Original Article

Chronic Hypoxia Accelerates the Progression of Atherosclerosis in Apolipoprotein E-Knockout Mice

Daisuke NAKANO, Tetsuya HAYASHI*, Naoko TAZAWA, Chika YAMASHITA, Sakiko INAMOTO*, Nobuaki OKUDA*, Tatsuhiko MORI*, Koichi SOHMIYA*, Yasushi KITAURA*, Yoshikatsu OKADA**, and Yasuo MATSUMURA

The aim of this study was to investigate the effect of chronic hypoxia on the development and progression of atherosclerosis in apolipoprotein E-knockout (apoE-KO) mice. Male and female apoE-KO mice (6 weeks old) and age- and sex-matched wild-type mice were kept under hypoxic conditions (10.0±0.5% O_2) in a gas chamber or in room air for 3 weeks. Aortic atherosclerotic plaque was not observed in wild-type mice under normoxic or hypoxic conditions. In the apoE-KO mice, however, hypoxia induced proliferation of smooth muscle cells and plaque formation in the aorta, which were not observed under normoxic conditions. Although sexual dimorphism of the response to hypoxia was not observed, these hypoxia-induced atherogenic changes were accompanied by a significant increase of plasma low density lipoprotein (LDL) cholesterol and NADPH-dependent vascular superoxide (O_2^-) production. Furthermore, matrix metalloproteinase (MMP)-9 was activated in the aorta of apoE-KO mice. In conclusion, chronic hypoxia accelerated the development of atherosclerosis in apoE-KO mice, along with increased O_2^- production and activated MMP-9 in the aorta. (*Hypertens Res* 2005; 28: 837–845)

***Key Words*: hypoxia, atherosclerosis, apolipoprotein E, superoxide, matrix metalloproteinase**

Introduction

Oxidative stress induced by reactive oxygen species (ROS) is known to be an important modulating factor in the progression of atherosclerosis (*1–3*). Several pathways may be involved when ROS contribute to the development of atherosclerosis. One possible mechanism is that ROS derived from macrophages (*4*), smooth muscle (SM) (*5*), adventitia (*6*) and endothelium (*7*) may induce the oxidation of low density lipoprotein (LDL). Oxidized LDL is phagocytosed by macrophages and this results in the formation of foam cells. Another possibility is that superoxide (O_2^-) reacts with endothelium-derived nitric oxide (NO) and reduces the bioavailability of NO, which then accelerates the development of atherosclerosis, including the promotion of platelet aggregation (*8*), SM cell (SMC) proliferation (*9*) and monocyte adhesion (*10*). Moreover, ROS also modulate the activity of matrix metalloproteinases (MMPs), which contribute to the degradation and remodeling of the extracellular matrix in atherosclerotic plaque, which may lead to rupture of atherosclerotic lesions (*11*, *12*).

Several *in vitro* studies have shown that hypoxic conditions increase cardiomyocytic generation of ROS despite the fact that the source of ROS (oxygen) is in low supply (*13*), and that antioxidants prevent hypoxia-induced inflammatory

From the Department of Pharmacology, Osaka University of Pharmaceutical Sciences, Takatsuki, Japan; and *Third Department of Medicine and **Department of Pathology, Osaka Medical College, Takatsuki, Japan.
Address for Reprints: Tetsuya Hayashi, M.D., Ph.D., Third Department of Medicine, Osaka Medical College, 2−7 Daigakumachi, Takatsuki 569−8686, Japan. E-mail: in3015@poh.osaka-med.ac.jp
Received April 8, 2005; Accepted in revised form August 29, 2005.

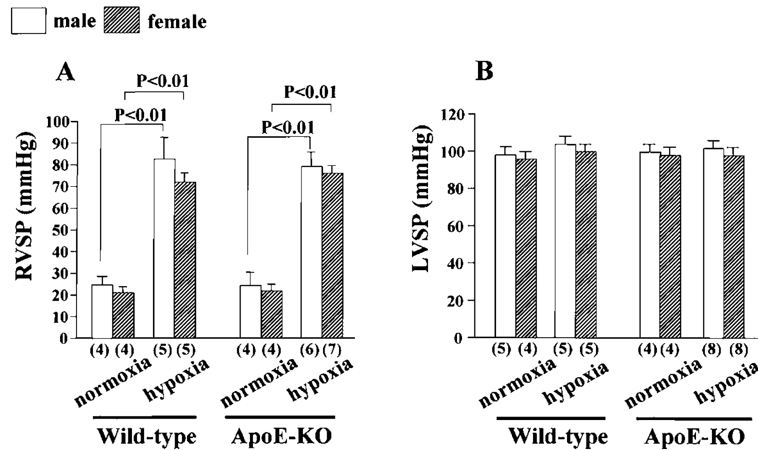

Fig. 1. *Effect of hypoxia on RVSP (A) and LVSP (B). Hypoxia markedly increased the RVSP without affecting the LVSP. Columns and bars represent the mean±SEM.*

responses, cell apoptosis and the activation of nuclear factor-κB in human aortic endothelial cells (*14*). Additionally, in rats, chronic hypoxia generated significant left ventricular hypertrophy and decreased left ventricular function, accompanying the increased oxidative stress in the left ventricular myocardium (*15*). Furthermore, in a clinical study, severe obstructive sleep apnea, a condition accompanied with hypoxic stress, increased the risk of fatal and non-fatal cardiovascular events, and effective medical treatment significantly reduced these outcomes (*16*). Thus, it can be suggested that a hypoxic condition aggravates oxygen catalytic activity, and that this leads to an increase of ROS generation and might induce the cardiovascular events. In this study, we tested the hypothesis that chronic hypoxia would accelerate the progression of atherosclerosis through increased ROS production and MMP activity in young apolipoprotein E-knockout (apoE-KO) mice that had not developed atherosclerosis.

Methods

Animals and Experimental Protocol

Male and female apoE-KO mice (n=45 each) that were 6 weeks old, and in whom atherosclerotic lesions had not yet developed in the aorta, as well as age- and sex-matched wild-type mice (n=40 each), were divided into two groups and housed in a normobaric hypoxic chamber (O_2; 10.0±0.5%) or under normoxic conditions for 3 weeks. The chamber was opened 3 times a week to provide the animals with fresh food (normal cholesterol diet), fresh water, and clean bedding.

After the 3-week experimental period, catheterization was done under anaesthesia with 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 jugular vein or the right carotid artery to measure right or left ventricular pressures, respectively. Blood samples were collected for measurement of plasma total cholesterol and plasma LDL. The aorta was removed and was freed from the adherent fat and connective tissue. All procedures were performed in accordance with the institutional guidelines for animal research.

Quantification of Atherosclerosis

The descending thoracic aorta harvested from each mouse was stained with oil red O, and atherosclerosis was quantitatively evaluated using an image analyzer (*17*).

Light Microscopy

For light microscopy, specimens were fixed in 10% formaldehyde, embedded in paraffin, and cut into 4-μm thick sections. The sections were stained with hematoxylin & eosin and elastica van Gieson before examination under a light microscope.

Immunohistochemistry for Proliferating Cell Nuclear Antigen (PCNA)

For immunohistochemical light microscopy, additional sections were obtained from the paraffin block and were incubated overnight at 4°C with a monoclonal antibody targeting PCNA (PC10; Santa Cruz Biotechnology Inc., Santa Cruz, USA). After incubation with a biotinylated secondary antibody, the sections were reacted with Vectastain Elite ABC reagent (Vector Laboratories, Burlingame, USA) and peroxidase substrate solution (Vectastain 3′3′-diaminobenzidine substrate kit; Vector Laboratories). The slides were counterstained with hematoxylin and mounted for light microscopy (*18*, *19*).

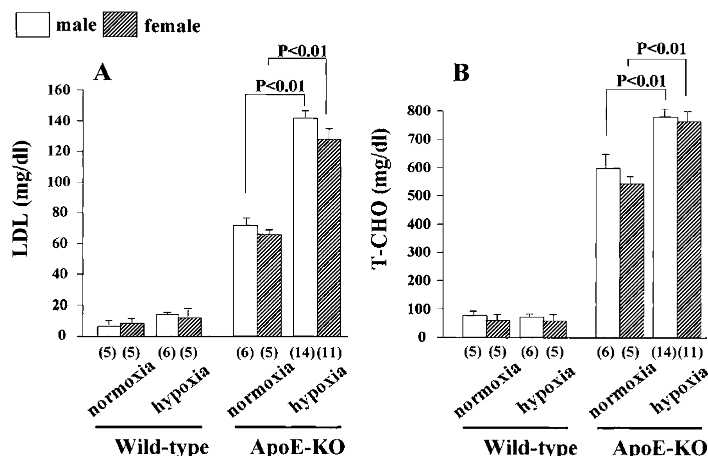

Fig. 2. *Effect of hypoxia on plasma LDL (A) and total cholesterol (T-CHO) (B). In wild-type mice, there were only slight differences in plasma lipid levels between the normoxia-treated group and the hypoxia-treated group. The plasma lipid levels of hypoxia-treated apoE-KO mice were higher than those of the normoxia-treated group. Columns and bars represent the mean±SEM.*

Dihydroethidium Labeling of Aortas

To detect ROS production *in situ*, fresh frozen sections of aortas were stained with dihydroethidium (DHE; Molecular Probes Inc., Eugene, USA) (*20*). Frozen sections (20 µm in thickness) were covered with 10 µmol/l of DHE solution and incubated in a light-protected humidified chamber at 37°C for 30 min. Then, sections were coverslipped and observed with a fluorescence microscope (Olympus BX 50; Tokyo, Japan).

Lucigenin Chemiluminescence Assay

NADPH-dependent O_2^- production was measured with a lucigenin-enhanced chemiluminescence assay (*21*). The lucigenin concentration was set at 5 µmol/l, which demonstrated that its own redox cycling is minimal (*22*, *23*). Aortic segments were placed in test tubes containing 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) and were incubated with NADPH (10 µmol/l) in the dark for 15 min at 37°C. Luminescence was measured using a luminometer (Sirius-2; Funakoshi, Tokyo, Japan). The background count was determined from identically treated vessel-free readings and was subtracted from the vessel readings. NADPH-dependent O_2^- production was expressed as relative light units (RLU) per min per dry aortic tissue weight.

Zymography

Vascular MMP activity in the gel was measured as described previously (*24*). Briefly, approximately 20 mg of frozen aorta was incubated in 0.4 ml of extraction buffer at 4°C with continuous agitation for 24 h. Then this process was repeated with fresh buffer. The extraction buffer (0.8 ml) was collected and the pH was raised to 7.5 by the addition of 0.1 mol/l Tris buffer (pH 7.6). The total tissue extract was concentrated in an Amicon Centricon YM-10 concentrator (Millipore Inc., Bedford, USA), after which 5 µl of concentrated extract was loaded onto gelatin containing SDS-polyacrylamide gel. Following electrophoresis, the gels were soaked in 2.5% Triton X-100 for 30 min at room temperature. Next, the gels were rinsed and incubated overnight at 37°C in developing buffer (50 mmol/l Tris-HCl, 200 mol/l NaCl, 5 mmol/l $CaCl_2$, and 0.02% Briji35). After incubation, the gels were stained in 0.5% Coomassie Blue R-250 in acetic acid:methanol:water (1:4:5 by volume), and were destained with the same solvent. MMP activity was indicated by clear zones on a blue background. The gels were scanned into a computer and analyzed with NIH image software (*25*).

Statistical Analysis

Results were expressed as the mean±SEM, except that MMP activity was reported as the value for each mouse. Statistical analysis was performed by one-way ANOVA. Post hoc analysis of multiple comparisons was corrected with Dunnett's test. Differences between pairs were determined by Student's *t*-test, and significance was recognized at $p<0.05$.

Results

Right and Left Ventricular Pressures

The right ventricular systolic pressures (RVSP) were significantly increased in groups treated with hypoxia (Fig. 1A).

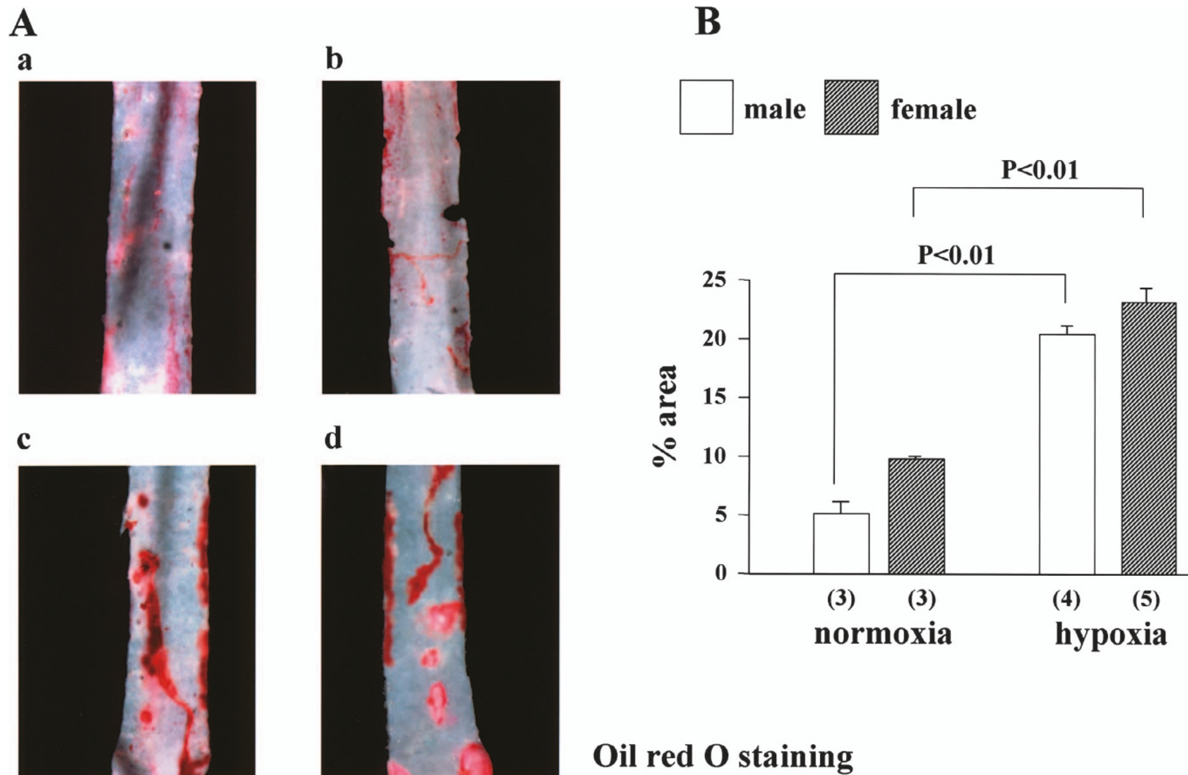

Fig. 3. *A: Representative examples of en face dissected thoracic aortas stained with oil red O from a normoxic male apoE-KO mouse (a), a normoxic female (b), a hypoxic male (c), and a hypoxic female (d). B: The percentage of the aortic area stained with oil red O. Hypoxia increased the oil red O-stained area in aorta of apoE-KO mice. Columns and bars represent the mean±SEM.*

However, 3-weeks of hypoxic stress did not affect the left ventricular systolic pressures (LVSP) (Fig. 1B).

Plasma Lipids

Plasma total cholesterol and LDL levels are shown in Fig. 2. In wild-type mice, there were no significant changes of plasma lipids due to hypoxia. However, apoE-KO mice housed under hypoxic conditions demonstrated significant elevation of the plasma total cholesterol and LDL levels compared with apoE-KO mice housed in a normoxic environment.

Quantification of Atherosclerosis

No atherosclerosis was found in the aortas of male and female wild-type mice. A small percent area of atherosclerosis was observed in the aorta of apoE-KO mice housed in a normoxic environment. On the other hand, a hypoxia-induced increase of atherosclerosis was observed in apoE-KO mice (Fig. 3).

Morphological Analysis

The descending aortas of male and female wild-type mice subjected to normoxia had a normal appearance, and hypoxia had little effect on the vascular architecture. On the other hand, hypoxia promoted SMC proliferation and induced plaque formation in both male and female apoE-KO mice (Fig. 4). Furthermore, hypoxia increased the DHE staining, which was observed not only in the atherosclerotic plaque of the intimal layer but also in the non-atherogenic medial layer, and PCNA-positive cells were observed in the aortas of apoE-KO mice exposed to hypoxia (Fig. 5A and B).

NADPH-Dependent O_2^- Production

In wild-type mice, hypoxia did not affect aortic tissue NADPH-dependent O_2^- production. Among apoE-deficient mice, however, hypoxia significantly increased NADPH-dependent O_2^- production in both males and females (Fig. 5C).

Vascular MMP Activity

MMP-9 activity was greater in apoE-KO mice than in wild-type mice, and it tended to be higher in females than males. The response to hypoxia was prominent in MMP-9, but not MMP-2, of female apoE-KO mice (Fig. 6).

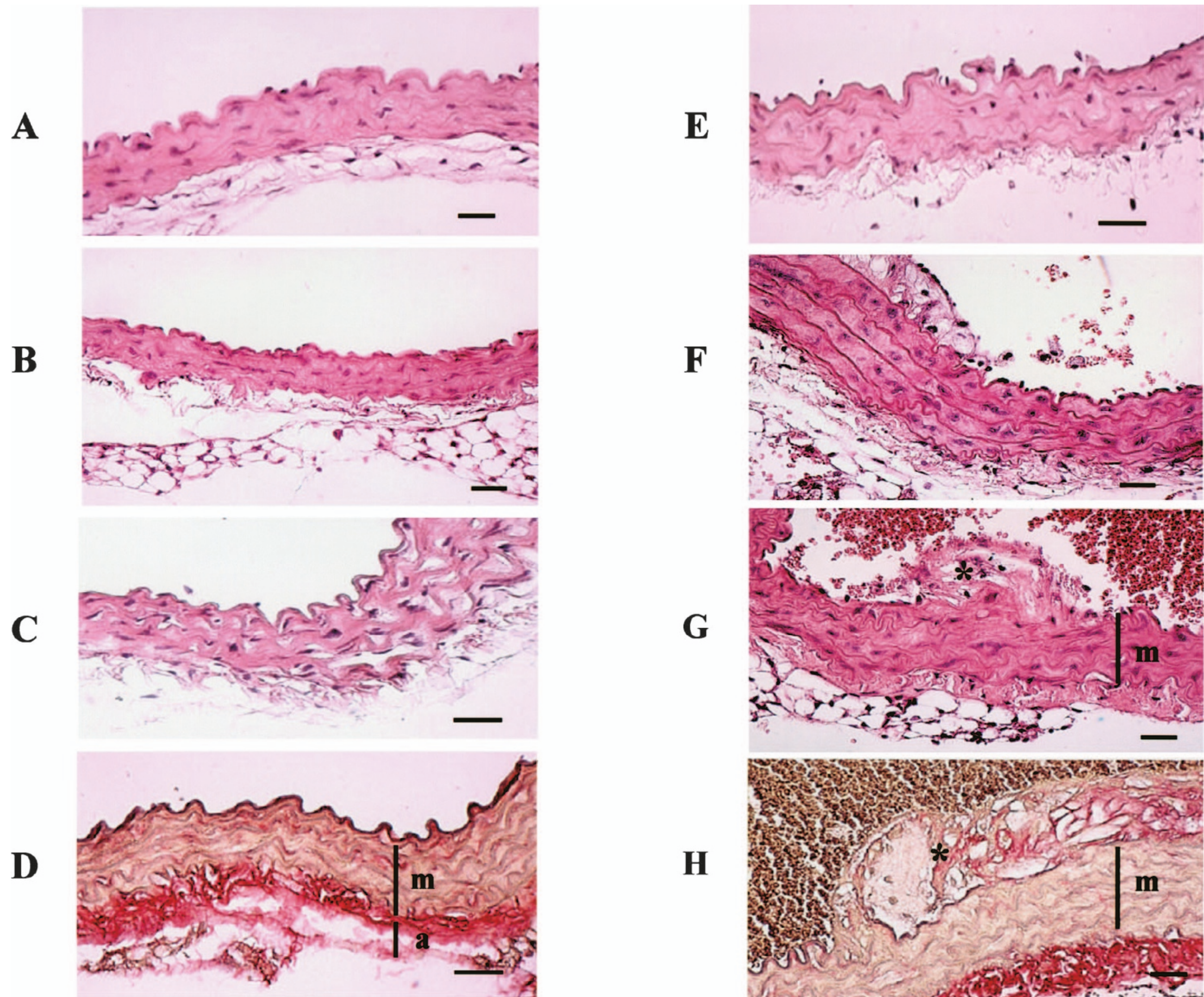

Fig. 4. *Representative examples of cross-sections (hematoxylin & eosin) of aortas from a normoxic wild-type mouse (A), male apoE-KO mouse (B), and female apoE-KO mouse (C and D), as well as a hypoxic wild-type mouse (E), male apoE-KO mouse (F), and female apoE-KO mouse (G and H). Although hypoxia caused only slight atherosclerotic changes in the aorta of wild-type mice, proliferation of smooth muscle cells and the plaque formation (*) were observed in the aorta of apoE-KO mice. A, B, C, E, F, G: hematoxylin-eosin staining; D, H: elastica van Gieson staining. m, tunica media; a, tunica adventitia. Scale bar, 50 μm.*

Discussion

Recently, several authors have reported that pulmonary vasculature exposed to chronic hypoxia shows structural remodeling and develops endothelial dysfunction (*26*). However, little is known about the effect of hypoxic stress on the arterial walls *in vivo*. In the present study, we showed that chronic hypoxia accelerated aortic atherosclerosis in apoE-KO mice without affecting the left ventricular function, and this pathological change was accompanied with an increase of NADPH-dependent O_2^- production and MMP activity in the aorta. This means that hypoxia might induce pathophysiological changes not only in the pulmonary vascular function but also in other arterial functions as well.

It is widely known that the plasma LDL and cholesterol concentrations are strongly correlated with the risk of atherosclerosis (*27*). In this study, hypoxic apoE-KO mice showed elevation of plasma LDL and cholesterol levels. Several researchers have reported that hypoxia modulates the handling of LDL by atherogenesis-related cells such as SMC and macrophages. Matsumoto *et al*. reported that there was less degradation of LDL by human monocyte-derived macrophages under hypoxic conditions than under normoxic condi-

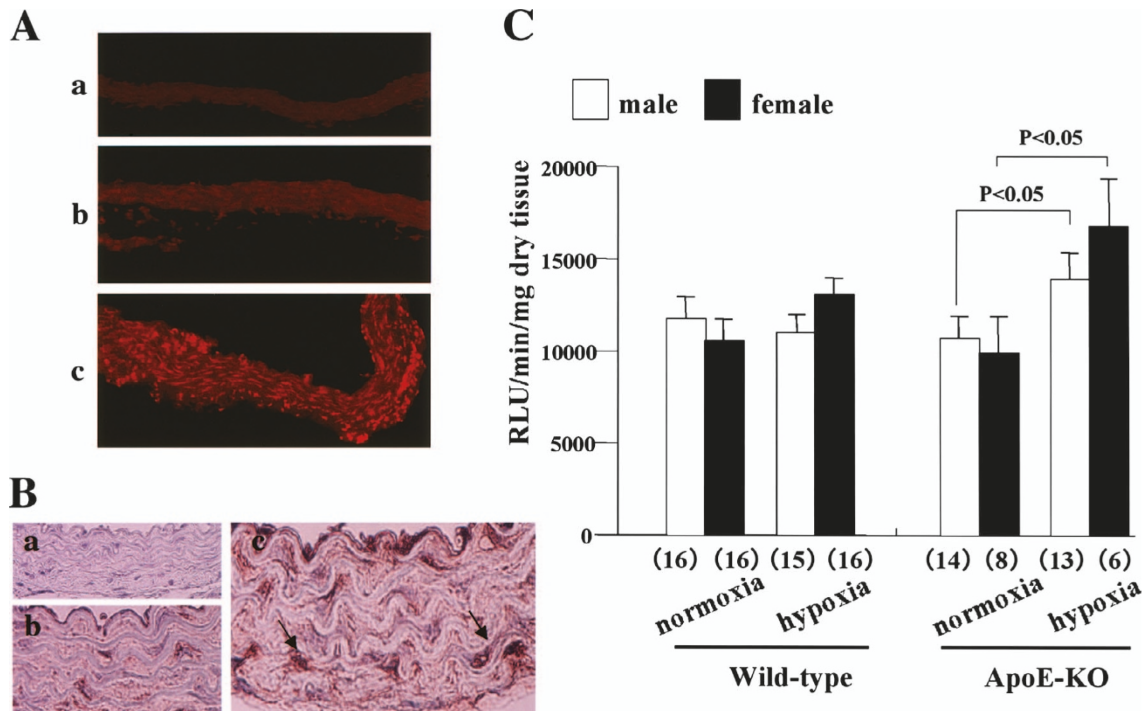

Fig. 5. *A: Representative examples of dihydroethidium (DHE) labeling of aortas. The DHE staining is increased by hypoxia in apoE-KO mice (c), compared with wild-type (a) and apoE-KO mice under normoxic conditions (b). B: Immunohistochemistry for PCNA. Increased PCNA-positive cells (arrows) in the medial wall are observed in the aortas of apoE-KO mice exposed to hypoxia (c), compared with wild-type (a) and apoE-KO mice under normoxic conditions (b). C: Effect of hypoxia on NADPH-dependent O_2^- production in aortic tissue of wild-type mice and of apoE-KO mice. Aortas were incubated with NADPH (10 μmol/l), and O_2^- production was determined by the lucigenin assay. Hypoxia did not affect the aortic O_2^- production in wild-type mice, in contrast, the production was significantly increased in apoE-KO mice. Columns and bars represent the mean±SEM.*

tions (*28*). Furthermore, Wada *et al*. reported that hypoxia increased LDL uptake and enhanced lipid accumulation in arterial SMC independent of LDL receptor activity (*29*).

Thus, it may be suggested that hypoxia modulates the development of atherosclerotic plaque by increasing the accumulation of LDL and inhibiting the degradation of LDL in atherogenesis-related cells. Taken together, such findings indicate that the development of atherosclerotic lesions in apoE-KO mice may be at least partly due to the alteration of LDL metabolism in atherogenesis-related cells. On the other hand, the synthesis and the metabolism of the cholesterol are mainly mediated in the liver. Therefore, the possibility that hypoxia affects the functions of hepatic cholesterol synthesis, metabolism, and excretion cannot be ruled out. The effect of hypoxia on plasma LDL and cholesterol concentrations should be examined in a future study.

In this study, chronic hypoxia also increased NADPH-dependent O_2^- production and DHE fluorescence. Several other studies have shown that exposure to hypoxic stress induces ROS production in the mitochondria of vascular cells, such as SMC (*30*) and endothelial cells (*31*), which might contribute to the development of various pathophysiological states. Schäfer *et al*. demonstrated that the increase of mitochondrial ROS production additionally activated non-phagocyte NADPH oxidase-dependent ROS production (*31*). Thus, hypoxia may promote intracellular ROS generation by stimulating the mitochondrial electron transport system in SMC and vascular endothelial cells, and the increase of ROS may have activated nonphagocyte NADPH oxidase in the aortas of apoE-KO mice.

Another possibility is that macrophages infiltrating the subendothelial spaces of the growing atherosclerotic plaques might produce additional ROS. It has been reported that foam cells may enhance oxidative stress through ROS production by phagocyte NADPH oxidase (*32*, *33*). Although massive infiltration of macrophages was not observed in this study, "burst"-like O_2^- production by these cells might contribute prominently to LDL oxidation and thus alter basic cellular functions such as adhesion and proliferation.

Recent reports indicated that activation of MMPs is regulated by oxidative stress, and that activated MMPs contribute to the aggravation of atherosclerosis (*11*, *12*, *34*). In particular, MMP-9 might contribute to atherogenesis by inducing plaque instability, plaque rupture, and platelet aggregation (*34–36*). In this study, we detected the activation of MMP-9 in hypoxic apoE-KO mice, especially females. These results

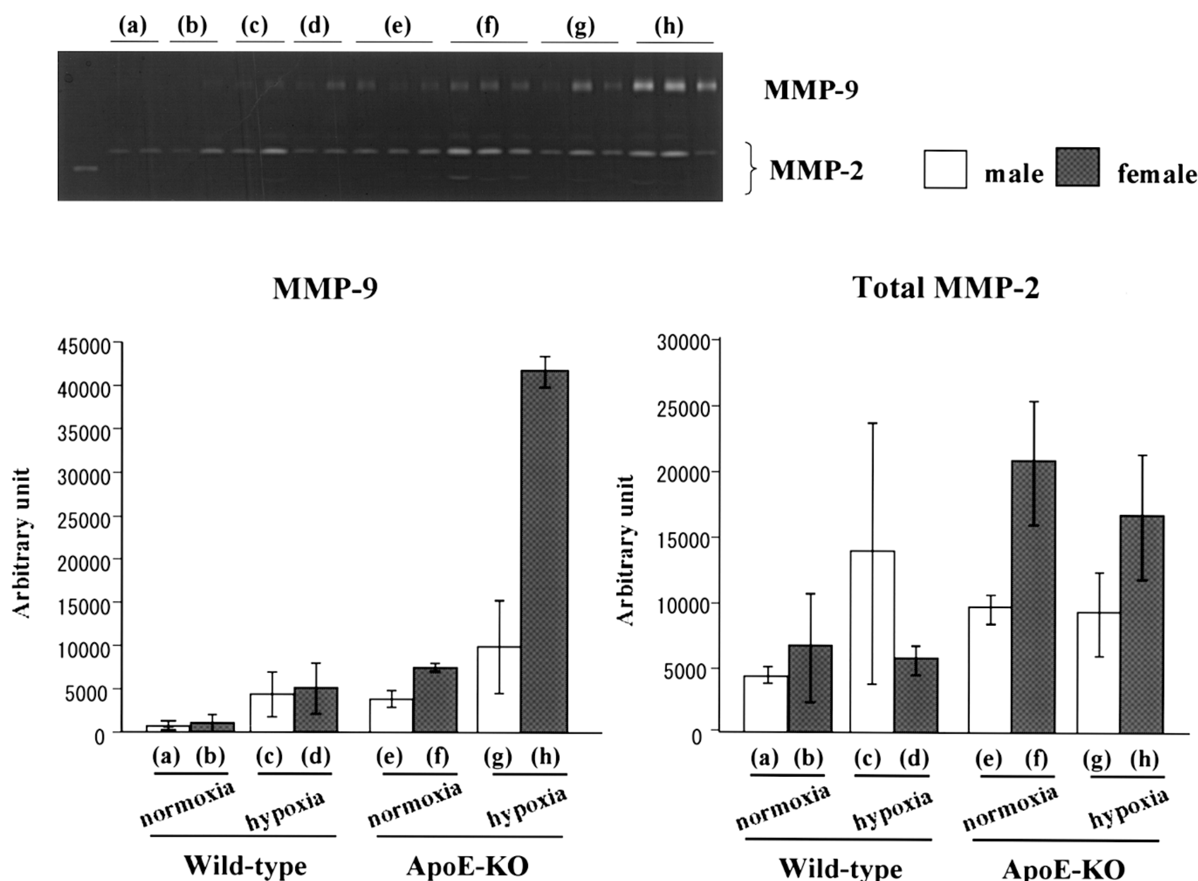

Fig. 6. *Effect of hypoxia on MMP-9 and total MMP-2 activity in aortic tissue. MMP-9 and total MMP-2 activities were assessed by gelatin zymography. The response to hypoxia was prominent in MMP-9, but not MMP-2, of female apoE-KO mice.*

agreed with the morphological features of atherosclerosis in apoE-KO mice, since atherosclerotic plaque was increased in hypoxic animals compared with normoxic animals. Thus, hypoxia might activate MMPs by increasing the oxidative stress in the aorta and thereby aggravate atherosclerosis.

On the other hand, Lemaître *et al*. reported that macrophage-specific MMP-1 transgenic adult apoE-KO mice showed less atherosclerotic plaque compared with control littermates (*37*). The role of MMPs in the development of atherosclerosis might vary according to the stage of the lesion, its localization, and the class of the activated enzyme(s). Therefore, we need to clarify the relationship between MMP activation and the development of atherosclerosis by studies using MMP inhibitors, and we need to determine the localization of activated MMP in vascular atherosclerotic lesions.

In the present study, aortic MMP-9 activity tended to be higher in female than in male apoE-KO mice under hypoxic conditions. Potier *et al*. have reported that 17β-estradiol increased both MMP-9 mRNA and MMP-9 activity in mesangial cells (*38*). Moreover, ovariectomy decreased left ventricular MMP-2 activity in female rats, and this change was ameliorated by estrogen replacement therapy (*39*). Thus, estrogen may modulate degradation of the extracellular matrix in vascular tissues and this may lead to more rapid progression of lesions in female mice. The effect of endogenous estrogen on apoE-KO mice exposed to hypoxia would be a suitable subject for future study.

In summary, the present study demonstrated that 3 weeks of hypoxia accelerated atherogenesis in the aortas of apoE-KO mice, accompanied by a significant increase of NADPH-dependent O_2^- production and MMP-9 activity. Thus, increased ROS generation and more rapid matrix degradation may induce the progression of atherosclerotic lesions.

Perspectives

The chronic hypoxic model used in this study might be adaptable to the breathing disorder diseases, such as the sleep apnea syndrome. One of the clinical characteristics of these diseases is hypoxia-induced ventricular dysfunction with pulmonary hypertension. A recent study has reported that the sleep apnea syndrome increased the risk of fatal and non-fatal cardiovascular events in accordance with ventricular dysfunction (*16*). Thus, the novel observation in this study that chronic hypoxia accelerated the development of atherosclerosis while also increasing O_2^- production and activating MMP-

9 in the aorta of apoE-KO mice highlights the potential benefit of blocking this pathway to prevent atherosclerosis and the subsequent cardiovascular events.

Acknowledgements

We would like to express our gratitude to Y. Kitaguni, C. Ohta, S. Uchida, Y. Ogami, and S. Imokawa for their expert technical assistance, and acknowledge the secretarial assistance of F. Maeda during the preparation of this manuscript.

References

1. Parthasarathy S, Young SG, Witztum JL, Pittman RC, Steinberg D: Probucol inhibits oxidative modification of low-density lipoprotein. *J Clin Invest* 1986; **77**: 641–644.
2. Barry-Lane PA, Patterson C, van der Merwe M, *et al*: p47phox is required for atherosclerotic lesion progression in ApoE−/− mice. *J Clin Invest* 2001; **108**: 1513–1522.
3. Williams RJ, Motteram JM, Sharp CH, Gallagher PJ: Dietary vitamin E and the attenuation of early lesion development in modified Watanabe rabbits. *Atherosclerosis* 1992; **94**: 153–159.
4. Rajagopalan S, Meng XP, Ramasamy S, Harrison DG, Galis ZS: Reactive oxygen species produced by macrophage-derived foam cells regulate the activity of vascular matrix metalloproteinases *in vitro*. *J Clin Invest* 1996; **98**: 2572–2579.
5. Yasunari K, Maeda K, Nakamura M, Yoshikawa J: Carvedilol inhibits pressure-induced increase in oxidative stress in coronary smooth muscle cells. *Hypertens Res* 2002; **25**: 419–425.
6. Pagano PJ, Clark JK, Cifuentes-Pagano ME, Clark SM, Callis GM, Quinn MT: Localization of a constitutively active, phagocyte-like NADPH oxidase in rabbit aortic adventitia: enhanced by angiotensin II. *Proc Natl Acad Sci U S A* 1997; **94**: 14483–14488.
7. Yin CJ, Xu JW, Takahashi K, Nara Y, Yamori Y: Tea polyphenols regulate nicotinamide adenine dinucleotide phosphate oxidase subunit expression and ameliorate angiotensin II-induced hyperpermeability in endothelial cells. *Hypertens Res* 2003; **26**: 823–828.
8. Pinsky DJ, Oz MC, Koga S, *et al*: Cardiac preservation is enhanced in a heterotopic rat transplant model by supplementing the nitric oxide pathway. *J Clin Invest* 1994; **93**: 2291–2297.
9. Griendling KK, Ushio-Fukai M: Redox control of vascular smooth muscle proliferation. *J Lab Clin Med* 1998; **132**: 9–15.
10. Gonzalez W, Fontaine V, Pueyo ME, *et al*: Molecular plasticity of vascular wall during N^G-nitro-L-arginine methyl ester-induced hypertension: modulation of proinflammatory signals. *Hypertension* 2000; **36**: 103–109.
11. Galis ZS, Sukhova GK, Lark MW, Libby P: Increased expression of matrix metalloproteinases and matrix degrading activity in vulnerable regions of human atherosclerotic plaques. *J Clin Invest* 1994; **94**: 2493–2503.
12. Damås JK, Wæhre T, Yndestad A, Gullestad L, Aukrust P: Atherosclerotic plaque stabilization—potential role for immunomodulatory therapy. *Vasc Dis Prevent* 2004; **1**: 17–31.
13. Duranteau J, Chandel NS, Kulisz A, Shao Z, Schumacker PT: Intracellular signaling by reactive oxygen species during hypoxia in cardiomyocytes. *J Biol Chem* 1998; **273**: 11619–11624.
14. Aoki M, Nata T, Morishita R, *et al*: Endothelial apoptosis induced by oxidative stress through activation of NF-κB. Antiapoptotic effect of antioxidant agents on endothelial cells. *Hypertension* 2001; **38**: 48–55.
15. Chen L, Einbinder E, Zhang Q, Hasday J, Balke WC, Scharf SM: Oxidative stress and left ventricular function with chronic intermittent hypoxia in rats. *Am J Respir Crit Care Med* 2005; **172**: 915–920.
16. Martin JM, Carrizo SJ, Vicente E, Agusti AGN: Long-term cardiovascular outcomes in men with obstructive sleep apnoea-hypopnoea with or without treatment with continuous positive airway pressure: an observational study. *Lancet* 2005; **365**: 1046–1053.
17. Takai S, Jin D, Sakaguchi M, *et al*: Comparative effects of candesartan and amlodipine in a monkey atherosclerotic model. *Hypertens Res* 2004; **27**: 517–522.
18. Okuda N, Hayashi T, Mori T, *et al*: Nifedipine enhances the cardioprotective effect of an angiotensin-II receptor blocker in an experimental animal model of heart failure. *Hypertens Res* 2005; **28**: 431–438.
19. Isoda K, Shiigai M, Ishigai N, *et al*: Deficiency of interleukin-1 receptor antagonist promotes neointimal formation after injury. *Circulation* 2003; **108**: 516–518.
20. Miller FJ, Cutterman DD, Rios DC, Heistad DD, Davidson BL: Superoxide production in vascular smooth muscle contributes to oxidative stress and impaired relaxation in atherosclerosis. *Circ Res* 1998; **82**: 1298–1305.
21. Nakano D, Itoh C, Ishii F, *et al*: Effects of sesamin on aortic oxidative stress and endothelial dysfunction in deoxycorticosterone acetate-salt hypertensive rats. *Biol Pharm Bull* 2003; **26**: 1701–1705.
22. Li Y, Zhu H, Kuppusamy P, Rouband V, Zweier JL, Trush MA: Validation of lucigenin (bis-*N*-methylacridinium) as a chemilumigenic probe for detecting superoxide anion radical production by enzymatic and cellular systems. *J Biol Chem* 1998; **273**: 2015–2023.
23. Skatchkov MP, Sperling D, Hink U, *et al*: Validation of lucigenin as a chemiluminescent probe to monitor vascular superoxide as well as basal vascular nitric oxide production. *Biochem Biophys Res Commun* 1999; **254**: 319–324.
24. Hayashi T, Sohmiya K, Ukimura A, *et al*: Angiotensin II receptor blockade prevents microangiopathy and preserves diastolic function in the diabetic rat heart. *Heart* 2003; **89**: 1236–1242.
25. Hayashi T, Nozawa M, Sohmiya K, *et al*: Efficacy of pancreatic transplantation on cardiovascular alterations in diabetic rats: an ultrastructural and immunohistochemical study. *Transplant Proc* 1998; **30**: 335–338.
26. Budhiraja R, Tuder RM, Hassoun PM: Endothelial dysfunction in pulmonary hypertension. *Circulation* 2004; **109**: 159–165.
27. Carmena R, Duriez P, Fruchart JC: Atherogenic lipoprotein particles in atherosclerosis. *Circulation* 2004; **109** (Suppl III): III-2–III-7.

28. Matsumoto K, Taniguchi T, Fujioka Y, Shimizu H, Ishikawa Y, Yokoyama M: Effects of hypoxia on cholesterol metabolism in human monocyte-derived macrophages. *Life Sci* 2000; **67**: 2083–2091.
29. Wada Y, Sugiyama A, Yamamoto T, *et al*: Lipid accumulation in smooth muscle cells under LDL loading is independent of LDL receptor pathway and enhanced by hypoxic conditions. *Arterioscler Thromb Vasc Biol* 2002; **22**: 1712–1719.
30. Sato H, Sato M, Kanai H, *et al*: Mitochondrial reactive oxygen species and c-Src play a critical role in hypoxic response in vascular smooth muscle cells. *Cardiovasc Res* 2005; **67**: 714–722.
31. Schäfer M, Schäfer C, Ewald N, Piper HM, Noll TH: Role of redox signaling in the autonomous proliferative response of endothelial cells to hypoxia. *Circ Res* 2003; **92**: 1010–1015.
32. Cathcart MK: Regulation of superoxide anion production by NADPH oxidase in monocytes/macrophages: contributions to atherosclerosis. *Arterioscler Thromb Vasc Biol* 2004; **24**: 23–28.
33. Kalinina N, Agrotis A, Tararak E, *et al*: Cytochrome b558-dependent NAD(P)H oxidase-phox units in smooth muscle and macrophages of atherosclerotic lesions. *Arterioscler Thromb Vasc Biol* 2002; **22**: 2037–2043.
34. Galis ZS, Khatri JJ: Matrix metalloproteinases in vascular remodeling and atherogenesis. The good, the bad, and the ugly. *Circ Res* 2002; **90**: 251–262.
35. Galia ZS, Sukhova GK, Kranzhöfer R, Clark S, Libby P: Macrophage foam cells from experimental atheroma constitutively produce matrix-degrading proteinases. *Proc Natl Acad Sci U S A* 1995; **92**: 402–406.
36. Brown DL, Hibbs MS, Kearney M, Loushin C, Isner JM: Identification of 92-kD gelatinase in human coronary atherosclerotic lesions. Association of active enzyme synthesis with unstable angina. *Circulation* 1995; **91**: 2125–2131.
37. Lemaître V, O’Byrne TK, Borczuk AC, Okada Y, Tall AR, D’Armiento J: ApoE knockout mice expressing human matrix metalloproteinase-1 in macrophages have less advanced atherosclerosis. *J Clin Invest* 2001; **107**: 1227–1234.
38. Potier M, Elliot SJ, Tack I, *et al*: Expression and regulation of estrogen receptors in mesangial cells: influence on matrix metalloproteinase-9. *J Am Soc Nephrol* 2001; **12**: 241–251.
39. Xu Y, Arenas IA, Armstrong SJ, Davidge ST: Estrogen modulation of left ventricular remodeling in the aged heart. *Cardiovasc Res* 2003; **57**: 388–394.