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Comparative Biochemistry and Physiology Part B 131 (2002) 201–207

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Prorenins activation by an enzyme from rat plasma (*PreR-Co*)

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Received 22 June 2001; received in revised form 5 October 2001; accepted 8 October 2001

Abstract

The aim of the present research was to explore the capacity of *PreR-Co* to process prorenin purified from kidney and corpora lutea (CL) and to study its action on extrarenal tissues. The *PreR-Co* was obtained from plasma as a single electrophoretic band by $(NH_4)_2SO_4$ precipitation, gel filtration, anti-rat albumin immunoaffinity, and ion-exchange chromatography. Prorenin free of renin was obtained after $(NH_4)_2SO_4$ precipitation, gel filtration, and ion-exchange chromatography by a passage through an affinity gel of H-77 Sepharose. SDS-PAGE of supernatant and of acidic elution from gel, exhibited a single band of 43 kDa and 35 kDa, respectively; both recognized by the specific anti rat renin antibody. The isolated renin was not attacked by *PreR-Co*; on the contrary prorenin was completely activated. The product of *PreR-Co*-activated prorenin showed an analogous MW to that of renin and was recognized by the specific antibody. In addition to processing kidney prorenin, *PreR-Co* was able to cleave inactive renin from ovary, CL, uterus and adrenal gland homogenates. However, the amount of active renin generated from these tissues was lower than those produced by trypsin activation. *PreR-Co* is a good candidate for the role of the enzyme involved in tissues prorenin activation.

Keywords: Inactive renin; Kidney; Prorenin; Renin; Renin activation; Renin-angiotensin system; Trypsin; Prorenin converting enzymes

1. Introduction

Prorenin is the inactive precursor of the fully active aspartyl protease renin (De Vito et al., 1970a), which is essential in the proteolytic cascade leading to the production of angiotensin II (Ang II). Despite the fact that prorenin has been detected in several tissues of mammals (Lumbers, 1993; Skinner, 1993), conversion of prorenin to renin appears to occur mainly in the storage granules of the renal juxtaglomerular (JG) cells. Prorenin is secreted constitutively but, in addition, a portion of the prorenin is sorted to dense core secretory granules where it is proteolytically cleave to active renin by removal of a 43-amino acid propeptide. Nevertheless, the processing enzyme and the precise mechanism of conversion in kidney and plasma (if that occurs) remains at

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1096-4959/02/$ - see front matter
PII: S1096-4959(01)00494-8

present unknown. Clarification of the mechanisms underlying these processes are considered essential to our understanding of the control of renin activity locally in the renal glomerulus, in plasma, and in the regulation of fluid and electrolyte balance.

Recently, we isolated from rat plasma a protein capable of activating renal prorenin (De Vito et al., 1996) This protein, which we called *PreR-Co*, has a molecular weight of 37 kDa and an N-terminal amino acid sequence that did not entirely match with any known proteolytic enzyme (Vincent and De Vito, 1999). Nevertheless, contrary to what was expected, *PreR-Co* was unable to interact with plasma prorenin leading us to speculate that plasma and renal prorenin may have different conformations.

The purposes of the present investigation are to study: (I) the action of *PreR-Co* on different tissue homogenates; and (II) the effect of *PreR-Co* on prorenin purified from different tissues.

2. Material and methods

2.1. Chemicals

Rat albumin (99% agarose electrophoresis, Lot 73H9320), Freund's complete adjuvant, ethylenediamine tetraacetic acid disodium salt (EDTA, ACS reagent), phenyl-methyl-sulfonylfluoride (PMSF), neomycin sulfate, trypsin (from bovine pancreas type III 7857 BAEE units/mg), soybean trypsin inhibitor (SBTI), (type I-S, inhibitor capacity 1.5 mg trypsin/mg, approx. 10 000 BAEE units/mg), H77 peptide (D-His-Pro-Phe-His-Le−^{R}Leu-Val-Tyr), bovine serum albumin (Cohn fraction V), glycine, ammonium sulfate, sodium azide, NEM, sodium chloride and $CaCl_2$ (ACS reagent), were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Sephacryl S-200 HR, Cyanogen Bromide-Activated Sepharose 4B and Sephadex G-25 were purchased from Pharmacia (Uppsala, Sweden). Ketamine chlorohydrate (Vetanarcol) was from Laboratories König SA BsAs-Argentina and Rompun [Hydrochloride 2-(2,6-xilidino)-5,6-dihidro.4-H-1,3 tiacina] from Bayer Argentina SA.

2.2. Collection and processing of samples

2.2.1. Rat kidney, ovaries, corpora lutea (CL), uterus and suprarenal gland

Rat kidney, ovaries, uterus and adrenal glands were rapidly removed under Ketamine 0.3 mg/kg − Rompun 16 mg/kg anesthesia and placed on ice-cooled Petri dishes. Luteal tissue was separated from the rest of the ovary by careful dissection under a stereoscopic microscope. Tissues were homogenized separately at 4 °C in 0.154 M NaCl (100 mg/ml) in an Ultra-Turrax homogenizer (Janke & Kunkel, Staufen, Germany) and centrifuged at $24\,000 \times g$ for 30 min. The clear supernatants were kept at −20 °C if not immediately used. Renin concentration before and after *PreR-Co* or trypsin activation was measured by incubation with homologous substrate and the angiotensin I (Ang I) generated was measured using a radioimmunoassay (RIA) kit (RIANEM) manufactured by DuPont Medical Products (Boston, MA, USA). Only the kidney cortex was used for purification of kidney renin and prorenin or to determine their concentration in renal tissue.

2.2.2. Renal prorenin purification

Purified rat prorenin was obtained from kidney homogenates following the methodology previously described for active renin purification (De Vito et al., 1997), but only up to the $(NH_4)_2SO_4$ precipitation step. This fraction (called R-3) was applied to a column containing Sephacryl S-200 gel, which was previously equilibrated with 50 mmol/l of phosphate buffer (pH 7.6), containing 150 mmol/l of NaCl and 0.02% of sodium azide. The elution was performed with the same buffer and proteins were monitored by absorbance at 280 nm. The peaks were concentrated in a Speed-vac evaporator and the renin activity was tested by incubation with homologous angiotensinogen obtained from 48-h nephrectomized male rats or hog plasma that was acquired from an authorized dealer as described previously for bovine serum angiotensinogen (De Vito et al., 1970b). The angiotensin I (Ang I) generated was measured using a radioimmunoassay. The peak with renin activity was named R4.

Prorenin free of renin was obtained by a passage through an affinity gel of H-77 Sepharose. In short: 1 mg of H77 was linked by its N-terminal residue to 6 aminohexanoic acid-activated

Sepharose 4B (McIntyre et al., 1983). The resin was stabilized with 50 mmol/l of Tris-acetate buffer (pH 7.4), containing 1 mmol/l of EDTA and 0.2 mmol/l of PMSF. Two hundred microliters of the R4 fraction were applied to 300 μl of the resin, incubated for 20 min at 4 °C and centrifuged at $27\,000 \times g$ for 3 min at 5 °C. The supernatant (RH5 fraction) contained prorenin that was not coupled to the resin. The resin with active renin fixed, was washed first with 3 ml of 50 mmol/l Tris-acetate buffer (pH 7.4), containing 1 mmol/l of EDTA, 0.2 mmol/l of PMSF and 0.5 mmol/l of NaCl, and thereafter with 2 ml of 0.1 mmol/l sodium acetate (pH 6), containing 1 mmol/l of EDTA, 0.2 mmol/l of PMSF and 0.5 mmol/l of NaCl. The elution of active renin was performed with 200 μl of 0.1 mmol/l sodium acetate (pH 4), containing 1 mmol/l EDTA, 0.2 mmol/l PMSF and 0.5 mmol/l NaCl. The mixture was centrifuged at $27\,000 \times g$ for 3 min at 5 °C and a second elution step was carried out, but the buffer pH was 3. The supernatants of the two-elution were pooled and quickly brought to pH 6.8 with 2 mmol/l Tris addition. This fraction containing the renin eluted from the column was named RH6.

To verify the purification grade and differences in its electrophoresis pattern, the SDS-PAGE of the fraction RH5 and RH6 was performed.

The same experimental procedure was applied to purify prorenin from hog corpora lutea (CL). Hog ovaries were collected in the slaughtering house and transported in a cool container. CL were removed from the rest of the ovary, homogenized at 4 °C in 0.154 M NaCl (100 mg/ml) in an Ultra-Turrax homogenizer, centrifuged at $24\,000 \times g$ for 30 min and kept at −20 °C if not immediately used.

2.2.3. PreR-Co purification

PreR-Co was purified from nephrectomized rat plasma by $(NH_4)_2SO_4$ precipitation, gel filtration on Sephacryl S-200 HR, immunoaffinity chromatography, and ion-exchange chromatography as previously described (Vincent and De Vito, 1999).

2.2.4. Effect of PreR-Co on R4, RH5 and RH6 fractions

Ten microliters of each fraction were incubated in a shaker water bath for 15 min at 37 °C with 50 μl of 50 mmol/l of phosphate buffer (pH 7.4) (control) or with 50 μl of *PreR-Co* solution (1 mg protein/ml). After that, renin concentration in the samples was measured by incubating with 420 μl of angiotensinogen solution (2 μg Ang I) and the Ang I generated was measured by RIA. Incubations at 4 °C were included to check for the presence of Ang I background. Results were expressed as ng Ang I/ml per h.

2.2.5. Identification of the product generated by PreR-Co

Forty microliters (10 μg protein) of RH5 fraction were incubated with 50 μl of *PreR-Co*, during 15 min at 37 °C and then applied to H77-Sepharose gel as previously described. The elution from the gel was dialyzed, concentrated and submitted to SDS-PAGE together with R4 and RH5 fractions. Gel was divided in two halves and the same profile was repeated in the other half of the gel and blotted onto a polyvinylidene fluoride membrane. One half of the membrane was stained with Coomassie Blue and the other half submitted to a Western Blot by using as primary antibody an anti-rat renin antibody and as secondary antibody an anti-rabbit IgG linked with alkaline phosphatase.

2.2.6. In vitro prorenin activation by PreR-Co

Prorenin was measured by a method of Vincent and De Vito (1999). Five-microliter sample (stock solution 100 mg tissues/ml) were placed into Eppendorf tubes containing 10 μl of 50 mmol/l phosphate buffer (pH 7.4), 5 μl of 40 mmol/l NEM and 5 μl of 80 mmol/l EDTA. Twenty-five μl (5 μg protein) of *PreR-Co* solution or phosphate buffer (control) were added and the mixtures were incubated in shaker water bath for 15 min at 37 °C. Renin was measured as described previously.

2.2.7. In vitro prorenin activation by trypsin

Prorenin was measured by incubating 22 μl of diluted extract (1 mg/ml tissue) during 10 min at 4 °C with 10 μl trypsin solution (168 μg/ml final concentration), in the presence of 0.75 mg/ml bovine serum albumin and 7 mmol/l $CaCl_2$ (final concentration). The reaction was stopped by adding SBTI (350 μg/ml) and incubated for 10 min at room temperature. After that, renin was measured in the samples with and without trypsin activation.

3. Results

3.1. Prorenin purification steps and effect of PreR-Co on R4, RH5 and RH6 fractions

Fig. 1 shows that the R4 fraction includes both active renin and an inactive precursor (prorenin), since a three-fold increase of active renin was observed after activation with *PreR-Co*. When R4 fraction was applied to H77-Sepharose chromatography, the supernatant (RH5 fraction) showed that the active renin was efficiently linked to the column since no renin activity was found and only the prorenin activated by *PreR-Co* remained. However, the acid elution of H-77 Sepharose gel (fraction RH6) showed that active renin was effectively eluted. In addition, the treatment of this fraction with *PreR-Co* did not produce an increase in renin concentration, indicating the absence of inactive precursors and also that *PreR-Co* was unable to interact with the active renin.

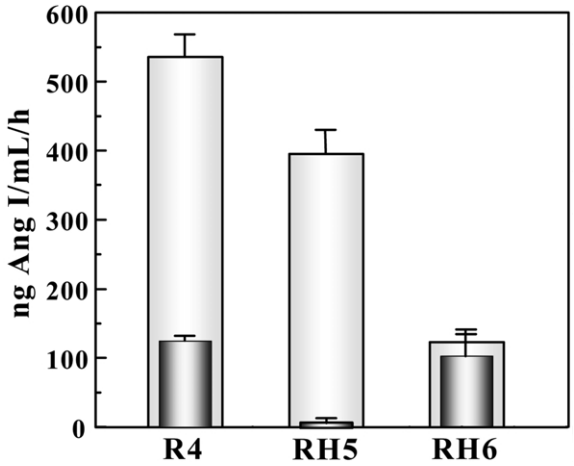

Fig. 1. Bars represent the amount of active renin before (inner, darker bars) and after treatment with *PreR-Co* (lighter bars). R4 is the protein fraction before the passage through the H77-Sepharose column. RH5 is the supernatant (prorenin not bound to gel) and RH6 is the fraction eluted from column by low pH treatment (renin bound to gel).

3.2. SDS-PAGE of fractions and identification of the product generated by PreR-Co

The SDS-PAGE of the RH5 and RH6 fractions showed 43 kDa and 35 kDa bands, respectively (Fig. 2a). The difference between the molecular masses of these bands is coincident with that of rat prorenin propeptide size.

The product resulting to the incubation of RH5 with *PreR-Co* and to the elution of H77-Sepharose showed a 35-kDa band (Fig. 2b), which was recognized by the rat renin antibody in the Western blot as well as a 43-kDa band (Fig. 2C). The R4 fraction showed two principal bands of 43 and 35 kDa that were recognized by the antibody.

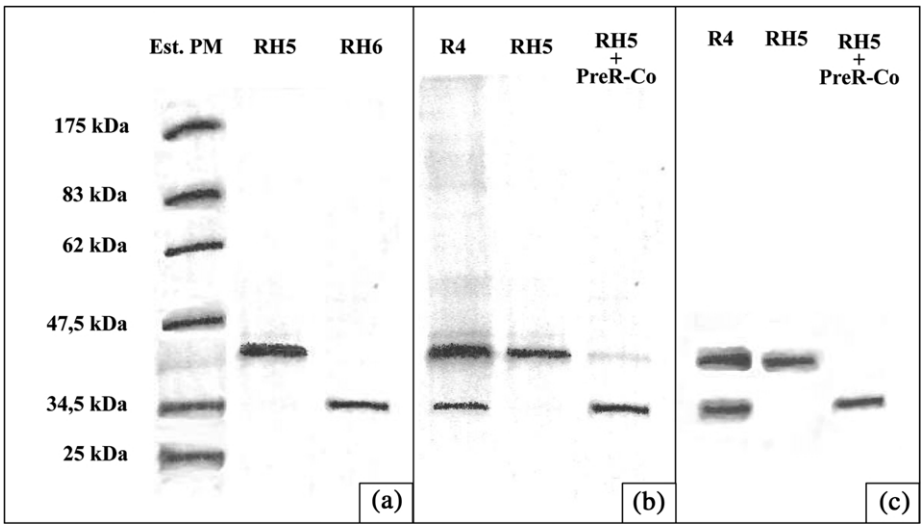

Fig. 2. (a) SDS-PAGE of different fractions from kidney renin purification. RH5 is the fraction not bound to H77 column. RH6 is the one bound and eluted at low pH. (b) SDS-PAGE of the R4, RH5 fractions and RH5 after incubation with *PreR-Co.* Panels a and b show the bands stained with Coomassie Blue. (c) The same profile that panel B but submitted to a Western blot.

3.3. Activation with PreR-Co of different tissues homogenates

Activation of inactive renin from tissues other than kidney was performed using ovary, corpora lutea, uterus and adrenal gland. 0.54 ± 0.06 and 0.86 ± 0.09 ng Ang I/mg tissue per h before and after treatment with *PreR-Co*, respectively (58.5% increase) in ovary; 0.51 ± 0.05 and 0.95 ± 0.14 ng Ang I/mg tissue/h before and after treatment with *PreR-Co*, respectively (87% increase) in corpora lutea; 0.83 ± 0.14 and 1.84 ± 0.32 ng Ang I/mg tissue per h before and after treatment with *PreR-Co*, respectively (120.7% increase) in uterus; 0.88 ± 0.14 and 1.17 ± 0.19 ng Ang I/mg tissue per h before and after treatment with *PreR-Co*, respectively (33.8% increase) in adrenal tissue and 80% increase in a single homogenate of Hog CL (Fig. 3a) were found. Nevertheless activation with trypsin of rat CL and adrenal gland, promoted higher activation (580% and 736% respectively). However, a purified fraction of prorenin from hog CL (RH5), was fully activated by *PreR-Co* (Fig. 3b). The active fraction was isolated in the purification procedure by monitoring the eluted aliquots with trypsin and *PreR-Co* activation. When the fraction RH5 (42 kDa) obtained from CL was incubated with *PreR-Co* and after elution from H77 column applied to SDS-PAGE, it showed a 32 kDa band (see insert of Fig. 3b) coincident with the mass of the RH6 fraction (active renin).

4. Discussion

Two main conclusions emerged from the present study. First, that the enzyme (*PreR-Co*) described in an earlier study from this laboratory (De Vito et al., 1996; Vincent and De Vito, 1999), which is capable of activating inactive renins in kidney homogenates, was competent to interact with purified prorenin. Second, this enzyme cleaves also inactive renin extracted from different tissues, such as ovary, corpora lutea, uterus and adrenal gland. Nevertheless, the amount of active renin generated by *PreR-Co* in these tissue homogenates, was lower than those produced by trypsin activation.

At present, we are unable to explain the fact that trypsin yield a larger active renin than *PreR-Co*. It is well known that activation of inactive renins by trypsin is a widely adopted procedure; however, trypsin is highly unspecific and, furthermore, could promote formation or products that may interfere with the results. In a previous report we, provided evidence that *PreR-Co* was unable to interact with plasma prorenin (Vincent

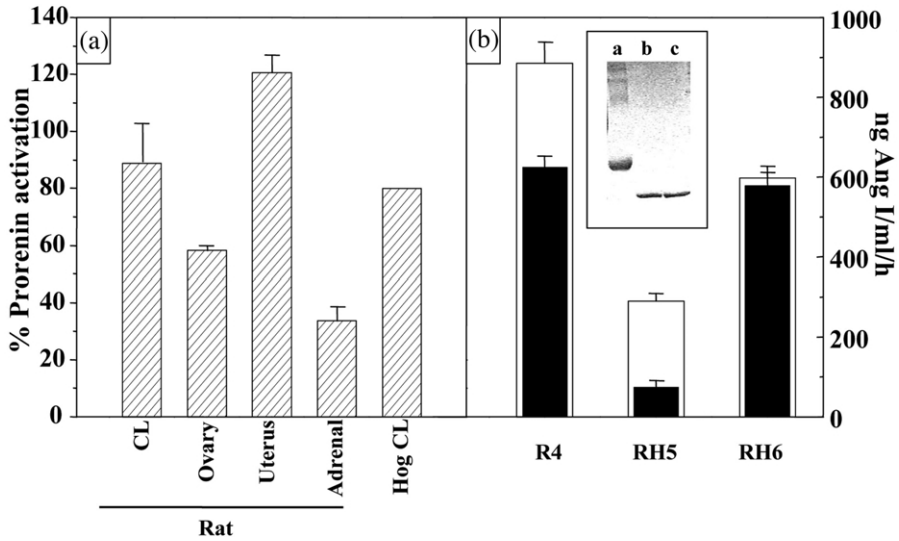

Fig. 3. (a) The mean ± S.E. percentage of inactive renins activation of different tissue homogenates: Corpora lutea, ovary, uterus and adrenal gland of rats ($n = 4$ of each one) and a single homogenate of corpora lutea of hog). (b) The amount of renin (Black bars) and prorenin activated by *PreR-Co* (White bars) in purified fractions of hog copora lutea. R4 is the fraction before H77 treatment, RH5 is the fraction not bound to column and RH6 the one eluted by low pH. The insert in panel b exhibits the SDS-PAGE of the RH5 (lane a), RH6 (lane b) fractions and RH5 incubated with *PreR-Co* (lane c).

and De Vito, 1999); hence, it is tempting to speculate that a high proportion of inactive renin in these tissues may be captured from plasma. Uptake of prorenin from the circulation by cardiac cells has been reported (van Kesteren et al., 1997; Saris et al., 2001) and it is not inappropriate to extend this concept to other cell membranes.

Renin has been found in tissues other than the kidney such as adrenal tissue (Mulrow, 1993), uterus, ovary (Lumbers, 1993) and corpora lutea (Cabrera et al., 1986), where its local synthesis was established. However, processing of renin in tissues appears to be less complete than that in the kidney; furthermore, inactive renin is a heterogeneous protein due mainly to variable glycosylation (Khalidi and McKenzie, 1991), and we cannot rule out the possibility that differential glycosylation of the constitutive pool protects the cleavage site from attack by the enzyme.

The present results reveal that the H77 column efficiently bound active renin that after elution was not attacked by *PreR-Co*. On the contrary, the supernatant (prorenin) not bound by the column was entirely activated by the enzyme as evidenced by the M.W. shift on SDS-PAGE. As expected, SDS-PAGE of fractions RH5 (supernatant, unbound prorenin) and RH6 (acidic elution from gel) exhibited a single band of 43 kDa and 35 kDa, respectively; both recognized by the specific anti rat renin antibody. The difference between the molecular weights is equivalent to approximately 70 amino acid, the size of the rat prorenin propeptide. Furthermore, the product of *PreR-Co*-activated prorenin showed a molecular weight analogous to that of renin, and was also recognized by the specific antibody, demonstrating that the final product of activation is true renin.

The precise identity of the enzyme involved in the conversion of prorenin into renin is at present unknown. Our results strongly support the concept that *PreR-Co* appears to be biologically more significant than the enzymes collectively known as 'prorenin converting enzymes' (Kim et al., 1990, 1991; Kikkawa et al., 1998; Hosoi et al., 1998) (which can only process prorenin from the mice submandibular gland) and more significant also than the 'proprotein convertases' (PC) (Jutras et al., 1997; Laframboise et al., 1997), whose activity on renal tissue is poorly supported. In addition, the enzyme-substrate kinetics for these enzymes has not been established. On the contrary, *PreR-Co* exhibits a high affinity for tissue prorenin and is able to transform it in a short period of time (Vincent and De Vito, 2000).

In conclusion, the identity of the enzyme involved in prorenin activation remains unknown. However, we believe that *PreR-Co* is a good candidate for that role, because it acts on all the tissue prorenins tested, including those isolated from corpora lutea. The fact that *PreR-Co* does not process plasma prorenin is in accordance with previous reports suggesting that plasma prorenin is not the source of circulating active renin (Mullins et al., 1990; Lenz et al., 1991). The role of plasma prorenin and extrarenal tissues prorenin is obscure and further investigations are necessary to clarify this matter.

Acknowledgements

This study was funded by grants from the Consejo Nacional de Investigaciones Científicas y Técnicas de la República Argentina (CONICET) and Consejo de Investigaciones de la Universidad Nacional de Tucumán (CIUNT).

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