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Ontogeny of prolyl endopeptidase and pyroglutamyl peptidase I in rat tissues

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Abstract

Prolyl endopeptidase and pyroglutamyl peptidase I are enzymes which participate in the degradation of thyrotropin-releasing hormone (TRH), a hormone which is thought to play an important role in the development of organs and tissues. Here, we have characterized the ontogeny of TRH degrading enzyme activity in the brain cortex, lung, heart, kidney and liver. Overall, prolyl endopeptidase activity was found to be 2 to 5 fold higher in newborn vs. adult rat tissues, with the exception of the soluble form in the liver and the particulate form in the lung. In contrast, the developmental profile of pyroglutamyl peptidase I activity was found to be more variable and tissue dependent. These results corroborate the idea that both enzymes play important, tissue-specific roles during the development and maturation of rat organs.

Keywords: Ontogeny; Peptidase; TRH; Intracrine

1. Introduction

Bio-active peptides are involved in the regulation of a large number of physiological functions [1]. In the central nervous system (CNS), the complexity of the role of neuropeptides in intercellular communication is based, in part, on their multiple effects as neurotransmitters or neuromodulators [2], acting via specific membrane receptors. When secreted into the systemic circulation, peptides can regulate the function of other tissues, thus acting as hormones [3–5]. Of all the functions assigned to peptides, one of the most remarkable is their involvement in the ontogeny of many different tissues [6–8]. Moreover, it has been described that during early stages of development, they can act in a paracrine or an autocrine manner, rather than as neurotransmitters [9–11]. Indeed, some recent work has even proposed an intracrine (inside the cell) mode of action for some peptides [12]. In this regard, evidence has been presented for the localization of receptors of some biologically active peptides in the cell nucleus [13–17]. Accordingly, the degradation of peptides inside the cell is thought to play important roles in developing tissues [18].

Thyrotropin-releasing hormone (TRH) is a tripeptide which acts as a hypothalamic peptide hormone. Besides its function as a hormone, it has been shown that TRH can also act as a neurotransmitter and/or neuromodulator in the CNS [19–23], but it has also been localized in a variety of other tissues [24–28]. The pattern of TRH expression and activity during the course of development is well characterized [29,30]. Indeed, of all biologically active peptides, TRH is the one best characterized in terms of its degradation due to its short chain length (pGlu-His-Pro-NH2).

Among the enzymes involved in the primary degradation of TRH are two ubiquitous peptidases: prolyl endopeptidase (PEP; EC 3.4.21.26), which is responsible for the deamidation of TRH [31], and pyroglutamyl peptidase (PGP; EC 3.4.19.3) I, which removes the N-terminal pyroglutamyl [32,33]. Both enzymes are widely distributed in most tissues and body fluids [6,34,35] and, although membrane-associated forms have also been reported [36,37], their localization is mainly cytosolic. PGP II, a membrane-bound enzyme which is mainly found in brain synaptosomes and in a limited number of tissues [38,39], is also capable of degrading TRH. However, with few exceptions, it is absent in extraneural tissues at birth and during the neonatal period.

In contrast, PEP [6,40] and PGP I [6,41,42] both appear to play vital functions during the development of the CNS and the activity of these enzymes in different brain areas is significantly higher (2–5 fold) during the first postnatal days of life compared to adulthood. In addition, PEP has been reported to be involved in the proliferation and differentiation of many

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types of cells [43,44]. TRH is developmentally regulated in a great variety of tissues other than the CNS. However, few studies have focused on characterizing the alterations in the activity of TRH degrading enzymes in peripheral tissues during development [6,43]. Here, we report the developmental changes in the activity of PEP and PGP I in several peripheral organs and in the brain cortex of the rat during the course of development.

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats bred in our university animal house and maintained under conditions of controlled light (light from 07:00 h to 19:00 h, dark from 19:00 h to 07:00 h) and temperature (24 °C), with food and water *ad libitum*, were used for the present studies. Adequate measures were taken to minimize pain or discomfort. The experiments were conducted in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and were approved by the Committee for Animal Ethics of the Faculty of Medicine of the University of the Basque Country.

Animals at the following stages of development were used (10 per age): embryonic day 22 (ED22), parturition day (PD0), postnatal days 2 (PD2), 4 (PD4), 10 (PD10), 15 (PD15), 20 (PD20), 30 (PD30), and 90 (PD90). The tissues of all the animals were obtained at 9:00 am.

2.2. Chemicals and instruments

All chemicals were obtained from The Sigma Chemical Company (St. Louis, Mo., USA), with the exception of Z-Gly-Pro-β-naphthylamide, which was obtained from Merck (Darmstadt, Germany) and Bachem (Bubendorf, Switzerland). The following fluorescence readers were employed: RF-540 (Shimadzu), UV-2401 (Shimadzu) and FL-500 (Bio-Tek Instruments).

2.3. Sample preparation

In order to avoid proteolytic contamination from blood, animals were perfused with phosphate buffered saline (150 mM NaCl, 0.2 mM Na2HPO4, 0.05 mM NaH2PO4.H2O; pH 7.4) for 10–15 min at a flow rate of 2.4–4.8 ml/min through the left cardiac ventricle under Equithensin anesthesia (2 ml/kg body wt. of the following solution: 42.5 g/l chloral hydrate, 9.72 g/l Nembutal, 22.74 g/l magnesium sulphate and 39.6% propylene glycol).

Dissected tissues (liver, kidney, heart and lung) were homogenized in 10 mM Tris-HCl buffer, pH 7.4, for 2 min at 800 rpm and ultracentrifuged at 100,000 *g* for 35 min. The resulting supernatants were used to measure soluble enzyme activity and protein concentrations. To avoid contamination with soluble enzymes and to remove any loosely bound proteins, the resulting pellet was washed three times by suspension in 10 mM Tris-HCl buffer, pH 7.4. Subsequently, pellets were homogenized for 30 s at 800 rpm in 4 M NaCl and ultracentrifuged at 100,000 *g* for 30 min. Finally, the pellet obtained in the last ultracentrifugation was washed three times by suspension in 10 mM Tris-HCl buffer, pH 7.4, homogenized for 30 s at 800 rpm in 10 mM Tris-HCl buffer, pH 7.4, plus 1% Triton X-100 and ultracentrifuged at 100,000 g for 30 min. The supernatants thus obtained were used to determine the solubilized particulate enzyme activity and protein concentrations. All steps were carried out at 4 °C.

2.4. Enzyme assays

Whole supernatants containing the soluble enzymes were diluted in 10 mM Tris-HCl buffer, pH 7.4, to a range between 0.4 and 1.2 mg protein/ml, while supernatants containing membrane-bound enzymes were diluted in 10 mM Tris-HCl buffer, pH 7.4 plus 1% Triton X-100 buffer, to a range between 0.4 and 1.2 mg protein/ml. Fifty μl of supernatant were incubated with 2 μl Z-Gly-Pro-β-naphthylamide [Yoshimoto et al., 1979] in dimethylsulphoxide and diluted to 0.125 mM in 0.05 M phosphate buffer, pH 7.4, containing 0.1 mg/ml bovine serum albumin, 2 mM EDTA and 2 mM dithiothreitol (DTT), at 37 °C for 30 min in order to measure PEP activity. In order to measure PGP I activity, pGlu-β-naphthylamide [36] (0.125 mM) in 0.05 M phosphate buffer, pH 7.4, containing 0.1 mg/ml bovine serum albumin was used as substrate. It also contained 5 mM DTT and 2 mM EDTA, which activate this enzyme, while they inhibit the activity of PGP II.

Blank samples consisted of incubation solution plus 50 μl of 10 mM Tris-HCl buffer. The amount of β-naphthylamine released was estimated fluorimetrically at 412 nm emission wavelength and 345 nm excitation wavelength in triplicate samples. Assays were linear with respect to time of hydrolysis and protein content. One unit of enzyme activity was considered to be the amount of enzyme that hydrolyzes one picomole of aminoacyl-β-naphthylamide per minute and per milligram of protein [45]. Protein concentration was measured in triplicate by the method of Bradford [46] using bovine serum albumin as a standard.

2.5. Statistical analysis

Data were analyzed statistically using SPSS. version 11.5. The results, recorded as means + SEM, were analyzed by the Student *t*-test for comparison between two groups and one-way analysis of the variance (ANOVA) followed by the Scheffé test, for comparisons between more than two groups.

3. Results

The activity of soluble PEP (sPEP) in samples of the liver, kidney, heart, lung and brain cortex at various developmental stages is shown in Fig. 1. In the rat liver, there are no significant differences. In the kidney, sPEP activity decreased during development (ANOVA $p<0.001$) to levels that were 5 fold lower than in the embryo. In this tissue, the change in enzyme activity was significantly noticeable from the first moments of development which we studied (Scheffé $p<0.001$, between E22 and PD2). In the heart and lung, sPEP activity was high