AT1b Receptor Predominantly Mediates Contractions in Major Mouse Blood Vessels

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Abstract—In rodents, angiotensin (Ang) II type-1 (AT1) receptors exist as two pharmacologically identical subtypes: AT1a and AT1b. Recent studies have utilized mouse models with specific subtype receptor deletions to differentiate the functional difference between AT1 subtypes. However, little information is available on AT1 subtype expression in mouse vasculature. Therefore, in this study, AT1a−/− mice and wild-type littermates (AT1a+/+) were used to examine AT1 subtype expression and its functional relevance in mouse arterial vessels. Using RT-PCR and restriction enzyme digestion, we showed that AT1b accounts for most of the total AT1 mRNA in mouse abdominal aorta and femoral artery. In contrast, AT1a is the predominant subtype in kidney. To study the functional role of AT1 subtypes, we measured the in vitro contractility in vessels from AT1a−/− and AT1a+/+ mice. The Ang II concentration response curves in abdominal aorta and femoral artery were comparable between the two mouse strains. Furthermore, the Ang II response in AT1a−/− mouse vessels was completely antagonized by losartan, an AT1 antagonist. These results demonstrate that AT1b receptor is a major mediator for Ang II contractile response in mouse vessels, such as abdominal aorta and femoral artery. (Circ Res. 2003;93:1089-1094.)

Key Words: angiotensin II ■ AT1 ■ subtype ■ vasoconstriction

Angiotensin (Ang) II is an effector peptide of the rennin-angiotensin system (RAS) that plays an important role in regulating cardiovascular function in health and disease.1,2 To date most of the actions induced by Ang II have been attributed to the type 1 receptor (AT1). The AT1 receptor belongs to the seven transmembrane G protein–coupled receptor family and is specifically antagonized by losartan.1,2 In blood vessels, AT1 is localized in the smooth muscle cells where it mediates vasoconstriction through increasing the intracellular Ca2+ and enhancing the Ca2+ sensitivity of the contractile apparatus.1–3

In rodents, AT1 receptors are further subdivided into two pharmacologically identical subtypes designated as AT1a and AT1b.4,5 Due to the lack of subtype-specific ligand, the pharmacologically identical subtypes designated as AT1a and AT1b. Recent studies have utilized mouse models with specific subtype receptor deletions to differentiate the functional contribution of each subtype among different tissue or cell types.6–11 Previous studies indicated that both AT1a and AT1b are present in mouse brain as well as some peripheral tissues.11–15 In addition, the disruption of AT1a receptor gene (AT1a+/−) has been found to cause a low basal blood pressure and an attenuated Ang II pressor response, which were accompanied by an altered renal function and salt sensitivity.8,16,17 In contrast, the AT1b-deficient mice (AT1b−/−) show a normal basal blood pressure.6 These results suggest that the AT1a receptor plays a primary role in the regulation of renal and cardiovascular functions. However, there are also suggestions that AT1b is functional in AT1a−/− mice. This is because the AT1 antagonist, losartan, still lowers blood pressure in mice that lack the AT1a receptor.18 Also, the in vivo and in vitro Ang II response still exists in AT1a−/− mice although to a lesser extent.18,19 Furthermore, the cultured vascular smooth muscle cells from AT1a−/− mice respond to Ang II with an unaltered Ca2+ transient.20 Therefore, it is also possible that the AT1b receptor might have a role in regulating vascular function or blood pressure. On the other hand, there has been little information on AT1 subtype expression in mouse vasculature.

In this study, AT1a−/− and wild-type littermates (AT1a+/+) mouse were used to investigate the expression and functional relevance of AT1 subtypes in mouse vessels. The subtype mRNA was analyzed by RT-PCR followed by restriction enzyme digestions. The in vitro contractile responses to Ang II were measured in isolated vessels using isometric force measurements.

Materials and Methods

Solution and Chemicals

The ionic composition of physiological saline solution (PSS) was as follows (in mmol/L): NaCl 123, KCl 4.7, NaHCO3 15.5, KH2PO4 1.2, MgCl2 1.2, CaCl2 1.25, and D-glucose 11.5. The 60 mmol/L K+-PSS (K+ replacement of NaCl with

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KCl. Chemicals such as the NO synthase (NOS) inhibitor, N’-nitro-l-arginine methyl ester (L-NAME) and Ang II were purchased from Sigma. Losartan was obtained from Merck & Co. All other chemicals were of the highest grade commercially available.

Animals and Tissue Preparation
Female AT1α+/– and AT1α−/− were bred and maintained in the Wright State University animal facility. The founder strains were produced by Coffman and colleagues.7 The mice were F2 progeny derived from crosses of (129XC57Bl/6) F1 AT1α heterozygous parents. This F2 generation of AT1α+/– and AT1α−/− mice posses similar random assortment of background genes making them appropriate matches for study. The genotypes were determined by PCR of DNA isolated from tail biopsies. Male wild-type C57/BL6J mice (WT) were purchased from The Jackson Laboratory (Bar Harbor, Maine). Animals were housed singly with free access to water and food.

Mice (8 to 12 weeks age) were euthanized with 95% CO2 inhalation. The kidney, thoracic, abdominal aorta, and femoral artery were excised rapidly, and the fat, connective tissues, or the adventitia were mechanically removed under a binocular microscope. For mRNA analyses, tissues were frozen in liquid nitrogen and stored at −80°C. For the functional in vitro studies, arterial segments of abdominal aorta and femoral artery were cut transversely into approximately 1.0-mm wide vascular rings. All experiments were approved by the Animal Research Ethics Committee of The Ohio State University.

Detection of mRNA for AT1 Subtype Receptors
Total RNA preparation and RT-PCR were performed using an Absolutely RNA RT-PCR Miniprep Kit (Stratagene), according to the manufacturer’s instruction. Primers for AT1 receptor were designed from common sequences of AT1 isoforms as previously described20,21: 5’-CCCAAAGTGCACCTGCATC-3’ (PCR sense) and 5’-CACAATGCCTAATTA TCTCA-3’ (RT and PCR antisense). The RT reaction was performed using 200 ng of total RNA in a volume of 20 µL. The PCR protocols were as follows: 94°C for 30 seconds, 56°C for 60 seconds, and 72°C for 60 seconds (31 cycles). The expected size of PCR products was 305 bp. To determine the subtype composition, the PCR products were digested with restriction enzymes based on mouse cDNA mappings. Briefly, the PCR products were precipitated with 50 µL of 2-propanol and washed with 100 µL 70% ethanol. The PCR products were then dissolved in 20 µL water and digested with EcoRI, HpaII, and BstXI, according to manufacturer’s instruction (NEB). The PCR products or digested fragments were separated with 2% agarose gel and detected with ethidium bromide staining. The band densities were quantified using a Biochemi System (UVP Inc).

Isometric Force Measurement
The isometric force developed in mouse arterial rings was measured as described previously.23 Briefly, the arterial ring was mounted onto two tungsten wires in a 37°C water-circulating bath filled with PSS by passing the tungsten wires through the lumen of the arterial ring. One of the wires was fixed and the other was connected to a force transducer (AE 801). During the equilibration period, the tissues were stimulated with 60 mmol/L K+ every 15 minutes (5 times), and the resting tension was increased in a stepwise manner. After the equilibration, the resting tension was adjusted to approximately 300 mg, at which the maximal 60 mmol/L K+ response was obtained.

Experimental Protocols
The physiological studies were conducted in mouse vessels at 37°C. Ang II was used only once in each specimen and was administered 15 minutes after the final 60 mmol/L K+ induced contraction had been relaxed with PSS. The force development caused by Ang II was expressed as a percentile of that obtained with 60 mmol/L K+, assuming the value in the PSS (5.9 mmol/L K+) and 60 mmol/L K+ to be 0% and 100% respectively. To eliminate the effect of endothelial NO, the vascular rings were treated with 1 mmol/L L-NAME 5 minutes before Ang II was applied.

Results
AT1 Subtype mRNA Expression in Major Mouse Vessels and Kidneys
To study AT1 subtype compositions in mouse tissues, we used RT-PCR amplification of the mRNAs for both AT1 subtypes, followed by restriction enzyme treatments. Figure 1 shows the results of analyses of PCR products amplified from AT1 mRNAs in abdominal aorta, femoral artery, and kidney. In the AT1α+/– tissues, PCR products of expected size (305 bp) were clearly identified (Figure 1, lane 1). To our surprise, EcoRI, which specifically cuts AT1a, only minimally digested the PCR products (15.7% or 1.3%, respectively) in abdominal aorta and femoral artery (Figure 1, lane 2). However, in the kidney, EcoRI produced a 92.2% digestion. All the digested fragments were in agreement with the sizes expected from the mouse AT1α cDNA sequences (128 and 177 bp).

On the other hand, HpaII cut the PCR products from both subtypes, producing fragments for AT1α (125 and 180 bp) and AT1b (51 and 254 bp) as expected. Similar to the results obtained with EcoRI, the density of the AT1b fragments accounted for 85.6% or 98.3% of the digested bands in
abdominal aorta and femoral artery, respectively. However, only 5.6% of the digested products corresponded to AT1b in kidney. As a control, BsuXI, which cuts both subtypes into fragments of 149 and 156 bp were also used. It produced a single band of approximately 150 bp in all tissues, well consistent with the sizes expected. These results clearly indicate that AT1b is the predominant subtype in mouse abdominal aorta and femoral artery, but not AT1a, which is predominantly expressed in the kidney.

To further prove that the PCR products in vessels of the AT1a−/− mice were mainly from AT1b, we conducted studies in tissues from AT1a−/− mice. The PCR products of expected size (305 bp) were also obtained in abdominal aorta and femoral artery, which were comparable to those of AT1a+/+ (Figure 1, lane 5). However, the PCR products in AT1a−/− mouse kidney were only 5.2% of those in AT1a+/+ mice (Figure 1, lane 5). In contrast, the PCR product amplified from β-actin mRNA was very similar (data not shown). EcoRI (which only cuts the PCR products for AT1a) did not digest the PCR products from the AT1a−/− tissues (Figure 1, lane 6). However, HpaII exhibited complete digestion, producing fragments consistent for AT1b (51 and 254 bp, Figure 1, lane 7). These results further confirm that AT1b is the major subtype in mouse vessels and demonstrate that AT1a was absent in AT1a−/− mice.

Ang II Induced Contractile Responses in Vessels From AT1a+/+ and AT1a−/− Mice

The difference in AT1 subtypes between the mouse vessels and the kidney (which is mostly composed of-noncontractile cells) suggests that AT1b might be an important mediator of vasoconstrictions in mouse. To test this hypothesis, we examined the Ang II responses in the major vessels, such as abdominal aorta and femoral artery from AT1a+/+ and AT1a−/− mice. As shown in Figure 2A, the response to 1, 10, 100, and 1000 nmol/L Ang II in abdominal aorta from AT1a+/+ mice (2.5 ± 0.6%, 44.0 ± 5.3%, 78.7 ± 9.6%, and 66.0 ± 8.4%, respectively, as compared with 60 mmol/L K+) were similar to that seen in AT1a−/− mice (2.6 ± 0.9%, 41.0 ± 3.4%, 77.5 ± 10.5%, and 69.1 ± 5.6%, respectively). Likewise in the femoral artery the contractile response to 1, 10, 100, and 1000 nmol/L Ang II (Figure 2B) in AT1a+/+ (3.4 ± 0.8%, 53.7 ± 1.3%, 83.9 ± 4.0%, and 75.4 ± 5.2%, respectively) was not different from those in AT1a−/− mice (2.7 ± 0.6%, 50.5 ± 5.7%, 86.4 ± 4.9%, and 77.3 ± 6.5%, respectively). These results indicated that the loss of AT1a does not affect Ang II contractile responses in isolated mouse abdominal aorta and femoral artery.

Effect of Losartan on the Ang II Contractile Response

To further confirm that the above-obtained Ang II responses in AT1a−/− were mediated by AT1b receptor, the effect of the AT1 antagonist, losartan, was examined. In abdominal aorta and femoral artery from AT1a−/− mice, the response to 100 nmol/L Ang II was blocked by treatment with 10 μmol/L of losartan (Figure 3). A similar effect of losartan was also observed in vessels from AT1a+/+ mice (data not shown). Therefore, the

AT1b subtype must be the mediator of Ang II responses in AT1a−/− mouse abdominal aorta and femoral artery.

AT1 Subtype mRNA in Male C57/BL6j Mouse Vessels

The above results were obtained from female AT1a+/+ and AT1a−/− mice. To determine whether they were related to a specific gender or genetic background, the abdominal aorta from male WT C57/BL6j mice was examined. To serve as a contrast, we also explored the thoracic aorta, which we had recently found to exhibit little Ang II response due to a lower AT1 receptor level.21 Consistent with this finding, in the thoracic aorta, which displayed only a minimal response to 100 nmol/L Ang II (Figure 4B), the density of PCR products for AT1 was lower, only 41.4% of that in abdominal aorta (Figure 4A, lanes 2 and 4). Similar to that in AT1a+/+ mice, EcoRI produced a 16% digestion in abdominal aorta (lane 3). On the other hand, the digestion amounted to 43.8% in thoracic aorta (lane 1). In addition, while digested bands (AT1a) were very similar, the density of the undigested (AT1b) in the thoracic was only 23.6% of that in abdominal aorta (lanes 1 and 3). These results confirm the predominance of AT1b in major mouse vessels and suggest that the lower AT1 receptor level in mouse thoracic aorta is mainly reflected in the amount of AT1b subtype.

Discussion

To date, the in vivo studies conducted using AT1 receptor null mouse models have suggested that AT1a is the major
AT1 receptor subtype in the renal and cardiovascular system. However, there has been little direct evidence to substantiate this hypothesis in mouse vasculature. In this study, we performed a direct examination on isolated mouse vessels to focus on AT1 subtype mRNA expression and its functional relevance. For this purpose, the total AT1 receptor mRNAs were amplified using RT-PCR with primers common to both AT1a and b subtypes. Then the PCR products were treated with restriction enzymes to analyze the contribution of each subtype. Using this approach, the mRNAs for both subtypes could be amplified and analyzed under the same experimental condition. In addition, we also measured the in vitro contractile response to Ang II in isolated vessels from AT1a/H11002 and AT1a/H11001 mice in order to assess the functional importance of each receptor subtype.

A significant finding of this study is that AT1b is the major source of AT1 mRNAs in major mouse vessels. This was clearly demonstrated by our analyses of PCR products amplified from AT1 mRNA with restriction enzymes in AT1a/H11001 or male C57/BL6j mouse specimens. The predominance of AT1b was especially obvious in abdominal aorta and femoral artery, where the treatments with EcoRI or HpaII showed that 85% and 98% of the PCR products were associated with the AT1b subtype, respectively. This result does not seem to be limited to a specific gender or genetic background, as a very similar result was also obtained from the abdominal aorta in male C57/BL6J mice. In contrast in mouse kidney, where AT1a has been proposed to be major form,7,8 we found that AT1a accounted for more than 90% specifically amplified PCR products, excluding that the AT1a mRNA could not be amplified with our RT-PCR protocols. These results indicate that AT1b receptor is the predominant subtype present in mouse vessels, such as abdominal aorta and femoral artery.

Of equal importance is our finding that the Ang II concentration response curves in abdominal aorta and femoral artery were very similar between AT1a^{-/-} and AT1a^{+/+} mice. It needs to be emphasized that the absence of AT1a receptor in AT1a^{-/-} mice was confirmed not only in vessels, but also in kidneys where AT1a is the predominant form. Therefore, the Ang II responses in AT1a^{-/-} mouse vessels must have been mediated through the AT1b receptors. This idea was further supported by the antagonistic effect of the AT1 receptor blocker, losartan.

**Figure 3.** Effect of AT1 antagonist, losartan on AT1a^{-/-} mouse vessels. Representative recordings (from 3 identical experiments) showing the complete inhibition of 100 nmol/L Ang II-induced response by 10 μmol/L of losartan in AT1a^{-/-} mouse abdominal aorta (A) and femoral artery (B). Upper panels represent the contractions induced by 100 nmol/L Ang II; lower panels, contractions induced in the presence of 10 μmol/L of losartan.

**Figure 4.** A, AT1 subtype expression in male WT C57BL/6j mouse abdominal (lanes 1 and 2) and thoracic aorta (lanes 3 and 4). Lanes 1 and 3, PCR products digested with EcoRI. Lanes 2 and 4, Undigested PCR products. M represents 100-bp ladder size markers (top to bottom: 500 to 100 bp). B, Representative recording (from 3 identical experiments) showing the contractile response to 100 nmol/L Ang II in male WT C57BL/6j mouse thoracic aorta.
the absence of AT1a, the AT1b is able to maintain a normal Ang II response in mouse abdominal aorta and femoral artery. In addition, as suggested by the small quantities of PCR products (5.2% of AT1a+/− mice, which is comparable to the percentile it represents in the total AT1 PCR products), the upregulation of AT1b in AT−/− mice was, if any, not to a substantial extent even in the kidney, where AT1a is the predominant form. Therefore, these unaltered contractile responses of mouse vessels mediated by AT1b receptor in AT1a−/− mice could not be simply interpreted as a compensation for the loss of minimal amounts of AT1a receptor.

These physiological studies performed on AT1a−/− mouse vessels may underscore the importance of AT1b receptor in regulating vascular function in mouse. In addition, using vessels from WT mice, we further found that the mouse thoracic aorta, although its AT1a mRNA level was comparable to that in abdominal aorta, exhibits little contractile response to Ang II. However, the femoral artery, in which the proportion of AT1a in the total AT1 mRNA was only minimal (less than 2%), showed potent contractile response to Ang II as abdominal aorta. It should be noted that the mouse thoracic aorta has the contractile property in response to agonist stimulation.21,22 Therefore, the unresponsiveness of mouse thoracic aorta to Ang II could only be explained by the low AT1b receptor level, which was found to be about 23% of that in abdominal aorta (Figure 4A). These observations further suggest that the proportion of AT1b receptor rather than AT1a receptor is the determinant of Ang II response in mouse vessels, such as abdominal aorta and femoral artery.

Similar to the findings of the present study, a previous study has reported that the loss of AT1a receptor does not affect Ang II–induced Ca2+ transient in cultured smooth muscle cells from mouse thoracic aorta.20 However, in contrast to the finding of this study, AT1b has been found to be only a minor form in cultured smooth muscle cells.20 This difference in AT1 subtype constitution might be a reflection of the functional alterations in the cultured smooth muscle cells. There are also studies suggesting that AT1a is the major vasoconstrictor receptor in the intrarenal microvessels as well.9,10,19 In these studies, Ang II response in the intrarenal vasculature of AT1a−/− mice was found to be significantly diminished.9,10,19 However, it needs to be pointed out that in AT1a−/− mouse intrarenal vasculatures, the response of another agonist, adenosine was also remarkably decreased, despite of a normal adenosine receptor level.23 Therefore, the depressed Ang II response could be a result of a general loss of contractility in AT1a−/− mouse intrarenal vasculature. As a result, it still remains to be determined whether the AT1a receptor directly mediates the vasoconstrictor effect of Ang II in mouse intrarenal vasculature.

While the present study suggests a predominant role for AT1b, the significance of AT1b-mediated vasoconstrictions may require further investigation. It has been reported that AT1b−/− mice do not exhibit abnormalities in basal blood pressure.6 In contrast, the AT1a−/− mice display a lower basal blood pressure as well as a remarkably reduced Ang II pressor response.6,8 One plausible explanation for this inconsistency could be that AT1b is a predominant form in the large arterial vessels, such as abdominal aorta or femoral artery, which do not contribute significantly to the overall vascular resistance. On the other hand, it should also be considered that the regulation of blood pressure in live animals is through an integration of inputs from neural, endocrine, renal, and cardiovascular systems. Therefore, it is possible that the loss of AT1b-mediated vasoconstrictions could be compensated by other regulatory mechanisms. However, the exact reason for the discrepancy between the findings of the present study and those from the in vivo study performed on AT1a−/− mice needs to be clarified. On the other hand, the lower blood pressure or diminished Ang II pressor response in AT1a−/− mice could be explained by the loss of AT1a mediated regulatory mechanisms, including its role in renal function18,19 and microvessels, like the intrarenal vasculature,8,10,19 although the later could be an indirect one as discussed above.

Interestingly, in humans, there are also AT1a and AT1b receptors.24–26 In addition, there is also data to suggest that in humans, AT1 exists as multiple spliced variants with distinct function.27,28 On the other hand, these AT1 subtypes or spliced forms are originated from the same gene, in contrast to two separate genes in mouse.4,15,27,28 Also, there has been no indication that these human AT1 subtypes or forms correspond to those in mouse. Therefore, it is premature to relate our findings in mouse to human tissues.

In summary, in the present study, using isolated mouse vessels, we provided the first direct evidence that AT1b is the predominant form in mouse abdominal aorta and femoral artery. We also found that Ang II response in these vessels of AT1a−/− mice was comparable to that in the WT littermate. Therefore, we propose that the AT1b is the predominant mediator of Ang II contractile response in mouse vessels, such as abdominal aorta and femoral artery.

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