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Sex Difference in Vascular Injury and the Vasoprotective Effect of Valsartan Are Related to Differential AT\textsubscript{2} Receptor Expression

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Abstract—The angiotensin II type 2 (AT\textsubscript{2}) receptor is upregulated in pathological conditions such as vascular injury and exerts antagonistic effects against AT\textsubscript{1} receptor–mediated actions. We examined the possibility that the sex difference in vascular remodeling is associated with altered AT\textsubscript{2} receptor expression, which is located on the X chromosome. In this study, we examined this possibility by using AT\textsubscript{2} receptor–null (Agtr2\textsuperscript{−/−}) mice. Vascular injury was induced by polyethylene cuff placement around the femoral artery of wild-type (Agtr2\textsuperscript{+/-}) and Agtr2\textsuperscript{−/−} mice. In Agtr2\textsuperscript{+/-} mice, AT\textsubscript{2} receptor expression in the injured artery was enhanced, and this increase was greater in female than in male mice, with no significant difference in AT\textsubscript{1} receptor expression between male and female mice. Increases in neointimal formation, DNA synthesis, expression of monocyte chemoattractant protein-1, production of superoxide anion, and NADPH oxidase activity in the injured artery were attenuated in female compared with male mice. These parameters were augmented in Agtr2\textsuperscript{−/−} mice, whereas the sex differences in these parameters were smaller in Agtr2\textsuperscript{−/−} than in Agtr2\textsuperscript{+/-} mice. Treatment with a nonhypotensive dose of the AT\textsubscript{1} receptor blocker valsartan decreased these parameters significantly in Agtr2\textsuperscript{+/-} mice, and these inhibitory effects of valsartan were greater in female mice. This sex difference in valsartan’s inhibitory effect was less marked in Agtr2\textsuperscript{−/−} mice. Our results suggest that the sex difference in response to vascular injury could be at least partially attributed to the exaggerated AT\textsubscript{2} receptor expression in the injured vessel in female mice. (Hypertension. 2005;46:577-583.)

Key Words: angiotensin II ■ inflammation ■ oxidative stress ■ receptors, angiotensin ■ remodeling

Recent evidence has revealed that actions of the angiotensin (Ang) II receptor subtypes AT\textsubscript{1} and AT\textsubscript{2} are mutually antagonistic.\textsuperscript{1,2} The AT\textsubscript{2} receptor is abundantly and widely expressed in fetal tissues, but its expression declines rapidly after birth.\textsuperscript{3,4} Interestingly, the AT\textsubscript{2} receptor is reexpressed in certain pathological conditions such as inflammation and vascular injury.\textsuperscript{1,2} These findings suggest that the AT\textsubscript{2} receptor plays an important role not only in vasculogenesis but also in vascular remodeling.

The effect of AT\textsubscript{1} receptor blockers (ARBs) may not be entirely due to the blockade of the AT\textsubscript{1} receptor. When the AT\textsubscript{1} receptor is blocked, increased Ang II may act on the AT\textsubscript{2} receptor, which could be involved in the effects of the ARB. The AT\textsubscript{2} receptor plays a role in the pathogenesis of cardiovascular and renal diseases. However, the results, though suggestive, are sometimes equivocal. A more extensive knowledge of the AT\textsubscript{2} receptor could therefore contribute to the understanding of the clinical effects of ARBs. We have previously reported that the AT\textsubscript{2} receptor exerts anti-inflammatory and antiproliferative effects by counteracting the AT\textsubscript{1} receptor in the process of neointimal formation after vascular injury in a mouse model of vascular disease induced by polyethylene cuff placement.\textsuperscript{5,6} We also demonstrated that treatment of mice with a selective AT\textsubscript{1} receptor blocker, valsartan, significantly decreased the inflammatory response and proliferation of vascular smooth muscle cells (VSMCs) in the injured artery, whereas these inhibitory effects of valsartan were less marked in AT\textsubscript{2} receptor–null (Agtr2\textsuperscript{−/−}) mice.\textsuperscript{5}

To examine the possibility that stimulation of the AT\textsubscript{2} receptor, located on the X chromosome in rodents and humans,\textsuperscript{2,7} may play more important roles especially in vascular remodeling in females, in this study we postulated the following: (1) Upregulation of the AT\textsubscript{2} receptor in the injured artery is more exaggerated in females, with decreased VSMC proliferation, inflammation, and oxidative stress. (2) The inhibitory effects of ARBs on vascular remodeling would be more effective in females, at least partly because of the enhanced AT\textsubscript{2} receptor expression in females. To evaluate these roles of potentially enhanced AT\textsubscript{2} stimulation in vascular remodeling, inflammation, and oxidative stress in females, we used a polyethylene cuff–induced vascular injury model in both wild-type (Agtr2\textsuperscript{+/-}) and Agtr2\textsuperscript{−/−} mice.
Methods

Animals and Operations

Adult male and female Agr2+ and Agr2− mice (10 to 12 weeks old) were used. The Animal Studies Committee of Ehime University approved the following experimental protocol. Mice were kept in a room in which lighting was controlled (12 hours on, 12 hours off). They were given a standard diet and water ad libitum. The surgical procedure for the cuff-induced vascular injury model of the femoral artery was performed. Valsartan (provided by Novartis Pharma AG) was administered with an osmotic minipump (Alzet model 1002, Durect Corp) implanted intraperitoneally at the same time as cuff placement. The pump delivered valsartan (1 mg·kg−1·d−1) continuously at a rate of 0.25 μL/hr. Female mice underwent bilateral ovariectomy or sham operation through a 1-cm abdominal incision, as previously described. 1 week before cuff placement.

Measurement of Plasma Estrogen Concentration

Estrogen concentration was measured as previously described9,10 with an enzyme immunoassay kit (Cayman Chemical Inc). This assay is based on competition between free estradiol and an estradiol tracer for estradiol-specific rabbit antiserum binding sites. Fifty microliters of serum or corresponding standards of estradiol was transferred to the antibody-coated wells. The estradiol tracer (estradiol linked to an acetylcholinesterase [ACHE] molecule) was added to the wells. The mixture was incubated at room temperature for 1 hour. After 3 washes with distilled water containing 0.05% Tween 20, Ellman’s reagent (containing the substance to AchE) was added, and the mixture was incubated at room temperature for 1 hour. The plate was immediately read with an enzyme immunoassay plate reader with a 420-nm filter.

Morphometric Analysis, Measurement of DNA Synthesis, and Immunohistochemical Study

Morphometric analysis, measurement of DNA synthesis, and immunohistochemical study were performed according to methods described previously. The sections were counterstained with hematoxylin, and the ratio of BrdU-positive nuclei to total nuclei was calculated as the BrdU index. The sections were also stained with antibodies were purchased from Santa Cruz Biotechnology Inc. Total RNA was extracted from the femoral arteries. Real-time quantitative RT-PCR was performed with a SYBR green I kit (MJ Research, Inc). PCR primers for the AT2 receptor were as follows: forward; for the AT1 receptor, they were 5′-GGACTGTGCTAGCCTCA-3′ (reverse); for MCP-1, they were 5′-TTAACGCCCCACTCAGCCTG-3′ (forward) and 5′-GCTTCGCTCTCGGCCCCAG-3′ (reverse); and for glyceraldehydes 3-phosphate dehydrogenase, they were 5′-ATGTAGGCAATGGAGCTC-3′ (forward) and 5′-TGCGACTTCACAG-CAACTC-3′ (reverse).

Superoxide Detection

Frozen, enzymatically intact, 10-μm-thick sections of sham-operated and injured femoral arteries were incubated at the same time with dihydroethidium (DHE; 10 μmol/L) in PBS for 50 minutes at 37°C in a humidified chamber protected from light. DHE is oxidized on reaction with superoxide to ethidium bromide, which binds to DNA in the nucleus and fluoresces red. Samples were examined with a Zeiss Axioskop microscope equipped with a computer-based imaging system and measured fluorescence intensity as superoxide production. For ethidium bromide detection, a 500- to 550-nm bandpass filter was used.

Measurement of NADPH Oxidase Activity

Arterial samples were homogenized in 500 μL of ice-cold Tris-sucrose buffer (10 mmol/L Tris, pH 7.1; 340 mmol/L sucrose, 1 mmol/L EDTA, 1 mmol/L PMSF, and 10 μg/mL aprotinin) and incubated for 30 minutes. Samples were centrifuged (15 000g, 10 minutes, 4°C), and the supernatant (20 μg protein) was added to reaction buffer (78 μmol/L cytochrome c, Sigma-Aldrich) with 100 μmol/L NADPH (Sigma-Aldrich), with or without 1000 μM superoxide dismutase (Sigma-Aldrich), and then incubated at 37°C for 60 minutes. NADPH oxidase activity was quantified from the absorbance with or without superoxide dismutase, as previously described.

Data Analysis

All values are expressed as mean±SEM. Data were analyzed by 2-way ANOVA. When a statistically significant effect was found, a Newman-Keuls test was performed to detect the difference between groups. Values of P<0.05 were considered statistically significant.

Results

Sex Difference in Vascular Remodeling Induced by Cuff Placement in Agr2− and Agr2+ Mice

We examined BrdU incorporation into VSMCs, as a marker of DNA synthesis, in the injured artery 7 days after cuff placement. As previously reported, cuff placement increased BrdU-positive VSMCs in both the media and neointima of the injured artery in Agr2+ mice, whereas the number of BrdU-positive cells was greater in male than in female mice (Figure 1). The proportion of BrdU-positive cells was higher in Agr2− than in Agr2+ mice in both males and females, however, the sex difference in BrdU-positive VSMCs in Agr2+ mice was attenuated in Agr2− mice (23.2% reduction in female Agr2+ mice and 13.6% reduction in female Agr2− mice compared with those in males, respectively; Figure 1). Ovariectomy significantly enhanced incorporation of BrdU into the injured arteries of Agr2+ mice, whereas the ovariectomy-induced increase in BrdU-positive VSMCs was less marked in Agr2− mice (33.3% increase in Agr2+ mice and 16.0% increase in Agr2− mice compared with those in intact female mice, respectively; Figure 1). We did not observe a significant difference in BrdU uptake in uninjured arteries between males and females. BrdU-positive cells were observed mainly in VSMCs stained with α-smooth muscle actin. Plasma estrogen concentration in Agr2+ mice was 53±12 pg/mL (n=4) in male mice, 210±30 pg/mL (n=4) in female mice, and 43±23 pg/mL (n=5) in ovariectomized mice, and that in Agr2− mice was 49±14 pg/mL in male mice (n=4), 230±58 pg/mL (n=5) in female mice, and 50±18 pg/mL (n=4) in ovariectomized mice. The plasma estrogen levels of each mouse were examined 7 days after cuff placement. We did not observe any apparent differences in estrogen concentrations before or after cuff placement. Cuff placement induced neointimal formation in the femoral artery in both male and female mice 14 days after the operation, whereas neointimal thickening in Agr2+ mice was significantly smaller in females than in males (Figure 2). Consistent with our previous observations, the area of neointima was larger in Agr2− than in Agr2+ mice. The sex difference in neointimal formation observed in Agr2+ mice...
mice was attenuated in Agtr2− mice (36.2% reduction in female Agtr2+ mice and 24.4% reduction in female Agtr2− mice; Figure 2). Ovariectomy significantly enhanced neointimal formation in the injured artery in Agtr2+ mice. The increase in neointimal formation by ovariectomy was different between Agtr2+ and Agtr2− mice (4303 ± 291 μm² in ovariectomized Agtr2+ mice, n=5, 29.9% increase; 926 ± 791 μm² in ovariectomized Agtr2− mice, n=6, 14.2% increase). We did not observe a significant difference in neointimal area in uninjured arteries between male and female mice. On the other hand, medial area was not significantly different between the experimental groups (data not shown).

**Sex Difference in Expression of AT2 Receptor Induced by Cuff Placement in Agtr2+ Mice**

In the uninjured arteries of Agtr2+ mice, AT2 receptor expression as assessed by quantitative RT-PCR was very low, and there was no significant difference between male and female mice (Figure 3A). As we previously reported,11 AT2 receptor mRNA was upregulated time-dependently in response to cuff placement and reached a peak 7 days after cuff placement; expression of the AT2 receptor gradually decreased in both male and female mice; and AT2 mRNA expression was significantly higher in female than in male Agtr2+ mice (Figure 3A). Similar results were obtained by immunohistochemical analysis, and the AT2 receptor was expressed mainly in VSMCs (Figure 3B). We examined the effect of ovariectomy on AT2 receptor expression and observed that its basal level expression in the femoral artery was similar, but it increased less in the injured arteries in ovariectomized mice, suggesting that some other factors such as cytokines might regulate AT2 receptor expression, at least in our experimental mouse model, in addition to estrogen and/or in concert with estrogen.

In contrast, AT1 receptor mRNA was also upregulated after cuff placement, and there was no significant sex difference in AT1 receptor expression (Figure 3D). We also observed that expression of the AT1 receptor in Agtr2− mice after vascular injury was comparable to that in wild-type mice, as we previously reported.6 Ovariectomy did not significantly decrease AT1 receptor mRNA expression in uninjured or injured arteries 7 days after cuff placement compared with that in nonovariectomized mice (Figure 3D). Treatment with valsartan did not affect the expression of AT1 and AT2 receptors in any experimental animal group (Figure 3C and 3D).

**Figure 1.** Sex difference in DNA synthesis and effect of valsartan on DNA synthesis in the injured artery 7 days after polyethylene cuff placement in Agtr2+ and Agtr2− mice. Inflammatory vascular injury was induced by polyethylene cuff placement around the femoral artery. BrdU was injected as described in Methods. Arterial samples were taken 7 days after cuff placement, and paraffin sections of artery were prepared for immunostaining of BrdU as described in Methods. DNA synthesis was expressed as the BrdU incorporation index (percentage of BrdU-positive nuclei/total nuclei) in the media and neointima. OVX indicates ovariectomized mice; Val, 1 mg · kg−1 · d−1 valsartan. Other abbreviations are as defined in text. n=5 to 8 for each group. *P<0.05 vs without valsartan. Values are mean±SEM.

**Figure 2.** Sex difference in neointimal formation and effect of valsartan on neointimal formation in injured arteries 14 days after polyethylene cuff placement in Agtr2+ and Agtr2− mice. Cuff placement was performed as described in text and the legend to Figure 1. Arterial samples were taken 14 days after cuff placement. Cross-sectional areas of the media and intima were determined by elastica–van Gieson’s staining as described in Methods. OVX indicates ovariectomized mice, Val, 1 mg · kg−1 · d−1 valsartan. Other abbreviations are as defined in text. n=5 to 8 for each group. *P<0.05 vs without valsartan. Values are mean±SEM.
Sex Difference in Vascular Inflammation and Oxidative Stress Induced by Cuff Placement in Agtr2+/H11001 and Agtr2+/H11002 Mice

We next examined MCP-1 expression as an index of the inflammatory response 7 days after cuff placement by quantitative RT-PCR and immunohistochemical study. As shown in Figure 4A, expression of MCP-1 mRNA was enhanced in both male and female mice after cuff placement, and MCP-1 expression in Agtr2+/H11001 mice was more exaggerated in male than in female mice. MCP-1 mRNA expression in Agtr2+/H11002 mice was higher compared with that in Agtr2+/H11001 mice, and the sex difference in MCP-1 expression observed in Agtr2+/H11002 mice was less marked in Agtr2+/H11002 mice. Similar results were obtained by immunohistochemical analysis (Figure 4B). Superoxide production determined by DHE staining followed by measurement of fluorescence intensity and NADPH oxidase activity in the injured arteries of Agtr2+/H11001 mice 7 days after operation were enhanced in both male and female mice, and these parameters were more exaggerated in female than in male mice (Figures 5 and 6). Superoxide production and NADPH oxidase activity in injured arteries of Agtr2− mice were higher than those in Agtr2+ mice, and the sex difference in these parameters in Agtr2+ mice was blunted in Agtr2− mice (Figures 5 and 6).

Difference in Sex- and AT2 Receptor–Related Inhibitory Effects of Valsartan on Vascular Remodeling, Inflammation, and Oxidative Stress

Administration of valsartan (1 mg · kg⁻¹ · d⁻¹) significantly suppressed the BrdU index, neointimal formation, expression of MCP-1, superoxide anion production, and NADPH oxidase activity, and these inhibitory effects of valsartan were exaggerated in female compared with male Agtr2+ mice (Figures 1, 2, 4A, and 5B). These inhibitory effects of valsartan were attenuated in Agtr2− mice, and the sex difference of valsartan’s effects was blunted in Agtr2− mice (Figures 1, 2, 4A, and 5B). This dose of valsartan did not change blood pressure, heart rate, or plasma estrogen concentration in these animals (data not shown).

Discussion

Epidemiologic and clinical evidence suggests that estrogen has protective effects on the cardiovascular system. However,
their mechanisms are incompletely understood. Consistent with previous reports,\textsuperscript{9,15,16} we demonstrated in this study that DNA synthesis in VSMCs, neointimal formation, inflammatory response, and oxidative stress in the injured artery were less marked in female than in male \textit{Agtr2}\textsuperscript{H11001} mice, suggesting that estrogen contributes at least partly to the improvement in vascular remodeling. Moreover, we demonstrated that sex differences in VSMC proliferation, neointimal formation, inflammatory response, and oxidative stress were blunted in \textit{Agtr2}\textsuperscript{H11002} mice. These results suggest that not only estrogen stimulation, but also AT2 receptor activation, could contribute to the sex difference in vascular remodeling. As previously reported,\textsuperscript{5,6,11} we observed an increase in AT2 receptor expression in the injured artery, which was more exaggerated in female mice. This result supports the notion that AT2 receptor stimulation exerts antiproliferative effects on VSMCs and anti-inflammatory and antioxidative effects in injured vessels. Moreover, the sex difference in AT2 receptor expression could contribute to the sex difference in vascular remodeling.

Consistent with previous studies,\textsuperscript{9,10} we demonstrated that ovariectomy increased neointimal formation, with an increase in VSMC proliferation. Ovariectomy removes both estrogen and progesterone. Progesterone has reported to block the effect of estrogen.\textsuperscript{17,18} Therefore, there is a possibility that progesterone plays some role in the enhancement of vascular remodeling in ovariectomized mice. However, our group has previously reported that increased neointimal formation, DNA synthesis, and atherosclerotic lesions in ovariectomized mice were reversed by treatment with estrogen to the physiologic (female) level,\textsuperscript{9,10} suggesting that estrogen contributes to improvements in vascular remodeling over and above the apparent opposite effects of progesterone. The effect of ovariectomy on vascular remodeling was attenuated in \textit{Agtr2}\textsuperscript{H11001} mice, however, suggesting the possibility that estrogen could exert its vascular protective effects at least in part in concert with AT2 receptor stimulation. It is possible that costimulation of the estrogen and the AT2 receptor accelerates growth-inhibitory signaling. We previously reported that 17\textbeta-estradiol attenuated AT1 receptor–mediated activation of extracellular signal–regulated kinases (ERKs) and \textit{c-fos} expression via activation of phosphatases such as Src homology 2-containing protein-tyrosine phosphatases (SHP)-1 and mitogen-activated protein kinase phosphatase-1 (MKP-1), thereby inhibiting VSMC proliferation.\textsuperscript{19} The growth-inhibitory effects of the AT2 receptor have been shown to be associated with activation and/or induction of a series of phosphatases, including the protein tyrosine phosphatase SHP-1, MKP-1, and serine/threonine phosphatase 2A, which results in inactivation of AT1 receptor– and/or growth factor–activated ERK.\textsuperscript{1,2} Moreover, we demonstrated that AT2 receptor stimulation attenuated MCP-1 expression via inhibitory protein (I) \textit{kB} stabilization, thereby increasing nuclear factor-\textit{kB} nuclear translocation, and SHP-1 might play a critical role in the transcriptional regulation of MCP-1 expression through the control of \textit{kB} protein stability in cultured fetal VSMCs,\textsuperscript{20} suggesting the possibility that estrogen-mediated SHP-1 activation could also enhance AT2 receptor–mediated anti-inflammatory effects.

A common mechanism by which cardiovascular risk factors initiate the disease process is oxidative stress, leading to vascular...
inflammation and excessive production of reactive oxygen species, which are involved in vascular inflammation and remodeling and cause hypertension, atherosclerosis, and restenosis.\textsuperscript{21–23} It has been reported that estrogen inhibits oxidative stress in VSMCs.\textsuperscript{24,25} In this study, we demonstrated that superoxide production and NADPH oxidase activity were enhanced in injured arteries of \textit{Agtr2\textsuperscript{-}} mice and that the sex difference in oxidative stress in \textit{Agtr2\textsuperscript{+}} mice was attenuated in \textit{Agtr2\textsuperscript{-}} mice, suggesting that AT\textsubscript{2} receptor stimulation could be pivotal for estrogen to exert its inhibitory effects on oxidative stress. The potential inhibitory effects of AT\textsubscript{2} receptor stimulation together with estrogen on oxidative stress need to be further clarified, and detailed analysis will contribute to further understanding of the pathogenesis of vascular inflammation, atherosclerosis, and vascular remodeling.

We have reported that treatment with an ARB, olmesartan, significantly inhibited neointimal formation and BrdU incorporation into VSMCs via inhibition of ERK and signal transducer and activator of transcription (STAT) activity, whereas the inhibitory effects of olmesartan were more marked in female than in male or ovariectomized mice, indicating that estrogen and ARB synergistically attenuate vascular remodeling and that

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**Figure 5.** Sex difference in superoxide production and the effect of valsartan on superoxide production in injured arteries after cuff placement in \textit{Agtr2\textsuperscript{+}} and \textit{Agtr2\textsuperscript{-}} mice. Vascular samples were taken 7 days after cuff placement operation. In situ production of superoxide was detected with DHE, as described in Methods. A, Representative images for detection of superoxide production in injured arteries. Magnification, \( \times 100 \). Similar results were obtained from 5 to 7 mice. B, Fluorescence intensity of superoxide production. Val, 1 mg \cdot kg\textsuperscript{-1} \cdot d\textsuperscript{-1} valsartan. Other abbreviations are as defined in text. \( n=5 \) to 8 for each group. \(* P<0.05 \) vs without valsartan. Values are mean\( \pm \)SEM.

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**Figure 6.** Sex difference in NADPH oxidase activity and effect of valsartan on NADPH oxidase activity in injured arteries after cuff placement in \textit{Agtr2\textsuperscript{+}} and \textit{Agtr2\textsuperscript{-}} mice. Arterial samples were taken 7 days after cuff placement operation. Protein samples were prepared from pooled arteries (\( n=5 \) for each pool), and enzyme activity was assayed as described in Methods. Val, 1 mg \cdot kg\textsuperscript{-1} \cdot d\textsuperscript{-1} valsartan. Other abbreviations are as defined in text. \( n=4 \) for each group. \(* P<0.05 \) vs without valsartan. Values are mean\( \pm \)SEM.
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