Role of Angiotensin II–Regulated Apoptosis Through Distinct AT₁ and AT₂ Receptors in Neointimal Formation

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Background—In vitro studies suggest that angiotensin II type 1 and type 2 (AT₁ and AT₂) receptors exert opposite effects in terms of vasoconstriction, natriuresis, and cell growth, but the role of these receptors in cardiovascular remodeling in vivo is still an enigma. In this study, we tested the hypothesis that AT₂ exerts an antiproliferative effect by inducing apoptosis, thereby antagonizing AT₁ in vascular remodeling.

Methods and Results—Vascular injury was induced by polyethylene cuff placement around the left femoral artery of AT₁a-null (AT₁aKO), AT₂-null (AT₂KO), and wild-type mice. Neointimal formation as well as DNA synthesis in vascular smooth muscle cells (VSMC) after vascular injury was exaggerated in AT₂KO mice, but they were both suppressed in AT₁aKO mice compared with those in wild-type mice. In contrast, the number of apoptotic cells in the injured artery in VSMC was significantly increased in AT₁aKO mice but decreased in AT₂KO mice. Reverse transcriptase–polymerase chain reaction analysis revealed that the expression of bax mRNA was attenuated in AT₁aKO mice. On the other hand, the expression of bcl-2 and bcl-xL mRNA was enhanced in AT₂KO mice but attenuated in AT₁aKO mice. Immunohistochemical staining with antibody to the bcl-2 protein family supported these results.

Conclusions—Our results suggest that AT₂ exerts antiproliferative effects and proapoptotic changes in VSMC by counteracting AT₁a in the process of neointimal formation after vascular injury. (Circulation. 2002;106:847-853.)

Key Words: angiotensin II receptors • apoptosis • arteries • remodeling

Apoptosis is characterized by a series of morphological events such as shrinkage of the cell, condensation of chromatin, fragmentation into apoptotic bodies, and rapid phagocytosis by neighboring cells. Apoptosis contributes to the physiological development and to the adaptation of the cardiovascular system to pathological states such as myocardial infarction and vascular injury. Therefore, the balance between cell proliferation and apoptosis may determine cardiovascular remodeling.

Angiotensin II (Ang II) is a principal vasoactive substance of the renin-angiotensin system, which has a variety of physiological actions including vasoconstriction, aldosterone release, and cell growth. Most of the known physiological actions of Ang II are thought to be mediated through the Ang II type 1 (AT₁) receptor but less is known about the function of the Ang II type 2 (AT₂) receptor. AT₁ is abundantly and widely expressed in fetal tissues, but its expression declines rapidly after birth. Interestingly, AT₂ is reexpressed in certain pathological conditions such as inflammation and vascular injury. These findings suggest that AT₂ plays an important role not only in vasculogenesis but also in vascular remodeling. Recent evidence suggests that AT₂ stimulation may exert antagonistic effects against AT₁. Moreover, Ang II–regulated apoptosis has been highlighted; however, its physiological roles in cardiovascular remodeling are still an enigma.

Recently, we have developed an inflammation-dependent vascular disease mouse model induced by polyethylene cuff placement around the femoral artery. We used AT₂-null (AT₂KO) mice and demonstrated that targeted deletion of AT₂ enhanced vascular inflammation and neointimal formation. However, the roles of Ang II–regulated apoptosis through the two distinct AT₁ and AT₂ receptors in vascular remodeling have not been elucidated despite their physiological importance. The bcl-2 protein family plays key roles in regulating apoptosis in response to various stimuli. Therefore, we postulated that antagonistic actions of AT₁ and AT₂ in apoptosis induction may contribute to inflammation-dependent vascular remodeling by regulating the bcl-2 protein family. To prove this hypothesis, we used AT₁a-null (AT₁aKO) and AT₂KO mice, which provide an opportunity to examine the roles of endogenous AT₁ and AT₂. We demonstrated that the proapoptotic effect of AT₁ and antiapoptotic effect of AT₂ oriented vascular remodeling and that the bcl-2 family is a critical factor that is regulated by the balance of AT₁ and AT₂ expression.
Methods

Animals and Treatment
Adult male wild-type (WT), AT<sub>1a</sub>KO<sup>8</sup> and AT<sub>2</sub>KO<sup>9</sup> mice, 10 to 12 weeks of age with a body weight ranging 25 to 30 g, were used in this study. AT<sub>1a</sub>KO mice were provided by one of us (T.S.). AT<sub>2</sub>KO mice were provided by Hein et al.<sup>9</sup> The animals were housed in a room where lighting was controlled (12 hours on, 12 hours off) and the temperature was kept at 25°C. They were given a standard diet and water ad libitum. The surgical procedure of cuff-induced vascular injury model in the femoral artery, morphometric analysis, and measuring DNA synthesis were performed according to the method described previously.<sup>3,10</sup> All experiments were approved by the Animal Studies Committee of Ehime University.

Detection of Apoptotic Cells
For the detection of DNA fragmentation, terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) staining of the injured artery was performed with the MEBSTAIN Apoptosis Kit II (MBL).<sup>11</sup> The TUNEL index was calculated as the ratio of TUNEL-positive nuclei to total nuclei in the neointima and media. Immunohistochemical staining with antibody against single-strand DNA (DAKO) was also performed according to the protocol previously described.<sup>12</sup> In addition, to examine the morphological changes of nuclear chromatin, chromatin dye staining was done with Hoechst 33342. The sections were stained with Hoechst 33342 (5 µg/mL in PBS) for 30 minutes at 37°C and viewed under fluorescence microscopy.

Reverse Transcriptase–Polymerase Chain Reaction
Total RNA was extracted from pooled arterial samples (n=7 to 10 for each group) through the use of TRizol reagent (Gibco-BRL). Reverse transcriptase–polymerase chain reaction (RT-PCR) was performed as previously described.<sup>3,10</sup> The PCR primers used for the AT<sub>1</sub>, AT<sub>2</sub>, bax, bcl-2, and bcl-x<sub>L</sub> were designed as previously described.<sup>3,10,13</sup>

Immunohistochemistry
Immunohistochemical staining was performed on formalin-fixed, paraffin-embedded sections by streptavidin-biotin-peroxidase technique with the use of a kit (Histostain-SP Kit, Zymed Laboratories).<sup>3,10</sup> Platelet-endothelial cell adhesion molecule-1 (PECAM-1), α-smooth muscle actin, CD45-positive leukocytes, macrophages, bax, bcl-2, and bcl-x<sub>L</sub> were stained with anti–PECAM-1 antibody (BD Biosciences), anti–α-smooth muscle actin (Sigma), anti–CD45/common leukocyte antigen antibody (PharMingen), anti–F4/80 antibody (BMA Biomedicals AG), anti–bax antibody (Santa Cruz Biotechnology), anti–bcl-2 antibody (Santa Cruz Biotechnology), and anti–bcl-x<sub>L</sub> antibody (Transduction Laboratories), respectively.

Statistical Analysis
All results are expressed as mean±SEM. Statistical analysis was performed by ANOVA followed by Bonferroni test for comparison between groups. Values of P<0.05 were considered significant.

Results

Neointimal Formation After Cuff Placement
To clarify the roles of the AT<sub>1</sub> and AT<sub>2</sub> in vascular remodeling, we used AT<sub>1a</sub>KO and AT<sub>2</sub>KO mice and examined the morphometric changes of the injured artery 14 days after cuff placement (Figure 1). There was no significant difference in medial area among the 3 groups. In contrast, neointimal area was 1.65-fold greater in AT<sub>2</sub>KO mice than in WT mice (P<0.01), but it was 57% smaller in AT<sub>1a</sub>KO mice than in WT mice (P<0.01). As shown in Figure 2, AT<sub>1</sub> expression was comparable in both WT and AT<sub>2</sub>KO mice, and AT<sub>2</sub> expression was similar in both WT and AT<sub>1a</sub>KO mice.

Apoptosis in Injured Artery After Cuff Placement
Both media and neointima were uniformly stained with anti–α-smooth muscle actin antibody, indicating that media
and neointima consisted primarily of VSMC (Figure 3 and 4). Moreover, neointimal area in AT1a KO mice was very small. Therefore, we examined DNA synthesis in the injured artery after cuff placement in endothelium, both media and neointima, respectively. The BrdU index in media and neointima was significantly higher in AT1 KO mice than in WT mice ($P<0.05$); conversely, it was lower in AT1a KO mice than in WT mice ($P<0.05$) 7 days after cuff placement (Figure 3). The BrdU index in endothelial cells and inflammatory cells did not differ among the 3 groups. Next we examined apoptotic changes in the injured artery. Figure 4 shows TUNEL staining of the injured artery 14 days after cuff placement. As shown in Figure 4, the TUNEL index in media and neointima was significantly higher in AT1 KO mice than in WT mice ($P<0.05$), whereas it was lower in AT1 KO mice than in WT mice ($P<0.05$). The TUNEL index in endothelial cells and inflammatory cells did not differ among the 3 groups. The TUNEL index in the media and neointima was lower in AT1a KO mice than in WT mice ($P<0.05$), whereas no significant difference was observed between AT1a KO and WT mice at 7 days after cuff placement (Figure 5). Similar results were obtained by immunohistochemical staining of single-stranded DNA (data not shown). Chromatin dye staining of the injured artery after cuff placement showed increase in chromatin condensation and fragmentation into apoptotic bodies mainly in neointima and media in AT1a KO mice, whereas these changes were rarely observed in AT1 KO mice (Figure 6).

Figure 2. Expression of mRNA for AT1 and AT2 in femoral arteries. Cuffed arteries and control intact arteries in WT, AT1aKO, and AT2KO mice were harvested and frozen at 7 days after cuff placement. One representative result of 4 independent RT-PCRs is shown.

Figure 3. Incorporation of BrdU into nuclei (A) and immunohistochemical staining of PECAM-1 (B) and α-smooth muscle actin (C) in media and neointima in WT, AT1a KO, and AT2 KO mice at 7 days after cuff placement. Result is representative of data obtained from 8 to 10 animals. Magnification ×200. Bar graph (D) shows BrdU index (BrdU-positive nuclei/total nuclei) ($n=8$ to 10 per group). *$P<0.05$ vs WT. Each bar represents mean±SEM.
Expression of bcl-2 Protein Family

To study the possibility that Ang II regulates bcl-2 protein family expression, we next examined the mRNA levels of bax, bcl-2, and bcl-xL in the injured artery (Figure 7). The expression of proapoptotic protein bax mRNA was increased in AT1a KO mice as well as WT mice after cuff placement, but this increase was attenuated in AT2 KO mice. In contrast, the change in expression of antiapoptotic protein bcl-2 mRNA after cuff placement was enhanced in AT2 KO mice but attenuated in AT1a KO mice. The expression of bcl-xL mRNA after cuff placement was enhanced in WT and AT2 KO mice, but this increase was attenuated in AT1a KO mice. Immunohistochemical staining of the bcl-2 protein family showed changes similar to those shown by RT-PCR (Figure 8).

Figure 4. In situ detection of apoptotic cells by TUNEL staining (A) and immunohistochemical staining of PECAM-1 (B) and α-smooth muscle actin (C) in media and neointima in WT, AT1a KO, and AT2 KO mice at 14 days after cuff placement. Sections were counterstained with hematoxylin. Result is representative of data obtained from 8 to 10 animals. Magnification ×200. Bar graph (D) shows TUNEL index (TUNEL-positive nuclei/total nuclei) (n=8 to 10 per group). *P<0.05 vs WT. Each bar represents mean±SEM.

Figure 5. Time course of apoptotic changes after cuff placement. Bar graph shows TUNEL index (TUNEL-positive nuclei/total nuclei) in media and neointima at 7 and 14 days after cuff placement (n=8 to 10 per group). *P<0.05 vs WT. Each bar represents mean±SEM.

Figure 6. Representative micrographs of Hoechst 33342 staining in AT1a KO and AT2 KO mice at 14 days after cuff placement. Result is representative of data obtained from 8 animals. Arrowheads indicate chromatin condensation and fragmentation into apoptotic bodies. Magnification ×200. E indicates endothelium; N, neointima; and M, media.
Bax
Bcl-2
Bcl-xL
GAPDH

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Figure 7. Expression of mRNA for bax, bcl-2, and bcl-xL in femoral arteries. Cuffed arteries and control intact arteries in WT, AT1aKO, and AT2KO mice were harvested and frozen at 7 days after cuff placement. One representative result of 4 independent RT-PCRs is shown.

Discussion

Cuff placement around the femoral artery induces neointimal thickening accompanied by enhanced expression of inflammatory cytokines as well as AT1 and AT2. This model may favor analysis of the inflammatory response involved in the pathogenesis of vascular injury and consequent remodeling mechanism. In the present study, we focused on the roles of the AT1a and AT2 in the regulation of apoptosis in vascular remodeling. We observed that neointimal formation as well as DNA synthesis in VSMC was exaggerated in AT2 KO mice, but they were both suppressed in AT1a KO mice. In contrast, the number of apoptotic cells in the injured artery in VSMC was significantly increased in AT1a KO mice, but it was decreased in AT2 KO mice. We did not observe any apparent difference in BrdU and TUNEL staining and chromatin condensation and fragmentation in endothelial cells and inflammatory cells among WT, AT1a KO, and AT2 KO mice. These findings support our hypothesis that the AT2 exerts antiproliferative effects and proapoptotic changes by counteracting AT1a-mediated signaling in VSMC in vascular remodeling by a cuff-induced vascular injury model.

Several studies have demonstrated that an AT1 blocker prevents neointimal formation after balloon injury in animal models. Moreover, a highly selective AT1 blocker, valsartan, reduced the restenosis rate after stent implantation in a recent human clinical trial. Consistent with these results, we observed that neointimal area after cuff placement was 57% smaller in AT1a KO mice than in WT mice; conversely, it was 1.65-fold greater in AT2 KO mice than in WT mice. The effects of an AT1 blocker appear not only to be due to blocking of the AT1-dependent pathway but also to stimulation of the unblocked AT1 by an increase in plasma Ang II. Very recently, we demonstrated that the inhibitory effects of valsartan on neointimal formation and inflammation were weaker in AT2 KO mice, suggesting that AT2 stimulation is important in the improvement of vascular remodeling by an AT1 blocker. However, Harada et al showed that no significant difference in the cross-sectional area of the neointima after vascular injury between WT and AT1 KO mice and infusion of Ang II enhanced the growth of neointima in only WT mice. Moreover, they showed that a selective AT2 blocker, PD-123319, had no effect on neointimal formation in both animal groups. They concluded that AT1-mediated Ang II signaling was not essential for the development of neointima, although it might modify it, and that AT2-mediated Ang II signaling was not involved in neointimal formation after vascular injury in their model. These apparently conflicting results could be partially due to the methods of vascular injury. Harada et al introduced an angioplasty wire (0.35-mm diameter) into the carotid artery and passed it along the vessel 3 times. Subsequently, they introduced a polyethylene tube (PE-10) into the artery and passed it along the vessel 3 times and then removed it. In contrast, we loosely placed a polyethylene tube (PE-90) around the isolated femoral artery and kept it in place during the experimental period. These two vascular injury models may differ in the magnitude of expression levels of AT1 and/or AT2 as well as their effects on vascular remodeling.

In a previous study, an AT1 blocker induced proapoptotic and growth-inhibitory effects in VSMC of spontaneously hypertensive rats, and these changes were prevented by an AT2 blocker. The role of VSMC proliferation as well as apoptosis has been emphasized in the process of neointimal formation after vascular injury. We evaluated the effects of AT1a and AT2 on apoptosis in an injured artery in this study. The biochemical hallmark of apoptosis is DNA fragmentation by endogenous DNase. With TUNEL staining, cells containing DNA strand breaks become visible on light microscopic analysis. It has been reported that immunohistochemical staining with antibody to single-strand DNA is also useful to detect DNA fragmentation. We observed a decrease in apoptotic cells in AT2 KO mice and an increase in apoptotic cells in AT1a KO mice by these methods. However, DNA fragmentation is observed not only in apoptotic cells but also...
in necrotic cells. Therefore, we confirmed the apoptotic changes based on morphological criteria by chromatin dye staining with Hoechst 33342. In contrast to BrdU-positive VSMC, the apoptotic VSMC in media and neointima in the injured artery was significantly increased in AT1a KO mice, but the number of apoptotic VSMC in this area was very low in AT2 KO mice. However, there was no significant difference in the relative level of apoptotic changes and DNA synthesis in endothelial cells in the injured artery among WT, AT2 KO, and AT1a KO mice. These results suggest that the AT1a plays crucial roles in neointimal formation by regulating apoptosis and DNA synthesis especially in VSMC but not in endothelial cells in our experimental vascular injury mouse model.

It has been reported that the AT2 exerts proapoptotic effects on PC12W cells, R3T3 mouse fibroblasts, AT2-transfected adult rat VSMC, and fetal VSMC. Ang II stimulation directly antagonized nitric oxide–donor–induced and cGMP analogue–induced apoptosis through activation of AT1 in VSMC in vitro. Consistent with these results, we demonstrated by using AT1a KO and AT2 KO mice that AT2 induced apoptosis and that AT1a suppressed apoptosis in VSMC in vivo in the cuff-injured artery. Differential regulation of apoptosis in different cell types is a well-established phenomenon. Indeed, apoptosis is often regulated in different cell types exposed to a given stimuli, although the underlying molecular mechanisms remain to be defined. In our experimental mode, there was no apparent difference in endothelial cell apoptosis among WT, AT1a KO, and AT2 KO mice. Cigola et al. reported that administration of Ang II induced apoptosis of neonatal rat ventricular myocytes and that Ang II–induced apoptosis was inhibited by an AT2 blocker but not by an AT1 blocker. Dimmelé et al. reported that Ang II induced apoptosis of human umbilical venous endothelial cells and that simultaneous blockade of both AT1 and AT2 prevented Ang II–induced apoptosis, whereas each individual receptor blocker alone was not effective and selective agonistic stimulation of the AT2 also induced apoptosis. These apparently conflicting results may provide evidence for heterogeneity of the effects of Ang II receptor stimulation in different tissues, cells, and/or different experimental conditions.

It has been reported that expression of the bcl-2 protein family plays a central role in the regulation of apoptosis. Therefore, to investigate the molecular determinants involved in regulating apoptosis, we also examined the expression of the bcl-2 protein family in the injured artery. In this study, expression of bcl-2 and bcl-xL was attenuated in AT1a KO mice. In contrast, the expression of bax was attenuated and the expression of bcl-2 and bcl-xL was enhanced in AT2 KO mice. These results suggest the possibility that AT2 exerts apoptotic changes through inhibition of bcl-2 and bcl-xL expression and that the AT1a exerts proliferative effects through both inhibition of bax expression and exaggeration of bcl-2 and bcl-xL expression in the injured artery. It has been demonstrated that AT1 stimulation inactivates bcl-2 and increases bax expression, thereby inducing apoptosis in PC12W cells. However, the effects of AT2 on other bcl-2 protein family members are still unknown. Moreover, it needs to be addressed whether Ang II receptor subtypes regulate bcl-2 protein family expression directly.

The intracellular signaling pathways leading to Ang II–mediated apoptosis are beginning to be defined. It is known that binding of Ang II to the AT1 activates extracellular signal-regulated kinase (ERK) and serine/threonine kinase (Akt). Activation of ERK and Akt exerts antiapoptotic and promitogenic effects. We previously reported that AT2 stimulation activates phosphatases, which results in the inhibition of ERK and Akt activity and antagonizes the antiapoptotic effect of the AT1. Moreover, AT2 stimulation increases ceramide production, an important mediator of apoptosis, in PC12W cells. These in vitro results support the notion that distinct AT1 and AT2 can regulate apoptosis through these counterbalancing actions.

In summary, we demonstrated that AT2 exerted antiproliferative and proapoptotic effects in VSMC and contributed to the decrease in neointimal formation in a cuff-induced vascular injury model by counteracting AT1. Our results provide new knowledge implicating AT1-regulated and AT2-regulated apoptosis in neointimal formation after vascular injury.

Acknowledgments

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References


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