being no change in the total AT1R level in the kidney (Fig. 10). Furthermore, the results of ANG II infusion experiments showed that the plasma membrane AT1R level was further decreased by ANG II stimulation in the kidney of ATRAP transgenic mice (Fig. 11), which was accompanied by a decreased response of renal angiotensinogen mRNA expression to ANG II. This is in contrast to there being no change in the plasma membrane AT1R level by ANG II in the kidney of wild-type mice (Fig. 9). These results collectively suggest that enhancement of renal ATRAP expression beyond baseline promotes AT1R internalization in response to ANG II. A recent study by Oppermann et al. (29) also showed that a genetic deficiency of ATRAP in mice caused an enhanced surface expression of AT1R in the kidney, which is consistent with the results in this study.

The results of the present study show that continuous ANG II infusion decreased intrarenal ATRAP expression through ANG II-mediated AT1R signaling, particularly in the outer medulla, with the lack of any decrease in plasma membrane AT1R expression in the kidney in C57BL/6 wild-type mice. On the other hand, transgenic overexpression of ATRAP reduced the plasma membrane AT1R level in the kidney at baseline and further decreased the plasma membrane AT1R expression in response to ANG II stimulation, concomitant with the decreased ANG II-induced response of the angiotensinogen gene, despite there being no change in the total AT1R level.

Nevertheless, a limitation of the present study is that our findings strongly suggest that ATRAP at supraphysiological levels can alter the plasma membrane levels of AT1R at baseline and in response to ANG II stimulation, but with no detectable physiological effect on blood pressure. Target organ effects such as proteinuria and degree of renal damage by histological examination were not analyzed in this study. The only detectable consequence was a difference in angiotensinogen mRNA expression in response to ANG II stimulation, but it remains unclear whether this would translate to a difference in angiotensinogen protein levels.

In conclusion, these results suggest that AT1R and ATRAP modulate each other, at least in the kidney, and that activation of AT1R signaling has a dominant effect over endogenous renal ATRAP under chronic ANG II infusion, but that renal ATRAP activation by a transgenic model that increases ATRAP expression beyond baseline may cause a constitutive reduction of plasma membrane AT1R expression and a promotion of AT1R internalization in response to ANG II. Further studies to analyze downstream signaling events mediated by activation of AT1R under the condition of ATRAP overexpression are warranted to elucidate the detailed molecular mechanisms and pathophysiological significance of ATRAP-mediated inhibition of AT1R signaling in vivo.

ACKNOWLEDGMENTS

The authors thank Emi Maeda and Hiroko Morinaga for technical assistance and helpful discussion. The authors also thank Dr. Kevin Boru for English editing of this manuscript.

GRANTS

This work was supported in part by grants from the Japanese Ministry of Education, Science, Sports, and Culture, by a Health and Labor Sciences Research grant, and by grants from the Salt Science Research Foundation (1033), the Mitsubishi Pharma Research Foundation, and the Strategic Research Project of Yokohama City University.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

REFERENCES

1. **Azuma K, Tamura K, Shigenaga A, Wakui H, Masuda S, Tsurumi-Ikeya Y, Tanaka Y, Sakai M, Matsuda M, Hashimoto T, Ishigami T, Lopez-Ilasaca M, Umemura S.** Novel regulatory effect of angiotensin II type 1 receptor-interacting molecule on vascular smooth muscle cells. *Hypertension* 50: 926–932, 2007.
2. **Beutler KT, Masilamani S, Turban S, Nielsen J, Brooks HL, Ageloff S, Fenton RA, Packer RK, Knepper MA.** Long-term regulation of ENaC expression in kidney by angiotensin II. *Hypertension* 41: 1143–1150, 2003.
3. **Block K, Eid A, Griendling KK, Lee DY, Wittrant Y, Gorin Y.** Nox4 NAD(P)H oxidase mediates Src-dependent tyrosine phosphorylation of PDK-1 in response to angiotensin II: role in mesangial cell hypertrophy and fibronectin expression. *J Biol Chem* 283: 24061–24076, 2008.
4. **Chabrashvili T, Kitiyakara C, Blau J, Karber A, Aslam S, Welch WJ, Wilcox CS.** Effects of ANG II type 1 and 2 receptors on oxidative stress, renal NADPH oxidase, and SOD expression. *Am J Physiol Regul Integr Comp Physiol* 285: R117–R124, 2003.
5. **Coffman TM, Crowley SD.** Kidney in hypertension: guyton redux. *Hypertension* 51: 811–816, 2008.
6. **Crowley SD, Gurley SB, Herrera MJ, Ruiz P, Griffiths R, Kumar AP, Kim HS, Smithies O, Le TH, Coffman TM.** Angiotensin II causes hypertension and cardiac hypertrophy through its receptors in the kidney. *Proc Natl Acad Sci USA* 103: 17985–17990, 2006.
7. **Cui T, Nakagami H, Iwai M, Takeda Y, Shiuchi T, Tamura K, Daviet L, Horiuchi M.** ATRAP, novel AT1 receptor associated protein, enhances internalization of AT1 receptor and inhibits vascular smooth muscle cell growth. *Biochem Biophys Res Commun* 279: 938–941, 2000.
8. **Daviet L, Lehtonen JY, Tamura K, Griese DP, Horiuchi M, Dzau VJ.** Cloning and characterization of ATRAP, a novel protein that interacts with the angiotensin II type 1 receptor. *J Biol Chem* 274: 17058–17062, 1999.
9. **Gonzalez-Villalobos RA, Satou R, Seth DM, Semprun-Prieto LC, Katsurada A, Kobori H, Navar LG.** Angiotensin-converting enzyme-derived angiotensin II formation during angiotensin II-induced hypertension. *Hypertension* 53: 351–355, 2009.
10. **Gonzalez-Villalobos RA, Seth DM, Satou R, Horton H, Ohashi N, Miyata K, Katsurada A, Tran DV, Kobori H, Navar LG.** Intrarenal angiotensin II and angiotensinogen augmentation in chronic angiotensin II-infused mice. *Am J Physiol Renal Physiol* 295: F772–F779, 2008.
11. **Guo S, Lopez-Ilasaca M, Dzau VJ.** Identification of calcium-modulating cyclophilin ligand (CAML) as transducer of angiotensin II-mediated nuclear factor of activated T cells (NFAT) activation. *J Biol Chem* 280: 12536–12541, 2005.
12. **Harrison-Bernard LM, El-Dahr SS, O'Leary DF, Navar LG.** Regulation of angiotensin II type 1 receptor mRNA and protein in angiotensin II-induced hypertension. *Hypertension* 33: 340–346, 1999.
13. **Harrison-Bernard LM, Zhuo J, Kobori H, Ohishi M, Navar LG.** Intrarenal AT_1 receptor and ACE binding in ANG II-induced hypertensive rats. *Am J Physiol Renal Physiol* 282: F19–F25, 2002.
14. **Hirose T, Satoh D, Kurihara H, Kusaka C, Hirose H, Akimoto K, Matsusaka T, Ichikawa I, Noda T, Ohno S.** An essential role of the universal polarity protein, aPKClambda, on the maintenance of podocyte slit diaphragms. *PLoS One* 4: e4194, 2009.
15. **Hong SW, Isono M, Chen S, Iglesias-De La Cruz MC, Han DC, Ziyadeh FN.** Increased glomerular and tubular expression of transforming growth factor-beta1, its type II receptor, and activation of the Smad signaling pathway in the db/db mouse. *Am J Pathol* 158: 1653–1663, 2001.
16. **Hunyady L, Bor M, Balla T, Catt KJ.** Identification of a cytoplasmic Ser-Thr-Leu motif that determines agonist-induced internalization of the AT1 angiotensin receptor. *J Biol Chem* 269: 31378–31382, 1994.
17. **Iwamoto T, Kita S, Zhang J, Blaustein MP, Arai Y, Yoshida S, Wakimoto K, Komuro I, Katsuragi T.** Salt-sensitive hypertension is triggered by Ca^{2+} entry via Na^+/Ca^{2+} exchanger type-1 in vascular smooth muscle. *Nat Med* 10: 1193–1199, 2004.

Downloaded from ajprenal.physiology.org on November 21, 2010

18. **Kagiyama S, Matsumura K, Fukuhara M, Sakagami K, Fujii K, Iida M.** Aldosterone-and-salt-induced cardiac fibrosis is independent from angiotensin II type 1a receptor signaling in mice. *Hypertens Res* 30: 979–989, 2007.
19. **Kobori H, Nangaku M, Navar LG, Nishiyama A.** The intrarenal renin-angiotensin system: from physiology to the pathobiology of hypertension and kidney disease. *Pharmacol Rev* 59: 251–287, 2007.
20. **Kobori H, Prieto-Carrasquero MC, Ozawa Y, Navar LG.** AT1 receptor mediated augmentation of intrarenal angiotensinogen in angiotensin II-dependent hypertension. *Hypertension* 43: 1126–1132, 2004.
21. **Lopez-Ilasaca M, Liu X, Tamura K, Dzau VJ.** The angiotensin II type I receptor-associated protein, ATRAP, is a transmembrane protein and a modulator of angiotensin II signaling. *Mol Biol Cell* 14: 5038–5050, 2003.
22. **Mogi M, Iwai M, Horiuchi M.** Emerging concepts of regulation of angiotensin II receptors: new players and targets for traditional receptors. *Arterioscler Thromb Vasc Biol* 27: 2532–2539, 2007.
23. **Mori T, Cowley AW Jr.** Angiotensin II-NAD(P)H oxidase-stimulated superoxide modifies tubulovascular nitric oxide cross-talk in renal outer medulla. *Hypertension* 42: 588–593, 2003.
24. **Mori T, O'Connor PM, Abe M, Cowley AW Jr.** Enhanced superoxide production in renal outer medulla of Dahl salt-sensitive rats reduces nitric oxide tubular-vascular cross-talk. *Hypertension* 49: 1336–1341, 2007.
25. **Navar LG, Harrison-Bernard LM, Nishiyama A, Kobori H.** Regulation of intrarenal angiotensin II in hypertension. *Hypertension* 39: 316–322, 2002.
26. **Nishiyama A, Nakagawa T, Kobori H, Nagai Y, Okada N, Konishi Y, Morikawa T, Okumura M, Meda I, Kiyomoto H, Hosomi N, Mori T, Ito S, Imanishi M.** Strict angiotensin blockade prevents the augmentation of intrarenal angiotensin II and podocyte abnormalities in type 2 diabetic rats with microalbuminuria. *J Hypertens* 26: 1849–1859, 2008.
27. **Nishiyama A, Seth DM, Navar LG.** Angiotensin II type 1 receptor-mediated augmentation of renal interstitial fluid angiotensin II in angiotensin II-induced hypertension. *J Hypertens* 21: 1897–1903, 2003.
28. **Niwa H, Yamamura K, Miyazaki J.** Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene* 108: 193–199, 1991.
29. **Oppermann M, Gess B, Schweda F, Castrop H.** Atrap deficiency increases arterial blood pressure and plasma volume. *J Am Soc Nephrol* 21: 468–477, 2010.
30. **Oshita A, Iwai M, Chen R, Ide A, Okumura M, Fukunaga S, Yoshii T, Mogi M, Higaki J, Horiuchi M.** Attenuation of inflammatory vascular remodeling by angiotensin II type 1 receptor-associated protein. *Hypertension* 48: 671–676, 2006.
31. **Prieto-Carrasquero MC, Kobori H, Ozawa Y, Gutierrez A, Seth D, Navar LG.** AT_1 receptor-mediated enhancement of collecting duct renin in angiotensin II-dependent hypertensive rats. *Am J Physiol Renal Physiol* 289: F632–F637, 2005.
32. **Reich HN, Oudit GY, Penninger JM, Scholey JW, Herzenberg AM.** Decreased glomerular and tubular expression of ACE2 in patients with type 2 diabetes and kidney disease. *Kidney Int* 74: 1610–1616, 2008.
33. **Rohrwasser A, Morgan T, Dillon HF, Zhao L, Callaway CW, Hillas E, Zhang S, Cheng T, Inagami T, Ward K, Terreros DA, Lalouel JM.** Elements of a paracrine tubular renin-angiotensin system along the entire nephron. *Hypertension* 34: 1265–1274, 1999.
34. **Sakai M, Tamura K, Tsurumi Y, Tanaka Y, Koide Y, Matsuda M, Ishigami T, Yabana M, Tokita Y, Hiroi Y, Komuro I, Umemura S.** Expression of MAK-V/Hunk in renal distal tubules and its possible involvement in proliferative suppression. *Am J Physiol Renal Physiol* 292: F1526–F1536, 2007.
35. **Shigenaga A, Tamura K, Wakui H, Masuda S, Azuma K, Tsurumi-Ikeya Y, Ozawa M, Mogi M, Matsuda M, Uchino K, Kimura K, Horiuchi M, Umemura S.** Effect of olmesartan on tissue expression balance between angiotensin II receptor and its inhibitory binding molecule. *Hypertension* 52: 672–678, 2008.
36. **Solis GP, Hoegg M, Munderloh C, Schrock Y, Malaga-Trillo E, Rivera-Milla E, Stuermer CA.** Reggie/flotillin proteins are organized into stable tetramers in membrane microdomains. *Biochem J* 403: 313–322, 2007.
37. **Tamura K, Tanaka Y, Tsurumi Y, Azuma K, Shigenaga A, Wakui H, Masuda S, Matsuda M.** The role of angiotensin AT1 receptor-associated protein in renin-angiotensin system regulation and function. *Curr Hypertens Rep* 9: 121–127, 2007.
38. **Tamura K, Umemura S, Nyui N, Yamakawa T, Yamaguchi S, Ishigami T, Tanaka S, Tanimoto K, Takagi N, Sekihara H, Murakami K, Ishii M.** Tissue-specific regulation of angiotensinogen gene expression in spontaneously hypertensive rats. *Hypertension* 27: 1216–1223, 1996.
39. **Tamura K, Umemura S, Yamakawa T, Nyui N, Hibi K, Watanabe Y, Ishigami T, Yabana M, Tanaka S, Sekihara H, Murakami K, Ishii M.** Modulation of tissue angiotensinogen gene expression in genetically obese hypertensive rats. *Am J Physiol Regul Integr Comp Physiol* 272: R1704–R1711, 1997.
40. **Tanaka Y, Tamura K, Koide Y, Sakai M, Tsurumi Y, Noda Y, Umemura M, Ishigami T, Uchino K, Kimura K, Horiuchi M, Umemura S.** The novel angiotensin II type 1 receptor (AT1R)-associated protein ATRAP downregulates AT1R and ameliorates cardiomyocyte hypertrophy. *FEBS Lett* 579: 1579–1586, 2005.
41. **Tang H, Guo DF, Porter JP, Wanaka Y, Inagami T.** Role of cytoplasmic tail of the type 1A angiotensin II receptor in agonist- and phorbol ester-induced desensitization. *Circ Res* 82: 523–531, 1998.
42. **Tsurumi Y, Tamura K, Tanaka Y, Koide Y, Sakai M, Yabana M, Noda Y, Hashimoto T, Kihara M, Hirawa N, Toya Y, Kiuchi Y, Iwai M, Horiuchi M, Umemura S.** Interacting molecule of AT1 receptor, ATRAP, is colocalized with AT1 receptor in the mouse renal tubules. *Kidney Int* 69: 488–494, 2006.
43. **Vila-Carriles WH, Kovacs GG, Jovov B, Zhou ZH, Pahwa AK, Colby G, Esimai O, Gillespie GY, Mapstone TB, Markert JM, Fuller CM, Bubien JK, Benos DJ.** Surface expression of ASIC2 inhibits the amiloride-sensitive current and migration of glioma cells. *J Biol Chem* 281: 19220–19232, 2006.
44. **Wakui H, Tamura K, Tanaka Y, Matsuda M, Bai Y, Dejima T, Masuda S, Shigenaga A, Maeda A, Mogi M, Ichihara N, Kobayashi Y, Hirawa N, Ishigami T, Toya Y, Yabana M, Horiuchi M, Minamisawa S, Umemura S.** Cardiac-specific activation of angiotensin II type 1 receptor-associated protein completely suppresses cardiac hypertrophy in chronic angiotensin II-infused mice. *Hypertension* 55: 1157–1164, 2010.
45. **Wesseling S, Ishola DA Jr, Joles JA, Bluyssen HA, Koomans HA, Braam B.** Resistance to oxidative stress by chronic infusion of angiotensin II in mouse kidney is not mediated by the AT_2 receptor. *Am J Physiol Renal Physiol* 288: F1191–F1200, 2005.
46. **Wolak T, Kim H, Ren Y, Kim J, Vaziri ND, Nicholas SB.** Osteopontin modulates angiotensin II-induced inflammation, oxidative stress, and fibrosis of the kidney. *Kidney Int* 76: 32–43, 2009.
47. **Ye M, Wysocki J, William J, Soler MJ, Cokic I, Batlle D.** Glomerular localization and expression of angiotensin-converting enzyme 2 and Angiotensin-converting enzyme: implications for albuminuria in diabetes. *J Am Soc Nephrol* 17: 3067–3075, 2006.
48. **Zhai P, Yamamoto M, Galeotti J, Liu J, Masurekar M, Thaisz J, Irie K, Holle E, Yu X, Kupershmidt S, Roden DM, Wagner T, Yatani A, Vatner DE, Vatner SF, Sadoshima J.** Cardiac-specific overexpression of AT1 receptor mutant lacking G alpha q/G alpha i coupling causes hypertrophy and bradycardia in transgenic mice. *J Clin Invest* 115: 3045–3056, 2005.
49. **Zhuo JL, Imig JD, Hammond TG, Orengo S, Benes E, Navar LG.** Ang II accumulation in rat renal endosomes during Ang II-induced hypertension: role of AT_1 receptor. *Hypertension* 39: 116–121, 2002.
50. **Zou LX, Imig JD, von Thun AM, Hymel A, Ono H, Navar LG.** Receptor-mediated intrarenal angiotensin II augmentation in angiotensin II-infused rats. *Hypertension* 28: 669–677, 1996.

Downloaded from ajprenal.physiology.org on November 21, 2010

Shin-ichiro Masuda, Kouichi Tamura, Hiromichi Wakui, Akinobu Maeda, Toru Dejima, Tomonori Hirose, Masao Toyoda, Koichi Azuma, Masato Ohsawa, Tomohiko Kanaoka, Mai Yanagi, Shin-ichiro Yoshida, Hiroshi Mitsuhashi, Miyuki Matsuda, Tomoaki Ishigami, Yoshiyuki Toya, Daisuke Suzuki, Yoji Nagashima and Satoshi Umemura
Am J Physiol Renal Physiol 299:720-731, 2010. First published Aug 4, 2010;
doi:10.1152/ajprenal.00667.2009

You might find this additional information useful...

This article cites 39 articles, 22 of which you can access free at:
http://ajprenal.physiology.org/cgi/content/full/299/4/F720#BIBL

Updated information and services including high-resolution figures, can be found at:
http://ajprenal.physiology.org/cgi/content/full/299/4/F720

Additional material and information about ***AJP - Renal Physiology*** can be found at:
http://www.the-aps.org/publications/ajprenal

This information is current as of November 21, 2010 .

AJP - Renal Physiology publishes original manuscripts on a broad range of subjects relating to the kidney, urinary tract, and their respective cells and vasculature, as well as to the control of body fluid volume and composition. It is published 12 times a year (monthly) by the American Physiological Society, 9650 Rockville Pike, Bethesda MD 20814-3991. Copyright © 2010 by the American Physiological Society. ISSN: 0363-6127, ESSN: 1522-1466. Visit our website at http://www.the-aps.org/.

Downloaded from ajprenal.physiology.org on November 21, 2010

Am J Physiol Renal Physiol 299: F720–F731, 2010.
First published August 4, 2010; doi:10.1152/ajprenal.00667.2009.

Expression of angiotensin II type 1 receptor-interacting molecule in normal human kidney and IgA nephropathy

Shin-ichiro Masuda,[1]* Kouichi Tamura,[1]* Hiromichi Wakui,[1] Akinobu Maeda,[1] Toru Dejima,[1] Tomonori Hirose,[2] Masao Toyoda,[3] Koichi Azuma,[1] Masato Ohsawa,[1] Tomohiko Kanaoka,[1] Mai Yanagi,[1] Shin-ichiro Yoshida,[1] Hiroshi Mitsuhashi,[1] Miyuki Matsuda,[1] Tomoaki Ishigami,[1] Yoshiyuki Toya,[1] Daisuke Suzuki,[3] Yoji Nagashima,[4] and Satoshi Umemura[1]

[1]*Department of Medical Science and Cardiorenal Medicine,* [2]*Department of Molecular Biology, and* [4]*Department of Molecular Pathology, Yokohama City University Graduate School of Medicine, Yokohama; and* [3]*Division of Nephrology and Metabolism, Department of Internal Medicine, Tokai University School of Medicine, Isehara, Japan*

Submitted 23 November 2009; accepted in final form 30 July 2010

Masuda S, Tamura K, Wakui H, Maeda A, Dejima T, Hirose T, Toyoda M, Azuma K, Ohsawa M, Kanaoka T, Yanagi M, Yoshida S, Mitsuhashi H, Matsuda M, Ishigami T, Toya Y, Suzuki D, Nagashima Y, Umemura S. Expression of angiotensin II type 1 receptor-interacting molecule in normal human kidney and IgA nephropathy. *Am J Physiol Renal Physiol* 299: F720–F731, 2010. First published August 4, 2010; doi:10.1152/ajprenal.00667.2009.—The intrarenal renin-angiotensin system plays a crucial role in the regulation of renal circulation and sodium reabsorption through the activation of vascular, glomerular, and tubular angiotensin II type 1 (AT_1) receptor signaling. We previously cloned a molecule that specifically interacted with the murine AT_1 receptor to inhibit AT_1 receptor signaling, which we named ATRAP (for AT_1 receptor-associated protein). Since murine ATRAP was shown to be highly expressed in the kidney, in the present study we investigated expression and distribution of human ATRAP in normal kidney and renal biopsy specimens from patients with IgA nephropathy. In the normal human kidney, both ATRAP mRNA and protein were widely and abundantly distributed along the renal tubules from Bowman's capsule to the medullary collecting ducts. In all renal tubular epithelial cells, the ATRAP protein colocalized with the AT_1 receptor. In renal biopsy specimens with IgA nephropathy, a significant positive correlation between ATRAP and AT_1 receptor gene expression was observed. There was also a positive relationship between tubulointerstitial ATRAP expression and the estimated glomerular filtration rate in patients with IgA nephropathy. Furthermore, we examined the function of the tubular AT_1 receptor using an immortalized cell line of mouse distal convoluted tubule cells (mDCT) and found that overexpression of ATRAP by adenoviral gene transfer suppressed the angiotensin II-mediated increases in transforming growth factor-β production in mDCT cells. These findings suggest that ATRAP might play a role in balancing the renal renin-angiotensin system synergistically with the AT_1 receptor by counterregulatory effects in IgA nephropathy and propose an antagonistic effect of tubular ATRAP on AT_1 receptor signaling.

renin-angiotensin system; immunohistochemistry; renal biopsy

THE RENIN-ANGIOTENSIN SYSTEM plays a critical role in the regulation of blood pressure and the maintenance of water-electrolyte metabolism. It acts through the production of the bioactive molecule angiotensin II (ANG II), exerting direct effects on the constriction of blood vessels, tubular sodium reabsorption, and the release of aldosterone. In addition, activation of the renin-angiotensin system at local sites is involved in the pathogenesis of hypertension and related target organ damage, as well as the development of renal inflammatory and fibrotic disease. For these actions of ANG II at local tissue sites, the ANG II type 1 (AT_1) receptor is the main receptor subtype. It is distributed in a variety of tissues, including the heart, brain, artery, adrenal gland, and kidney.

The AT_1 receptor is a member of the G protein-coupled receptor superfamily, having a seven-transmembrane spanning structure which activates G proteins through the third intracellular loop and the intracellular carboxyl-terminal (C-terminal) tail of the receptor (23, 24). The C-terminal cytoplasmic domain of the AT_1 receptor is involved in the control of AT_1 receptor internalization and plays an important role in linking receptor-mediated signal transduction to the specific biological responses to ANG II, such as hypertension, cardiovascular remodeling, and renal injury (12, 21). To further investigate the pathophysiological molecular mechanisms of AT_1 receptor signaling, we performed a yeast two-hybrid system screening of a murine kidney cDNA library. We identified the molecule angiotensin II type 1 receptor-associated protein (ATRAP), which interacts specifically with the C-terminal cytoplasmic domain of the AT_1 receptor (4). Murine ATRAP is predicted to have a three-transmembrane domain structure and is localized to the plasma membrane and intracellular trafficking vesicles in cultured cells (1, 18, 33). Functionally, several in vitro studies have shown that overexpression of murine ATRAP induces the internalization of the AT_1 receptor and decreases both the generation of inositol lipids and the cellular hypertrophic and proliferative responses, suggesting that ATRAP may be a negative regulator of AT_1 receptor signaling (1, 18, 33).

With respect to a human homolog of murine ATRAP, a previous study reported the cloning of human ATRAP cDNA, with its DNA and amino acid sequences 85 and 75% identical to the murine ATRAP gene, respectively (37). Human ATRAP was also shown to specifically interact with the C terminal of the human AT_1 receptor in vitro (37). To date, almost all of the studies on the physiological roles of the AT_1 receptor in the kidney have been performed in rat or mouse models, and there have been few studies on the distribution and function of the AT_1 receptor in the human kidney (15). A few previous studies showed that AT_1 receptor immunohistochemical staining was broadly observed in the blood vessels, renal tubules, and glomeruli in the normal human kidney and disclosed a

* S.-I. Masuda and K. Tamura contributed equally to this work.

Address for reprint requests and other correspondence: K. Tamura, Dept. of Medical Science and Cardiorenal Medicine, Yokohama City Univ. Graduate School of Medicine, 3-9 Fukuura, Kanazawa-ku, Yokohama 236-0004, Japan (e-mail: tamukou@med.yokohama-cu.ac.jp).

0363-6127/10 Copyright © 2010 the American Physiological Society

Downloaded from ajprenal.physiology.org on November 21, 2010

marginal increase in immunostaining for the AT_1 receptor in renal tubules in biopsy specimens from patients with IgA nephropathy (3, 5, 22).

Furthermore, at present there is no information available concerning the distribution of ATRAP in the normal and diseased human kidneys. Thus, to explore the possible pathophysiological role of ATRAP in human kidney disease, it is important to examine the expression of ATRAP in human renal tissues. Therefore, in the present study we examined the precise localization of renal human ATRAP expression at the mRNA and protein level by in situ hybridization and immunohistochemistry to determine whether the ATRAP protein coincided with the AT_1 receptor in the normal human kidney. Furthermore, we evaluated the relationship between ATRAP and AT_1 receptor protein expression, as well as the relationship between clinical variables and renal tissue expression of ATRAP and AT_1 receptor proteins in biopsy specimens from patients with IgA nephropathy.

METHODS

Materials. ANG II was purchased from Sigma. The AT_1 receptor-specific blocker candesartan (CV11974) and ANG II type 2 receptor (AT_2 receptor)-specific blocker PD123319 were kindly supplied by Takeda Pharmaceutical Company and the Parke-Davis division of Pfizer, respectively.

Cell culture and transient transfection. The immortalized cell line H9c2, which expresses the endogenous AT_1 receptor, was cultured as described previously (33). The human ATRAP cDNA expression plasmid (PCMV6XL5), the NH_2-terminal heme agglutinin (HA) epitope-tagged murine ATRAP in pcDNA3 (pc-HA-mATRAP), or the empty vector pCAGGS plasmid was transiently transfected into H9c2 cells according to the Lipofectamine 2000 protocol (Invitrogen) (4). Forty-eight hours after the transfection, whole cellular extracts were prepared from the transfected cells (18).

Mouse distal convoluted tubule (mDCT) cells were kindly provided by Dr. Peter A. Friedman (University of Pittsburgh School of Medicine, Pittsburgh, PA). The cells had been previously isolated and functionally characterized as described (9). Cells were grown on 100-mm dishes (Corning) in DMEM/HAM F-12 media (1:1, Sigma-Aldrich) supplemented with 5% heat-inactivated fetal calf serum (MBL), 2 mM L-glutamine (GIBCO), 0.5 mg/ml streptomycin, 0.5 mg/ml penicillin, and 1 mg/ml neomycin (GIBCO), in a humidified atmosphere of 5% CO_2-95% air. For the stimulation with ANG II, mDCT cells were subcultured in 6-cm-diameter dishes (1.5×10^5/ml), incubated overnight, and further incubated in serum-free medium for 24 h.

Preparation of normal human renal tissue and biopsy specimens with IgA nephropathy. Normal, uninvolved human male renal tissue ($n = 1$) obtained from resected renal carcinoma kidneys was perfusion-fixed with 4% paraformaldehyde and used for in situ hybridization and immunohistochemistry. Needle renal biopsy specimens were also obtained from 22 patients with IgA nephropathy and were used for immunohistochemistry. The diagnosis of IgA nephropathy was

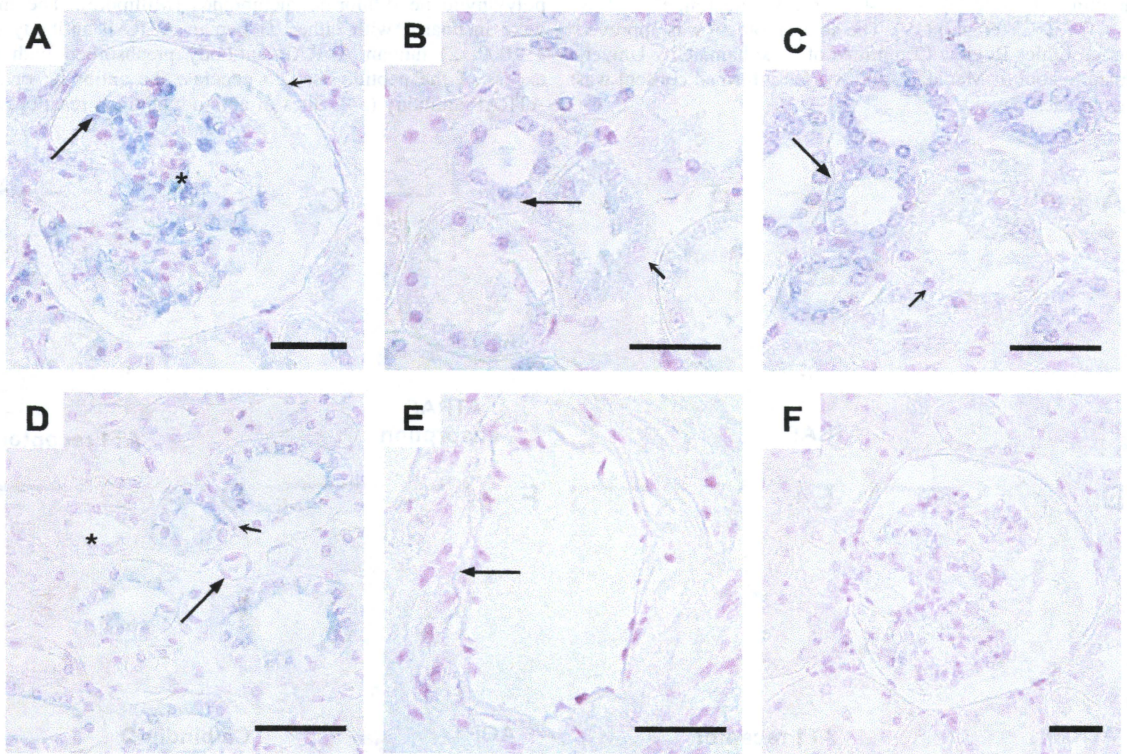

Fig. 1. Localization of ANG II type 1 (AT_1) receptor-associated protein (ATRAP) mRNA in a normal human kidney by in situ hybridization. *A*: glomerular section showing human ATRAP mRNA (positive staining blue) in Bowman's capsule (short arrow), podocytes (long arrow), and mesangial cells (asterisk). *B*: cortical section showing ATRAP in the proximal convoluted tubule (PCT; short arrow) and distal convoluted tubule (DCT; long arrow). *C*: cortical section showing ATRAP in the proximal straight tubule (PST; short arrow) and cortical collecting duct (CCD; long arrow). *D*: medullary section showing ATRAP in the medullary collecting duct (MCD; short arrow), thin limb (TL; long arrow), and thick ascending limb of Henle's loop (TAL; asterisk). *E*: interlobular artery showing weak ATRAP mRNA staining. *F*: consecutive section of that in *A* hybridized with the ATRAP sense probe as a negative control. ATRAP mRNA-positive staining is shown in blue, nuclear staining in pink, and overlapped staining in purple. Original magnification ×400. Bars = 50 μm.

Downloaded from ajprenal.physiology.org on November 21, 2010

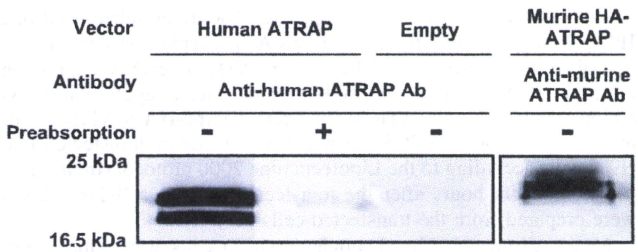

Fig. 2. Specificity of purified polyclonal anti-human ATRAP antibody. H9c2 cells were transfected with the human ATRAP cDNA expression plasmid (Human ATRAP) or an empty pCAGGS vector (Empty), and Western blot analysis was performed using an anti-human ATRAP antibody (Anti-human ATRAP Ab). The specificity of the anti-human ATRAP antibody was confirmed by preabsorption of the anti-human ATRAP antibody with the competing antigenic peptide (preabsorption +). As a reference, H9c2 cells were also transfected with the NH_2-terminal heme agglutinin (HA) epitope-tagged murine ATRAP in pcDNA3 (Murine HA-ATRAP), and Western blot analysis was performed using the anti-murine ATRAP antibody (Anti-murine ATRAP Ab).

confirmed by pathological evaluation of renal biopsy specimens, such as light microscopy, electron microscopy, and immunofluorescence staining. No patients received steroids or immunosuppressive drugs before renal biopsy. The following clinical parameters were examined at the time of needle renal biopsy: age, gender, body mass index, blood pressure, serum creatinine, total protein, urinary protein, and estimated glomerular filtration ratio (eGFR). We calculated eGFR with an application of a revised equation for the Japanese population: eGFR ($ml \cdot min^{-1} \cdot 1.73\ m^{-2}$) = 194 × serum $creatinine^{-1.094}$ × $age^{-0.287}$ × 0.739 (if female) (19). The study protocol was approved by the Human Ethics Review Committee of Yokohama City University Graduate School of Medicine, and written informed consent was obtained from each patient.

In situ hybridization. In situ hybridization was performed as described previously (34). After fixation, the normal human renal tissue (n = 1) was embedded in paraffin and sectioned. The riboprobes were generated complementary to the human ATRAP cDNA fragments (981 bases, +132 to +1112). Digoxygenin-labeled antisense and sense probes were prepared by the transcription of linearized human ATRAP cDNA in a pcDNA3.1 vector (Invitrogen) using a digoxygenin RNA labeling kit (Roche, Basel, Switzerland). Hybridized digoxygenin-labeled probes were detected using anti-digoxygenin-alkaline phosphatase conjugate. The probe was visualized using 4-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate.

Production of rabbit polyclonal anti-human ATRAP antibody. A 16-amino acid synthetic peptide corresponding to amino acids 144–158 of the C-terminal tail of human ATRAP was produced using standard solid-phase peptide synthesis techniques. Analysis using the BLAST computer program showed no significant overlap of the immunizing peptide with any known eukaryotic protein. The peptide was purified, conjugated, and injected five times intradermally into rabbits at 2-wk intervals for the production of the polyclonal antiserum. The rabbits developed enzyme-linked immunosorbent assay titers >1:128,000 before exsanguination. The selectivity of the antiserum was validated by the recognition of the PCMV6XL5-transfected H9c2 cells by Western blot analysis. Anti-ATRAP polyclonal antibodies were affinity-purified. This study was performed in accordance with the National Institutes of Health guidelines for the use of experimental animals. All of the animal studies were reviewed and approved by the animal studies committee of Yokohama City University.

SDS-PAGE and immunoblotting. Whole cellular extracts (20 μg/lane) from H9c2 cells were subjected to SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore). The membranes were incubated with either *1*) human ATRAP antibody diluted to 1:1,000; *2*) human ATRAP antibody preabsorbed with a 10-fold excess of the peptide used to generate the antibody; or *3*) murine ATRAP antibody (34). Sites of antibody-antigen reaction were visu-

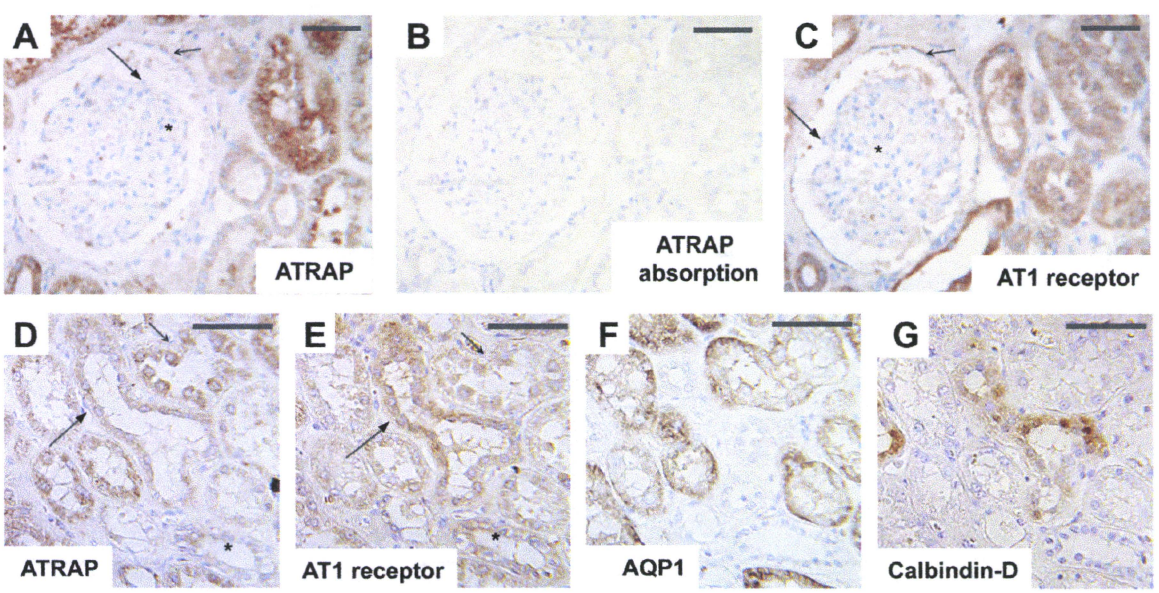

Fig. 3. Localization of ATRAP and AT_1 receptor proteins in the cortical region of a normal human kidney by immunohistochemistry. *A*: glomerular section showing human ATRAP in Bowman's capsule (short arrow), podocytes (long arrow), and mesangial cells (asterisk). *B*: consecutive section stained with ATRAP antibody preabsorbed with a 10-fold excess of immunizing peptide as a negative control. *C*: consecutive section showing human AT_1 receptor in Bowman's capsule (short arrow), podocytes (long arrow), and mesangial cells (asterisk). Consecutive tubular sections show human ATRAP (*D*) and the AT_1 receptor (*E*) in the PCT (short arrow), DCT (long arrow), and CCD (asterisk). The consecutive sections were also stained with a polyclonal antibody against aquaporin-1 (AQP1; *F*), a specific marker of proximal tubules, and a monoclonal antibody against calbindin-D (*G*), a specific marker of DCT and connecting tubule (CNT). Both the ATRAP- and AT_1 receptor protein-positive staining patterns are in brown. Original magnification ×400. Bars = 50 μm.

Downloaded from ajprenal.physiology.org on November 21, 2010

alized by enhanced chemiluminescence (Amersham Biosciences). The images were analyzed with an LAS-3000 imaging system (Fuji Film, Tokyo, Japan).

Immunohistochemistry. Immunohistochemistry was performed essentially as described previously (13, 34). After fixation, the renal tissues were embedded in paraffin, and sectioned at 4-μm thickness. The sections were treated for 60 min with 10% normal goat serum in phosphate-buffered saline and blocked for endogenous biotin activity using an Avidin/Biotin Blocking kit (Vector Laboratories). For the study of ATRAP, the sections were incubated with one of the following: *1*) ATRAP antibody diluted at 1:100; *2*) ATRAP antibody preabsorbed with a 10-fold excess of the peptide used to generate the antibody; or *3*) nonimmune rabbit IgG. For the study of AT_1 receptor, the sections were incubated with *1*) AT_1 receptor antibody [AT_1 (N-10), sc-1173, Santa Cruz Biotechnology, Santa Cruz, CA] diluted at 1:100; or *2*) nonimmune rabbit IgG. The characterization and specificity of the anti-AT_1 receptor antibody was described previously (34). For the study of specific nephron markers, the sections were incubated with either *1*) aquaporin-1 antibody (ab9566, Abcam) diluted at 1:100; *2*) calbindin D-28K antibody (C9848, Sigma-Aldrich) diluted at 1:3,000; *3*) Tamm-Horsfall glycoprotein antibody (sc-20631, Santa Cruz Biotechnology) diluted at 1:100; or *4*) nonimmune rabbit IgG. The sections were incubated for 60 min with biotinylated goat anti-rabbit IgG or biotinylated goat anti-murine IgG (SAB-PO kit, Nichirei, Tokyo, Japan), blocked for endogenous peroxidase activity by incubation with 0.3% H_2O_2 for 20 min, treated for 30 min with the streptavidin and biotinylated peroxidase (DAKO, Heidelberg, Germany), and then exposed to diaminobenzidine. The sections were counterstained with hematoxylin, dehydrated, and mounted.

Continuous high-resolution and high-magnification immunohistochemical images were obtained by using a BZ-9000 (KEYENCE, Osaka, Japan), and immunoreactivity was semiquantitatively evaluated in a blind manner. Examination was performed using a microscope with ×400 magnification (Olympus, Tokyo, Japan) and an integrated digital camera system (Olympus). Image Pro-plus computer

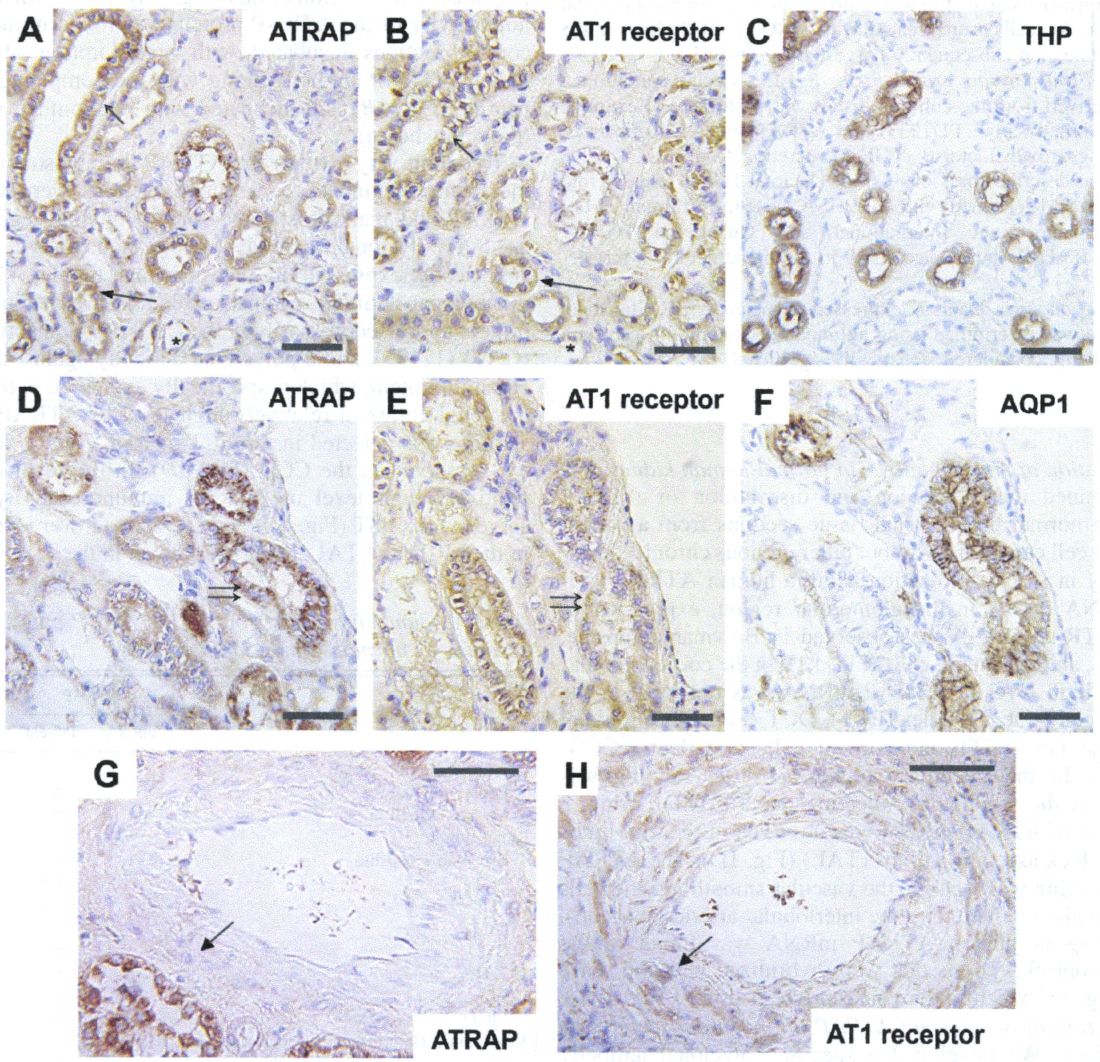

Fig. 4. Localization of ATRAP and AT_1 receptor proteins in the medullary region and the vasculature of a normal human kidney by immunohistochemistry. Consecutive medullary sections (*A–C* and *D–F*) show human ATRAP (*A* and *D*; positive staining brown) and AT_1 receptor (*B* and *E*; positive staining brown) in the MCD (short arrow), TAL (long arrow), TL (asterisk), and PST (double arrow). The consecutive sections were also stained for a polyclonal antibody against Tamm-Horsfall protein (THP; *C*), a TAL-specific marker, and a polyclonal antibody against AQP1 (*F*), a specific marker of the proximal tubules. Consecutive sections of the interlobular artery exhibit very weak human ATRAP immunostaining (*G*) and moderate AT_1 receptor staining (*H*) in the vascular smooth muscle cells. Original magnification ×400. Bars = 50 μm.

Downloaded from ajprenal.physiology.org on November 21, 2010

image-analysis software (Media Cybernetics, Bethesda, MD) was used to analyze the brown staining pixel density and to quantify the protein levels, as described previously (6, 10, 14, 28, 39).

To further assess the colocalization of the AT_1 and ATRAP proteins, 4-μm-thick mirror image paraffin sections were subjected to immunohistochemistry. Mirror image immunohistochemical staining was performed as described above. To demonstrate colocalization in the identical nephron section, ATRAP was detected by peroxidase-3-amino-9-ethylcarbazole and AT_1 receptor by alkaline phosphatase-nitroblue tetrazolium.

Preparation of recombinant adenoviral vectors and gene transfer. Adenoviral vectors were prepared using cDNAs coding for the NH_2-terminal HA epitope-tagged ATRAP (Ad.HA-ATRAP) and bacterial β-galactosidase (Ad.LacZ) using a commercially available system (Adeno X Expression System, Clontech), and the virus titer was determined with a plaque assay (33). For the adenoviral gene transfer experiments, mDCT cells were subcultured in 6 cm-diameter dishes (5×10^4/ml), incubated overnight, infected with recombinant adenovirus (Ad.HA-ATRAP or Ad.LacZ) at 50 multiplicity of infection for 24 h, and further incubated in a serum-free medium for an additional 24 h. Then, the cells were treated with ANG II (10^{-6} M) for the indicated time, and subsequently harvested for analysis.

ELISA of transforming growth factor-β. ELISA using conditioned medium derived from the cultured dishes was performed to examine the effect of candesartan, PD123319, or ATRAP on ANG II-mediated transforming growth factor-β (TGF-β) secretion from mDCT cells, essentially as described previously (30). The total TGF-β released into the media was determined with an ELISA system by Immuno Mini NJ-2300 (Nalge Nunc International) and Quantikine TGF-β1 (MB100B, R&D Systems), according to the manufacturer's instructions.

Statistical analysis. Data are expressed as means ± SE. Statistical significance was determined using an unpaired Student's *t*-test, with $P < 0.05$ being considered statistically significant.

RESULTS

Distribution of ATRAP mRNA in normal human kidney. We first examined the expression and distribution of ATRAP mRNA in normal human renal tissue sections from a patient with renal cell carcinoma without other obvious chronic kidney disease by in situ hybridization using a human ATRAP antisense cRNA probe. In the glomerular region, expression of human ATRAP mRNA was observed in Bowman's capsule, podocytes, and mesangial cells (Fig. 1*A*). In the cortical tubular region, a high level of ATRAP mRNA was observed in the proximal convoluted tubules (PCT), DCT (Fig. 1*B*), proximal straight tubules (PST), and cortical collecting ducts (CCD) (Fig. 1*C*). In the medullary region, positive staining was observed in the medullary collecting ducts (MCD), while a lower level of ATRAP mRNA was observed in the thin limbs (TL) and thick ascending limbs (TAL) (Fig. 1*D*). The ATRAP mRNA staining was weak in the vascular smooth muscle cells of the vasculature, including the interlobular arteries (Fig. 1*E*). No labeling of human ATRAP mRNA was found in the negative control sections that were hybridized with the sense probe (Fig. 1*F* which is the consecutive section of Fig. 1*A*).

Production of anti-human ATRAP antibody and validation of specificity. We developed a specific polyclonal antibody against human ATRAP. Western blot analysis of human ATRAP cDNA-transfected H9c2 cells revealed that the antiserum for human ATRAP recognized a prominent band of 17 kDa, which was consistent with the predicted molecular weight of human ATRAP, and also with the murine ATRAP band (= 18 kDa) derived from murine ATRAP cDNA-transfected cells, which was detected by an anti-murine ATRAP antibody (Fig. 2). This band was not observed when the antiserum was replaced with the preimmune serum (data not shown), or when empty vector pCAGGS-transfected H9c2 cells were used instead of human ATRAP-transfected cells (Fig. 2). These data indicate that the anti-human ATRAP antibody is able to recognize the human ATRAP protein specifically.

Distribution of ATRAP protein in normal human kidney. We next examined the expression and distribution of ATRAP protein in the normal human kidney of the same patient, who had been analyzed for the distribution of ATRAP mRNA expression, by immunohistochemistry using the anti-human ATRAP antibody. In the renal cortex, a relatively high level of ATRAP immunoreactivity was observed in the tubular nephron segments (Fig. 3*A*), and a lower level of ATRAP immunostaining was detected in Bowman's capsule, podocytes, and mesangial cells in the glomerulus (Fig. 3*A*). No significant immunoreactivity of the ATRAP protein was found in the negative control sections incubated with preabsorption of the anti-human ATRAP antibody along with the competing antigenic peptide (Fig. 3*B*) and incubated with nonimmune rabbit IgG (data not shown).

To definitively identify the ATRAP immunostaining sites in the tubular segments, consecutive sections were stained for ATRAP and markers specific for the tubular segments. We used a polyclonal antibody against aquaporin-1, which is specifically expressed in the proximal tubules (20, 25), a monoclonal antibody against calbindin-D, a calcium-binding protein expressed primarily in the DCT and connecting tubules (CNT) (17, 35), and a polyclonal antibody against the Tamm-Horsfall protein, which is specifically expressed in the TAL. In the renal cortex, a relatively high level of ATRAP immunostaining was detected in the PCT and DCT and a lower level was observed in the CCD (Fig. 3*D*). In the renal medulla, a relatively high level of ATRAP immunoreactivity was observed in the PST (Fig. 4, *A* and *D*), with a lower level detected in the MCD and TAL, and a weak level in the TL (Fig. 4*A*). In

Table 1. *Summary of distribution of ATRAP and AT_1 receptor in normal human kidney tissue*

	ATRAP		AT_1 Receptor
	mRNA	Protein	Protein
Glomerulus			
Podocyte	+	±	±
Mesangial cell	+	±	±
Renal tubule			
Bowman's capsule	+	±	++
PCT	+	++	++
PST	+	++	++
DCT	+	++	++
CCD	+	±	++
TAL	±	+	++
TL	±	±	±
MCD	+	+	++
Vasculature (interlobular artery, arteriole)			
Vascular smooth muscle cell	±	±	+
Vascular endothelial cell	−	−	−

ATRAP, ANG II type 1 receptor (AT_1)-associated protein; PCT, proximal convoluted tubule; PST, proximal straight tubule; DCT, distal convoluted tubule; CCD, cortical collecting duct; TAL, thick ascending limb of Henle's loop; TL, thin limb; MCD, medullary collecting duct.

Downloaded from ajprenal.physiology.org on November 21, 2010

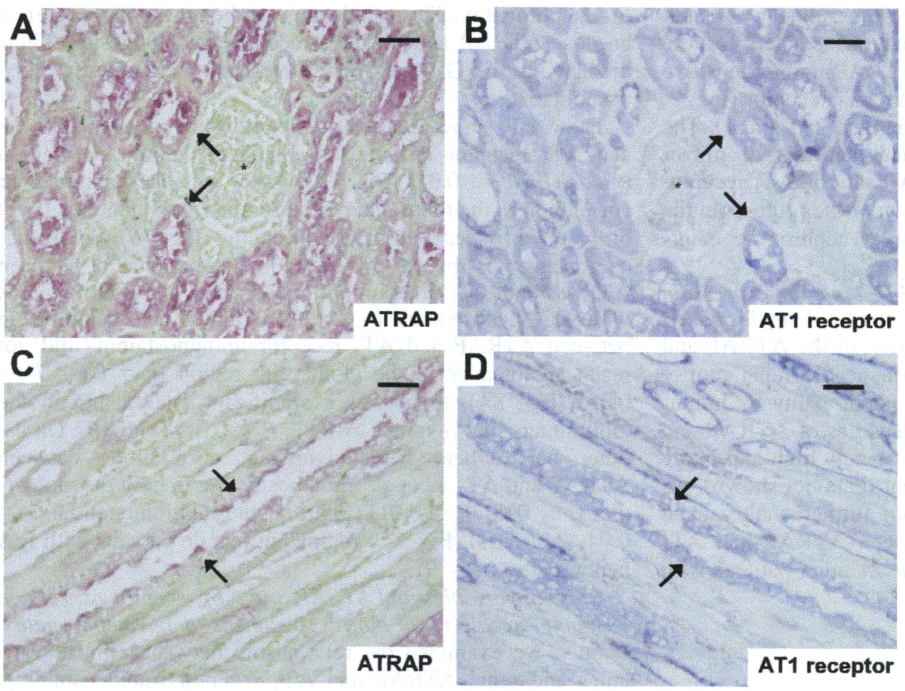

Fig. 5. Mirror-image analysis of ATRAP and AT_1 receptor proteins in a normal human kidney by immunohistochemistry. Mirror sections of the renal cortex (*A* and *B*) and medulla (*C* and *D*) show substantial colocalization of the human ATRAP (*A* and *C*; positive staining red) and AT_1 receptor (*B* and *D*; positive staining purple) proteins in the glomerulus of the renal cortex (asterisk) and in the renal tubules of both the renal cortex and medulla (arrow). Original magnification ×132. Bars = 50 μm.

vascular smooth muscle cells, including the interlobular arteries, ATRAP immunostaining was very weak (Fig. 4*G*).

Colocalization of ATRAP with the AT_1 receptor in human normal kidney. We also examined the intrarenal distribution of human AT_1 receptor protein by immunohistochemistry using the anti-AT_1 receptor antibody. The results of immunohistochemistry using the AT_1 receptor antibody revealed intense staining in Bowman's capsule (Fig. 3*C*), PCT, PST, DCT, CCD, TAL, and MCD (Fig. 3*E* and Fig. 4, *B* and *E*), and moderate staining in the vascular smooth muscle cells (of the vasculature), including the interlobular arteries (Fig. 4*H*). A lower level of AT_1 receptor immunostaining was also detected

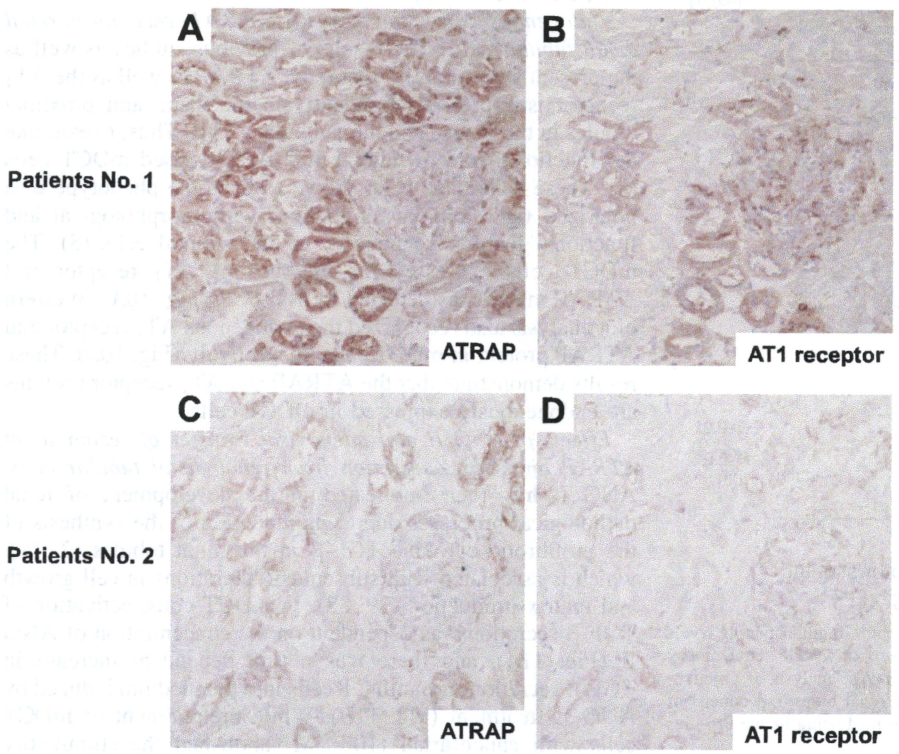

Fig. 6. Representative immunostaining of ATRAP and AT_1 receptor in needle renal biopsy specimens of IgA nephropathy. Consecutive sections from *patient 1* show a relatively high expression of ATRAP (*A*; positive staining brown) and AT_1 receptor (*B*; positive staining brown), while those from *patient 2* show a relatively low expression of ATRAP (*C*) and AT_1 receptor (*D*). Original magnification ×200. Bars = 50 μm.

Downloaded from ajprenal.physiology.org on November 21, 2010

in podocytes and mesangial cells in the glomeruli (Fig. 3*C*) and TL (Fig. 4*B*). In the consecutive sections stained for the ATRAP and AT_1 receptor, all of the ATRAP-immunopositive tubular nephron segments expressed the AT_1 receptor (Fig. 3, *D* and *E*, Fig. 4, *A*, *B*, *D*, and *E*; summarized in Table 1). The results of further immunohistochemical analysis of mirror sections which were stained for ATRAP and the AT_1 receptor also disclosed a significant colocalization of the proteins in the glomerulus and especially in the tubular nephron segments (Fig. 5).

Relationship between ATRAP and AT_1 receptor expression in human needle renal biopsy specimens of IgA nephropathy. The intrarenal expression and distribution of the ATRAP and AT_1 receptor genes were evaluated on needle renal biopsy specimens from 22 patients with IgA nephropathy by immunohistochemistry (Fig. 6). Since the densities of ATRAP and AT_1 receptor immunostaining were extremely high and likely to lie outside the range limit in 1 patient (data not shown), we excluded the data of this patient from further analysis and evaluated a potential correlation between ATRAP and AT_1 receptor gene expression patterns in the 21 other patients with IgA nephropathy. This relationship is thought to be of interest because ATRAP and the AT_1 receptor have opposite effects mediated by ANG II, i.e., opposite effects with regard to renal tissue damage.

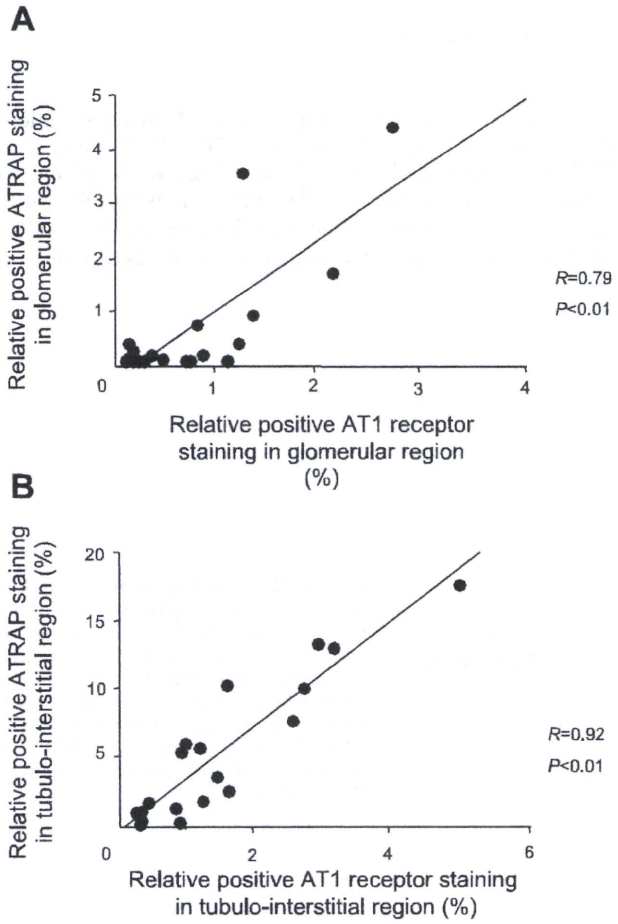

Fig. 7. Relationship between ATRAP and AT_1 receptor immunostaining levels in glomerular (*A*) and tubulointerstitial (*B*) regions of needle renal biopsy specimens of IgA nephropathy. Semiquantitative image analysis of immunostaining for ATRAP and AT_1 receptor in needle renal biopsy specimens of patients with IgA nephropathy ($n = 21$) was performed using Image Pro-plus computer software.

The results showed a positive relationship between ATRAP and AT_1 receptor immunostaining in the glomerular region ($r = 0.79$, $P < 0.01$) (Fig. 7*A*). There was also a significant correlation in the tubulointerstitial region ($r = 0.92$, $P < 0.01$) of the renal tissues with IgA nephropathy (Fig. 7*B*). We further examined a possible sex difference in the relationships between ATRAP and AT_1 receptor immunostaining. In male patients with IgA nephropathy ($n = 12$), although the relationship between ATRAP and AT_1 receptor immunostaining did not reach significance in the glomerular region ($r = 0.60$, $P = 0.09$) (Fig. 8*A*), there was a significant relationship in the tubulointerstitial region ($r = 0.82$, $P < 0.01$) (Fig. 8*B*). On the other hand, there were significant correlations between ATRAP and AT_1 receptor immunostaining in both the glomerular ($r = 0.90$, $P < 0.01$) (Fig. 8*C*) and tubulointerstitial ($r = 0.94$, $P < 0.01$) (Fig. 8*D*) regions in female patients with IgA nephropathy ($n = 9$).

Clinical variables and ATRAP and AT_1 receptor expression in human needle renal biopsy specimens of IgA nephropathy. Finally, we examined the relationship between clinical variables and renal ATRAP and AT_1 receptor immunostaining. The clinical parameters examined included age, body mass index, mean blood pressure, serum creatinine, total protein, urinary protein, and eGFR (Table 2). There was a significant positive relationship between eGFR and tubulointerstitial ATRAP immunostaining ($r = 0.44$, $P = 0.046$) in the needle renal biopsy specimens of IgA nephropathy (Fig. 9*D*). However, no significant correlation was observed between any other variables and the staining of the two proteins (Fig. 9, *A–C*; data not shown).

Endogenous expression of ATRAP and AT_1 receptor in renal distal tubular cells. The results of previous studies as well as the present study showed that renal ATRAP as well as the AT_1 receptor is expressed abundantly in the distal and proximal tubules in the mouse and human kidney (34). Thus, to examine the functional role of tubular ATRAP, we used mDCT cells (8). These cells have been shown to have the phenotype of a polarized tight junction epithelium with morphological and functional features retained from the parental cells (8). The mDCT cells expressed the endogenous AT_1 receptor and ATRAP mRNA, as detected by RT-PCR (Fig. 10*A*). Western blot analysis also recognized the endogenous AT_1 receptor and ATRAP protein in mDCT cells, respectively (Fig. 10*B*). These results demonstrate that the ATRAP and AT_1 receptor proteins are endogenously expressed in mDCT cells.

Effects of ANG II and adenoviral transfer of recombinant ATRAP on TGF-β secretion from renal distal tubular cells. ANG II has been implicated in the development of renal pathological processes due to its induction of the synthesis of the profibrotic cytokine TGF-β in the renal tubules (2, 11), which is associated with subsequent alterations in cell growth and matrix production (29, 38). In mDCT cells, activation of TGF-β secretion was dependent on the concentration of ANG II (Fig. 11*A*), and there was a time-dependent increase in TGF-β secretion from mDCT cells into the medium induced by ANG II treatment (Fig. 11*B*). While pretreatment of mDCT cells with candesartan (10^{-5} M) abolished the stimulatory

Downloaded from ajprenal.physiology.org on November 21, 2010

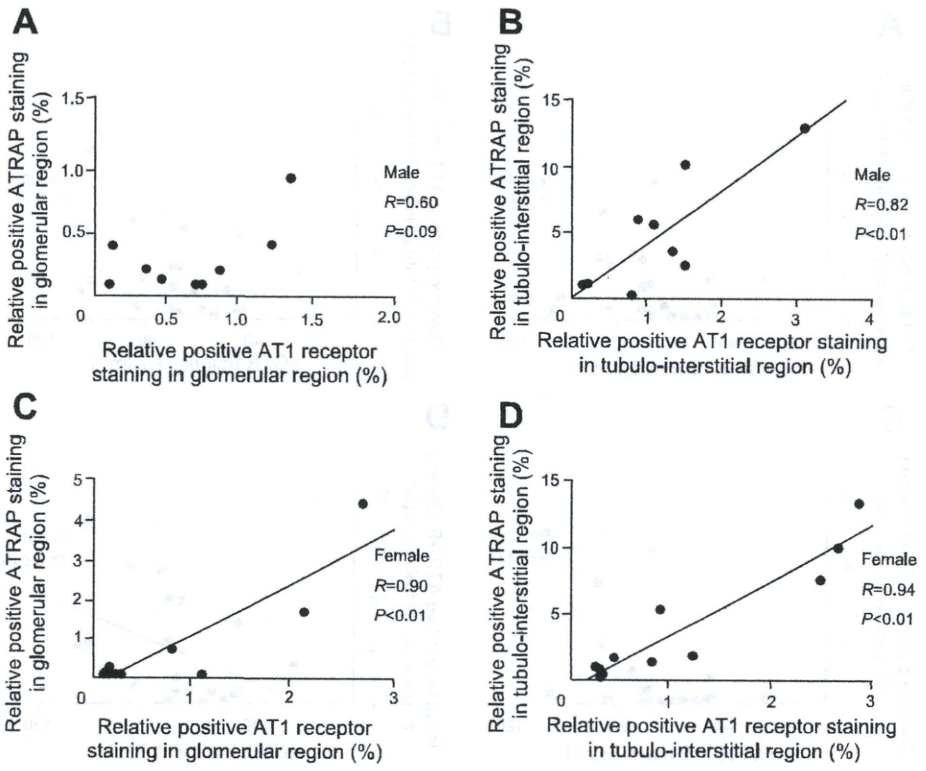

Fig. 8. Sex differences in relationship between ATRAP and AT_1 receptor immunostaining levels in glomerular (*A* and *C*) and tubulointerstitial (*B* and *D*) regions of needle renal biopsy specimens of IgA nephropathy. Semiquantitative image analysis of immunostaining for ATRAP and AT_1 receptor in needle renal biopsy specimens was performed, respectively, in male (*A* and *B*; $n = 12$) and female (*C* and *D*; $n = 9$) patients with IgA nephropathy using Image Pro-plus computer software.

effect of ANG II (10^{-6} M) on TGF-β secretion into the medium, preincubation of cells with PD123319 (10^{-5} M) did not affect the response to ANG II (Fig. 11*C*). These results indicate that the endogenously expressed AT_1 receptor is responsible for the ANG II-mediated activation of TGF-β production in mDCT cells.

To investigate the possible role of ATRAP in the functional modulation of distal tubular cells, we examined the effects of overexpression of ATRAP on the downstream effectors of the AT_1 receptor-signaling pathway in mDCT cells by performing adenoviral transfer of recombinant ATRAP. The mDCT cells were infected with an adenoviral vector containing ATRAP cDNA (Ad.HA-ATRAP) or control bacterial β-galactosidase cDNA (Ad.LacZ), and an ELISA of TGF-β was performed. Although ANG II (10^{-6} M) treatment of mDCT cells infected with Ad.LacZ increased the secretion of TGF-β protein into the medium, mDCT cells infected with Ad.HA-ATRAP exhibited an inhibition of the ANG II-induced enhancement (Fig. 11*D*), thereby indicating that ATRAP suppressed the ANG II-mediated activation of the downstream effectors of AT_1 receptor signaling in mDCT cells.

Table 2. *Clinical parameters in patients with IgA nephropathy at the time of needle renal biopsy*

Age, yr	37.4 ± 2.5
Body mass index, kg/m^2	22.8 ± 0.7
Mean BP, mmHg	95.3 ± 3.6
Serum creatinine, mg/dl	0.93 ± 0.06
Serum total protein, g/dl	6.8 ± 0.1
Urinary protein, g/g creatinine	0.97 ± 0.19
eGFR, ml·$^{-1}$·1.73 m^{-2}	66.7 ± 4.2

Values are means ± SE; $n = 21$, 12 men and 9 women. eGFR, estimated glomerular filtration rate.

DISCUSSION

The renin-angiotensin system is a well-coordinated hormonal cascade which critically regulates cardiovascular and renal homeostasis by maintaining fluid and electrolyte balance. In addition, stimulation of the renin-angiotensin system, particularly activation of AT_1 receptor signaling at local tissue sites, plays an important role in the pathophysiology of cardiovascular and renal disease. We previously cloned ATRAP as a novel molecule which interacts with the AT_1 receptor and showed that ATRAP suppressed ANG II-induced pathological responses by inducing constitutive AT_1 receptor internalization (4, 18). Thus ATRAP is suggested to be a potent counterregulator of the AT_1 receptor. We previously showed that ATRAP is expressed in a variety of murine tissues, as is the AT_1 receptor (34). Because activation of the AT_1 receptor at local tissue sites is thought to be a pivotal step in cardiovascular and renal injury, ATRAP has emerged as a potential inhibitor of tissue AT_1 receptor activation (24, 32).

There are previous reports on the human intrarenal renin-angiotensin system showing abundant expression of the human AT_1 receptor in the glomeruli, tubules, and vasculature and its upregulation in the renal tubules in IgA nephropathy (3, 5, 22). However, to date there has been no report on renal expression and distribution of ATRAP other than in the murine kidney (34). The role and significance of ATRAP in the regulation of the renin-angiotensin system in human renal tissue have yet to be elucidated. Moreover, the clinical and pathological factors modulating the expression of ATRAP in the human kidney

Downloaded from ajprenal.physiology.org on November 21, 2010

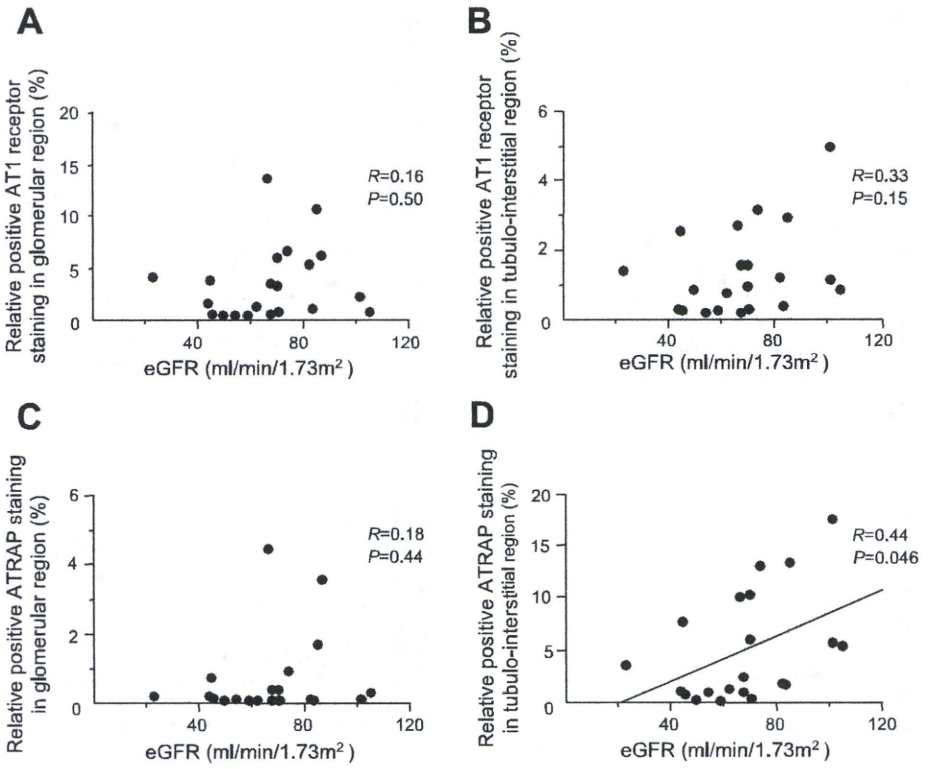

Fig. 9. Relationship between estimated glomerular filtration rate (eGFR) and immunostaining levels of ATRAP and AT_1 receptor in glomerular (*A* and *C*) and tubulointerstitial (*B* and *D*) regions of needle renal biopsy specimens from patients with IgA nephropathy. Semiquantitative image analysis of immunostaining for ATRAP and AT_1 receptor in renal biopsy specimens of patients with IgA nephropathy ($n = 21$) was performed using Image Pro-plus computer software. The clinical parameters examined included age, body mass index, mean blood pressure, serum creatinine, total protein, urinary protein, and eGFR. No significant correlation was observed between any of the clinical variables other than eGFR and the expression of the 2 proteins.

remain to be determined. Along these lines, a polyclonal anti-human ATRAP antibody was developed proven to be useful for the analysis of ATRAP protein expression in human tissue. Employing in situ hybridization and immunohistochemistry for ATRAP within human kidney sections, we showed for the first time that renal ATRAP expression, at both the mRNA and protein level, was widely and abundantly distributed in the renal tubules from Bowman's capsule to the MCD, with the relative abundance similar in each nephron segment. Furthermore, expression of ATRAP mRNA and protein was also detected in the glomerulus and vasculature, although the signals for both were comparatively weak in these areas.

With respect to the intrarenal distribution of the AT_1 receptor, specific immunoreactivity was observed in the vasculature and along the nephron segments, which was consistent with the findings of a previous study performed using normal kidney sections (22). From the immunohistochemical results of the present study, all ATRAP-immunopositive tubules from Bowman's capsule to the MCD expressed the AT_1 receptor in the consecutive sections which also stained for ATRAP. Thus the results of the present study demonstrate that human ATRAP is expressed along almost the entire nephron segment, as is the AT_1 receptor, with ATRAP abundantly expressed in the renal tubules. However, there was some dissociation between the two proteins in the pattern of intrarenal distribution. In particular, at the protein level, renal AT_1 receptor expression was more abundant than ATRAP expression in the vasculature. Previous studies have indicated that pathological vascular remodeling of the small renal arteries is critically involved in the development of hypertension and renal damage (7, 16, 31). They have also suggested that the protective effects of renin-angiotensin system inhibitors on the renal vasculature are, at least in part, responsible for prevention and amelioration of hypertension and renal injury (7, 16, 31).

On the other hand, the renal tubulointerstitial AT_1 receptor is also reported to play an important role in the regulation of sodium handling and epithelial-mesenchymal transition, as well as the pathogenesis of hypertension and renal fibrosis (15, 26, 27, 29). The results of the present study showed abundant renal tubular ATRAP expression in addition to tubular AT_1 receptor expression, while ATRAP expression in the vasculature was low. Interestingly, a significant correlation was detected between ATRAP and AT_1 receptor expression in the tubulointerstitial region, mainly in the tubular segments and not the vasculature, of the human needle renal biopsy specimens from patients with IgA nephropathy (Figs. 7 and 8). A previous study reported a similar synergistic expression of angiotensin-converting enzyme (ACE) and ACE2, a counterregulator of ACE in generating ANG II as one of the organ-protective factors in human renal tissue (36). Thus we consider this finding to be important in the effort to elucidate the mechanisms of the renal interplay between the AT_1 receptor and ATRAP and for establishing the role of renal ATRAP as a counterregulator of the AT_1 receptor.

In particular, the interpretation of the results showing a positive but not negative correlation between ATRAP and the AT_1 receptor in the diseased kidney might be controversial. The present results suggest that ATRAP protein expression does not actively alter the status of the renal renin-angiotensin system. We speculate that AT_1 receptor and ATRAP protein expression may be regulated in a coordinated manner, or synergistically, which is mediated via the local activation of AT_1 receptor signaling. A notable point here is that not only the AT_1 receptor, but also ATRAP, may be responsible for

Downloaded from ajprenal.physiology.org on November 21, 2010

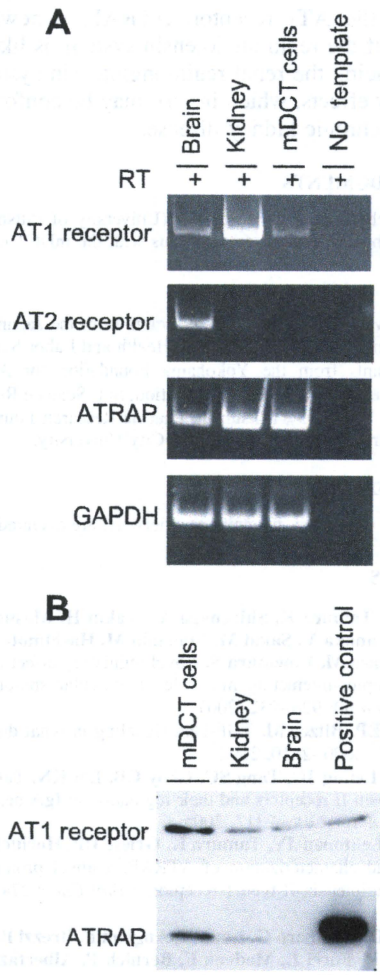

Fig. 10. Expression of endogenous AT_1 receptor and ATRAP in renal distal tubular cells. *A*: representative RT-PCR analysis of mDCT cells using the AT_1 receptor-, AT_2 receptor-, and ATRAP-specific primers. The negative control, consisting of RT-PCR reactions lacking a template (no template), are also shown. *B*: representative Western blot analysis of endogenous AT_1 receptor and ATRAP proteins with anti-AT_1 receptor and anti-ATRAP polyclonal antibodies, respectively, in extracts from mouse tissues and mDCT cells. The positive control using cellular extracts from H9c2 cells transiently transfected with the HA-tagged ATRAP in pcDNA3 (positive control) is also shown.

determination of the renal activity of AT_1 receptor signaling at the local tissue sites, with a coordinated expression of ATRAP exerting a countereffect against AT_1 receptor activity by constitutive stimulation of AT_1 receptor internalization. It is suggested that the role of ATRAP must be taken into consideration for the pathogenesis of renal tissue injury.

However, since the present study is a cross-sectional study examining the relationship between ATRAP and the AT_1 receptor in IgA nephropathy, but not a prospective study to examine effects of intervention to affect AT_1 receptor signaling, the exact mechanism for this regulation is not clear. We were not able to determine whether the AT_1 receptor influences ATRAP expression in the kidney at this stage. Additional studies with repeated biopsies and intervention in AT_1 receptor signaling such as by means of AT_1 receptor-specific blockers are needed to elucidate the mechanism of the relationship between ATRAP and the AT_1 receptor in the kidney of IgA nephropathy.

We also tested the possibility that the clinical characteristics are substantially related to renal ATRAP expression. Indeed, semiquantitative analysis of the relationship between the clin-

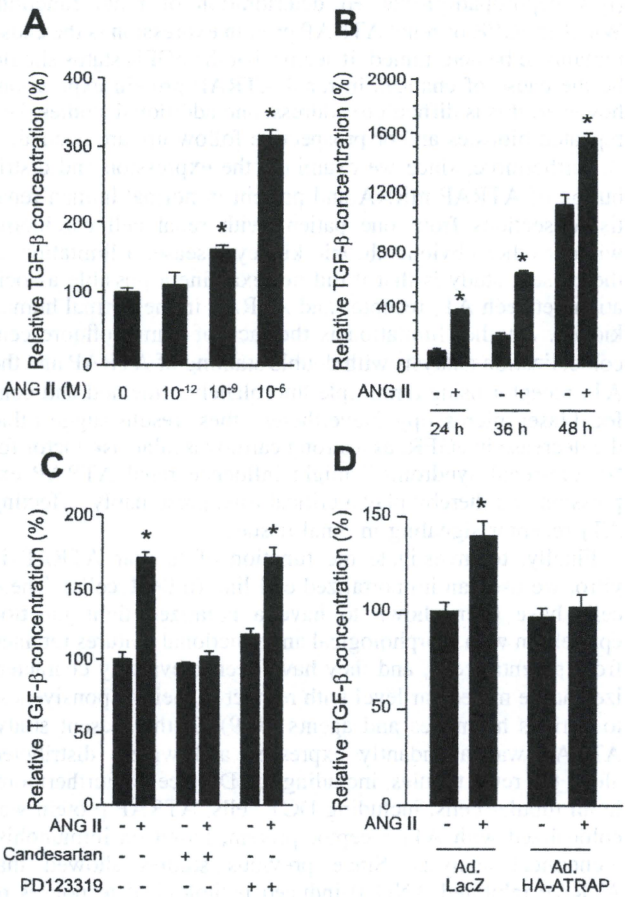

Fig. 11. Effects of ANG II and adenoviral transfer of recombinant ATRAP on transforming growth factor (TGF)-β secretion from renal distal tubular cells. (*A*) ELISA showing the relative TGF-β concentrations in the cultured medium of mouse (m) DCT cells stimulated with vehicle (0 M ANG II) or ANG II at the indicated concentrations (10^{-12} M~10^{-6} M) for 36 h. Values are calculated relative to those achieved with extracts from mDCT cells stimulated with vehicle and are expressed as means ± SE ($n = 7$ in each group). *$P < 0.05$ vs. 0 M ANG II. *B*: ELISA showing relative TGF-β concentrations in cultured medium of mDCT cells stimulated with vehicle (ANG II, −) or ANG II at 10^{-6} M for the indicated time (24 h~48 h). The values are calculated relative to those achieved with extracts from mDCT cells stimulated with vehicle (ANG II, −) for 24 h and are expressed as means ± SE ($n = 7$ in each group). *$P < 0.05$ vs. ANG II, −. *C*: ELISA showing relative TGF-β protein levels in cultured medium of mDCT cells pretreated with an AT_1 receptor-specific blocker (candesartan; 10^{-5} M) or AT_2 receptor-specific blocker (PD123319; 10^{-5} M) followed by stimulation with vehicle (ANG II, −) or ANG II at 10^{-6} M (ANG II, +) for 36 h. Values are calculated relative to those achieved with extracts from mDCT cells stimulated with vehicle without AT_1 receptor- or AT_2 receptor-specific blockers and are expressed as means ± SE ($n = 7$ in each group). *$P < 0.05$ vs. ANG II, −. *D*: ELISA showing relative TGF-β protein levels in cultured medium of mDCT cells infected with the ATRAP adenoviral vector (Ad.HA-ATRAP) or LacZ adenoviral vector (Ad.LacZ). Forty-eight hours after infection, the cells were stimulated with vehicle (ANG II, −) or ANG II at 10^{-6} M (ANG II, +) for 36 h. The values are calculated relative to those achieved with extracts from mDCT cells infected with Ad.LacZ and stimulated with vehicle and are expressed as means ± SE ($n = 9$ in each group). *$P < 0.05$ vs. ANG II.

Downloaded from ajprenal.physiology.org on November 21, 2010

ical variables and the renal AT_1 receptor and ATRAP immunostaining revealed ATRAP protein expression to be significantly and positively correlated with eGFR. The other parameters of renal function, e.g., urinary protein and serum creatinine, were not significantly associated with ATRAP protein expression (Fig. 7). The eGFR values may reflect disease (IgA nephropathy)-induced deterioration of renal function. Whether eGFR or renal ATRAP protein expression is the cause remains to be determined. It seems that the eGFR status should be the cause of changes in renal ATRAP protein expression; however, this is difficult to address, and additional studies, i.e., repeated biopsies and/or prospective follow-up, are needed.

Furthermore, since we examined the expression and distribution of ATRAP mRNA and protein in normal human renal tissue sections from one patient with renal cell carcinoma without other obvious chronic kidney disease, a limitation of the present study is that it did not examine a possible association between AT_1 receptor and ATRAP in the normal human kidney. Another limitation is the lack of immunofluorescent colocalization analysis with double staining of ATRAP and the AT_1 receptor using a multiple fluorolabeling method and confocal laser microscopy. Nevertheless, these results suggest that the decrease in eGFR, as a strong cardiovascular risk factor for "cardio-renal syndrome," might influence renal ATRAP expression and thereby play a critical role, presumably, affecting AT_1 receptor signaling in renal tissues.

Finally, to investigate the function of tubular ATRAP in vitro, we used an immortalized cell line (mDCT cells). These cells have been shown to have a polarized tight junction epithelium with morphological and functional features retained from parental cells, and they have been previously characterized at the molecular level with respect to their responsiveness to various hormones and agents (8, 9). In the present study, ATRAP was abundantly expressed and widely distributed along the renal tubules, including the DCT cells. Furthermore, in all tubular cells, including DCT cells, ATRAP protein was colocalized with AT_1 receptor protein, based on immunohistochemical analysis. Since previous studies showed that ATRAP inhibited ANG II-induced pathological responses of cardiovascular cells by promoting a constitutive internalization of the AT_1 receptor (24, 32), we examined whether renal tubular ATRAP antagonizes the pathological activation of the tubular AT_1 receptor using mDCT cells. The results showed that the overexpression of ATRAP suppresses the AT_1 receptor-mediated activation of TGF-β production in response to ANG II stimulation, thereby suggesting that tubular ATRAP is an endogenous suppressor of the activation of tubular AT_1 receptor signaling.

In summary, the results of the present study demonstrate the abundant expression of ATRAP mRNA and protein and their distribution in the human kidney. ATRAP is broadly distributed along the nephron, with a substantial colocalization of ATRAP and the AT_1 receptor. The results using human needle renal biopsy specimens of IgA nephropathy show a significant relationship between renal ATRAP and AT_1 receptor protein levels, and renal ATRAP protein expression appears to be influenced by renal functional status. Furthermore, the findings obtained by in vitro experiments using renal distal tubular cells also showed the functional significance of renal tubular AT_1 receptor signaling, as well as the antagonistic effect of tubular ATRAP on this signaling. These findings suggest that in addition to the AT_1 receptor, ATRAP, a newly emerging component of the renin-angiotensin system, is likely to play a role in balancing the renal renin-angiotensin system by counterregulatory effects, which in turn may be confounded by the presence of chronic kidney disease.

ACKNOWLEDGMENTS

We are indebted to Dr. P. Friedman (University of Pittsburgh School of Medicine, Pittsburgh, PA) for providing us with the mDCT cells.

GRANTS

This work was supported in part by grants from the Japanese Ministry of Education, Science, Sports, and Culture, Health and Labor Sciences Research grants; and grants from the Yokohama Foundation for Advancement of Medical Science, Takeda Science Foundation, Salt Science Research Foundation (nos. 0911 and 1033), Mitsubishi Pharma Research Foundation, and the Strategic Research Project of Yokohama City University.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

REFERENCES

1. **Azuma K, Tamura K, Shigenaga A, Wakui H, Masuda S, Tsurumi-Ikeya Y, Tanaka Y, Sakai M, Matsuda M, Hashimoto T, Ishigami T, Lopez-Ilasaca M, Umemura S.** Novel regulatory effect of angiotensin II type 1 receptor-interacting molecule on vascular smooth muscle cells. *Hypertension* 50: 926–932, 2007.
2. **Bottinger EP, Bitzer M.** TGF-beta signaling in renal disease. *J Am Soc Nephrol* 13: 2600–2610, 2002.
3. **Chan LY, Leung JC, Tang SC, Choy CB, Lai KN.** Tubular expression of angiotensin II receptors and their regulation in IgA nephropathy. *J Am Soc Nephrol* 16: 2306–2317, 2005.
4. **Daviet L, Lehtonen JY, Tamura K, Griese DP, Horiuchi M, Dzau VJ.** Cloning and characterization of ATRAP, a novel protein that interacts with the angiotensin II type 1 receptor. *J Biol Chem* 274: 17058–17062, 1999.
5. **Del Prete D, Gambaro G, Lupo A, Anglani F, Brezzi B, Magistroni R, Graziotto R, Furci L, Modena F, Bernich P, Albertazzi A, D'Angelo A, Maschio G.** Precocious activation of genes of the renin-angiotensin system and the fibrogenic cascade in IgA glomerulonephritis. *Kidney Int* 64: 149–159, 2003.
6. **Faulkner JL, Szcykalski LM, Springer F, Barnes JL.** Origin of interstitial fibroblasts in an accelerated model of angiotensin II-induced renal fibrosis. *Am J Pathol* 167: 1193–1205, 2005.
7. **Folkow B.** "Structural factor" in primary and secondary hypertension. *Hypertension* 16: 89–101, 1990.
8. **Friedman PA, Gesek FA.** Stimulation of calcium transport by amiloride in mouse distal convoluted tubule cells. *Kidney Int* 48: 1427–1434, 1995.
9. **Gesek FA, Friedman PA.** Sodium entry mechanisms in distal convoluted tubule cells. *Am J Physiol Renal Fluid Electrolyte Physiol* 268: F89–F98, 1995.
10. **Gonzalez-Villalobos RA, Seth DM, Satou R, Horton H, Ohashi N, Miyata K, Katsurada A, Tran DV, Kobori H, Navar LG.** Intrarenal angiotensin II and angiotensinogen augmentation in chronic angiotensin II-infused mice. *Am J Physiol Renal Physiol* 295: F772–F779, 2008.
11. **Gore-Hyer E, Shegogue D, Markiewicz M, Lo S, Hazen-Martin D, Greene EL, Grotendorst G, Trojanowska M.** TGF-beta and CTGF have overlapping and distinct fibrogenic effects on human renal cells. *Am J Physiol Renal Physiol* 283: F707–F716, 2002.
12. **Hein L, Meinel L, Pratt RE, Dzau VJ, Kobilka BK.** Intracellular trafficking of angiotensin II and its AT1 and AT2 receptors: evidence for selective sorting of receptor and ligand. *Mol Endocrinol* 11: 1266–1277, 1997.
13. **Hirose T, Satoh D, Kurihara H, Kusaka C, Hirose H, Akimoto K, Matsusaka T, Ichikawa I, Noda T, Ohno S.** An essential role of the universal polarity protein, aPKClambda, on the maintenance of podocyte slit diaphragms. *PLoS One* 4: e4194, 2009.
14. **Hong SW, Isono M, Chen S, Iglesias-De La Cruz MC, Han DC, Ziyadeh FN.** Increased glomerular and tubular expression of transforming growth factor-beta1, its type II receptor, and activation of the Smad

Downloaded from ajprenal.physiology.org on November 21, 2010

signaling pathway in the db/db mouse. *Am J Pathol* 158: 1653–1663, 2001.

15. **Kobori H, Nangaku M, Navar LG, Nishiyama A.** The intrarenal renin-angiotensin system: from physiology to the pathobiology of hypertension and kidney disease. *Pharmacol Rev* 59: 251–287, 2007.
16. **Lever AF, Harrap SB.** Essential hypertension: a disorder of growth with origins in childhood? *J Hypertens* 10: 101–120, 1992.
17. **Loffing-Cueni D, Flores SY, Sauter D, Daidie D, Siegrist N, Meneton P, Staub O, Loffing J.** Dietary sodium intake regulates the ubiquitin-protein ligase nedd4–2 in the renal collecting system. *J Am Soc Nephrol* 17: 1264–1274, 2006.
18. **Lopez-Ilasaca M, Liu X, Tamura K, Dzau VJ.** The angiotensin II type I receptor-associated protein, ATRAP, is a transmembrane protein and a modulator of angiotensin II signaling. *Mol Biol Cell* 14: 5038–5050, 2003.
19. **Matsuo S, Imai E, Horio M, Yasuda Y, Tomita K, Nitta K, Yamagata K, Tomino Y, Yokoyama H, Hishida A.** Revised equations for estimated GFR from serum creatinine in Japan. *Am J Kidney Dis* 53: 982–992, 2009.
20. **Maunsbach AB, Marples D, Chin E, Ning G, Bondy C, Agre P, Nielsen S.** Aquaporin-1 water channel expression in human kidney. *J Am Soc Nephrol* 8: 1–14, 1997.
21. **Mehta PK, Griendling KK.** Angiotensin II cell signaling: physiological and pathological effects in the cardiovascular system. *Am J Physiol Cell Physiol* 292: C82–C97, 2007.
22. **Mifune M, Sasamura H, Nakazato Y, Yamaji Y, Oshima N, Saruta T.** Examination of angiotensin II type 1 and type 2 receptor expression in human kidneys by immunohistochemistry. *Clin Exp Hypertens* 23: 257–266, 2001.
23. **Miura S, Saku K, Karnik SS.** Molecular analysis of the structure and function of the angiotensin II type 1 receptor. *Hypertens Res* 26: 937–943, 2003.
24. **Mogi M, Iwai M, Horiuchi M.** Emerging concepts of regulation of angiotensin II receptors: new players and targets for traditional receptors. *Arterioscler Thromb Vasc Biol* 27: 2532–2539, 2007.
25. **Nagy G, Szekeres G, Kvell K, Berki T, Nemeth P.** Development and characterisation of a monoclonal antibody family against aquaporin 1 (AQP1) and aquaporin 4 (AQP4). *Pathol Oncol Res* 8: 115–124, 2002.
26. **Navar LG, Harrison-Bernard LM, Nishiyama A, Kobori H.** Regulation of intrarenal angiotensin II in hypertension. *Hypertension* 39: 316–322, 2002.
27. **Nishiyama A, Seth DM, Navar LG.** Angiotensin II type 1 receptor-mediated augmentation of renal interstitial fluid angiotensin II in angiotensin II-induced hypertension. *J Hypertens* 21: 1897–1903, 2003.
28. **Reich HN, Oudit GY, Penninger JM, Scholey JW, Herzenberg AM.** Decreased glomerular and tubular expression of ACE2 in patients with type 2 diabetes and kidney disease. *Kidney Int* 74: 1610–1616, 2008.
29. **Ruiz-Ortega M, Ruperez M, Esteban V, Rodriguez-Vita J, Sanchez-Lopez E, Carvajal G, Egido J.** Angiotensin II: a key factor in the inflammatory and fibrotic response in kidney diseases. *Nephrol Dial Transplant* 21: 16–20, 2006.
30. **Sakai M, Tamura K, Tsurumi Y, Tanaka Y, Koide Y, Matsuda M, Ishigami T, Yabana M, Tokita Y, Hiroi Y, Komuro I, Umemura S.** Expression of MAK-V/Hunk in renal distal tubules and its possible involvement in proliferative suppression. *Am J Physiol Renal Physiol* 292: F1526–F1536, 2007.
31. **Sasamura H, Hayashi K, Ishiguro K, Nakaya H, Saruta T, Itoh H.** Prevention and regression of hypertension: role of renal microvascular protection. *Hypertens Res* 32: 658–664, 2009.
32. **Tamura K, Tanaka Y, Tsurumi Y, Azuma K, Shigenaga A, Wakui H, Masuda S, Matsuda M.** The role of angiotensin AT1 receptor-associated protein in renin-angiotensin system regulation and function. *Curr Hypertens Rep* 9: 121–127, 2007.
33. **Tanaka Y, Tamura K, Koide Y, Sakai M, Tsurumi Y, Noda Y, Umemura M, Ishigami T, Uchino K, Kimura K, Horiuchi M, Umemura S.** The novel angiotensin II type 1 receptor (AT1R)-associated protein ATRAP downregulates AT1R and ameliorates cardiomyocyte hypertrophy. *FEBS Lett* 579: 1579–1586, 2005.
34. **Tsurumi Y, Tamura K, Tanaka Y, Koide Y, Sakai M, Yabana M, Noda Y, Hashimoto T, Kihara M, Hirawa N, Toya Y, Kiuchi Y, Iwai M, Horiuchi M, Umemura S.** Interacting molecule of AT1 receptor, ATRAP, is colocalized with AT1 receptor in the mouse renal tubules. *Kidney Int* 69: 488–494, 2006.
35. **Vekaria RM, Shirley DG, Sevigny J, Unwin RJ.** Immunolocalization of ectonucleotidases along the rat nephron. *Am J Physiol Renal Physiol* 290: F550–F560, 2006.
36. **Wakahara S, Konoshita T, Mizuno S, Motomura M, Aoyama C, Makino Y, Kato N, Koni I, Miyamori I.** Synergistic expression of angiotensin-converting enzyme (ACE) and ACE2 in human renal tissue and confounding effects of hypertension on the ACE to ACE2 ratio. *Endocrinology* 148: 2453–2457, 2007.
37. **Wang W, Huang Y, Zhou Z, Tang R, Zhao W, Zeng L, Xu M, Cheng C, Gu S, Ying K, Xie Y, Mao Y.** Identification and characterization of AGTRAP, a human homolog of murine Angiotensin II Receptor-Associated Protein (Agtrap). *Int J Biochem Cell Biol* 34: 93–102, 2002.
38. **Wolf G, Jablonski K, Schroeder R, Reinking R, Shankland SJ, Stahl RA.** Angiotensin II-induced hypertrophy of proximal tubular cells requires p27Kip1. *Kidney Int* 64: 71–81, 2003.
39. **Ye M, Wysocki J, William J, Soler MJ, Cokic I, Batlle D.** Glomerular localization and expression of Angiotensin-converting enzyme 2 and Angiotensin-converting enzyme: implications for albuminuria in diabetes. *J Am Soc Nephrol* 17: 3067–3075, 2006.

Downloaded from ajprenal.physiology.org on November 21, 2010

Hypertension
JOURNAL OF THE AMERICAN HEART ASSOCIATION
American Heart Association®
Learn and Live℠

Cardiac-Specific Activation of Angiotensin II Type 1 Receptor Associated Protein Completely Suppresses Cardiac Hypertrophy in Chronic Angiotensin II Infused Mice

Hiromichi Wakui, Kouichi Tamura, Yutaka Tanaka, Miyuki Matsuda, Yunzhe Bai, Toru Dejima, Shin-ichiro Masuda, Atsu-ichiro Shigenaga, Akinobu Maeda, Masaki Mogi, Naoaki Ichihara, Yusuke Kobayashi, Nobuhito Hirawa, Tomoaki Ishigami, Yoshiyuki Toya, Machiko Yabana, Masatsugu Horiuchi, Susumu Minamisawa and Satoshi Umemura

Hypertension 2010;55;1157-1164; originally published online Mar 15, 2010;
DOI: 10.1161/HYPERTENSIONAHA.109.147207
Hypertension is published by the American Heart Association. 7272 Greenville Avenue, Dallas, TX 72514
Copyright © 2010 American Heart Association. All rights reserved. Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/cgi/content/full/55/5/1157

Data Supplement (unedited) at:
http://hyper.ahajournals.org/cgi/content/full/HYPERTENSIONAHA.109.147207/DC1

Subscriptions: Information about subscribing to Hypertension is online at
http://hyper.ahajournals.org/subscriptions/

Permissions: Permissions & Rights Desk, Lippincott Williams & Wilkins, a division of Wolters Kluwer Health, 351 West Camden Street, Baltimore, MD 21202-2436. Phone: 410-528-4050. Fax: 410-528-8550. E-mail:
journalpermissions@lww.com

Reprints: Information about reprints can be found online at
http://www.lww.com/reprints

Downloaded from hyper.ahajournals.org at Yokohama City University on April 26, 2010

Cardiac-Specific Activation of Angiotensin II Type 1 Receptor–Associated Protein Completely Suppresses Cardiac Hypertrophy in Chronic Angiotensin II–Infused Mice

Hiromichi Wakui, Kouichi Tamura, Yutaka Tanaka, Miyuki Matsuda, Yunzhe Bai, Toru Dejima, Shin-ichiro Masuda, Atsu-ichiro Shigenaga, Akinobu Maeda, Masaki Mogi, Naoaki Ichihara, Yusuke Kobayashi, Nobuhito Hirawa, Tomoaki Ishigami, Yoshiyuki Toya, Machiko Yabana, Masatsugu Horiuchi, Susumu Minamisawa, Satoshi Umemura

Abstract—We cloned a novel molecule interacting with angiotensin II type 1 receptor, which we named ATRAP (for angiotensin II type 1 receptor–associated protein). Previous in vitro studies showed that ATRAP significantly promotes constitutive internalization of the angiotensin II type 1 receptor and further attenuates angiotensin II–mediated hypertrophic responses in cardiomyocytes. The present study was designed to investigate the putative functional role of ATRAP in cardiac hypertrophy by angiotensin II infusion in vivo. We first examined the effect of angiotensin II infusion on endogenous ATRAP expression in the heart of C57BL/6J wild-type mice. The angiotensin II treatment promoted cardiac hypertrophy, concomitant with a significant decrease in cardiac ATRAP expression, but without significant change in cardiac angiotensin II type 1 receptor expression. We hypothesized that a downregulation of the cardiac ATRAP to angiotensin II type 1 receptor ratio is involved in the pathogenesis of cardiac hypertrophy. To examine this hypothesis, we next generated transgenic mice expressing ATRAP specifically in cardiomyocytes under control of the α-myosin heavy chain promoter. In cardiac-specific ATRAP transgenic mice, the development of cardiac hypertrophy, activation of p38 mitogen-activated protein kinase, and expression of hypertrophy-related genes in the context of angiotensin II treatment were completely suppressed, in spite of there being no significant difference in blood pressure on radiotelemetry between the transgenic mice and littermate control mice. These results demonstrate that cardiomyocyte-specific overexpression of ATRAP in vivo abolishes the cardiac hypertrophy provoked by chronic angiotensin II infusion, thereby suggesting ATRAP to be a novel therapeutic target in cardiac hypertrophy. (***Hypertension.*** **2010;55:1157-1164.)**

Key Words: basic science ■ receptors ■ gene expression/regulation ■ hypertrophy/remodeling ■ angiotensin receptors

Evidence suggests that the activation of angiotensin II (Ang II) type 1 receptor (AT_1R) through the tissue renin-angiotensin system may play an important role in the development of cardiac hypertrophy. The carboxyl-terminal portion of AT_1R is involved in the control of AT_1R internalization independent of G protein coupling, and it plays an important role in linking receptor-mediated signal transduction to the specific biological response to Ang II.[1,2]

We previously cloned a novel AT_1R-associated protein (ATRAP) that specifically interacts with the carboxyl-terminal domain of AT_1R.[3–6] We showed that ATRAP is broadly expressed in many tissues, as is AT_1R, and suppresses Ang II–mediated pathological responses in cardiomyocytes and vascular smooth muscle cells by promoting the constitutive internalization of AT_1R.[7–9] However, the function of ATRAP in cardiac hypertrophy in vivo still remains to be demonstrated. Thus, the present study was carried out to investigate whether there is a role for ATRAP in the cardiac hypertrophy induced by chronic Ang II treatment in vivo. We first examined an effect of chronic Ang II infusion on endogenous cardiac expression of ATRAP in C57BL/6J wild-type (Wt) mice. Next, we examined whether cardiac ATRAP attenuates the pathological hypertrophic response provoked by chronic Ang II infusion using transgenic (Tg) mice with cardiomyocyte-specific overexpression of ATRAP.

Materials and Methods

This study was performed in accordance with the National Institutes of Health guidelines for the use of experimental animals. All of the animal studies were reviewed and approved by the animal studies committee of Yokohama City University.

Received November 4, 2009; first decision November 23, 2009; revision accepted February 11, 2010.

From the Department of Medical Science and Cardiorenal Medicine (H.W., K.T., M.M., Y.B., T.D., S.M., A.S., A.M., N.I., Y.K., N.H., T.I., Y.T., M.Y., S.U.), Yokohama City University Graduate School of Medicine, Yokohama, Japan; Department of Molecular Cardiovascular Biology and Pharmacology (M.M., M.H.), Ehime University, Graduate School of Medicine, Ehime, Japan; Department of Life Science and Medical Bio-science (S.M.), Waseda University, Tokyo, Japan.

Correspondence to Kouichi Tamura, Department of Medical Science and Cardiorenal Medicine, Yokohama City University Graduate School of Medicine, 3-9 Fukuura, Kanazawa-ku, Yokohama 236-0004, Japan. E-mail tamukou@med.yokohama-cu.ac.jp

© 2010 American Heart Association, Inc.

Hypertension **is available at http://hyper.ahajournals.org** **DOI: 10.1161/HYPERTENSIONAHA.109.147207**

Downloaded from hyper.ahajournals.org at Yokohama City University on April 26, 2010

Animals and Treatment

Male Wt mice were purchased from Charles River Laboratories. Tg mice expressing the ATRAP specifically in cardiomyocytes were generated on a C57BL/6J background using standard techniques. Littermates genotyped as Wt were used as the littermate control (LC) mice in this study. Mice aged 8 to 12 weeks were used in the present study.

Ang II (200 ng/kg per minute) or vehicle was continuously infused into mice subcutaneously via an osmotic minipump (model 1003D, 2001, 2001D, 2002; ALZET) for 0, 15, 30, and 60 minutes and 3 and 14 days. Olmesartan (RNH6270) was provided by Sankyo Pharmaceuticals. It was dissolved in drinking water for oral administration and given to Wt mice for 2 weeks. The olmesartan dosage (10 mg/kg per day) was determined from previous reports.[10]

Blood Pressure Measurements by Tail-Cuff Method and Echocardiography

Systolic blood pressure (BP; SBP) and heart rate were measured indirectly by the tail-cuff method (BP-monitor MK-2000; Muromachi Kikai Co), as described.[7,11] Under anesthesia with an intraperitoneal injection of Avertin, transthoracic echocardiography was performed with an echo cardiographic system equipped with a 12.0-MHz phase-array transducer (Aplio SSA-700A; Toshiba), as described previously.[12,13] Left ventricular (LV) diameter, wall thickness, and the ejection fraction were measured using M-mode tracings and averaged for 3 cycles.

BP Measurements by Radiotelemetry

Direct BP measurement was performed by a radiotelemetric method in which a BP transducer (PA-C10, Data Sciences International) was inserted into the left carotid artery. Ten days after transplantation, each mouse was housed individually in a standard cage on a receiver under a 12-hour light-dark cycle. Direct BP was recorded every minute by radiotelemetry, as described previously.[14]

Western Blot Analysis of ATRAP and AT_1R

The characterization and specificity of the antimouse ATRAP antibody was described previously.[7–9] The anti-AT_1R antibody (sc-1173, lot E2508) was purchased from Santa Cruz Biotechnology, Inc.[7] To examine the specificity of the antibody, an AT_1R-selective blocking peptide (sc-1173p) was used. Western blot showed a single protein band of ≈42 kDa, which was abolished by an AT_1R-selective blocking peptide (Figure S1, available in the online Data Supplement at http://hyper.ahajournals.org). Western blot analysis was performed as described previously.[7,8] Briefly, tissue extracts were used for electrophoresis, and membranes (Millipore) were incubated with an anti-ATRAP antibody or an anti-AT_1R antibody and subjected to enhanced chemiluminescence (Amersham Biosciences). The images were analyzed quantitatively using FUJI LAS3000 Image Analyzer (FUJI Film) for determination of the ATRAP and AT_1R protein levels. To measure the cardiac expression ratio of ATRAP/AT_1R, each ATRAP protein level was divided by the corresponding AT_1R protein level obtained by reprobing and, thus, derived from the same extract.

Histological Analysis

After 2 weeks of vehicle or Ang II infusion, both the LC and Tg mice hearts were cleared by perfusion with PBS at 70 mm Hg through the coronary arteries and then fixed by perfusion with 4% paraformaldehyde. Tissue sections were stained with hematoxylin/eosin and immunohistochemical antibody (antidystrophin monoclonal antibody, Novocastra) for cell size measurement, because this antibody binds to myocardial cellular membranes. Cross-sectional area of cardiomyocytes in the LV free wall was measured digitally using Image-Pro Plus software, as described previously.[15]

Real-Time Quantitative RT-PCR Analysis

Total RNA was extracted from the LV with ISOGEN (Nippon Gene), and cDNA was synthesized using the SuperScript III First-Strand System (Invitrogen). Real-time quantitative RT-PCR was performed by incubating the reverse transcription product with TaqMan PCR Master Mix and a designed TaqMan probe (Applied Biosystems).[11] RNA quantity was expressed relative to the 18S rRNA control.

Determination of Mitogen-Activated Protein Kinase Activity

Western blot analysis was performed for phosphorylated p38, extracellular signal-regulated protein kinase 1/2 (ERK), and c-Jun N-terminal kinase (JNK) using antiphospho-p38 antibody (V1211, Promega), antiphospho-ERK antibody (4370, Cell Signaling Technology), and antiphospho-JNK antibody (4668, Cell Signaling Technology), which recognize only activated p38, ERK1/2, and JNK, respectively, as described previously.[8] To detect total p38, ERK, and JNK, the anti-p38 mitogen-activated protein kinase (MAPK) antibody (sc-728, Santa Cruz Biotechnology), anti-ERK antibody (4695, Cell Signaling Technology), and anti-JNK antibody (sc-571, Santa Cruz Biotechnology), were used.

Statistical Analysis

For the statistical analysis of differences among groups, unpaired Student t test or ANOVA followed by Scheffe F test was used. All of the quantitative data are expressed as mean±SE. Values of $P<0.05$ were considered statistically significant.

Results

Effects of Ang II Infusion on Cardiac Hypertrophy in Wt Mice

In the first experiment, age-matched Wt mice were divided into 3 groups: (1) a vehicle-infused group; (2) an Ang II (200 ng/kg per minute)–infused group without ARB treatment; and (3) an Ang II (200 ng/kg per minute)–infused group with ARB treatment. Ang II infusion significantly increased diastolic intraventricular septum and diastolic LV posterior wall thickness, as estimated by echocardiography and heart weight (HW)/body weight (BW) ratio, and these hypertrophic responses to Ang II treatment were completely prevented by angiotensin receptor blocker (ARB) treatment (Table 1). Ang II infusion also increased cardiac hypertrophy-related gene

Table 1. BP, BW, Heart Rate, Tissue Weight, and Echocardiographic Measurements 14 Days After Ang II Infusion in Wt Mice

Variable	Vehicle	Ang II	Ang II+ARB
SBP, mm Hg	112±6	120±5	110±7
BW, g	27.0±0.6	27.0±0.4	26.9±0.4
HR, bpm	685±19	693±32	715±22
HW/BW, mg/g	4.05±0.05	4.58±0.13*	3.94±0.06
KW/BW, mg/g	5.59±0.07	5.80±0.10	5.85±0.18
Echocardiography			
IVSd, mm	0.60±0.01	0.75±0.03*	0.58±0.02
LVPWd, mm	0.58±0.01	0.75±0.05*	0.60±0.02
LVEDD, mm	3.96±0.07	3.78±0.12	3.95±0.12
LVESD, mm	2.70±0.06	2.43±0.12	2.81±0.12
EF, %	68.4±0.9	73.3±3.1	65.6±2.9

HR indicates heart rate; IVSd, intraventricular septum, diastolic; LVPWd, left ventricular posterior wall, diastolic; LVEDD, left ventricular end diastolic diameter; LVESD, left ventricular end systolic diameter; EF, ejection fraction. All of the values are mean±SE (n=6 to 8).

*$P<0.05$ vs vehicle group.

Downloaded from hyper.ahajournals.org at Yokohama City University on April 26, 2010

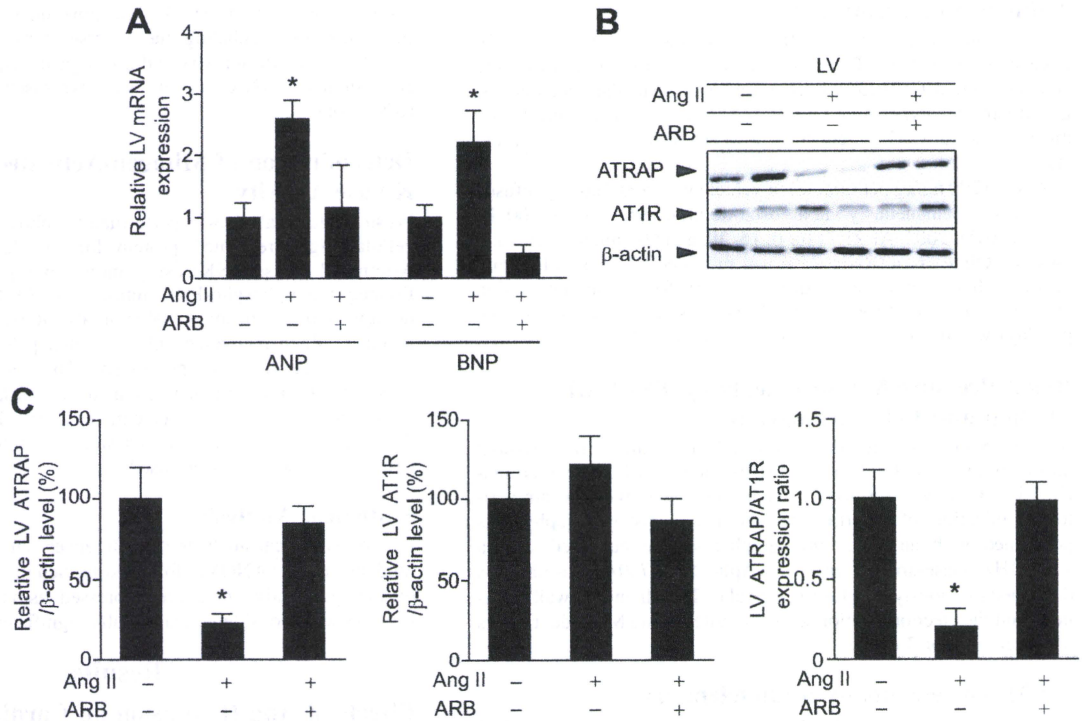

Figure 1. Expression of cardiac hypertrophy-related mRNAs and cardiac ATRAP and AT_1R proteins by Ang II infusion into Wt mice. A, Effects of Ang II infusion and the AT_1R antagonist olmesartan (ARB) on cardiac atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) mRNA expression in Wt mice. Values are calculated relative to those achieved with extracts in the vehicle-infused group (Ang II and ARB) and expressed as the mean±SE (n=6 in each group). *$P<0.05$ vs vehicle. B, Representative Western blot analysis of the effects of Ang II infusion and the AT_1R antagonist olmesartan (ARB) on cardiac ATRAP and AT_1R protein expression in Wt mice. C, Quantitative analysis of the effects of Ang II infusion and the AT_1R antagonist olmesartan (ARB) on cardiac ATRAP and AT_1R protein expression in Wt mice. Values are calculated relative to those achieved with extracts in the vehicle-infused group (Ang II and ARB) and expressed as the mean±SE (n=6 in each group). *$P<0.05$ vs vehicle.

expression in Wt mice (Figure 1A). LV mRNA levels of atrial natriuretic peptide (ANP) and brain natriuretic peptide were increased 2.2- and 2.6-fold by Ang II infusion, respectively, and the mRNA upregulation of these peptides was abolished by ARB treatment.

Effects of Ang II Infusion on Cardiac ATRAP and AT_1R Expression in Wt Mice

We also examined the effects of Ang II infusion on endogenous ATRAP and AT_1R protein expression in the hearts of Wt mice. With respect to the regulation of cardiac AT_1R

Table 2. BP, BW, Heart Rate, Tissue Weight, and Echocardiographic Measurements 14 Days After Ang II Infusion in LC, Tg46, and Tg52 Mice

	LC		Tg46		Tg52	
Variable	Vehicle	Ang II	Vehicle	Ang II	Vehicle	Ang II
BW, g	23.5±0.4	23.8±0.9	24.5±0.9	23.7±0.6	24.1±1.0	24.7±0.6
HW/BW, mg/g	4.15±0.11	4.79±0.12*	4.19±0.21	4.24±0.13	4.17±0.15	3.97±0.13
KW/BW, mg/g	5.88±0.27	5.85±0.23	5.40±0.23	5.89±0.26	5.75±0.17	5.69±0.06
Echocardiography						
IVSd, mm	0.56±0.02	0.75±0.03*	0.61±0.02	0.63±0.03	0.64±0.05	0.67±0.02
LVPWd, mm	0.59±0.02	0.74±0.07*	0.64±0.03	0.61±0.03	0.63±0.05	0.63±0.02
LVEDD, mm	4.05±0.12	4.0±0.15	3.85±0.15	3.86±0.13	4.18±0.60	4.25±0.11
LVESD, mm	2.64±0.09	2.65±0.21	2.42±0.17	2.56±0.12	2.95±0.10	2.99±0.11
EF, %	68.4±0.9	67.5±1.6	71.3±2.9	70.7±1.8	65.8±1.9	63.8±1.7

IVSd indicates intraventricular septum, diastolic; LVPWd, left ventricular posterior wall, diastolic; LVEDD, left ventricular end diastolic diameter; LVESD, left ventricular end systolic diameter; EF, ejection fraction. All of the values are mean±SE (n=6 to 8).

*$P<0.05$ vs vehicle-infused LC mice.

Downloaded from hyper.ahajournals.org at Yokohama City University on April 26, 2010

expression by Ang II infusion, previous studies reported increased, decreased, or unaltered cardiac AT_1R levels after Ang II infusion, probably because of differences in the dose and duration of the Ang II infusion.[16–18] In the present study, treatment with Ang II did not affect cardiac AT_1R protein or mRNA levels (Figure 1B and 1C and data not shown). On the other hand, Ang II infusion significantly decreased the cardiac ATRAP protein level, thereby resulting in downregulation of the cardiac ATRAP/AT_1R ratio (Figure 1B and 1C). However, ARB treatment by olmesartan (10 mg/kg per day) recovered the cardiac expression ratio of ATRAP to AT_1R so as to be comparable with the vehicle-infused group.

Effects of Ang II Infusion on Cardiac Hypertrophy in Cardiac-Specific ATRAP Tg Mice

Because chronic Ang II treatment significantly decreased the endogenous ATRAP expression in the heart concomitant with the development of cardiac hypertrophy in Wt mice, we hypothesized that an increase in cardiac ATRAP expression might suppress it in vivo. Thus, to validate the antihypertrophic properties of ATRAP in vivo, we generated Tg mice with cardiac-specific overexpression of ATRAP by the use of mouse ATRAP cDNA linked to the α-major histocompatibility complex promoter.[19] Quantitative analysis of ATRAP expression at the protein level revealed the highest and a moderate expression level of ATRAP in lines 52 and 46 (Tg52 and Tg46), respectively, among 10 obtained lines of Tg mice, and these 2 lines of Tg mice are characterized in Figure S2.

Age-matched LC and 2 independent lines of Tg mice (Tg46 and Tg52) were divided into 6 groups: (1) vehicle-infused LC mice; (2) Ang II (200 ng/kg per minute)–infused LC mice; (3) vehicle-infused Tg46 mice; (4) Ang II (200 ng/kg per minute)–infused Tg46 mice; (5) vehicle-infused Tg52 mice; and (6) Ang II (200 ng/kg per minute)–infused Tg52 mice. Although Ang II infusion significantly increased the diastolic intraventricular septum and diastolic LV posterior wall thickness, as estimated by echocardiography and HW/BW ratio in LC mice, these cardiac hypertrophic responses to Ang II infusion were completely suppressed in both Tg52 mice and Tg46 mice (Table 2). Thus, Tg52 mice were further characterized in comparison with LC mice.

The results of SBP measurement by the tail-cuff method did not result in significant Ang II–mediated BP responses in the LC or Tg mice (Table S1). Thus, to examine diurnal BP profiles and strictly compare the effects of Ang II infusion on BP, direct BP measurement by radiotelemetric devices was performed in LC and Tg mice. In LC mice, Ang II infusion for 2 weeks tended to increase SBP in the light period (118.2±4.0 versus 132.7±3.7 mm Hg; $P=0.075$) and significantly increased SBP in the dark period (129.3±2.7 versus 144.7±4.1 mm Hg; $P<0.05$; Figure 2A). Similarly, in Tg52 mice, Ang II infusion significantly increased SBP in both the light (115.2±2.8 versus 130.5±5.0 mm Hg; $P<0.05$) and dark (128.7±2.0 versus 143.3±4.6 mm Hg; $P<0.05$) periods. Although radiotelemetric SBP of the vehicle-infused Tg52 mice was significantly lower than that of the vehicle-infused LC mice at 12:00 AM (107.2±2.7 versus 116.2±4.5 mm Hg; $P<0.05$) and 3:00 PM (112.0±2.0 versus 123.7±3.5 mm Hg; $P<0.05$; Figure 2B), SBP of the Ang II–infused Tg52 mice was comparable to that of the Ang II–infused LC mice throughout the light-dark cycle (Figure 2C). Regarding other parameters obtained by radiotelemetry, the mean BP and heart rate of Tg52 mice were comparable to those of LC mice with or without Ang II infusion (Figure S3).

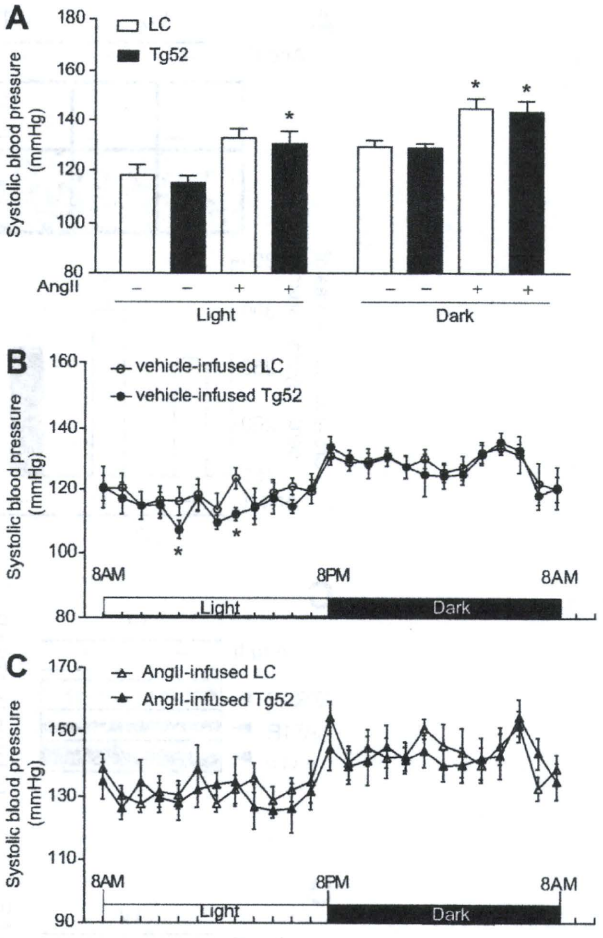

Figure 2. Diurnal BP profiles and effects of Ang II infusion analyzed by the radiotelemetric method in LC mice and cardiac-specific ATRAP transgenic (Tg52) mice. A, Effects of Ang II infusion on SBP measured by the radiotelemetric method in LC and Tg52 mice in the light and dark periods. Values are expressed as the mean±SE (n=6 in each group). *$P<0.05$ vs vehicle. B, Diurnal SBP profile of LC and Tg52 mice infused with vehicle. Values are expressed as the mean±SE (n=6 in each group). *$P<0.05$ vs LC mice. C, Diurnal BP profile of LC and Tg52 mice infused with Ang II. Values are expressed as the mean±SE (n=6 in each group).

These results of direct BP measurement by the radiotelemetric method confirmed no significant BP difference between the LC and Tg52 mice after Ang II infusion. With respect to histological analysis, Ang II infusion significantly increased the cross-sectional area of LC mice (251.5±6.7 versus 302.0±10.4 μm^2; $P<0.01$) but not Tg52 mice (267.7±11.2 versus 277.3±11.5 μm^2; Figure 3A). There were no significant increases in interstitial fibrosis in either the LC mice or Tg52 mice on Masson staining at this stage (data not shown). These results indicate that the cardiac hypertrophy effects induced by Ang II infusion were com-

Downloaded from hyper.ahajournals.org at Yokohama City University on April 26, 2010