*Original Article*

# The Long-Term Effect of Angiotensin II Type 1a Receptor Deficiency on Hypercholesterolemia-Induced Atherosclerosis

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**Angiotensin II type 1 receptor may contribute to atherogenesis by facilitating the proliferative and inflammatory response to hypercholesterolemia. In the present study, we investigated the long-term effect of angiotensin II type 1a receptor (AT1a) deficiency on hypercholesterolemia-induced atherosclerosis by the use of AT1a-knockout (AT1a-KO) mice and apolipoprotein E–knockout (apoE-KO) mice. AT1a-KO were crossed with apoE-KO, generating double-knockout (D-KO) mice. Mice were fed a standard diet and analyzed at 25- or 60-weeks-old. The quantification of atherosclerotic volume in the aortic root revealed that the atherosclerotic lesions of D-KO mice were significantly smaller than those of apoE-KO mice at 25-week-old ($0.81 \pm 0.16$ mm$^2$ *vs.* $1.05 \pm 0.21$ mm$^2$, $p<0.001$) and at 60-week-old ($0.89 \pm 0.11$ mm$^2$ *vs.* $2.44 \pm 0.28$ mm$^2$, $p<0.001$). Surprisingly, there was no significant difference in atherosclerotic lesion size of D-KO mice at 25- and 60-week-old, suggesting that AT1a deficiency completely protected against the age-related progression of atherosclerosis. The amounts of collagen and elastin, the expression of p22phox, serum amyloid P (SAP), matrix metalloproteinase (MMP)-2, and MMP-9, and the number of apoptotic cells of D-KO mice were lower than those of apoE-KO mice. Furthermore, we confirmed that the expression of procollagen α1(I), procollagen α1(III), tropoelastin, p22phox, SAP, MMP-2, and MMP-9 decreased in cultured vascular smooth muscle cells from D-KO mice compared with those of apoE-KO mice. In conclusion, AT1a deficiency reduces atherosclerotic lesion size of apoE-KO mice and protects against the age-related progression of atherosclerosis. Reduction of oxidative stress, apoptosis, and MMP expression in atherosclerotic lesions by AT1a deficiency may contribute to plaque size. (*Hypertens Res* 2008; 31: 1631–1642)**

***Key Words*: angiotensin, apoptosis, atherosclerosis, hypercholesterolemia, oxidative stress**

## Introduction

Angiotensin II (Ang II) is a potent vasoconstrictor, and, apart from its effects on blood pressure, it is strongly implicated in the pathogenesis of atherosclerosis. Oxidative stress and inflammation, triggered by Ang II, are involved in the initiation and progression of atherosclerosis (*1–3*). Ang II elicits production of superoxide anion, a reactive oxygen species (ROS), from arterial endothelial cells (EC) and smooth muscle cells (SMC) (*4*). Ang II can also increase expression of proinflammatry cytokines such as interleukin (IL)-6 and monocyte chemoattractant protein (MCP)-1 in arterial SMC and the leukocyte adhesion molecule, VCAM-1, in EC (*5–7*).

Angiotensin II type 1 receptor (AT1) may contribute to atherogenesis by facilitating the proliferative and inflammatory response to hypercholesterolemia. AT1 blockers (ARB) and angiotensin-converting enzyme (ACE) inhibitors normal-

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ize oxidative stress and endothelial dysfunction, and they reduce the progression of atherosclerosis (*8*, *9*). Several studies using various animal models have shown that blocking the actions of Ang II by ACE inhibitors or ARB is effective in preventing the progression of atherosclerosis (*9*). Recently, it was reported that AT1 deficiency significantly reduced hypercholesterolemia-induced atherosclerosis in apolipoprotein E (apoE)–knockout (apoE-KO) mice at 19-weeks-old (*10*) or in low density lipoprotein (LDL) receptor deficient mice at 20-weeks-old (*11*). However, the long-term effect of angiotensin II type 1a receptor (AT1a) deficiency on hypercholesterolemia-induced atherosclerosis has not been reported. AT1-KO mice did not show any atherosclerotic lesion themselves. Therefore, AT1a-KO mice were crossed with apoE-KO mice in order to generate double-knockout (D-KO) mice. We compared the atherosclerotic lesion and expression of factors that are related to plaque vulnerability between the apoE-KO and D-KO mice at 25- and 60-week-old. Furthermore, to address the role of AT1 in atherogenesis, we performed cell culture experiments using vascular smooth muscle cells (VSMCs) explanted from the medial layer of aortas from the D-KO or apoE-KO mice.

## Methods

### Generation of AT1a$^{-/-}$/ApoE$^{-/-}$ D-KO Mice

ApoE-KO mice on a C57BL/6 background were donated by Dr. Jan L Breslow (Rockefeller University, New York, USA), and AT1a-KO mice on a C57BL/6 background were obtained from Tanabe Seiyaku Co. Ltd. (Osaka, Japan). ApoE-KO mice were crossed with AT1a-KO mice, and heterozygous knockout mice were crossed until homozygous D-KO mice were obtained. Genotypes for apoE and AT1a were determined by polymerase chain reaction amplification of DNA isolated from the tail. These mice were fed a standard diet, and water was available ad libitum. The study protocol was approved by the ethics committee of the Graduate School of Medicine, Kagoshima University. This study conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

### Measurements of Heart Rate, Blood Pressure, Cholesterol, and 8-Isoprostane

D-KO and apoE-KO mice were compared in this study. Before sacrifice, the heart rate and blood pressure of conscious restrained mice were measured using a tail-cuff system. At the age of 25 or 60 weeks, mice were anesthesized with pentobarbital (80 μg/kg, i.p.) after measuring body weight, and blood was drawn from the left ventricle of anesthesized mice. The mean blood pressure was calculated from the systolic and diastolic blood pressure. Plasma total- and high density lipoprotein (HDL)-cholesterol were measured with an enzymatic kit (Cat.#DR-2100, DR-2210, Kainos Laboratories, Inc., Tokyo, Japan).

To clarify the production of oxidative stress, 8-isoprostane (a plasma indicator of oxidative stress) was determined using plasma from the apoE-KO and D-KO mice using an 8-Isoprostane EIA Kit (Cat.#516351, Cayman Chemical, Ann Arbor, USA) as described previously (*12*).

### Genotyping by PCR

AT1a genotyping used the following primers: antisense, 5′-ATCCTAAAGATGTCATCATTTC-3′ and sense, 5′-ATG GATTTTGAACAGTGTTG-3′. Resultant wild-type and deficient allele bands were 770 bp and 1,134 bp, respectively (data not shown). ApoE genotyping used the following primers: antisense, 5′-GCCTAGCCGAGGGAGAGCCG-3′ and sense, 5′-GCCGCCCCGACTGCATCT-3′. Resultant wild-type and deficient allele bands were 155 bp and 245 bp, respectively (data not shown).

### Tissue Preparation and Lesion Assessment

Animals were anesthesized with pentobarbital (80 μg/kg, i.p.) and perfused with phosphate-buffered saline (pH 7.4) followed by 10% neutral-buffered formalin through a catheter placed in the left ventricle. The heart with aortic root was fixed in 10% neutral-buffered formalin, embedded in O.C.T. compound (O.C.T.; Sakura Finetechnical Co., Tokyo, Japan) and frozen at −80°C. Frozen sections were cut into 10-μm sections and fixed to glass slides. The slides were stained with oil red O. All sections were examined under a microscope, and the lipid-staining aorta and total area of the histological sections were measured (*13*).

For en face preparations, the aorta was dissected from the aortic valve to the iliac bifurcation and fixed in 10% neutral-buffered formalin. The aorta was opened longitudinally and pinned on a board. To identify atherosclerotic lesions, the aorta was stained with oil red O. It has been reported that the extent of atherosclerosis in the entire aorta (expressed as percent of surface area) reflects the extent of atherosclerosis in the aortic root (expressed as average lesion area per section) of apoE-KO mice and LDL receptor deficient mice (*14*).

For histological analysis of the extracellular matrix (ECM) and immunohistochemical staining, the hearts and aortas of mice were carefully removed, fixed in 10% neutral-buffered formalin, and embedded in paraffin. Azan staining for collagen and Victoria blue staining for elastin were carried out to assess matrix production.

### Immunohistochemical Analysis

Immunohistochemical staining was carried out on paraffin-embedded sections as described previously (*15*). After deparaffinization and hydration of specimens, endogenous peroxidase activity was blocked, and the specimens were fixed by

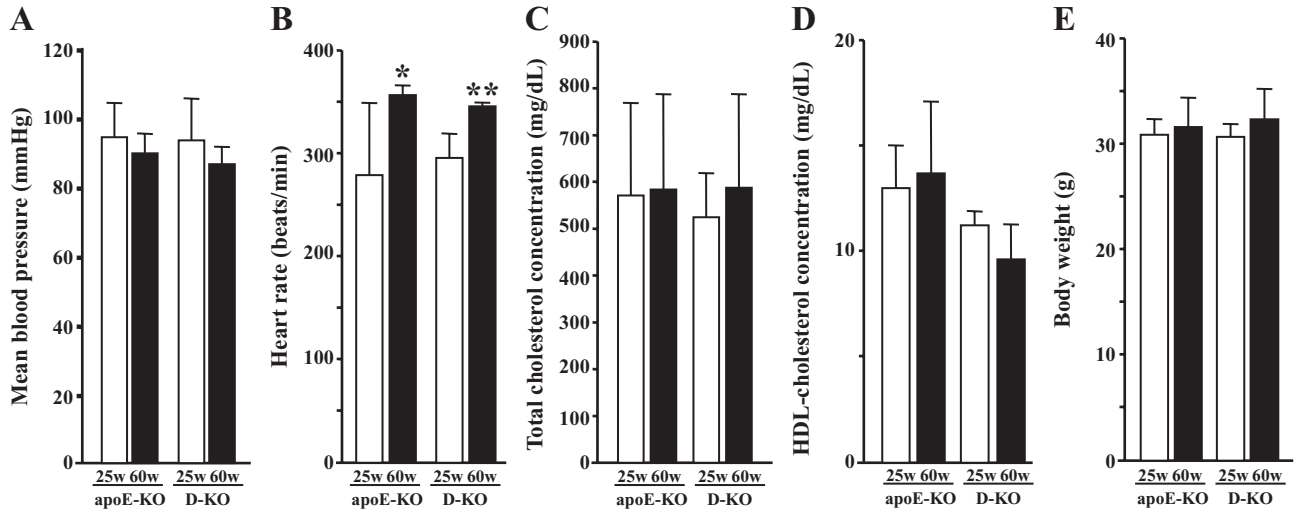


**Fig. 1.** *Hemodynamic parameters, body weight, and cholesterol concentration. There was no significant difference in mean blood pressure (A), heart rate (B), total cholesterol concentration (C), HDL-cholesterol concentration (D), or body weight (E) between apoE-KO and D-KO mice at both 25- and 60-week-old. There was no significant difference in mean blood pressure, total- and HDL-cholesterol concentration, or body weight between 25- and 60-week-old in both apoE-KO and D-KO mice. However, heart rate at 60-week-old was significantly higher than that at 25-week-old. *$p < 0.05$ vs. 25-week-old apoE-KO mice, **$p < 0.05$ vs. 25-week-old D-KO mice.*

immersion in 0.3% $H_2O_2$ in methanol for 20 min. Immunohistochemical staining was performed with a goat polyclonal antibody against human muscle actin (Cat.#sc-1615, Santa Cruz Biotechnology, Santa Cruz, USA), a goat polyclonal antibody against human CD-64 (Cat.#sc-7642, Santa Cruz Biotechnology), a goat polyclonal antibody against human p22phox (Cat.#sc-11712, Santa Cruz Biotechnology), a goat polyclonal antibody against mouse serum amyloid P (SAP; Cat.#sc-18312, Santa Cruz Biotechnology), a goat polyclonal antibody against human matrix metalloproteinase (MMP)-2 (Cat.#sc-6838, Santa Cruz Biotechnology), a rabbit polyclonal antibody against human MMP-9 (Cat.#sc-10737, Santa Cruz Biotechnology), a goat polyclonal antibody against human angiotensin II type 2 receptor (AT2; Cat.#sc-7420, Santa Cruz Biotechnology), a rabbit polyclonal antibody against human activated caspase-3 (Cat.#557035, Santa Cruz Biotechnology), and a goat polyclonal antibody against human apoptosis inducing factor (AIF; Cat.#sc-9416, Santa Cruz Biotechnology) using the labeled streptavidin biotin complex method (Cat.#424141, 424151, Simple-stain MAX-PO kit, Nichirei, Tokyo, Japan). After blocking with 10% rabbit or goat serum, slides were incubated overnight with a primary antibody at 4°C in a moisture chamber. Slides were washed with Tris-buffered saline (TBS) and incubated with a biotinylated secondary antibody at room temperature for 30 min. After washing with TBS, slides were incubated with streptavidin at room temperature for 30 min and visualized with 3,3′-diaminobenzidine.

## Cell Experiments

Primary VSMCs were explanted from the medial layer of apoE-KO or D-KO aortas as described previously (*16*). They were cultured in 75 $cm^2$ flasks at 37°C in a humidified atmosphere of 95% air and 5% $CO_2$, and the medium was changed every 3 d. The growth medium consisted of Dulbecco's modified Eagle Medium (D-MEM; Cat.#31600-034, Invitrogen, Carlsbad, USA) supplemented with 10% fetal bovine serum (FBS; Cat.#12303-500M, SAFC Biosciences, Wicklow, Ireland), 100 units/mL penicillin, and 100 mg/mL streptomycin (Cat.#15140-122, Invitrogen). VSMCs used for experiments were from the third to the fifth passages.

Reverse-transcription polymerase chain reaction (RT-PCR) was performed as described previously (*17*). Total RNA for use in RT-PCR was isolated using the Mini RNA isolation kit (Cat.#R1005, ZYMO RESEARCH, Orange, USA). For cDNA synthesis, 1 μg of total RNA was reverse-transcribed with random hexamers using SuperScript II RT (Cat.#18064-022, Invitrogen). The transcribed cDNA was amplified by PCR with specific primers for procollagen α1(I), procollagen α1(III), tropoelastin, p22phox, SAP, MMP-2, MMP-9, and glyceraldehydes-3-phosphate dehydrogenase (GAPDH). Two specific primer pairs corresponding to published sequences were used to amplify procollagen α1(I) (5′-CAGCCGCTTCACCTACAGC-3′ and 5′-AATCACTGT CTTGCCCCAGG-3′) (*18*), procollagen α1(III) (5′-TCCAACTGCTCCTACTCGCC-3′ and 5′-GAGGGCCTG GATCTCCCTT-3′) (*18*), tropoelastin (5′-GGTGCGGTG GTTCCTCAGCCTGG-3′ and 5′-GGGCCTTGAGATACC

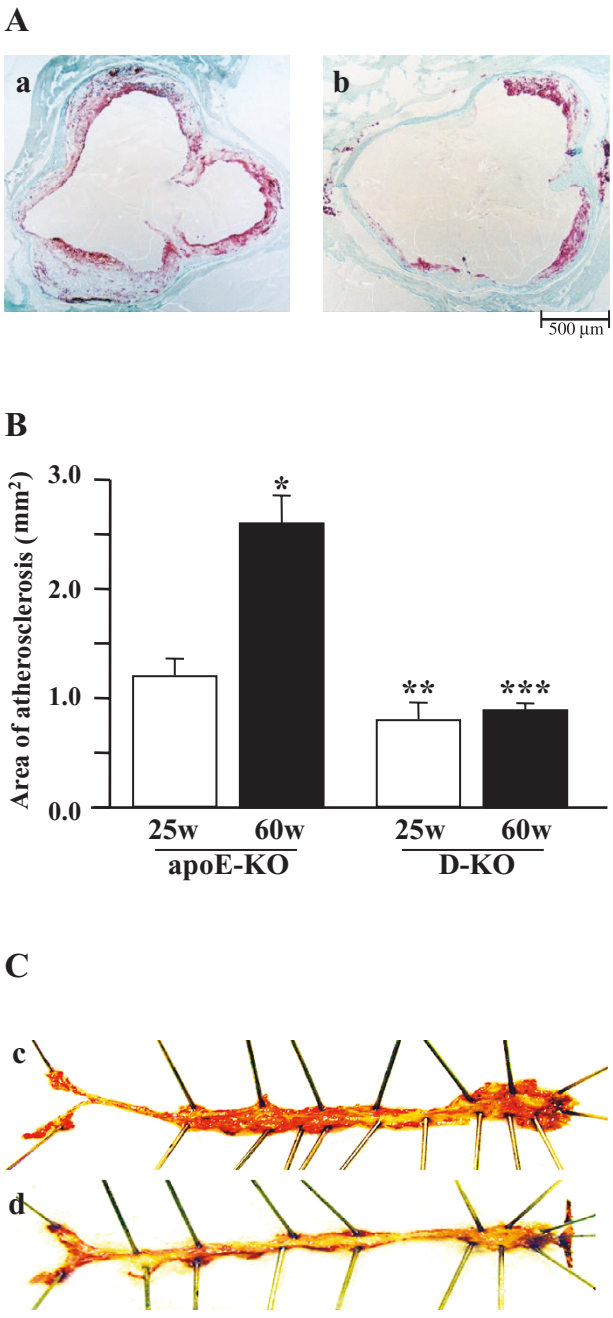


**Fig. 2.** *Atherosclerotic lesions. A: Representative oil red O stained cross-sections of the aortic root of apoE-KO mice (a) and D-KO mice (b) at 60-week-old. B: Quantification of atherosclerotic volume in aortic root. Atherosclerotic lesions in D-KO mice were significantly smaller than those in apoE-KO mice at 25- and 60-week-old. *p < 0.001 vs. 25-week-old apoE-KO mice, **p < 0.001 vs. 25-week-old apoE-KO mice, ***p < 0.001 vs. 60-week-old apoE-KO mice. C: Oil red O stained en face preparation of longitudinally opened aortas of a 60-week-old apoE-KO mouse (c) and a D-KO mouse (d).*

CCAGTG-3′) (*19*), p22phox (5′-TGGGCGGCTGCT TATGGT-3′ and 5′-GTTTGTGTGCCTGCTGGAGT-3′) (*20*), SAP (5′-CTCAGACAGACCTCAATCAG-3′ and 5′-TCAGCAATACCAGAGGAGGA-3′) (*21*), MMP-2 (5′-ACCCAGATGTGGCCAACTAC-3′ and 5′-TACTTT TAAGGCCCGAGCAA-3′) (*22*), MMP-9 (5′-ATGATG GAGGAGAAGCAGTC-3′ and 5′-AGGTGAAGGGAA AGTGACAT-3′) (*23*), and GAPDH (5′-CAGGAATTCGGT GAAGGTCGGAGTCAAGGG-3′ and 5′-AGTGGATCC GGTCATGAGTCCTCCCAGGAT-3′) (*24*). The PCR amplification protocol included 35 cycles of denaturing, annealing, and elongation with *Taq* polymerase (Cat.#R001A, TAKARA BIO Inc., Shiga, Japan). Equal amounts of PCR products were subjected to electrophoresis through 1.5% agarose gels and visualized with ethidium bromide. The quantification of procollagen α1(I), procollagen α1(III), tropoelastin, p22phox, SAP, MMP-2, MMP-9, and GAPDH mRNA was analyzed by real-time PCR using LightCycler FastStart DNA Master SYBR Green I Kit (Cat.#3003230, Roche Diagnostics K.K., Tokyo, Japan) as described previously (*25*). GAPDH expression was used as a reference for quantification of the respective mRNAs.

## Western Blotting

For Western blotting of collagen type I and elastin, proteins were extracted from apoE-KO and D-KO aortas as reported previously (*17*). The aorta was ground to a fine powder under liquid nitrogen and incubated in ice cold 0.1% Triton lysis solution (10 mmol/L HEPES [pH 7.4], 50 mmol/L sodium pyrophosphate, 50 mmol/L NaF, 5 mmol/L EDTA, 5 mmol/L EGTA, 50 mmol/L NaCl, 100 mmol/L $Na_3VO_4$, 0.1% Triton X-100, 500 mmol/L PMSF, and 10 mg/mL leupeptin) for 30 min.

For Western blotting of p22phox, SAP, MMP-2, MMP-9, activated caspase-3, and AIF, cytoplasmic and nuclear proteins were extracted from cultured VSMCs of apoE-KO and D-KO using the Protein and RNA Isolation System (Cat.#1921, Ambion Inc., Austin, USA).

Insoluble matter was removed by centrifugation, and the protein concentration was measured by a bicinchoninic acid assay (PIERCE Biotechnology Inc., Rockford, USA). Western blotting was performed with a NuPAGE™ Electrophoresis System (Cat.#NPO322BOX, Invitrogen) as reported previously (*17*). Briefly, 10-μg protein samples were resuspended in reduced sample buffer, electrophoresed on a 4–12% Bis-Tris gel (Invitrogen) with MOPS running buffer, blotted to nitrocellulose membrane, and sequentially probed with a goat polyclonal antibody against human collagen type I (Cat.#sc-27954, Santa Cruz Biotechnology), a goat polyclonal antibody against elastin (Cat.#sc-17581, Santa Cruz Biotechnology), a goat polyclonal antibody against human p22phox (Cat.#sc-11712, Santa Cruz Biotechnology), a goat polyclonal antibody against mouse SAP (Cat.#sc-18312, Santa Cruz Biotechnology), a goat polyclonal antibody

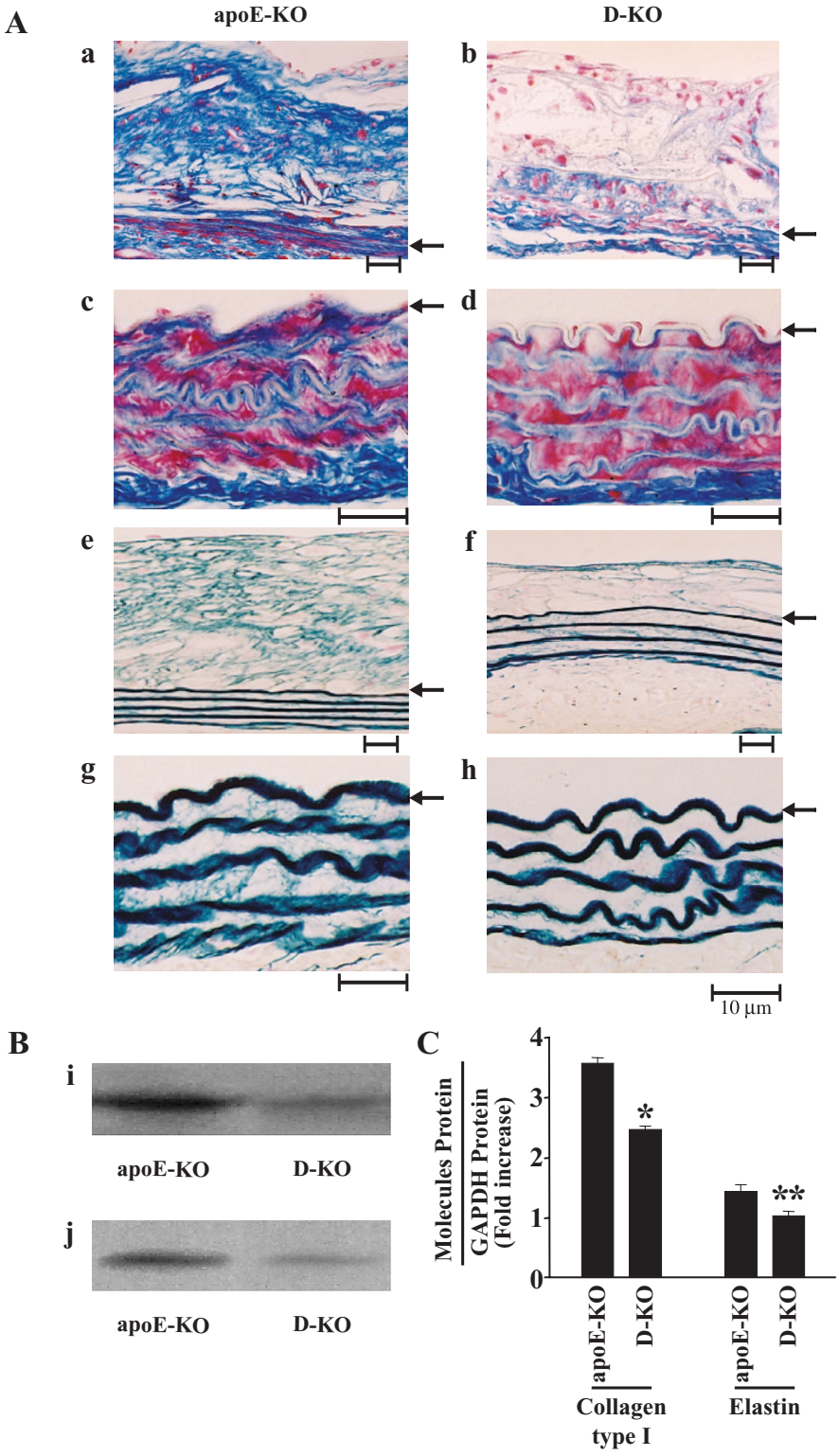


**Fig. 3.** *Extracellular matrix. A: Extracellular matrix was analyzed in atherosclerotic lesions of the aortic root in apoE-KO (a, e) and D-KO mice (b, f) at 60 weeks of age. The amount of collagen detected by Azan staining (b, d) and the amount of elastin detected by Victoria blue staining (f, h) in atheromas or non-atherosclerotic aortas of D-KO mice were less than those of apoE-KO mice. The arrows indicate internal elastic lamina. B: Western blotting for collagen type I (i) and elastin (j) of aorta. C: Densitometry of Western blots for collagen type I and elastin protein of aorta. The amounts of collagen type I and elastin proteins of D-KO mice were less than those of apoE-KO mice. *$p < 0.01$ vs. apoE-KO mice, **$p < 0.05$ vs. apoE-KO mice.*

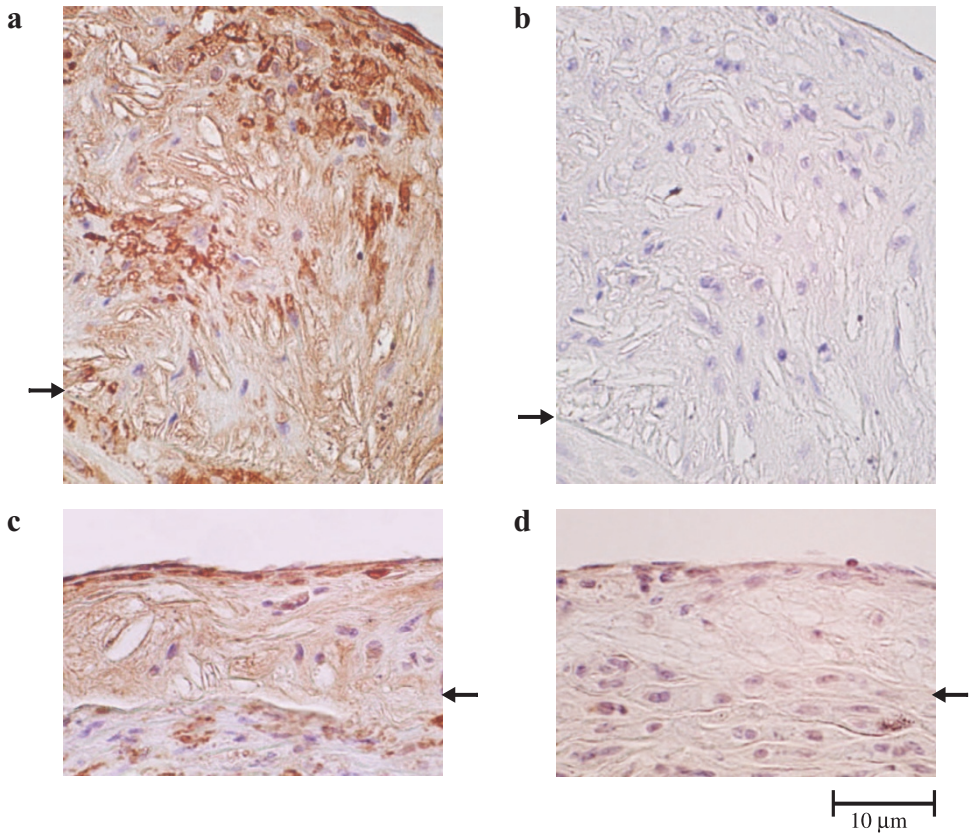


**Fig. 4.** *Immunohistochemical staining for muscle actin (a, c) and CD-64 (b, d) of apoE-KO (a, b) and D-KO mice (c, d) at 60 weeks of age. Muscle actin is a marker for VSMCs, and CD-64 is a marker for macrophages. The immunoreactivity of muscle actin was detected in apoE-KO and D-KO at 60-week-old, but the immunoreactivity of CD-64 was only slightly apparent. The arrows indicate internal elastic lamina.*

against human MMP-2 (Cat.#sc-6838, Santa Cruz Biotechnology), a rabbit polyclonal antibody against human MMP-9 (Cat.#sc-10737, Santa Cruz Biotechnology), a rabbit polyclonal antibody against human activated caspase-3 (Cat.#557035, Santa Cruz Biotechnology), and a goat polyclonal antibody against human AIF (Cat.#sc-9416, Santa Cruz Biotechnology). Either horseradish peroxidase–conjugated rabbit anti-goat antibody (Santa Cruz Biotechnology) or donkey anti-rabbit antibody (Santa Cruz Biotechnology) was then added, and the secondary antibody was detected by autoradiography using enhanced chemiluminescence (Cat.#RPN2132, ECL Plus, GE Healthcare UK Ltd., Little Chalfont, UK). GAPDH expression was used as a reference for quantification of the respective proteins.

### Statistical Analysis

All calculated data are presented as the mean±SD. Statistical significance was evaluated using unpaired Student's *t*-test for comparisons between two groups. A probability value of $p<0.05$ was considered statistically significant.

## Results

### Hemodynamic Parameters, Body Weight, Cholesterol Concentration, and 8-Isoprostane

At both 25- and 60-week-old, there were no significant differences in mean blood pressure, heart rate, total- and HDL-cholesterol concentrations, or body weight between the apoE-KO and D-KO mice. In both apoE-KO and D-KO mice, although there was no significant difference in mean blood pressure, total- and HDL-cholesterol concentrations, or body weight between 25- and 60-week-old, the heart rate at 60-week-old was significantly higher than in those at 25-week-old (Fig. 1).

The plasma concentration of 8-isoprostane in the D-KO mice was significantly lower than that in the apoE-KO mice at 60-week-old (135.7±18.1 *vs*. 230.7±36.0 pg/mL, $p<0.05$).

### Atherosclerosis and Extracellular Matrix

We analyzed atherosclerotic lesions in aortic roots and ECM production in both the aortic root and aorta. Figure 2A shows

representative oil red O stained cross-sections of the aortic root of apoE-KO and D-KO at 60-week-old. Figure 2C demonstrates oil red O stained en face preparations of longitudinally opened aortas of 60-week-old apoE-KO and D-KO mice. The atherosclerotic area of D-KO mice was smaller than of apoE-KO mice. The quantification of atherosclerotic volume in the aortic root revealed that the atherosclerotic lesion of D-KO mice was significantly smaller than that of apoE-KO mice at 25-week-old ($0.81 \pm 0.16$ *vs*. $1.05 \pm 0.21$ $mm^2$, $p < 0.001$) and at 60-week-old ($0.89 \pm 0.11$ *vs*. $2.44 \pm 0.28$ $mm^2$, $p < 0.001$). Surprisingly, there was no significant difference in atherosclerotic lesion size of D-KO mice at 25- and 60-week-old, and AT1a deficiency completely protected against the age-related progression of atherosclerosis (Fig. 2B).

We analyzed the ECM in atherosclerotic lesions of the aortic root. Azan staining for collagen and Victoria blue staining for elastin were carried out to assess matrix production. Expression of collagen type I and elastin proteins in aorta was quantitatively analyzed by Western blotting. The amount of collagen and elastin in atheromas of D-KO mice was less than that of apoE-KO mice (Fig. 3).

### Immunohistochemistry

In order to analyze factors that are related to plaque vulnerability, immunohistochemical staining was performed using primary antibodies against muscle actin, CD-64, p22phox, SAP, MMP-2, MMP-9, or AT2. Muscle actin is a marker for VSMCs, and CD-64 is a marker for macrophages. p22phox is an essential component of nicotinamide adenine dinucleotide phosphate (NAD(P)H) oxidase, which causes oxidative stress. SAP is highly inducible during the acute-phase response in mice and is a marker for inflammation. MMP-2 and MMP-9 may be co-related with plaque stability in atherosclerotic lesions.

The immunoreactivity of muscle actin was detected in atherosclerotic lesions of apoE-KO and D-KO mice at 60-week-old, but the immunoreactivity of CD-64 was only slightly apparent (Fig. 4). Thus, the majority of cells in the atherosclerotic lesions in old mice were found to be VSMCs, not macrophages. The immunoreactivities of p22phox, SAP, MMP-2, and MMP-9 in atherosclerotic lesions of D-KO mice were lower than those of apoE-KO mice (Fig. 5a–h). Furthermore, using immunohistochemistry, we analyzed the expression of AT2, which can act as an antagonist of AT1 responses such as blood pressure, vascular reactivity, and apoptosis. We hypothesized that the absence of AT1 may lead to a compensatory up-regulation of AT2. However, AT2 expression in atherosclerotic lesions of D-KO mice did not differ from those of apoE-KO mice (Fig. 5i, j).

### Cell Experiments

To confirm the precise effect of AT1 on the expression of procollagen $\alpha$1(I), procollagen $\alpha$1(III), tropoelastin, p22phox, SAP, MMP-2, and MMP-9 in VSMCs, we performed cell culture experiments using VSMCs explanted from the medial layer of aortas from apoE-KO or D-KO mice. mRNA expression of these molecules was evaluated by RT-PCR and real-time PCR. Decreases in mRNA expression of procollagen $\alpha$1(I), procollagen $\alpha$1(III), tropoelastin, p22phox, SAP, MMP-2, and MMP-9 were found in VSMCs of D-KO mice compared with apoE-KO mice (Fig. 6A, B).

We also performed Western blotting to analyze the protein expression of p22phox, SAP, MMP-2, and MMP-9 in cultured VSMCs. The level of protein expression of these molecules decreased in D-KO mice compared with apoE-KO mice (Fig. 6C).

### Apoptosis

We analyzed apoptosis in atherosclerotic lesions of D-KO and apoE-KO mice at 60-week-old by immunohistochemistry. The expression of activated caspase-3 and AIF in atherosclelotic lesions decreased in D-KO mice compared with apoE-KO mice. Moreover, expressions of activated caspase-3 and AIF of cultured VSMCs from D-KO mice were lower than those from the apoE-KO mice (Fig. 7).

## Discussion

AT1 may contribute to atherogenesis by facilitating proliferative and inflammatory responses to hypercholesterolemia. To clarify the long-term effect of AT1 in atherogenesis, AT1a-KO mice were crossed with apoE-KO mice, resulting in D-KO mice. Although there was no significant difference in blood pressure or total- and HDL-cholesterol concentrations between D-KO and apoE-KO mice, the atherosclerotic lesions of D-KO mice were significantly smaller than those of apoE-KO mice. The amount of collagen and elastin, the expression of p22phox, SAP, MMP-2 and MMP-9, and the number of apoptotic cells in atherosclerotic lesions of D-KO mice were lower than those of apoE-KO mice. However, AT2 expression in atherosclerotic lesions of D-KO mice did not differ from AT2 expression in the lesions of apoE-KO mice. Furthermore, we confirmed that the expression of procollagen $\alpha$1(I), procollagen $\alpha$1(III), tropoelastin, p22phox, SAP, MMP-2, and MMP-9 decreased in cultured VSMCs of D-KO mice compared with apoE-KO mice.

Ang II activates NAD(P)H oxidase, which is a major source of ROS production by vascular cells (*26*). Oxidative stress initiates several processes involved in atherogenesis, including expression of adhesion molecules, stimulation of VSMC proliferation and migration, apoptosis in the endothelium, oxidation of lipids, activation of matrix metalloproteinases, and altered vasomotor activity (*27*, *28*). We confirmed that AT1 deficiency reduced the plasma concentration of 8-isoprostane.

Vascular NAD(P)H oxidase consists of a cytochrome b558,

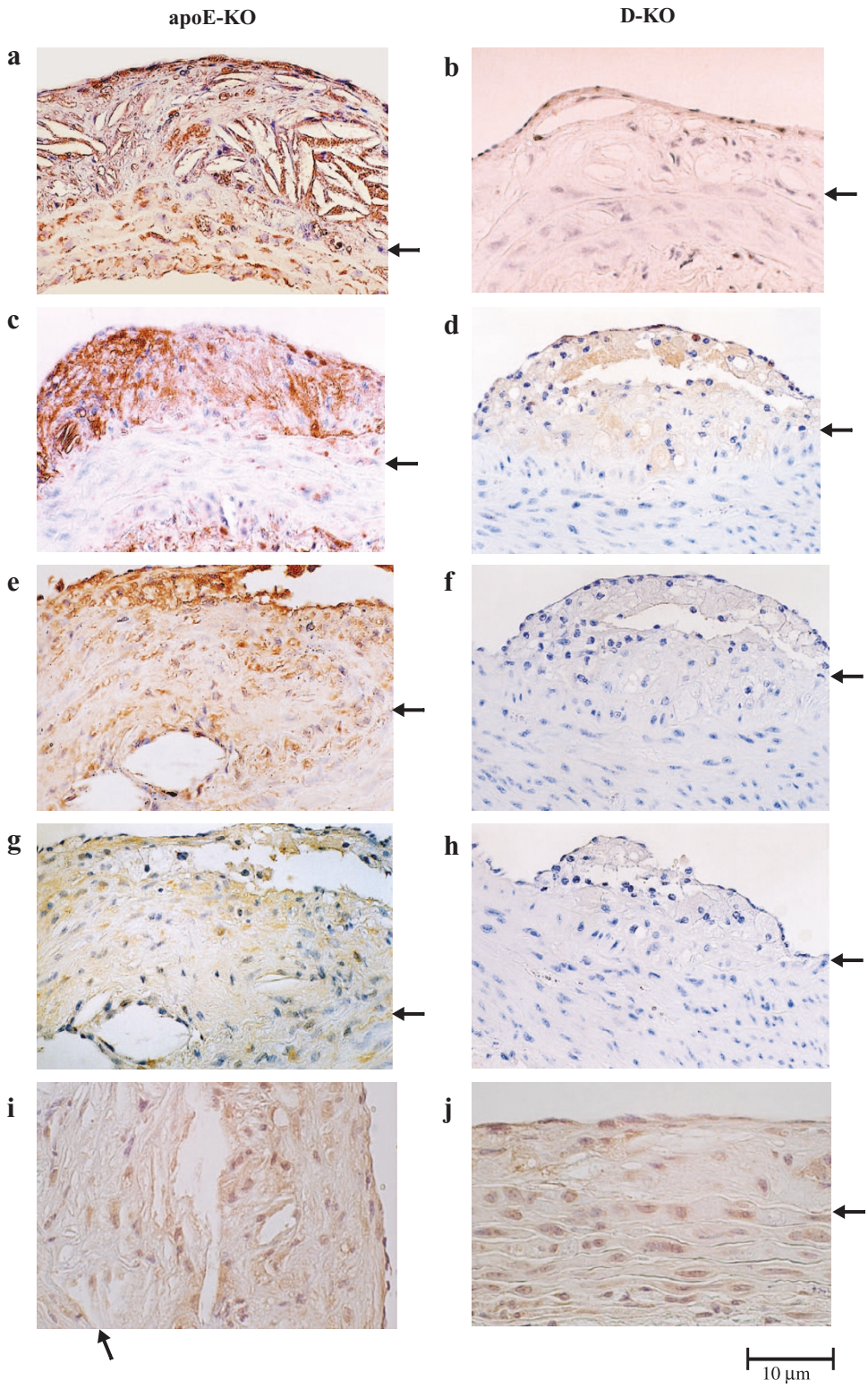


**Fig. 5.** *Immunohistochemical staining for p22phox (a, b), SAP (c, d), MMP-2 (e, f), MMP-9 (g, h), and AT2 (i, j) of apoE-KO (a, c, e, g, i) and D-KO mice (b, d, f, h, j) at 60-week-old. The immunoreactivities of p22phox, SAP, MMP-2, and MMP-9 of D-KO mice (b, d, f, h) in atherosclerotic lesions were less than those of apoE-KO mice (a, c, e, g). However, the immunoreactivities of AT2 of D-KO mice (j) in atherosclerotic lesions did not differ from those of apoE-KO mice (i). The arrows indicate internal elastic lamina.*

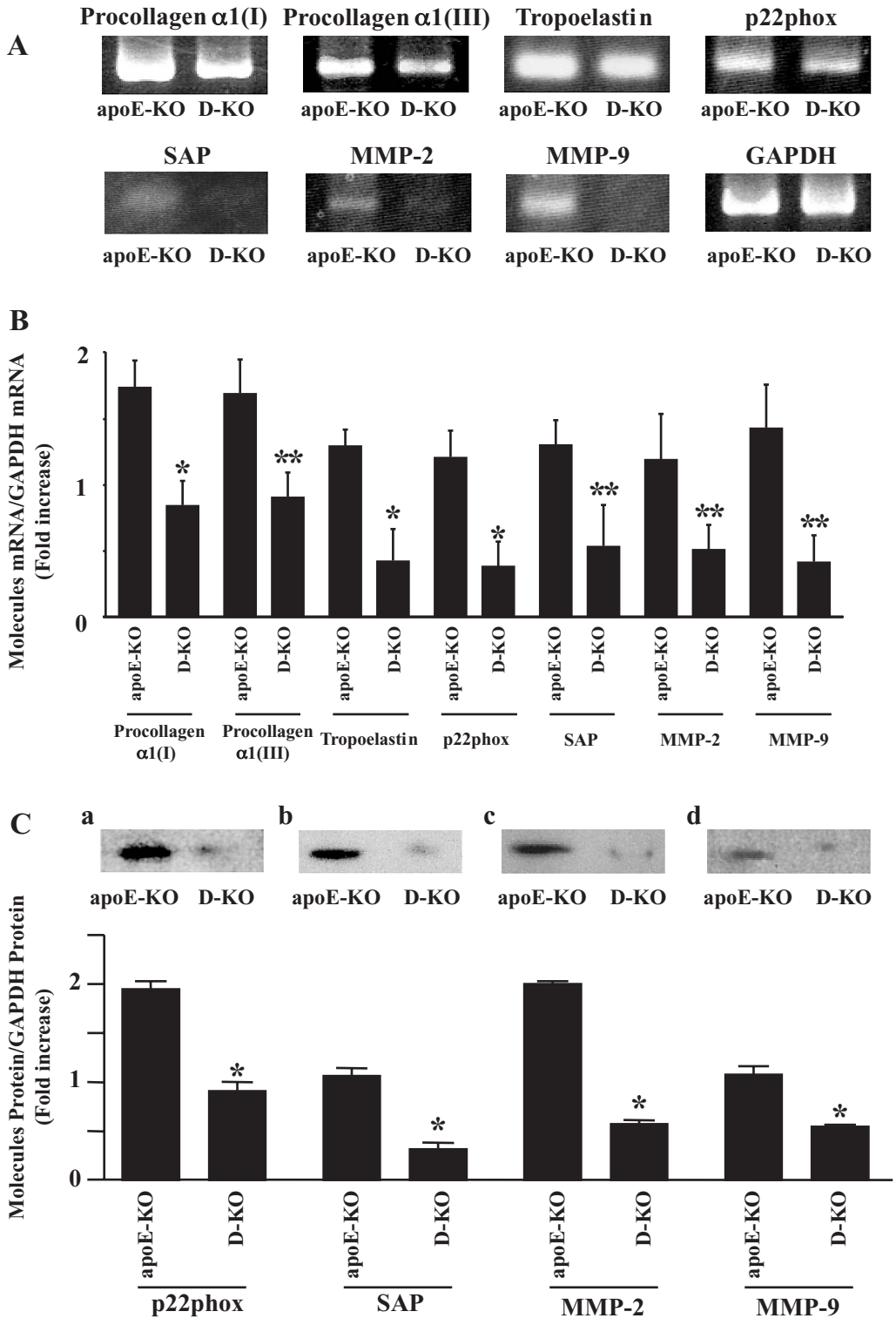


**Fig. 6.** *A: The expression of mRNA for procollagen α1(I), procollagen α1(III), tropoelastin, p22phox, SAP, MMP-2, and MMP-9 in cultured VSMCs from apoE-KO and D-KO mice by RT-PCR. B: Expression of mRNA for procollagen α1(I), procollagen α1(III), tropoelastin, p22phox, SAP, MMP-2, and MMP-9 in cultured VSMCs from apoE-KO and D-KO mice by real-time PCR. Decreases in the mRNA expression of these molecules were seen in VSMCs from D-KO mice in comparison with apoE-KO mice. C: Western blotting for p22phox (a), SAP (b), MMP-2 (c), and MMP-9 (d) in cultured VSMCs from apoE-KO or D-KO mice. C shows that the level of protein expression of these molecules decreased in the D-KO mice in comparison with the apoE-KO mice. *$p < 0.01$ vs. apoE-KO mice, **$p < 0.05$ vs. apoE-KO mice.*

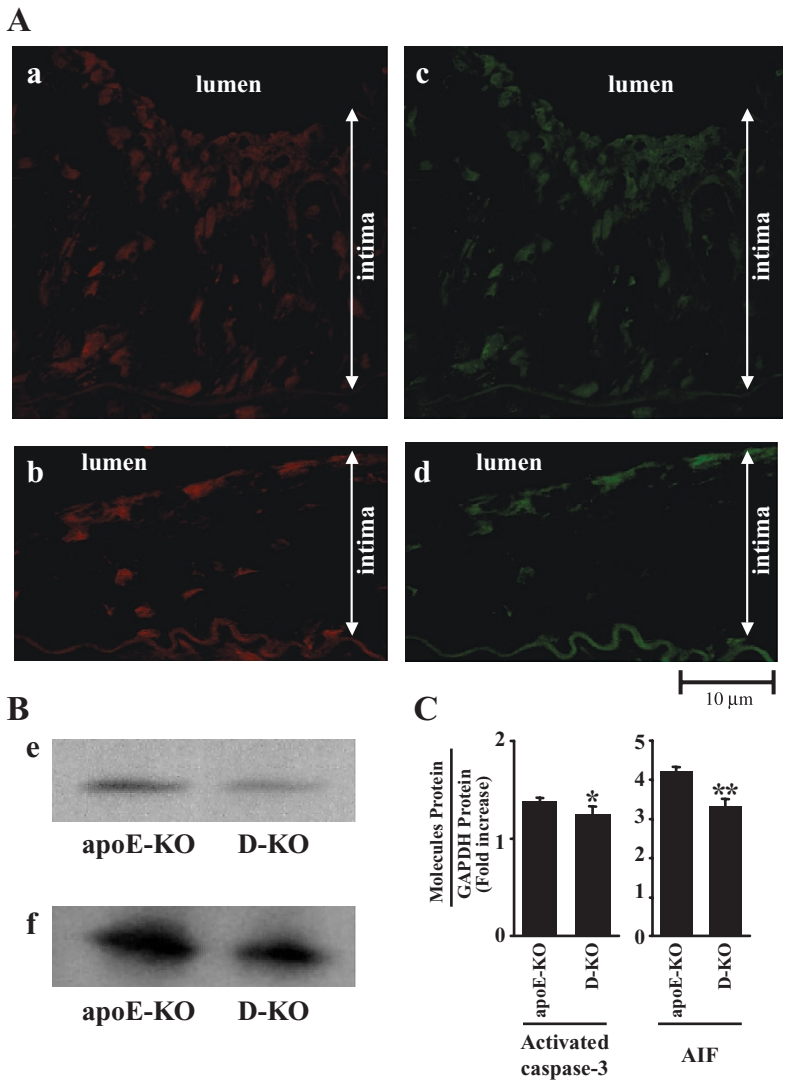


**Fig. 7.** *A: Immunofluorescence staining for the analysis of apoptosis in atherosclerotic lesions of apoE-KO (a, c) and D-KO mice (b, d) at 60-week-old. The expression of activated caspase-3 (a, b) and AIF (c, d) decreased in D-KO mice compared with apoE-KO mice. B: Western blotting for activated caspase-3 (e) and AIF (f) of cultured VSMCs from apoE-KO or D-KO mice. C: Densitometry of Western blots for activated caspase-3 and AIF of cultured VSMCs from apoE-KO or D-KO mice. The level of protein expression of these molecules decreased in D-KO mice in comparison with the apoE-KO mice. *$p < 0.05$ vs. apoE-KO mice, **$p < 0.01$ vs. apoE-KO mice.*

which is composed of p22phox and gp91phox subunits and three cytosolic components, rac, p47phox, and p67phox (*29*). Ang II was found to activate p47phox *via* AT1 (*30*). In addition, ARB was reported to inhibit p22phox mRNA expression caused by mechanical stretching and in patients with diabetes mellitus (*31*, *32*). We also showed that AT1a deficiency in apoE-KO mice inhibited the expression of p22phox at 60 weeks of age.

Superoxide dismutase (SOD) prevents increases in vascular ROS. In addition, activation of AT1 has been reported to contribute to a decrease in the expression of extracellular SOD, and ARB increased the serum SOD activity in patients with hypertension (*33*, *34*). Although we did not measure the SOD activity in the present study, AT1a deficiency might increase the SOD activity and prevent age-related atherosclerosis in the apoE-KO mice.

AT1 deficiency was reported to have a striking effect in reducing hypercholesterolemia-induced atherosclerosis in apoE-deficient mice at 19-week-old (*10*) and LDL receptor deficient mice at 20-week-old (*11*). However, the effect of AT1a deficiency on apoptosis, matrix production, or MMP in atherosclerosis of apoE-KO mice has not been previously determined. The present study is the first report demonstrating that AT1 deficiency reduces atherosclerosis in apoE-KO

mice *via* a reduction of oxidative stress, apoptosis, matrix production, and MMP expression at 60-week-old.

Ang II stimulation of AT1 has been reported to inhibit apoptosis in cultured VSMCs, and we expected that AT1 deficiency would increase apoptosis in atherosclerotic lesions. However, the expression of activated caspase-3 and AIF decreased in atherosclerotic lesions of D-KO mice in comparison with apoE-KO mice at 60-week-old. In addition, there is no report that demonstrates stimulation of apoptosis in atherosclerotic lesions by the clinical use of ARB. We speculate that the reduction of oxidative stress may lead to the inhibition of apoptosis in the atherosclerotic lesions of D-KO mice.

Acute coronary syndromes result from fissure, erosion, or rupture of vulnerable atherosclerotic plaques. The characteristics of a vulnerable plaque include a large lipid pool, an abundance of inflammatory cells and mediators, a reduced smooth muscle cell and collagen content, and a thin overlying fibrous cap (*35*). Not only do lipids in the atheroma create mechanical instability, but biologically active lipids participate in promoting oxidative stress and inflammatory responses. Keidar *et al*. (*36*) reported that an AT1 antagonist inhibited LDL lipid peroxidation and atherosclerosis in apoE-KO mice. Blocking AT1 may play an important role in the attenuation of atherogenesis.

Although the reduction of ECM production in the aorta leads to aortic distensibility, decreased ECM production in atheromas may cause plaque vulnerability. However, a reduction of oxidative stress, apoptosis, and MMP in atheromas by AT1a deficiency may contribute to plaque stabilization.

Daugherty *et al*. (*11*) reported that AT1 deficiency reduces hypercholesterolemia-induced atherosclerosis in LDL receptor deficient mice. Absence of AT1a has the potential to lead to a compensatory change in AT2 abundance. However, AT2 mRNA expression was not altered, and Ang II type 1b receptor (AT1b) mRNA expression was increased in the aorta of AT1a-deficient mice. They showed that AT1a deficiency in LDL receptor deficient mice had no significant effect on systolic blood pressure. Our study also demonstrated that AT1a deficiency in apoE-KO mice had no significant effect on blood pressure. We hypothesize that AT1a deficiency may reduce hypercholesterolemia-induced atherosclerosis independent of blood pressure changes.

Similar to the results reported by Daugherty *et al*. (*11*), we also observed no compensatory up-regulation of the AT2 mRNA expression in the atherosclerotic lesions of D-KO mice. Moreover, lack of any effect of AT2 deficiency on the atherosclerotic lesion area in the LDL receptor deficient mice has also been reported (*11*). Therefore, we speculate that AT2 does not contribute significantly to atherosclerosis in mice.

In conclusion, AT1a deficiency reduced atherosclerotic lesion size of apoE-KO mice and protected against the age-related progression of atherosclerosis. Reduction of oxidative stress, apoptosis, and MMP in atheromas by AT1a deficiency may contribute to plaque size.

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