Original Article

Tissue Inhibitor of Metalloproteinase-3 Deficiency Inhibits Blood Pressure Elevation and Myocardial Microvascular Remodeling Induced by Chronic Administration of N^{ω}-Nitro-L-Arginine Methyl Ester in Mice

Masayoshi HIGUCHI[1)], Osamu YASUDA[1)], Hidenobu KAWAMOTO[1)], Takamori YOTSUI[1)], Yoshichika BABA[1)], Tohru OZAKI[2)], Nobuyo MAEDA[3)], Keisuke FUKUO[4)], Hiromi RAKUGI[1)], and Toshio OGIHARA[1)]

Hypertension is a major risk factor for cardiovascular disease. Thus, prevention of hypertension and consequent organ damage is important for reducing its incidence. In the present study, we examined the involvement of tissue inhibitor of metalloproteinase-3 (Timp-3) in N^{ω}-nitro-L-arginine methyl ester (L-NAME)–induced hypertension and accompanying vascular remodeling in mice. L-NAME was orally administered to wild-type (WT) and Timp-3 knockout (KO) mice for 6 weeks, blood pressure was monitored, and histological changes in myocardial arteries were examined. After L-NAME administration, blood pressure was lower in Timp-3 KO mice than in WT mice. The coronary arteries of WT and Timp-3 KO mice were similar after L-NAME treatment and showed no differences compared to untreated control mice. However, cardiac microvessels differed histologically between WT and Timp-3 KO mice. Vascular walls were less thickened in Timp-3 KO than in WT mice, and fibrotic changes were significantly reduced in Timp-3 KO mice. Moreover, the L-NAME–induced production of reactive oxygen species in cardiac microvessels was lower in Timp-3 KO than in WT mice. These results indicate that Timp-3 plays an important role in L-NAME–induced hypertension and myocardial vascular remodeling. Our findings suggest that Timp-3 may be a novel therapeutic target for the treatment of hypertension and consequent organ damage. (*Hypertens Res* 2007; 30: 563–571)

***Key Words*: tissue inhibitor of metalloproteinase-3, N^{ω}-nitro-L-arginine methyl ester, hypertension, vascular remodeling**

From the [1)]Department of Geriatric Medicine, Osaka University Graduate School of Medicine, Suita, Japan; [2)]Astellas Pharma Inc., Osaka, Japan; [3)]Department of Pathology and Laboratory Medicine, School of Medicine, The University of North Carolina at Chapel Hill, Chapel Hill, USA; and [4)]Department of Food Sciences and Nutrition, School of Human Environmental Sciences, Mukogawa Women's University, Mukogawa, Japan.

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Address for Reprints: Osamu Yasuda, M.D., Ph.D., Department of Geriatric Medicine, Osaka University Graduate School of Medicine, 2–2 Yamadaoka, Suita 565–0871, Japan. E-mail: yasuda@geriat.med.osaka-u.ac.jp

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Introduction

Hypertension is one of the major risk factors for atherosclerosis, which is accelerated in combination with other risk factors such as diabetes mellitus, hyperlipidemia, and smoking. Therefore, it is clinically important to control blood pressure for the prevention of cardiovascular diseases (*1–4*).

Blood pressure is determined mainly by cardiac output and peripheral vascular resistance, which is regulated by the constriction and relaxation of peripheral arteries. The sympathetic nervous system, angiotensin II, and endothelin are vasoconstrictive factors, whereas nitric oxide (NO), originally identified as an endothelium-dependent relaxing factor (*5*), is a vasodilator. The production of NO in vascular systems is catalyzed mainly by endothelial NO synthase (eNOS). Transgenic mice overexpressing eNOS have hypotension (*6*), whereas mice lacking the eNOS gene or carrying a mutated eNOS gene have elevated blood pressure (*7–9*).

Chronic administration of N^{ω}-nitro-L-arginine methyl ester (L-NAME), an artificial inhibitor of NO synthase (NOS), raises blood pressure in animals, which are used as an important hypertensive model (*10*, *11*). In this model, chronic administration of L-NAME induces a proinflammatory phenotype in the arterial wall (*12*, *13*), and both increases the wall-to-lumen ratio and promotes perivascular fibrosis in coronary microvessels. Recent studies indicate the involvement of the renin-angiotensin system and the sympathetic nervous system in the mechanism to elevate blood pressure in this model (*14–16*).

Matrix metalloproteinases (MMP) are believed to play a significant role in the degradation of extracellular matrix components (*17*). MMP activity is strictly regulated to maintain tissue homeostasis at a minimum of three steps: transcription, activation, and inhibition. The tissue inhibitor of metalloproteinase (Timp), which binds to MMP with a 1:1 molar stoichiometry, is an endogenous inhibitor of MMP. Four Timp members (Timp-1 to -4) have been reported (*18*). Among the four Timp members, Timp-3 is unique in that it exists bound to the extracellular matrix (*19*, *20*), which may localize its action.

Furthermore, Timp-3 is unique in that it inhibits the tumor necrosis factor (TNF)-α–converting enzyme (TACE) (*21*), a member of the ADAM (A disintegrin and metalloproteinase) family also known as ADAM17. This action of Timp-3 is important for TNF-dependent systemic inflammation (*22*). Timp-3 expression induces apoptosis of human colon carcinoma cell lines by stabilizing the TNF receptor, possibly due to protection from the proteolytic activities of MMP (*23*, *24*). On the other hand, Timp-3 inhibits angiogenesis by binding to the vascular endothelial growth factor (VEGF) receptor VEGFR2 and blocking the action of VEGF (*25*).

Mice deficient in Timp-3 gene expression have been reported to develop dilated cardiomyopathy (*26*) and alveolar enlargement (*27*), indicating that Timp-3 plays important roles in the maintenance of cardiac and alveolar tissue homeostasis. We have recently reported that Timp-3 is important for the maintenance of the kidney macrostructure after unilateral ureteral obstruction (*28*). On the other hand, in humans, mutations in the Timp-3 gene cause the autosomal dominant hereditary disease, Sorsby's fundus dystrophy, which is characterized by central vision loss in the third or fourth decade of life (*29–31*). The role of Timp-3 in atherosclerosis has been reported. Timp-3 expression was higher in human atheroma than in non-atherosclerotic tissue, suggesting that Timp-3 influences plaque stability by counteracting MMP activity (*32*). Adenoviral expression of Timp-3 inhibited neointima formation in porcine coronary arteries (*33*). Therefore, it is predicted that Timp-3 is involved in vascular remodeling and in perivascular fibrosis through the regulation of MMP.

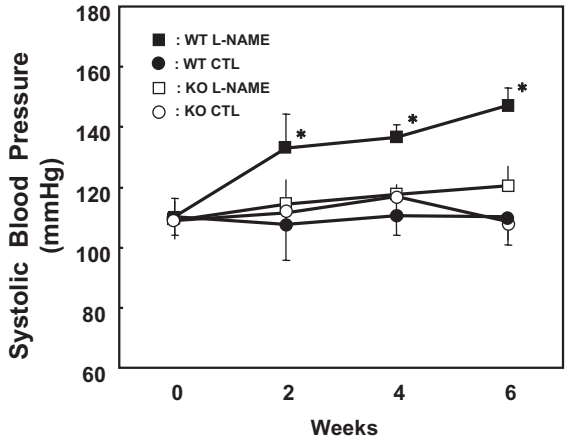

Fig. 1. *Systolic blood pressure of L-NAME–treated (L-NAME) or untreated (CTL) wild-type (WT) and Timp-3 knockout (KO) mice. Data are means±SD. *$p < 0.05$ vs. L-NAME–treated KO mice.*

In this study, we hypothesized that Timp-3 is involved in vascular remodeling and in blood pressure control. We investigated the role of Timp-3 in L-NAME–induced hypertension and perivascular fibrosis using Timp-3 knockout (KO) mice. Furthermore, we examined the influence of Timp-3 on perivascular oxidative stress, which is thought to be involved in the pathogenesis of atherosclerosis.

Methods

Animals

Timp-3 KO mice were generated using a gene-targeting technique as described previously (*28*). Mice carrying the mutant allele were backcrossed with C57BL/6 mice more than seven times to produce the genetic background of C57BL/6. Genotypes of mice were determined by polymerase chain reaction (PCR) using tail DNA as described (*28*).

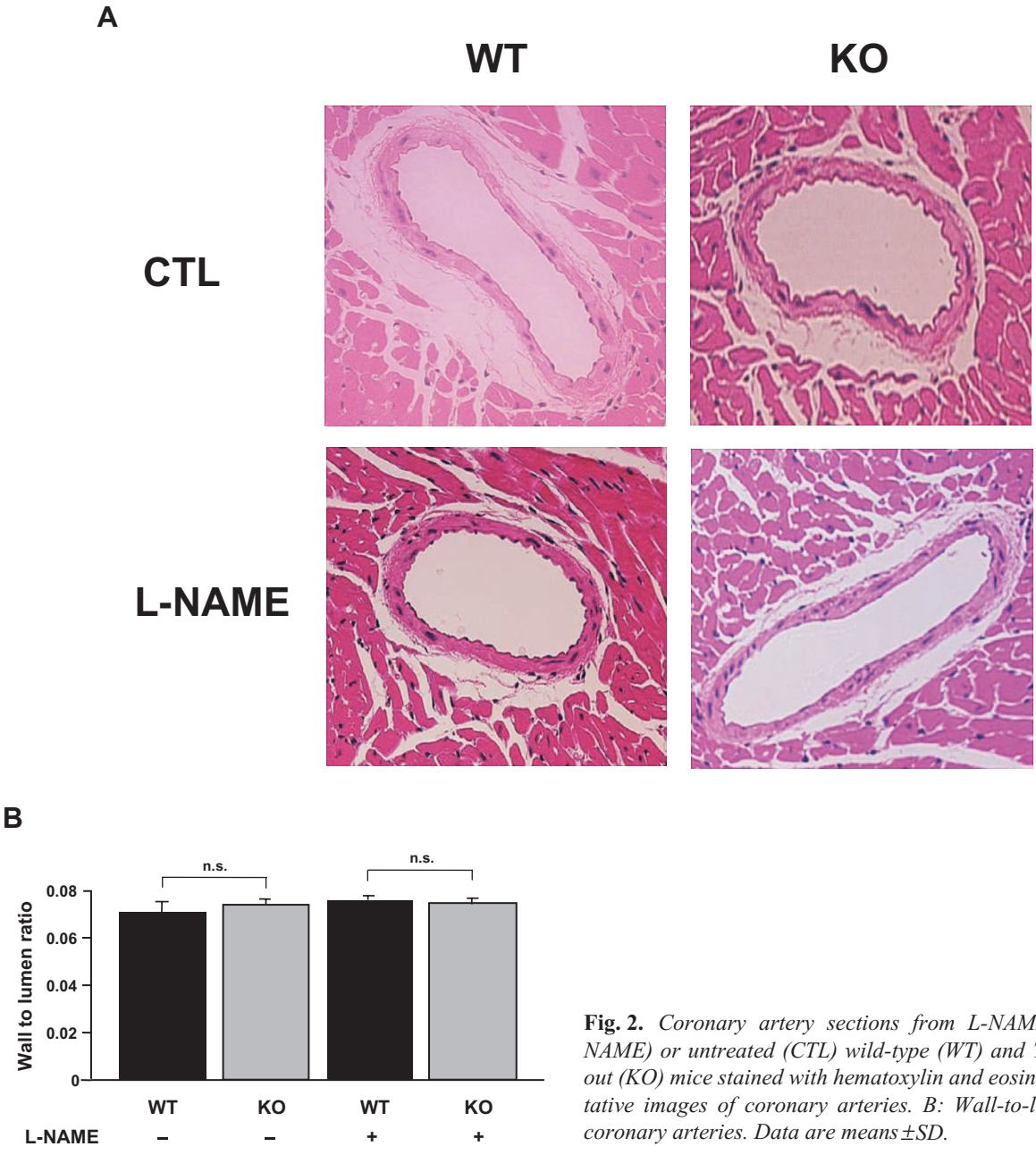

Fig. 2. *Coronary artery sections from L-NAME–treated (L-NAME) or untreated (CTL) wild-type (WT) and Timp-3 knockout (KO) mice stained with hematoxylin and eosin. A: Representative images of coronary arteries. B: Wall-to-lumen ratio of coronary arteries. Data are means ±SD.*

L-NAME Administration and Blood Pressure Measurement

Eight-week-old wild-type (WT) and Timp-3 KO mice weighing 25–30 g were used. C57BL/6 mice were used as a WT control. Mice were randomly assigned to a control group or an L-NAME group and fed a standard diet. L-NAME (Sigma Chemical, St. Louis, USA) was administered to the L-NAME group mice at a concentration of 3 mg/mL through drinking water for 6 weeks. Each group consisted of 10 mice. Blood pressure was serially determined in conscious, trained mice using a noninvasive tail-cuff device by a researcher blinded to the genotypes and groups. The experimental protocols were approved by the Osaka University Medical School Animal Care and Use Committee and were performed in accordance with the Osaka University Medical School Guidelines for the Care and Use of Laboratory Animals.

Histological Analysis

Mice were sacrificed with an overdose of pentobarbital after 6 weeks of L-NAME administration. The hearts were removed and fixed in 4% paraformaldehyde (pH 7.4). Fixed tissues were placed in Tissue-Tek OCT embedding medium

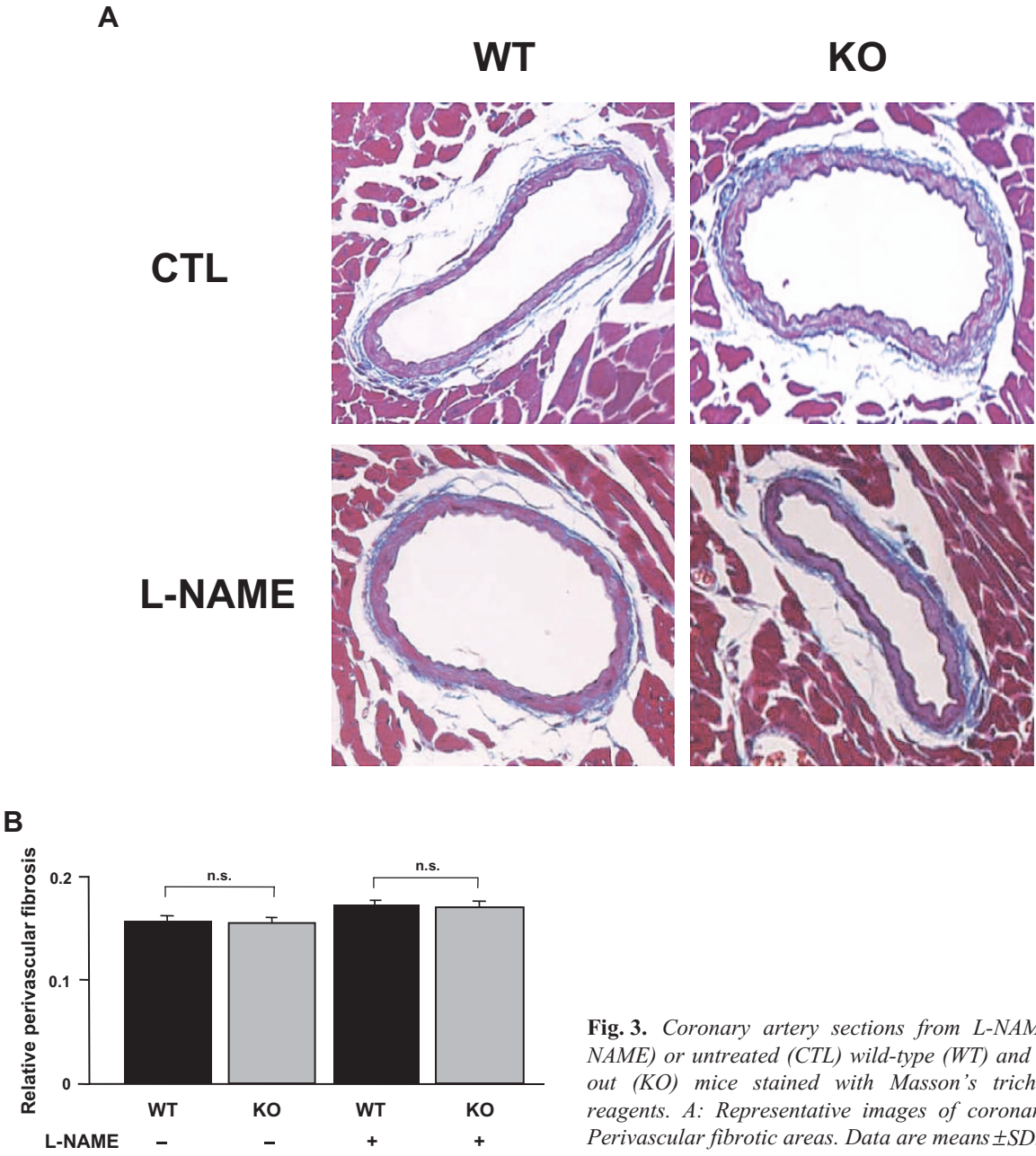

Fig. 3. *Coronary artery sections from L-NAME–treated (L-NAME) or untreated (CTL) wild-type (WT) and Timp-3 knockout (KO) mice stained with Masson's trichrome staining reagents. A: Representative images of coronary arteries. B: Perivascular fibrotic areas. Data are means ± SD.*

(Miles, Elkhart, USA), sectioned, and stained with hematoxylin and eosin for the evaluation of vascular wall thickness or with Masson's trichrome staining reagents for the assessment of perivascular fibrosis. The extents of medial thickening and of perivascular fibrosis of arteries were evaluated by the ratio of medial thickness to internal diameter and by the ratio of perivascular fibrosis area to total vascular area, respectively. The thicknesses and areas were calculated by using NIH Image version 1.55.

Detection of Oxidative Stress

Reactive oxygen species (ROS) production in the myocardial microvessels was detected by dihydroethidium (DHE) staining. Frozen, enzymatically intact, 10-μm–thick sections of WT and Timp-3 KO hearts were incubated at the same time with 10 μmol/L DHE in PBS for 30 min at 37°C in a light-protected, humidified chamber. DHE is oxidized by ROS and converted to ethidium, which binds to DNA in the nucleus and fluoresces red. ROS production was examined under a fluorescent microscope.

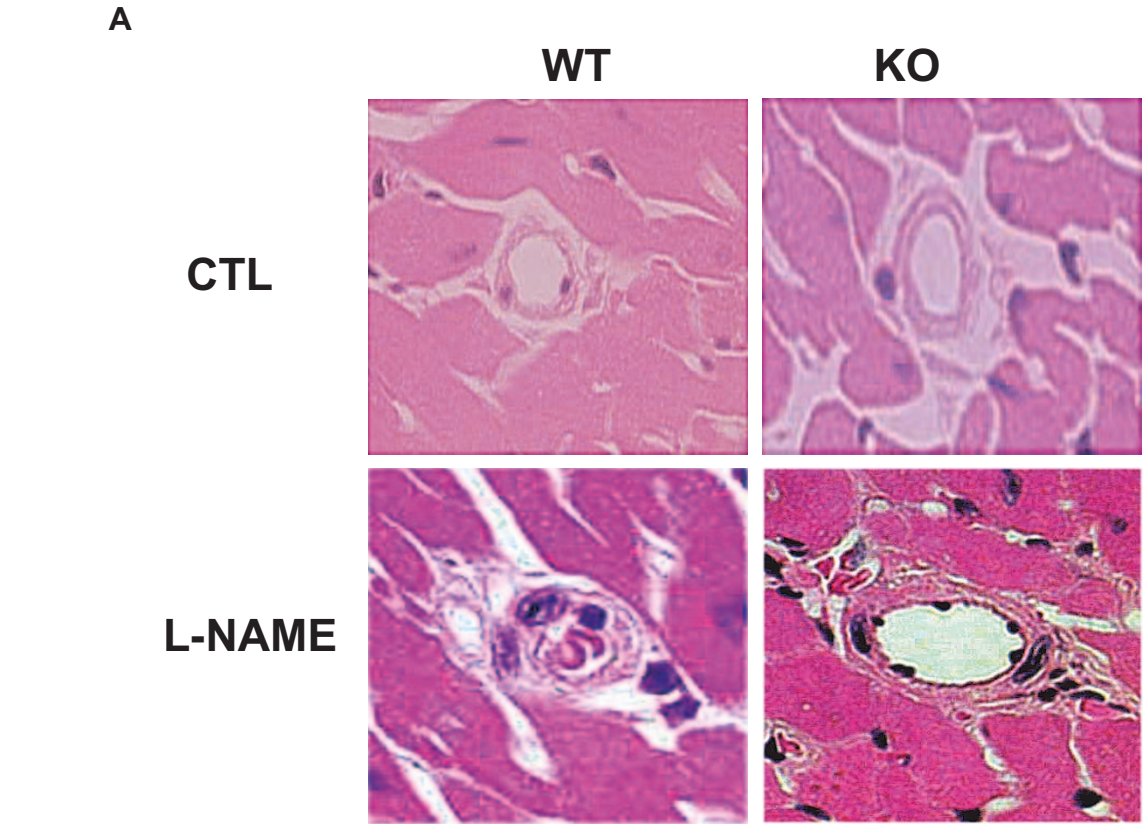

Fig. 4. *Myocardial microvessel sections from L-NAME–treated (L-NAME) or untreated (CTL) wild-type (WT) and Timp-3 knockout (KO) mice stained with hematoxylin and eosin. A: Representative images of myocardial microvessels. B: Wall-to-lumen ratio of myocardial microvessels. Data are means ± SEM. *$p < 0.01$.*

Statistical Analysis

Statistical analysis was performed using Student's unpaired *t*-test. Results are expressed as means ± SD.

Results

Blood Pressure

Systolic blood pressure data are shown in Fig. 1. Systolic blood pressure did not differ between WT and Timp-3 KO mice at baseline. However, it was significantly different after the initiation of L-NAME treatment. Blood pressure was increased in WT mice after 2 weeks of L-NAME (3 mg/mL) administration. Timp-3 KO mice had much lower blood pressure than WT mice at every time point of blood pressure monitoring after the initiation of L-NAME treatment, although Timp-3 KO blood pressure was significantly elevated after 4 weeks of L-NAME administration compared to the pretreatment level. For the untreated control groups, blood pressure did not differ significantly between WT and Timp-3 KO groups during the 6 weeks of monitoring. Systolic blood pressure was not elevated in either untreated group throughout the observation period.

Histological Analysis of Coronary Arteries

To investigate the effect of L-NAME treatment on coronary

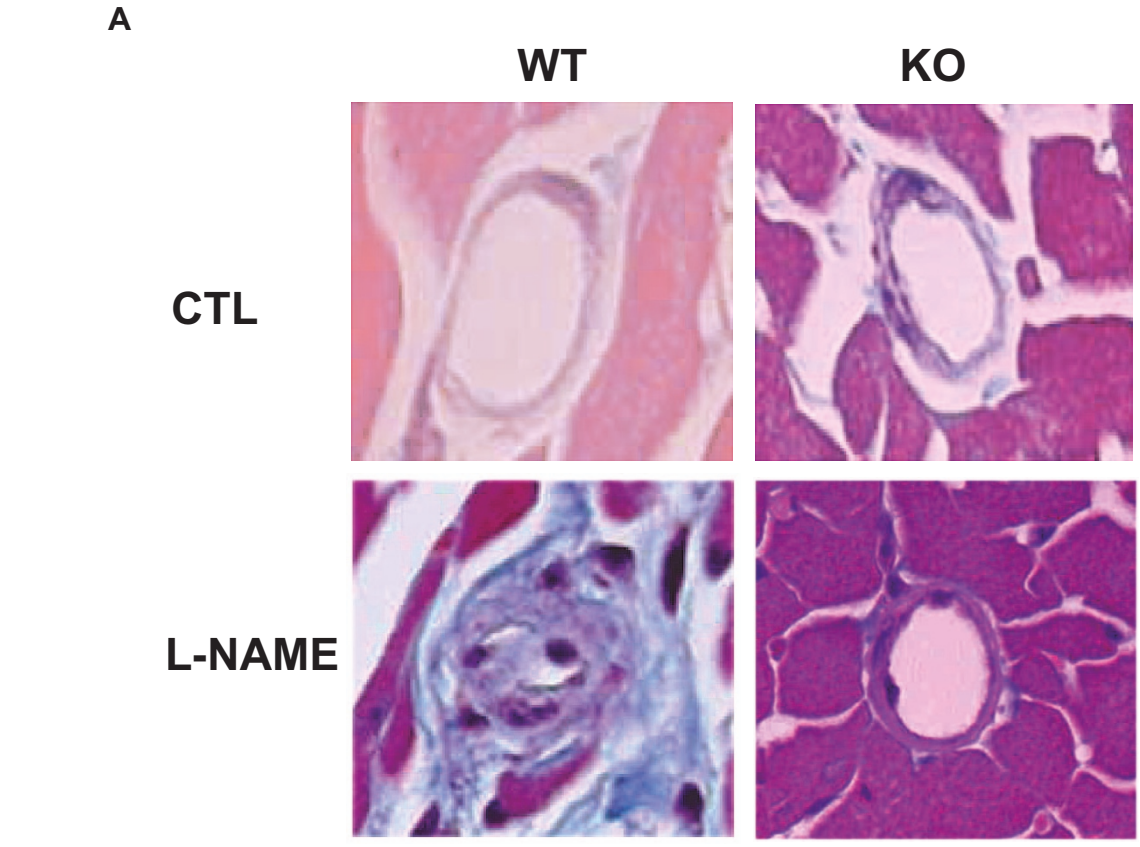

Fig. 5. *Myocardial microvessel sections from L-NAME–treated (L-NAME) or untreated (CTL) wild-type (WT) and Timp-3 knockout (KO) mice stained with Masson's trichrome staining reagents. A: Representative images of myocardial microvessels. B: Perivascular fibrotic areas. Data are means±SEM. *$p < 0.01$.*

arteries, we investigated vascular wall thickness and perivascular fibrosis after 6 weeks of L-NAME administration. Coronary arterial wall thickness and perivascular fibrosis surrounding coronary arteries were evaluated in sections stained with hematoxylin and eosin (Fig. 2) and with Masson's trichrome staining reagents (Fig. 3), respectively. L-NAME–treated coronary arteries did not show wall thickening or progression of perivascular fibrosis in WT mice. Timp-3 KO mice also had no significant vascular wall thickening or fibrosis compared to the untreated control.

Histological Analysis of Myocardial Microvessels

We next investigated the histological changes in coronary microvessels after 6 weeks of L-NAME treatment. The vascular wall thickness was similar between WT and Timp-3 KO mice in the untreated control groups (Fig. 4). However, after treatment with L-NAME, vascular wall thickness was significantly less in Timp-3 KO mice than in WT mice. In control untreated mice, perivascular fibrotic changes were similar in WT and Timp-3 KO mice (Fig. 5). After L-NAME treatment, fibrosis surrounding coronary microvessels was significantly less in Timp-3 KO than in WT animals.

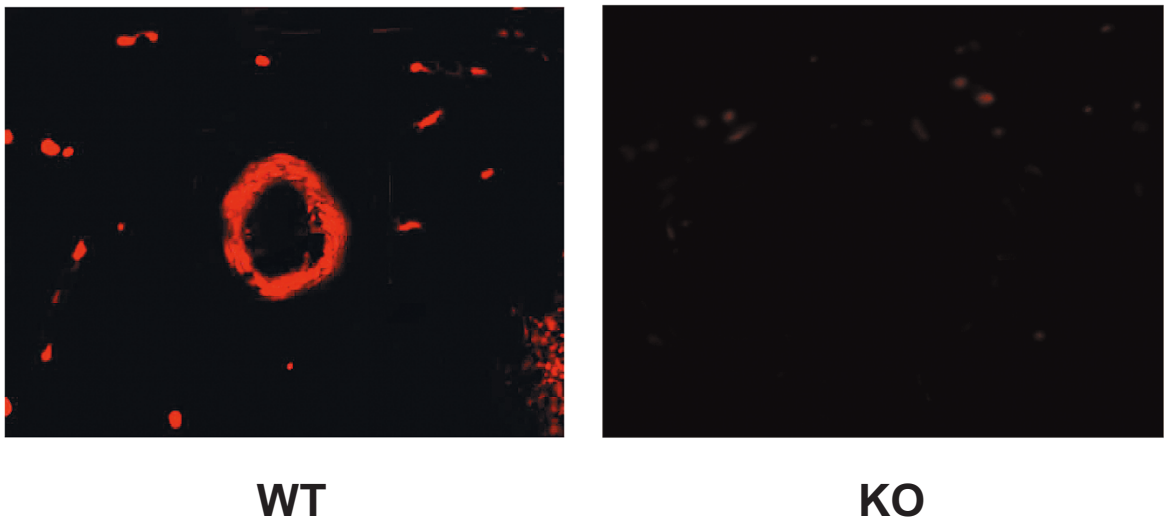

Fig. 6. *Reactive oxygen species production in myocardial microvessels from L-NAME–treated wild-type (WT) and Timp-3 knockout (KO) mice. Sections were stained with dihydroethidium. Representative fluorescent microscopic images are shown.*

Oxidative Stress in Myocardial Microvessels

To elucidate the effect of L-NAME treatment in myocardial microvessels, we assessed ROS generation. Dihydroethidium staining showed obvious ROS generation in WT microvessels. However, little or no oxidative stress was detected in Timp-3 KO microvessels (Fig. 6). This indicates that Timp-3 plays an important role in L-NAME–induced oxidative stress.

Discussion

In this study, we used mice lacking the Timp-3 gene to investigate whether or not Timp-3 is involved in L-NAME–induced hypertension and vascular remodeling. During the 6 weeks of L-NAME administration, WT mice developed marked increases in blood pressure and coronary microvascular wall thickening and fibrosis, although coronary arteries showed almost no changes compared to L-NAME–untreated controls. However, changes caused by L-NAME treatment were not apparent in Timp-3 KO mice, indicating that Timp-3 is involved in L-NAME–induced elevation of blood pressure and cardiac microvascular remodeling. Moreover, a marked decrease in ROS generation in coronary microvessels was observed in Timp-3 KO mice, implicating Timp-3 in microvascular oxidative stress.

Timp-3 expression is induced by the inflammatory cytokine transforming growth factor (TGF)-β, which mediates tissue fibrosis, and is observed in human atheroma (*32*). Inflammation and ROS production play roles in the remodeling of myocardial microcirculation in pigs in early hypertension (*34*). Therefore, it seems likely that Timp-3 is involved in vascular remodeling and in ROS production induced by hypertension.

In L-NAME–treated animals, the synthesis of NO, a vasodilatory molecule, is prevented through the inhibition of NOS, causing blood pressure to rise. The sympathetic nervous system and renin-angiotensin system are also involved in L-NAME–induced hypertension (*15*). Furthermore, L-NAME promotes vascular remodeling including wall thickening and fibrosis.

The mechanism by which Timp-3 deficiency influences L-NAME–induced hypertension remains to be elucidated. One possible explanation is that activated MMP in Timp-3 KO mice degrades extracellular matrix components surrounding peripheral vessels, reducing vascular resistance and preventing the elevation of blood pressure. It has been reported that plasminogen activator inhibitor-1 (PAI-1) deficiency prevents hypertension induced by long-term L-NAME treatment (*35*). This result seems consistent with our work because PAI-1 is a plasmin inhibitor that activates MMP and contributes to the formation of aneurysms (*36*, *37*).

Another possibility is that the tissue renin-angiotensin system is involved in L-NAME–induced hypertension and perivascular fibrosis. This hypothesis is supported by the fact that the effects of long-term L-NAME treatment on the vascular system are mediated not only by NOS inhibition but also by the local renin-angiotensin system and by ROS production (*14*, *38*). It seems likely that diminished oxidative stress in Timp-3 KO mice reduced vascular resistance and fibrotic changes surrounding microvessels.

Although blood pressure was lower in Timp-3 KO mice than in WT mice after L-NAME treatment, the activity and appearance of Timp-3 KO mice were indistinguishable from those of WT mice in this study. This indicates that the lowered blood pressure in Timp-3 KO mice was not due to impaired systemic conditions that reduce blood pressure, such

as cardiac failure and blood volume loss. Moreover, the basal blood pressure before L-NAME treatment was not different between Timp-3 KO and WT mice. These findings indicate that Timp-3 is an important factor in blood pressure elevation by L-NAME.

In this study, we showed for the first time that Timp-3 plays essential roles in L-NAME–induced elevation of blood pressure and cardiac vascular remodeling. These findings indicate that inhibition of Timp-3 can help protect the cardiovascular system and may prevent hypertension. Therefore, Timp-3 is a novel target for the treatment of hypertension and organ damage due to high blood pressure.

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