Effect of Olmesartan on Tissue Expression Balance Between Angiotensin II Receptor and Its Inhibitory Binding Molecule


Abstract—We previously cloned a novel molecule interacting with angiotensin II (Ang II) type 1 receptor protein (ATRAP) and showed it to be an endogenous inhibitor of Ang II type 1 receptor signaling in cardiovascular cells. In this study, we tested a hypothesis that the balance of tissue expression of ATRAP and Ang II type 1 receptor is regulated in a tissue-specific manner during the development of hypertension and related cardiac hypertrophy. Concomitant with blood pressure increase and cardiac hypertrophy in spontaneously hypertensive rats, there was a constitutive decrease in the ratio of cardiac expression of ATRAP to Ang II type 1 receptor. However, treatment with olmesartan, an Ang II type 1 receptor–specific antagonist, either at a depressor or subdepressor dose, recovered the suppressed cardiac ATRAP to Ang II type 1 receptor ratio, which was accompanied by a decrease in Ang II type 1 receptor density, an inhibition of p38 mitogen-activated protein kinase activity, and a regression of cardiac hypertrophy. Furthermore, Ang II stimulation suppressed the ATRAP to Ang II type 1 receptor ratio with hypertrophic responses in both the cardiomyocytes and rat hearts. These findings show a tissue-specific regulatory balancing of the expression of ATRAP and Ang II type 1 receptor during the development of hypertension and cardiac remodeling and further suggest that the upregulation of the tissue ATRAP to Ang II type 1 receptor ratio may be one of the therapeutic benefits of olmesartan beyond its blood pressure-lowering effect. (Hypertension. 2008;52:672-678.)

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

The renin-angiotensin system has been implicated in the pathogenesis of hypertension and cardiovascular remodeling based on the generation of angiotensin II (Ang II), a key regulator of cardiovascular homeostasis. The pathophysiological actions of Ang II are mediated by the Ang II type 1 receptor (AT1R). AT1R activates G proteins through the third intracellular loop and the intracellular carboxyl-terminal tail of the receptor.1 The carboxyl-terminal end of AT1R is involved in the control of AT1R internalization independently of G protein coupling and plays an important role in linking receptor-mediated signal transduction to the specific biological response to Ang II such as cardiovascular remodeling.2

We previously cloned a novel AT1R-associated protein (ATRAP) that specifically interacts with the carboxyl-terminal domain of AT1R.3,4 We showed that ATRAP is expressed in many tissues such as the kidney, heart, and liver.3 Previous studies showed that ATRAP exerts its inhibitory action on AT1R signaling by promoting a constitutive internalization of AT1R and decreasing the cell surface AT1R number.6,7 In this study, we examined whether the expression of ATRAP is regulated in a tissue-specific manner in spontaneously hypertensive rats (SHR). We further investigated whether the balance of tissue expression of ATRAP and AT1R in SHR is modulated during the development of hypertension and by the treatment with the AT1R-specific blocker olmesartan at either a depressor or subdepressor dose.

Methods

Cell Culture, Animals, and Treatment
The Animal Studies Committee of Yokohama City University approved all the animal experimental protocols.

Expression of ATRAP and AT1R in Wistar-Kyoto Rats and SHR
Male Wistar-Kyoto (WKY) and SHR were purchased from Oriental Yeast Kogyo (Tokyo, Japan). The rats were housed and systolic blood pressure was measured by the tail cuff method (BP-monitor MK-2000; Muromachi Kikai Co) at 6 and 14 weeks of age as described previously.8 The BP-monitor MK-2000 made it possible to measure blood pressure without preheating animals to avoid very

Received May 30, 2008; first decision June 25, 2008; revision accepted July 27, 2008.
From the Department of Cardiorenal Medicine (A.S., K.T., H.W., S.M., K.A., Y.T.-I., M.O., M. Mogi, K.U., S.U.), Yokohama City University Graduate School of Medicine, Yokohama, Japan; the Department of Molecular Cardiovascular Biology and Pharmacology (M. Matsuda, M.H.), Ehime University, Graduate School of Medicine, Japan; and the Division of Cardiology (K.K.), Yokohama City University Medical Center, Yokohama, Japan. Correspondence to Kouichi Tamura, MD, PhD, Department of 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

© 2008 American Heart Association, Inc.

Hypertension is available at http://hyper.ahajournals.org

DOI: 10.1161/HYPERTENSIONAHA.108.117341

672
Western Blot Analysis of ATRAP and AT1R

Neonatal cardiomyocytes were prepared from the ventricles of 1-day-old Sprague-Dawley rats (Oriental Yeast Kogyo) as previously described. Serum-starved cardiomyocytes were treated with Ang II (100 nmol/L) for 24 hours. For the Ang II infusion experiments, male Sprague-Dawley rats (7 weeks of age; Oriental Yeast Kogyo) were divided into 2 groups (N=6 rats per group) for the subcutaneous administration of either vehicle or Ang II at a dose of 200 ng/kg per minute by osmotic minipump (model 2002; Alza Corp) for 6 days.

Determination of Mitogen-Activated Protein Kinase Activity

Western blot analysis was performed for phosphorylated p38 mitogen-activated protein kinase (MAPK) using antiphospho-p38 MAPK antibody (Promega), which recognizes only activated p38 MAPK, as described previously. To detect p38 MAPK, antiphospho-p38 MAPK antibody (Santa Cruz Biotechnology) was used.

Determination of [3H]-Phenylalanine Incorporation

Cardiomyocytes were incubated with 4 μCi/mL of [3H]-phenylalanine in culture medium containing 0.36 mmol/L unlabeled phenylalanine for 1 hour. Membrane preparations from left ventricles and measurement of [3H]-Phenylalanine Incorporation were performed as described in the Methods.

Membrane Preparations and Radioligand Receptor Binding Assay

Membrane preparations from left ventricles and measurement of AT1R binding using [125I]-[Sar1, Ile8]-Ang II were performed as described previously. AT1R binding was calculated as the difference between the total count and the count from samples incubated with extracts from WKY at 6 weeks of age, and expressed as the mean±SE (%). *P<0.05 vs WKY, †P<0.05, vs 6 weeks of age.

Effect of Olmesartan on Expression of ATRAP and AT1R

Male SHR (10 weeks of age; Oriental Yeast Kogyo) were divided into 2 groups (N=6 rats per group) for oral administration of vehicle or olmesartan (either 0.6 or 6 mg/kg per day) in drinking water for 4 weeks. The olmesartan dosages were determined from previous reports. Male Sprague-Dawley rats (7 weeks of age; Oriental Yeast Kogyo) were divided into 2 groups (N=6 rats per group) for the subcutaneous administration of either vehicle or Ang II at a dose of 200 ng/kg per minute by osmotic minipump (model 2002; Alza Corp) for 6 days.

Determination of ATRAP to AT1R Expression

To analyze the balance of the tissue expression of ATRAP and AT1R, each ATRAP protein level was divided by the corresponding AT1R protein level obtained by reprobing and thus derived from the same cellular or tissue extract.

Membrane Preparations and Radioligand Receptor Binding Assay

Membrane preparations from left ventricles and measurement of AT1R binding using [125I]-[Sar1, Ile8]-Ang II were performed as described previously. AT1R binding was calculated as the difference between the total count and the count from samples incubated with olmesartan.

Effect of Olmesartan on Expression of ATRAP and AT1R

Male SHR (10 weeks of age; Oriental Yeast Kogyo) were divided into 2 groups (N=6 rats per group) for oral administration of vehicle or olmesartan (either 0.6 or 6 mg/kg per day) in drinking water for 4 weeks. The olmesartan dosages were determined from previous reports. Male Sprague-Dawley rats (7 weeks of age; Oriental Yeast Kogyo) were divided into 2 groups (N=6 rats per group) for the subcutaneous administration of either vehicle or Ang II at a dose of 200 ng/kg per minute by osmotic minipump (model 2002; Alza Corp) for 6 days.

Determination of Mitogen-Activated Protein Kinase Activity

Western blot analysis was performed for phosphorylated p38 mitogen-activated protein kinase (MAPK) using antiphospho-p38 MAPK antibody (Promega), which recognizes only activated p38 MAPK, as described previously. To detect p38 MAPK, antiphospho-p38 MAPK antibody (Santa Cruz Biotechnology) was used.
stimulated with Ang II for 24 hours, and [3H]-phenylalanine incorporation was measured in a liquid-scintillation counter as described previously.6

Statistical Analysis
For the statistical analysis of differences among groups, unpaired Student t test or analysis of variance followed by Scheffe’s F test were used. All the quantitative data are expressed as means±SE. Values of P<0.05 were considered statistically significant.

Results
Expression of ATRAP and AT1R in WKY and SHR
Blood pressure, body weight, and the wet tissue weight of the kidney and heart in SHR and WKY are summarized in Table S1 (available online at http://hyper.ahajournals.org). The systolic blood pressure of SHR at 6 weeks of age was statistically higher than that of age-matched WKY (P<0.05) and was increased further at 14 weeks of age as compared with that of WKY (P<0.05). Concomitant with the blood pressure increase, the wet tissue weight of the heart-to-body weight ratio (HW/BW ratio) in SHR became significantly greater at 14 weeks of age, whereas no difference in the HW/BW ratio was detected at 6 weeks of age.

As shown in Figure 1, the relative expression ratio of ATRAP to AT1R in the liver was similar between SHR and WKY at 6 and 14 weeks of age. Although the kidney ATRAP to AT1R expression ratio in SHR was higher than that in WKY at 6 weeks of age, the ratio in SHR decreased toward value similar to that in WKY at 14 weeks of age. With respect to the cardiac expression of ATRAP and AT1R, although the ATRAP protein levels did not evidently exhibit any changes

<table>
<thead>
<tr>
<th>Character</th>
<th>WKY Vehicle</th>
<th>Vehicle</th>
<th>0.6 mg/kg per day</th>
<th>6 mg/kg per day</th>
</tr>
</thead>
<tbody>
<tr>
<td>SBP, mm Hg</td>
<td>105±2*</td>
<td>187±2</td>
<td>185±3</td>
<td>130±2*</td>
</tr>
<tr>
<td>HW, g</td>
<td>1.19±0.02*</td>
<td>1.66±0.03</td>
<td>1.42±0.01*</td>
<td>1.36±0.03*</td>
</tr>
<tr>
<td>HW/BW ratio, ×10^-3</td>
<td>3.39±0.06*</td>
<td>4.91±0.06</td>
<td>4.18±0.02*</td>
<td>4.14±0.03*</td>
</tr>
<tr>
<td>KW, g</td>
<td>1.25±0.03</td>
<td>1.42±0.12</td>
<td>1.23±0.04</td>
<td>1.50±0.19</td>
</tr>
<tr>
<td>KW/BW ratio, ×10^-3</td>
<td>3.34±0.09</td>
<td>4.26±0.43</td>
<td>3.86±0.12</td>
<td>4.08±0.52</td>
</tr>
</tbody>
</table>

All of the values are mean±SE (n=7). SBP indicates systolic blood pressure; KW, wet tissue weight of kidney. *P<0.01 vs SHR, vehicle group.

Figure 2. Effects of olmesartan on the balance of tissue expression of ATRAP and AT1R. A, Representative Western blots of ATRAP and AT1R in WKY and SHR treated with vehicle (0 mg/kg olmesartan per day) or olmesartan (either 0.6 or 6 mg/kg olmesartan per day) are shown. B, A quantitative analysis of the relative expression ratio of ATRAP to AT1R by treatment with olmesartan. Measurement of the ATRAP to AT1R ratio was performed as described in the Methods. Values are calculated relative to those achieved with extracts from WKY treated with vehicle and expressed as the mean±SE (N=7 in each group). *P<0.05 vs WKY with vehicle, †P<0.05, vs SHR with vehicle.
in either WKY or SHR, the AT1R protein levels in SHR were higher than those in WKY at 6 and 14 weeks of age. As a result, the relative expression ratio of ATRAP to AT1R in the heart was suppressed significantly in SHR compared with WKY at both ages.

**Effect of Olmesartan on Expression of ATRAP and AT1R**
As shown in the Table, olmesartan at a dose of 6 mg/kg per day significantly lowered blood pressure and improved the elevated HW/BW ratio in SHR. Furthermore, olmesartan at a dose of 0.6 mg/kg per day, which did not influence blood pressure, also significantly suppressed cardiac hypertrophy in SHR. The results of Western blot analysis showed that administration of olmesartan caused no apparent changes in the relative expression ratio of ATRAP to AT1R in the liver and kidney (Figure 2). However, olmesartan at doses of both 0.6 and 6 mg/kg per day significantly decreased the AT1R protein levels and recovered the repressed cardiac expression ratio of ATRAP to AT1R, independently of changes in blood pressure in SHR, to the value in WKY (Figure 2).

To determine the change in AT1R number on the cardiac membrane in response to olmesartan, AT1R binding assay using ventricular membrane fractions was performed (Figure 3A). Although the cardiac membrane AT1R density levels in SHR were higher than those in WKY at baseline, olmesartan at doses of both 0.6 and 6 mg/kg per day significantly decreased the cardiac membrane AT1R density levels to values similar to those in WKY. We also examined the cardiac activity of p38 MAPK as one of the important downstream effectors of AT1R signaling (Figure 3B). Again, although the expression levels of cardiac-activated p38 MAPK in SHR were higher than those in WKY at baseline, olmesartan at doses of both 0.6 and 6 mg/kg per day significantly decreased the expression levels of cardiac activated p38 MAPK to values similar to those in WKY.

**Effect of Ang II on Cardiac ATRAP and AT1R Expression**
Finally, we examined the effects of Ang II on the expression ratio of ATRAP to AT1R in vitro and in vivo by performing Western blot analysis using primary cultured rat cardiomyocytes and Ang II-infused rats, respectively. Ang II (100 nmol/L) treatment of cardiomyocytes for 24 hours resulted in a suppression of ATRAP protein expression and a decreased relative expression ratio of ATRAP to AT1R with an activation of protein synthesis as evaluated by the incorporation of [3H]-phenylalanine into cardiomyocytes (Figure 4A–B).

Systolic blood pressure and the HW/BW ratio in Ang II-infused rats were significantly increased compared with vehicle-infused rats after continuous infusion by an osmotic minipump for 6 days (Table S2). With respect to the cardiac expression of ATRAP and AT1R, the ATRAP protein levels were decreased and the relative expression ratio of ATRAP to AT1R in the heart was suppressed significantly in Ang II-infused rats compared with vehicle-infused rats (Figure 4C).

**Discussion**
An important role for the renin-angiotensin system in promoting hypertension and related end-organ damage, including cardiac hypertrophy, is well established. These actions are mediated primarily by the activation of tissue AT1R signaling. One of the major findings in this study is that the expression of ATRAP, a novel endogenous inhibitor of AT1R signaling, is regulated in a tissue-specific manner in SHR, an established rat model of human essential hypertension. It is also shown that olmesartan, at either a subdepresor or depressor dose, recovered the repressed cardiac expression ratio of ATRAP to AT1R along with an inhibition of the
Activation of cardiac AT1R signaling played a pivotal role in the pathogenesis of cardiac hypertrophy and remodeling. Previous studies have shown that increases in cardiac membrane AT1R density and p38 MAPK activity through the activation of AT1R signaling are profoundly involved in cardiac hypertrophy and the damage incurred in genetic and experimental hypertension models, including SHR and Ang II infusion.19–25 The results of our previous in vitro studies also showed that ATRAP suppresses Ang II–induced hypertrophic and proliferative responses by promoting a constitutive internalization of AT1R and decreasing the p38 MAPK activity in cardiovascular cells, thereby suggesting ATRAP to be an endogenous inhibitor of AT1R signaling.5,12

In this study, it is revealed that the incubation of cardiomyocytes with Ang II as well as the chronic infusion of Ang II into Sprague-Dawley rats significantly decreased the ratio of ATRAP to AT1R through a suppression of ATRAP protein expression concomitantly with the hypertrophic responses in vitro and in vivo. We observed that olmesartan abolished Ang II–mediated decreases in the ATRAP to AT1R ratio through an upregulation of ATRAP protein expression in mice cardiomyocytes and hearts of Ang II–infused mice (Wakui and Tamura, unpublished observation). We also showed that an increase in the cardiac expression ratio of ATRAP to AT1R by treatment with olmesartan at a subdepressor dose through a suppression of AT1R protein expression was actually accompanied by decreases in cardiac membrane AT1 receptor density and p38 MAPK activity in SHR. Thus, the molecular mechanisms responsible for the regulation of cardiac ATRAP to AT1R ratio may differ between Ang
II-infused rats and SHR. Furthermore, cardiomyocyte membrane expression of AT1R assayed by receptor binding, in addition to whole cell expression of AT1R analyzed by Western blot, was increased in SHR but reversed by olmesartan. Therefore, it is possible that AT1R membrane expression levels are regulated by the whole cell expression as well as by independent intracellular trafficking due to the level of AT1R activation affected by ATRAP expression.

Taken together, the results of the present study indicate that Ang II-mediated activation of AT1R signaling is one of the important pathways for the pathological downregulation of cardiac ATRAP to AT1R ratio, which provokes cardiac hypertrophy. Furthermore, these results suggest that the upregulation of the cardiac ATRAP to AT1R ratio by olmesartan through its direct AT1R blockade at a subpressor dose may be another mechanism involved in the therapeutic effect of olmesartan on cardiac hypertrophy, one that occurs in addition to stimulation of the angiotensin-converting enzyme-2/Ang-(1-7) pathway.26,27 The precise functions of ATRAP in vivo remain to be determined, but this is the first report to specifically show that an increase in the cardiac expression ratio of ATRAP to AT1R was actually accompanied by a decrease in cardiac membrane AT1 receptor density and improvement in cardiac hypertrophy in hypertensive rats. Further studies are needed to elucidate the molecular mechanism of ATRAP-mediated inhibition of AT1R signaling in cardiovascular physiology by such means as cardiac-specific transgenic mice or gene knockout mice, and these are currently being planned and will be undertaken in due course. In summary, the present study shows the tissue-specific regulation of the balanced expression of ATRAP and AT1R during the development of hypertension and cardiac hypertrophy and by treatment with olmesartan.

**Perspectives**

Because the results obtained in this study are essentially derived from genetically hypertensive rats, it is important to use caution in applying the findings to the pathophysiology of human essential hypertension and hypertension-mediated organ damage. Nevertheless, the findings of the present study may provide important information for the further investigation of possible functional roles of ATRAP in vivo in the pathogenesis of hypertension and cardiovascular remodeling and suggest a possible advantage of ATRAP activation strategy over currently used antihypertensive drugs like β-blockers or angiotensin-converting enzyme inhibitors. A recent study has shown that stimulation of β-adrenoceptors inhibits ATRAP expression through JAK/STAT and PKA signaling in adipocytes and suggested adrenergic regulation of fat ATRAP in such as obesity-related hypertension.28 Further studies may enable us to promote clinical application of ATRAP such as the use of putative activating ligands for more efficient inhibition of the AT1R pathway in combination with inhibitors of the renin-angiotensin system in the near future.

**Sources of Funding**

This study was supported by grants from the Japan Society for the Promotion of Science. This study was also supported by the grants of Yokohama Foundation for Advancement of Medical Science and of Strategic Research Project of Yokohama City University.

**Disclosures**

None.

**References**


Effect of Olmesartan on Tissue Expression Balance Between Angiotensin II Receptor and Its Inhibitory Binding Molecule

_Hypertension_. 2008;52:672-678; originally published online August 25, 2008;
doi: 10.1161/HYPERTENSIONAHA.108.117341

_Hypertension_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2008 American Heart Association, Inc. 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/content/52/4/672

Data Supplement (unedited) at:
http://hyper.ahajournals.org/content/suppl/2008/08/25/HYPERTENSIONAHA.108.117341.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Hypertension_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

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

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

Effect of Olmesartan on Tissue Expression Balance between Angiotensin II Receptor and Its Inhibitory Binding Molecule

Short Title: Ang II Receptor-Binding Protein in Hypertension


From the Department of Cardiorenal Medicine (A. S., K. T., H. W., S. M., K. A., Y. T-I., M. O., M. M., K. U., S. U.), Yokohama City University Graduate School of Medicine, Japan; Department of Molecular Cardiovascular Biology and Pharmacology (M. M., M. H.), Ehime University, Graduate School of Medicine, Japan; and Division of Cardiology (K. K.), Yokohama City University Medical Center, Japan.

* To whom correspondence should be addressed:
Kouichi Tamura, M.D., Ph.D.
Department of Cardiorenal Medicine,
Yokohama City University Graduate School of Medicine,
3-9 Fukuura, Kanazawa-ku, Yokohama 236-0004, Japan.
Tel: 81-45-787-2635, Fax: 81-45-701-3738,
E-mail: tamukou@med.yokohama-cu.ac.jp
Table S1. Characteristics of WKY and SHR at 6 and 14 weeks of age

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>WKY</th>
<th>SHR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6 wk</td>
<td>14 wk</td>
</tr>
<tr>
<td>SBP, mmHg</td>
<td>96 ± 4</td>
<td>109 ± 2</td>
</tr>
<tr>
<td>HW, g</td>
<td>0.58 ± 0.05</td>
<td>0.71 ± 0.10</td>
</tr>
<tr>
<td>HW/BW ratio, x 10⁻³</td>
<td>4.09 ± 0.31</td>
<td>2.52 ± 0.10</td>
</tr>
<tr>
<td>KW, g</td>
<td>1.27 ± 0.03</td>
<td>2.17 ± 0.05</td>
</tr>
<tr>
<td>KW/BW ratio, x 10⁻³</td>
<td>9.57 ± 0.24</td>
<td>6.76 ± 0.11</td>
</tr>
</tbody>
</table>

SBP indicates systolic blood pressure; wk, weeks of age; WKY, Wistar-Kyoto rats; SHR, spontaneously hypertensive rats; BW, body weight; HW, wet tissue weight of heart; HW/BW, heart/body weight ratio; KW, wet tissue weight of kidney; KW/BW, kidney/body weight ratio. All of the values are mean±SE (n=7). * P<0.05, † P<0.01 vs WKY.
Table S2. Characteristics of vehicle- and Ang II-infused rats

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Vehicle-infused rats</th>
<th>Ang II-infused rats</th>
</tr>
</thead>
<tbody>
<tr>
<td>SBP, mmHg</td>
<td>109 ± 1</td>
<td>133 ± 3 *</td>
</tr>
<tr>
<td>BW, g</td>
<td>257.3 ± 4.7</td>
<td>246.5 ± 3.7</td>
</tr>
<tr>
<td>HW, g</td>
<td>0.78 ± 0.16</td>
<td>0.96 ± 0.33 *</td>
</tr>
<tr>
<td>HW/BW ratio, x 10^{-3}</td>
<td>3.02 ± 0.08</td>
<td>3.88 ± 0.10 *</td>
</tr>
</tbody>
</table>

Ang II indicates angiotensin II; SBP, systolic blood pressure; BW, body weight; HW, wet tissue weight of heart; HW/BW, heart/body weight ratio. All of the values are mean±SE (n=6). * P<0.01 vs vehicle.