In Vivo Enzymatic Assay Reveals Catalytic Activity of the Human Renin Precursor in Tissues

Danielle Methot, David W. Silversides, Timothy L. Reudelhuber

Abstract—The aspartyl protease renin is secreted into the circulation of mammals in 2 forms: the proteolytically processed active form of the enzyme and the precursor form, prorenin. Prorenin has no detectable enzymatic activity in the circulation, but it is the exclusive form of the enzyme produced by several tissues that also produce the other components of the renin enzymatic cascade (renin-angiotensin system). To test whether prorenin might be enzymatically active in these tissues, transgenic mice expressing the human renin substrate (angiotensinogen) exclusively in the pituitary gland were mated to mice expressing either active human renin or prorenin in the same tissue. Measurement of in vivo product formation in pituitary glands of double-transgenic mice revealed that human prorenin was enzymatically active, and Western blot analysis demonstrated that this prorenin was in the precursor form with its prosegment attached. This in vivo enzymatic assay demonstrates for the first time that human prorenin can be activated within tissues by nonproteolytic means, where it could contribute to the activity of a localized renin-angiotensin system. (Circ Res. 1999;84:1067-1072.)

Key Words: renin ■ prorenin ■ angiotensin

Many proteases are first synthesized as precursors containing a prosegment that serves not only as a folding catalyst or scaffold for the nascent protein, but also as an endogenous inhibitor to restrain the proteolytic activity of the protein until it has reached its target destination. Likewise, the aspartyl protease renin is synthesized as a proenzyme precursor (prorenin), and the renin present in the circulation is derived by the proteolytic removal of a 43-amino acid N-terminal prosegment exclusively in the kidney. Renin plays a key role in the regulation of blood pressure and in numerous cardiovascular pathologies, such as hypertension, cardiac hypertrophy, and renal defects, through the cleavage of angiotensinogen into angiotensin I (Ang I), which is the rate-limiting step in the renin-angiotensin system (RAS; Figure 1A).

Prorenin is also present in the circulation of mammals at levels that often largely exceed the concentration of renin. This prorenin is secreted not only from the kidney but also from numerous other tissues, including adrenal and pituitary glands, brain, eyes, ovaries, testes, uterus, and placenta. Moreover, these tissues express the other components of the RAS, raising the possibility that local RASs may exist that can contribute to tissue RAS activity and cardiovascular pathologies.

We have developed a double-transgenic mouse approach to test for the enzymatic activity of human prorenin (Figure 1B). In this system, the renin substrate (human angiotensinogen) and its potential protease (either human active renin, human native prorenin, or human noncleavable prorenin) are expressed in a specific cell type (pituitary somatotrophs) of individual lines of mice. Selected lines of single-transgenic mice were mated, and pituitary glands were excised from double-transgenic offspring expressing both human angiotensinogen and I of the human prorenins. Tissue content of Ang I (the product of the reaction) was determined as an indicator of renin enzymatic activity.

Materials and Methods

Plasmid Constructions and Generation of Transgenic Mice

Human prorenin14 and human angiotensinogen15 cDNAs were expressed under the transcriptional control of a 320-bp fragment of rat...
Expression of Transgenes

RNA was isolated from whole tissues of male mice by the acid guanidinium thiocyanate-phenol chloroform method. To prepare labeled RNA probes, human prorenin from nucleotides 401 to 650 of the cdNA and human angiotensinogen from nucleotides 178 to 377 of the cdNA were subcloned in the Bluescript II KS+ plasmid (Stratagene). RNase protection assays were carried out by using the Promega Riboprobe Gemini System (Promega Corp) according to the manufacturer’s protocol. To compare prorenin expression levels in the different transgenic lines, unquantified total RNA from 2 pooled pituitary glands was hybridized with 5×10⁷ cpm ³²P-labeled RNA probe for human prorenin and 5×10⁶ cpm ³²P-labeled RNA probe for human angiotensinogen overnight at 45°C in hybridization buffer containing 80% formamide. Yeast RNA (5 µg) and RNA from nontransgenic mouse pituitary glands were treated similarly to negative controls, and RNA products from in vitro transcription of human prorenin and human angiotensinogen were hybridized as positive controls. To test for tissue specificity of transgene expression, 5 µg of total RNA from brain, liver, kidney, and heart were also hybridized under the same conditions. Protected fragments after RNase A and T1 digestion were fractionated on a 6% polyacrylamide gel and were then exposed to x-ray film for 5 days at −80°C using intensifying screens. The protected fragments of prorenin and angiotensinogen were 250 and 200 nucleotides in length, respectively. Relative expression of prorenin in the various double-transgenic lines was estimated by comparing the expression of human prorenin with that of human angiotensinogen (all animals were bred against the same human angiotensinogen-producing line). Animals were classified as expressing more prorenin than angiotensinogen (+ + +), equivalent prorenin and angiotensinogen (+ +), or less prorenin than angiotensinogen (+ −)/−).

Determination of Pituitary Ang I Content

Pituitary glands surgically excised from 5 male mice were pooled and immediately sonicated in cold 1% trifluoroacetic acid (TFA) solution (pH 1.5) and cleared by centrifugation, and their peptide content was separated by HPLC. The HPLC separation was performed on a C18 column (Waters Corp). Solvent A consisted of 0.1% TFA in water; solvent B, 0.1% TFA in acetonitrile. Flow rate was 1 mL per 47 minutes, and 40 fractions were collected using a gradient of 20% to 40% solvent B over 40 minutes. Pituitary Ang I content of the eluted fractions was measured by an Ang I RIA (DuPont NEN) and was detected in the identical fraction as a commercial Ang I standard. The acid extraction used was designed to avoid in vitro activation of prorenin, activation of released proteases, and degradation of angiotensin peptides. Ang I recovery using this method was determined to be ~70%. Statistical analysis was performed by 1-way ANOVA with Dunnett posttest.

Western Blot Analysis of Human Prorenin

Pituitary glands from 2 male transgenic mice were surgically excised and lysed in 450 µL of a buffer containing (in mmol/L) Tris (pH 8.0) 10, NaCl 10, and EDTA 1 and 0.1% SDS. The lysates were passed repeatedly through a 25-gauge needle and were immunoprecipitated with a rabbit polyclonal antibody to prorenin and renin. Immunoprecipitated proteins were separated by SDS−10% PAGE, and human prorenin and renin were detected by Western blot with a mouse monoclonal antibody specific for human prorenin and renin (a gift from D. Lamarre, Bio-Mega, Quebec, Canada) using a chemiluminescence kit (SuperSignal, Pierce).

Renin Activity in Transfected Cells

Rat pituitary GH₄C₁ cells were grown in DMEM supplemented with 5% (vol/vol) FCS, 0.1% serXtend (Irvine Scientific), and 10 µg/mL gentamicin (Life Technologies, Inc) in a humidified incubator (5% CO₂, 95% air) at 37°C. GH₄C₁ cells, plated at 1×10⁶ cells per 35-mm dish, were transfected 20 hours after plating with lipofectin (Life Technologies, Inc) in serum-free medium using 18 µg of human angiotensinogen plasmid DNA alone or in combination with either 2 µg of human active renin plasmid DNA or 2 µg of human active renin promoter. 16 Intron and polyadenylation signals for the transcribed RNA are provided by a portion of the simian virus 40 growth hormone promoter. 16 Double-transgenic mice expressing human angiotensinogen (the renin substrate) and human active renin, human native prorenin, or human noncleavable prorenin in the pituitary gland were generated, and the mice were assayed for increased pituitary content of Ang I.

Figure 1. A, Schematic representation of the RAS. AGEN indicates angiotensinogen; ANG I, angiotensin; and ACE, angiotensin-converting enzyme. B, Schematic representation of the approach used to test for enzymatic activity of prorenin in vivo. Double-transgenic mice expressing human angiotensinogen (the renin substrate) and human active renin, human native prorenin, or human noncleavable prorenin in the pituitary gland were generated, and the mice were assayed for increased pituitary content of Ang I.

Figure 2. Diagram of transgenes used in this study. Mutations relative to the native prorenin cleavage site are underlined. rGH indicates rat growth hormone promoter; PRO, human prorenin prosegment; AOGEN, angiotensinogen; and SV40, simian virus 40 T antigen splice and polyadenylation signal. Arrow indicates expected and putative cleavage sites for the various prorenins; question mark indicates questionable cleavage; and crossed-out arrow indicates no cleavage. Drawings are not to scale. See text for details.
native prorenin plasmid DNA per dish. After 20 hours, cells were transferred to 12-well plates in medium with serum. Twenty-four hours later the supernatants were collected and boiled for 2 minutes, and the cells were lysed in a solution of 0.5% Triton X-100 in Tris-buffered saline and boiled. Ang I content was measured by an Ang I RIA (DuPont NEN).

Results

Transgene expression was targeted specifically to the somatotrophs of the pituitary gland by putting the human renin and angiotensinogen cDNAs under the transcriptional control of the rat growth hormone gene promoter. Human prorenin and human angiotensinogen mRNA were monitored in various tissues (brain, heart, kidney, liver, and pituitary gland) and were detectable only within the pituitary gland (Figure 3), confirming the anterior pituitary gland–specific activity of the rat growth hormone promoter.16,20,21 We have studied 8 independent lines of animals expressing the 3 different types of human prorenin transgenes. The relative levels of transgene expression in each line were compared by quantifying mRNA transcripts in the pituitary glands of transgenic mice (Figure 4). Because all of these lines were crossed with the same human angiotensinogen-expressing line, the angiotensinogen mRNA signal was used as an internal reference to compare the level of prorenin mRNA expression in the various lines. Because of the species-specific reactivity between renin and angiotensinogen,22,23 it is possible to express the 2 human proteins in the mouse without producing compounding effects from the endogenous mouse RAS components. Measurement of pituitary Ang I in this system (Figure 1B) is a direct indicator of renin activity within the same tissue, given that Ang I released into the circulation would be rapidly diluted and converted to Ang II by the angiotensin-converting enzyme present in vascular endothelium (Figure 1A). Human prorenin and human renin were undetectable in the plasma of transgenic lines (data not shown).

To test for the Ang I–generating activity of human active renin in this system, we engineered a mutation in human prorenin (Figure 2) that renders the prosegment sensitive to removal by a ubiquitous protease (furin) present in the secretory pathway.24–26 The advantage of this approach for generating active renin is the conservation of the prosegment during the early stages of protein biosynthesis, when it is required for efficient protein folding,14 while allowing the removal of the prosegment before secretion in a broad variety of cell types17 (also D. Methot et al, unpublished results, 1998). Mice expressing both human active renin and human angiotensinogen have marked increases in pituitary Ang I content (Figure 5).

Figure 3. Pituitary gland–specific expression of the human renin transgene. Total RNA from the designated tissues of transgenic line 34 (expressing native human prorenin) was hybridized with a human prorenin-specific antisense RNA probe. *Nonspecific bands arising from digestion of the probe. PRO indicates the expected position for the human prorenin-protected fragment after RNase digestion.

Figure 4. Relative expression of the human transgenes in various transgenic lines. Comparison of the mRNA levels of 8 independent double-transgenic lines of animals expressing human angiotensinogen (AOG1N) and 1 of the 3 different human prorenins (active renin, native prorenin, or noncleavable prorenin) by RNase protection assay. Each of the lines generated with the different human prorenin transgenes was crossed with the same angiotensinogen-expressing line, and the angiotensinogen mRNA signal was used as an internal reference to compare the different prorenin mRNA levels. M indicates size marker; NT, nontransgenic control; and ST, standards. Expected positions for the human prorenin (PRO)–protected and human angiotensinogen-protected fragments are designated at the right. *Non-specific bands.

Figure 5. Pituitary content of Ang I in double-transgenic mice. Pituitary Ang I content was determined in pituitary glands of transgenic mice expressing either human angiotensinogen alone (open bar) or in combination with human active renin (hatched bar), human native prorenin (solid bar), or human noncleavable prorenin (stippled bar). Ang I content was not detectable from either nontransgenic mice or individual single-transgenic mice expressing only the various prorenins. Relative levels of prorenin transgene expression in the various lines (derived from Figure 4) are indicated by + and − signs shown above the bars (see Materials and Methods for classifications). Each data point is derived from a pool of 5 pituitary glands, and results are expressed as the quantity in picograms of Ang I/pituitary gland. Data are mean±SE. **P<0.01; *P<0.05 vs mice expressing only human angiotensinogen by 1-way ANOVA with Dunnett post-test. AOG1N indicates angiotensinogen.
content (Figure 5, hatched bars) as compared with single-transgenic mice expressing only human angiotensinogen (Figure 5, open bar). Pituitary Ang I was not detectable in any of the transgenic mice expressing only the various human prorenins (not crossed with angiotensinogen-expressing mice) or in nontransgenic controls. Repeated assays within the same transgenic lines yielded highly reproducible results and demonstrate that transgenic mice can be used as a sensitive measure for tissue-specific activity of human renin.

We then tested whether prorenin itself could generate Ang I under these conditions by crossing the same line of angiotensinogen-expressing mice with lines of mice expressing human native prorenin. Interestingly, these double-transgenic mice also have clearly elevated pituitary Ang I content (Figure 5, solid bars), which suggests that human native prorenin is enzymatically active in the pituitary gland. The relative amounts of Ang I generated in the pituitary glands of the various crosses show that both native prorenin and noncleavable prorenin are capable of generating Ang I in the in vivo assay (Figure 5). Second, enzymatically active prorenin shows the capability of the enzyme to cleave its substrate in the pituitary glands of double-transgenic mice is that the 2 proteins meet in the secretory pathway of expressing cells before the prorenin has completed its folding to the repressed state. To test whether Ang I generation could occur intracellularly, cultured GH4C1 (rat somatotroph) cells were cotransfected with expression vectors for human angiotensinogen either alone or with one of the human prorenins. The results (Table) show that coexpression of human angiotensinogen and human active renin leads to the detection of Ang I in the supernatant of transfected cells, but not in cell lysates. In contrast, cotransfection of human angiotensinogen and human native prorenin does not lead to generation of Ang I either in the cell culture supernatant or within the transfected cells. These results suggest that angiotensinogen cleavage does not occur within the cell and that, unlike renin and prorenin expressed in the in vivo model, prorenin is unable to carry out the cleavage of angiotensinogen after secretion into the serum-containing culture supernatant.

Discussion

Using a sensitive and specific in vivo enzymatic assay, we have obtained 2 independent lines of evidence that human prorenin does not need to be proteolytically cleaved to display enzymatic activity within the pituitary gland. First, both native prorenin and noncleavable prorenin are capable of generating Ang I in the in vivo assay (Figure 5). Second, Western blot analysis of enzymatically active prorenin shows that it retains its prosegment (Figure 6). These findings strongly suggest that prorenin can be activated within tissues by means other than proteolytic cleavage. Although the actual mechanism whereby prorenin is activated in tissues remains to be elucidated, it is highly likely that the prosegment, which is predicted to fold over the substrate-binding cleft of the enzyme, is able to unfold and to transiently expose the active site of the enzyme. The conditions favoring this activation must be particular to the extracellular space in tissues, because prorenin catalytic activity is not detectable in either the circulation or in the lysates or supernatants of

<table>
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<tr>
<th>Transfected Expression Plasmid</th>
<th>Supernatant Ang I, pg/mL</th>
<th>Cellular Ang I, pg/mg Protein</th>
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<tbody>
<tr>
<td>Angiotensigen alone</td>
<td>173</td>
<td>ND</td>
</tr>
<tr>
<td>Angiotensigen and active renin</td>
<td>1645</td>
<td>ND</td>
</tr>
<tr>
<td>Angiotensigen and prorenin</td>
<td>191</td>
<td>ND</td>
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*Cell lysates and supernatants were collected 24 hours after transfection. Results represent the mean of 2 samples derived from a single experiment. Findings were confirmed in 4 independent transfection assays. ND indicates nondetectable.
cells cotransfected with prorenin and angiotensinogen (Table).

Ang II has been proposed to play a role in the modulation of secretion of several pituitary hormones, including adrenocorticotropic hormone, prolactin, and growth hormone. However, in our study, mice with dramatically elevated levels of pituitary Ang I (the immediate peptide precursor of Ang II) show no reproducible effects on growth, reproduction, blood pressure, or pituitary hormone content or distribution by immunostaining (data not shown). These results might be explained by one of the following factors. First, we have targeted expression of the transgenes to somatotropes in this study, whereas in rodents the endogenous renin gene is expressed in gonadotropes, perhaps leading to a different tissue distribution of produced peptides. Second, we have noted that pituitary Ang II levels were not reproducibly elevated in mice expressing very high amounts of Ang I, which suggests that the necessary Ang II–generating enzyme (see Figure 1) might be limiting. Finally, our results could point to physiological differences in the role of angiotensin peptides in pituitary hormone secretion between the mice used in this study and the much more thoroughly characterized rat model.

Prorenin is the only human aspartyl protease that does not cleave its own prosegment. Autocatalysis in other aspartyl proteases is thought to be initiated by a transient unfolding of the prosegment on contact of the precursors with the acid pH pro tease is thought to be initiated by a transient unfolding of the prosegment of its substrate. In vitro, purified prorenin can be induced to exhibit reversible enzymatic activity by prolonged exposure to acid pH or cold storage. Thus, I possible explanation for the prorenin activity seen in tissues could be the slightly more acidic pH found in tissue interstitial spaces as compared with the circulation. Alternatively, serum proteins might be responsible for maintaining the inactive conformation of prorenin in both the circulation and in tissue culture supernatants.

In certain body fluids, such as ovarian follicular fluid, chorionic fluid, amniotic fluid, and seminal fluid, as well as in the ovarian and adrenal veins, prorenin concentrations reach many times the levels found in plasma. Ang II is also found in higher concentrations than in circulation in tissues such as the adrenal gland, the pituitary gland, and others, and the current study suggests that it may be produced locally by tissue prorenin. A transient unfolding of the prosegment of prorenin to exhibit enzymatic activity within tissues might also allow modulation by local physiological and pathophysiological conditions.

Heart and vascular tissues have also been shown to take up renin and prorenin from the circulation, and a specific receptor for these has been reported. The ability of tissues to activate prorenin by a nonenzymatic mechanism, as well as the ability to capture circulating prorenin and renin, strengthen the possibility that tissue RASs function in a locally restricted manner.

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