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) *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 [4].

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[5, 6]. 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[7]. 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[7].

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**)[9].

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[10-12] 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.

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