Identification of bona fide alternative renin transcripts expressed along cortical tubules and potential roles in promoting insulin resistance in vivo without significant plasma renin activity elevation

Tomoaki Ishigami, Tabito Kino, Lin Chen, Shintaro Minegishi, Naomi Araki, Masanari Umemura, Kaito Abe, Rie Sasaki, Hisako Yamana, Satoshi Umemura

Abstract—Renin belongs to a family of aspartyl proteases and is the rate-limiting enzyme in the synthesis of the potent vasoactive peptide angiotensin II. Processing of renal renin has been extensively investigated in juxtaglomerular granular cells, in which prorenin and active renin are present in secretory condensed granules. Previous studies demonstrated alternative renin transcription in rat adrenal glands. Different studies reported novel intracellular forms of renin deduced from novel 5′ variants derived from renin mRNA in both mice and humans. Comprehensive detailed studies in genetically engineered mice showed that both a secreted and an intracellular form of renin plays divergent mechanism regulating fluid intake and metabolism by the brain renin–angiotensin system; however, the presence, regulation, and functions of these renin isoforms in kidney and adrenal gland are not fully understood in mice. To investigate the characteristics of renin isoforms in mice, we performed a systematic inventory of renin transcripts of mice with and without a duplication of the renin gene alternatively from previous studies. We discovered a novel isoform of renin of the Ren2 gene, which conserved functionally important residues of the prosegment and incomplete isoforms of the Ren1C/D gene lacking a pre-pro segment. In situ hybridization assays revealed alternative renin isoforms expressed along cortical tubules. Newly generated transgenic mice with systemic overexpression of alternative renin transcript showed enhanced local angiotensin II generation without elevation of plasma renin activity and systemic insulin resistance in vivo, providing new pathophysiological insights into insulin resistance exaggerated by bona fide renin isoform. (Hypertension. 2014;64:125-133.) ● Online Data Supplement

Key Words: alternative transcript ■ insulin resistance ■ renin

Renin belongs to a family of aspartyl proteases and is the rate-limiting enzyme in the synthesis of the potent vasoactive peptide A-II. Renin is expressed and secreted into the blood stream by the juxtaglomerular cells of the kidney (Goormaghtigh cells). However, renin is also expressed in several other tissues, including brain, pituitary gland, adrenal gland, heart, arterial smooth muscle, and testis. Renin-producing cells in extrarenal tissue may play a part in local autocrine or paracrine tissue RAS. Such paracrine RAS contributes to fluid and electrolyte balance and may be important pathological mediators of cellular entities associated with hypertension and electrolyte abnormalities.

Renin is synthesized as preprorenin; enzymatic liberation of the prosegment generates the secreted inactive form. Removal of the prosegment generates active renin. Processing of renal renin has been extensively investigated in juxtaglomerular granular cells, in which prorenin and active renin are present in secretory condensed granules. However, the precise activating mechanisms of prorenin are not well understood. Extrahepatic angiotensinogen expression prompted extensive studies of potential renin receptors that mediate uptake of renin to regulate A-II generation in extrarenal tissue.

Previous studies demonstrated alternative renin transcription in rat adrenal glands. Different studies reported novel intracellular forms of renin deduced from novel 5′ variants derived from renin mRNA in both mice and humans. Comprehensive detailed studies in genetically engineered mice showed that both a secreted and an intracellular form of renin plays divergent mechanism regulating fluid intake and metabolism by the brain RAS as demonstrated by Sigmund. The presence,

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regulation, and functions of these renin isoforms in kidney and adrenal gland are not fully understood in mice. To investigate the characteristics of renin isoforms in mice, we performed a systematic inventory of renin transcripts in adrenal gland and kidney of mice with and without a duplication of the renin gene alternatively for previous studies.

Materials and Methods

Cloning and Identification of Alternative Renin Transcripts

Animals

All animals were purchased from Oriental Yeast Company (Mihama, Chiba, Japan) and were housed and maintained under conditions approved by the Institution Animal Care and Use Committee of Yokohama City University.

Rapid Amplification of cDNA Ends Protocol

To investigate whether alternative renin expression is found in mouse adrenal glands, we performed 5′ rapid amplification of cDNA ends (RACE) analysis in adrenal tissue from 129 mice that endogenously have both Ren1D and Ren2 renin and C57BL/6j mice with Ren1C renin. RNA was isolated from the mice with the use of Trizol reagent (Invitrogen, Carlsbad, CA). 5′ RACE reactions were performed with a SMART II RACE cDNA amplification kit (Clontech, Palo Alto, CA) according to the protocol of manufacturer. First-strand cDNA synthesis was performed with Power Script reverse transcriptase (Clontech, Palo Alto CA). After reverse transcription, first-strand cDNA was directly used in 5′ RACE experiments. Gene-specific polymerase chain reaction (PCR) products were obtained by using a nested PCR approach. Subsequently, products were cloned into pGEM-T Easy vector with pGEM-T Easy Vector Systems (Promega, Madison, WI) and were sequenced. In addition, PCR products excised from agarose gels were also sequenced directly. Sequences of gene-specific primers such as gene-specific primer 1 and gene-specific primer 2 were generated as follows according to the sequence of both Ren1C and Ren2 as demonstrated by Holm et al.: gene-specific primer 1 (Ex2-2) 5′ CAG GTC TGT GTG GAG GAC CAC G C 3′ (213–234) and gene-specific primer 2 (Ex2) 5′ GG/AT AAG ATT/ C AGT CAA GGA AGG/A 3′ (187–207), respectively.

Quantitative Analyses of Renin and Alternative Renin in Kidney and Adrenal Gland and In Situ Hybridization for Mouse Kidney of C57BL/6J

Expressions of renin and alternative renin transcripts in mouse kidney and adrenal glands were examined by quantitative reverse transcription (RT) PCR with GAPDH as an internal control using absolute calibration method. A 66-bp segment of the mouse ARen1C gene was generated by performing RT-PCR on total RNA prepared from mouse kidney, using an RNaseasy system (Qiagen, Valencia, CA). Primers for RT reactions for quantifications and in situ hybridization were as follows: 5′ TATAACGACTCATAAGGGCTGACGGCTGACCTGGCAGA 3′ (up primer with T7 promoter sequence underlined) and 5′ ATTTAGGGACATACTCCCGGAGAGGATTT 3′ (rp primer with Sp6 promoter sequence underlined). DIG oxigenin-labeled sense and antisense probes were generated using 1 ng of purified cDNA template, T7 or Sp6 RNA polymerase, and a digoxigenin RNA labeling kit (Roche Diagnostics, Basel, Switzerland). Dilutions of probes (1:100) were prepared in hybridization buffer and used for hybridization. Paraffin-embedded sections of mouse kidneys were deparaffinized and dehydrated. Subsequent hybridization was performed following standard protocols with the use of digoxigenin-labeled RNA probes. After washing, digoxigenin was detected using an alkaline phosphatase-conjugated antibody. The sections were stained with nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate, counterstained with nuclear green staining, and photographed.

Cloning of Alternative Renin Gene and Transient Expression in Cercopithecus Aethiops 7 Cells

cDNAs of CREN1C, AREn1C, and alternative Ren2 renin (AREn2) were synthesized by PCR using high-fidelity Taq polymerase (KOD plus, Toyobo, Tokyo, Japan) and were subsequently cloned into Gateway shuttle vector pENTR/SD/D-TOPO (Life Technologies Japan, Shibaura, Tokyo, Japan) according to the protocol of manufacturer. The entire sequences of the 3 isoforms were confirmed by sequencing using fluorescence technology. The vectors with the 3 isoforms were subsequently transferred from the entry clone into pcDNA-40 and pcDNA-47 with green fluorescent protein fusion protein in its 3′ terminus using LR clonase (Life Technologies Japan, Shibaura, Tokyo, Japan). These vectors were transiently transfected into Cercopithecus aethiops 7 cells using FuGENE 6 (Roche Diagnostics, Basel, Switzerland). After 48 hours, whole cellular extracts (25 μg per lane) from cultured cells were loaded on 7.5% SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore Corporation, Billerica, MA). The membranes were incubated with anti–V5-horseradish peroxidase antibody (Invitrogen, Carlsbad, CA) in a 1:500 dilution in Tris-buffered saline and Tween 20, Triton X with 5% skim milk. Sites of antibody–antigen reactions were visualized by enhanced chemiluminescence using an LAS 3000 imaging system (Fujifilm, Nishizabu, Japan). In addition, AREn2-containing pcDNA-DEST47 vector transfected into the cells was visualized with the use of an Inverted Research Microscope System, TE2000-E (Nikon Instrument Inc, Melville, NY). Sequences of primers used for PCR-based cloning were as follows: 5′ CACATGTCGCTCACTCAATCGAA 3′ (gwAREn2up), 5′ CACATGAGATCAGAGAGGATG 3′ (gwCREn1Cup), 5′ CAGCAGCTCTGATGACTGAGA 3′ (gwAREn1Cup), and 5′ CGCGGGCAAGGGCATTCAT 3′ (common for rp), respectively.

Generation of Transgenic Mice With Alternative Renin (AREn2) Overexpression and Phenotypic Examinations

To investigate in vivo enzymatic activity and the pathological implications of AREn2 renin, we generated transgenic mice harboring AREn2 with CAGGS promoter. Constructs of transgene (AREn2) with pCAGGS promoter are shown in Figure 5A in the online-only Data Supplement. We performed experiments to compare AREn2TG mice with wild-type littermates in terms of following phenotypes: measurement of blood pressure,\textsuperscript{16,17} measurement of plasma renin activity,\textsuperscript{16,18,19} the results of in vivo near-infrared imaging using the renin substrate analog ReninSense 680 FAST (Perkin Elmer, Waltham, MA),\textsuperscript{20} the results of insulin tolerance tests,\textsuperscript{21} fasting plasma glucose levels, the expressions of inflammatory molecules in various tissues on quantitative RT-PCR analyses,\textsuperscript{16,18,19} and in situ histological examinations for livers of both transgenic mice and wild-type littermate using rabbit polyclonal anti-angiotensin II antibodies (NBPI-30027; Novus Biologicals, LLC, Littleton, CO).\textsuperscript{22,23} All experiments were performed according to the protocols of manufacturer, the references, and our reports as specified above.

Results

Analyses of Alternative Transcripts of Renin Expressed in Adrenal Glands of 129 Mice

First-step PCR reactions of synthesized cDNA derived from adrenal tissues from 129 mice possessing both Ren1D and Ren2 showed products of 3 different sizes (Figure 1A). Subsequent nested PCR reactions were performed for each gel-purified product, and nested PCR products were sequenced. Sequence analysis showed 2 different renin isoforms, Ex1 and AREx1A (Figure 1A), for both Ren1D and Ren2. We observed a 14-bp deletion in the identified product (Figure 1A), subsequently identified as AREx1A of Ren1D.
To determine whether this isoform of renin is also expressed in mice harboring Ren1C (C57BL/6J), we performed RACE analysis for total RNA extracted from the kidneys and adrenal glands of C57BL/6J mice (Figure 1C). Sequence analysis revealed a shorter AREx1a product in kidney and adrenal gland of C57BL/6J mice (Figure 1C). Different 5′ ends indicated independently generated RACE products and excluded the possibility of PCR artifacts. Multiple comparisons of conserved sequences of alternative exons among rodents and humans, including the results of previous studies,8,9 are shown in Figure S1.

Quantitative Analyses of Renin and Alternative Renin in Kidney and Adrenal Gland and In Situ Hybridization for Mouse Kidney of C57BL/6J

Quantitative RT-PCR for both CRen1C and AREn1C in kidney and adrenal gland revealed that expressions of CRen1C in kidneys are ≈10× greater than those of AREn1C in kidney and adrenal glands and those of CRen1C in adrenal glands (Figure 1C). In situ hybridization of AREn1C for kidney and adrenal gland of C57BL/6J mice (Figure 1C). Different 5′ ends indicated independently generated RACE products and excluded the possibility of PCR artifacts. Multiple comparisons of conserved sequences of alternative exons among rodents and humans, including the results of previous studies,8,9 are shown in Figure S1.

Cloning of 3 Renin Genes and Transient Expression in Cercopithecus Aethiops 7 Cells

Figure 3A shows the representative results of immunoblotting of whole cellular extracts with transiently transfected expression vectors of 3 renin genes, AREn1C, CRen1C, and AREn2, respectively. Three mouse renin isoforms were successfully cloned, and transcriptions and translations of the genes were also successfully confirmed in vivo in a cultured cell line. Figure 3B shows green fluorescent protein fusion AREn2 expressed in Cercopithecus aethiops 7 cells, suggesting intracellular protein expression.

Plasma Renin Activity for AREn2TG Mice and Wild-Type Littermates

Plasma renin activities were measured by radio-immuno assay16 for both AREn2TG and wild-type littersmates in 8-week-old males. Mean plasma renin activity levels in AREn2TG mice and wild-type littermates were 22.7±1.7 ng/mL per hour and 29.0±2.3 ng/mL per hour, respectively. There were no significant differences between the 2 groups (Figure 4; P=0.10).

In Vivo Near-Infrared Imaging Using the Renin Substrate Analog ReninSense 680 FAST

AREn2TG mice (n=4) and wild-type littersmates (n=4) were fed a normal diet or high sodium diet (8% NaCl) plus amiloride for 48 hours before ReninSense 680 FAST was injected intravenously. Imaging was performed using an IVIS Lumina ll system (Caliper Life Science, Hopkinton, MA) to quantify the signals 3, 6, and 21 hours after injection (Figure 5A–5C) for 6× at each observation point. Quantitative data are presented in Figure 5D. There were significant differences among the 4 groups in the renin activity (P<0.001).

Insulin Tolerance Tests and Measurements of Fasting Plasma Glucose

Intraperitoneal insulin tolerance tests were performed for both AREn2TG and wild-type littersmates, 12 weeks old, according
Intraperitoneal insulin tolerance tests showed significantly impaired insulin-induced hypoglycemic reactions in ARen2TG mice (Figure 6A) and elevated fasting plasma glucose concentrations in ARen2TG mice (Figure 6B).

Quantitative RT-PCR Analyses of Inflammatory Molecules for Kidney, Heart, and Liver and In Situ Hybridization Using Rabbit Polyclonal Anti-Angiotensin II Antibodies

Our quantitative RT-PCR analyses of inflammatory molecules such as transforming growth factor β1, collagen 1, collagen 2, and macrophage chemotactic protein 1 for kidney, heart, and liver of both ARen2TG and wild-type littermates showed significantly enhanced expressions of these molecules in ARen2TG mice (Figure 7A). In addition, in situ hybridization using rabbit polyclonal anti-angiotensin II antibodies for liver of both ARen2TG and wild-type littermates showed enhanced immunohistochemical staining in liver of transgenic mice, suggesting enhanced A-II production in the organs (Figure 7B).

Discussion

We described novel renin isoforms identified in mice different from previous reports. Bona fide alternative transcription is initiated from AREx1a localized in the first intron of the renin gene as shown previously, with no identical sequence for the mice
with and without a duplication of the renin gene. We also demonstrated the promoter activity of 5′ of AREx1a by promoter activity assays (Figure S2A and S2B). Demonstration of promoter activity and confirmation by RT-PCR excluded the possibility of RACE artifacts. Because both adrenal glands and kidneys play pivotal roles in fluid and electrolyte homeostasis, the expression of alternative isoforms of renin is key to tissue-specific generation of A-II. Our quantitative analyses using RT-PCR showed that relative abundances of alternate transcripts were ≈10% of classical renin transcripts in kidneys (Figure 2A).

The identification of alternative renin isoforms raises several interesting questions on local A-II formation.10,12,13 Partitioning of A-II formation in the circulation, as well as several organ systems such as brain, provides response mechanisms to a variety of physiological and pathological stimuli.11–13 Renin is produced and secreted into the circulation by the juxtaglomerular cells of the kidney. However, renin expression has also been observed in a variety of extrarenal tissues. The function of renin expression at these sites, as well as the mechanisms by which tissue angiotensinogen is processed by renin of systemic or tissue origin, remains poorly understood and controversial except brain.13 Uptake of circulating prorenin of renal origin by the mannose-6-phosphate/insulin-like growth factor receptor into cardiomyocytes was proposed as an alternative pathway for the local generation of A-II.7 The (pro)renin receptor was initially characterized as a component of the RAS.24 (Pro)renin receptor–bound renin and prorenin display increased enzymatic activity, and binding activates intracellular signaling, upregulating the expression of profibrotic proteins.25,26 However, new data now suggest that (pro)renin receptor is functionally linked to the vacuolar proton-ATPase27–29; therefore, the role of (pro) renin receptor as a part of the RAS remains controversial. The independent identification of possible intracellular isoforms of renin in rodents and humans provides further support for alternative mechanisms that act to locally generate A-II and to partition the local and systemic effects of the hormone.4–10,30

In Figure S1, we summarize all identified and deduced renin isoforms (Ex1-Intron 1-Ex2) in rodents and humans. We found that the genomic sequence of the identified alternative exon is conserved in all species. In contrast to this sequence homology, the actually transcribed isoforms differed among all species, including humans. Interestingly, a common observation on all currently known isoforms except AREn2 was the use of a translation initiation codon in exon 2, generating a truncated isoform. Initiation of renin transcription in exon 2 was predicted on the basis of extensive sequence analyses as demonstrated by Morris.31

Previous studies,18,19 including ours,16 showed that the RAS in renal tubules plays a key role in the development of sodium sensitivity and hypertension. Angiotensinogen in proximal tubular cells and renin in connecting tubules are major components of the tubular RAS, acting to maintain sodium balance via epithelial sodium channels along aldosterone-sensitive distal nephrons. Because the tubular lumen is compartmentalized by single epithelial cells and tight junctions, the tubular RAS is regulated independent of the changes in the circulating RAS.16,18,19,32 Our in situ hybridization and diuretic treatment analyses of bona fide renin isoform revealed alternative renin transcripts expressed along cortical tubules, regulated by salt-water balance in tubules (Figure S3). Our analyses by in situ hybridization remain anatomic features of cortical tubules; therefore, further detailed analyses using specific tubular marker might be of help to understand the role of bona fide renin transcripts in tubules in detail.

Because of a gene rearrangement, inbred mice strains can be classified into 2 groups, either with or without a duplicated renin gene (Ren2).8 Sequence analyses have revealed that evolutionally Ren 2 is the result of a gene duplication of the ancestral Ren 1 (Ren1C and Ren1D) renin gene.33,34 We observed a 14-bp deletion in both Ren1C and Ren1D in the vicinity of AREx1a. Although this deletion generates an AREx1a renin isoform lacking an in-frame methionine for both Ren1C and Ren1D, it is surprising that AREx1a of

Figure 3. Representative results of immunoblotting of Cercopithecus aethiops 7 whole cellular extracts with transiently transfected expression vectors of 3 renin genes, AREn1C, CRen1C, and ARen2, respectively (A). B, Expression of green fluorescent protein fusion ARen2 in Cercopithecus aethiops 7 cells.

Figure 4. The results of plasma renin activity measured by plasma renin activity16 are shown for both ARen2TG and wild-type littermates (8-week-old males). The mean plasma renin activity (PRA) levels of ARen2TG mice and wild-type littermates were 22.7±1.7 ng/mL per hour and 29.0±2.3 ng/mL per hour, respectively. There was no significant difference between the groups.
Ren 2 conserves a functionally significant arginine/lysine residues (Arg10P, Lys15P, and Arg20P)\(^3\) and leucine residue (Leu13P)\(^3\) in its prosegment.\(^7\) The transgenic rat (TGR(mREN2)27), harboring the mouse Ren 2 gene, shows fulminant hypertension without elevated circulating concentrations of renin.\(^3\) The mechanisms of

**Figure 5.** Representative results of in vivo near-infrared imaging using the renin substrate analogue ReninSense 680 FAST are shown in A–C. D, Results of measuring signals with an IVIS Lumina II system (Caliper Life Science, Hopkinton, MA) \(^3,\) \(^6,\) and 21 hours after injection. Quantitative data are presented with statistical calculations by repeated measured ANOVA. Statistical significance: *\(P<0.05\) and **\(P<0.001.\)

**Figure 6.** The results of intraperitoneal insulin tolerance tests and measurements of fasting blood glucose are shown. Relative glucose levels after intraperitoneal insulin injection are shown in A. There were significant differences of relative glucose levels at both 60 and 120 minutes after insulin injections between ARen2TG and wild-type littermates. *\(P<0.05\) compared with wild-type littermate. In addition, fasting blood sugar (FBS) levels were significantly higher in ARen2TG than wild-type littermates. **\(P=0.024\) compared with wild-type littermate. B, Repeated measure ANOVA, post hoc analyses, and unpaired t test were performed.
hypertension in this genetically unique rat model have been debated for a long time. In earlier days, it was demonstrated that enhanced kinetics of the reaction between mouse renin and rat angiotensinogen are one of pathophysiological mechanism for this model.\cite{39,40} Recently, this animal model was reported to have various pathophysiological features such as insulin resistance,\cite{41-43} left ventricular dysfunction,\cite{44,45} nonalcoholic fatty liver disease-like significant hepatic steatosis with increased hepatic reactive oxygen species, lipid peroxidation, steatohepatitis, and fibrosis\cite{46} as a result of tissue RAS overactivation. We generated transgenic ARen2 overexpression mice with a C57BL/6J background, which endogenously have only the Ren1C renin gene. Our analyses showed that ARen2TG mice are phenotypically characterized by insulin resistance with mild elevation of blood pressure (Figure S5A and S5B), without plasma renin activity elevation (Figure 4). Using in vivo quantitative imaging techniques, we found significant enhancement of systemic fluorescence activity for ARen2TG mice in vivo (Figure 5). Quantitative RT-PCR analyses of inflammatory molecules in kidney, heart, and liver revealed significantly elevated expressions of such molecules especially in kidney and liver which angiotensinogen expressed endogenously in proximal tubular cells and

Figure 7. A, The results of quantitative reverse transcription polymerase chain reaction analyses of inflammatory molecules in heart, kidney, and liver of both ARen2TG and wild-type littermates are shown as relative abundance, using GAPDH expression as an internal control. *, **, #, ##Significantly elevated compared with the expressions of wild-type littermates. Repeated measure ANOVA and subsequent post hoc analyses were performed. B, Representative results of in situ histological examinations for livers of both transgenic mice and wild-type littermate using rabbit polyclonal anti-angiotensin II antibodies (NBP1-30027; Novus Biologicals, LLC, Littleton, CO) were shown. a–c, Enhanced immunohistochemical staining in livers of transgenic mice compared with those of wild-type littermates (Bd), suggesting enhanced angiotensin II production in the organs.
hepatocytes (Figure 7A). Our observations on the novel isoform derived from the Ren 2 gene might be of functional relevance for hypertension and insulin resistance in TGR(mREN2)/27. These findings suggest that bona fide alternative renin products potentially have intracellular A-II–generating activity, promoting local inflammatory changes leading to systemic insulin resistance. This finding might provide new pathophysiological insight into insulin resistance exaggerated by bona fide renin isoform.

In summary, we identified bona fide renin isoforms in mice as shown in Figure S6. However, concepts of alternative exon usage in intronic sequence were reported previously, and promoter activity and tissue-specific distribution in cortical tubules of the identified isoforms suggest functional significance of the isoforms. Discoveries of bona fide alternative renin transcripts and subsequent current analyses including detailed genomic sequence analyses, molecular cloning, transcriptional regulations, tubular-specific expressions, and transgenic mice experiments will provide not only new molecular pathophysiological insights into hypertension and insulin resistance because of enhancements of A-II production but also important clues and hints in the fields of biologically potent RASs for further understanding of the role of tubular RASs for developing sodium sensitivity, the molecular basis for well-known TGR(mREN2)/27 rats showing fulminating hypertension without plasma renin activity elevations, and the genetic originations of mouse tandem renins that have been considered to be because of gene duplication to date. Because our observations were limited, in part, for expressions, regulations, and transgenic animal analyses of bona fide renin products and transcripts, further analyses for the components of RAS cascade such as angiotensinogen, angiotensin-converting enzyme, and A-II receptors should be performed in future.

Perspectives

Physiological, pathophysiological, and genetic analyses about bona fide renin transcripts and proteins would be important to understand the as-yet-unrecognized roles of RASs for homeostasis of body fluids and electrolytes and development of hypertension including potential therapeutic targets for various cardiovascular diseases.

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Disclosures

None.

References


What Is New?

• We identified bona fide renin isoforms in mice, which are different from previous report. We successfully cloned these renin transcripts and found tissue-specific distribution in cortical tubules.

What Is Relevant?

• Systemic overexpressions of one of renin transcripts, ARe2, showed elevated blood pressure with insulin resistance without significant plasma renin activity elevations.

Novelty and Significance

Summary

Our findings will provide new insights into angiotensin II generation by bona fide renin transcripts, tubular renin–angiotensin systems, molecular basis of TGR(mRen2)27 rats, and the genetic origins of tandem duplication of renin gene in mice.
Identification of Bona Fide Alternative Renin Transcripts Expressed Along Cortical Tubules and Potential Roles in Promoting Insulin Resistance In Vivo Without Significant Plasma Renin Activity Elevation

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Identification of bona fide alternative renin transcripts expressed along cortical tubules and potential roles in promoting insulin resistance in vivo without significant PRA elevation.


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Short title: Alternative transcripts of renin gene expressed in kidney and potential roles in development of insulin resistance.

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Expanded materials and methods

Cell culture and transient transfection assay  The mouse adrenal-gland-derived cell line Y-1 was maintained as recommended by ATCC (Ham's F12K medium with 2 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate, 82.5%; horse serum, 15%; fetal bovine serum, 2.5%). Cell lines were kept in 5% CO₂ in a 37°C incubator and were plated approximately 24 hr before transfection at a density of 5 X10⁴ cells in a Multiwell 24 well insert system (Becton Dickinson Labware, Franklin Lakes, NJ, USA). Transient transfection assays were performed in triplicate, using Tfx-50 Reagent according to the manufacturer's protocol (Promega, Madison, WI, USA). 0.75 ug of plasmid DNA with 0.015 ug of pRL TK vector were used for transfection. Cells were harvested 48 hrs after transfection and assayed for both firefly luciferase activity and Renilla luciferase activity using a Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) on a TD-20e Luminometer (Turner, Sunnyvale, CA, USA). Firefly luciferase activity was expressed relative to Renilla luciferase activity.

Functional promoter analysis of the AREx1a 5’ leading sequence for alternative Ren1C (ARen1C) To test for promoter activity of the 5’ leading sequence of the AREx1a isoform of Ren1C, we performed functional promoter activity assays using luciferase reporter gene constructs and 5’ sequences of Ex1 (served as positive control) and AREx1a of Ren1C (S2A).

Regulations of mouse renin with high-sodium diet and diuretic treatment To test the regulations of both classic renin Ren1C (CRen1C) and alternative renin Ren1C (ARen1C) in kidney, we performed pharmacological experiments using furosemide (100 mg/kg in chew), tolvaptan (V2 receptor antagonist, 10 mg/kg in chew), hydrochlorothiazide (1 mg/kg), and amiloride (10 mg/kg) with a high-sodium diet (8% NaCl) in male C57BL/6J mice. A total of 16 mice (aged 10-12 weeks) were divided into six groups and given the following diets for 2 weeks: (1) a high-salt diet containing 8% NaCl (HS group, n=3), (2) a high-salt diet containing 8% NaCl and furosemide (100 mg/kg, furosemide group, n=3), (3) a high-salt diet containing 8% NaCl and tolvaptan (10 mg/kg, tolvaptan group, n=3), (4) a high-salt diet containing 8% NaCl with amiloride/hydrochlorothiazide treatments for 1 latter week (HS and A/T group, n=3), (5) a high-salt diet containing 8% NaCl and furosemide with amiloride/hydrochlorothiazide treatments for 1 latter week (furosemide and A/T group, n=4), (6) a high-salt diet containing 8% NaCl and tolvaptan with amiloride/hydrochlorothiazide treatments for 1 latter week (tolvaptan and A/T group, n=3). On day 14, the mice were anesthetized, and the kidneys were removed for subsequent quantitative RT-PCR analyses of both CRen1C and ARen1C. Relative abundances of both isoforms to the mice with high
sodium diets alone were calculated by \( \Delta \Delta \text{CT} \) methods using GAPDH as an internal control.

**Generation of transgenic mice with alternative renin (AREn2) overexpression and phenotypic examinations.**

To produce AREn2 transgenic mice, AREn2 cDNA was subcloned into pCAGGS expression vector, which contained a cytomegalovirus enhancer and chicken \( \beta \)-actin (CAG) promoter, and the resultant transgene construct was microinjected into the pronuclei of fertilized mouse embryos at the single-cell stage to generate transgenic mice (C57BL/6J strain). The AREn2 transgene positive (+) mice were mated with C57BL/6J wild-type mice to obtain AREn2 transgene positive (+) mice and littermate control mice for the experiments. Transgenic mice were identified by PCR using 5′-TAATACGACTCACTATAGGGAGAC-3′ as the forward primer and 5′-ATACGTCCCATTCAGCACTG-3′ as the reverse primer. Finally, two transgenic strains, named AREn2TG52 (with relative high transgene expression) and AREn2TG15, were established. We decided to examine the phenotypes of AREn2TG52 for subsequent experiments. We performed experiments to compare AREn2TG mice with wild littermates in terms of following phenotypes in addition to the methods described in our manuscript itself: AREn2 transgene products in various tissues on Western blot analyses, measurement of blood pressure. Blood pressure was measured 10 to 12 times by the tail-cuff method, using a non-invasive blood pressure monitor (MK-2000, Muromachi Kikai Co., Ltd., Osaka, Japan) as described previously.
Figures and supporting information

S1

Multiple genomic sequence alignment of conserved sequences of alternative exons in rodents and humans were shown.

S1

Multiple comparisons for conserved sequences of alternative exons among rodents and human.
S2A. Schematic presentations of chimeric Exon 1 and ARExon 1a renin promoter constructs are shown. Constructs no. 1, 3, 5, and 7 show “sense” leading sequence with luciferase gene fusion; in contrast, no. 2, 4, 6, and 8 show “anti-sense” leading sequence with luciferase gene fusion.
S2B. Summary of two times triplicate transfection experiments in Y1 cell line. Results are shown as the mean ± SEM. The results of two times triplicate transfection experiments are shown in S2B. The 5’ leading sequence of AREx1a showed significant firefly luciferase activity, suggesting that this region has functional promoter activity in the cell culture system used. Interestingly, shorter constructs without a GATA consensus sequence (construct No. 5 in S2A) showed significantly less luciferase activity than the longer constructs containing the GATA consensus element (construct No. 3). The 5’ region of Ex 1 renin showed the highest luciferase activity in this assay system (construct No. 7 in S2A), whereas all anti-sense constructs lacked significant luciferase activity (constructs No. 2, 4, 6, 8 in S2A). Overall ANOVA p<0.0001 * indicates p<0.0001 for difference between NC(pGL3-basic) and corresponding anti-sense constructs by repeated measure ANOVA with post-hoc analyses.
High-sodium diets with furosemide treatment significantly enhanced both ARen1C and CRen1C expressions in kidney, and concurrent treatment with amiloride and hydrochlorothiazide significantly abolished this enhancement, while treatment with vasopressin V2 receptor antagonists was not associated with enhanced expression of either ARen1C or CRen1C in kidneys (S3). The results of quantitative RT-PCR for ARen1C and CRen1C isoforms in whole kidney are shown in S3. High-sodium diet with furosemide treatment significantly enhanced the expressions of both ARen1C and CRen1C isoforms, and combined treatment with amiloride and hydrochlorothiazide abolished such enhancement. Treatment with selective vasopressin type 2 receptor antagonists, which suppresses water reabsorption in collecting ducts via AQP2 channels, did not enhance the expression of either ARen1 or CRen1C in kidney. Statistical calculations were performed by repeated measured ANOVA among the groups. # indicates p<0.0001 compared to others. * indicates p<0.0001 compared to the groups. ** indicates p<0.001 compared to HS, HS+Tolvaptan and *** indicates p<0.001 compared to HS.
**S4 a, b, c**

*Generation of transgenic mice with alternative renin 2 (ARen2) overexpression and phenotypic examinations* Constructs of ARen2 transgene with pCAGGS promoter are shown in the right-hand panel of S4c. Expressions of ARen2 in the organs and tissues of the transgenic mice were examined by Western blotting. Western blot showed ARen2 expression in the organs and tissues of transgenic mice (S4a), but not in wild-type mice (S4b).
S5A, 5B shows the results of blood pressure measurements for F1-ARen2 TG mice with or without high-sodium diets (S5A), and the results of comparisons of SBP and body weight between F2-ARen2TG and wild littermates (S5B). Although sodium sensitivity of blood pressure responses was not observed, blood pressure elevations were seen in 30-week-old F1-ARen2TG mice with statistical calculations by repeated measure ANOVA (S5A). F2-ARen2TG mice showed significantly higher blood pressure than wild littermates despite no difference in body weight with unpaired t-test (S5B).
Exon-intron interaction and formations of three mouse renin gene with bona fide AREx1a were schematically summarized. Mice such as C57BL/6, BALB/c with Ren1C have a single renin locus on chromosome 1 with two different transcripts, CRen1C and ARen1C, respectively. Mice such as 129, DBA/2J with both Ren1D and Ren2 have tandem renin loci on chromosome 1 with four different transcripts, CRen1D, ARen1D, CRen2, and ARen2, respectively.