Angiotensin II Destabilizes Coronary Plaques in Watanabe Heritable Hyperlipidemic Rabbits

Shen Li,* Yan-Ning Wang,* Manabu Niimi, Bo Ning, Yajie Chen, Dedong Kang, Ziyun Wang, Qi Yu, Ahmed Bilal Waqar, Enqi Liu, Jifeng Zhang, Masashi Shiomi, Y. Eugene Chen, Jianglin Fan

Objective—Increased plasma concentrations of angiotensin II (Ang II) have been implicated in many cardiovascular diseases, such as atherosclerosis, aortic aneurysms, and myocardial infarction, in humans. However, it is not known whether high levels of plasma Ang II affect coronary plaque stability and subsequent myocardial infarction. This study was designed to examine whether elevated plasma Ang II can directly induce coronary events, such as acute coronary syndrome.

Approach and Results—To examine the above hypothesis, we infused Ang II (100 ng/min per kg [low group] and 200 ng/min per kg [high group]) or saline vehicle via osmotic minipumps into Watanabe heritable hyperlipidemic rabbits, a model of human familial hypercholesterolemia and atherosclerosis. Infusion of Ang II resulted in mortality rates of 50% and 92% in the low- and high-Ang II groups, respectively, whereas there were no deaths in the vehicle group. Pathological analysis revealed that Ang II–infused Watanabe heritable hyperlipidemic rabbits that died showed myocardial infarction. Furthermore, Ang II–infused Watanabe heritable hyperlipidemic rabbits exhibited coronary plaque erosion and rupture that were associated with thrombosis.

Conclusions—These findings suggest that increased blood levels of Ang II can destabilize coronary plaques and trigger the thrombosis, which possibly induces myocardial infarction. The model described in this study provides a novel means for the study of human acute coronary syndrome. (Arterioscler Thromb Vasc Biol. 2016;36:00-00. DOI: 10.1161/ATVBAHA.115.306871.)

Key Words: acute coronary syndrome ■ angiotensin II ■ hypercholesterolemia ■ myocardial infarction ■ thrombosis
Ang II infusion increases MI

Histological examinations revealed that 80% (8/10) of low-Ang II and 100% (12/12) of high-Ang II groups exhibited histological features of fresh MI (MI), including myocardial eosinophilic degeneration, disappearance of striation, coagulation necrosis, edema, neutrophil infiltration, and hemorrhage (Figure 2). In addition, focal or diffuse fibrosis and calcification accompanied by mononuclear cell infiltration were observed (Figure VA in the online-only Data Supplement). MI changes were found in the right, left ventricle wall, interventricular septum, and papillary muscles. In some areas, subendomyocardial infarction, transmural infarction, subepicardial infarction, and pericarditis were observed (Figure VB and VC in the online-only Data Supplement). MI changes were also observed in 1 rabbit in the vehicle group.

Coronary Atherosclerotic Lesions

Because Ang II infusion led to a high prevalence of MI and a high mortality rate, we examined whether coronary plaque erosion/rupture was present, which may be responsible for MI observed in Ang II–infused WHHL rabbits. The whole hearts were dissected into 5 blocks, and the coronary lesions were investigated extensively as described in the Materials and Methods section of this article and shown in Figure VI in the online-only Data Supplement. Although coronary atherosclerosis was observed in different-sized arteries varying from large epicardial arteries (blocks I and II) to small arteries and arterioles (blocks III–V), the lesions of epicardial arteries in blocks I (left coronary artery) and II (right coronary artery) were consistently present in all rabbits. These lesions were characterized either by fibrosis with more smooth muscle cells and few macrophages (Figure VIIA in the online-only Data Supplement) or by the accumulation of foam cells on the lumen surface or a typical necrotic core covered by a thin fibrous cap (Figure VIIIB and VIIIC in the online-only Data Supplement). To examine whether Ang II can affect coronary complications, we observed all sections under a light microscope and found that erosion along with superimposed lumen thrombi was indeed present in Ang II–infused WHHL rabbits (Figure 3A–C). The close association of coronary plaque erosion/rupture with thrombosis could be further illustrated using step sections (Figure VIII in the online-only Data Supplement). To show the lesion distribution, we quantified the number of coronary plaques with erosion, rupture, and thrombosis in each block. As shown in Table, coronary erosion/rupture along with thrombosis were found in both low- and high-Ang II groups but not in the vehicle group.

Materials and Methods

Materials and methods are available in the online-only Data Supplement.

Results

Ang II Infusion Induces a High Prevalence of WHHL Rabbit Death

The first striking finding after Ang II infusion was gradual death of WHHL rabbits: the mortality rates reached 50% (5/10) in the low-Ang II group at 4 weeks and 92% (11/12) in the high-Ang II group by 16 days (Figure 1). Many rabbits looked inactive, showed dyspnea, and ate less before they died, but 2 healthy rabbits died suddenly. These rabbits died at various times, whereas none of the vehicle rabbits died. Surviving rabbits of the Ang II groups at 4 weeks showed high blood pressure along with an increased number of blood neutrophils and monocytes compared with the vehicle group (Figure III in the online-only Data Supplement), but plasma lipid levels were unchanged (Table III in the online-only Data Supplement). Autopsy examinations revealed that all dead rabbits in both low-Ang II and high-Ang II groups showed severe pulmonary edema, congestion, and hemorrhage (Figure IV in the online-only Data Supplement), which was not present in all surviving rabbits euthanized at 4 weeks. There were no abnormalities in other organs, such as liver, kidneys, brain, adrenals, stomach, and intestines. The pulmonary pathological features suggested that the death of the WHHL rabbits was possibly caused by acute left heart failure.

Coronary plaque erosion and thrombosis are frequently observed in the lesions of Ang II–infused WHHL rabbits, whereas such lesions were rarely found in the vehicle group. These results indicate that increased plasma levels of Ang II can lead to coronary complications.

Nonstandard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>Ang II</td>
<td>Angiotensin II</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>MI</td>
<td>Myocardial infarction</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>WHHL</td>
<td>Watanabe heritable hyperlipidemic</td>
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</table>

Kaplan–Meier analysis of cumulative rates of survival in WHHL rabbits after Ang II infusion.
These coronary erosions/rupture were mainly in the main
trucks (epicardial arteries) of the left (blocks I and III) and the
right (block II) but not seen in the small arteries and arterioles
(blocks IV and V). Because matrix metalloproteinases (MMP)
may be involved in the plaque rupture,26 we examined MMP
expression in the erosion/rupture area by immunohistochemi-
cal staining. MMP-1, MMP-2, MMP-9, and MMP-12 immu-
noreactive proteins were stained in these areas especially where
macrophages were present but not in the normal-appearance
area of the same coronary artery (Figure 4A and 4B). This was
also seen in the lesions, which were associated with thrombo-
sis (Figure 4C). Regardless of these morphological features, all
coronary lesions resulted in various degrees of coronary lumen
stenosis although coronary stenosis of the Ang II groups was
not significantly different from that of the vehicle group (Figure
IX in the online-only Data Supplement).

Escalation Increase of Plasma Ang II Also
Induces Coronary Complications and MI
Experiment 1 described above showed that the abrupt increase
of plasma Ang II resulted in high prevalence of MI and death,
which may possibly be caused by or related with coronary
atherosclerosis. To investigate whether escalation increase of
plasma Ang II also exhibited such effects, we performed a
second experiment in which WHHL rabbits were first infused
with relatively low doses of Ang II for 4 weeks and then fur-
ther infused with high doses of Ang II for another 4 weeks
(Figure I in the online-only Data Supplement). Except for 1
rabbit in the low-Ang II group that died at 2 weeks, all rabbits
survived until 4 weeks and further received another infusion of
Ang II for another 4 weeks (Figure X in the online-only Data
Supplement). Similar to experiment 1, coronary plaque rupture/erosion or with thrombosis were found in Ang II
(groups Table IV in the online-only Data Supplement), but
coronary stenosis of the Ang II groups was not significantly different from that of the vehicle group (Figure
IX in the online-only Data Supplement).

Aortic Atherosclerosis
Grossly, we did not observe aortic aneurysms (such as bul-
ous dilation of the aorta) in Ang II groups, as reported in Ang
II–infused apolipoprotein E knockout mice.20 Extensive aortic
atherosclerotic lesions were observed in both Ang II and vehi-
cle groups and occupied for >80% of the aortic surface, but
there was no significant difference between Ang II–infused
groups and the vehicle group in both experiments (data not
shown). Histological examinations showed that luminal sur-
face macrophages of the aortic arch were increased in Ang II–
infused groups (data not shown). Moreover, RT-PCR showed
that aortic lesions of Ang II groups had significantly higher
mRNA expression of interleukin-6 (IL-6) and plasminogen
activator inhibitor-1. Expression of monocyte chemoattractant
protein-1, IL-1β, tumor necrosis factor-α, and MMP-9 were
also increased, whereas expression of collagens I and III were
reduced compared with those in the vehicle group, although
the differences were not statistically significant (Figure XIII
in the online-only Data Supplement).

Ang II Infusion Does Not Cause Coronary Plaque
Erosion and MI in Wild-Type JW Rabbits
To rule out the possibility that Ang II infusion had any effects
on the wild-type JW rabbits, we performed experiments using

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Figure 2. Representative micrographs of fresh myo-
cardial infarction (MI) in Watanabe heritable hyper-
lipidemic (WHHL) rabbits induced by angiotensin II
(Ang II) infusion. Compared with the normal heart
structure (top left), diffuse myocardial eosinophilic
degeneration and coagulation necrosis, edema and
hemorrhage (indicated by arrowheads) were seen in
the hearts of Ang II–infused WHHL rabbits. These
features represent those of fresh MI. All sections are
stained with hematoxylin and eosin (H&E) staining.
the same protocol. Although Ang II infusion led to the elevation of blood pressure in JW rabbits, as may be expected, there was no discernible atherosclerosis in both aortas and coronary arteries or MI (data not shown).

Discussion

In this study, we demonstrated for the first time that increased plasma levels of Ang II led to a high prevalence of death in WHHL rabbits. All dead rabbits showed pathological features of MI and many of them also showed culprit coronary lesions, suggesting that excessive levels of Ang II can trigger coronary plaque erosion/rupture and possibly cause subsequent MI because plaque erosion/rupture was predominantly present in epicardial arteries. Although both abrupt (experiment 1) and escalation (experiment 2) increases of Ang II in the circulation increased the coronary complications, the death rate in the experiment 2 was lower than in the experiment 1, suggesting that both doses and durations of Ang II infusion affect the severity of coronary complications and MI. Nevertheless, this study indicates that WHHL rabbits treated with Ang II may become a new model for investigation of human coronary atherosclerosis and its complications, such as acute coronary syndromes of unstable angina, MI, and sudden cardiac death.27 Vulnerable plaques have been reported in the innominate
brachiocephalic) arteries of apolipoprotein E knockout mice. Aortic plaque rupture has been also attempted in cholesterol-fed rabbits injected with catecholamines and Russel viper venom. Although these models are valuable for the study of lesion development, these models bear little resemblance to human coronary plaque rupture or thrombosis. Metal stress or hypoxia can induce MI in hypercholesterolemic mice, but plaque rupture and thrombosis have not been shown to be the underlying mechanism. Furthermore, SR-B1 and apolipoprotein E–double knockout mice developed high prevalence of myocardial death or MI because of the coronary occlusions, rather than plaque rupture and subsequent thrombosis. Therefore, Ang II–treated WHHL rabbits may be useful not only for disclosing many aspects of human ACS mechanisms but also for the development of new therapeutics. In the future studies, it will be interesting to investigate whether different therapeutics (such as lipid-lowering drugs, anti-hypertensive drugs, and anticoagulant drugs) will prevent coronary complications or MI or death in this model.

In spite of this, the molecular mechanisms for Ang II–induced coronary complications in WHHL rabbits are not fully understood. Several possible mechanisms may operate in concert for Ang II–induced coronary events in WHHL rabbits. Essential functions of Ang II are to elevate the blood pressure; therefore, it is reasonable to consider that the hemodynamic influence on the plaques is the first cause of the coronary events in WHHL rabbits. To verify this possibility, we generated hypertensive WHHL rabbits by unilateral removal of a kidney. Although these WHHL rabbits indeed had high blood pressure, all of them could survive for >30 weeks (Waqar et al, unpublished data), suggesting that hypertension alone is insufficient to trigger the plaque erosion/rupture in WHHL rabbits. The second possible mechanism may be associated with the multiple local effects of Ang II on the arterial wall. Ang II infusion elevated blood leukocytes in WHHL rabbits, indicating the presence of systemic inflammation. Ample evidence showed that Ang II affects several functions of the arterial wall cells, such as endothelial cells, smooth muscle cells, and macrophages, thereby provoking vascular inflammation. In support of this notion, aortic lesions of Ang II–treated WHHL rabbits showed significantly higher expression of plasminogen activator inhibitor-1 and IL-6 along with increased trend of other proinflammatory cytokines and MMP-9 (presumably derived from accumulated macrophages). Furthermore, our preliminary studies revealed that Ang II treatment increases the mRNA of tissue factor and MMP-9 in THP-1 macrophages and reduces the expression of eNOS of human umbilical vein endothelial cells (data not shown). Taken together, these observations prompted us to envision that an Ang II–induced proinflammatory state facilitates the transformation of a stable plaque into a vulnerable plaque prone to rupture. Previous studies have shown that MMPs produced by macrophages may cause the rupture of vulnerable plaques. Increased tissue factor along with plasminogen activator inhibitor-1 activity in the lesions renders lesions, rather than thrombogenic, which further promotes the formation of thrombosis in case plaques rupture. The third possibility for Ang II–induced plaque rupture is associated with the fact that Ang II is notorious for inducing vascular oxidative stress because of the generation of reactive oxygen species, such as superoxide and hydrogen peroxide, by stimulating the activity of nicotinamide adenine dinucleotide phosphate and xanthine oxidase from a variety of vascular cells, which has been considered to play an important role in the pathogenesis of atherosclerosis. It should be pointed out that it is currently unknown whether MI was totally caused by coronary complications induced by Ang II infusion or whether MI is responsible for rabbit death because Ang II can directly exert detrimental effects on the heart or other organs. Therefore, it remains to be investigated.
in future whether Ang II can also cause myocardial injury or myocardial death. In addition, we observed that many eroded areas were not associated with occlusive thrombosis in Ang II–infused rabbits. We speculate that this may be because of

(1) plaques with occlusive thrombi were overlooked, (2) luminal thrombosis superimposed on the eroded area was quickly dissolved by the fibrinolytic system, or (3) MI could be caused by coronary spasm induced by Ang II,19–41 which cannot be

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Figure 4. Immunohistochemical staining of macrophages and matrix metalloproteinases (MMPs). A representative eroded lesion (arrowheads) with a thrombus on the surface shows accumulation of a few macrophages and stained with MMP-1, MMP-2, and MMP-12 Abs (A). In the normal area adjacent to the erosion of the same artery, there are only a few macrophages that are stained with MMP Abs (B). Another representative ruptured lesion (arrowheads) associated with an occlusive thrombus shows accumulated macrophages with MMPs staining at the rupture area (C).
evaluated by pathological methods. Therefore, it is necessary to evaluate cardiac functions induced by Ang II infusion by other methods such as ECG, plasma cardiac markers, ultrasonic cardiogram, and angiography of this model. Regardless of this, the current animal model may become a novel means to investigate many facets of human coronary syndrome and to develop new therapeutics for MI in the future.

In conclusion, we showed herein that Ang II infusion induces coronary complications and increases the prevalence of MI and mortality in WHHL rabbits. Although the molecular mechanisms are not yet completely clear, vigorous treatment of Ang II-induced deleterious effects may be beneficial for the prevention of cardiovascular events.

Acknowledgments

We thank A. Suiimi and Y. Nakagawa for their technical assistance in making pathological specimens.

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Disclosures

None.

References

31. Sukhova GK, Schinella U, Rubkin E, Schoon FL, Poole AR, Billinghurst A, Federman M, Gaboury CL, Conlin PR, Seely EW, Williams GH, Vaughn DE. Stimulation of plasminogen activator inhibitor in vivo by infusion of...
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<th>Annealing Temp. (°C)</th>
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## Supplemental Table II. List of antibodies used in the current study

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<td>M0633</td>
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<td>(36.2 µg/ml)</td>
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<td>Muscle actin (HHF35)</td>
<td>Dako Co., Carpinteria, CA</td>
<td>M0635</td>
<td>1:300 (122.6 ng/ml)</td>
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<td>(36.8 µg/ml)</td>
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<td>F-67</td>
<td>1:400 (1.25 µg/ml)</td>
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<td>MMP-2 (500 µg/ml)</td>
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<td>MMP-9 (500 µg/ml)</td>
<td>Daiichi Fine Chemical, Toyama, Japan</td>
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<td>1:25 (20 µg/ml)</td>
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<td>MMP-12 (500 µg/ml)</td>
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<td>Mouse IgG (1.4 mg/ml)</td>
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<td>i8765</td>
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### Supplemental Table III. The plasma levels of TC, TG and HDL-C.

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<th>High (n=9)</th>
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<tr>
<td>TC (mg/dL)</td>
<td>940±130</td>
<td>839±111</td>
<td>1154±80</td>
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<tr>
<td>TG (mg/dL)</td>
<td>228±47</td>
<td>262±88</td>
<td>310±81</td>
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<tr>
<td>HDL-C (mg/dL)</td>
<td>7.1±0.48</td>
<td>8±1.12</td>
<td>8±0.33</td>
</tr>
</tbody>
</table>

|          | Vehicle (n=8) | Low (n=5) | High (n=1) |
| TC (mg/dL) | 825±131      | 868±274    | 1400       |
| TG (mg/dL) | 265±64       | 379±166    | 308        |
| HDL-C (mg/dL) | 7.8±0.64 | 8±1.1      | 7.8        |

| Experiment | Vehicle (n=6) | Low (n=7) | High (n=7) |
| TC (mg/dL) | 1249±183     | 1172±74    | 1416±162   |
| TG (mg/dL) | 298±81       | 183±48     | 362±128    |
| HDL-C (mg/dL) | 8.47±0.74 | 7.7±1.20   | 8.87±0.59  |

|          | Vehicle (n=6) | Low (n=6) | High (n=7) |
| TC (mg/dL) | 975±187      | 999±89     | 1057±202   |
| TG (mg/dL) | 185±47       | 243±58     | 257±159    |
| HDL-C (mg/dL) | 6.25±0.79 | 7.25±1.60  | 6.85±1.01  |

|          | Vehicle (n=6) | Low (n=5) | High (n=2) |
| TC (mg/dL) | 1039±53      | 1005±118   | 1035±134   |
| TG (mg/dL) | 198±30       | 227±60     | 196±16     |
| HDL-C (mg/dL) | 7.78±0.75 | 8.1±2.42   | 7.9±0.07   |

Values are mean ± SD. TC, Total cholesterol; TG, triglycerides; HDL-C, High-density lipoprotein cholesterol. We compared plasma lipids at different time points (0, 4, and 8 weeks) using one way ANOVA with post-hoc Tukey test. In the experiment 1, comparison was only made between the low group and vehicle group using Student’s t-test because the number of high group was 1 (not enough for comparison). No statistical significance was found between Ang II groups and the vehicle group.
Supplemental Table IV. The number of coronary erosions, rupture and thrombosis in each block of experiment 2.

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<th>Block III</th>
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<tr>
<th>Ruptures</th>
<th>Block I</th>
<th>Block II</th>
<th>Block III</th>
<th>Block IV</th>
<th>Block V</th>
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<tr>
<td>Vehicle (n=6)</td>
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<td>0 ± 0</td>
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<tr>
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<table>
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<th>Block III</th>
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<td>Vehicle (n=6)</td>
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<tr>
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<tr>
<td>Ang II-H (n=7)</td>
<td>1.14 ± 0.69**</td>
<td>0.71 ± 0.49</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
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<tr>
<td>(6/7)</td>
<td>(5/7)</td>
<td>(0/7)</td>
<td>(0/7)</td>
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The number of each type of lesions was calculated from all sections of each block. The largest number on the section was used to represent each block. The data are expressed as the mean ± SD. *P<0.05, **P<0.01 vs vehicle group using the Mann-Whitney U test. Parentheses indicate the ratio of animals with the lesions to total animal number. Thrombosis includes both partial and occluded thrombi.
**Supplemental figure I. Schematic diagram showing the experimental design.**

Two experiments were designed to illustrate the effects of Ang II infusion administered differently: acute increase of Ang II in circulation by a single high-dose infusion (upper panel) and escalation increase of Ang II in circulation by two different doses (from low- to high-dose infusion) (lower panel).
Supplemental figure II. Immunohistochemical staining specificity of each antibody used for the current study.
Serial sections of WHHL rabbit aorta were immunohistochemically stained with antibodies against rabbit macrophage (Mφ), smooth muscle cells (SMC), MMP-1, -2, -9, -12 (See S-Table II) along with non-specific mouse IgG as a negative control staining (bottom right).

Supplemental figure III. Blood pressure and blood leukocytes at 4 weeks.
Blood pressure (upper) and blood leukocyte count (lower panel) were measured at 4 weeks after Ang II infusion. Data are expressed as mean ± SEM. * P<0.05 or ** P<0.01 versus the vehicle. n=7 for the vehicle, n=5 for low-Ang II and n=1 for high-Ang II groups.
Supplemental figure IV. Pulmonary pathological changes in Ang II-infused rabbits.
Representative photographs of rabbit lungs of the vehicle group (left) and Ang II group (right) are shown. The lungs of a rabbit that died at 10 days after Ang II infusion showed pulmonary congestion (upper right). Microscopically, alveolar space was filled with exudate fluid and blood cells (lower right) compared with normal alveoli (lower left). Paraffin sections were stained with HE staining.
Supplemental figure V. Micrographs of myocardial infarction in Ang II-infused WHHL rabbits.

A. Necrotic cardiac myocytes were partly replaced by fibrosis (upper panels, HE and Masson’s trichrome staining) and calcification (lower panels, HE staining), suggesting that healing process after myocardial infarction. Fibrosis (stained as blue) is more distinctive when the specimens are stained with Masson’s trichrome staining.

B. Representative micrograph of transmural myocardial infarction (HE staining). The whole layer of the left ventricle wall of a dead WHHL rabbit of the Ang II group showed diffuse myocardial infarction and many necrotic foci (an asterisk indicates the ventricle cavity).

C. Pathological features of pericarditis and subepicardial infarction. In some areas, pericardium was either infiltrated by chronic inflammatory cells (top, arrow head) or the presence of fibrinous exudative stained pink (middle, arrow head). Beneath the subepicardial area, there are foci of necrosis (indicated by an arrow), indicating the presence of subepicardial infarction (bottom).
Supplemental figure VI. Representative micrographs of 5 blocks of the WHHL rabbit heart dissected for the analysis (HE staining).

Block I usually contains one large left coronary main trunk (highlighted by a square) located in the epicardium and several small arteries and arterioles (indicated by arrowheads) in the heart muscle (interventricular septum). The diameter of the epicardial arteries is arranged from 1.0 to 3.0 mm (n=10). Small arteries and arterioles are ~0.8 mm in diameter (arterioles less than 300 \( \mu \)m are not calculated in the current study because they do not have any lesions). In general, 1-2 arteries have atherosclerosis in epicardial arteries or small arteries and arterioles in this block.

Similar to the block I, block II also contains one large right coronary main trunk located in the epicardium with several small arteries and arterioles (indicated by arrowheads) in the heart muscle (interventricular septum). The diameter of the epicardial arteries is up to 2.0 mm. In general, 1-2 arteries have atherosclerosis in epicardial arteries or small arteries and arterioles in this block.

Block III is a part of the left ventricle. The number of small arteries and arterioles are varying from 4 to 9 but the number of the arteries with lesions is 0-2.

Block IV contains the right ventricle and part of the interventricular septum. The number of small arteries and arterioles are varying from 4 to 9 but the number of the arteries with lesions is 0-2.

Block V shows both left and right ventricle. The number of small arteries and arterioles are varying from 6 to 18 but the number of the arteries with lesions is 0-3. Arteriolosclerosis can be seen in both the septum and left ventricle but rarely seen in the right ventricle. Arrowheads indicate small arteries.
Supplemental figure VII. Representative micrographs of coronary atherosclerotic lesions showing either various histological features in WHHL rabbits.

A. Step sections (the distance from coronary artery orifice to each section: ① orifice, ② 50μm, ③ 100μm, ④ 150μm, ⑤ 200μm) of “stable” lesions show that the lesions contain few macrophage-derived foam cells and almost totally consist of fibrotic tissue with smooth muscle cells sparsely. Immunohistochemical staining using Abs against either macrophages (Mφ) or smooth muscle cells (SMC) was performed using the serial sections of No. ① and is shown on the right.

B. Step sections (the distance from coronary artery orifice to each section: ① 50μm, ② 100μm, ③ 150μm, ④ 300μm, ⑤ 400μm) of “unstable” lesions reveal that the lesions show not only severe stenosis but also accumulation of foam cells in both lumen and peripheral area (beneath the adventitia). The surface foam cells seems to be exposed to the lumen (①~③), which is filled with a blood clot (④~⑤). Immunohistochemical staining using Abs against either macrophages (Mφ) or smooth muscle cells (SMC) was performed using the serial sections of No. ② and shown on the right.

C. Step sections (the distance from coronary artery orifice to each section: ① orifice, ② 350μm, ③ 400μm, ④ 900μm) of another “unstable” lesions contain a thin cap (arrowheads, ①~④) and calcification (asterisk, ④). A blood clot is either attached to the surface of the lesions or filled with the lumen.
S-Figure VIII-①

Hemorrhage
Rupture
Thrombus
Figure VIII-②

- Rupture
- Thrombus
- Erosion
S-Figure VIII-③
S-Figure VIII-⑤ 

- Rupture
- Thrombus
- Erosion
- Hémorrhage
Figure VIII-6

- Thrombus
- Erosion
- Hemorrhage
- Rupture
S-Figure VIII-⑨

Rupture

Thrombus
S-Figure VIII-10

- Rupture
- Thrombus
Supplemental figure VIII. Representative micrographs showing the continuity of the coronary plaque rupture/erosion and thrombosis. Step sections (numbered from ① to ⑩) of the left coronary artery from a dead WHHL rabbits infused with Ang II were cut at 50 μm interval and stained with HE staining.

The plaque shows a distinctive disruption (indicated by arrows, from ① to ⑩ on the left, bottom panel) in which many red blood cells are contained. Accumulation of foam cells associated with the surface erosion is present (indicated by arrows, ②-⑧). Discernible hemorrhage within the lesions is seen in sections ①, ④-⑥. Thrombi are present in sections ①-⑩.

Supplemental figure IX. Comparison of coronary stenosis of Ang II groups with the vehicle group.

Data are expressed as mean ± SEM. n=8 for the vehicle, n=10 for low-Ang II and n=9 for high-Ang II groups. No statistical significance was found between Ang II groups and the vehicle group.
Supplemental figure X. Kaplan-Meier analysis of cumulative rates of survival in WHHL rabbits in experiment two.
Supplemental figure XI. Blood pressure and blood leukocytes in experiment two.

Blood pressure (upper) at 0, 4, 8 wks and blood leukocyte count (lower panel) were measured at 6 wks. We compared the blood pressure at different time points (0, 4, and 8 weeks) using one way ANOVA with post-hoc Tukey test (0 and 4 weeks) or Student’s t-test (8 weeks, low group vs vehicle). Data are expressed as mean ± SEM. *P<0.05 or **P<0.01 versus the vehicle. n=6 for the vehicle, n=5-7 for low-Ang II and n=1-7 for high-Ang II groups.
Supplemental figure XII. Quantitative analysis of the coronary stenosis of experiment 2.
Coronary stenosis of both left (LCA) and right coronary arteries (RCA) was compared between Ang II and vehicle groups. N=6 for vehicle group, n=7 for low- and high-Ang II groups.

Supplemental figure XIII. RT-PCR analysis of mRNA expression of aortic lesions of the aortic arch.
Total RNA was extracted as described in the Materials and Methods and quantitative real-time RT-PCR was used to quantify each gene expression. N=5 for each group. *P<0.05 vs. vehicle group.
Materials and Methods

Animals

We used Watanabe heritable hyperlipidemic (WHHL) rabbits at an age of 8 months, in which atherosclerosis was well established\(^1\). Osmotic pumps (Alzet Model 2ML4; Durect Corporation, Cupertino, CA) were placed into the subcutaneous space of ketamine/medetomidine-anesthetized rabbits through a small incision on the back of the neck that was closed with surgical sutures. All incision sites healed rapidly without any treatment. The pumps were filled with either saline vehicle as a control or different doses of angiotensin II (Ang II) (CSBio Com. Inc., Menlo Park, CA). Rabbits were fed with a standard chow diet (CR-3) (100 g/day), containing 18.6% protein, 3.2% fat and 14.2% fiber (CLEA Japan, Inc., Tokyo) \textit{ad libitum}. All animal experiments were performed with the approval of the Animal Care Committee of University of Yamanashi and also conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

Experimental design

We designed two experiments as shown in S-Fig. 1. In the first experiment, WHHL rabbits were infused with two single doses of Ang II for 4 weeks: 100 ng/min/kg (n=10, designated as Ang II L) and 200 ng/min/kg (n=12, designated as Ang II H) along with the saline vehicle (n=8). The purpose of this experiment was to examine the effects of rapid elevation of plasma Ang II on WHHL rabbits. The doses of Ang II were selected according to a previous publication\(^2\) and our own studies. Infusion of Ang II at 60 ng/min/kg did not elevate the blood pressure of the wild-type NZW rabbits, but Ang II at 200 ng/min/kg increased the mean blood pressure by 25 mmHg\(^2\). In our pilot study, we infused Ang II at 40 ng/min/kg into WHHL rabbits for 8 wks and found that systolic pressure was increased by 40 mmHg, suggesting that WHHL rabbits are more sensitive to Ang II infusion compared with NZW rabbits. However, there were no other deteriorating effects on the WHHL rabbits’ general health or mortality (data not shown).

In the second experiment, WHHL rabbits were first infused with 50 ng/min/kg (n=7, low Ang II) or 75 ng/min/kg (n=7, high Ang II) for 4 wks and then further infused with 100 or 150 ng/min/kg for another 4 wks in order to evaluate the effects of the gradual elevation of plasma Ang II (in contrast to the first experiment, in which Ang II was raised abruptly without an adaptation period) on WHHL rabbits (S-Fig. 1). The vehicle group (n=6) was continuously infused with saline for 8 wks with osmotic pumps changed at 4 wks.

Determination of blood pressure and plasma lipids

The blood pressure (BP) was examined weekly for those rabbits that survived (see below). To minimize the influence of stress, conscious rabbits were placed in a quiet room for 30 min before BP measurement. The medial auricular artery was cannulated with a 23G cannula, and BP were simultaneously recorded using a transducer positioned at the heart level, as described previously\(^3\). The data were collected for 15~25 minutes after rabbits
had become completely calm using a BP amplifier (ADInstruments, Tokyo, Japan) attached to the digital Powerlab data acquisition system (ML870 PowerLab; ADInstruments). BP was calculated using Chart 5 Pro v5.5 software (ADInstruments). Blood was collected from rabbits after 16 h of food deprivation. Plasma lipids were determined using Wako assay kits (Wako Pure Chemical Industries, Osaka, Japan) and hematological examinations were performed as reported previously.

**Pathological examinations**

Rabbits that died during the experiment were autopsied (3~10 hours after death). Procedures of autopsy included gross and microscopic examinations such as the body appearance examination, thoracic and abdominal cavity examination, and all important organs (liver, adrenal, spleen, kidneys, stomach and intestines, heart and lung, brain). After gross examinations of these organs, all organs were fixed in 10% neutral buffered formalin solution and specimens were routinely made for microscopic examinations. These tissues were embedded in paraffin and sections (3 μm thick) were routinely stained with hematoxylin and eosin (H&E). We did not see any pathological changes in these organs except lung and heart (see below). Those that survived to the end of the experiment were euthanized by an overdose injection of sodium pentobarbital (100 mg/kg); the following tissues were then collected for pathological examinations: lung, heart and aorta, brain, liver, and kidney as those dead rabbits.

The aortic trees were isolated and opened out. After fixing in 10% formalin, they were stained with Sudan IV and analyzed as described previously. For the microscopic quantification of the lesion areas and lesion features, aorta was divided into arch, thoracic and abdominal segments, and each segment was further cut into cross sections (eight for the aortic arch and 20 each for the thoracic and abdominal aorta). All sections were stained with H&E, Masson’s trichrome (MT) and immunohistochemically stained with Abs against rabbit macrophages (Dako Co., RAM-11). In addition, a small piece of the aortic arch was collected for extraction of total RNA and the mRNA expression of cytokines: plasminogen activator inhibitor-1 (PAI-1), interleukin-1β (IL-1β), interleukin-6 (IL-6), tumor necrosis factor (TNF-α), Monocyte chemoattractant protein-1 (MCP-1), matrix metalloproteinase-9 (MMP-9), collagen I and III was analyzed by real-time RT-PCR (see S-Table 1).

For the analysis of coronary lesions, we used a method reported recently. The whole protocol can be obtained from the Appendix A. Supplementary data published through the following website: http://www.sciencedirect.com/science/article/pii/S0163725814001855.

In brief, the hearts were sectioned into 5 blocks. Blocks I and II contain the main trunks of the left and right coronary arteries. To undertake an extensive examination of coronary lesions, these two blocks were cut into 4 serial sections (3 μm thick) at 50 μm intervals (20 cuts for block I and 10 cuts for block II). Blocks III-V were cut into 3 serial sections at 500 μm intervals. In total,
39 sections from each heart were examined under a light microscope. These sections were stained routinely with H&E and their histological features (presence of myocardial infarction and atherosclerosis, see below) were examined under the light microscopy. Coronary lesions of blocks I-V were quantified for (1) the number of plaque erosion and rupture (which contains an apparent cleft or split) or with or without thrombosis in each block; (2) lumen stenosis (%) in blocks I and II. Serial sections adjacent to sections with erosion or rupture features confirmed by HE staining were selected and immunohistochemically stained with Abs against rabbit macrophage (RAM-11), α-smooth muscle actin (HHF-35) (Dako Co.), and MMP-1 (41-1E5, Daiichi Fine Chemical Co.), MMP-2 (42-5D11) (Daiichi Fine Chemical Co.), MMP-9 (56-2A4) (Daiichi Fine Chemical Co.), MMP-12 (82902) (R&D Systems Inc.) (S-Table 2, S-Fig. 2).

In the current study, ruptures refer to those lesions which show apparent split or disruption of the fibrous cap accompanied by thrombosis or the intrusion of blood cells as proposed by others whereas erosion refers to those lesions in which the intimal surface is eroded (with endothelial cells detached) but the maximal depth is less than 15 μm. Due to lack of specific CD31 Abs against rabbit endothelial cells, endothelial cells were evaluated by pathologists based on morphological structures.

Pathology of myocardial infarction is defined based on the following histological features. In the acute phase of MI (that can be seen with ~3 days after MI occurs), cardiac myocytes show diffuse coagulation degeneration and necrosis (loss of striations) accompanied by nuclear changes such as karyopyknosis and karyorrhexis. Edema, hemorrhage and neutrophil infiltration are often seen. In the later stage (healing process), while the above features are still present, the following features predominate, including fibroblast cell proliferation, fibrosis, mononuclear cell infiltration, calcification and angiogenesis. For evaluation of myocardial infarction and fibrosis, the heart sections were also subjected to Masson's trichrome staining. In the current study, we assessed the presence of MI pathology for each animal.

Statistical analysis
All values are expressed as the mean ± SEM. The Kaplan-Meier estimator along with the log-rank test was used to evaluate the survival rate after Ang II infusion. All other data were examined by Shapiro-Wilk test to verify their distribution. For those data which are normally distributed, we analyzed them using parametric tests (One-way ANOVA test with Tukey's multiple comparison for three groups and Student’s t test for two groups). Otherwise, there were analyzed using non-parametric tests (Kruskal-Wallis rank test for three-group comparison and Mann-Whitney U test for two-group comparison). In all cases, a P value of less than 0.05 was considered statistically significant.
References:


