fractionation successfulness in both hippocampal (HC) and hypothalamic (HT) tissues, since it is well-recognized that LDH is a soluble enzyme [10,51] and APM is a puromycin-insensitive alanyl aminopeptidase activity restricted to membrane-bound fraction in brain tissues [28].

Distinct ($P < 0.05$) susceptibilities of each enzyme activities (residual activities) to the employed concentrations of inhibitors were: HC S CAP, amastatin (90.9 ± 2.3) > bestatin (95.8 ± 0.8) > puromycin (96.1 ± 0.4) > control (100 ± 1); HT S

Table 1 – Body mass, glycemia, hematocrit and plasma osmolality in control (C), streptozotocin-diabetic (STZ) and STZ rats treated with insulin (STZ + I)

Treatments	Body mass (g)	Glycemia (mg/dL)	Hematocrit (%)	Osmolality (mOsm/kg)
C	388 ± 17 (12)	74 ± 3 (12)	44 ± 2 (12)	310 ± 5 (12)
STZ	257 ± 19**** (12)	348 ± 22**** (12)	51 ± 2* (12)	335 ± 7** (12)
STZ + I	353 ± 22 (7)	71 ± 7 (5)	53 ± 1*** (7)	323 ± 3 (6)

Values are means ± S.E.M. Number of animals per group is given in brackets. Measurements were made in triplicate. Values in the same line are measurements from the same animals (11–14 weeks old). *$P < 0.03$; **$P < 0.01$; ***$P < 0.005$; ****$P < 0.0001$ in comparison to C in the same column (two-sided unpaired Student's *t*-test).

Table 2 – Activities of lactate dehydrogenase (LDH) (mmol NADH oxidized min^{-1} mg $protein^{-1}$) and aminopeptidase M (APM) (pmol substrate hydrolyzed min^{-1} mg - $protein^{-1}$) in soluble (S) and membrane-bound (M) fractions of hippocampus (HC) and hypothalamus (HT) of control rats

Fraction	LDH		APM	
	HC	HT	HC	HT
S	6.22 ± 1.31	5.91 ± 1.12	<50	<50
M	1.24 ± 0.33	1.02 ± 0.32	3011 ± 297	1630 ± 191

Values are means ± S.E.M. from four animals (assays made in triplicate). Values in the same column are measurements from the same animals (11–14 weeks old). S and M values are different in the same structure (two-sided paired Student's *t*-test, $P < 0.001$).

CAP, amastatin (83.6 ± 1.3) > bestatin (90.5 ± 1.3) > puromycin (93.0 ± 2.0) > control (100 ± 2); HC M CAP, only amastatin (92.4 ± 0.9) > control (100 ± 0.7); HT M CAP, all inhibitors without effect; HC S PAP-I, puromycin (84.5 ± 1.1) > bestatin (85.2 ± 2.5) > amastatin (87.5 ± 2.5) > control (100 ± 1.5); HT S PAP-I, amastatin (57.5 ± 1.3) > bestatin (85.6 ± 2.6) > puromycin (86.2 ± 3.7) > control (100 ± 2.3); HC M PAP-I, only puromycin (88.5 ± 1.0) > control (100 ± 1.7); HT M PAP-I, only amastatin (91.2 ± 2.1) > control (100 ± 2.9). Compared with the respective controls (100 ± 2.3, 100 ± 2.2), DPPIV was inhibited by diprotin A in soluble HC (88.7 ± 1.4) and HT (90.7 ± 1.8) but not in membrane-bound fraction of both structures. POP activity was reduced from 100 ± 3.6 to 59.7 ± 2.2 in the presence of Z-prolinal. All enzyme activities in S and M were inhibited by Zn^{2+} in the HT and HC (except M PAP-I). In the presence of serine protease inhibitor PMSF, only M and S DPPIV were significantly inhibited in the HT, while in the HC only POP and M PAP-I were resistant.

As shown in Table 3, among examined S peptidases the activity levels of PAP-I decreased about 28% in both the HC and HT, while POP increased about 78% in the HT when compared to controls. Table 4 shows that M PAP-I activity also decreased between 40% and 45% in the HC and HT relative to controls. When compared to controls, the activity levels of M CAP increased about 414% in the HC and about 155% in the HT (Table 4). Tables 3 and 4 also show that all these changes were mitigated by concomitant treatment of STZ animals with insulin.

As shown in Fig. 1, relative to the incubation in the CSF control, CAP activity increased about 359% in M fraction from HT slices incubated in the CSF with insulin. PAP-I activity decreased about 69% in S fraction from HT slices incubated in the CSF with insulin and additional glucose in comparison to incubation in the CSF control (Fig. 1). PAP-I activity in M

Table 3 – Soluble cystyl (CAP) and pyroglutamyl (type I) (PAP-I) aminopeptidase, dipeptidyl-peptidase (DPPIV), and prolyl oligopeptidase (POP) activities (pmol substrate hydrolyzed min^{-1} mg $protein^{-1}$) in the hippocampus (HC) and hypothalamus (HT) of control (C), streptozotocin-diabetic (STZ) and STZ rats treated with insulin (STZ + I)

Brain structure	Treatment	Peptidase activity			
		CAP	PAP-I	DPPIV	POP
HC	C	253 ± 33 (9)	155 ± 10 (11)	296 ± 15 (8)	5224 ± 308 (10)
	STZ	346 ± 46 (10)	112 ± 15* (9)	330 ± 21 (7)	5254 ± 340 (6)
	STZ + I	–	132 ± 22 (7)	–	–
HT	C	314 ± 40 (11)	139 ± 9 (11)	274 ± 20 (10)	3707 ± 228 (9)
	STZ	266 ± 32 (8)	100 ± 14* (8)	230 ± 21 (8)	6582 ± 370** (6)
	STZ + I	–	110 ± 19 (7)	–	4189 ± 1223 (7)

Values are means ± S.E.M. Number of animals per group is given in brackets (assays made in triplicate). Only the peptidase activities changed in STZ relative to the controls were measured in STZ + I. Values in the same line are measurements from the same animals (11–14 weeks old). *$P < 0.05$; **$P < 0.0001$ in comparison to C in the same structure (two-sided unpaired Student's *t*-test).

Table 4 – Membrane-bound cystyl (CAP) and pyroglutamyl (type I) (PAP-I) aminopeptidases, and dipeptidyl-peptidase (DPPIV) activities (pmol substrate hydrolyzed min^{-1} mg $protein^{-1}$) in the hippocampus (HC) and hypothalamus (HT) of control (C), streptozotocin-diabetic (STZ) and STZ rats treated with insulin (STZ + I)

Brain structure	Treatment	Peptidase activity		
		CAP	PAP-I	DPPIV
HC	C	28 ± 2 (8)	95 ± 7 (7)	85 ± 7 (12)
	STZ	144 ± 10*** (8)	57 ± 9* (7)	76 ± 6 (9)
	STZ + I	21 ± 4 (5)	69 ± 17 (7)	–
HT	C	47 ± 3 (7)	58 ± 4 (6)	344 ± 24 (9)
	STZ	120 ± 6*** (7)	32 ± 3** (6)	306 ± 22 (7)
	STZ + I	36 ± 7 (7)	85 ± 17 (7)	–

Values are means ± S.E.M. Number of animals per group is given in brackets (assays made in triplicate). Only the peptidase activities changed in STZ relative to the controls were measured in STZ + I. Values in the same line are measurements from the same animals (11–14 weeks old). *$P < 0.01$; **$P < 0.005$; ***$P < 0.0001$ in comparison to C in the same structure (two-sided unpaired Student's *t*-test).

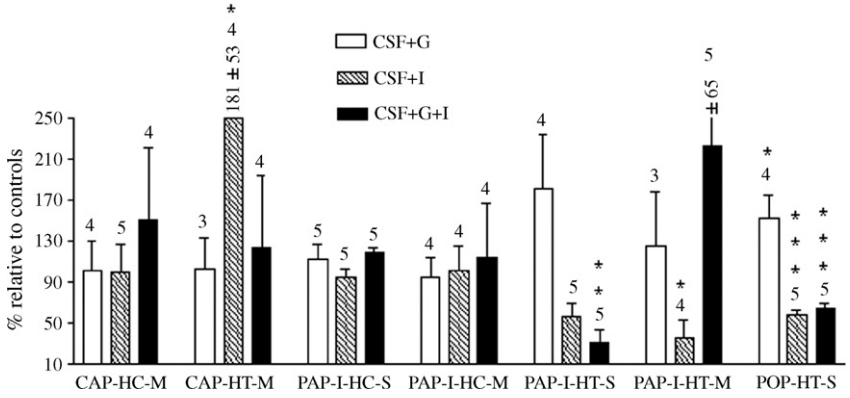

Fig. 1 – Soluble (S) and membrane-bound (M) cystyl aminopeptidase (CAP), pyroglutamyl aminopeptidase (type I) (PAP-I) and prolyl oligopeptidase (POP) activities in the hippocampus (HC) and hypothalamus (HT) of normoglycemic rats (11–14 weeks old) after incubation in artificial cerebrospinal fluid, Krebs-phosphate (CSF) with additional 10 mM glucose (CSF + G), with insulin 4 IU/mL (CSF + I), or with 10 mM glucose and insulin 4 IU/mL (CSF + G + I) in relation to those after incubation in CSF (control—100%). Values are means ± S.E.M. Numbers of animals are given horizontally over the bars. Measurements were made in triplicate. *P < 0.05, **P < 0.02, ***P < 0.003 in comparison to controls (two-tailed unpaired Student's *t*-test).

fraction from HT slices incubated in the CSF with insulin also decreased about 65% in comparison to incubation in the CSF control (Fig. 1). Fig. 1 shows that incubation of the HT in the CSF with insulin and/or additional glucose promoted changes in soluble POP activity levels. An increase of about 52% on this POP activity was detected after incubation in the CSF supplemented with glucose, while a decrease between 36% and 42% was detected after incubation in the CSF with insulin and with both insulin and additional glucose (Fig. 1).

4. Discussion

Peripheral administration of STZ has been recognized to cause hyperglycemia, plasma hyper-osmolality, hypoinsulinemia, hyperphagia and polydipsia [54]. Our results confirmed the marked effects of STZ treatment on glycemia, as well on blood volume and osmolality. The dysfunction of pancreatic beta cells caused by STZ injection is recognized to play a key role in the physiopathology of diabetes mellitus and it is responsible for the hyperglycemic state even in insulin-resistant diabetes [45].

Several data indicate that different substrate hydrolysis measured by the use of xenobiotic aminoacyl derivatives do not necessarily represent the specific activity of different proteins. Particularly to aminopeptidases, it is a limitation difficult to solve. There are different proteins with similar aminopeptidase-hydrolysing activity, pH-specificity and protease-inhibitor-sensitivity [23,32,35,50,59]. Furthermore, the selectivity of available DPPIV, LAP/IRAP, PAP-I [47] and POP [12] inhibitors is poorly defined. We confirmed here the occurrence of certain selectivity with the studied inhibitors on peptidase activities. For example, POP activity was inhibited by Z-prolinal, and diprotin A inhibited soluble DPPIV activity in the hippocampus and hypothalamus, as well membrane-bound PAP-I was preferentially inhibited by puromycin and amastatin, respectively in the hippocampus and hypothalamus. However, the effect of Zn^{2+} was quite similar on all peptidases in both brain structures (except hippocampal membrane-bound PAP-I), as well the inhibition profile in the hippocampus and hypothalamus was quantitatively similar for soluble CAP activity and qualitatively similar for soluble PAP-I. Thus, this selectivity level is not enough to associate only one inhibitor to only one peptidase activity within a short range of inhibitor concentrations, and does not permit the standard practice of using specific inhibitors in the enzyme assays to determine the proportion of the enzyme activity, which cannot be ascribed to a certain protein. However, the validation of physiopathological significance of the enzyme activity results obtained from whole tissues or cells does not have a direct relationship with the difficult to clearly attribute one enzyme activity to a given protein. Anyhow, considering the hydrolytic activities under study, as exerted or not by different proteins in the brain, they could play a role in the regulation of functionality of their peptide substrates and respective neuroendocrine functions through being affected by neuroendocrine challenges, such those represented by STZ-diabetes mellitus.

The catalytic activities on prolyl, cystyl and pyroglutamyl naphthylamide derivative substrates, as evaluated in the present study, mimic the activities of a group of peptidases on AVP, OT, NPY, GLP-1, peptide YY, LHRH, TRH, neurotensin and bombesin. Our present results (summarized on Table 5) show that some of these peptidase activity levels in rat hypothalamus and hippocampus can be modified in STZ-diabetes mellitus and/or by direct effects of insulin and/or glucose. Thus, these peptidases might be part of the common pathways that play the interactions between certain diabetic-related pathologies and the functionality of their correspondent peptide substrates. The comparison between the relative degrees that these enzyme activities were affected in different locations demonstrates their compartmentalization and tissue-specific responses to STZ-diabetes mellitus. In the intracellular compartment (secretory pathway), peptide levels depend on a balance between biosynthetic and release rates, and soluble peptidases can play a role in the biosynthesis and

Table 5 – Summary of changes observed on the levels of peptidase activities of the hippocampus and hypothalamus from streptozotocin(STZ)-diabetic in relation to normoglycemic rats or from normoglycemic rats after incubation *in vitro* in artificial cerebrospinal fluid

STZ treatment		Incubation *in vitro*	
Effect	Implication	Condition	Effect
Hippocampus			
Increased membrane-bound CAP	Pro-homeostatic decreased levels of AVP/OT	Insulin and/or additional glucose	Without effect on any peptidase activities
Decreased soluble and membrane-bound PAP-I	Deleterious increased levels of TRH analogs and indirect TRH overactivation of OT/AVP neurons		
Unchanged DPPIV	Unchanged NPY/peptide YY/GLP-1		
Hypothalamus			
Increased POP and membrane-bound CAP	Pro-homeostatic decreased levels of AVP/OT and TRH analogs	Additional glucose	Increased POP
Decreased soluble and membrane-bound PAP-I	Deleterious increased levels of TRH analogs and indirect TRH overactivation of OT/AVP neurons	Insulin	Decreased POP and membrane-bound PAP-I, and increased membrane-bound CAP
Unchanged DPPIV	Unchanged NPY/peptide YY/GLP-1	Insulin with additional glucose	Decreased POP and soluble PAP-I

consequently in the levels of synthesized peptides. In the extracellular compartment, peptide levels are defined in part by the electrical activity of the neurons that release them, an activity that is independent of the peptidase activity; however, there is a body of evidence that the peptide actions in the CNS can be also limited by the presence of the peptidases under study [18,25,44,55–57,67]. This last function can be considered as predominantly exerted by membrane-bound enzymes as seems to occur in the human cerebral cortex [27]; or soluble peptidases could be exocytosed and then act on released peptides [49], besides their capability to cleave peptides excised from endocytosed peptide receptor complexes.

We found unchanged levels of the soluble form and increased levels of the membrane-bound CAP activity in the hippocampus and hypothalamus of STZ-diabetic rats. The activity levels of soluble hypothalamic POP increased in STZ-diabetics. A slight reduction in the activity of soluble and membrane-bound PAP-I was also observed in both brain structures of STZ-diabetics. Since AVP, OT, TRH, LHRH, neurotensin and bombesin share a functional role on the balance of fluid volume and osmolality [13,16,20,21,54,63] and some of these peptides have also been associated with cognitive deficits and neurophysiological and structural changes in the CNS of diabetics, conditions that may be referred to as diabetic encephalopathy [11], it is tempting to speculate that changes on brain cell volume caused by diabetes melittus could be related to this pathological condition due to changes on the functionality of these peptides and to changes observed here on their related peptidase activities. Furthermore, direct and indirect effects of insulin and glucose on these enzyme activities can be hypothesized on the basis of obtained data from *in vitro* assay. Insulin has been related to induce decreased proteolysis in cells [30]. The effect of insulin (inhibition) and glucagon (stimulation) on cell proteolysis has been reported as completely dependent on their influence on cell volume [65].

Although many regulatory pathways are tissue-specific, hypothalamic membrane-bound CAP activity measured in the present study can be attributable in part to IRAP enzyme, since insulin has been reported to increase IRAP activity in fat and muscle cells [36], as we observed here for this CAP activity *in vitro*. IRAP, like GLUT4, is predominantly localized into intracellular vesicles under basal conditions. The glucose transporter (GLUT) family comprises highly related 12 transmembrane domain-containing proteins, activated in response to insulin, and can be divided into three classes based upon conserved structural characteristics. GLUT4 is the predominant member of this family [31]. Many studies indicate that insulin causes translocation of GLUT4 from intracellular stores to the plasma membrane, where it can promote glucose uptake into the cell [31]. In response to insulin, IRAP, like GLUT4, has been found to be translocated to the plasma membrane [36]. Furthermore, in insulin- and OT-treated cells *in vitro*, the appearance of IRAP at the cell surface occurs concomitantly with the increase of aminopeptidase activity [36]. Although GLUT4 is not the main glucose transporter isoform in the brain, its expression also changes in the brain under conditions associated with changes of glycemia and insulinemia [5]. By using immunohistochemistry, LAP/IRAP was found to be highly expressed exclusively in neurons in selected olfactory regions, in septal and hypothalamic nuclei, throughout the hippocampal formation and cerebral cortex, and in motor and motor associated nuclei. At the cellular level, LAP/IRAP was localized within cell bodies, excluding the nucleus, in a punctate vesicular pattern of expression. IRAP-positive immunoreactivity was also found in some proximal processes, but was not detected in synaptic nerve terminals. IRAP was expressed in cholinergic cell bodies of the medial septum, a source of cholinergic projections to the hippocampus and cerebral cortex [25]. Thus, like IRAP, a physiological function for CAP activity, as observed in the present study in the hypothalamus and hippocampus, may be the processing of extracellular peptides. These extracellular substrates would be processed efficiently only when the enzyme gets access to them, after translocation to the cell surface upon stimulation of cells. In this way, it has also reported that P-LAP is converted to a soluble form through proteolytic cleavage. ADAM (a disintegrin and metalloproteinase) members, such as ADAM 12, were suggested to be responsible for P-LAP shedding [33].

However, taking into account that the STZ causes hypoinsulinemia and our *in vivo* data show that the hypothalamic membrane-bound CAP activity is increased in the treatment with STZ, the CAP activity measured in the present study cannot be attributable only to IRAP enzyme. Anyhow, since upregulated AVP/OT availability in diabetes mellitus has beneficial effects in the acute setting but this long-term change is maladaptive and leads off to end organ damages such as neurodegeneration and apoptosis [38], the increment of membrane-bound CAP observed in the present study could be a pro-homeostatic response against the deleterious presence of sustained increase in extracellular levels of AVP/OT at these areas. As an additional evidence, it has been reported that OT proteolysis in brain decreases during aging. Such decrease might counterbalance the impairment of central oxytocinergic transmission caused by the age-related decrease of OT content in the brain [55].

The increase of POP activity induced by STZ-diabetes mellitus might contribute to the regulation of AVP/OT at intracellular level only in the hypothalamus. POP activity in the hypothalamus was also affected *in vitro* by stimulatory effect of glucose and prevalent depressant effect of insulin, which suggests that promnesic and anti-amnesic properties and enhancement of cholinergic neurotransmission [60] promoted by POP inhibitors could be exerted indirectly through modulation of hippocampal function by hypothalamic neuropeptides susceptible to POP hydrolysis. It can be hypothesized that neuroendocrine modulation involves the balance between CAP and POP activities under influence of cholinergic neurotransmission. By using RT-PCR and *in situ* hybridization, POP was found in most hypothalamic nuclei, notably in the lateral and posterior hypothalamic areas and the ventromedian, paraventricular, and arcuate nuclei, where most of the AVP and TRH receptors are abundant [7]. POP gene is primarily expressed in neurons but not in astrocytes, suggesting that hydrolysis of neuropeptides catalyzed by POP predominantly occurs on postsynaptic neurons that also express neuropeptide receptors. POP mRNA is also abundant in brain regions that are clearly implicated in the promnesic effects of neuropeptides [7]. POP has been reported to display age-specific differences in expression levels, with increased enzyme activity in the brain of aged mouse [52]. Our present data also reinforce the hypothesis that POP can be a valuable target for the treatment of memory impairment associated with neurodegenerative diseases, which can occur in diabetic individuals [34].

The results obtained here about PAP-I are in agreement with data of CAP and also with those about the decrease of PAP-I with age [2]. The hypothalamus and amygdala showed changes on pro-TRH and TRH receptor R1 mRNA levels related to the Morris water maze task, but not specific to spatial learning, while in the hippocampus, these levels were specifically increased in those animals trained to find a hidden platform, suggesting the involvement of TRH hippocampal neurons in memory formation processes [3]. On the other hand, the TRH neurons of the hypothalamic paraventricular nucleus project to the median eminence where TRH is released, in response to neural stimuli, into the portal vessels that irrigate the pituitary, where it exerts a hypophysiotropic role [24]. Upon neuronal stimulation, the releasing of TRH increases hypothalamic TRH mRNA levels in a fast and transient manner [53]. In the adenohypophysis, TRH modulates the expression and activity of TRH receptor R1 and PAP type II [58,64]. Although PAP type II, but not PAP-I, might be dominantly involved in the degradation of TRH in rats [1], we have to consider that its TRH-hydrolyzing activity could be physiologically relevant in addition to the activity of PAP type II. Furthermore, since hypothalamic PAP-I seems to play a part in the normal development of the CNS [2], and some of its substrates are involved in the regulation of OT and AVP [13,16,20,63], its slight downregulation in diabetic rats, as observed in the present work, can be linked to the overactivation of OT/AVP neurons, which modulate hippocampal function [6], leading ultimately to cell death of some of them [38] and contributing to cognitive deficits. However, since glucose alone does not seem to have an effect on PAP-I activity *in vitro*, it is difficult to interpret a possible relationship between the reduction of this enzyme activity *in vivo* and that *in vitro* observed in the hypothalamic membrane-bound and soluble fractions, respectively promoted by insulin alone or insulin with additional glucose.

On the other hand, in general our results *in vitro* also show that the effects of STZ-diabetes on hippocampal soluble and membrane-bound peptidases seem to be indirect, while the regulation of hypothalamic peptidases under study is largely determined by direct interaction between insulin and glucose on the hydrolytic ability of these enzymes. In addition to be in accordance with its functionality, these data agree with the known susceptibility of the hypothalamus to directly sense modifications in the extracellular fluid composition [46].

Finally, much attention has recently been given to DPPIV due to its ability to cleave GLP-1 [22]. DPPIV-resistant GLP-1 analogues, and DPPIV inhibitors such as sitagliptin [9] have currently being tested in clinical trials for diabetes mellitus therapies. Our data showing that DPPIV activity levels in the hippocampus and hypothalamus were unaffected by STZ-diabetes mellitus might be a useful information in the evaluation of these drugs.

5. Conclusion

The STZ-induced diabetes mellitus directly and/or indirectly affects the levels of hippocampal and hypothalamic CAP, PAP-I and POP activities, which may change the metabolic control of their susceptible physiological peptide substrates in the brain. In addition to bring to light new targets for diabetic-related effects on CNS, this information should help to understand how chronic diabetes mellitus affects the hippocampus and hypothalamus and their functions.

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