Deletion of the Angiotensin II Type 1a Receptor Prevents Atherosclerotic Plaque Rupture in Apolipoprotein E−/− Mice

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Objective—Angiotensin II is involved in the genesis of atherosclerosis. As the role of the angiotensin II type 1a (AT1a) receptor in plaque rupture is poorly understood, we assessed the hypothesis that the AT1a receptor contributes to atherosclerotic plaque rupture.

Methods and Results—Atherosclerotic plaque rupture was induced by carotid artery ligation for 4 weeks followed by polyethylene cuff placement around the carotid in apolipoprotein E (ApoE)−/− and ApoE−/− AT1a−/− mice. The incidence of plaque rupture at 4 days after cuff placement was 72% in ApoE−/− mice compared with 24% in ApoE−/− AT1a−/− mice (P<0.01). Lipid accumulation, macrophage infiltration, expression of inflammatory cytokines, nicotinamide adenine dinucleotide phosphate-oxidase activity, and matrix metalloproteinase-9 activity in atherosclerotic plaque were markedly attenuated in ApoE−/− AT1a−/− compared with ApoE−/− mice. Oxidized low-density lipoprotein inhibited macrophage migration in ApoE−/− macrophages. In contrast, oxidized low-density lipoprotein-induced macrophage trapping was abolished in ApoE−/− AT1a−/− macrophages, and this was associated with decreased CD36 expression and focal adhesion kinase activity.

Conclusion—These results suggest that blocking the AT1 receptor may reduce atherosclerotic plaque rupture and that AT1a receptor-mediated macrophage trapping, inflammation, oxidative stress, and matrix metalloproteinase activation may play crucial roles in plaque vulnerability. (Arterioscler Thromb Vasc Biol. 2012;32:1453-1459.)

Key Words: angiotensin II ■ lipids ■ macrophage ■ metalloproteinases ■ plaque rupture

Atherosclerotic plaque rupture and subsequent thrombus formation in the culprit lesion are recognized to be the seminal events in the majority of cases of acute coronary syndrome. Plaque stability is determined by many factors, including lipid deposits, oxidative stress, inflammation, and extracellular matrix degradation. Accumulation of lipid in the atherosclerotic lesion increases mechanical stress within the fibrous cap. In addition, modification of low-density lipoprotein (LDL) by oxidative stress results in the avid uptake of these lipoproteins via infiltrating macrophages, which induces macrophage activation and foam cell formation. The activation of macrophages in atherosclerotic plaques leads to the secretion of inflammatory cytokines and proteolytic enzymes capable of degrading the extracellular matrix, and these changes consequently increase plaque vulnerability.

Angiotensin II (Ang II) is well known to mediate cardiovascular disease pathogenesis by regulating oxidative stress, inflammation, and cell proliferation. We previously demonstrated that the angiotensin II type 1a (AT1a) receptor enhances neointimal formation after cuff-induced vascular injury as well as high-fat diet–induced atherosclerotic lesion formation in apolipoprotein E (ApoE)−/− mice, and that the AT2 receptor counterregulates AT1a receptor-mediated lesion formation. However, the role of the AT1a receptor in atherosclerotic plaque rupture is poorly understood. In a previous study, we demonstrated that expression of the AT1 receptor in atherosclerotic lesions was increased by feeding a high-cholesterol diet to ApoE−/− mice. Ang II receptor blockade significantly inhibited lipid accumulation and oxidative stress in the atherosclerotic lesions of ApoE−/− mice fed a high-cholesterol diet. Moreover, Ang II receptor deletion prevents vascular oxidative stress, endothelial dysfunction and atherosclerotic lesion formation in ApoE−/− mice, irrespective of blood pressure and plasma cholesterol levels. These results suggest that the AT1a receptor may play a critical role in atherosclerotic plaque vulnerability.

Several models of plaque rupture in ApoE−/− mice have been used for investigating the mechanisms responsible for plaque vulnerability. These models have included chow feeding for about a year,13 high-fat feeding for about 2 months,14 and cuff placement and subsequent adenovirus-mediated transfer of p53. Recently, carotid artery ligation and cuff placement in ApoE−/− mice was shown to be a simple and highly efficient method of inducing plaque rupture. This
plaque rupture model partially overlaps with human disease as cracks in the neointima and thrombus formation were consistently observed in the section of the carotid artery surrounded by the cuff.

In this study, we tested the hypothesis that the AT$_1$ receptor contributes to plaque vulnerability by analyzing the incidence of plaque rupture after carotid artery ligation and cuff placement in ApoE$^{-/-}$, ApoE$^{-/-}$ AT$_{1a}$, ApoE$^{-/-}$ AT$_{1a}^{-/-}$, and ApoE$^{-/-}$ mice treated with valsartan. We showed that deletion of the AT$_1$ receptor as well as treatment with valsartan prevented plaque rupture in ApoE$^{-/-}$ mice, and this was associated with decreased lipid accumulation, macrophage infiltration, oxidative stress, and matrix metalloproteinase (MMP) activity. Moreover, we showed that the AT$_1$ receptor modulates CD36-dependent macrophage migration.

**Materials and Methods**

An expanded Materials and Methods section is available in the online-only Data Supplement.

**Animals**

All animal studies were approved by the Animal Studies Committee of Ehime University. Male ApoE$^{-/-}$ mice were purchased from Jackson Laboratory (Bar Harbor, ME). ApoE$^{-/-}$ AT$_{1a}$ with the same C57BL/6J genetic background were generated by crossing ApoE$^{-/-}$ and AT$_{1a}$ mice (donated by Tanabe Seiyaku Co. Ltd., Osaka, Japan). ApoE$^{-/-}$ AT$_{1a}$ mice were generated by crossing ApoE$^{-/-}$ and ApoE$^{-/-}$ AT$_{1a}^{-/-}$ mice. During the experimental period, they were provided standard chow (MF, Oriental Yeast Co. Ltd., Tokyo, Japan) and water ad libitum.

**Murine Model of Atherosclerotic Plaque Rupture**

We generated a murine model of atherosclerotic plaque rupture as previously reported. In brief, 9-week-old mice were anesthetized with an intraperitoneal injection of pentobarbital sodium (50 mg/kg), and then their common carotid artery was ligated just proximal to the bifurcation. Four weeks after ligation, a polyethylene cuff (length 2 mm; inside and outside diameter of 0.580 mm and 0.965 mm, respectively; IMI Isamura Co. Ltd., Tokyo, Japan) was placed around the common carotid artery, just proximal to the ligated site. The intracuff lesions were evaluated at 4 days after cuff placement. Valsartan (purchased from Tokyo Chemical Industry Co. Ltd., Tokyo, Japan; 1mg/kg per day) was administered intraperitoneally via an implanted osmotic minipump starting 1 week before cuff placement.

**Statistical Analysis**

All values for continuous variables are expressed as the mean±SEM and represent data from at least 3 independent experiments. Values for categorical variables are expressed as the number of animals or incidence of events. For continuous variables, groups were compared with a 1-way ANOVA followed by Scheffe post hoc test. For categorical variables, groups were compared with a $\chi^2$ test. A value of $P<0.05$ was considered statistically significant.

**Results**

**Incidence of Atherosclerotic Plaque Rupture Was Reduced in ApoE$^{-/-}$ AT$_{1a}^{-/-}$ and ApoE$^{-/-}$ Mice Treated With Valsartan**

To test the hypothesis that the AT$_1$ receptor plays a crucial role in atherosclerotic plaque rupture, we performed carotid artery ligation and subsequent cuff placement in ApoE$^{-/-}$, ApoE$^{-/-}$ AT$_{1a}^{-/-}$, ApoE$^{-/-}$ AT$_{1a}^{-/-}$, and ApoE$^{-/-}$ mice treated with valsartan. Reproducible neointimal formation was induced by carotid artery ligation for 4 weeks, but no intraplaque hemorrhage or plaque rupture was observed. At 4 days after cuff placement, a variety of morphological changes including intraplaque hemorrhage, and plaque rupture with mural or occlusive thrombus were observed in both strains of mice (Figure 1A–1D). The incidence of plaque rupture with thrombus was significantly reduced in ApoE$^{-/-}$ AT$_{1a}^{-/-}$ mice compared with ApoE$^{-/-}$ mice (72% versus 24%, respectively; $P<0.01$; Figure 1E). Similarly, valsartan significantly reduced plaque rupture in ApoE$^{-/-}$ mice from 72% to 25% ($P<0.01$; Figure 1E). There was no significant difference in the incidence of plaque rupture between ApoE$^{-/-}$ and ApoE$^{-/-}$ AT$_{1a}^{-/-}$ mice (72% versus 50%, respectively; Figure 1E).

**Lipid Accumulation and Macrophage Infiltration in the Carotid Artery Are Attenuated in ApoE$^{-/-}$ AT$_{1a}^{-/-}$ and ApoE$^{-/-}$ Mice Treated With Valsartan**

Because high lipid content and increased numbers of inflammatory cells are implicated in plaque vulnerability, we next examined lipid accumulation and macrophage infiltration into plaques. There were no significant differences in plasma total cholesterol and LDL cholesterol levels between ApoE$^{-/-}$ and ApoE$^{-/-}$ AT$_{1a}^{-/-}$ mice (data not shown). The expression of AT$_1$ receptors was examined in the carotid arteries by immunohistochemical staining before and after ligation, and cuff placement, and AT$_1$ receptor expression increased to the same extent in both ApoE$^{-/-}$ and ApoE$^{-/-}$ AT$_{1a}^{-/-}$ mice (Figure I in the online-only Data Supplement). To visualize lipid-rich atherosclerotic plaques, frozen sections of the aortas were stained with oil red O. At 4 days after cuff placement, abundant lipid accumulation in plaques and infiltration of F4/80-positive macrophages were observed in ApoE$^{-/-}$ mice (Figure 2A and 2D). In contrast, these changes were clearly attenuated in ApoE$^{-/-}$ AT$_{1a}^{-/-}$ mice (Figure 2B and 2E) and ApoE$^{-/-}$ mice treated with valsartan (Figure 2C and 2F). Because inflammation is central at all stages of atherosclerosis and inflammatory cytokines involved in atherogenesis, we examined the expression of proinflammatory cytokines in the carotid lesions to determine the role of the AT$_1$ receptor in mediating inflammation in rupture-prone plaques. The protein expression of both tumor necrosis factor-$\alpha$ and interleukin 6 was significantly attenuated in ApoE$^{-/-}$ AT$_{1a}^{-/-}$ and ApoE$^{-/-}$ mice treated with valsartan compared with ApoE$^{-/-}$ mice (Figure II in the online-only Data Supplement). These results suggest that the AT$_1$ receptor blockade contributes to atherosclerotic plaque stability by abolishment of lipid accumulation, macrophage infiltration, and inflammation.

**Superoxide Anion Production, Nicotinamide Adenine Dinucleotide Phosphate Oxidase Activity and MMP Activity in the Carotid Artery Are Attenuated in ApoE$^{-/-}$ AT$_{1a}^{-/-}$ and ApoE$^{-/-}$ Mice Treated With Valsartan**

Superoxide anion generated by lipid-laden macrophages has been shown to induce expression of matrix-degrading proteases, including MMP-2 and MMP-9 that directly contribute to the instability of atherosclerotic plaques. Therefore, we next examined superoxide anion production, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity, and MMP
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**Figure 1.** Deletion and pharmacological blockade of the angiotensin II type 1a (AT<sub>1a</sub>) receptor attenuate lipid accumulation and macrophage infiltration into the plaque. Representative cross sections of the carotid artery stained with hematoxylin-eosin (X100) before (A) and after (B-D) ligation and cuff placement (scale bar=50 µm). Ligation just proximal to the bifurcation of the common carotid artery was performed in 9-week-old apolipoprotein E (ApoE)<sup>−/−</sup>, ApoE<sup>−/−</sup> AT<sub>1a</sub><sup>−/−</sup>, ApoE<sup>−/−</sup> AT<sub>1a</sub><sup>−/+</sup>, and ApoE<sup>−/−</sup> mice treated with valsartan (Val). Four weeks after ligation, the common carotid artery just proximal to the ligated site was wrapped with a polyethylene cuff. Four days after cuff placement, intracuff lesions were analyzed. A variety of morphological changes including no rupture (B), intraplaque hemorrhage (C), and plaque rupture with thrombus (D) were observed in both strains of mice. The arrows indicate intraplaque hemorrhage. The arrowhead indicates neointimal disruption. A bar graph (E) shows the incidence of plaque rupture (n=21-25 for each group). *P<0.01 versus ApoE<sup>−/−</sup>. Values are means±SEM.

**Figure 2.** Deletion and pharmacological blocking of angiotensin II type 1a (AT<sub>1a</sub>) receptor attenuate lipid accumulation and macrophage infiltration into the plaque. Representative fresh-frozen cross sections with oil red O staining of the carotid artery in apolipoprotein E (ApoE)<sup>−/−</sup> (X100) (A), ApoE<sup>−/−</sup> AT<sub>1a</sub><sup>−/−</sup> (B), and ApoE<sup>−/−</sup> mice treated with valsartan (C). Representative immunohistochemical staining of fresh-frozen cross sections of the carotid artery with F4/80 antibody in ApoE<sup>−/−</sup> (D), ApoE<sup>−/−</sup> AT<sub>1a</sub><sup>−/−</sup> (E), and ApoE<sup>−/−</sup> mice treated with valsartan (F). Sections were counterstained with hematoxylin-eosin. Bar=50 µm.

ApoE<sup>−/−</sup> mice (Figure III in the online-only Data Supplement). Active and proform MMP-2 levels tended to be lower in ApoE<sup>−/−</sup> AT<sub>1a</sub><sup>−/−</sup> and ApoE<sup>−/−</sup> mice treated with valsartan compared with ApoE<sup>−/−</sup> mice, but the difference was not statistically significant (Figure III in the online-only Data Supplement). These results suggest that the AT<sub>1a</sub> receptor is crucial for superoxide anion production, NADPH oxidase activation, and MMP-9 activation.

**Deletion of the AT<sub>1a</sub> Receptor Prevents Oxidized LDL-Induced Macrophage Trapping**

Excessive uptake of lipid-rich particles by scavenger receptors results in transformation of macrophages into foam cells, and the trapping of the cells in the atherosclerotic plaque is a critical step in atherosclerosis. An increased number of monocyte-derived cells in progressive atherosclerotic plaques is associated not only with robust recruitment of monocytes into lesions, but also with a depressed rate of their migratory clearance from the lesions. To investigate the mechanism by which AT<sub>1a</sub> receptor deficiency decreases macrophage infiltration into carotid lesions (Figure 2E), we next examined the migration activity of mouse peritoneal macrophages. We harvested resident (not thioglycolate-elicited) mouse peritoneal macrophages to avoid basal activation. Macrophage migration was assayed by a modified Boyden chamber method, and this assay revealed that ApoE<sup>−/−</sup> AT<sub>1a</sub><sup>−/−</sup> macrophages migrated to the same extent as ApoE<sup>−/−</sup> macrophages under basal conditions (Figure 5). Furthermore, monocyte chemoattractant protein-1 (10 ng/mL) enhanced migration of ApoE<sup>−/−</sup> and ApoE<sup>−/−</sup> AT<sub>1a</sub><sup>−/−</sup> macrophages to the same degree (Figure 5). We next confirmed the observation of Park et al<sup>20</sup> that oxidized LDL induces macrophage trapping by showing that oxidized LDL significantly inhibited monocyte chemoattractant protein-1–induced macrophage migration in ApoE<sup>−/−</sup> mice (Figure 5). In contrast, the inhibition of macrophage migration by oxidized LDL (50 µg/mL) was completely abolished in ApoE<sup>−/−</sup> AT<sub>1a</sub><sup>−/−</sup> mice.
Deletion of the AT₁R Receptor Decreases Oxidized LDL-Induced CD36 Expression, Focal Adhesion Kinase, and c-Jun-N-Terminal Kinase Activation

CD36, a member of the scavenger receptor class B family with a capacity to bind oxidized LDL as well as various other ligands, has been shown to transduce signals that regulate inflammatory responses in macrophages. Oxidized LDL can stimulate its own uptake by induction of CD36 gene expression. Moreover, CD36 signaling in response to oxidized LDL alters cytoskeletal dynamics to inhibit macrophage migration. To investigate the mechanism by which AT₁a receptor deficiency inhibits oxidized LDL-induced macrophage trapping, we next examined the time course of CD36 protein expression after oxidized LDL stimulation in ApoE⁻/⁻ and ApoE⁻/⁻ AT₁a⁻/⁻ resident mouse peritoneal macrophages by Western blot analysis. Enhanced CD36 expression was observed following 6 hours of exposure to oxidized LDL (50 µg/mL) with abundant expression at 8 hours in ApoE⁻/⁻ macrophages, whereas the CD36 protein level did not change in ApoE⁻/⁻ AT₁a⁻/⁻ macrophages (Figure 6). The mechanism of oxidized LDL-induced macrophage trapping has been postulated to involve both CD36- and Src kinase-dependent direct activation of focal adhesion kinase (FAK) and indirect activation of FAK by Src homology 2 domain-containing phosphotyrosine phosphatase (SHP-2) inactivation via CD36-mediated reactive oxygen species. To investigate the effect of AT₁a receptor deficiency for activation of FAK and inhibition of SHP-2, we examined the time course of FAK and SHP-2 phosphorylation after oxidized LDL stimulation in ApoE⁻/⁻ and ApoE⁻/⁻ AT₁a⁻/⁻ resident mouse peritoneal macrophages. FAK phosphorylation was significantly attenuated in ApoE⁻/⁻ AT₁a⁻/⁻ macrophages compared with ApoE⁻/⁻ macrophages (Figure VA and VB in the online-only Data Supplement). Phosphorylation of Tyr-580 of SHP-2 was detected by dihydroethidium (DHE; 10 µmol/L). A bar graph (D) shows the intensity of fluorescence analyzed and quantified using imaging software for densitometry (n=7–8 for each group). P<0.05 versus ApoE⁻/⁻. Values are means±SEM.

Figure 4. Deletion and pharmacological blockade of the angiotensin II type 1a (AT₁a) receptor attenuate the activity of matrix metalloproteinases (MMPs). Fresh-frozen cross sections of the intracuff lesion were prepared and gelatinolytic activity in apolipoprotein E (ApoE)⁻/⁻ (A), ApoE⁻/⁻ AT₁a⁻/⁻ (B), and ApoE⁻/⁻ mice treated with valsartan (C) was detected by DQ-Gelatin (Invitrogen, Carlsbad, CA). A bar graph (D) shows the intensity of fluorescence analyzed and quantified using imaging software for densitometry (n=7–8 for each group). P<0.01 versus ApoE⁻/⁻; †P<0.05 versus ApoE⁻/⁻. Values are means±SEM.

Figure 3. Deletion and pharmacological blockade of the angiotensin II type 1a (AT₁a) receptor attenuate superoxide production and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity. Fresh-frozen sections of the carotid lesions were prepared and superoxide anion production in apolipoprotein E (ApoE)⁻/⁻ (A), ApoE⁻/⁻ AT₁a⁻/⁻ (B), and ApoE⁻/⁻ mice treated with valsartan (C) was detected by dihydroethidium (DHE; 10 µmol/L). A bar graph (D) shows the intensity of fluorescence analyzed and quantified using imaging software for densitometry (n=5–6 for each group). A bar graph (E) shows changes in NADPH oxidase activity in the carotid lesion of ApoE⁻/⁻, ApoE⁻/⁻ AT₁a⁻/⁻, and ApoE⁻/⁻ mice treated with valsartan. Protein samples were prepared, and NADPH oxidase activity was measured, as described in the Materials and Methods. The data were obtained from 3 independent experiments. P<0.01 versus ApoE⁻/⁻; †P<0.05 versus ApoE⁻/⁻. Values are means±SEM.
Deletion of angiotensin II type 1a (AT1a) receptor abolishes oxidized low-density lipoprotein (LDL)-induced macrophage trapping. Resident peritoneal macrophages from apolipoprotein E (ApoE)−/− (A) and ApoE−/− AT1a−/− (B) mice were added to the upper chamber of the transwell with or without 50 µg/mL of oxidized LDL. Medium alone or medium containing 10 ng/mL of monocyte chemoattractant protein (MCP)-1 was added in the lower chamber and incubated for 24 hours at 37°C. Migratory cells on the lower side of filter were stained with Giemsa (X100). Bar=100 µm. The number of migratory cells was counted under a microscope (C).

With ApoE−/− macrophages, but the difference was not statistically significant (Figure VC and VD in the online-only Data Supplement). These results suggest that CD36- and Src kinase-dependent direct activation of FAK is a dominant pathway for oxidized LDL-induced macrophage trapping in our experimental model. C-Jun N-terminal kinase (JNK) 2 is a key downstream kinase of CD36-dependent signaling. It has been reported that exposing peritoneal macrophages from wild type but not CD36−/− mice to oxidized LDL leads to activation of JNK2 and that pharmacological blockade of JNK inhibits CD36-dependent foam cell formation in vitro and in vivo. Therefore, we also examined the effect of AT1a receptor deficiency on oxidized LDL-induced JNK2 activation in resident mouse peritoneal macrophages. As shown in Figure VI in the online-only Data Supplement, phosphorylation of JNK2 was observed following 10 minutes of exposure to oxidized LDL (50 µg/mL) and was sustained for 60 minutes in ApoE−/− macrophages. In contrast, oxidized LDL-induced phosphorylation of JNK2 was significantly attenuated in ApoE−/− AT1a−/− macrophages. Moreover, we confirmed that the phosphorylation of JNK2 in the carotid lesions was also significantly attenuated in ApoE−/− AT1a−/− mice as compared with ApoE−/− mice (Figure VII in the online-only Data Supplement). The lipoygenase (LO) pathway is involved in LDL oxidation and the pathogenesis of atherosclerosis. Angiotensin II has been shown to upregulate 12-LO activity and expression in human aortic smooth muscle cells. Moreover, it has been reported that the LO pathway mediates AngII-induced JNK activation and impairs the macrophage ATP-binding cassette transporter G1 for cholesterol efflux.

In this study, expression of 12/15 LO protein in the carotid lesions tends to be lower in ApoE−/− AT1a−/− mice compared with ApoE−/− mice, but the difference was not statistically significant (Figure VIII in the online-only Data Supplement). The expression of ATP-binding cassette transporter G1 also did not significantly differ between ApoE−/− and ApoE−/− AT1a−/− mice (Figure IX in the online-only Data Supplement). On the other hand, expression of the ATP-binding cassette transporter A1 (ABCA1) in carotid lesions was significantly increased in ApoE−/− AT1a−/− mice compared with ApoE−/− mice (Figure IX in the online-only Data Supplement). Taken together, these results strongly suggest that the AT1a receptor plays a crucial role in oxidized LDL-induced CD36 upregulation, and FAK and JNK2 activation. These changes may cause the transformation of macrophages into foam cells and the trapping of lipid-laden macrophages in atherosclerotic plaques.

**Discussion**

The major findings of this study were that AT1a receptor deficiency and AT1a receptor pharmacological blockade in vivo significantly reduced the incidence of atherosclerotic plaque rupture. We showed that the inhibitory effect of the AT1a receptor on plaque rupture was associated with attenuation of lipid accumulation, macrophage infiltration, inflammation, superoxide anion production, NADPH oxidase activation, and MMP activation in the lesion. We also showed that inhibition of CD36 expression, and FAK and JNK activation are important mechanisms by which AT1a receptor deficiency attenuates macrophage burden, macrophage trapping into the
atherosclerotic plaque and foam cell formation. To our knowledge, these findings are the first to demonstrate that AT1a receptor deficiency can prevent atherosclerotic plaque rupture.

Two large randomized clinical trials have shown that angiotensin-converting enzyme inhibition or Ang II receptor blockade reduces the risk of major events including cardiovascular death, myocardial infarction, or stroke.30,31 However, few studies have investigated the effect of the AT1a receptor on atherosclerotic plaque rupture. In a previous study, we showed that Ang II receptor blockade significantly inhibited lipid accumulation and oxidative stress in atherosclerotic lesions in ApoE–/– mice fed a high-cholesterol diet, suggesting that AT1a receptor-mediated signaling enhances plaque vulnerability. Moreover, Ang II receptor blockade with irbesartan has been reported to decrease inflammation and MMP expression in human carotid plaques.32 These observations led us to study the effect of AT1a receptor deficiency on atherosclerotic plaque rupture.

MMPs can degrade the major components of the vascular extracellular matrix including the fibrous cap of atherosclerotic plaque. Activation of MMPs by monocyte/macrophages could therefore promote plaque instability. Ang II has been reported to increase the activity of MMPs through AT1a receptor stimulation.33 Because reactive oxygen species enhance matrix degradation by activating type IV collagenases, oxidative stress might be involved in the mechanism of activation of MMPs by the AT1a receptor. Indeed, we have previously reported that AT1a receptor stimulation by Ang II induces oxidative stress through phosphorylation of p47phox and translocation of p47phox and Rac1 to the plasma membrane fraction.34 In the present study, we showed that superoxide anion production, NADPH oxidase activity and the activity of type IV collagenases in carotid lesions were significantly attenuated in ApoE–/– AT1a−/− and ApoE–/– mice treated with valsartan compared with ApoE–/– mice. These results suggest that AT1a receptor blockade contributes to plaque stability in part by attenuating oxidative stress and activation of MMPs.

Macrophage CD36 has been shown to transduce signals that regulate inflammatory responses and foam cell formation by mediating macrophage migration and the uptake of oxidized LDL.25,34,35 CD36 signaling in response to oxidized LDL has also been shown to alter cytoskeletal dynamics to inhibit macrophage migration.21 Thus, CD36 plays a critical role in oxidized LDL-induced macrophage trapping in atherosclerotic lesions, foam cell formation, and inflammatory responses. To understand the mechanism by which AT1a receptor deficiency attenuates macrophage infiltration into the lesion, we performed a macrophage migration assay. Consistent with a previous report,21 we showed that monocyte chemotactant protein-1–induced migration was significantly inhibited by oxidized LDL in ApoE–/– macrophages. Interestingly, the inhibitory effect of oxidized LDL on migration was completely abolished in ApoE–/– AT1a−/− macrophages. In addition, we showed that oxidized LDL-induced CD36 expression, FAK, and JNK2 phosphorylation were significantly attenuated in ApoE–/– AT1a−/− macrophages compared with ApoE–/– macrophages. These results suggest that AT1a receptor deficiency contributes to the inhibition of foam cell formation as well as macrophage emigration from lesions by attenuating CD36 expression, FAK, and JNK2 activation. Recently, deletion of the ATP-binding cassette transporters ABCA1 and ATP-binding cassette transporter G1 has been reported to impair macrophage migration through increased Rac1 signaling.36 We confirmed that ABCA1 protein expression in carotid lesions was significantly increased in ApoE–/– AT1a−/− mice compared with ApoE–/– mice. Indeed, Ang II has been shown to transcriptionally repress ABCA1 gene expression via the AT1a receptor.37 Thus, ABCA1 could be involved in AT1a–/– receptor-regulated macrophage migration. Inflammation at sites of atherosclerotic plaques is thought to be a major factor for the progression of atherosclerotic disease, and proinflammatory cytokine may reflect inflammatory activity in the plaques. In agreement with this notion, we showed that AT1a receptor deletion reduced the expression of proinflammatory cytokines (interleukin 6 and tumor necrosis factor α) in the carotid lesions. The LO pathway also regulates inflammatory responses in the atherosclerotic plaque, and has been reported to contribute to atherogenesis via initiation of LDL oxidation and foam cell formation. The expression of 12/15 LO protein in the carotid lesions tended to be lower in ApoE–/– AT1a−/− mice as compared with ApoE–/– mice, although the difference did not reach statistical significance. This tendency suggests that inhibition of the LO pathway may represent 1 of the vascular protective effects of AT1a receptor blockade.

Several limitations of this study should be noted. First, this study was carried out in a mouse model of atherosclerotic plaque rupture that mimics only some aspects of the human disease such as reduced collagen content, increased MMP activity, and apoptotic cells.16 Second, the mechanism by which AT1a receptor deficiency suppresses oxidized LDL-induced CD36 expression is unclear. Although oxidized LDL38 as well as Ang II99 have been shown to upregulate CD36 expression, further investigations are necessary to understand the signaling crosstalk between the AT1a receptor and CD36.

In summary, the present study suggests that the AT1a receptor modulates oxidized LDL-induced macrophage CD36 expression, and FAK and JNK2 activity, and deletion of this receptor increases plaque stability. Furthermore, pharmacological blockade of the AT1a receptor may have similar beneficial effects on atherosclerotic plaque stability via attenuation of macrophage trapping in atherosclerotic lesions, foam cell formation, inflammation, superoxide production, and activation of MMPs. These results provide new insight into the mechanisms responsible for atherosclerotic plaque rupture.

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Disclosures
None.
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emigration from lesions by attenuating CD36 expression, FAK, and JNK2 activation. In a previous study, we showed that Ang II has been shown to transcriptionally repress ABCA1 in macrophages. Interestingly, the inhibitory effect of the AT1a receptor on macrophage migration and the uptake of oxidized LDL in ApoE−/− macrophages. In addition, we showed that AT1a receptor stimulation by Ang II induces degradation through p38- and JNK2-dependent pathways. Effects of the angiotensin-receptor blocker telmisartan on cardiovascular events in high-risk patients intolerant to angiotensin-converting enzyme inhibitors: a randomised controlled trial. Lancet 2008;372:1174–1183.


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In the article by Aono et al, which appeared in the June 2012 issue of the journal (*Arterioscler Thromb Vasc Biol*. 2012;32:1453–1459. DOI: 10.1161/ATVBAHA.112.249516), corrections are needed.

Panels A and B of Figure 5 were incorrect. The corrected panels are below.

The authors apologize for the error.

The online version of the article has been corrected and is available at http://atvb.ahajournals.org/content/32/6/1453.
Supplemental Material

Supplemental Methods

Tissue Collection, Processing, and Lesion Analysis

Anesthetized mice were perfused through the left ventricle with PBS followed by 10% formalin under physiological pressure. The carotid arteries in the cuff were removed and embedded in paraffin. Fixed tissues were cut into 5 μm sections. To obtain fresh-frozen sections, the carotid arteries were embedded in optimal cutting temperature compound (Sakura Finetechnical, Tokyo, Japan), snap-frozen in liquid nitrogen, and stored at -80°C until use. Intracuff lesions were evaluated by analysis of three serial cross-sections (5 μm thickness) that were obtained at an interval of 0.3 mm. For morphometric analysis, hematoxylin-eosin staining and Oil red O staining were performed, according to standard methods. Plaque rupture was defined as an area of neointimal disruption with thrombus. Immunohistochemistry to detect macrophages and AT2 receptors was performed using fresh-frozen sections. Sections were incubated with antibodies against F4/80 (1:500, BMA Biomedicals, Augst, Switzerland), AT2 receptor (1:500, Abcam, MA) and stained using appropriate Histofine Simple Stain MAX-PO (Nichirei Biosciences, Inc., Tokyo, Japan) following the protocol recommended by the manufacturer.

Superoxide Detection

Histological detection of superoxide anion in situ was performed using fresh-frozen sections stained with dihydroethidium (DHE; 10 μmol/L) in PBS for 30 minutes at 37°C in a moisture
box.\textsuperscript{1} The intensity of the fluorescence signal was analyzed and quantified using Densitograph Imaging Software (ATTO Corporation, Tokyo, Japan).

**NADPH oxidase activity**

The carotid arteries in the cuff were homogenized in 150 µl of ice-cold Tris-sucrose buffer (10 mmol/L Tris, pH 7.1, 340 mmol/L sucrose, 1 mmol/L EDTA) and incubated for 30 minutes on ice. Samples were centrifuged (15000 g, 10 minutes, 4°C), and the supernatant (20 µg protein) was added to reaction buffer (78 mmol/L cytochrome c [Sigma, MO], 10mmol/L NADPH [Sigma, MO], with or without 1000 U/mL superoxide dismutase [Sigma, MO]), and then incubated at 37°C for 15 minutes. NADPH oxidase activity was quantified from the absorbance with or without superoxide dismutase, as previously described.\textsuperscript{1}

**In Situ Gelatin Zymography**

In situ detection of gelatinolytic activity was performed with a fluorogenic gelatin substrate, DQ-gelatin (Invitrogen, CA).\textsuperscript{2} Fresh-frozen sections (10 µm) of carotid artery were incubated with DQ-gelatin for 30 minutes at 37°C in a moisture box according to the manufacturer’s instructions. This procedure enabled localization of MMP-2 and MMP-9 activity in the carotid artery. Gelatinolytic activity was visualized under a fluorescence (530 nm) microscope. Negative control sections were incubated in the same way as described above, but without DQ-gelatin. Quantitative analysis was performed with Densitograph Imaging Software (ATTO Corporation, Tokyo, Japan).
Gelatin Zymography

The carotid arteries in the cuff were homogenized in lysis buffer (Cell Signaling Technology, MA) containing protease inhibitor cocktail (Sigma, MO). Protein (5 µg/well) electrophoresis was performed in 10% SDS polyacrylamide gels containing 1 mg/ml gelatin. Subsequently, SDS was removed from the gels by two washes (30 minutes) with 2.5% Triton X-100. Gels were incubated for 12 hours (37°C) in zymography buffer (50 mmol/l Tris (pH 8.0), 10 mmol/l CaCl2, 0.05% Brij 35). After the incubation, gels were stained with Coomassie brilliant blue and then destained in 7% methanol / 5% acetic acid.

Resident Macrophage Isolation and Cell Culture

Resident mouse peritoneal macrophages were harvested by peritoneal lavage with 5 ml of ice-cold PBS. The cells were then suspended in RPMI 1640 medium containing 10% FBS and 1% L-glutamine-penicillin-streptomycin solution. The nonadherent cells were removed after 2 hours, and the adherent macrophages were cultured for 24 hours in culture medium.

Western Blot Analysis

Macrophages were washed twice with ice-cold PBS and harvested in cell lysis buffer (Cell Signaling Technology, MA) containing protease inhibitor cocktail (Sigma, MO). Carotid arteries were homogenized on ice in lysis buffer as described above. Equal amounts of protein were subjected to SDS-PAGE and immunoblotted with antibodies against CD36 (Abcam, MA), β-actin (Cell Signaling Technology, MA), Thr183/Tyr185 phospho-JNK (Cell Signaling Technology, MA), JNK (Cell Signaling Technology, MA), Tyr576/577 phospho-FAK (Cell Signaling Technology, MA), FAK (Cell Signaling Technology, MA), Tyr580 phospho–SHP-2
(Cell Signaling Technology, MA), and SHP-2 (Cell Signaling Technology, MA), 12-lipoxygenase (LO) (Santa Cruz Biotechnology, Inc.), 15-LO (Santa Cruz Biotechnology, Inc.), TNF-α (Hycult Biotech Inc), IL-6 (R&D Systems, Inc.), ABCA-1 (Abcam, MA) or ABCG-1 (Abcam, MA). Immunoreactive bands were visualized using ECL (GE Healthcare, Buckinghamshire, UK). Densitometric analysis was performed using NIH image software.\textsuperscript{1,3}

**Macrophage Migration Assay**

Migration of mouse peritoneal macrophages was determined with a modified Boyden chamber that consisted of a 24-well chemotacticell chamber (Kurabo, Osaka, Japan) with a polycarbonate filter (5 μm pore size).\textsuperscript{4} Macrophages in serum free medium were placed in the upper chamber of the transwell with or without 50 μg/ml of oxidized LDL (Intracel, MD). Medium alone or medium containing 10 ng/ml of MCP-1 (AbD Serotec, Oxford, UK) was added to the lower chamber and incubated for 24 hours at 37°C. The noninvading cells on the upper surface of the filter were completely removed with a cotton swab, and then the migratory cells on the lower side of filter were stained with Giemsa. The number of migratory cells was counted under a microscope.

**References**


**Supplemental Figure Legends**

**Supplemental Figure I**

Expression of AT2 receptors in carotid lesions of ApoE−/− and ApoE−/− AT1a−/− mice.

Representative immunohistochemical staining of fresh-frozen cross sections of the carotid artery with AT2 receptor antibody in ApoE−/− ((A) before ligation and (B) after ligation and cuff placement) and ApoE−/− AT1a−/− ((C) before ligation and (D) after ligation and cuff placement) mice. Sections were counterstained with hematoxylin.

**Supplemental Figure II**

Deletion of the AT1a receptor attenuates proinflammatory cytokine expression in carotid lesions. Western blot analysis was performed with an antibody against TNF-α (A) or IL-6 (B). β-Actin was used to demonstrate equal protein loading. A bar graph shows densitometric measurements
from 5 independent experiments (C and D). *P<0.05 vs ApoE\(^{-/-}\); †P<0.01 vs ApoE\(^{-/-}\). Values are means ± SEM.

**Supplemental Figure III**

Deletion of the AT\(_{1a}\) receptor attenuates the activity of MMP-9. MMP activities were examined by gelatin zymography and quantified by densitometric analysis. Representative gelatin zymograms of active and proform MMP-9 (A) and MMP-2 (C) levels are shown. Protein extracts (5 µg/lane) from intracuff lesions in ApoE\(^{-/-}\) (n=6), ApoE\(^{-/-}\) AT\(_{1a}\)\(^{-/-}\) (n=6) and ApoE\(^{-/-}\) mice treated with valsartan (n=6) were analyzed by gelatin zymography for MMPs. The data from 5 independent experiments are displayed in box plots (B and D). *P<0.05 vs ApoE\(^{-/-}\); †P<0.01 vs ApoE\(^{-/-}\). Values are means ± SEM.

**Supplemental Figure IV**

Time course of macrophage infiltration in carotid lesions of ApoE\(^{-/-}\) and ApoE\(^{-/-}\) AT\(_{1a}\)\(^{-/-}\) mice. Representative immunohistochemical staining of fresh-frozen cross sections of the carotid artery with F4/80 antibody in ApoE\(^{-/-}\) ((A) day 0, (B) day 2 and (C) day 4 after cuff placement) and ApoE\(^{-/-}\) AT\(_{1a}\)\(^{-/-}\) ((D) day 0, (E) day 2 and (F) day 4 after cuff placement) mice. Sections were counterstained with hematoxylin.

**Supplemental Figure V**

Deletion of AT\(_{1a}\) receptor attenuates oxidized LDL-induced phosphorylation of FAK and SHP-2 in mouse peritoneal macrophages. Resident peritoneal macrophages from ApoE\(^{-/-}\) and ApoE\(^{-/-}\) AT\(_{1a}\)\(^{-/-}\) mice were exposed to 50 µg/ml oxidized LDL for the indicated time. Western blot
analysis of cell lysates was performed with an antibody against Tyr576/577 phospho-FAK (A) or Tyr580 phospho–SHP-2 (C). Immunoblotting with anti-FAK and SHP-2 antibodies were used for loading control. A bar graph shows densitometric measurements from 3 independent experiments (B and D). * $P<0.05$ vs ApoE$. Values are means ± SEM.

**Supplemental Figure VI**

Deletion of the AT$_{1a}$ receptor attenuates oxidized LDL-induced JNK2 phosphorylation in mouse peritoneal macrophages. Resident peritoneal macrophages from ApoE$^+/−$ and ApoE$^+/−$ AT$_{1a}^{−/−}$ mice were exposed to 50 $\mu$g/ml oxidized LDL for the indicated time. Western blot analysis of cell lysates was performed with an antibody against Thr183/Tyr185 phospho-JNK (A). Immunoblotting with anti-JNK antibody was used for loading control. A bar graph shows densitometric measurements from 3 independent experiments (B). * $P<0.05$ vs ApoE$. Values are means ± SEM.

**Supplemental Figure VII**

Deletion of the AT$_{1a}$ receptor attenuates phosphorylation of JNK2 in carotid lesions. Western blot analysis was performed with an antibody against Thr183/Tyr185 phospho-JNK (A). Immunoblotting with anti-JNK and β-Actin antibodies were used for loading control. A bar graph shows densitometric measurements from 3 independent experiments (B). * $P<0.05$ vs ApoE$. Values are means ± SEM.
**Supplemental Figure VIII**

Deletion of the AT$_{1a}$ receptor attenuates 12/15-lipoxygenase protein expression in carotid lesions. Western blot analysis was performed with an antibody against 12-lipoxygenase (A) or 15-lipoxygenase (B). β-Actin was used to demonstrate equal protein loading. A bar graph shows densitometric measurements from 5 independent experiments (C and D). Values are means ± SEM.

**Supplemental Figure IX**

Deletion of the AT$_{1a}$ receptor enhances ABCA1 protein expression in carotid lesions. Western blot analysis was performed with an antibody against ABCA-1 (A) or ABCG1 (B). β-Actin was used to demonstrate equal protein loading. A bar graph shows densitometric measurements from 5 independent experiments (B). *$P<0.05$ vs ApoE$^{-/-}$. Values are means ± SEM.
Supplemental Figure II
Supplemental Figure III
Supplemental Figure IV
Supplemental Figure VI
Supplemental Figure VII
Supplemental Figure VIII
Supplemental Figure IX