Published in final edited form as:

Am J Physiol Renal Physiol. 2007 September ; 293(3): F956–F960. doi:10.1152/ajprenal.00090.2007.

Novel sandwich ELISA for human angiotensinogen

Akemi Katsurada^{1,*}, Yoshiaki Hagiwara², Kazuya Miyashita², Ryousuke Satou¹, Kayoko Miyata¹, Naro Ohashi¹, L. Gabriel Navar¹, and Hiroyuki Kobori^{1,*}

¹Department of Physiology, and Hypertension and Renal Center of Excellence, Tulane University Health Sciences Center, New Orleans, Louisiana;

²Immuno-Biological Laboratories Co., Ltd., Fujioka, Gunma, Japan

Abstract

We recently reported that urinary excretion rates of angiotensinogen (U_{AGT}) provide a specific index of intrarenal renin-angiotensin (ANG) system (RAS) status in ANG II-dependent hypertensive rats. When this is shown to be applicable to human subjects, a diagnostic test to identify those hypertensive patients most likely to respond to an RAS blockade could provide useful information to allow a mechanistic rationale for selection of an optimized approach to treatment of hypertensive patients. However, simple and accurate methods to measure human angiotensinogen (hAGT) are unavailable. For future studies of human subjects, we developed antibodies and a sensitive and specific quantification system for hAGT using a sandwich ELISA. We raised two antibodies against hAGT: a mouse monoclonal antibody and a rabbit polyclonal antibody. The standard curve of this ELISA exhibited a high linearity (0.31–20 ng/ml). The correlation coefficient was >0.99. Plasma angiotensinogen concentrations of healthy volunteers ranged from 28 to 71 $\mu\text{g/ml}$ ($n = 10$). The ratio of U_{AGT} to urinary creatinine concentration ranged from 5.0 to 30 $\mu\text{g/g}$ ($n = 7$). Intra- and interassay coefficients of variation ranged from 4.4 to 5.5% and from 4.3 to 7.0%, respectively. This ELISA system had no cross-reactivity with major proteins in proteinuric urine samples, such as human albumin, immunoglobulin, or transferrin. Moreover, the cross-reactivity of the system with angiotensin peptides was also negligible. This hAGT ELISA will be a useful tool to investigate the relationship of U_{AGT} and reactivity to antihypertensive drugs in hypertensive patients.

Keywords

renin-angiotensin system; plasma; urine

The Renin-Angiotensin System (RAS) is well known to play an important role in blood pressure regulation and fluid and electrolyte homeostasis (18). In recent years, the focus of interest on the RAS has shifted to a main emphasis on the role of the local/tissue RAS in specific tissues (5). Emerging evidence has demonstrated the importance of the tissue RAS in the brain (1), heart (4), adrenal glands (15), and vasculature (3,6), as well as the kidneys (18). There is substantial evidence that the major fraction of angiotensin II in renal tissues is generated locally from angiotensinogen delivered to the kidney, as well as from

Copyright © 2007 the American Physiological Society

Address for reprint requests and other correspondence: H. Kobori, Tulane Univ. Health Sciences Center, 1430 Tulane Ave., #SL39, New Orleans, LA 70112-2699 (e-mail: hkobori@tulane.edu).

*A. Katsurada and H. Kobori contributed equally to this work.

Portions of this study were presented in abstract form at the 17th Scientific Sessions of the Inter-American Society of Hypertension, Miami Beach, FL, May 6–10, 2007.

angiotensinogen locally produced by proximal tubule cells (7). Renin secreted by juxtaglomerular apparatus cells into the renal interstitium and vascular compartment also provides a pathway for the local generation of angiotensin I (17). Angiotensin-converting enzyme is abundant in the rat kidney and is present in the proximal and distal tubules, collecting ducts, and renal endothelial cells (2). Angiotensin I delivered to the kidney can also be converted to angiotensin II (14). Therefore, all the components necessary for generation of intrarenal angiotensin II are present along the nephron (18).

The presence of all components of the RAS in the kidney provides a great flexibility and independence in regulating intrarenal levels of angiotensin II, and the differential regulation of angiotensin peptide levels in plasma and kidney has been established (19). Recently, we reported that urinary excretion rates of angiotensinogen provide a specific index of intrarenal RAS status in angiotensin II-dependent hypertensive rats (8-11,13). When this is shown to be applicable to human subjects, a diagnostic test to identify those hypertensive patients most likely to respond to blockade of the RAS could provide useful information to allow a mechanistic rationale for selection of an optimized approach to treatment of hypertensive patients. ELISA for human angiotensinogen has been reported (16,21). However, simple and accurate methods to measure human angiotensinogen are unavailable. For future studies of human subjects, we developed antibodies and a sensitive and specific quantification system for human angiotensinogen using a microtiter plate-based sandwich-type ELISA.

MATERIALS AND METHODS

Protocol

The experimental protocol of this study was approved by the Institutional Review Board of Tulane University. All samples were obtained from healthy volunteers, who provided written informed consent.

Preparation of expression vectors for human angiotensinogen

The full length, except for the signal peptide (1-33 aa), of the human angiotensinogen (34-485 aa) gene (gene identification no. 73622269) was amplified by PCR using a high-fidelity *Pyrococcus furiosus* DNA polymerase (Promega) with sense (5'-CGG GAT CCG ACC GGG TGT ACA TAC ACC CC-3') and antisense (5'-CGG TTG GGC GAC TCG TGT CGT GAG CTC GCC-3') primers from the human adult liver cDNA library (Clontech). Then this fragment was inserted into pGEX4T1 expression vectors (Promega) with the glutathione *S*-transferase (GST) tag at the 5'-end by the restriction enzymes *Bam*H I and *Sal* I.

Preparation of recombinant proteins for human angiotensinogen

The recombinant constructs were transformed to a high-efficiency expression bacterial strain (Takara). Large-scale bacterial cultures were induced with isopropyl- β -D-thiogalactopyranoside (Takara) and harvested for protein purification. GST-tagged proteins were purified using glutathione beads (Upstate) in native conditions.

Antibody preparation

We raised two antibodies for human angiotensinogen: a mouse monoclonal and a rabbit polyclonal antibody. The monoclonal antibody was raised in mouse against recombinant protein of human angiotensinogen (see above). The polyclonal antibody was raised in rabbit against synthetic oligopeptide corresponding to human angiotensinogen (72-89 aa). Both antibodies were affinity purified.

Western blot

Western blot analysis was performed as previously described (12,20,22) using the LI-COR Odyssey infrared imaging system.

Epitope mapping

The epitopes of these antibodies were determined by Western blot analysis using a variety of lengths of recombinant proteins of human angiotensinogen (34-124, 34-223, 34-303, 34-393, and 34-485 aa).

Plate preparation

The ELISA plates were coated with the polyclonal antibody against human angiotensinogen (100 μ l/well in 100 mmol/l carbonate buffer, pH 9.5) at 4°C overnight. The plates were washed with PBS and blocked with 1% bovine serum albumin (200 μ l/well) in PBS containing 0.05% NaN₃ at 4°C overnight. The plates were stored at 2–8°C.

Sample collections

Peripheral blood samples from healthy volunteers were collected into EDTA-containing tubes, and plasma samples were separated after centrifugation. First-morning-urine samples were also collected from healthy volunteers.

Development of sandwich ELISA

Highly purified angiotensinogen from human plasma was used as the standard. Human angiotensinogen standards (0.31–20 ng/ml diluted in ELISA buffer), plasma (1:8,000 dilution in ELISA buffer), and urine (1:8 dilution in ELISA buffer) samples (100 μ l/well) were added to each well of the plates and incubated at 37°C for 1 h. Then the plates were washed a total of seven times with a washing buffer (PBS containing 0.05% Tween 20, pH 7.5). After the plates were incubated with horseradish peroxidase-labeled monoclonal antibody against human angiotensinogen (100 μ l/well, 1:30 dilution in antibody solution) at 37°C for 30 min, they were washed a total of nine times with the washing buffer. Then the plates were incubated with 3,3',5,5'-tetramethylbenzidine solution (100 μ l/well) under light-protected conditions at room temperature for 30 min. The reaction was stopped by treatment of the plates with sulfuric acid (100 μ l/well, 0.5 mol/l). The absorbance values were measured at 450 nm.

Measurement of urinary creatinine

The urinary creatinine concentrations were measured by an automated machine (model DCA 2000+, Bayer) with microalbumin/creatinine reagent kits (Bayer). The urinary angiotensinogen concentrations were normalized to urinary creatinine concentrations.

Statistical analysis

Statistical analysis was performed using a one-way factorial ANOVA with post hoc Scheffé's *F* test. Values are means $\pm$ SE. *P* < 0.05 was considered significant.

RESULTS AND DISCUSSION

Epitope mapping of antibodies

An equal amount (10 ng) of various lengths of recombinant proteins of human angiotensinogen (34-124, 34-223, 34-303, 34-393, and 34-485 aa) was blotted on nitrocellulose membranes, and epitope mapping was done by Western blot. The polyclonal antibody against human angiotensinogen recognized GST-tagged recombinant proteins of

human angiotensinogen (34-124, 34-223, 34-303, 34-393, and 34-485 aa), but not mock protein or GST protein (Fig. 1A). The monoclonal antibody against human angiotensinogen recognized GST-tagged recombinant proteins of human angiotensinogen (34-485 aa), but not GST-tagged recombinant proteins of human angiotensinogen (34-124, 34-223, 34-303, and 34-393 aa), mock protein, or GST protein. An antibody against GST protein recognized GST-tagged recombinant proteins of human angiotensinogen (34-124, 34-223, 34-303, 34-393, and 34-485 aa), as well as GST protein, but not mock protein. These data clearly indicate that the epitope for the polyclonal antibody against human angiotensinogen is located on the NH₂ terminus of human angiotensinogen (34-124 aa) and that the epitope for the monoclonal antibody against human angiotensinogen is on the COOH terminus of human angiotensinogen (394-485 aa).

Plate stability

To address their stability, we incubated the polyclonal antibody-coated ELISA plates at 37°C for 1, 8, or 15 days. Then different concentrations of human angiotensinogen standards were applied to the plates and processed, and the absorbance values were obtained. Each concentration of human angiotensinogen standards ($n = 6$) exhibited similar absorbance values beyond 15 days (Fig. 1B). These data clearly indicate that the polyclonal antibody-coated ELISA plates are stable ≥ 15 days after antibody coating.

Standard curves

The corresponding standard curve exhibited a high linearity (0.31–20 ng/ml), with absorbance values ranging from 0.03 to 2.34 ($n = 6$ at each concentration; Fig. 1C). The correlation coefficient was >0.99 .

Detection limit

The lowest sensitivity for this system (0.31 ng/ml) was determined using the guidelines of the National Committee for Clinical Laboratory Standards Evaluation Protocols.

Plasma and urinary angiotensinogen concentrations

Angiotensinogen concentrations in plasma obtained from 10 healthy volunteers ranged from 28 to 71 $\mu\text{g/ml}$ (Fig. 2A). Angiotensinogen concentrations in urine obtained from seven healthy volunteers ranged from 7.1 to 35 ng/ml (Fig. 2B). Urinary creatinine concentrations of seven healthy volunteers were 0.93–2.2 mg/ml. The ratio of urinary angiotensinogen concentration to urinary creatinine concentration was 5.0–30 $\mu\text{g/g}$ (Fig. 2C).

Addition and recovery test

Different known concentrations of human angiotensinogen standards were added to a human plasma sample or a human urine sample, and the added angiotensinogen concentrations were calculated as the difference between angiotensinogen concentrations in samples with and without added angiotensinogen. Recovery rates were $>82.9\%$ for plasma and $>87.6\%$ for urine (Table 1).

Sample stability

To address stability of samples, we performed two tests: a freeze-and-thaw test and an accelerated test. In a freeze-and-thaw test, one plasma sample was frozen and thawed 0, 1, 2, and 3 times, and plasma angiotensinogen concentrations were measured by the ELISA system. No significant differences were observed (Fig. 3A). In an accelerated test, one plasma sample was incubated at 37°C for 0, 1, 3, and 7 days, and plasma angiotensinogen concentrations were measured by the ELISA system. No differences were observed (Fig.

3B). These data clearly indicate that angiotensinogen in plasma samples is stable without preservatives or proteinase inhibitors, except EDTA.

Intra-assay test

Intra-assay tests were performed using three different concentrations of human angiotensinogen standards. Coefficients of variation among the wells ranged from 4.4 to 5.5% (Table 2).

Interassay test

Interassay tests were performed using three different concentrations of human angiotensinogen standards. Coefficients of variation among the plates ranged from 4.3 to 7.0% (Table 3).

Specificity

To confirm cross-reactivity with other proteins, several human proteins (20 µg/ml) were applied to the ELISA system and processed. Cross-reactivity for other human proteins in this ELISA system was negligible (Table 4). In addition, downstream products of angiotensinogen (20 µg/ml) were also applied to the ELISA system. The cross-reactivity for angiotensin peptides in this ELISA system was also negligible (Table 4).

Effect of pH of test samples on sensitivity of ELISA

As described above, plasma samples were diluted 1:8,000 and urine samples 1:8 in PBS-based dilution buffer (pH 7.0); therefore, pH of diluted plasma and urine samples should be ~7.0. To address this issue, seven urine samples were prepared (pH 5.5–7.0, 6.3 ± 0.6). After the samples were diluted with the dilution buffer, pH values of the samples were integrated (pH 6.7–7.0, 6.8 ± 0.2). Moreover, diluted urine samples worked well with ELISA (15.9 ± 6.4 ng/ml). These data clearly indicate that the change of urine sample pH does not affect the sensitivity of ELISA.

Conclusion

We developed antibodies and a sensitive and specific quantification system for human angiotensinogen using a sandwich ELISA. This human angiotensinogen ELISA will be a useful tool to investigate the relationship between urinary angiotensinogen excretion rate and reactivity to anti-hypertensive drugs in hypertensive subjects.

Acknowledgments

The authors acknowledge the excellent technical assistance of My-Linh Rauv, Duy V. Tran, and Dale M. Seth (Tulane University).

GRANTS

This study was supported by National Institutes of Health Grants R01 DK-072408, P20 RR-017659, and R01 HL-026371, the Health Excellence Fund from the Louisiana Board of Regents, and Sankyo Co., Ltd.

REFERENCES

1. Baltatu O, Silva JA Jr, Ganten D, Bader M. The brain renin-angiotensin system modulates angiotensin II-induced hypertension and cardiac hyper-trophy. *Hypertension*. 2000; 35:409–412. [PubMed: 10642333]
2. Casarini DE, Boim MA, Stella RC, Krieger-Azzolini MH, Krieger JE, Schor N. Angiotensin I-converting enzyme activity in tubular fluid along the rat nephron. *Am J Physiol Renal Physiol*. 1997; 272:F405–F409.

3. Danser AH, Admiraal PJ, Derkx FH, Schalekamp MA. Angiotensin I-to-II conversion in the human renal vascular bed. *J Hypertens.* 1998; 16:2051–2056. [PubMed: 9886896]
4. Dell'Italia LJ, Meng QC, Balcells E, Wei CC, Palmer R, Hageman GR, Durand J, Hankes GH, Oparil S. Compartmentalization of angiotensin II generation in the dog heart: evidence for independent mechanisms in intravascular and interstitial spaces. *J Clin Invest.* 1997; 100:253–258. [PubMed: 9218500]
5. Dzau VJ, R Re. Tissue angiotensin system in cardiovascular medicine. A paradigm shift? *Circulation.* 1994; 89:493–498. [PubMed: 8281685]
6. Griendling KK, Minieri CA, Ollerenshaw JD, Alexander RW. Angiotensin II stimulates NADH and NADPH oxidase activity in cultured vascular smooth muscle cells. *Circ Res.* 1994; 74:1141–1148. [PubMed: 8187280]
7. Ingelfinger JR, Pratt RE, Ellison K, Dzau VJ. Sodium regulation of angiotensinogen mRNA expression in rat kidney cortex and medulla. *J Clin Invest.* 1986; 78:1311–1315. [PubMed: 3533999]
8. Kobori H, Harrison-Bernard LM, Navar LG. Enhancement of angiotensinogen expression in angiotensin II-dependent hypertension. *Hypertension.* 2001; 37:1329–1335. [PubMed: 11358949]
9. Kobori H, Harrison-Bernard LM, Navar LG. Expression of angiotensinogen mRNA and protein in angiotensin II-dependent hypertension. *J Am Soc Nephrol.* 2001; 12:431–439. [PubMed: 11181790]
10. Kobori H, Harrison-Bernard LM, Navar LG. Urinary excretion of angiotensinogen reflects intrarenal angiotensinogen production. *Kidney Int.* 2002; 61:579–585. [PubMed: 11849399]
11. Kobori H, Nishiyama A, Harrison-Bernard LM, Navar LG. Urinary angiotensinogen as an indicator of intrarenal angiotensin status in hyper-tension. *Hypertension.* 2003; 41:42–49. [PubMed: 12511528]
12. Kobori H, Ozawa Y, Suzuki Y, Nishiyama A. Enhanced intrarenal angiotensinogen contributes to early renal injury in spontaneously hyper-tensive rats. *J Am Soc Nephrol.* 2005; 16:2073–2080. [PubMed: 15888567]
13. Kobori H, Prieto-Carrasquero MC, Ozawa Y, Navar LG. AT₁ receptor mediated augmentation of intrarenal angiotensinogen in angiotensin II-dependent hypertension. *Hypertension.* 2004; 43:1126–1132. [PubMed: 15037565]
14. Komlosi P, Fuson AL, Fintha A, Peti-Peterdi J, Rosivall L, Warnock DG, Bell PD. Angiotensin I conversion to angiotensin II stimulates cortical collecting duct sodium transport. *Hypertension.* 2003; 42:195–199. [PubMed: 12835330]
15. Mazzocchi G, Malendowicz LK, Markowska A, Albertin G, Nussdorfer GG. Role of adrenal renin-angiotensin system in the control of aldosterone secretion in sodium-restricted rats. *Am J Physiol Endocrinol Metab.* 2000; 278:E1027–E1030. [PubMed: 10827004]
16. Mizrahi J, Coezy E, Auzan C, Corvol P, Menard J. Monoclonal antibodies to human angiotensinogen: development of an ELISA for measurement of hepatocyte cultured cells content. *Clin Exp Hypertens.* 1987; 9:1479–1491.
17. Moe OW, Ujiiie K, Star RA, Miller RT, Widell J, Alpern RJ, Henrich WL. Renin expression in renal proximal tubule. *J Clin Invest.* 1993; 91:774–779. [PubMed: 7680667]
18. Navar LG, Harrison-Bernard LM, Nishiyama A, Kobori H. Regulation of intrarenal angiotensin II in hypertension. *Hypertension.* 2002; 39:316–322. [PubMed: 11882566]
19. Navar, LG.; Prieto-Carrasquero, MC.; Kobori, H. Renal renin-angiotensin system. In: Kastin, AJ., editor. *Handbook of Biologically Active Peptides*. 1st ed.. Elsevier; Burlington, MA: 2006. p. 1235-1242.chapt. 170
20. Ozawa Y, Kobori H, Suzuki Y, Navar LG. Sustained renal interstitial macrophage infiltration following chronic angiotensin II infusions. *Am J Physiol Renal Physiol.* 2007; 292:F330–F339. [PubMed: 16804106]
21. Rubin I, Lykkegaard S, Olsen AA, Selmer J, Ballegaard M. Monoclonal antibodies against human angiotensinogen, their characterization and use in an angiotensinogen enzyme linked immunosorbent assay. *J Immunoassay.* 1988; 9:257–274. [PubMed: 2466873]
22. Suzuki Y, Ozawa Y, Kobori H. Intrarenal oxidative stress and augmented angiotensinogen are precedent to renal injury in Zucker diabetic fatty rats. *Int J Biol Sci.* 2007; 3:40–46. [PubMed: 17200690]

Fig. 1.

A: epitope mapping. GST, glutathione *S*-transferase; hAGT, human angiotensinogen; Poly and Mono, poly- and monoclonal antibodies. *B*: absorbance values. Polyclonal antibody-coated ELISA plates were incubated at 37°C for 1 (open bars), 8 (gray bars), or 15 (solid bars) days. *C*: a corresponding standard curve.

Fig. 2.

A: angiotensinogen concentrations in plasma obtained from 10 healthy volunteers. Samples were applied to the ELISA system in triplicate, and averaged values were obtained. *B:* angiotensinogen concentrations in urine obtained from 7 healthy volunteers. Samples were applied to the ELISA system in triplicate, and averaged values were obtained. *C:* ratio of urinary angiotensinogen concentration to urinary creatinine concentration.

Fig. 3.
A: freeze-and-thaw test. B: accelerated test.

Table 1

Addition-and-recovery test

Theoretical Values, ng/ml	Measured Values, ng/ml	%
<i>Human plasma</i>		
9.29	7.70	82.9
6.79	6.50	95.7
5.54	4.98	89.9
<i>Human urine</i>		
7.47	6.54	87.6
4.97	4.69	94.4
3.72	3.38	90.9

Different known concentrations of human angiotensinogen standards were added to human plasma or urine sample, and added angiotensinogen concentrations were calculated as the difference between angiotensinogen concentrations in samples with and without added angiotensinogen.

Table 2

Intra-assay test

Measured Values, ng/ml	SD	%CV	<i>n</i>
8.81	0.39	4.4	24
2.14	0.11	5.1	24
0.73	0.04	5.5	24

Intra-assay tests were performed using 3 different concentrations of human angiotensinogen standards. CV, coefficient of variation.

Table 3

Interassay test

Measured Values, ng/ml	SD	%CV	<i>n</i>
8.77	0.38	4.3	32
2.14	0.10	4.7	32
0.71	0.05	7.0	32

Interassay tests were performed using 3 different concentrations of human angiotensinogen standards.

Table 4

Specificity

Human Proteins	Cross-Reactivity, %
Human angiotensinogen	100
Human tenascin C	<0.1
Human growth-regulated oncogene- α	<0.1
Human growth-regulated oncogene- β	<0.1
Human angiopoietin-like 3	<0.1
Human osteopontin	<0.1
Human stem cell factor	<0.1
Human leptin	<0.1
Human granulocyte-colony-stimulating factor	<0.1
Human albumin	<0.1
Human immunoglobulin	<0.1
Human transferrin	<0.1
Angiotensin I	<0.1
Angiotensin II	<0.1
Angiotensin III	<0.1
Angiotensin IV	<0.1
Angiotensin-(1-7)	<0.1
Angiotensin-(1-9)	<0.1