Evidence Supporting a Functional Role for Intracellular Renin in the Brain

Julie L. Lavoie, Xuebo Liu, Robert A. Bianco, Terry G. Beltz, Alan Kim Johnson, Curt D. Sigmund

Abstract—The brain renin–angiotensin system is implicated in the regulation of blood pressure (BP) and fluid homeostasis. Recent studies reveal that 2 forms of renin are expressed in the brain of rodents and humans: secreted prorenin and a nonsecreted intracellular form of active renin (icREN). Although the intracellular action of renin has long been postulated, no data supporting its role in BP regulation has been reported. Therefore, we directly evaluated whether this form of renin has physiological implications for BP regulation by characterizing transgenic mice expressing human icREN driven by the glial fibrillary acidic protein (GFAP) promoter and comparing it with similar mice expressing the secreted form of renin. GFAP-icREN mice express hREN primarily in the brain and at the same level of expression as GFAP-secreted prorenin. Unlike the secreted form, which can be detected in cerebrospinal fluid, no human renin could be detected in the cerebrospinal fluid of GFAP-icREN mice. GFAP-icREN mice were then bred with transgenic mice expressing human angiotensinogen, also driven by the GFAP promoter. Double-transgenic mice expressing either the intracellular renin (2.0 ± 0.12 mL/10 g/day) or secreted renin (2.8 ± 0.3 mL/10 g/day) exhibited an increase in drinking volume compared with nontransgenic littermates (1.5 ± 0.1 mL/10 g/day). Both models exhibited an increase in mean arterial pressure (137 ± 5 and 133 ± 8 mm Hg, respectively) compared with control littermates (115 ± 3 mm Hg), which could be rapidly reduced after ICV injection of losartan. These data support the concept of an intracellular form of renin in the brain, which may provoke functional changes in fluid homeostasis and BP regulation. (Hypertension. 2006;47:461-466.)

Key Words: renin-angiotensin system  ■  brain

The renin–angiotensin system (RAS) is well known for its effects on blood pressure (BP) and fluid homeostasis. Although these actions were long thought to be caused primarily by the circulating RAS, in the past decade, it has become apparent that local RAS have an important physiological role. For instance, all components of the RAS are present in the heart, kidney, adrenal gland, and brain (reviewed in Reference 1). It is now becoming clear that cells expressing renin and angiotensinogen (AGT) are in close proximity to each other and to cells expressing angiotensin type 1 (AT1) receptors in a number of tissues including the proximity to each other and to cells expressing angiotensin type 1 (AT1) receptors in a number of tissues including the proximal tubules of the kidney, the outer medulla of the adrenal gland, and the brain.2,3 This has led us and others to question whether the RAS can function intracellularly either through the intracellular production or intracellular action of angiotensin II (Ang II). Interestingly, the intracellular action of Ang II was proposed as early as 1971 when Robertson and Khairallah4 reported nuclear localization of the peptide in perfused rat liver. More recently, Re5 and others6 have described Ang II as part of a class of peptide hormones that act intracellularly, as an “intracrine.”7-6

Circulating Ang II may act intracellularly by binding to AT1 receptors, which are subsequently internalized,7,8 and Ang II–dependent internalization of the AT1 receptor has been reported in neurons.9 This suggests that extracellular Ang II is first internalized after ligation of the AT1 receptor. However, there has been the suggestion that Ang II may be synthesized within the cell and, thus, acts intracellularly. First, it has been reported that renin can be internalized using the mannos-6-phosphate receptor, although some have suggested that this might act simply as a clearance mechanism.10 A receptor for renin and prorenin has also been identified recently.11 Other compelling evidence in support of intracellular production of Ang II comes from studies reported by our laboratory and others that an alternative form of renin mRNA exists in several tissues.12-14 This altered form of the protein lacks the signal peptide and, thus, is not secreted and the first 15 amino acids of the prosegment (and is, therefore, constitutively active) suggesting the formation of an intracellular form of active renin. The alternative REN (intracellular form of active renin; icREN) mRNA start site is located ∼6.2 kb upstream of the classic promoter and encodes a new exon 1 (termed exon 1b) that splices directly to exon 2. This forms a mRNA, which lacks the normal translational initiation

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codon in exon I, thus forcing the use of an in-frame ATG present in exon II. Alternative transcripts have been identified for REN in the adrenal gland and heart, as well as in the brain; and biochemical studies suggest that this form of REN is active enzymatically. Increased expression of the icREN was reported in the heart after myocardial infarction. This would support the hypothesis that there may be intracellular formation of Ang II in these different tissues. However, it is unknown whether this form of REN has any physiological relevance. We, therefore, directly tested the hypothesis that intracellular production of a nonsecreted active renin in the brain can participate in the regulation of BP and electrolyte homeostasis.

Methods

Generation of Glial Fibrillary Acidic Protein-icREN Transgenic Mice

The generation of the glial fibrillary acidic protein (GFAP)-secreted prorenin (sREN) and GFAP-icREN constructs, production of transgenic mice, and their genotyping is described in the online supplement to the Methods (available at http://hyper.ahajournals.org). Double-transgenic mice carrying either GFAP-icREN or GFAP-sREN and GFAP-human AGT (hAGT) were generated by breeding heterozygous mice. Nontransgenic age- and sex-matched littermates, sREN and GFAP-human AGT (hAGT) were generated by breeding transgenic mice, and their genotyping is described in the online supplement previously.

Plasma Renin Assay, Cerebrospinal Fluid Collection, and Western Blot Analysis

Plasma human renin concentration and plasma mouse renin activity (PRA) were determined in single transgenic mice as described previously. Radioimmunoassay was performed on plasma using Ang I-labeled radioimmunoassay kit (DiaSorin). Samples were diluted with reagent blank to remain on the linear portion of the standard curve. Collection of cerebrospinal fluid (CSF) from the brain was pooled together to harvest a sufficient amount of protein. The generation of the glial fibrillary acidic protein (GFAP)-secreted prorenin (sREN) and GFAP-icREN constructs, production of transgenic mice, and their genotyping is described in the online supplement previously. Mice used for these experiments were 15 to 20 weeks of age. All of the mice received standard mouse chow (LM-485; Teklab Premier Laboratory Diets) and water ad libitum unless specified. Care of the mice used in the experiments met the standard set forth by the National Institutes of Health in their guidelines for the care and use of experimental animals, and all of the procedures were approved by the University Animal Care and Use Committee at the University of Iowa.

Plasma Renin Assay, Cerebrospinal Fluid Collection, and Western Blot Analysis

Plasma human renin concentration and plasma mouse renin activity (PRA) were determined in single transgenic mice as described previously. Radioimmunoassay was performed on plasma using Ang I-labeled radioimmunoassay kit (DiaSorin). Samples were diluted with reagent blank to remain on the linear portion of the standard curve. Collection of cerebrospinal fluid (CSF) from the mouse was detailed in the online supplement. The CF of cerebrospinal fluid (CF) from the mouse is shown. The start site of transcription is indicated as ATG. The sREN transgene expresses full-length preprorenin that becomes active by virtue of a mutation that alters the normal prorenin-converting enzyme site to a furin (F) cleavage site. The icREN construct initiates translation at the ATG inside exon II making a truncated preprorenin protein lacking a signal peptide. The construct retains the normal prorenin-converting enzyme cleavage site (P). The transgene was excised as a BssHII fragment for microinjection. Insert shows a genotyping assay distinguishing GFAP-sREN (S) from GFAP-icREN (IC). 100-bp markers are shown (M).

Biological Analysis

BP and daily drinking measurements for individual mice were averaged to obtain each individual number. Data were then ex-

Figure 1. Schematic map of the GFAP-icREN construct. A schematic map of the transgene is shown. The start site of transcription is indicated as ATG. The sREN transgene expresses full-length preprorenin that becomes active by virtue of a mutation that alters the normal prorenin-converting enzyme site to a furin (F) cleavage site. The icREN construct initiates translation at the ATG inside exon II making a truncated preprorenin protein lacking a signal peptide. The construct retains the normal prorenin-converting enzyme cleavage site (P). The transgene was excised as a BssHII fragment for microinjection. Insert shows a genotyping assay distinguishing GFAP-sREN (S) from GFAP-icREN (IC). 100-bp markers are shown (M).
pressed as mean±SE. All of the comparisons were made with nontransgenic littermates. PRA was compared using Student’s t test, whereas BP and drinking were compared by ANOVA. A value of \( P<0.05 \) was considered statistically significant.

**Results**

Transgenic mice were generated using a human GFAP promoter fused to a cDNA encoding the alternative intracellular form of renin (Figure 1). To do so, we modified the classical hREN cDNA by PCR amplifying a fragment using a 5’ primer just distal to the internal ATG present in exon-2 and a 3’ primer downstream of the termination codon. Hence, this hREN does not contain any of the renin signal peptide and is missing the first third of the prosegment and, thus, should remain intracellular and be constitutively active as reported previously.13 Interestingly, an analysis of renin protein sequences revealed that the ATG in exon II encoding the initiator methionine of icREN is perfectly conserved in human, mouse, rat, sheep, and dog renin and is also retained in Zebrafish and Fugu renin, suggesting that an important function has been conserved evolutionarily (Figure 2).

Tissue-specific expression of the GFAP-icREN transgene was evaluated using RNase protection (Figure 3A). Although transgene mRNA was present in brain from mice from all 4 transgenic lines (data not shown), ectopic expression in other tissues varied considerably. Expression of icREN in line 19560/3 was almost exclusively localized to the brain with only a low level detected in the lung (Figure 3A). We recognized the importance of sacrificing the level of expression (which was higher in other lines) in favor of a model with brain-specific expression. We next measured plasma hREN concentration in the presence of excess exogenous hAGT to determine whether any ectopic expression of the transgene in tissues outside the brain resulted in a significant increase in hREN in the systemic circulation. We found no significant differences in human plasma renin concentration (which were at the background of the assay) when comparing transgenic with nontransgenic mice (Figure 4). Accordingly, it is unlikely that a significant amount of hREN protein is released into the systemic circulation in this transgenic model. We previously reported the similar absence of circulating hREN in mice expressing the secreted form of renin specifically in glial cells.16 Interestingly, there was a modest increase in endogenous PRA in the GFAP-icREN mice compared with nontransgenic littermates. The mechanism causing this increase remains unclear, especially provided that the measurement of plasma mouse renin was performed in single icREN transgenic mice where the level of systemic and brain Ang II should remain unaltered.

We next felt it was crucial to confirm that renin in GFAP-icREN was not secreted. To accomplish this, we measured hREN in the CSF collected from GFAP-icREN and compared this with CSF from nontransgenic control mice. As an additional control, we also measured CSF renin in transgenic mice expressing secreted renin encoded from the same promoter (GFAP-sREN),16 after first comparing the level of GFAP-icREN and GFAP-sREN mRNA expression in the brain. RNase protection assay revealed equivalent levels of transgene expression in both transgenic lines allowing us to make a direct comparison between the 2 mouse models (Figure 3B). As predicted, hREN, migrating at the same position as purified recombinant hREN, could be detected in the CSF of the GFAP-sREN mice (Figure 5). Conversely, no
specific hREN band was detected in the CSF of either GFAP-icREN or nontransgenic mice. This observation was replicated in separate experiments using independent CSF samples (Figure 5 left and right panels provide examples).

In order to evaluate the physiological consequence of icREN overexpression, we generated 2 double-transgenic mouse models and measured drinking, urine volume, and BP. The intracellular model was generated by cross-breeding GFAP-icREN with mice expressing hAGT from the GFAP promoter (GFAP-hAGT), whereas the secreted model was made by cross-breeding GFAP-sREN with GFAP-hAGT.16,21 The generation of the double-transgenic mice was necessary because of the species specificity of the renin-AGT reaction.22 Double-transgenic mice expressing icREN exhibited a significant increase in drinking volume compared with nontransgenic littermates, although the increase tended to be less than that observed in double-transgenic mice expressing sREN (Figure 6A). On the contrary, there was only a modest increase in urinary volume in the mice expressing icREN, which did not reach statistical significance, whereas a significant increase in urine volume was detected in mice expressing sREN (Figure 6B). There was no significant difference in the urine osmolality or concentration of sodium or potassium when comparing all 3 of the groups (Supplemental Table, available online at http://hyper.ahajournals.org). In addition, there was no significant difference in salt preference when comparing GFAP-icREN/GFAP-hAGT (31.8±10.0%), GFAP-sREN/GFAP-hAGT (35.4±9.4%), and nontransgenic control (31.5±6.7%)

mice. As expected, all of the single transgenic mice were phenotypically similar to nontransgenic littermates (data not shown). It is noteworthy that double-transgenic mice expressing either icREN or sREN exhibited a significant increase in mean AP compared with nontransgenic littermates (Figure 7A). Mean AP significantly decreased in both models after ICV injection of losartan (Figure 7B), whereas there was no effect of either ICV ACSF or IV losartan in any group of mice.

Discussion

We first became interested in the concept of intracellular Ang II synthesis after we and others identified an alternative form of renin mRNA in the mouse, rat, and human brain.12–14 This alternative mRNA lacks the normal translational initiation codon in exon 1, because the alternative first exon splices directly to exon 2 and, thus, forces the use of an in-frame ATG initiation codon in exon 2.16,21 The generation of the double-transgenic mice was necessary because of the species specificity of the renin-AGT reaction.22 Double-transgenic mice expressing icREN exhibited a significant increase in drinking volume compared with nontransgenic littermates, although the increase tended to be less than that observed in double-transgenic mice expressing sREN (Figure 6A). On the contrary, there was only a modest increase in urinary volume in the mice expressing icREN, which did not reach statistical significance, whereas a significant increase in urine volume was detected in mice expressing sREN (Figure 6B). There was no significant difference in the urine osmolality or concentration of sodium or potassium when comparing all 3 of the groups (Supplemental Table, available online at http://hyper.ahajournals.org). In addition, there was no significant difference in salt preference when comparing GFAP-icREN/GFAP-hAGT (31.8±10.0%), GFAP-sREN/GFAP-hAGT (35.4±9.4%), and nontransgenic control (31.5±6.7%)
after myocardial infarction.15 Interestingly, the first glimpse of a potential role of mitochondrial renin in steroid biosynthesis was suggested, there is no evidence supporting its function in adrenal or in any other tissue at this time.

In order to provide proof-of-principal that this alternative form of renin could play a role in BP and fluid homeostasis, we developed a transgenic mouse model that overexpresses intracellular renin specifically in astrocytes. Cellular specificity was provided by the GFAP promoter. Importantly, whereas a significant amount of human renin could be detected by Western blot in the CSF of mice expressing a secreted form of renin (GFAP-sREN), there was no CSF renin detected in mice expressing the intracellular form (GFAP-icREN). We felt it was necessary to confirm this experimentally, although the icREN lacked a signal peptide. We also confirmed that the sREN and icREN transgenes were being expressed at equivalent levels. This assured us that any differences in phenotypes measured would be because of the differences in secreted versus intracellular renin and not the level of expression of the transgenes.

We observed that the BP increase in mice expressing icREN was similar to the increase in mice expressing sREN. BP in both models was normalized by an ICV injection of losartan, whereas the same dose given systemically had no effect. This suggests that the effect observed was because of the local actions of Ang II in the brain, not from leakage of Ang II into the systemic circulation. This, coupled with our biochemical data showing no renin in CSF, suggests that icREN could cleave AGT intracellularly to produce angiotensin I (Ang-I), thus confirming the enzymatic activity of icREN tested previously in vitro.13 Still, what remains unclear is the fate of Ang I once it is produced inside the cell; for example, is it released into the extracellular space or converted to Ang II inside the cell? Intracellular dialysis of Ang I in myocytes from cardiomyopathic hamsters results in intracellular Ang II formation, which can be blunted by dialysis with an angiotensin-converting enzyme inhibitor.25 Also supporting intracellular Ang II generation is the observation that adult rat cardiomyocytes can internalize unglycosylated prorenin with subsequent intracellular formation of Ang II.26 In our experimental system, BP dropped rapidly after the addition of losartan, suggesting that the BP rise is because of activation of cell surface AT1 receptors. This would tend to favor a model of intracellular generation of Ang I (and perhaps Ang II) followed by release of the peptide (with subsequent conversion of Ang-I to Ang II) and ligation of AT1 receptors on adjacent neurons. One piece of data perhaps refuting this explanation is that the time required for the maximal depressor response to ICV losartan was longer in mice expressing icREN (73 ± 17 s) than sREN (41 ± 11 s), although this difference was not statistically significant. Does this signify intracellular uptake of losartan? It has been reported that losartan can be internalized by cell-surface receptor-mediated endocytosis.27,28 Other reports suggest that extracellular losartan cannot block the growth-promoting actions of intracellular Ang II in the heart.29

In addition to the effect on BP, double-transgenic mice expressing sREN and icREN both exhibited a significant increase in drinking volume compared with nontransgenic littermates. Because it has been reported that the effects of Ang II on thirst are mediated by the stimulation of AT-1 receptors present in organum vasculosum lamina terminalis, subfornical organ, and median preoptic nucleus, it is possible that overexpression of the transgenes in these specific areas might be involved.30

Perspectives
To our knowledge, this is the first observation describing a functional role for the alternative form of intracellular active renin in the brain. Consequently, this offers the possibility that an intracellular or intracrine RAS exists in this tissue. Re31 defines an intracrine as “a peptide hormone or factor that acts in the intracellular space either after internalization or retention in its cell of synthesis.” One implication of this definition is that Ang II has an intracellular function distinct from those caused by activation of cell surface AT1 or AT2 receptors. Intracellular Ang II has been implicated to stimulate the transcription of AGT, renin, platelet-derived growth factor, and the oncogene c-myc in response to binding to nuclear Ang II AT1 receptors.32 This definition also implies...
that extracellular Ang II is either internalized or directly synthesized in the same cell in which it acts. AT₁-receptor-dependent internalization of Ang II has been reported in a number of cell types, including neurons.⁷–⁹ Our studies using reporter genes indicate that some cells in the brain may coexpress renin and AGT.³ By extension, therefore, our data suggest the possibility that Ang-I, and perhaps Ang II, is formed intracellularly. Although this concept has been suggested previously to occur in the heart,²⁹ these are the first data suggesting that it may also occur in the brain. Additional studies will have to be performed to determine the exact mechanisms by which intracellular RAS regulates BP and electrolyte homeostasis and the relative importance of the classical and intracrine pathways. It will be important to determine whether this mechanism exists in neurons, in particular those neurons containing intracellular Ang II that project between nuclei controlling cardiovascular function and use Ang II as a neurotransmitter.

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