Increased aortic stiffness assessed by pulse wave velocity in apolipoprotein E-deficient mice

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Wang, Yi-Xin, Meredith Halks-Miller, Ron Vergona, Mark E. Sullivan, Richard Fitch, Cornell Mallari, Baby Martin-McNulty, Valdecia Da Cunha, Ana Freay, Gabor M. Rubanyi, and Katalin Kauser. Increased aortic stiffness assessed by pulse wave velocity in apolipoprotein E-deficient mice. Am. J. Physiol. Heart Circ. Physiol. 278: H428–H434, 2000.—Atherosclerosis develops and progresses spontaneously in apolipoprotein E-knockout (apoE-KO) mice. A direct consequence of atherosclerosis is an increase in vascular stiffness. Pulse wave velocity (PWV) has been used to assess the stiffness of large vessels and was found to be increased in patients with atherosclerosis. In the present study, aortic stiffness was assessed by PWV in 4- and 13-mo-old apoE-KO mice and age-matched controls (C57BL/6J). In 13-mo-old apoE-KO mice with extensive atherosclerotic lesions in the aorta (61 ± 4%), PWV increased significantly (3.8 ± 0.2 m/s) compared with controls (2.9 ± 0.2 m/s). Endothelial nitric oxide (EDNO)-mediated vasorelaxation in response to ACh was markedly diminished in the aortic rings isolated from 13-mo-old apoE-KO mice compared with age-matched controls. In contrast, in 4-mo-old apoE-KO mice with only moderate atherosclerotic lesions in the aorta (23 ± 5%), there were no significant changes in PWV and EDNO-mediated relaxation compared with controls. Blood pressure was not different among the four groups of mice. There were no significant differences in endothelium-independent vascular responses to sodium nitroprusside among different groups investigated. Histological evaluation revealed focal fragmentation of the elastic laminae in the aortic walls of 13-mo-old apoE-KO mice. These results demonstrate for the first time that aortic stiffness determined by PWV increases in 13-mo-old apoE-KO mice. Endothelial dysfunction and elastic destruction in vascular wall caused by atherosclerosis may have contributed.

A common consequence of atherosclerosis is an increase in the stiffness of the aorta and major arteries, resulting in decreased vascular elasticity and compliance (16, 18). The stiffness of conduit vessels can be estimated by pulse wave velocity (PWV). The stiffer the vessel, the faster the pulse wave moving along the aorta. PWV is an accepted parameter for estimating elasticity and compliance of large vessels in humans (2), nonhuman primates (12, 13), dogs (22), rabbits (30), rats (3), and mice (15). It has been used as a surrogate marker for vascular diseases (4, 18), including atherosclerosis (30). For example, PWV has been used in laboratory animal studies to evaluate the antiatherosclerotic effect of a fish oil in rabbits fed high cholesterol (30) and in clinical studies to evaluate the therapeutic effects of antihypertensive agents on the elastic properties of vascular wall (4).

A properly functioning endothelium produces nitric oxide (NO) and other vasoactive substances that are able to protect the vascular wall against noxious stimuli by multiple mechanisms of action (11). NO has been shown to inhibit platelet aggregation and adhesion, to attenuate leukocyte and monocyte adhesion and transmigration, to suppress smooth muscle proliferation, and, most well known, to induce vasorelaxation (6). NO has also been shown to react with oxygen free radicals (27) and interferes with redox-sensitive transcription of proinflammatory molecules (vascular cell adhesion molecule 1, intercellular adhesion molecule 1, monocyte chemoattractant protein 1, macrophage colony-stimulating factor, and others) by inhibiting the activation of nuclear factor-kB (25).

Apolipoprotein E (apoE) is an important mediator for hepatic metabolic clearance of circulating cholesterol. When apoE polypeptide is dysfunctional or absent, severe hyperlipidemia occurs in humans or animal models. Atherosclerosis develops and progresses spontaneously in apolipoprotein E-knockout (apoE-KO) mice. Histological changes in atherosclerotic plaques in the aorta and other large vessels of apoE-KO mice have been extensively investigated. However, the changes in physical properties as well as endothelial function of the aorta in connection to the progression of atherosclerosis have not been well characterized in this animal model of atherosclerosis. Therefore, the present study was designed to investigate the relationship among the aortic elasticity, as measured by PWV; the degree of endothelial dysfunction, as measured by ACh-induced relaxation of isolated aortic rings; and the morphological changes of the aorta, as determined by plaque area and histological examination.

MATERIALS AND METHODS

Animals

Experiments were carried out in 4- and 13-mo-old apoE-KO and age-matched control (C57BL/6J) mice obtained from...
Jackson Laboratories (Bar Harbor, ME). Animals were kept in a room at controlled temperature (24°C) and lighting (14:10-h light-dark cycle) with free access to food and tap water.

At death, blood samples were collected by cardiac puncture from the animals into serum separator tubes (Microtainer, Becton Dickinson, NJ) and centrifuged at 1,000 g for 10 min. Serum total cholesterol levels were measured by Consolided Veterinary Diagnostics (West Sacramento, CA).

In Vivo Measurement of PWV As an Index for Aortic Elasticity

Surgical preparation. Mice were anesthetized with a mixture of ketamine (120 mg/kg, Ketaset; Fort Dodge Laboratories, Fort Dodge, IA) and xylazine (7 mg/kg, Rompun; Bayer, Shawnee Mission, KS) injected intramuscularly. A Millar Mikro-tip pressure transducer (1.4-Fr, Millar Instruments, Houston, TX) was inserted via the left carotid artery into the aortic arch for measuring intravascular arterial blood pressure (proximal pressure wave). The abdominal aorta was exposed through a suprapubic incision. Another Millar catheter (1.8-Fr) was positioned underneath the abdominal aorta at a site just above the iliac bifurcation and was oriented to record extravascular tonometric pressure wave outside the vessel (distal pressure wave). The best signals were obtained by orienting the catheter tip at a right angle to the vessel axis and the side-mounted sensor flat against the vessel. By taking simultaneous measurements of extravascular tonometric pressure and Doppler velocity waveforms at the same site on the mouse aorta, Hartley et al. (15) demonstrated that upstrokes of the pressure and velocity waves occur simultaneously within the resolution of the measurement technique. When the surgery was completed, all wounds were closed to minimize evaporative loss of fluid.

Experimental procedures. The mice were placed on a heating board at 37°C to maintain body temperature. When blood pressure and heart rate had been stable for ~30 min, the pressure waves from the aortic arch and abdominal aorta were recorded simultaneously for at least 30 min. The signals were digitized and stored using a data acquisition system (PowerLab 16/s, ADInstruments, Castle Hill, New South Wales, Australia). Samples were acquired at a rate of 1,000 Hz. Because the Millar pressure transducer has a frequency response from flat to 10,000 Hz, it is able to provide high-fidelity measurements of the pressure waveforms of the mice.

Data analysis. Systolic arterial pressure (SAP), diastolic arterial pressure (DAP), pulse pressure (PP), mean arterial blood pressure (MAP), and heart rate (HR) were calculated by the data acquisition system from the proximal pressure wave. The propagation time for the pulse wave moving from the aortic arch to the abdominal aorta was measured by the time delay between the upstrokes (foot) of each pressure wave front. Measurements reported in the text were made by averaging at least 10 normal consecutive cardiac cycles. After the experiments were completed, the animals were euthanized with an overdose of pentobarbital sodium (200 mg/kg) and the full length of the aorta was exposed. The distance between the two measurement sites was determined using a damp cotton thread stuck onto the aorta between the tips of the two pressure transducers and marked. The thread was then removed and laid straight for measurement of the distance between the two marks. PWV was then calculated by dividing the distance by the pulse wave propagation time in units of meters per second.

Ex Vivo Measurements of Endothelium-Dependent and -Independent Vasorelaxation of the Aortic Rings in Organ Chambers

Mice were euthanized with CO₂. The thoracic aortas were removed from the animals and cut into 2-mm segments. The aortic rings were placed inside a 15-ml tissue bath filled with Krebs (composition in mM: 118 NaCl, 4.7 KCl, 2.5 CaCl₂, 1.2 MgSO₄, 1.17 KH₂PO₄, 20 NaHCO₃, 0.026 EDTA, and 11 glucose) at 37°C, bubbled with 95% O₂-5% CO₂, and attached to a force transducer (model F30, Hugo Sachs Electronic) coupled to a data acquisition system (MP100, Biopac system) for measurement of isometric tension.

After the initial equilibration period of 1 h at a resting tension of 0.5 g, aortic rings were contracted twice with KCl (40 mM) followed by U-46619 (3 × 10⁻⁸ M), a dose that submaximally contracted the vessel to ~70% of the maximal response. After the plateau was reached, cumulative concentration-response curves to ACh and sodium nitroprusside (SNP) were sequentially generated. Responses are expressed in percent relaxation. The log molar concentration of the drug required to produce 50% of the maximum response was determined by computer-assisted interactive nonlinear regression analysis (GraphPad Prism, San Diego, CA).

Morphological Examination of the Aorta

Atherosclerotic plaque analysis. The proximal portion of the thoracic aorta up to the aortic origin was isolated, cleaned from the adherent connective tissue, fixed with 10% Formalin, cut open longitudinally, and pinned down individually on silicon-coated petri dishes. Atherosclerotic plaque areas are visible without staining. The images of the open luminal surface of the vessels were captured at a resolution of 512 × 512 by a red-green-blue three-chip charge-coupled device digital camera (Sony) mounted on a dissecting microscope (Nikon SMZ-2T) and recorded on an attached computer in 24-bit true image format. The analyses of the images were performed using C-Simple software (C. Imaging 1208, Compix, PA). The atherosclerotic plaque area was quantified and expressed as a percentage of the total luminal surface area of the thoracic aorta.

Histological examination of the aortic wall. Cross sections of the proximal aorta were obtained from 4- and 13-mo-old...
Table 1. Mean, systolic, diastolic, and pulse arterial blood pressure in control and apoE-KO mice

<table>
<thead>
<tr>
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<th>4 mo</th>
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<tr>
<td></td>
<td>Control</td>
<td>apoE-KO</td>
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<tr>
<td>No. of mice</td>
<td>6</td>
<td>6</td>
<td>7</td>
<td>7</td>
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<tr>
<td>MAP, mmHg</td>
<td>68 ± 3.2</td>
<td>71 ± 3.2</td>
<td>71 ± 6.4</td>
<td>75 ± 6.3</td>
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<tr>
<td>SAP, mmHg</td>
<td>82 ± 6.3</td>
<td>88 ± 3.8</td>
<td>90 ± 7.8</td>
<td>92 ± 6.3</td>
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<tr>
<td>DAP, mmHg</td>
<td>52 ± 4.6</td>
<td>57 ± 3.1</td>
<td>53 ± 4.1</td>
<td>62 ± 6.2</td>
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<tr>
<td>PP, mmHg</td>
<td>30 ± 3.3</td>
<td>30 ± 1.3</td>
<td>37 ± 4.5</td>
<td>31 ± 1.0</td>
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Values for pressure are means ± SE in 4- and 13-mo-old mice. MAP, mean arterial blood pressure; SAP, systolic arterial blood pressure; DAP, diastolic arterial blood pressure; PP, pulse pressure; apoE-KO, apolipoprotein E-knockout.

Statistical Analysis

Results are presented as means ± SE for the number of animals (n) indicated. Multiple comparisons of the mean values were performed by a two-way (group and age) ANOVA followed, if significance was indicated, by a subsequent Student-Newman-Keuls test for repeated comparisons. The data were tested for homogeneity of variances before the two-way ANOVA was performed. Differences were considered to be statistically significant when the P value was <0.05. The statistical analysis was processed with Statistica software (StatSoft, Tulsa, OK).

RESULTS

PWV in the Aorta: In Vivo Measurement

PWV was slightly, but not significantly, higher in 4-mo-old apoE-KO than in control mice (Fig. 1).

Table 2. Maximal vasorelaxation and half-maximal dose in response to ACh and SNP in control and apoE-KO mice

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<tr>
<td></td>
<td>Control</td>
<td>apoE-KO</td>
<td>Control</td>
<td>apoE-KO</td>
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<tr>
<td>Response to ACh</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>No. of mice</td>
<td>6</td>
<td>5</td>
<td>6</td>
<td>6</td>
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<tr>
<td>pD2, -log M</td>
<td>6.90 ± 0.14</td>
<td>6.96 ± 0.14</td>
<td>6.87 ± 0.12</td>
<td>6.43 ± 0.28</td>
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<tr>
<td>E_max, %</td>
<td>69 ± 3.7</td>
<td>72 ± 3.6</td>
<td>74 ± 5.9</td>
<td>42 ± 12*</td>
</tr>
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Response to SNP

<table>
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<th>4 mo</th>
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<th>13 mo</th>
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<tr>
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<td>Control</td>
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<td>No. of mice</td>
<td>6</td>
<td>6</td>
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<td>5</td>
</tr>
<tr>
<td>pD2, -log M</td>
<td>8.17 ± 0.09</td>
<td>8.31 ± 0.14</td>
<td>7.94 ± 0.19</td>
<td>8.27 ± 0.09</td>
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<tr>
<td>E_max, %</td>
<td>87 ± 3.7</td>
<td>94 ± 3.5</td>
<td>89 ± 0.1</td>
<td>92 ± 7.2</td>
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</table>

Values are means ± SE for maximal vasorelaxation (E_max) and half-maximal dose (pD2) in response to ACh and sodium nitroprusside (SNP) in 4- and 13-mo-old mice. *P < 0.05 vs. control.

Fig. 2. Endothelium-dependent vasorelaxation of aortic rings in response to ACh in 4-mo-old (A) and 13-mo-old (B) apoE-KO and control mice. Data are expressed as means ± SE.

Fig. 3. Endothelium-independent vasorelaxation of aortic rings in response to sodium nitroprusside in 4-mo-old (A) and 13-mo-old (B) apoE-KO and control mice. Data are expressed as means ± SE.
13-mo-old control mice, PWV was not statistically different from that in the 4-mo-old controls. PWV of the 13-mo-old apoE-KO was significantly increased compared with both age-matched controls and 4-mo-old apoE-KO mice. There were no statistically significant differences in MAP, SAP, DAP, and PP among the four experimental groups (Table 1). Although the age of the animals at the time of the experiments was the same for apoE-KO and control mice, body weight was lower in apoE-KO (28 ± 6 and 34 ± 1 g) than in control (30 ± 6 and 40 ± 1 g) mice of 4 and 13 mo of age, respectively. However, the distance between the proximal and distal measurement sites was not different between apoE-KO (38 ± 1 and 42 ± 1 mm) and control (38 ± 1 and 41 ± 1 mm) mice of 4 and 13 mo of age, respectively. Serum cholesterol levels were high in apoE-KO mice of 4 (480 ± 45 mg/dl) and 13 (730 ± 78 mg/dl) mo of age compared with controls of 4 (87 ± 4 mg/dl) and 13 (89 ± 7 mg/dl) mo of age.

Vasorelaxation of the Aortic Rings: In Vitro Organ Chamber Assay

Endothelial NO (EDNO)-mediated relaxation of the aortic rings in response to ACh was not significantly

Fig. 4. Photographs of plaque-covered luminal surface of aortas isolated from representative animals of 4-mo-old control (left) and apoE-KO (middle) and 13-mo-old apoE-KO (right) mice. Aortas were opened longitudinally to expose lumens surface and atherosclerotic plaques (arrows).

Fig. 5. Hematoxylin and eosin-stained sections of aortas from control mice of 4 (A) and 13 (C) mo of age appear completely normal. In a section from a 13-mo-old apoE-KO mouse (D), focal atherosclerotic plaque development is evident, whereas a section from a 4-mo-old apoE-KO mouse (B) has no visible lesion. All sections are shown at same magnification. Bar, 100 µm.
different between 4-mo-old apoE-KO and control mice (Fig. 2A). The maximal response and calculated half-maximal dose of ACh also were not significantly different between these two groups (Table 2). In 13-mo-old mice, however, the relaxation to ACh was significantly attenuated in apoE-KO mice compared with age-matched controls (Fig. 2B). The maximal response was significantly lower in apoE-KO than in control mice, whereas the half-maximal dose of ACh was not different between these two groups (Table 2). In contrast, endothelium-independent relaxation in response to SNP in both 4- and 13-mo-old apoE-KO mice was not affected compared with that in age-matched controls (Fig. 3). The maximal relaxation and half-maximal dose of SNP also were not significantly different among different groups (Table 2).

Morphological Analysis of the Aorta

Atherosclerotic plaques in the aorta. Image analysis of the open luminal surface of the Formalin-fixed aortic arch and thoracic aorta showed no detectable vascular lesions in either 4- or 13-mo-old controls (Fig. 4, left). In the 4-mo-old apoE-KO mice (n = 8), 23 ± 5% of the luminal surface area of the aortic arch and thoracic aorta was covered by plaques (Fig. 4, middle). In the 13-mo-old apoE-KO group (n = 6), over 61 ± 4% of the luminal surface area was covered by atherogenic plaques (Fig. 4, right).

Histological findings in the aortic wall. Microscopic examination revealed normal-appearing thoracic aorta in all the sections from control mice of both 4 (Fig. 5A) and 13 mo of age (Fig. 5C) and in the section from an apoE-KO mouse of 4 mo of age (Fig. 5B). There was no evidence of endothelial damage or intimal hyperplasia. In contrast, atherosclerotic plaques were identified in sections obtained from 13-mo-old apoE-KO mice as evidenced by intimal thickening, foam cell accumulation, and a thin collagen cap (Fig. 5D). As shown in Fig. 6, the elastic laminae underneath the plaque from the aorta of a 13-mo-old apoE-KO mouse had focal fragmentation as well as local destruction. Cells of apparent smooth muscle origin can be seen bridging the region from the vessel media to the plaque area.

DISCUSSION

The results of this study demonstrate for the first time that changes in PWV and endothelial function develop during the progression of atherosclerosis in apoE-KO mice. At 13 mo of age, PWV was significantly higher and EDNO-mediated vasorelaxation was attenuated in apoE-KO mice, with extensive atherosclerotic lesions and elastic laminar fragmentation in the aortic wall compared with age-matched controls as well as the 4-mo-old apoE-KO mice, without visible elastic laminar fragmentation in the aorta.

PWV can be influenced by several factors such as age, blood pressure, and hypercholesterolemia. It has been reported that the aorta becomes stiffer and less distensible with age (10). However, we found no significant difference in PWV between old and young control mice, suggesting that in the 9-mo interval that we examined, age-related vascular stiffening could not explain the increase in PWV in the 13-mo-old apoE-KO mice. High blood pressure also has been known to increase PWV (22). Because blood pressure was not significantly different among the four experimental groups under the conditions in which PWV was determined, it is not a contributing factor in this study. Hypercholesterolemia has also been shown to correlate to PWV increases (19). However, in the present study, cholesterol level alone is unlikely to influence our results directly because there were no measurable differences in PWV between the 4-mo-old apoE-KO and control mice despite the high levels of circulating lipids in the apoE-KO group. In support of this view, Farra et al. (12) reported that resuming a normal chow diet in cynomolgus monkeys after a high-cholesterol diet for 18 mo did not reverse the increase in PWV in the first 6 mo. After the atherogenic diet had been removed for 12 mo in these monkeys, PWV significantly decreased, and this decrease was also accompanied by a decrease in the ratio of intimal (plaque) to medial area in the aorta by that time (12). Therefore, high cholesterol does not seem to directly affect PWV. It is more likely that it increases vascular stiffness by promoting the progression of atherosclerotic lesions and vascular remodeling.
The pathology of atherosclerosis is characterized by massive infiltration of macrophages and foam cells into the aortic wall. Increased production of active matrix metalloproteinases from these cells triggers degradation of insoluble elastin and fibrillar collagen in the aortic wall (17). Indeed, the present study demonstrated focal breakdown of the elastic laminae in the media underneath the atherosclerotic plaques in the older apoE-KO mice. This result is consistent with the findings by Carmeliet et al. (8), who also reported fragmentation of elastic laminae and rupture of all elastic layers across the media of the aortic wall in apoE-KO mice. The integrity of the medial elastin in the conduit vessels is a major determinant of the arterial elasticity and the capacitive function of these arteries. Therefore, we postulate that the increase in PWV in the old apoE-KO mice could largely be caused by the breakdown of the elastin in the aortic wall of these animals.

One of the earliest events in atherogenesis is increased low-density lipoprotein (LDL) accumulation in the artery wall. Rutledge et al. (29) demonstrated that lipoprotein lipase bound to glycosaminoglycans increases LDL permeating across the endothelial layer, thus increasing retention and decreasing efflux of LDL from the artery wall. The trapped LDL becomes oxidized and has been found to decrease arterial elasticity, probably by affecting the matrix cross-link structure and contents in the vessel wall (31). This may also contribute to the aortic stiffening in old apoE-KO mice.

Endothelial dysfunction is characterized by impaired EDNO-mediated relaxation in atherosclerosis. Numerous studies have demonstrated impaired EDNO-mediated vasodilation in the coronary arteries (20, 26, 32–34) as well as systemically (1, 24) in patients with atherosclerosis or risk factors for atherosclerosis. The present study also demonstrated significant endothelial dysfunction in isolated aortic rings of the 13-mo-old apoE-KO mice. In contrast, endothelium-independent vasorelaxation in response to NO donor was not significantly affected in the same animals, indicating that the responsiveness of the vascular smooth muscle to NO is preserved. EDNO is believed to be an antiatherogenic molecule that antagonizes several pathophysiological processes contributing to plaque development simultaneously (5–7). Lack of EDNO can be considered a key progression factor in the development of atherosclerosis. The pathogenic link between decreased EDNO production and atherosclerosis is proven by animal experiments demonstrating accelerated atherosclerosis after chronic inhibition of NO formation by N-nitro-L-arginine methylester (L-NAME) (9). The increase in PWV may be a consequence of endothelial dysfunction. In a separate study in rats, we observed that acute inhibition of NO synthase (NOS) by L-NAME increased PWV independent of the increase in blood pressure, suggesting that lack of NO production contributes to the decrease in vascular elasticity (14). In support of this hypothesis, Matz et al. (21) demonstrated that inhibition of NOS in rabbits is also associated with augmentation of the arterial pressure wave reflection, a consequence of increased arterial stiffness. Infusion of ACh, a stimulant of EDNO release, had the opposite effect, which can be blocked by NOS inhibition (21).

In addition, we also demonstrated that chronic inhibition of NOS by L-NAME for 3 wk further increased PWV compared with that in acute L-NAME-treated rats (14). This additional increase in PWV is probably the result of abnormal vascular remodeling. Numaguchi et al. (23) demonstrated that chronic treatment with L-NAME in rats resulted in a significantly abnormal remodeling of the aortic wall. In addition, studies with endothelial constitutive NOS-deficient mice also showed abnormal vascular remodeling (28). Thus, it is possible that abnormal vascular remodeling caused by chronic attenuation of EDNO production in atherosclerosis contributes to the increase in PWV.

In summary, with the use of PWV measurement, the present study revealed for the first time an increase in aortic stiffness in 13-mo-old apoE-KO mice, accompanied by severe atherosclerosis and fragmentation of the elastic laminae in the aortic wall as well as attenuated EDNO-mediated relaxation of the aortic rings. The decrease in the aortic elasticity is probably related to both the lack of EDNO and elastic destruction caused by atherosclerotic lesions. Thus our results demonstrate that PWV can be used as a surrogate end point or marker for estimating the progression of atherosclerosis in apoE-KO mice.

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