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Carboxypeptidases A1 and A2 from the perfusate of rat mesenteric arterial bed differentially process angiotensin peptides

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ABSTRACT

Here we report the isolation of carboxypeptidases A1 and A2 (CPA1 and CPA2) from the rat mesenteric arterial bed perfusate, which were found to be identical with their pancreatic counterparts. Angiotensin (Ang) I, Ang II, Ang-(1-9) and Ang-(1-12) were differentially processed by these enzymes, worthy mentioning the peculiar CPA1-catalyzed conversion of Ang II to Ang-(1-7) and the CPA2-mediated formation of Ang I from Ang-(1-12). We detected gene transcripts for CPA1 and CPA2 in mesentery and other extrapancreatic tissues, indicating that these CPAs might play a role in the renin–angiotensin system in addition to their functions as digestive enzymes.

1. Introduction

The renin–angiotensin system (RAS) consists of a number of peptide ligands and receptors whose distributions vary between species and, within species between individuals, according to the developmental stage, integrity and functional status of their different tissues. Such complexity reflects the many physiological and physiopathological functions carried out by the RAS which, in addition, require a network of intertwined enzymatic pathways to produce the different angiotensin (Ang) peptides that act as effector molecules of the system. At first the RAS was thought as a typical endocrine system in which the effector hormone Ang II would be formed by a two-step reaction, whereby the Ang I initially released from angiotensinogen by circulating renin would then be converted into Ang II by the action of the metalloprotease angiotensin converting enzyme (ACE). Despite the central role of this angiotensinogen–renin-ACE-Ang II axis for many of the functions carried out by the RAS, it became clear over the years that in some tissues Ang II could also be formed from Ang I by ACE-independent [5] or from Ang-(1-12) by renin-independent [20] pathways. The serine protease chymase, for instance, is the major enzyme that converts Ang I to Ang II in the human heart [32], while in the rat heart infused with Ang-(1-12) this enzyme appears to be responsible for most of the hemodynamic effects caused by the released Ang II [26]. Moreover, several Ang peptides shorter than Ang II were described as functionally active components of the RAS [9], implying the participation of proteases other than ACE to account for the required processing of the respective Ang precursors. Among these, neprilysin generates C-terminally modified Ang fragments, releasing Ang-(1-7) from both Ang I and Ang-(1-9) [28]. A variety of enzymes displaying CPA-like activity have also been implicated in the proteolytic processing of Ang peptides. Cathepsin A of human heart generates Ang-(1-7) and Ang-(1-9), two molecules that act as bradykinin potentiator and ACE inhibitor, respectively [12]. Besides, in the human heart a mast cell CPA-like enzyme has been proposed to regulate the local Ang II formation by releasing the ACE inhibitor Ang-(1-9) into the interstitial fluid [13]. In porcine kidney, cathepsin A seems to participate in the local RAS by forming Ang-(1-9) and Ang II, but not Ang-(1-7) [19]. The identification of ACE2 by genomic approaches as a human homolog of ACE that displays carboxypeptidase activity [6,30] reinforces the current awareness of the functional complexity of the multifaceted, multicomponent RAS. ACE2 can act upon Ang I and Ang II to generate Ang-(1-9) and Ang-(1-7), respectively, two metabolites that

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oppose the action of Ang II either by regulating the formation of Ang II by ACE [13,29] or triggering opposing biological responses mediated by distinct receptors [7]. In previous investigations we showed that the perfused ex vivo preparation of the rat mesenteric arterial bed (MAB), known as the McGregor's preparation [18], secretes a multiplicity of Ang I- and Ang II-processing CPs potentially relevant to the metabolism of vasoactive and other peptides in the rat mesentery [22,25]. To further characterize these enzymes, in the present study we aimed at: (1) identifying the CPs that constitute major Ang processing pathways in the rat MAB perfusate; (2) investigating the enzymatic activities of purified CPs obtained from rat MAB perfusate toward Ang I, Ang-(1-9), Ang II and Ang-(1-12); and (3) determining the expression profile of the mRNAs for the different CPAs in representative rat tissues, in which RAS is believed to play a functional role in the local circulatory system.

2. Material and methods

2.1. Material

Potato carboxypeptidase inhibitor (PCI), N-carbobenzyloxy-Val-Phe (Z-Val-Phe), Ang I (Asp^1-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu^{10}), Ang II (Asp^1-Arg-Val-Tyr-Ile-His-Pro-Phe^8), bradykinin (BK; Arg^1-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg^9), DL-2-mercaptomethyl-3-guanidinoethylthiopropanoic acid (MGTA),1,10-phenanthroline, soybean trypsin inhibitor (SBTI) and DEAE-Sepharose fast flow were obtained from Sigma Chemical Co. (St. Louis, MO). Ang-(1-9) and Ang-(1-12) were synthesized by conventional Fmoc solid phase peptide synthesis [8] and purified by C-18 reversed-phase HPLC. Packed MonoQ 5/5 column was from Pharmacia Fine Chemicals (Uppsala, Sweden). All other reagents used were of analytical grade.

2.2. Isolation of the rat mesentery and harvesting of MAB perfusate

All animal protocols were approved by the School of Medicine of Ribeirão Preto Institutional Animal Care and Use Committees. The surgical procedures for the isolation of the rat mesentery and the perfusion of the respective arterial system with Krebs solution were performed as previously described [24]. High molecular mass substances of pooled rat MAB perfusates were concentrated 80-fold by ultrafiltration under N_2 pressure using Amicon YM-10 membrane and stored at 4 °C until use.

2.3. Enzyme assays

All rat MAB CPA assays were carried out at 37 °C by incubating the specified substrate with the enzyme in 150 μL of 20 mM Tris–HCl buffer pH 8.1, and the reactions terminated by the addition of 10 μL of 5% TFA. One unit of enzyme activity was defined as the amount of enzyme capable of releasing 1.0 μmol of product per min from the indicated substrate solution; unless otherwise specified, the concentration of Z-Val-Phe in the reaction mixture was 10 mM and those of angiotensin peptides and bradykinin were 0.25 mM. The cleavage of Z-Val-Phe and other peptides was assessed by reversed-phase HPLC analysis on a Shimadzu SCL-6B equipment fitted with a 0.46 cm × 15 cm Vydac ODS column; Phe and peptide fragments were eluted with a linear gradient of acetonitrile concentration ranging from 0 to 10% (10 min) and 12 to 45% (33 min) in 0.1% TFA, respectively, at a flow rate of 1.0 mL/min, and monitored by absorbance at 215 nm; peptides were identified by comparison of their retention times with those of the respective cognate synthetic peptides. Whenever used, the protease inhibitors MGTA, PCI, 1,10-phenanthroline and SBTI were preincubated for 10 min, at the indicated concentrations, with the enzyme solution.

2.3.1. Estimation of kinetic constants

To estimate the kinetic parameters for the rat CPA1 and CPA2-catalyzed hydrolyses of Ang II, initial velocities were determined, in duplicate, under conditions adjusted to limit substrate consumption to less than 10% of its initial concentration. Thus, samples of rat MAB CPA1 (0.45 mU, based on Z-Val-Phe hydrolysis) and CPA2 (9.2 mU, based on Z-Val-Phe hydrolysis) were incubated at 37 °C for 20 min and 150 min, respectively, in a final volume of 0.5 mL of 20 mM Tris–HCl buffer, pH 8.1, with seven concentrations of Ang II ranging from 10 to 200 μM for CPA1 and 25 to 500 μM for CPA2. Reactions were terminated by the addition of 20 μL of 5% TFA and the respective products were assayed by HPLC analysis. The kinetic parameters Michaelis constant, K_m, and catalytic constant, k_{cat}, were derived from initial velocity data ($N = 2$) using GraFit version 3.0 software [15], which performed non-linear regression analysis of data plotted according to the Michaelis–Menten equation.

2.4. Enzyme purification

The initial step in the purification of the two major rat MAB Ang-processing carboxypeptidases was carried out by anion exchange chromatography, as detailed in a previous work [25]. Further purification leading to the isolation of CPA1 and CPA2 was achieved by chromatographing the major Ang-(1-7)-forming enzyme and the most conspicuous Ang-(1-9)-forming enzyme of the rat MAB perfusate [25], respectively, over MonoQ 5/5 column; each chromatography was developed at room temperature with a flow rate of 6 mL/h using 20 mL of the indicated linear gradient of NaCl concentration in 20 mM Tris–HCl buffer, pH 8.1. Enzyme activity was measured using either Z-Val-Phe or Ang II as the substrate, as indicated in the respective figures. Contaminant kininase activity was removed from pooled CPA-containing fractions by affinity chromatography over arginine-Sepharose column (1.5 cm × 3.5 cm) equilibrated and developed with 1 M NaCl solution buffered with 30 mM Tris–HCl, pH 7.2, as previously described [23]. The CPA-containing fractions were pooled and stored at 4 °C until use.

2.5. SDS-PAGE

Analytical SDS-PAGE was carried out on 15% polyacrylamide gels essentially as described [14], using a Mini-Protean 3 electrophoresis system (BioRad, Hercules, CA, USA). The Mr standard proteins (14.4–116 kDa) were from Fermentas Inc. (Hoover, MD, USA); protein bands were stained with Coomassie Blue R-250. SDS-PAGE separations intended for preparing proteins to be digested in-gel and further characterized by LC–MS/MS were performed on precast 4–12% gradient polyacrylamide gels using an Invitrogen NuPage system (Carlsbad, CA, USA). Proteins bands were stained with Coomassie Blue G-250.

2.6. RNA extraction and reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was extracted from rat mesentery, pancreas, kidney, liver, lung, heart, aorta and carotid using the Trizol reagent in RNAse-free labware, following the manufacturer instructions (Invitrogen, Carlsbad, CA, USA). RNA integrity was confirmed by agarose gel electrophoresis and then treated with DNAse for 15 min at room temperature to remove any potential genomic DNA contamination. Four micrograms of total RNA from each tissue, based on $A_{260\,nm}$ measurements, and oligo-d(T) were used to generate cDNAs by reverse transcription following SuperScript II protocols (Invitrogen).