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Tissue distribution of CysAP activity and its relationship to blood pressure and water balance

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ABSTRACT

Aims: To better understand the functional role of soluble (Sol) and membrane-bound (MB) cystinyl-aminopeptidase (CysAP) activities, we studied differentially their organ distribution in adult male Wistar–Kyoto (WKY) and spontaneously hypertensive rats (SHR) with or without treatment with captopril. We searched for a possible tissue-specific association of CysAP with water balance and blood pressure.
Main methods: We used twenty WKY rats distributed in ten controls and ten captopril-treated, and sixteen SHR divided in eight controls and eight captopril-treated. Captopril (100 mg/kg/day) was administered in drinking water for 4 weeks. Systolic blood pressure, water intake and diuresis were measured individually. CysAP was assayed fluorometrically using L-cystine-di-β-naphthylamide as substrate.
Key findings: Sol or MB activities were generally higher in SHR compared to WKY notably in hypothalamus and kidney than in the other tissues. Captopril mainly decreased CysAP in SHR whereas it increased in WKY. The distribution of Sol CysAP was more homogeneous among tissues of WKY than SHR. In contrast, the distribution of MB CysAP was more heterogeneous than Sol CysAP in both WKY and SHR. This suggests that MB CysAP activity acts in a more tissue-specific manner than Sol CysAP. The majority of the significant correlations between tissue activities and the measured physiological parameters were observed mostly in renal medulla and hypothalamus.
Significance: Sol and MB CysAP activities, acting separately or in concert and mainly in renal medulla, regulate the function of their susceptible endogenous substrates, and may participate meaningfully in the control of blood pressure and fluid balance.

1. Introduction

Cystinyl aminopeptidase activity (CysAP), which exhibits broad substrate specificity, hydrolyzes amino-terminal cysteine residues of various peptides and polypeptides. The same enzyme has been reported under various terms such as insulin-regulated aminopeptidase (IRAP), placental-leucyl aminopeptidase, vasopressinase, oxytocinase or the Ang IV receptor (AT_4), which refers to the same protein [3,26,30]. This enzyme hydrolyzes oxytocin, vasopressin, angiotensins and opioid peptides [17,29]. However, the assumption that the AT_4 receptor is IRAP has also been challenged and it was proposed that this molecule is the c-Met tyrosine kinase receptor [36]. Cystinyl aminopeptidase is a protein involved in a large variety of functions including parturition, milk ejection, blood pressure, water balance, local blood flows, glucose homeostasis, and cognitive functions [1,6,10,28,37]. However, depending on the method used for its determination, the connection of CysAP activity with these different functions is not totally understood. In addition, its response to diverse physiological or pathological conditions differs markedly depending on whether its activity is analyzed in plasma or in the soluble (Sol) or membrane-bound (MB) fraction of tissues in diverse experimental conditions [12,25,32]. In addition, a close relationship between the distribution/enzyme activity of CysAP and the renin angiotensin aldosterone system (RAAS) (whose key role in regulating hydro mineral balance and blood pressure is well-known), has been observed [37] as well.

The spontaneously hypertensive rat SHR, in comparison with WKY, is a commonly used animal model for both water balance and blood pressure studies [23]. However, rarely has been used this strain to analyze the behavior of CysAP [25,32]. Although there are reports providing the local regional distribution of CysAP in selected tissues such as the central nervous system [9] or kidney [1], no comprehensive studies have been performed to establish the inter-tissue distribution of both Sol and MB forms of CysAP activities in WKY and SHR. Therefore, to examine its tissue relative importance in basal conditions and to search

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a possible connection of L-cystine-di-β-naphthylamide hydrolyzing activity with water balance and blood pressure (functions in which RAAS plays a major role), we analyzed CysAP activities, using L-cystine-di-β-naphthylamide as substrate, in plasma and in the Sol and MB fractions of diverse tissues in WKY and SHR, treated or not with the ACE inhibitor captopril.

2. Material and methods

2.1. Animals and treatments

The study was performed in twenty WKY rats distributed in ten controls and ten captopril-treated, and in sixteen SHR divided in eight controls and eight captopril-treated. These rats were age-matched groups of adult male aged 12 weeks at the beginning of the experiment and obtained from Charles River, Barcelona, Spain. All experimental protocols performed with animals agreed with the European Communities Council Directive 86/609/EEC, being approved by the ethics committee of the University of Jaén. 100 mg of captopril (Sigma-Aldrich) per kilogram and day was administered in the drinking water for a period of 4 weeks [31]. To measure water intake (WI) and diuresis (DIU) individually, 24 h prior to performing the sacrifice, rats were kept in metabolic cages and water balance (WB) was calculated as the difference between water intake and diuresis.

2.2. Blood pressure measurement

The tail-cuff plethysmographic method was used to measure systolic blood pressure (SBP) in un-anesthetized rats (LE 5001-Pressure Meter, Letica SA, Barcelona, Spain). A minimum of fifteen measurements were performed per rat, being the mean of similar values (within a range of 5 mm Hg) the selected SBP level. The first and last determinations of each recording session were discarded [32].

2.3. Surgical procedure and collection of tissue samples

After the period of four weeks of treatment with captopril and immediately after measuring SBP, the animals were anesthetized with equithensin (2 ml per kg body weight) (42.5 g/l chloralhydrate dissolved in 19.76 ml ethanol, 0.396 l/l propylenglycol, 21.3 g/l magnesium sulfate and 9.72 g/l Nembutal© in distilled water) injected intraperitoneally. Depth of anesthesia was verified, by the absence of corneal reflex as well as the absence of response when pinching the tail while breathing was slow but regular. Blood samples were obtained from the left cardiac ventricle and centrifuged for 10 min at 2000 *g* to isolate the plasma (PL) which was stored at −20 °C. Rats were then perfused injecting saline solution through the left cardiac ventricle and samples from the anterior hypothalamus (HT) (pooled left and right), pituitary (pooled anterior and posterior) (PT), left ventricle (VT), adrenal glands (pooled left and right) (AG), renal cortex (RC) and renal medulla (RM) (both pooled left and right) were obtained as described elsewhere [31,32]. Briefly, once the animals were perfused in full, their brains were rapidly removed and cooled in dry ice. The anterior part of the hypothalamus was obtained following the coordinates of the atlas of Paxinos & Watson [20]. The hypothalamic samples were between 7.7 mm and 3.7 mm anterior to the interaural line. Additionally, samples from renal cortex and medulla and a left ventricular sample from the heart were rapidly dissected and cooled in dry ice.

2.4. Procedures for enzymatic and protein assays

Plasmatic, Sol and MB CysAP activities were measured by fluorimetry using the arylamide derivative L-cystine-di-β-naphthylamide (Sigma) as substrate as formerly was described [32]. Shortly, in order to obtain the Sol fraction, samples from tissues were homogenized in a hypoosmolar medium (10 mM HCl–Tris buffer, pH 7.4). The homogenates were ultracentrifuged at 100,000 *g* for 30 min at 4 °C and the obtained supernatants and PL were used to measure the protein content and enzymatic activities. To get the membrane-bound protein fraction, the pellets obtained in the above ultracentrifugation were homogenized again in HCl–Tris buffer (pH 7.4) but now adding 1% of the detergent Triton X-100. After a new ultracentrifugation (100,000 *g*, 30 min, 4 °C), the obtained supernatants were used to determine MB enzymatic activities and proteins in triplicate. We used the adsorbent polymeric Bio-beads SM^{-2} (100 mg/ml) (shaking the samples for 2 h at 4 °C) to remove the detergent from the medium and fully recover enzyme activity. Cystinyl aminopeptidase activity was measured using L-cys-di-ß-naphthylamide as substrate: 25 μl of plasma or 10 μl of each supernatant was incubated (30 min at 25 °C) with 1 ml of the substrate solution: 5.53 mg/100 ml of L-cys-di-ß-naphthylamide, 10 mg/100 ml dithiothreitol (DTT) and 10 mg/100 ml bovine serum albumin (BSA) in 50 mM HCl–Tris buffer, pH 6. To stop the enzymatic reaction, 1 ml of 0.1 mol/l of acetate buffer, pH 4.2 was added to the medium. Because of the enzymatic activity, the ß-naphthylamine was released by hydrolysis from the substrate and then determined by fluorimetry at 412 nm emission wavelength with an excitation wavelength of 345 nm. Quantification of proteins was performed colorimetrically at 595 nm by the method of Bradford [4], using the dye Coomassie Brilliant Blue G250 and BSA as a standard. Specific Sol and MB CysAP activities were expressed as pmol of L-cys-di-ß-naphthylamide hydrolyzed per min per mg of protein. We confirmed the linearity of fluorogenic determinations regarding time of hydrolysis and protein content.

2.5. Statistical analysis

The difference between tissues and between groups was evaluated by two-way analysis of variance. LSD-tests were used for post-hoc comparisons. We considered significant *p*-values below 0.05. To analyze the relationship between CysAP activities of the samples analyzed with WI, DIU, WB and SBP, the Pearson's coefficient of correlation was used [5]. Calculations were performed using SPSS 13.0 and STATA 9.0. Correlations with *p*-values under 0.05 were considered significant. The ratio of lowest to highest activity, as a reflection of the homogeneity of the tissue-distribution, was also calculated.

3. Results

Results are shown in Figs. 1 and 2 and Table 1. Water intake was significantly higher ($p < 0.001$) in control SHR compared to control WKY but no differences were observed between both strains in captopril-treated animals. While WI increased in WKY after treatment with captopril ($p < 0.001$), no such differences were observed in SHR. Captopril increased DIU in WKY ($p < 0.001$) and SHR ($p < 0.05$). Nevertheless, significant modifications between WKY and SHR were not detected in the levels of DIU of control and rats treated with captopril. Water balance, as the difference between water intake and diuresis, was significantly higher ($p < 0.001$; 200% higher) in control SHR when compared to WKY. This increase was lower ($p < 0.05$; 100% higher) in captopril-treated SHR. However, there were no significant differences in WB between control and captopril-treated WKY or SHR rats. Compared to WKY, SBP was significantly higher ($p < 0.001$) in control and captopril treated SHR. Captopril reduced significantly ($p < 0.001$) SBP in both WKY and SHR.

Regarding the inter-tissue distribution, Sol or MB activities were usually significantly higher in the hypothalamus and kidney (particularly renal medulla) than those in the other tissues (Fig. 2). Briefly, considering the hypothalamus and kidney, while Sol CysAP activity in HT and RM was slightly higher (around 1-fold) than those in PT and PL, and did not differ with those in the other tissues in control WKY, both the HT and kidney (RC and RM) demonstrated 0.5–4 fold higher levels of this activity (than the rest) in control SHR. After captopril treatment, Sol CysAP displayed a similar inter-tissue distribution than control