Roles of Angiotensin II Type 2 Receptor Stimulation Associated With Selective Angiotensin II Type 1 Receptor Blockade With Valsartan in the Improvement of Inflammation-Induced Vascular Injury

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Background—To investigate the effect of angiotensin (Ang) II type 1 receptor (AT1) blocker on vascular remodeling and explore the possibility of the involvement of Ang II type 2 receptor (AT2) stimulation in this process, we examined the effects of the selective AT1 blocker valsartan on the vascular injury in wild-type (Agtr2+/+) and AT1-null (Agtr2−/−) mice.

Methods and Results—Neointima formation and the proliferation of vascular smooth muscle cells (VSMCs) induced by cuff placement on the femoral artery were greater in Agtr2−/− mice than those in Agtr2+/+ mice. Treatment of mice with valsartan at a dose of 1 mg·kg−1·day−1, which did not influence systolic blood pressure, significantly decreased neointima formation and the proliferation of VSMCs, whereas the valsartan was less effective in Agtr2−/− mice. Moreover, cuff placement increased the expression of monocyte chemotactractant protein-1 (MCP-1); inflammatory cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-6, and IL-1β; and infiltration of CD45-positive leukocytes and macrophages in the injured arteries and further enhanced them in Agtr2−/− mice, suggesting the antagonistic effects of AT1 and AT2 for vascular inflammation. Valsartan attenuated the expression of MCP-1, TNF-α, IL-6, IL-1β, and infiltration of leukocytes and macrophages in the injured arteries; however, these effects of valsartan were less prominent in Agtr2−/− mice.

Conclusions—These results suggest that the stimulation of the AT2 receptor after AT1 blockade is important in the improvement of the inflammatory vascular injury. (Circulation. 2001;104:2716-2721.)

Key Words: angiotensin receptors arteries inflammation remodeling

Recent evidence has revealed that the functions of angiotensin (Ang) II type 1 (AT1) and type 2 (AT2) receptors are mutually antagonistic.1,2 Monocyte infiltration into the vessel wall, a key initial step in the process of atherosclerosis, is mediated in part by monocyte chemoattractant protein-1 (MCP-1). It appears that AT1 stimulation increases inflammation and stimulates atherosclerosis by stimulating MCP-1 expression.3–5 Moreover, Ruiz-Ortega et al6 suggested that Ang II could participate in the recruitment of mononuclear cells through nuclear factor (NF)-κB activation and MCP-1 expression by renal cells in immune-complex nephritis. We could speculate that AT1 might exert anti-inflammatory and anti-atherogenic effects by inhibiting the expression of MCP-1. In contrast, Ang II–mediated induction of the chemokine RANTES was shown to be transduced by AT2 in cultured rat glomerular endothelial cells and in vivo.7

The effect of AT1 blocker may not be entirely due to the blockade of the AT1. When AT1 is blocked, increased Ang II may act on AT2 and Ang(1–7), and Ang IV, via the AT2 receptor, might be involved in the effects of AT1 blockade. Obviously, the AT2 plays a role in the pathogenesis and remodeling of cardiovascular and renal diseases.1,2 The results, however, although suggestive, are sometimes equivocal. Harada et al8 demonstrated that neointima formation in AT1−/− mice was comparable to that in wild-type mice and that AT2 was not involved in injury-induced neointima formation. If the AT2 contributes to the pathogenesis and consequent remodeling of cardiovascular diseases in humans, AT1 blocker may have some specific effects in the treatment of cardiovascular diseases. A more extensive knowledge of the AT2 could therefore contribute to the understanding of the clinical beneficial effects of the AT1 blockers. To evaluate the potential roles of AT2 stimulation in vascular inflammation and remodeling when we use AT1 blockers, we used Agtr2−/− mice in this study.

Methods

Animals and Treatment

Adult male Agtr2+/+ and Agtr2−/− mice9 (10 to 12 weeks of age) were used. The Animal Studies Committee of Ehime University approved the following experimental protocol. The surgical procedure of

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cuff-induced vascular injury in the femoral artery, morphometric analysis, and measurement of DNA synthesis were performed according to methods described previously. Some Agtr2+ and Agtr2− mice were treated with the AT blocker valsartan (provided by Novartis Pharma AG) by Alzet micro-osmotic minipumps (model 1002. Alza Corp) implanted intraperitoneally at the same time as cuff placement. The pumps delivered valsartan (1 mg · kg⁻¹ · d⁻¹) continuously for 7 or 14 days at a rate of 0.25 μL/h.

Reverse Transcription–Polymerase Chain Reaction
RNA was prepared from the pooled arteries (n=7 to 10 for each group) with the use of TRIzol Reagent (Gibco-BRL). Reverse transcription–polymerase chain reaction (RT-PCR) was performed as previously described. PCR primers for MCP-1 are 5’-ACTGAAGCCAGCTCTCTCTTCCTC-3’ (forward) and 5’-TTCTTCTTTGGGTCAGCACAGAC-3’ (reverse). The PCR reactions were optimized and carried out with 30 cycles for interleukin (IL)-1β, IL-6, tumor necrosis factor (TNF)-α, and MCP-1 and 25 cycles for GAPDH. To verify the identity of the PCR products, we sequenced PCR products and confirmed that the sequences of PCR products matched the predicted sequences. PCR-amplified DNA of MCP-1 was subcloned into pGEM-T Easy Vector (Promega Corp), and cDNA probe for Northern blotting was prepared from this plasmid vector.

Northern Blot Analysis
RNA was prepared from the pooled arteries (n=24 to 30 for each group). After size-fractionation on a denaturing agarose-formaldehyde gel, total RNA (30 μg) was transferred to a nylon membrane (Hybond-N+, Amersham Pharmacia Biotech). Hybridization was carried out with 32P-labeled probes of (1) a HindIII-NsiI fragment of mouse AT_1 cDNA, (2) mouse AT_2 cDNA and MCP-1 cDNA, or (3) a 0.78-kb PstI-XbaI fragment of human GAPDH. Densitometric analysis was performed with an image scanner (EPSON GT-8000) and NIH Image software.

Western Blot Analysis
Total proteins were prepared from the pooled arteries after cuff placement (n=6 to 8 for each group), and Western blot was performed as previously described. Immunoblotting was done with anti-MCP-1 antibody (Santa Cruz Biotechnology Inc), anti–TNF-α antibody (Genzyme-Tecne), and anti–α-smooth muscle (α-SM) actin antibody (clone 1A4; Sigma).

Immunohistochemistry
MCP-1, macrophage (F4/80), CD45-positive leukocytes, and TNF-α were stained as described previously. MCP-1, leukocytes, macrophages, and TNF-α were stained with anti–MCP-1 antibody, anti-CD45/common leukocyte antigen antibody (clone 30f11, BD PharMingen), anti-F4/80 antibody (Cl: A-1, BMA Biomedicals AG), and anti-mouse TNF-α antibody (Genzyme-Tecne), respectively. The infiltrated CD45-positive leukocytes and macrophages in the media and neointima in 3 transverse sections per artery were counted, and the number of infiltrated cells is expressed as the mean of values obtained from 3 independent sections per artery of 8 to 10 mice.

Statistical Analysis
Values are expressed as mean±SEM in the text and figures. The data were analyzed by 2-way ANOVA. If a statistically significant effect was found, post hoc analysis was performed to detect the difference between the groups. A value of P<0.05 was considered to be statistically significant.

Results
Vascular Remodeling Induced by Cuff Placement
The area of neointima 14 days after the cuff placement around the femoral artery was 2-fold larger in the Agtr2− mouse than in the Agtr2+ mouse, whereas medial area was not different in the 2 groups (Figure 1a). Indexes of bromodeoxyuridine (BrdU)-positive cells and proliferating cell nuclear antigen (PCNA) labeling index (PCNA-positive nuclei/total nuclei) were analyzed at 7 days after cuff placement. Open bars, without valsartan; solid bars, with valsartan. n=8 to 10 for each group. *P<0.05 vs without valsartan. §P<0.05 vs Agtr2− mice. Values are mean±SEM.

Figure 1. Effects of valsartan treatment (Val) on morphological changes, DNA synthesis, and cell proliferation after cuff placement. a, Morphometric analysis of cuffed femoral artery in Agtr2+ and in Agtr2− mice with or without valsartan at 14 days after cuff placement. b, BrdU uptake in media and neointima of cuffed femoral artery 7 days after operation. c, Proliferating cell nuclear antigen (PCNA) labeling index (PCNA-positive nuclei/total nuclei) was higher in the Agtr2− mice than in the Agtr2+ mouse. As previously reported, AT_1 mRNA was upregulated in response to cuff placement, and the expression of AT_1 in Agtr2− mice after vascular injury is comparable to those in wild-type mice. AT_2 expression was stimulated markedly from the baseline undetectable level by Northern blot. The peak effect was observed at 5 days after cuff placement, then the expression of AT_2 declined gradually. Consistent with our previous results, Northern blot shows that the expression of AT_1 was increased and the AT_1 reexpressed 7 days after cuff placement in the Agtr2+ mice (Figure 2). In the Agtr2− mice, the mRNA of AT_1 appears to be different from that in the Agtr2+ mice (Figure 2). As previously reported, immunohistochemistry using the antibody to AT_2 demonstrated that AT_2 expression was observed mainly in the media and neointima and was associated with α-SM actin, indicating that AT_2 was...
Effects of Valsartan on Neointima Formation, BrdU Index, and PCNA Labeling Index

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<td>Agtr2+</td>
<td>87.8 ± 4.3</td>
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<td>46.3 ± 6.5</td>
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Arteries were taken 14 days after cuff placement for measurement of neointima formation and 7 days after for staining of BrdU- and PCNA-positive cells. n = 8–10 for each group. Values are expressed as a percentage of the value without valsartan. *P < 0.05 vs Agtr2+.

Effects of AT1 Blockade on Vascular Remodeling

As shown in Figure 1a and the Table, the area of neointima after cuff placement was decreased by valsartan in the Agtr2+ mice. The inhibition by valsartan was significantly smaller in the Agtr2− mice than in the Agtr2+ mice. This dose of valsartan did not change blood pressure and heart rate in these animals (data not shown). BrdU index in the Agtr2+ mice was significantly suppressed in both media and neointima by valsartan, whereas this inhibition by valsartan was smaller in the Agtr2− mice than in the Agtr2+ mice (Figure 1b and Table). The ratio of PCNA-positive cells was also decreased with valsartan, whereas the effect of valsartan was significantly weaker in the Agtr2− mice. We observed that treatment with valsartan did not influence the mRNA levels of AT1 and AT2 (Figure 2).

Effects of AT1 Blockade on MCP-1 Expression

Determined with Northern blot, the basal mRNA level of MCP-1 was similar in the Agtr2+ and Agtr2− mice, and the mRNA level was increased 2.8 ± 0.3-fold 7 days after cuff placement in the Agtr2+ mice; this increase appeared to be greater in the Agtr2− mice (4.5 ± 0.4-fold, P < 0.05 versus Agtr2+) (Figure 3). The increase in mRNA of MCP-1 was inhibited by valsartan in the Agtr2+ mice (63.3 ± 6.2% inhibition), but this inhibitory effect of valsartan was attenuated in the Agtr2− mice (35.2 ± 4.1% inhibition versus Agtr2+, P < 0.05). Consistent with these results, the protein contents of MCP-1 determined by Western blot were increased after cuff placement, with a higher increase in Agtr2− mice (2.4 ± 0.3-fold increase in Agtr2+ mice versus 5.2 ± 0.5-fold increase in Agtr2− mice, P < 0.05), and valsartan effectively inhibited the increase in protein contents of MCP-1, whereas the inhibitory effect of valsartan on MCP-1 protein level was weaker (65.2 ± 7.1% inhibition in Agtr2+ mice versus 35.5 ± 3.9% inhibition in Agtr2− mice, P < 0.05) (Figure 4a). In immunohistochemical staining 7 days after operation, MCP-1 was localized mainly in media and neointima in both the Agtr2+ and Agtr2− mice after cuff placement (Figure 4b). In the Agtr2− mice, MCP-1, especially in the media, was stained more strongly in the Agtr2− than the Agtr2+ mice.

Effect of AT1 Blockade on Inflammatory Response

Immunohistochemical study shows that the infiltrations of CD45-positive leukocytes and macrophages in the injured vessels 7 days after cuff placement were enhanced, whereas infiltration of these cells was less in Agtr2+ mice (CD45-positive leukocytes: 10 ± 2 in Agtr2+ mice versus 30 ± 5 in Agtr2− mice per transverse section, P < 0.05; macrophages: 15 ± 4 in Agtr2+ mice versus 26 ± 5 in Agtr2− mice per transverse section, P < 0.05) (Figure 5). Valsartan effectively inhibited the inflammatory cell infiltration in the injured vessels in both strains; however, this inhibitory effect of valsartan was less in Agtr2+ mice (CD45-negative leukocytes: 48 ± 5% inhibition in Agtr2+ mice versus 32 ± 3% inhibition in Agtr2− mice, P < 0.05; macrophages: 52 ± 5% inhibition in Agtr2+ mice versus 29 ± 4% inhibition in Agtr2− mice, P < 0.05) (Figure 5). We also examined the expression of inflammatory cytokines, such as TNF-α, IL-6, and IL-1β, in the injured artery as determined by RT-PCR 7 days after operation (Figure 6). The expressions of TNF-α,
IL-6, and IL-1β were upregulated in Agtr2+/ mice and further enhanced in Agtr2− mice. Valsartan inhibited the increase in the expression of these cytokines, but these inhibitory effects of valsartan were weaker in Agtr2− mice (Figure 6). We examined the expression of TNF-α at the protein level by Western blot and immunohistochemical staining (Figure 7a). As shown in Figure 7a, expression of TNF-α in noninjured arteries was comparable in Agtr2+ and Agtr2− mice and was enhanced in Agtr2+ mice and further exaggerated in Agtr2− mice (3.4±0.4-fold increase in Agtr2+ mice versus 6.5±0.6-fold increase in Agtr2− mice, P<0.05). AT1 blockade by valsartan decreased TNF-α expression in Agtr2+ mice, and this effect of valsartan was weaker in Agtr2− mice (51.2±5.9% inhibition in Agtr2+ mice versus 24.6±3.1% inhibition in Agtr2− mice, P<0.05). Similar results were obtained by immunohistochemical analysis (Figure 7b).

**Discussion**

Most of the beneficial effects provided by AT1 blocker appear to be related to a more complete blockade of AT1. Costimulation of the uninhibited AT2, however, appears to play some role in the improvement of cardiovascular remodeling and decrease blood pressure.13,14 To further clarify the possibility of the potential roles of AT2 stimulation associated with AT1 blocker in vascular remodeling, we used AT2-null mice, and we chose the inflammation-induced nonconstricting cuff model because it is very reproducible, whereas vascular injury induced by intraluminal balloon or wire in the mouse is quite variable. We demonstrated that treatment with even a...
small dose of valsartan effectively attenuated neointima formation, along with a decrease in the DNA synthesis in the media and the neointima, whereas these effects of valsartan were significantly weaker in Agtr2− mice.

Previous in vitro studies have shown that the AT2 can mediate growth inhibition in various cells.Using in vivo gene transfer, we have demonstrated that overexpression of the AT2 inhibited the subsequent development of the neointima. Moreover, Liu et al demonstrated that AT2 expressed in failed rat heart decreased interstitial collagen deposition and cardiomyocyte size. In contrast, Levy et al reported that chronic blockade of AT2 in Ang II-induced hypertensive rats had no effect on arterial pressure but antagonized the effect of Ang II on arterial hypertrophy and fibrosis. We reported that neointima formation in response to vascular injury, coronary artery thickening, and perivascular fibrosis after aortic banding were exaggerated in Agtr2− mice. In contrast, Senbonmatsu et al observed recently that the targeted deletion of mouse AT2 prevented the left ventricular hypertrophy resulting from pressure overload. Harada et al demonstrated that there were no significant differences between wild-type and AT1r-null mice in the extent of histological findings, such as increased cross-sectional areas of the neointima and the media in the vascular injured model, and that treatment with subpressor doses of Ang II after injury enhanced the growth of neointima in wild-type mice but not in AT1r-null mice. Moreover, they reported that treatment with the selective AT1 blocker CV-11974 significantly decreased the formation of neointima only in wild-type mice, whereas treatment with the selective AT2 antagonist PD-123319 had no effects in either animal group. They suggested that AT1-mediated Ang II signaling is not essential for the development of neointima formation, although it may modify it, and that AT2 was not involved in neointima formation. In contrast, we demonstrated that valsartan effectively attenuated neointima formation in the injured vessels in both Agtr2+ and Agtr2− mice, whereas neointima formation was exaggerated in Agtr2− mice and the inhibitory effect of valsartan on neointima formation was weaker in Agtr2− mice. Moreover, we previously demonstrated that the AT2 antagonist PD123319 increased neointima formation in the cuff-induced vascular injury model in wild-type mice, suggesting that both AT1 and AT2 are important for regulating neointima formation in this cuff-induced vascular injury model. These apparently conflicting results are at least partially due to the methods used to introduce vascular injury. Harada et al introduced the curved flexible angioplasty wire into the left common carotid artery via the external carotid artery and passed it along the artery 3 times; subsequently, a polyethylene tube (PE-90) was immediately introduced into the artery and was passed along the vessel 3 times. The tube was then removed, and the external carotid artery was tied off around the artery, and kept the tube. We also have to take the difference of genetic background of the mice into account, because the genetic background of our Agtr2− mice is FBV/N, and theirs are C57BL/6. These apparently conflicting results may provide evidence for heterogeneity in the effects of AT1 and/or AT2 stimulation in different tissues, cells, experimental conditions, species, and/or genetic backgrounds in vascular remodeling. Taken together, these results raised the question of whether the stimulation of AT1 as well as AT2 is essential in regulation of neointima formation in response to vascular injury in different disease states. These issues have to be addressed in the near future. In this context, our results provide new insights that stimulation of uninhibited AT2 might play some role in the beneficial effects of AT1 blocker in vascular inflammation and vascular smooth muscle cell proliferation in the cuff-induced vascular injury mouse model.

The cuff injury model stimulates an inflammatory reaction that appears to play an important role in mediating the formation of neointimal hyperplasia. MCP-1 induction was higher in Agtr2− mice, especially in the media and neointima cells, where AT2 was specifically expressed. These results raise the possibility that AT2 stimulation exerts an inhibitory effect against MCP-1 expression. The expression of inflammatory cytokines, such as TNF-α, IL-6, and IL-1β, increased in cuff-injured artery and was higher in Agtr2− mice. These results provide us with the new idea that AT2 has anti-inflammatory effects against AT1 in the injured vessel.

AT1-mediated MCP-1 expression is decreased by tyrosine kinase inhibitor and the mitogen-activated protein kinase inhibitor. Accumulating evidence revealed opposite intracellular effects of AT1 and AT2. AT2 receptors are reported to activate phosphatases such as MKP-1, SHP-1, and PP2A, which results in the inactivation of AT1- and/or growth factor–activated extracellular signal–regulated kinase (ERK). Therefore, it seems conceivable that the specific phosphatase(s) may antagonize the pathway of AT1-mediated MCP-1 production. MCP-1 gene expression has been reported to be regulated by the cooperative action of NF-κB and AP-1. NF-κB activation consists of the dissociation of inhibitor-κB (IκB), and phosphorylation of IκB is a prerequisite for its degradation and subsequent liberation of active NF-κB. Therefore, it seems possible that the specific phosphatase(s) activated by AT2 may antagonize the pathway of AT1-mediated MCP-1 production. Indeed, Hoshi et al reported that PD98059, an inhibitor of ERK, suppressed NF-κB transcriptional activity. We also observed that AT1-induced c-fos expression is inhibited by AT2 stimulation, suggesting that the transcriptional activity of AP-1 might be decreased by AT2 stimulation and result in the inhibition of MCP-1 expression. In contrast, recent reports demonstrated that AT2, as well as AT1 stimulation increased NF-κB transcriptional activity. It might be possible, however, that increased cytokine expression in the injured arteries in Agtr2− mice would enhance NF-κB activation, because cytokines such as TNF-α and IL-1β are potent inducers of NF-κB activation.

Valsartan significantly decreased MCP-1 expression, the production of inflammatory cytokines, and infiltration of inflammatory cells in cuff-injured artery, whereas valsartan was less effective in the inhibition of inflammation in Agtr2− mice. This result indicates that costimulation of uninhibited AT1 is important in mediating the beneficial effect of valsar-
tanning on the improvement of vascular inflammation and remodeling. In conclusion, specific AT₁ blockade with valsartan is associated with AT₂ stimulation, attenuates neointima formation, and inhibits inflammation in the injured artery.

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