Angiotensin Type 1 Receptor Blocker Reduces Intimal Neovascularization and Plaque Growth in Apolipoprotein E–Deficient Mice

Xian Wu Cheng, Haizhen Song, Takeshi Sasaki, Lina Hu, Aiko Inoue, Yasuko K. Bando, Guo-Ping Shi, Masafumi Kuzuya, Kenji Okumura, Toyoaki Murohara

Abstract—The interactions between the renin-angiotensin system and neovascularization in atherosclerotic plaque development are unclear. We investigated the effects of angiotensin II type 1 receptor antagonism in the pathogenesis of atherosclerosis in apolipoprotein E–deficient (ApoE<sup>−/−</sup>) mice with a special focus on plaque neovascularization. ApoE<sup>−/−</sup> mice fed a high-fat diet were randomly assigned to 1 of 2 groups and administered vehicle or olmesartan for 12 weeks. Quantification of plaque areas at the aortic root and in the thoracic and abdominal aorta revealed that, in all 3 of the regions, olmesartan reduced intimal neovessel density and the mRNA levels of toll-like receptor (TLR) 2 and TLR4. Olmesartan increased the levels of collagen and elastin, reduced the level of macrophages in the aortic root, and reduced the mRNA and the activity of matrix metalloproteinase (MMP) 2 in aortic roots and thoracic aortas. Aortic ring assay revealed that olmesartan-treated ApoE<sup>−/−</sup> mice had a markedly lower angiogenic response than that of untreated ApoE<sup>−/−</sup> mice. Bone marrow–derived endothelial progenitor cell–like c-Kit<sup>+</sup> cells from olmesartan-treated ApoE<sup>−/−</sup> mice showed marked impairment of cellular functions and lower expression of TLR2/TLR4 and MMP-2 compared with those of untreated controls. MMP-2 deficiency reduced intimal neovessel density and atherosclerotic lesion formation. Olmesartan and small-interfering RNA targeting TLR2 reduced the levels of TLR2, and MMP-2 mRNA induced angiotensin II in cultured endothelial cells. Angiotensin II type 1 receptor antagonism appears to inhibit intimal neovascularization in ApoE<sup>−/−</sup> mice, partly by reducing TLR2/TLR4-mediated inflammatory action and MMP activation, thus decreasing atherosclerotic plaque growth and increasing plaque instability. (Hypertension. 2011;57:981-989.)

Key Words: plaque growth • angiogenesis • toll-like receptor • matrix metalloproteinases

The renin-angiotensin system is thought to play a role in the pathogenesis of atherosclerosis by stimulating a series of coordinated cellular and molecular events in atherosclerotic lesions. Previous studies showed that angiotensin II (AT II) produced by vascular tissues induces the production of reactive oxygen species and stimulates the expression of adhesion molecules and chemokines through activation of the AT II type 1 receptor (AT<sub>1</sub>R), leading to the accumulation of inflammatory cells and the proliferation or migration of vascular smooth muscle cells (SMCs). These findings suggest that local effects of AT<sub>1</sub>R activation in the vessel wall play a central role in the pathogenesis of chronic vascular inflammation by directly acting on resident vascular cells. Recent research in the field of cardiovascular research has generated increasing interest in the family of toll-like receptors (TLRs). Supporting a role for TLRs in human atherosclerotic plaque development, the gene expression of TLR1, TLR2, TLR6, TLR7, and TLR8 was increased significantly in diseased vessels compared with nondiseased controls, and that of TLR4 was higher but without statistical significance. Immunohistochemistry analysis revealed that TLR1, TLR2, and TLR4 are highly expressed, particularly in endothelial cells (ECs) and macrophages, and that TLR3 and TLR5, by comparison, are more weakly expressed. In apolipoprotein E–deficient (ApoE<sup>−/−</sup>) mice, a model for atherosclerosis, the expression of TLR2 and TLR4 was increased over time in diseased tissues. TLR2 plays a critical role in monocyte activation and in stimulating the release of inflammatory cytokines and chemokines, which are crucial processes in the progression of atherosclerosis. Several lines of evidence suggest that AT II/AT<sub>1</sub>R induces vascular inflammation through the TLR4-dependent signaling pathway; however, the precise mechanisms of crosstalk between Ang II and the TLR signaling pathways in the development of atherosclerosis remain unknown.
In a previous study, Moulton et al\(^9\) demonstrated that neovascularization is associated with advanced atherogenesis in ApoE\(^{-/-}\) mice, and they further showed that the angiogenesis inhibitors endostatin and TNP-470 significantly reduced plaque neovascularization and growth. Conversely, stimulators of angiogenesis, such as vascular EC growth factor (VEGF), have been shown to induce lesion progression.\(^10\) Recent in vitro studies have shown that AT II induces stimulators of angiogenesis, such as vascular EC growth and matrix metalloproteinase (MMP) activation.

**Methods**

Methods in detail are available as an online Data Supplement at http://hyper.ahajournals.org.

**Animals and Treatment**

All of the animal studies were conducted in accordance with the animal care guidelines of Nagoya University Graduate School of Medicine. Male ApoE\(^{-/-}\) mice (C57BL/6 genetic background) were purchased from the Jackson Laboratory (Bar Harbor, ME). Until required for experiments, the mice were provided with a standard diet (Oriental Yeast) and tap water ad libitum. For experiments, 10-week-old male ApoE\(^{-/-}\) mice were fed a Western-type diet\(^14\) containing 21.00% fat from lard and 0.15% cholesterol and were assigned to either the control group (n=26) or the olmesartan group (n=27). The control group was administered 0.5% carboxymethyl-cellulose, whereas the olmesartan group was administered olmesartan (donated by Daiichi-Sankyo Pharmaceutical Co) at 3 mg/kg of body weight per day, in chow. After 12 weeks of consuming a Western-type diet, mice underwent trial recordings to accustom them to being measured, and then 3 reliable recordings were taken and used for the determination of blood pressure (BP) and heart rate. Mice were heavily sedated with Avertin, and blood was collected from the inferior vena for biological analysis.

**Quantitative Real-Time PCR**

Total RNA was isolated from the aortic tissues and the lysates from ECs with the use of an RNeasy Fibrous Tissue minikit (Qiagen, Inc) and was subjected to reverse transcription. The resulting cDNA was subjected to quantitative real-time PCR analysis, as described previously.\(^15,16\)

**Histological Characterization of Atherosclerotic Lesions in the Aortic Root**

Paraffin sections (3 \(\mu\)m) from aortic roots were deparaffinized in xylene, rehydrated with decreasing alcohol concentrations, and stained routinely with hematoxylin-eosin, Elastica van Gieson staining for elastin, and picrosirius red for collagen, as described previously.\(^14,17\)

**Mouse EC Culture and St excitations**

Mouse ApoE\(^{-/-}\) mouse aortic ECs were isolated as described previously.\(^15\) The ECs were cultured in endothelial basal medium 2 (Chambrex) containing growth factors and supplements (Clonetics). Positive 1,1',3,3'-tetramethylindocarbocyanine perchlorate-labeled acetylated low-density lipoprotein uptake and CD31 expression indicated that there was no contamination of SMCs (Figure S2, available in the online data Supplement). After being cultured in serum-free endothelial basal medium 2 for 24 hours, the ECs were subjected to the stimulation assays.

**Small-Interfering RNA Transfection**

Specific small-interfering RNA (siRNA) duplexes (ONTARGETplus siRNA) for TLR2 (ID: L-062583-02-0005), TLR4 (ID: L-047487-00-0005), lamin A/C (D0010500105, as a positive control), and a nontargeted control siRNA (D-001810-01-20) were purchased from Dharmacon (Brebieres, France). siRNA was transfected into cells as described previously.\(^14,19\)

**Statistical Analysis**

All of the measurements were conducted by 2 observers blinded to the treatment of the mice. Data were expressed as the mean±SEM. Student t test (for comparison between 2 groups) or 1-way ANOVA (for comparison among \(\geq3\) groups) followed by Tukey post hoc test were used for statistical analysis by using SPSS software version 17.0 (SPSS Inc, Chicago, IL). A P value of \(<0.05\) was considered statistically significant.

**Results**

**Effect of Olmesartan on Systolic BP, Heart Rate, Plasma Lipid Profile, and the Levels of AT II and Inflammatory Cytokines**

As shown in Table S1, after 12 weeks of treatment, orally administered olmesartan had no significant effect on systolic BP, body weight, or heart rate. Olmesartan had no effect on triglycerides, total cholesterol, or high-density lipoprotein. ELISA analysis of the concentrations of inflammatory cytokines in plasma showed that olmesartan significantly reduced the levels of tumor necrosis factor (TNF)-\(\alpha\) and interleukin (IL)-1\(\beta\), whereas AT II levels were numerically but not significantly higher.

**Olmesartan Reduces the Extent of Atherosclerotic Lesions and Neovessel Plaque Density and Changes Plaque Composition**

The effect of olmesartan on atherosclerotic lesion formation was assessed by measuring the intima area and by determining the ratio of the intima area to the media area in lesional cross-sections of the heart aortic roots, as well as those of the thoracic and abdominal aortas. Olmesartan treatment reduced the area of the neointima in these lesional regions compared with that of the controls (Table). Olmesartan also lowered the ratio of the intima area to the media area in these lesions compared with that of the controls (Table). Immunohistochemistry revealed that olmesartan reduced the neovessel density of the atherosclerotic plaques in the aortic roots (total neovessel numbers: 2.5±0.5 versus 6.3±1.7; plaque base: 0.6±0.1 versus 1.4±0.2 mm\(^2\) in the olmesartan and control groups, respectively; \(P<0.001\); Figure 1).

Cross-sectional analysis of the aortic roots was performed to characterize the constituents of atherosclerotic plaques. Olmesartan treatment increased the intimal collagen content of the neointima (17.8±2.1% versus 35.2±2.2% in the olmesartan and control groups, respectively; \(P=0.011\)) as determined by picrosirius red staining analyzed with polarized light. In addition, the elastin level of the neointimas determined by Elastica van Gieson staining remained significantly higher in olmesartan-treated mice compared with that of control mice (8.1±1.2% versus 17.2±2.0%, respectively; \(P=0.004\). Immunohistochem-
Aortic roots of apolipoprotein E–deficient (ApoE
Figure 1.

We evaluated macrophage accumulation and chemokine expression in atherogenic plaques of the aortic roots. We detected extensive immunostaining of Mac-3 for macrophages in the intima of atherosclerotic plaque in control ApoE−/− mice (Figure 2A). By comparison, this Mac-3–positive area was significantly diminished in ApoE−/− mice treated with olmesartan (Figure 2C). Immunostaining against monocyte chemoattractant protein 1 or osteopontin revealed that olmesartan attenuated the levels of monocyte chemoattractant protein 1 and osteopontin protein (Figure 2D and 2E). TLR2/TLR4 gene expression and that of stromal-derived factor 1 (SDF-1) and a CXC chemokine receptor (CXC4R) were then evaluated in the aortic roots and in the thoracic and abdominal aortas (Figure 3A). Quantitative PCR revealed that olmesartan attenuated the expression of TLR2 and TLR4 mRNAs compared with the expression in control mice (Figure 3B). Olmesartan-treated mice also showed a significant decrease in the expression of SDF-1 and CXCR4 mRNAs compared with the expression in the controls. In addition, the levels of AT1R protein were lower in the aortic roots and thoracic aortas of the olmesartan group than in those of the control group (Figure 3C and 3D).

Figure 1A. Immunofluorescence staining for neovessel density in aortic roots of apolipoprotein E–deficient (ApoE−/−) mice not treated (control, CONT; n = 7) or treated with olmesartan (OLM; n = 8) for 12 weeks. A, through C, Neovessel density of the atherosclerotic plaques was analyzed by immunofluorescence with anti-von Willebrand factor antibody (red). Results are expressed as total numbers of neomicrovessels divided by the plaque area (in millimeters squared). L indicates lumen; A, atheroma; M, media. Arrowhead indicate microvessel. Scale bar: 50 μm.

Table. Histological Characterization of Atherosclerotic Lesions in the Aortic Roots and in the Thoracic and Abdominal Aortas of Control and Olmesartan-Treated Mice

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Aortic Root</th>
<th>Thoracic Aorta</th>
<th>Abdominal Aorta</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CONT n = 7</td>
<td>OLM n = 8</td>
<td>CONT n = 7</td>
</tr>
<tr>
<td>Intima (×10²/μm²)</td>
<td>459±32</td>
<td>288±30*</td>
<td>221±28</td>
</tr>
<tr>
<td>Media (×10²/μm²)</td>
<td>567±56</td>
<td>575±45</td>
<td>525±18</td>
</tr>
<tr>
<td>Intima/media ratio</td>
<td>0.81±0.17</td>
<td>0.51±0.15*</td>
<td>0.42±0.14</td>
</tr>
<tr>
<td>IEL (×10²/μm²)</td>
<td>70±6</td>
<td>72±8</td>
<td>71±6</td>
</tr>
</tbody>
</table>

The ratio of intima to media was calculated as the ratio of the intimal area to the media area in lesional cross-sections of the heart aortic root and those of the thoracic and abdominal aortas of control (CONT) and olmesartan-treated (OLM) groups. All of the results are presented as mean±SEM. IEL indicates internal elastin lamina.

*P<0.05 vs corresponding controls.

The effects of olmesartan on MMP expression were then evaluated in the aortic roots and in the thoracic and abdominal aortas of control (CONT) and olmesartan-treated (OLM) groups. All of the results are presented as mean±SEM.

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EPC-like c-Kit⁺ cells from olmesartan-treated mice compared with untreated mice (Figure 5E through 5G).

MMP-2 Deficiency Reduced Intimal Neovessel Density and Plaque Growth

Compared with control mice, MMP-2⁻/⁻ mice had reduced fat accumulation around the aortas and also reduced intimal neovessel density and plaque growth in the aortic roots, as determined by fluorescein isothiocyanate–lectin–labeled CD31⁺ and oil-red O staining (Figure 6A through 6E). Compared with the controls, MMP-2⁻/⁻ mice had a lower level of plasma TNF-α (74±11 versus 102±12 pg/mL; \( P=0.045 \)), and we observed a lower level of macrophage accumulation in atheroma plaques of ApoE⁻/⁻ mice (Figure 6F), which is in agreement with the results of our previous study.\(^{15,20} \)

Quantitative real-time PCR analysis revealed that MMP-2 deficiency reduced the levels of TLR2 and TLR4 (Figure 7A). Next, we investigated the mechanism underlying AT₁R antagonism–mediated MMP suppression in ECs by examining the effect of several inhibitors on intracellular signaling pathways. Pretreatment with the phosphatidylinositol 3-kinase inhibitor LY294002 reduced the expression of MMP-2 and MMP-9 mRNAs stimulated by AT II in ECs (Figure 7C). By comparison, the extracellular signal–regulated kinase inhibitor U0124 and the janus kinase/signal transducer and activator of transcription 3 inhibitor AG490 had little effect on the level of gene expression of MMP-2 and MMP-9 (Figure 7C). Furthermore, transfection with small-interfering TLR (siTLR) 2 and siTLR4 significantly reduced the gene expression of MMP-2 and MMP-9 (Figure 7C). However, nontarget control siRNA had no effect on TLR2 or TLR4 gene expression induced by AT II (data not shown).

Discussion

We found that antagonism of the actions of AT₁R not only lessened the progression of atherosclerotic lesions but also changed the composition of the vascular wall such that it contained more extracellular matrix protein (collagen and elastin) and SMCs, changes that predict greater stability of the atherosclerotic plaque. AT₁R antagonism reduced diet-induced intimal neovascularization in atherosclerotic plaques,
which was accompanied by reduced accumulation of macrophages and reduced gene expression and subsequent activity of MMP-2 and MMP-9 associated with the reduction in TLR2/TLR4 gene expression. MMP-2 deficiency lessened diet-induced intimal neovascularization and atherosclerotic lesion formation, as well as the levels of TLR2 and TLR4 mRNA, the extent of macrophage infiltration, and the levels of plasma TNF-α and IL-1β in ApoE−/− mice. Furthermore, an in vitro experiment demonstrated that pretreatment with AT1R antagonism abolished AT II–induced MMP-2 and MMP-9 gene expression via the TLR2/TLR4–mediated phosphatidylinositol 3-kinase signaling pathway.

Engagement of TLRs on the cell surface by specific ligands leads to an increase in the expression of proinflam-

Figure 3. Inhibitory effects of olmesartan on inflammatory reactions and proteolysis. A and B, Representative images of PCR blots and combined quantitative gene expression data for toll-like receptor (TLR) 2, TLR4, CXCR4, and stromal-derived factor 1 (SDF-1) in aortic roots and in thoracic and abdominal aortas of the control (CONT) and olmesartan (OLM) groups (n=5). C and D, Representative images of Western blot and combined quantitative data for expression of angiotensin II type 1 receptor (AT1R) protein in aortic root (AR) and thoracic aorta (TR) are shown (n=4). E and F, Representative images of gelatin zymography and combined quantitative data for gelatinolytic activities of matrix metalloproteinase (MMP) 2 and MMP-9 in the AR and TA are shown (n=4). Values are mean±SEM. P<0.05 was considered statistically significant.

Figure 4. Angiotensin II type 1 receptor (AT1R) antagonism reduced the angiogenic activity of aortic explants. A, Photomicrographs showing unreversed and reversed aortic explants (left) and the sprouted vessels before and after incorporation of 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate-labeled acetylated low-density lipoprotein (Dil-acLDL) in the aortic ring assay (right panels). Magnified images show microvessel networks formed from cells that have incorporated Dil-acLDL. B, Representative images of tubulogenesis in the aortas of the control (CONT) and olmesartan (OLM) groups (n=4). C, Combined quantitative data from the images in B. Scale bars: 50 μm.
matory mediators, such as IL-1β, TNF-α, monocyte chemotactrant protein 1, and IL-6.21 Recent studies in humans and animals have shown that, among the TLRs, TLR1, TLR2, and TLR4 contribute to atherosclerotic plaque accumulation in ECs and macrophages.6,22,23 Here, we found that olmesartan attenuates the mRNA levels of AT1R, TLR2, TLR4, SDF-1, and CXCR4 in aortic roots and in thoracic and abdominal aortas. Concomitantly, the levels of monocyte chemoattractant protein 1 and osteopontin protein, which are inflammatory mediators, were suppressed by olmesartan in aortic roots. This result is in agreement with findings from a previous study on cardiac tissues.16 Thus, AT1R antagonism appears to reduce and stabilize atherosclerotic lesions in ApoE−/− mice. Data from the present and previous studies show that a deficiency in MMP-2 and MMP-9 impairs neovascularization under conditions of ischemia or exercise.15,20,26 Our observations that inhibition of AT II reduces the mRNA and activity of MMP-2 and MMP-9 in the aortic roots and thoracic aortas of ApoE−/− mice are in agreement with previous reports.16 Furthermore, we showed that atherosclerotic plaque neovessel density and lesions were also reduced in ApoE−/− MMP-2−/− mice, and AT1R antagonism had no significant effect on atherosclerotic lesion formation in ApoE−/− MMP-2−/− mice. These findings suggest that attenuation of neovascularization by AT1R via inhibition of MMP-2 and MMP-9 expression and activity represents a common mechanism for the reduction of diet-induced atherosclerotic plaque growth and stability.

Recent observations indicate that plaque neovascularization can cause plaque instability and rupture of advanced atherosclerotic lesions in human aortas.27 Further supporting this role, the angiogenesis inhibitor endostatin was shown to reduce intimal neovascularization and plaque growth in ApoE−/− mice.28 Inhibition of AT II, or AT1R deficiency, reduces ischemia- and tumor-related angiogenesis in animal models.12,13 Here, we...
showed that AT₁R antagonism reduced the angiogenic response of cultured aorta rings and bone marrow–derived EPC-like c-Kit⁺ cells to VEGF. Thus, attenuation of neovascularization by AT₁R antagonism has a potential vasculoprotective effect that could prevent atherosclerotic plaque growth and disruption, the process often responsible for myocardial infarction and ischemic stroke. It should be noted that daily administration of olmesartan at the dose used in this study and a previous study had no significant effect on BP. This result supports our hypothesis that olmesartan-mediated atherosclerotic plaque stabilization is not attributable to BP reduction but rather to the pleiotropic effects of olmesartan.

It is clear that TLRs regulate monocyte/macrophage activation, and inflammatory responses and activation of TLRs induce MMPs in animal tissues and cultured human acute monocytic leukemia cells. The present data show that AT₁R antagonism reduces the mRNA and protein levels of MMP-2/MMP-9 or/and TLR2/4 in aortic tissues and EPC-like c-Kit⁺ cells. Consistent with this, we have also confirmed in vitro for the first time that pretreatment with AT₁R antagonist reduces the expression of TLR2/4 mRNAs induced by Ang II in ECs. Recent lines of evidence highlight the importance of protease induction through activation of TLRs by molecules other than by specific ligands. We further confirmed that AT₁R antagonism and both siTLR2 and siTLR4 reduced AT II–induced MMP-2/MMP-9 gene expression in ECs. Taken together, these findings suggest that there might be cross-talk between the AT II/AT₁R and TLR2/4 signaling pathways in ECs and EPCs and that AT₁R antagonism-mediated reduction of TLR2/4 expression might be involved, at least in part, in the inhibitory effect of AT₁R antagonists on MMP-2/MMP-9 gene expression and activity. A previous study reported that TLR activation is involved in the induction of the cysteine protease cathepsin K and MMP-9 by several intracellular signaling pathways. Here, we showed that LY294002 reduced AT II–induced MMP-2 and MMP-9 gene expression in ECs, whereas U0126 and AG490 had no effects. Thus, the ability of AT₁R antagonism to abolish AT II–induced MMP-2 and MMP-9 gene expression is not attributable to the inhibition of the extracellular signal–regulated kinase and janus kinase/signal transducer and activator of transcription 3 signaling pathways but rather to the phosphatidylinositol

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Figure 6. Intimal neovessel density and atherosclerotic lesion formation are reduced in apolipoprotein E–deficient (ApoE⁻/⁻) matrix metalloproteinase (MMP) 2⁻/⁻ mice. A, Representative images of fat accumulation around aortas in ApoE⁻/⁻ MMP-2⁻/⁻ and ApoE⁻/⁻ MMP-2⁻/⁻ mice. A indicates atheroma; M, media. Scale bar: 50 μm. B through E, Representative images of immunostaining and combined quantitative data for neovessel density (B and D) and lipid content (C and E) in aortic roots. The neovessel density (D, n=6) and lipid content (E, n=5) were analyzed in aortic roots by staining with fluorescein isothiocyanate–labeled lectin antibody and oil red O in the intima of each section. Results are expressed as plaque-based neovessel density (in millimeters squared) and oil-red O–positive area (in millimeters squared). Macrophages were identified in aortic roots by immunostaining with a Mac-3 antibody. F, Macrophage content was quantified in the intima of each section, and the results are expressed as the Mac-3–positive area (in millimeters squared). G, Real-time PCR to detect toll-like receptor (TLR) 2 and TLR4 mRNA levels in the aortic roots (n=4).
Recent evidence indicates that SDF-1/CXCR4 is expressed in human atherosclerotic plaque, whereas high plasma levels are clinically associated with plaque stability.32 SDF-1/CXCR4 has been shown to modulate recruitment of hematopoietic cells and angiogenesis.33 The authors of a previous study reported that SDF-1/CXCR4 plays an instrumental role in neointimal formation in response to injury via the recruitment of circulating SMC progenitor cells.32 Our observations here show that AT1R antagonism reduced the expression of SDF-1 and CXCR4 mRNAs in the aortic atherosclerotic plaques. We have also observed in vitro that AT1R antagonism attenuated bone marrow–derived EPC-like c-Kit+ cell functions. These findings raise the possibility that the beneficial effects of AT1R antagonism are likely attributable, in part, to the attenuation of bone-marrow–derived vascular progenitor cell mobilization induced by SDF-1α/CXCR4 signaling pathway activation. This notion is further supported by the recent study from Zernecke et al34 that the SDF-1α/CXCR4 axis is pivotal for vascular remodeling by recruiting a subset of SMC progenitor cells in response to apoptosis in an animal model.

**Clinical Perspectives**

Pathological neovascularization occurs concurrently with the development of atherosclerotic plaque and must be considered when devising therapeutic strategies to treat atherosclerotic disease. Our findings show a cross-talk between the AT II and the TLR signaling pathways in cardiovascular cells in the neovascularization associated with atherogenesis. During the early stages of atherosclerosis, AT1R antagonism decreases the intimal neovessel density and lessens the growth and destabilization of the atherogenic plaques. Inhibition of neovascularization resulting from a reduction in TLR-mediated inflammatory action and MMP expression and activity is possibly a key mechanism for stabilizing atherosclerotic plaques that might underlie the decreased mortality observed in patients who have established atherosclerotic disease and are treated with AT1R antagonists.28,35

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**Disclosures**

None.
References


19. Cheng et al ARB Inhibits TLR Expression and Angiogenesis

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MATERIALS AND METHODS

Animals and treatment
Male ApoE⁻/⁻ mice (C57BL/6 genetic background) were purchased from The Jackson Laboratory (Bar Harbor, Maine). Until required for experiments, the mice were provided with a standard diet (Oriental Yeast) and tap water *ad libitum*. For experiments, ten-week-old male ApoE⁻/⁻ mice were fed a Western-type diet¹ containing 21% fat from lard and 0.15% cholesterol and were assigned to either the control group (*n* = 26) or the olmesartan group (*n* = 27). The control group was administered 0.5% carboxymethylcellulose, whereas the olmesartan group was administered olmesartan (donated by Daiichi-Sankyo Pharmaceutical Co.) at 3 mg/kg body weight per day, in chow. The dose of olmesartan was established by reference to a previous report.² Systemic blood pressure (BP) and heart rate were determined by using a tail-cuff pressure analysis system (Softron BP-98A, Softron) in conscious mice. After 12 weeks of consuming a Western-type diet, mice underwent trial recordings to warm up, and then three reliable recordings were taken and used for the determination of BP and heart rate. Mice were heavily sedated with avertin, and blood was collected from the inferior vena for biological analysis.

In separate experiments, MMP-2-deficient mice (MMP-2⁻/⁻) on a C57BL/6 background (kindly given by S. Itohara³) were crossed with ApoE⁻/⁻ mice to generate ApoE⁻/⁻MMP-2⁻/⁻ mice. Male mice from both genetic backgrounds (*n* = 6 for each group) consumed an atherogenic Western-type diet from 10 weeks of age for 12 weeks to allow the development of atherosclerotic lesions until sacrifice. In separate experiments, olmesartan (3 mg/kg daily; *n* = 5) or vehicle (0.5% carboxymethylcellulose; *n* = 4) was administered orally to ApoE⁻/⁻MMP-2⁻/⁻ mice until sacrifice by supplementation of the Western-type diet. All animal studies were conducted in accordance with the animal care guidelines of Nagoya University Graduate School of Medicine.

Preparation and quantification of atherosclerotic lesions
Mice were euthanized by intraperitoneal injection of pentobarbital, then perfused with phosphate buffered saline (PBS) under physiological pressure. The entire mouse aorta together with the heart was dissected from the proximal ascending aorta to the bifurcation of the iliac artery by using a dissecting microscope (Figure S1A). Following removing of adventitial fat, the entire aorta connected together with heart was isolated and separated into the aortic root, thoracic aorta, and abdominal aorta and stored at −80°C for biological analyses, or fixed with 4% paraformaldehyde for 16 h (4°C) and subsequently embedded in paraffin for pathological analysis. For oil red O staining,
some aortic tissues were snap-frozen in OCT compound (Tokyo, Japan).

The cross-sections of the aortic root were analyzed according to the modified method of Matsumoto et al. Each heart was cut in a plane between the lower tips of the right and left atria. The upper portion was embedded in paraffin. Then, the aortic root was sectioned (3-μm) serially at 5-μm intervals from the start of the aortic valve to the start of the ascending aorta until the valve cusps were no longer visible. The thoracic and abdominal aortas were sectioned (3-μm) serially at 45-μm intervals. Five cross-sections of vessels in each aortic tissue (Figure S1B) were quantified for neointima, media, and internal elastin length, and the results for each mouse were averaged. All morphometric analyses were made on hematoxylin and eosin (H&E) stained sections.

**Quantitative real-time polymerase chain reaction (PCR)**

Total RNA was isolated from the aortic tissues and the lysates from macrophages with the use of an RNeasy Fibrous Tissue Mini-Kit (Qiagen Inc) and was subjected to reverse transcription. The resulting cDNA was subjected to quantitative real-time PCR analysis with primers specific for MMP-2, MMP-9, TLR-2, TLR-4, stromal-derived factor-1 (SDF-1), and a CXC chemokine receptor (CXCR4) and with the use of an ABI 7300 Real-Time PCR System (Applied Biosystems), as previously described. The primer sequences for MMP-2 and MMP-9 were described previously. The remaining primers were purchased from AB Applied Biosystems (Gene expression assay IDs: Mn00442346_ml, Mn00445273_ml, and Mn00445553_mL, and Mn01292123_mL). The expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was measured in parallel with that of the genes of interest and was used as an internal standard for quantitative comparison of mRNA levels.

**Histological characterization of atherosclerotic lesions in the aortic root**

Paraffin sections (3 μm) from aortic roots were deparaffinized in xylene, rehydrated with decreasing alcohol concentrations and stained routinely with H&E, Elastica van Gieson (EVG) staining for elastin, and picrosirius red (PSR) for collagen. Corresponding sections on separate slides were immunostained with goat polyclonal antibodies against MMP-2 (1:100, Fuji Chemical Co) and MMP-9 (1:100, Chemicon), rabbit polyclonal antibodies against α-SMC actin (ASMA; 1:100, Neo Marker), rat monoclonal antibodies against macrophages (Mac 3; 1:40, BD Pharmingen), and mouse monoclonal antibodies against monocyte chemoattractant protein-1 (MCP-1; 1:100, Neo Marker) and osteopontin (1:100, Sigma-Aldrich). The sections were preincubated with 5% normal serum and then incubated with primary antibodies for 16 h at 4°C. The sections were then reacted with an alkaline phosphatase (AP)-conjugated secondary
antibody against goat, rabbit, or mouse IgG (1:200, all from Vector Laboratories) for 2 h at 4°C. These sections were visualized with an AP substrate kit (Vector Laboratories) in accordance with the manufacturer’s instructions. Levamisole (Vector Laboratories) was used as an inhibitor of endogenous AP. Oil red O staining was performed to assess the lipid content of atherosclerotic plaques in ApoE−/−MMP-2−/− mice. For the negative control, the primary antibody was replaced with nonimmune immunoglobulin G.

Collagen and elastin contents were evaluated using the PSR- and EVG-stained positive areas, respectively. Images of sections stained for collagen, elastin, MMP-2, MMP-9, Mac-3, ASMS, MCP-1, and osteopontin were analyzed with the use of WinROOF software version 5.0 (Mitani, Tokyo, Japan). Five cross-sections of vessels in each aorta were quantified and averaged for each animal. The results are reported as the percentage of the intima area that contained the lesions. We set a threshold to automatically compute the positively stained area for each antibody or histochemical stain and then computed the ratio (percent) of the positively stained area to the total cross-sectional vessel wall area or intimal plaque lesion area studied.

**Biochemical analysis**

The plasma concentration of Ang II was measured by radioimmunoassay (human NEX-105 [125]I-Try4-Angiotensin II, Perkin Elmer Life and Analytical Sciences). Plasma levels of tumor necrosis factor (TNF)-α and interleukin (IL)-1β were measured with commercially available kits. All assays were performed in triplicate.

**Gelatin zymography**

For gelatin zymography, 20 μg of aortic protein extract was mixed with SDS sample buffer without reducing agent and loaded onto a 10% SDS-polyacrylamide gel containing 1 mg/mL gelatin as previous described.

**Aortic ring culture for angiogenesis assay**

We performed the aortic ring assay for quantification of angiogenesis as previously described, with minor modifications. Briefly, the rings from the aortas of control and olmesartan-treated mice were inserted between two layers of type I collagen gel (BD Biosciences) and cultured in endothelial basal medium-2 (EBM-2) (Chambrex) in the presence or absence of VEGF (20 ng/mL, Genzyme/techne) and olmesartan (1 μmol/L) for 14 days. Microvascular sprouting was assessed by measuring the incorporation of 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate-labeled acetylated low-density lipoprotein (Dil-ac-LDL, 10 μg/mL, Molecular Probes, shown in Figure 4A right panels), which is selectively uptaken by microvessel endothelial cells. Quantitative analysis of endothelial sprouts at two randomly selected points at the edge of the aortic
ring was performed with WinROOF version 5.0 image processing software. Endothelial cell sprouting density was expressed as a percentage of pixels per image occupied by vessels within a defined area. Four separate aortic sections were quantified for each aorta, and the results were averaged for each animal.

**Endothelial progenitor cell (EPC)–like c-Kit+ cell isolation**

We obtained bone marrow–derived mononuclear cells from control and olmesartan-treated mice as previously described. Following isolation of mononuclear cell lineages, we isolated EPC–like c-Kit+ cells by using CD117 microbeads in MACS separation columns (Milteniyli Biotec GmbH) according to the manufacturer’s instructions. Isolated C-Kit+ BM cells were >90% positive for CD31 (Figure 5A). These cells also expressed the characteristic endothelial cell-surface markers flt-related receptor tyrosine kinase (flk-1), lectin, and the acetylated low density lipoprotein (acLDL) receptor (data not shown), and they were used for the gene expression, cell invasion, and tubulogenesis assays as previously described.

**EPC invasion and tube formation assays**

To investigate the effect of olmesartan on EPC function, invasion assays were performed used EPCs from ApoE−/− mice treated with or without olmesartan in 24-well tissue culture plates with Transwell membrane supports (Costar), as previously described. Cells that had invaded the outer side of the Transwell membrane were stained and counted in six to eight randomly chosen fields in duplicate chambers at a magnification of ×200 for each sample.

The tube formation assay was performed as described previously. EPCs (2.5 × 10^5 cells/well) were seeded into 12-well tissue culture plates pre-coated with Matrigel (BD Biosciences) and cultured overnight in endothelial basal medium-2 containing VEGF (20 ng/mL) to induce tube formation. Matrigel-induced tube formation was observed under a phase-contrast microscope (Olympus IIX 70, Tokyo Japan). WinRF version 5.0 image processing software (Mitani, Tokyo, Japan) was used to quantify the number of endothelial cell tubules in 5 fields (100×) in each well.

**Mouse endothelial cell (EC) culture and stimulations**

Male ApoE−/− mice (n = 3) aortic ECs were isolated as previously described. The ECs were cultured in endothelial basal medium-2 (EBM-2, Chambrex) plus 10% fetal bovine serum and EGM SingleQuotes (Clonetics). Positive 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate-labeled acetylated low-density lipoprotein (Dil-acLDL) uptake and CD31 expression indicated that there was no contamination of SMCs (Figure S2).
After being cultured in serum-free EBM-2 for 24 h, the ECs were used for the following experiments. First, the cells were treated for 24 h with and without several stimulators, including lipopolysaccharide (LPS, 50 pg/mL; Escherichia coli 026:B6, Sigma), Ang II (10⁻⁷ mol/L, Sigma-Aldrich), and recombinant mouse TNF-α (10 ng/mL; R&D Systems). The cells were then subjected to quantitative real-time PCR for examination of TLR gene expression. Second, to examine whether olmesartan had an effect on TLR expression, cells were pretreated with olmesartan (1 μmol/L) for 30 min, then cultured in presence or absence of LPS (50 pg/mL) and Ang II (10⁻⁷ mol/L) for 24 h and subjected to quantitative real-time PCR for examination of TLR gene expression. Third, to explore the mechanism of Ang II–induced MMP expression, macrophages were pretreated with or without several AT1R inhibitors, including olmesartan (1 μmol/L), the phosphatidylinositol-3-kinase (PI3K) inhibitor 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002, 20 μmol/L), the extracellular signal-regulated kinase (ERK) inhibitor 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene (U0126, 5 μmol/L), and the janus kinase (JAK)/STAT3 pathway inhibitor tyrphostin (AG490; 5 μmol/L; all 3 from Cell Signaling Technology, MA). Cells were then cultured in the presence or absence of Ang II (10⁻⁷ mol/L) for 24 h and subjected to quantitative PCR for examination of MMP gene expression. The ECs were also transfected with siTLR2 and siTLR4 (both 20 nmol/L; Invitrogen) for 24 h, and were then subjected to quantitative PCR for examination of MMP gene expression. For all cell culture assays, at least 3 independent experiments were performed in triplicate.

Statistical analysis
All measurements were conducted by two observers blinded to the treatment of the mice. Data were expressed as means ± standard error of the mean (SEM). The student’s t-test (for comparison between 2 groups) or 1-way ANOVA (for comparison of 3 or more groups) followed by Tukey’s post-hoc test were used for statistical analysis by employing SPSS software version 17.0 (SPSS Inc, Chicago, III). A P-value of <0.05 was considered statistically significant.

References
2. Tsuda M, Iwai M, Li JM, Li HS, Min LJ, Ide A, Okumura M, Suzuki J, Mogi M, Suzuki H, Horiuchi M. Inhibitory effects of AT1 receptor blocker, olmesartan,


**Table S1: Systolic blood pressure, plasma lipid content, and other parameters in ApoE<sup>−/−</sup> mice treated with and without olmesartan for 12 weeks**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>CONT</th>
<th>OLM</th>
<th>P value</th>
</tr>
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<tbody>
<tr>
<td>BW (g)</td>
<td>22</td>
<td>35.1 ± 2.0</td>
<td>23</td>
</tr>
<tr>
<td>BP (mm Hg)</td>
<td>22</td>
<td>90.3 ± 2.4</td>
<td>23</td>
</tr>
<tr>
<td>HR (bpm)</td>
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</tr>
<tr>
<td>TG (mg/dl)</td>
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<td>102.9 ± 9.3</td>
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</tr>
<tr>
<td>TCh (mg/dl)</td>
<td>11</td>
<td>554.9 ± 20.1</td>
<td>12</td>
</tr>
<tr>
<td>HDL (mg/dl)</td>
<td>11</td>
<td>23.8 ± 2.0</td>
<td>10</td>
</tr>
<tr>
<td>TNF-α (pg/dl)</td>
<td>9</td>
<td>102.5 ± 12.3</td>
<td>11</td>
</tr>
<tr>
<td>IL-1β (ng/dl)</td>
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<td>13</td>
</tr>
<tr>
<td>Ang II (pg/mL)</td>
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<td>201.2 ± 23.1</td>
<td>8</td>
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</table>

BW: body weight; BP: blood pressure; HR, heart rate; TG: triglycerides; TCh: total cholesterol; HDL: high density lipoprotein; TNF-α, tumor necrosis factor-α; IL-1β, interleukin-1α. Data are means ± SEM. P < 0.05 was considered statistically significant.
ARB inhibits TLR expression and angiogenesis

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**Figure S1:**
A. Representative images of the whole aorta together with the heart in control (aortas stain primrose-yellow) and olmesartan-treated ApoE−/− mice (aortas stain saffron-yellow).
B. Image shows locations of serial sections made of atherosclerotic lesions and components in the aortic root as well as in the thoracic and abdominal aorta.
Figure S2: Aorta-derived EC expressed surface markers (PECAM/CD31 and Dil-acLDL receptor). Following isolation from the aorta of ApoE−/− mice, the ECs were cultured in EGM-2 for 7 days and identified as positive cells to CD31 (green) and Dil-acLDL uptake (red) reactive. Bar = 100 μm.
Figure S3: MMP-2 and MMP-9 expression and characterization in aortic roots. (A and B) Representative images of immunostaining and combined quantitative data for expression of MMP-2 and MMP-9 proteins are shown. MMP-2 and MMP-9 proteins were immunostained with goat polyclonal antibodies against MMP-2 or MMP-9, respectively. MMP-2- and MMP-9-positive areas in atherosclerotic intima lesions in the aortic roots of control (CONT, n = 6) and olmesartan-treated mice (OLM, n = 7) were quantified for each section. Results are expressed as the ratio (percent) of the positively stained area to the neointimal area. Values are means ± SEM. Scale bars: 50 μm. P < 0.05 was considered statistically significant (Student’s t-test).