*Original Article*

# Angiotensin II Receptor Blocker Reduces Oxidative Stress and Attenuates Hypoxia-Induced Left Ventricular Remodeling in Apolipoprotein E–Knockout Mice

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**Elevated superoxide formation in cardiac extracts of apolipoprotein E–knockout (apoE-KO) mice has been reported. In addition, we previously reported that hypoxia increased oxidative stress in the aortas of apoE-KO mice, although we did not examine the effect of hypoxia on the heart. The aim of this study was to investigate the effect of chronic hypoxia on the left ventricular (LV) remodeling in apoE-KO mice treated with or without an angiotensin II receptor blocker. Male apoE-KO mice (*n*=83) and wild-type mice (*n*=34) at 15 weeks of age were kept under hypoxic conditions (oxygen, 10.0±0.5%) and treated with olmesartan (3 mg/kg/day) or vehicle for 3 weeks. Although LV pressure was not changed, hypoxia caused hypertrophy of cardiomyocytes and increased interstitial fibrosis in the LV myocardium. Furthermore, nuclear factor-κB (NF-κB) and matrix metalloproteinase (MMP)-9 activities were increased in apoE-KO mice exposed to chronic hypoxia. Olmesartan effectively suppressed the 4-hydroxy-2-nonenal protein expression and NF-κB and MMP-9 activities, and preserved the fine structure of the LV myocardium without affecting the LV pressure. In conclusion, olmesartan reduced oxidative stress, and attenuated the hypoxia-induced LV remodeling, in part through the inhibition of NF-κB and MMP-9 activities, in apoE-KO mice. (*Hypertens Res* 2007; 30: 1219–1230)**

***Key Words*: hypoxia, oxidative stress, apolipoprotein E, heart, angiotensin II receptor blocker**

## Introduction

Hypoxic stress can be induced by chronic obstructive pulmonary disease (COPD) and sleep apnea syndrome. Both COPD and sleep apnea have been known to increase the risk of cardiovascular disease (*1*, *2*). In addition, several studies have shown that intermittent hypoxia due to sleep apnea has an association with left ventricular (LV) dysfunction (*3*, *4*). The LV remodeling, accompanied by hypertrophy of cardiomyocytes and interstitial fibrosis, is a contributory factor in the progression to heart failure (*5*). On the other hand, sleep apnea has been reported to contribute to hypercholesterolemia (*6–8*), and abnormalities in lipid regulation that occur in response to hypoxia may also act to increase the cardiovascular risk.

Recently, several studies have explored the relation of the apolipoprotein E (apoE) genotype to obstructive sleep apnea, with conflicting results. Among the middle-aged adult patients in the Wisconsin Sleep Cohort Study and the Sleep

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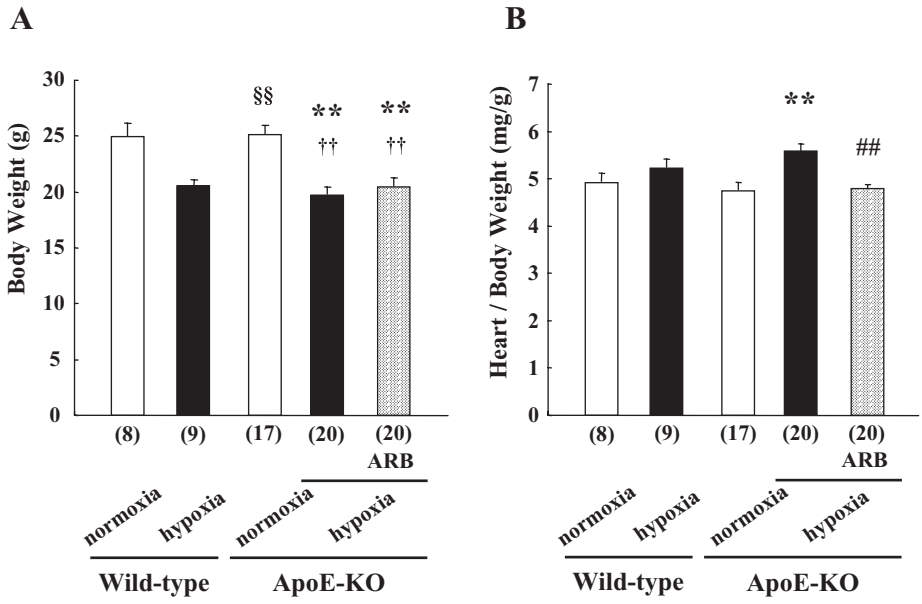


**Fig. 1.** *Effect of hypoxia on the body weight and the ratio of heart weight to body weight (Hw/Bw). A: Hypoxia significantly decreased the body weight in both wild-type and apolipoprotein E–knockout (apoE-KO) mice. Treatment with an angiotensin II receptor blocker (ARB) had no effect on body weight. B: Hypoxia did not change the Hw/Bw in wild-type mice. However, the Hw/Bw was significantly increased in apoE-KO mice exposed to hypoxia, and this increase was suppressed by treatment with an ARB. Columns and bars represent the mean±SEM.* $^{\dagger}p<0.05$, *and* $^{\dagger\dagger}p<0.01$ *vs. normoxic wild-type mice.* $^{\S\S}p<0.01$ *vs. hypoxic wild-type mice.* $^{**}p<0.01$, *and* $^{\#\#}p<0.01$ *vs. normoxic and hypoxic apoE-KO mice, respectively.*

**Table 1. Effect of Hypoxia on Right and Left Ventricular Systolic Pressure and Plasma Low-Density Lipoprotein (LDL)**

| | Wild-type | | apoE-KO | | |
|---|---|---|---|---|---|
| | Normoxia | Hypoxia | Normoxia | Hypoxia | Hypoxia+ARB |
| *n* | 6 | 6 | 6 | 10 | 5 |
| RVsys (mmHg) | 22±2 | 72±5$^{\dagger\dagger}$ | 23±2$^{\S\S}$ | 59±8$^{\dagger\dagger,**}$ | 40±3$^{\S}$ |
| LVsys (mmHg) | 99±4 | 95±3 | 96±4 | 98±3 | 95±9 |
| *n* | 10 | 10 | 16 | 15 | 13 |
| LDL (mg/dL) | 6.6±0.3 | 14.1±1.3 | 87.7±8.6$^{\dagger\dagger,\S\S}$ | 148.5±13.4$^{\dagger\dagger,\S\S,**}$ | 106.2±22.3$^{\dagger\dagger,\S\S}$ |

Values are means±SEM. RVsys and LVsys, right and left ventricular systolic pressure respectively. $^{\dagger\dagger}p<0.01$ *vs*. normoxic wild-type mice. $^{\S}p<0.05$, $^{\S\S}p<0.01$ *vs*. hypoxic wild-type mice. $^{**}p<0.01$ *vs*. normoxic apoE-KO mice.

Heart Health Study, the presence of an apoE ε4 allele was associated with increased risk of obstructive sleep apnea, although no such association was observed in a group of Finnish sleep apnea patients (*9–11*). On the other hand, the Cleveland Family Study found evidence suggesting a linkage to the apnea-hypopnea index in a region of chromosome 19 that includes the apoE gene (*12*).

We previously reported that chronic hypoxia accelerated the progression of atherosclerosis in apoE-knockout (apoE-KO) mice (*13*), accompanied by significant increases of oxidative stress and matrix metalloproteinase (MMP)-9 activity in aortic tissues. However, the effect of hypoxia on the heart was not examined, and the role of apoE in cardiac function remains obscure. Wu *et al*. have suggested that apoE might play an important role in modulating LV hypertrophy (*14*). In addition, the ratio of heart weight to body weight (Hw/Bw) is significantly increased, and superoxide formation, which might induce cardiac remodeling, is elevated in cardiac extracts in apoE-KO mice (*15*, *16*). On the other hand, increased MMP activities as well as oxidative stress might contribute to the development of LV remodeling. MMP activities are regulated through a number of pathways, including reactive oxygen species (ROS) (*17*) and nuclear factor-κB (NF-κB) (*18*). NF-κB activation plays an important role in the hypertrophic response of cardiomyocytes (*19*, *20*).

Angiotensin II receptor blockers (ARBs) are widely known to exert protective effects against heart failure. Harada *et al*. demonstrated that angiotensin II type 1 ($AT_1$) receptor signal-

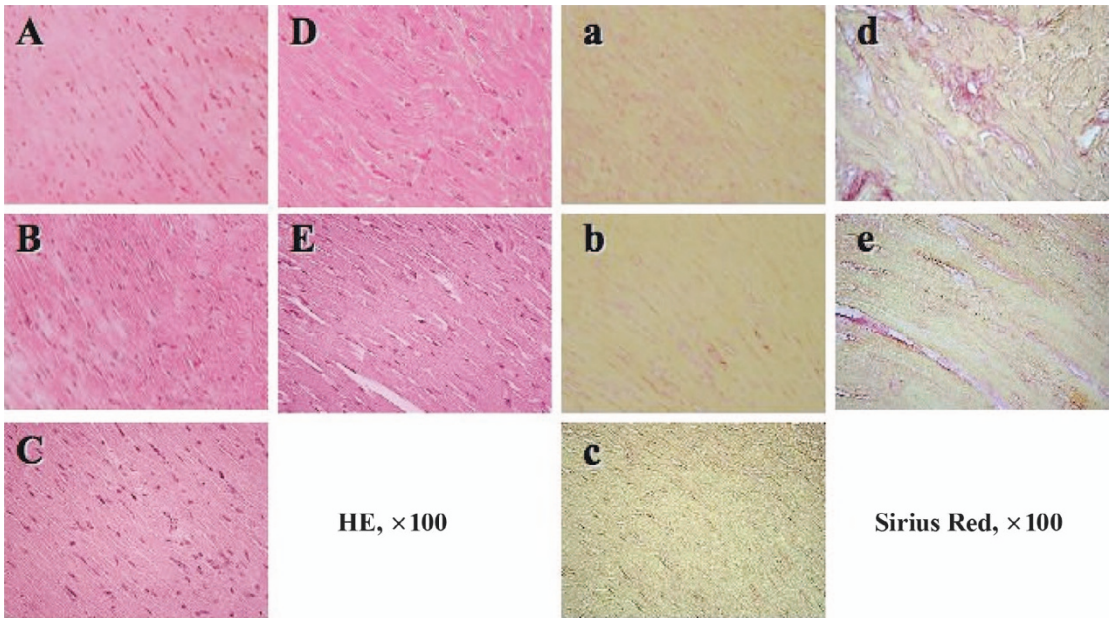


**Fig. 2.** *Representative light micrographs of the left ventricular (LV) myocardium in wild-type (A, B, a, b) and apolipoprotein E–knockout (apoE-KO) mice (C–E, c–e). The histology of wild-type and apoE-KO mice kept under normoxia looks normal (A, C, a, c). Although hypoxia showed little effect on wild-type mice (B, b), disarrangement of myofibers, hypertrophy of cardiomyocytes, and increased interstitial fibrosis were observed in apoE-KO mice (D, d). These changes were attenuated by treatment with an ARB (E, e).*

ing plays a pivotal role in the progression of LV remodeling after myocardial infarction (*21*). We reported that ARBs reduced hypoxia inducible factor-1α and were cardioprotective in the LV myocardium of the diabetic rat heart (*22*). However, the precise mechanisms of cardioprotection by ARBs are complicated, and whether ARBs can exert the same cardioprotective effect in apoE-KO mice is unclear. Therefore, the aim of this study was to examine the effect of chronic hypoxia on the heart in apoE-KO mice with or without treatment with an ARB, and to investigate whether an ARB would influence NF-κB and MMP-9 activities under hypoxic conditions.

## Methods

### Experimental Protocol

Male apoE-KO mice at 15 weeks of age and age-matched male wild-type mice were used. The mice were divided into two groups and were housed in a normobaric hypoxic chamber ($O_2$; 10.0±0.5%) or under normoxic conditions. Furthermore, apoE-KO mice exposed to hypoxia were randomized for treatment with the ARB olmesartan at a dose of 3 mg/kg/day or with vehicle by gavage for 3 weeks. The chamber was opened three times a week to provide the animals with fresh food (normal cholesterol diet), fresh water, and clean bedding.

After the 3-week experimental period, mice were anesthetized by intraperitoneal injection of sodium pentobarbitone (50 mg/kg, i.p.). A 1.4 French Micro-tip® catheter (model SPR-671; Millar Instruments, Houston, USA) was introduced through the right carotid artery to measure LV pressures. Blood samples were collected for measurement of low-density lipoprotein (LDL) and lipid peroxide (LPO) in plasma. Then the LV myocardium was excised for use in histological examination, electrophoretic mobility shift assay (EMSA), zymography, and immunohistochemistry.

All procedures were performed in accordance with our institutional guidelines for animal research.

### Light and Electron Microscopy

For light microscopy, a part of the LV myocardium was fixed in 10% formaldehyde, embedded in paraffin and cut into 4-μm–thick sections. To evaluate the mean diameter of cardiomyocytes, the shortest diameters of cardiomyocytes were measured only in nucleated transverse sections stained with hematoxylin-eosin under a light microscope at a magnification of ×400. After Sirius red staining, digital images of each section were obtained with a digital camera (Fujix Digital Camera HC-300Z, Fujifilm, Tokyo, Japan) mounted on a Nikon Microphot-FXA (Nikon, Tokyo, Japan). Color images were obtained from 5 randomly selected separate high-power fields (×200) in 5 sections per mouse. Each image covered an

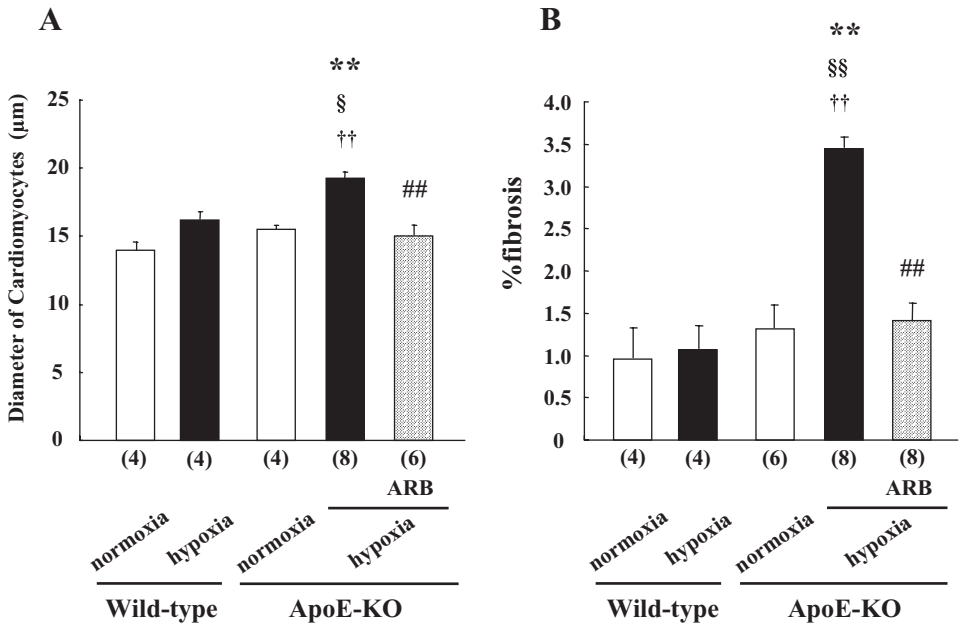


**Fig. 3.** *Mean diameter of cardiomyocytes and percentage of interstial fibrosis (%fibrosis) in the left ventricular (LV) myocardium. A: Hypoxia significantly increased the mean diameter of cardiomyocytes in apolipoprotein E–knockout (apoE-KO) mice, an effect which was attenuated by treatment with an angiotensin II receptor blocker (ARB). B: The %fibrosis was significantly increased by hypoxia. Treatment with an ARB significantly suppressed this increase. Columns and bars represent the means±SEM.* $^{\dagger\dagger}p<0.01$ *vs. normoxic wild-type mice.* $^{\S}p<0.05$*, and* $^{\S\S}p<0.01$ *vs. hypoxic wild-type mice.* $^{**}p<0.01$*, and* $^{\#\#}p<0.01$ *vs. normoxic and hypoxic apoE-KO mice, respectively.*

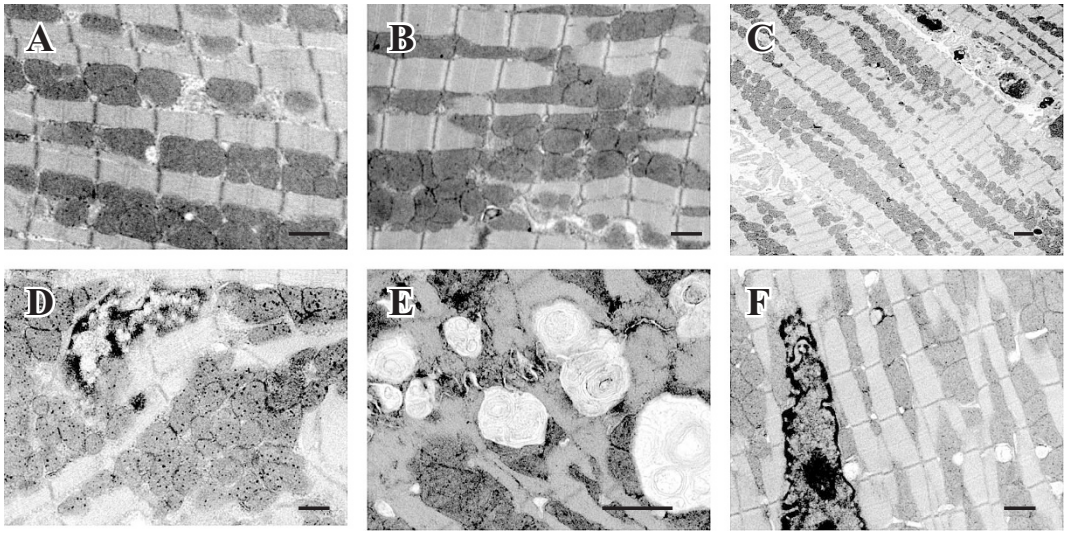


**Fig. 4.** *Representative electron micrographs of the left ventricular (LV) myocardium in wild-type (A, B) and apolipoprotein E–knockout (apoE-KO) mice (C–F). The ultrastructure of the wild-type mice is normal (A). Although deformed mitochondria tended to increase, no apparent abnormality was observed in wild-type mice exposed to hypoxia (B). The cardiomyocytes in apoE-KO mice kept under normoxia showed a nearly normal ultrastructure (C). In apoE-KO mice exposed to hypoxia, however, nuclear invagination and pores, and increased electron-dense deposits in mitochondria were observed (D). Furthermore, swelling and vacuolation of the sarcoplasmic reticulum and T-tubules were seen (E). In mice treated with an ARB, these hypoxia-induced changes were modest (F). Scale bar, 1 μm.*

area of 367,500 pixels and was analyzed using NIH Image 1.60 software. Both the intensity and area were analyzed by the method published previously (*23*). The data from all the examined sections were used to calculate the percent area of positive pixels relative to the total examined pixel area.

For electron microscopy, the specimens were fixed in 4% paraformaldehyde containing 0.25% glutaraldehyde and 4.5% sucrose. Ultrathin sections obtained from the embedded blocks were stained with uranyl acetate and lead citrate, and were examined with a Hitachi H-7650 electron microscope (Hitachi, Tokyo, Japan) (*24*).

## Immunohistochemistry for 4-Hydroxy-2-Nonenal Protein

For immunohistochemical light microscopy, additional sections were obtained from the paraffin block. Immunostaining was performed with a Vector M.O.M. Immunodetection kit (Vector Laboratories, Burlingame, USA) according to the manufacturer's instructions. Briefly, the sections were deparaffinized, hydrated, and incubated with 3% $H_2O_2$ to reduce endogenous peroxidase activity, and incubated with monoclonal antibody against 4-hydroxy-2-nonenal (4-HNE, No. MHN-20; Japan Institute for the Control of Aging, Shizuoka, Japan). After incubation with a biotinylated secondary antibody, the sections were allowed to react with Vectastain Elite ABC reagent (Vector Laboratories). Then the sections were reacted with the peroxidase substrate solution (Vectastain 3′,3′-diaminobenzidine substrate kit; Vector Laboratories). The slides were counterstained with Mayer-hematoxylin, and the percent area of 4-HNE staining was measured by quantitative analysis (*25*, *26*).

## NADPH Oxidase Activity

NADPH-dependent superoxide production was measured by a lucigenin-enhanced chemiluminescence assay (*13*, *27*). Briefly, LV tissues were homogenized with Multi-Beads Shocker (YASUI KIKAI, Osaka, Japan) in modified Krebs-HEPES buffer (pH 7.4, 99.01 mmol/L NaCl, 4.69 mmol/L KCl, 1.87 mmol/L $CaCl_2$, 1.20 mmol/L $MgSO_4$, 1.03 mmol/L $K_2HPO_4$, 25 mmol/L Na-HEPES, 11.1 mmol/L glucose) containing 10 mmol/L dithiothreitol (DTT), 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 3 μmol/L pepstatin and 10 μmol/L leupeptin. The tissue homogenates were centrifuged at 5,000 × *g* for 30 min at 4°C in an Eppendorf centrifuge. The supernatants were transferred into test tubes containing modified Krebs-HEPES buffer and were incubated with NADPH (100 μmol/L) in the dark for 15 min at 37°C. Luminescence was measured using a luminometer (Sirius-2; Funakoshi, Tokyo, Japan). The lucigenin concentration in the final reaction mixture was 5 μmol/L. NADPH-dependent superoxide production was expressed as relative light units (RLU) per min per mg of protein.

## Assessment of NF-κB Activation by EMSA

A part of the LV myocardium (30 mg) was washed in precooled phosphate buffered saline (PBS) (pH 7.4) containing 2.5 mmol/L EDTA, 2 mmol/L β-glycerophosphate, 10 mmol/L NaF and 1 mmol/L $Na_3VO_4$ and homogenized with Multi-Beads Shocker in 500 μL of cold buffer A (10 mmol/L HEPES [pH 7.9], 10 mmol/L KCl, 0.1 mmol/L NaF, 1 mmol/L $Na_3VO_4$, 0.5 mmol/L PMSF, 1 mmol/L DTT, 20 mmol/L β-glycerophosphate). The tissue homogenates were incubated on ice for 15 min, vortexed for 15 s after addition of 44 μL of 10% Nonidet P-40, and then centrifuged at 5,000 rpm for 1 min at 4°C in an Eppendorf centrifuge. The supernatant was removed, and the nuclear pellet was extracted with 150 μL of cold buffer C (10 mmol/L HEPES [pH 7.9], 0.4 mol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA 1.5 mmol/L $MgCl_2$, 20% glycerol, 10 mmol/L NaF, 1 mmol/L $Na_3VO_4$, 0.5 mmol/L PMSF, 0.2 mmol/L DTT, 20 mmol/L β-glycerophosphate) by shaking at 4°C for 15 min. Nuclear extracts were then centrifuged at 15,000 rpm for 10 min at 4°C, stored at −80°C, and used (10 μg protein) for EMSA. Double-stranded oligonucleotide containing the most common NF-κB consensus binding site (5′-AGTTGAGGGGACTTTCCCAGGC-3′) (Promega, Madison, USA) was end-labeled with [$\gamma$-$^{32}$P]ATP using T4 polynucleotide kinase (Promega). The binding reaction was performed for 20 min at 25°C in a total volume of 10 μL of binding buffer that contained 10 mmol/L Tris-HCl (pH 7.5), 50 mmol/L NaCl, 0.5 mmol/L EDTA, 1 mmol/L $MgCl_2$, 0.5 mmol/L dithiothreitol, 4% glycerol, and 0.5 μg of double-stranded poly(dI-dC). DNA-protein complexes were resolved by electrophoresis on 6% nondenaturing polyacrylamide gels at 150 V for 1 h in 0.5× Tris-borate–EDTA (1× Tris-borate–EDTA, which consists of 90 mmol/L Tris-borate, pH 8.3, 2 mmol/L EDTA) at 4°C. After electrophoresis, gels were dried and exposed to imaging plates (Fujifilm). The protein-DNA complexes were visualized by autoradiography, and then the relative intensities of bands were analyzed using NIH image 1.60 software. A supershift assay using antibodies to P65 and P50 was performed to confirm NF-κB–binding specificity.

## Zymography for MMP Activity

Cardiac samples were pulverized under liquid nitrogen, and proteins were extracted in buffer (1:10 w/v) containing 0.15 mol/L NaCl, 20 mmol/L $CaCl_2$, 1 μmol/L $ZnCl_2$, 1.5 mmol/L $NaN_3$, 10 mmol/L cacodylic acid, and 0.01% Triton X-100. Extracts were incubated on ice for 30 min and then centrifuged at 12,000 rpm for 15 min at 4°C. The supernatants were retained, and the protein concentration of the extracts was measured using a Bio-Rad Protein Assay Kit. Equal amounts (5 μg) of protein extracts were denatured in a non-reducing sample buffer (63 mmol/L Tris-HCl pH 6.8, 10% glycerol, 2% SDS and 0.1% bromophenol blue, final concentrations) and subjected to electrophoresis on 10% SDS polyacrylamide gels containing 0.1% gelatin. Human MMP-2 and MMP-9

(CC073; Chemicon, Temecula, USA) were used as standards to verify the identity of MMP species. After electrophoresis, the gels were renatured by incubation in 2.5% Triton X-100 and incubated overnight at 37°C in 50 mmol/L Tris-HCl pH 7.6, 5 mmol/L $CaCl_2$, 200 mmol/L NaCl, and 0.02% Briji 35. After incubation, gels were stained with 0.5% (w/v) Coomassie blue R-250 in a glacial acetic acid:methanol:distilled $H_2O$ mixture (1:4:5) and subsequently destained. The gels were scanned and lytic activity (clear bands on blue background) was analyzed using NIH image 1.60 software (*28*).

### Statistical Analysis

Data were expressed as the mean±SEM. For statistical analysis, we used one-way analysis of variance followed by Tukey-Kramer multiple comparison tests. Significance was recognized at $p<0.05$.

## Results

### Heart and Body Weight, and LDL

In wild-type mice, hypoxia did not affect the Hw/Bw. In apoE-KO mice, however, hypoxia significantly decreased the body weight (Fig. 1A) but not the heart weight, and consequently increased the Hw/Bw. Treatment with olmesartan decreased the heart weight and suppressed the increase of the Hw/Bw (Fig. 1B).

The plasma LDL level in apoE-KO mice was significantly increased by hypoxia (Table 1). Treatment with olmesartan showed no significant effect on the LDL level in apoE-KO mice.

### Hemodynamic Data

The right ventricular systolic pressure (RVsys) significantly increased in groups treated with hypoxia (Table 1). However, 3 weeks of hypoxic stress did not affect the LV systolic pressure (LVsys). Treatment with olmesartan tended to decrease RVsys but did not change LVsys (Table 1).

### Light and Electron Microscopy

Hypoxia caused disarrangement of myofibers, hypertrophy of cardiomyocytes and interstitial fibrosis in apoE-KO mice but not in wild-type mice. The mean diameter of the cardiomyocytes and percentage of interstitial fibrosis in the LV myocardium were significantly increased in hypoxic apoE-KO mice, and these increases were significantly suppressed by treatment with olmesartan (Figs. 2, 3).

Under the electron microscope, the ultrastructure of the LV myocardium in apoE-KO mice under normoxia appeared nearly normal. However, increased nuclear pores and invagination and many mitochondrial electron-dense deposits were found in the LV myocardium of hypoxic apoE-KO mice (Fig. 4). Furthermore, swelling and vacuolation of sarcoplasmic reticulum and T-tubules were observed. These hypoxia-induced changes were modest in olmesartan-treated apoE-KO mice.

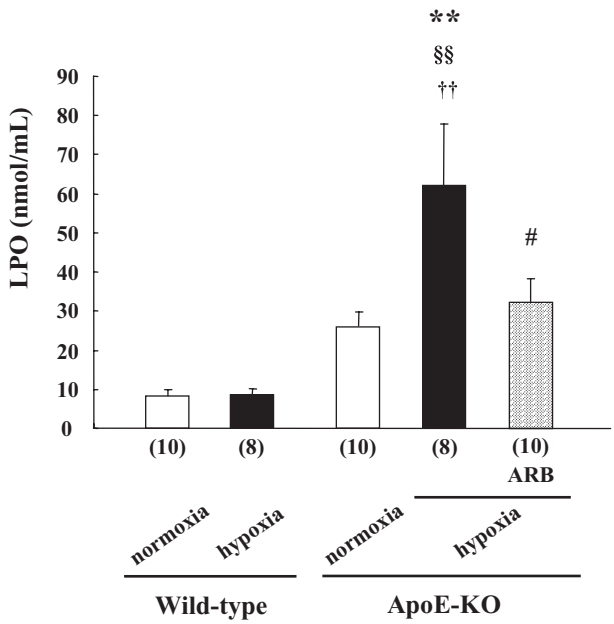


**Fig. 5.** *Plasma lipid peroxide (LPO) levels in wild-type and apolipoprotein E–knockout (apoE-KO) mice. Plasma LPO levels were significantly increased in apoE-KO mice exposed to hypoxia, and this effect was significantly decreased in the mice treated with an angiotensin II receptor blocker (ARB). Columns and bars represent the means±SEM. $^{\dagger\dagger}p<0.01$, and $^{\S\S}p<0.01$ vs. normoxic and hypoxic wild-type mice, respectively. $^{**}p<0.01$, and $^{\#}p<0.05$ vs. normoxic and hypoxic apoE-KO mice, respectively.*

### Plasma LPO Levels

There was no significant difference between apoE-KO and wild-type mice in plasma LPO levels before the exposure to hypoxia. Hypoxia significantly increased plasma LPO levels in apoE-KO mice, although there were no changes between normoxic and hypoxic wild-type mice. Treatment with olmesartan significantly decreased plasma LPO levels in apoE-KO mice (Fig. 5).

### 4-HNE Expression

The expression of 4-HNE protein was significantly increased in the LV myocardium of hypoxic apoE-KO mice compared with normoxic apoE-KO mice, although there was no significant difference between normoxic and hypoxic wild-type mice. Treatment with olmesartan significantly decreased 4-HNE protein, which means that olmesartan reduced oxidative stress in the LV myocardium (Fig. 6).

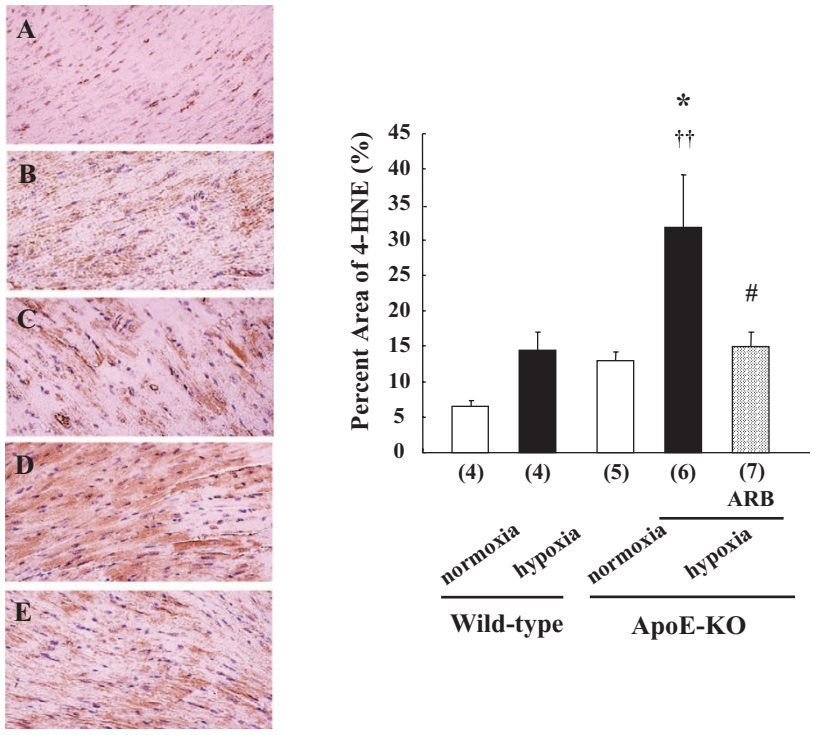


**Fig. 6.** *Immunochemistry and percent area of 4-hydroxy-2-nonenal (4-HNE) protein. The left panels show representative light micrographs of the left ventricular myocardium (original magnification, ×100). A: Wild-type mice kept under normoxia. B: Wild-type mice exposed to hypoxia. C: Apolipoprotein E–knockout (apoE-KO) mice under normoxia. D: apoE-KO mice exposed to hypoxia. E: apoE-KO mice exposed to hypoxia with ARB treatment. Hypoxia tended to increase the 4-HNE expression in wild-type mice. In contrast, the expression of 4-HNE was significantly increased in apoE-KO mice exposed to hypoxia, and this effect was significantly suppressed by an ARB. Columns and bars represent the means±SEM.* $^{\dagger\dagger}p<0.01$ *vs. normoxic wild-type mice.* $^{*}p<0.05$, *and* $^{\#}p<0.05$ *vs. normoxic and hypoxic apoE-KO mice, respectively.*

### NADPH-Dependent Superoxide Production

In wild-type mice, hypoxia did not affect NADPH-dependent superoxide production in the LV myocardium. However, NADPH-dependent superoxide production in the LV myocardium of apoE-KO mice was markedly increased by hypoxia (Fig. 7). Treatment with olmesartan significantly suppressed NADPH-dependent superoxide production in apoE-KO mice (Fig. 7).

### NF-κB–Binding Activity

Hypoxia significantly increased NF-κB–binding activity in the LV myocardium of apoE-KO mice. And the NF-κB activation was attenuated by olmesartan treatment (Fig. 8).

### MMP Activity

MMP-9 was activated in the LV myocardium of hypoxic apoE-KO mice, whereas MMP-2 showed no change. Treatment with olmesartan tended to suppress the MMP-9 activity in the LV myocardium of apoE-KO mice (Fig. 9).

## Discussion

Hypoxia due to sleep apnea or COPD has been reported as a risk factor of cardiovascular events (*1*, *2*, *29*, *30*). Recently, significant associations between coronary heart disease or myocardial infarction and single nucleotide polymorphisms in replication genes including apoE have been reported (*31*). Furthermore, a relation between the apoE genotype and obstructive sleep apnea has been suggested. Although the mechanism of the effect of the apoE genotype on sleep apnea is unknown, the potential importance of a causal association would be substantial.

In our previous report, 3 weeks of hypoxic stress significantly increased RVsys but not LVsys in apoE-KO mice (*13*). Treatment with olmesartan tended to decrease RVsys, and we could not rule out an effect of RVsys on the LV remodeling. In our subsequent study, however, in which diabetic rats were exposed to hypoxia for the same period, the effect of pulmonary hypertension on LV remodeling was slight (*22*). In the present study, therefore, we were able to exclude pulmonary hypertension as a possible cause of the LV remodeling.

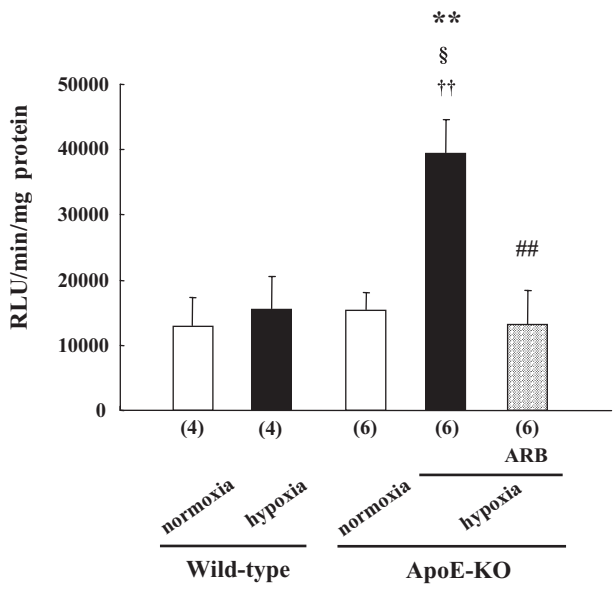


**Fig. 7.** *NADPH-dependent superoxide production in homogenates from left ventricular (LV) tissues of wild-type and apolipoprotein E–knockout (apoE-KO) mice. NADPH-dependent superoxide production in the LV myocardium significantly increased in apoE-KO mice exposed to hypoxia. Treatment with an angiotensin II receptor blocker (ARB) significantly decreased the production.* $^{\dagger\dagger}p < 0.01$, *and* $^{\S}p < 0.05$ *vs. normoxic and hypoxic wild-type mice, respectively.* $^{**}p < 0.01$, *and* $^{\#\#}p < 0.01$ *vs. normoxic and hypoxic apoE-KO mice, respectively.*

Cardiac remodeling is defined as genomic expression that results in molecular, cellular, and interstitial changes in the heart (*32*). In the present study, we demonstrated that hypoxia caused hypertrophy of cardiomyocytes, increased interstitial fibrosis, and consequently induced LV remodeling in apoE-KO mice. The dose of olmesartan used in this study (3 mg/kg per day) was based on previous studies and might be suitable for protection against oxidative stress without a change in blood pressure (*33*, *34*). Treatment with olmesartan prevented hypertrophy of cardiomyocytes and interstitial fibrosis without a significant change of LVsys and plasma LDL level in this study. This might suggest that the renin-angiotensin-aldosterone system (RAAS) plays an important role in the development of hypoxia-induced LV remodeling in apoE-KO mice.

The activation of RAAS has been reported in patients with sleep apnea (*35*). Increases in plasma renin activity, aldosterone level and angiotensin converting enzyme (ACE) activity have also been observed in patients with low arterial oxygen saturation due to high-altitude pulmonary edema (*36*). In the present study, apoE-KO mice exposed to hypoxia showed hypertrophy of cardiomyocytes, increased interstitial fibrosis and various types of degeneration in the LV myocardium, and these pathological changes were attenuated by treatment with olmesartan (Figs. 2, 3). Thus, hypoxia accelerated the development of LV remodeling in apoE-KO mice, and $AT_1$ receptor might play an important role in this mechanism.

Plasma LPO levels, a marker of systemic oxidative stress, were significantly increased in apoE-KO mice exposed to hypoxia. Furthermore, hypoxia significantly enhanced 4-HNE expression and NADPH-dependent superoxide production in the LV myocardium of apoE-KO mice, indicating that there was an increased oxidative stress in the LV myocardium. We have reported that chronic hypoxia increased NADPH-dependent superoxide production and dihydroethidium labeling in the aortas of apoE-KO mice (*13*). Chen *et al*. noted that chronic intermittent hypoxia increased oxidative stress in the rat heart (*37*). However, the precise mechanisms by which hypoxia increases oxidative stress in apoE-KO mice, but not in wild-type mice, have been obscure.

Adamy *et al*. have shown that expression of cardiac $AT_1$ and angiotensin II type 2 ($AT_2$) receptors is elevated in rats exposed to hypoxia (*38*). In addition, chronic hypoxia has been reported to increase the circulating levels of angiotensin II (*39*). Recently, El-Sokkary *et al*. indicated that hypoxia induced severe morphological changes and oxidative stress in the liver (*40*). In addition, angiotensin II has been shown to augment the aneurysms formation in apoE-KO mice, but not in wild-type mice (*41*). Therefore, it might be suggested that apoE-KO mice are more sensitive than wild-type mice to hypoxia, and thus experience greater oxidative stress. In the present study, treatment with olmesartan significantly suppressed plasma LPO levels, 4-HNE expression and NADPH-dependent superoxide production, indicating that the oxidative stress in hypoxia-induced LV remodeling might occur at least partly through NADPH oxidase activation and $AT_1$ receptor–dependent signaling (*38*). However, another possibility is that the beneficial effects of olmesartan might be mediated by angiotensin II through activated $AT_2$ receptors. The tissue angiotensin II system, including the $AT_1$ receptor expression, should be evaluated in both apoE-KO and wild-type mice in a future study (*42*).

Oxidative stress is also a potent stimulus for inflammation. ROS can promote NF-κB activation and inflammatory cytokine production in the myocardium. Intermittent hypoxia activates NF-κB and results in the expression of inducible nitric oxide synthase (iNOS) in cardiovascular tissues of mice (*43*). Thus, it may be that hypoxia promotes NF-κB activation *via* oxidative stress, leading to the expression of inflammatory cytokines and iNOS, which contribute to the development of LV remodeling.

NF-κB plays a crucial role in the transcriptional activation of multiple genes. Li *et al*. suggested that NF-κB activation was required for the development of cardiac hypertrophy *in vivo* (*20*). In addition, NF-κB has been reported to regulate the activity of MMPs (*18*), and increased MMPs in the failing heart have been demonstrated in both clinical and animal studies (*19*, *20*, *44*, *45*). Vellaichamy *et al*. reported that MMP-9 activity in *Npr1* gene (encoding guanylyl cyclase/

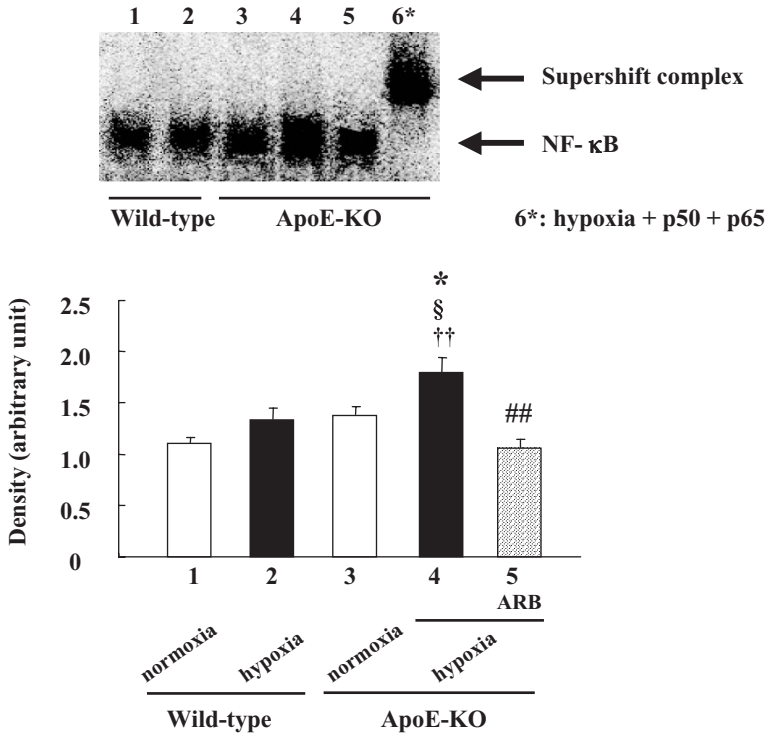


**Fig. 8.** *Effect of hypoxia on nuclear factor-κB (NF-κB)–binding activity in the left ventricular (LV) myocardium. Representative examples of NF-κB–binding activity are shown at the top. A supershift band is shown with specific anti-p50 and anti-p65 antibodies (lane 6). Hypoxia significantly increased NF-κB–binding activity in the LV myocardium of apolipoprotein E–knockout (apoE-KO) mice (lane 4). The NF-κB activation was attenuated by treatment with an angiotensin II receptor blocker (ARB) (lane 5). Values were normalized by the average of normoxic wild-type mice (n = 5). $^{\dagger\dagger}p < 0.01$, and $^{\S}p < 0.05$ vs. normoxic and hypoxic wild-type mice, respectively. $^{*}p < 0.05$, and $^{\#\#}p < 0.01$ vs. normoxic and hypoxic apoE-KO mice, respectively.*

natriuretic peptide receptor A)–disrupted homozygous null mutant mice was positively correlated with an increase in interstial fibrosis (*46*). Thus, activated MMPs might contribute to the development of LV remodeling. In our study, hypoxia significantly increased the mean diameter of cardiomyocytes, in addition to increasing NF-κB and MMP-9 activities in the LV myocardium of apoE-KO mice (Figs. 3, 8, 9). Taken together, these facts suggest that hypoxia might activate NF-κB, resulting in increased MMP-9 activity, and thereby to the development of LV remodeling in apoE-KO mice.

Interstitial fibrosis is a major contributor to the development of LV remodeling. In the present study, hypoxia significantly increased interstitial fibrosis in apoE-KO mice, and this effect was clearly suppressed by treatment with an ARB (Figs. 2, 3). Suppression of MMP-9 by ACE inhibition has been reported to result in degradation of collagens and to prevent collagen accumulation (*47*). MMPs are known to degrade all components of the extracellular matrix. Therefore, it is expected that an elevation of MMP activity would result in a reduction of MMP substrate (collagens). However, an elevation of myocardial MMP activity is accompanied by increased fibrosis, while suppression of MMP activity is associated with less deposition of fibrotic tissues (*47*, *48*). This phenomenon suggests that the total matrix collagen content is a function of both synthesis and degradation, and degraded products of matrix proteins might stimulate collagen synthesis (*48*, *49*). In the present work, we also observed the activation of MMP-9 after hypoxic exposure, and the suppression of such activation by an ARB (Fig. 9). Thus, treatment with olmesartan might prevent the increase of interstitial fibrosis through the suppression of MMP-9. Further examinations using an MMP inhibitor might clarify the role of ARBs in preventing LV remodeling.

We did not evaluate the atherosclerotic lesions of apoE-KO mice in this study. The results of our previous study suggest that the effect of hypoxia-induced atherosclerosis on the LV myocardium is modest. However, quantitative measurement of the atherosclerotic lesions in apoE-KO mice may be critical. Further investigations using apoE-KO mice with accelerated atherosclerosis should be done to compare hypoxia-induced LV remodeling with the remodeling due to increased afterload. We understand that the hypoxic condition adopted in this study is different from that occurring in humans with sleep apnea syndrome, and thus have made plans to examine the effect of repetitive hypoxic stress on the cardiovascular

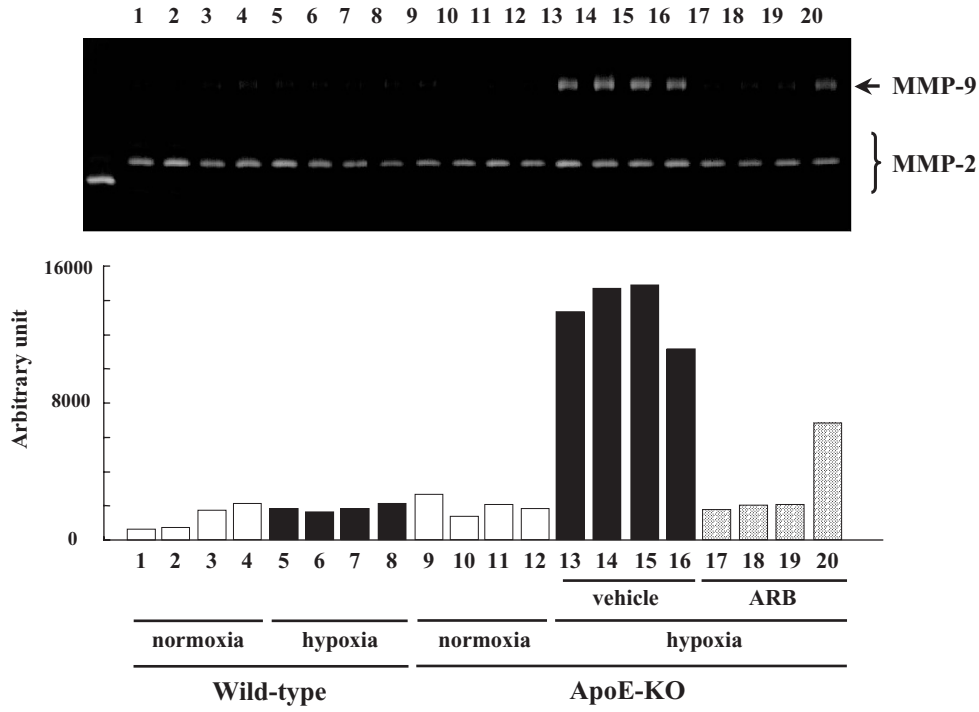


**Fig. 9.** *Zymography for matrix metalloproteinase (MMP) activity in the left ventricular (LV) myocardium of wild-type and apolipoprotein E–knockout (apoE-KO) mice. The lower panel shows MMP-9 activity quantitatively evaluated using NIH image software. Although MMP-2 showed no change, hypoxia increased MMP-9 activity in the LV myocardium of apoE-KO, but not in that of wild-type mice. Treatment with an angiotensin II receptor blocker (ARB) tended to suppress the increased MMP-9 activity.*

system in a future study.

In conclusion, chronic hypoxia accelerated LV remodeling in apoE-KO mice genetically disposed to atherosclerosis, and olmesartan reduced oxidative stress and attenuated the LV remodeling at least partly through the inhibition of NF-κB and MMP-9 activities.

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