BBA - Molecular Basis of Disease 1865 (2019) 2379–2392

Contents lists available at ScienceDirect

BBA - Molecular Basis of Disease

journal homepage: www.elsevier.com/locate/bbadis

5-Lipoxagenase deficiency attenuates L-NAME-induced hypertension and vascular remodeling

Jia-xiang Chen[a,1], Kun-yue Xue[a,1], Juan-juan Xin[a,1], Xin Yan[a,b,1], Ru-Li Li[a], Xiao-Xiao Wang[a], Xu-Lei Wang[a,c], Ming-ming Tong[a], Lu Gan[a], He Li[a], Jie Lan[a], Xue Li[a], Cai-li Zhuo[a], Ling-yu Li[a], Zi-jie Deng[a], Heng-Yu Zhang[d], Wei Jiang[a,*]

[a] Molecular Medicine Research Center, State Key Laboratory of Biotherapy, West China Hospital, Sichuan University, Chengdu, Sichuan 610041, PR China
[b] Department of Critical Care Medicine, West China Hospital, Sichuan University, Chengdu, Sichuan 610041, PR China
[c] School of Life Sciences and Bioengineering, Southwest Jiaotong University, Chengdu, Sichuan 610031, PR China
[d] Department of Cardiology, West China Hospital, West China Medical School, Sichuan University, Chengdu, Sichuan 610041, PR China

ARTICLE INFO

Keywords:
5-lipoxygenase
Leukotriene B4
Cysteinyl leukotriene
Hypertension
Vascular remodeling

ABSTRACT

Background: Abnormalities of the L-arginine-nitric oxide pathway induce hypertension. 5-Lipoxygenase (5-LO) is the key enzyme involved in synthesis of leukotrienes (LTs). However, whether nitricoxide synthase dysfunction induces hypertensive vascular remodeling by regulating 5-LO activity and its downstream inflammatory metabolites remains unknown.

Methods and results: Six-week L-NAME treatment significantly induced hypertension and vascular remodeling in both wild-type (WT) and 5-LO–knockout (5-LO–KO) mice, and blood pressure in caudal and carotid arteries was lower in 5-LO–KO than WT mice with L-NAME exposure. On histology, L-NAME induced less media thickness, media-to-lumen ratio, and collagen deposition and fewer Ki-67–positive vascular smooth muscle cells (VSMCs) but more elastin expression in thoracic and mesenteric aortas of 5-LO–KO than L-NAME–treated WT mice. L-NAME significantly increased LT content, including LTB4 and cysteinyl LT (CysLTs), in plasma and neutrophil culture supernatants from WT mice. On immunohistochemistry, L-NAME promoted the colocalization of 5-LO and 5-LO–activating protein on the nuclear envelope of cultured neutrophils, which was accompanied by elevated LT content in culture supernatants. In addition, LTs significantly promoted BrdU incorporation, migration and phenotypic modulation in VSMCs.

Conclusion: L-NAME may activate the 5-LO/LT pathway in immune cells, such as neutrophils, and promote the products of 5-LO metabolites, including LTB4 and CysLTs, which aggravate vascular remodeling in hypertension. 5-LO deficiency may protect against hypertension and vascular remodeling by reducing levels of 5-LO downstream inflammatory metabolites.

1. Introduction

Hypertension is a serious disease that can damage blood vessel walls and increase the risk of atherosclerosis, heart attack, stroke and other conditions [1,2]. Under hypertensive pathological conditions, elevated blood pressure acutely changes blood vessel tone and also alters the structure of vessels chronically [1,3]. The structure alteration or rearrangement is mediated by vascular remodeling, considered an adaptive process in response to a long-term abnormal hemodynamic condition, which is also regulated by humoral factors [3]. The mechanisms of vascular remodeling, characterized by abnormal function, media growth, extracellular matrix deposition and inflammation, are complex [1–3]. Recent investigation revealed that inflammatory processes are important in the pathogenesis of hypertension and further

Abbreviations: 5-LO, 5-lipoxygenase; FLAP, 5-lipoxygenase activating protein; AA, arachidonic acid; HPETE, hydroperoxyeicosatetraenoic acid; LTs, leukotrienes; LTB4, leukotriene B4; CysLTs, Cysteinyl Leukotriene; GPCR, G protein-coupled receptor; NO, Nitric oxide; NOS, nitric oxide synthase; eNOS, endothelial nitric oxide synthase; iNOS, the calcium-independent nitric oxide synthase; cNOS, the calcium-dependen nitric oxide synthase; L-NAME, N^G-nitro-$_L$-arginine methyl ester; VSMC, vascular smooth muscle cell; LV, left ventricle; DBP, diastolic blood pressure; MBP, mean arterial blood pressure; SBP, systolic blood pressure; EEL, external elastic lamina; IEL, internal elastic lamina; SNOG, S-nitrosoglutathione; LPS, lipopolysaccharide

* Corresponding author.
E-mail address: wcumsjw@scu.edu.cn (W. Jiang).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.bbadis.2019.05.021
Received 8 January 2019; Received in revised form 22 May 2019; Accepted 31 May 2019
Available online 02 June 2019
0925-4439/ © 2019 Elsevier B.V. All rights reserved.

trigger vascular fibrosis to exacerbate remodeling [4]. Currently, the precise role of inflammation in vascular remodeling related to hypertension is unclear [1–4].

Leukotrienes (LTs) are a series of derivatives of arachidonic acid (AA) that are synthesized at the nuclear membrane in inflammatory cells including neutrophils, macrophages, eosinophils and mast cells in response to diverse immune and inflammatory stimuli [5]. 5-Lipoxygenase (5-LO) is one of the key enzymes in LT biosynthesis [6]. With the aid of 5-LO–activating protein (FLAP), it catalyzes the reaction that converts AA to hydroperoxyeicosatetraenoic acid (HPETE) [5]. HPETE continually forms the unstable intermediate leukotriene A4 (LTA4), which can further be hydrolyzed into leukotriene B4 (LTB4), a potent neutrophil chemotaxin, or conjugated with glutathione to yield cysteinyl leukotrienes (CysLTs), including leukotriene C4 (LTC4), D4 (LTD4) and E4 (LTE4) [5,6]. LTB4 and CysLTs mediate their actions via G protein-coupled receptors (GPCRs), LTB4 receptors BLT1 and BLT2, and CysLT receptors CysLT1 and CysLT2 [6]. These receptors are expressed on different target cells, including leukocytes, smooth muscle cells and endothelial cells [5,6].

LTs play important roles in maintaining the functions of the cardiovascular system [6,7]. Both LTB4 and CysLTs can cause smooth muscle constriction and increase post-capillary venule permeability, and CysLTs exert negative inotropic action on the myocardium and decrease coronary blood flow, with no effect on heart rate [6,7]. The 5-LO/LT pathway is involved in cardiovascular diseases because it can promote atherosclerosis, abdominal aortic aneurysm, and myocardial infarction/reperfusion injury by inducing leucocyte chemotaxis, vascular inflammation, permeability, and subsequent matrix degeneration [7]. Currently, inflammation, which may be low grade, is strongly implicated as an important trigger of vascular fibrosis in hypertension [4,8]. However, the pathophysiological action of the 5-LO/LT pathway in hypertension and vascular remodeling is unknown [5–8].

Nitric oxide (NO), produced by endothelial nitric oxide synthase (eNOS), is a vital signaling molecule maintaining vascular homeostasis [9]. Endothelial dysfunction, especially impaired NO/NOS bioavailability in endothelial cells, is an important component of both hypertension and cardiovascular diseases [9,10]. Given that inhibition or gene disruption of eNOS leads to increased blood pressure in animal hypertension models, a tight link between impaired NO/NOS bioactivity and hypertension was demonstrated [10,11]. Previous studies indicated a negative regulation between NOS and 5-LO activity in vessels [11]. Laszlo et al. reported that a selective 5-LO inhibitor effectively alleviated the acute microvascular injury caused by NO inhibition in the early stage of endotoxin-induced sepsis in rat [12]. Further evidence revealed that L-NAME increased the contribution of 5-LO and NADPH oxidase to reactive oxygen/nitrogen species production in the rat aorta [13], and NOS inhibition activated the 5-LO/LT pathway in human mast cells [14].

However, few studies have investigated the role of 5-LO/LT pathway in regulating hypertension induced by endothelial or NOS dysfunction [6,11–14]. Here, we found that 5-LO deficiency, decreasing plasma LTB4 and CysLT contents, could effectively attenuate elevated blood pressure and vascular remodeling in hypertensive mice under L-NAME induction. NOS inhibition due to L-NAME treatment induced 5-LO activity and also LTB4 and CysLT production in neutrophils, the main cellular source of LT production, and these inflammatory mediators further induced remodeling phenotypes, including proliferation and migration, in vascular smooth muscle cells (VSMCs).

2. Methods

2.1. Reagents

L-NAME, collagenase I, elastase VI, lipopolysaccharide (LPS), insulin-like growth factor 1 (IGF−I), hemotoxylin-eosin, histopaque-1083 and -1119 gradients, dextran T-500, calmodulin and actinomycin D were from Sigma Aldrich (St Louis, MO, USA); LTB4, CysLT mixture, anti-5-lipoxygenase antibody, Griess Reagent kit, and ELISA kits for LTB4 (No. 520111), CysLT (No. 10009291), LTC4 (No. 501070) and LTE4 (No. 501060) content assay were from Cayman Chemical (Ann Arbor, MI, USA); ELISA kits for mouse LTD4 (Catalog #, MBS263057) content assay were from MyBioSource (MyBioSource, San Diego, CA, USA); NO donor S-nitrosoglutathione (SNOG), anti-FLAP and anti-Ki-67 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA); anti-SMC α-actin antibody was from Cell Signaling Technology (Danvers, MA, USA); anti-CD11b FITC and anti-CD45 PerCP antibodies were from BD Biosciences (San Diego, CA, USA); Dulbecco's modified Eagle's medium, Hank's Balance Salt Solution, RPMI 1640 medium (phenol red free) and fetal bovine serum (FBS) were from GIBCO BRL (Carlsbad, CA, USA); TRIzol Reagent and Alexa Fluor 488-, Alexa Fluor 594- and FITC-conjugated secondary antibodies were from Invitrogen (Carlsbad, CA, USA); SuperScript™ II reverse transcriptase was from Promega (Hercules, CA, USA); iQ™ SYBRH Green Supermix was from Bio-Rad Laboratories (Hercules, CA, USA); dNTP was from Takara Bio Inc. (Shiga, Japan); Masson trichrome and elastin staining kits were from Baso Biological Technology (Zhuhai, China); cell proliferation ELISA and BrdU (colorimetric) kits were from Roche (Indianapolis, IN, USA); DAF-FM kit was from Molecular Probes (Carlsbad, CA, USA); and L-[^{3}H]arginine was from PerkinElmer (St. Waltham, MA, USA). Oligonucleotides were synthesized by Shenggong Biotechnology (Shanghai). Other chemicals and reagents were of analytical grade of internal standards.

2.2. Animals

Alox5$^{-/-}$(5-LO–KO) mice on a C57BL/6J background and wild-type C57BL/6J mice (WT) were purchased from the Jackson Laboratory (Bar Harbor, ME, USA); the genotype of 5-LO–KO mice was confirmed by PCR analysis (data not shown). Male Sprague–Dawley (SD) rats, 8–12 weeks old and weighing 250–300 g, were supported by the animal center, Health Sciences Center, Sichuan University. All mice were bred 4 per cage at 20–24 °C, humidity 60 ± 10%, on a 10-h dark/14-h light cycle (from 6:00 to 20:00). All experimental procedures were performed in accordance with the Guidelines for Animal Experiments from the Committee of Medical Ethics, National Health Department of China.

2.3. Experimental hypertension model

Male 5-LO–KO and WT mice, at 8 weeks old, weighing 20–23 g, were randomly assigned to L-NAME-treated or control groups. L-NAME-treated mice were administered 80 mg/kg/day L-NAME in regular drinking water for 6 weeks to develop hypertension [15], and 5-LO–KO and WT control mice received drinking water without any inducer. The drinking water was replaced with fresh water every other day. In addition, the water intake per day did not differ between L-NAME-treated 5-LO–KO and WT mice or 5-LO–KO and WT control mice (3.06 ± 0.55, 3.02 ± 0.61, 3.08 ± 0.73, and 3.05 ± 64 mL/day/mouse, respectively).

2.4. Blood pressure measurement

Systolic blood pressure (SBP), mean arterial blood pressure (MBP), diastolic blood pressure (DBP) and heart rate were detected by two different methods. A noninvasive computerized tail-cuff system [15] of the Softron BP2010 Series Blood Pressure Meter (Softron, Tokyo) was used to measure mouse caudal artery blood pressure weekly at about 17:00. After 6-week L-NAME treatment, mice were anesthetized with isoflurane (2%) in oxygen, and hemodynamic parameters were measured from the left common carotid artery by using an eight-channel physiological recorder (iWorx 308, iWorx/CB Sciences, Dover, NH, USA) with a carotid artery catheter [16]. The blood was then collected from the abdominal aorta with heparin anticoagulation, and the plasma

was further separated by centrifugation (1600 ×*g* for 15 min), and aliquots of plasma were immediately frozen and stored at −80 °C for NO and LT assays. Finally, mice were euthanized by intraperitoneal injection of a lethal dose of pentobarbital, then perfused intracardiacally with cold 4% paraformaldehyde (in PBS), and thoracic aortas (2 mm from the descending aorta) and mesenteric arteries were excised and fixed in 4% paraformaldehyde for histopathology studies [17].

2.5. Histology and morphometric analysis

Thoracic aortas and mesenteric arteries were isolated and processed for paraffin embedding, then cut into 4-μm sections. Sections from each cluster were stained with hemotoxylin-eosin, collagen and elastin following the manufacturer's instructions [17]. Images were captured and analyzed by using a Zeiss AxioCam Microscope Camera (Zeiss, Germany). Measurements included luminal radius and media thickness. The media-to-lumen ratio was calculated as described [17]. In brief, elastin-stained slides were used for morphometric analysis. Thoracic aorta thickness was determined by measuring the distance from the internal elastic lamina (IEL) to the external elastic lamina (EEL). For each slide, 4 points (12, 3, 6 and 9 o'clock positions) were measured and averaged. The media area was determined by measuring the area between the IEL and EEL. Then the media-to-lumen ratio was calculated on the basis of the measured lumen area and media areas. In addition, the intensity of collagen and elastin staining in aortas was detected as described [18]. In brief, all images were taken at 10× magnification and then the collagen and elastin staining was converted into gray-scale images. Image-Pro Plus (Media Cybernetics, USA) was used to analyze the density of collagen and elastin staining in the vessel wall. Densitometric data were collected as with western blot densitometry, in which a vessel wall fragmentation was analyzed as a band on a western blot. Five fragmentations, with almost the same size, were randomly chosen from a vessel cross section, then the collagen and elastin densities were averaged for each vessel. Elastin-to-collagen ratio was calculated by dividing the densitometric data for elastin by that for collagen [18].

To identify the proliferation of aortic VSMCs, immunofluorescence staining for Ki-67, a marker of proliferating cells, was used [19]. Aorta sections (4–5 mice/group) were permeabilized with 0.02% Triton-X100, then stained anti-mouse Ki-67 primary antibody (1:100) and anti-SMC α-actin antibody (1:150) for 15 h at 4 °C. Alexa Fluor 594–conjugated secondary antibody (1:500) to indicate Ki-67 and FITC-conjugated secondary antibody (1:500) to indicate SMC α-actin were then added and incubated for another 1 h at room temperature. DAPI was used to stain the nuclei. Images of aortic media were taken under a Zeiss fluorescence microscope (Zeiss, Germany). The number of Ki-67–positive cells was counted in five random different fields per section by an investigator blinded to the study. A Ki-67–labeling index was calculated by the ratio of Ki-67–positive VSMCs to total number of VSMCs in aortic media of each of the 5 fields. An averaged Ki-67–labeling index was then obtained for each group.

2.6. Rat primary VMSC isolation and culture

Two steps of enzyme disaggregation process were used to isolate rat VMSCs [20]. Briefly, rat aortas were cleaned of fat and adventitial tissue and then minced in sterile culture dishes. In the first digestion step, the medial layer pieces were incubated in enzyme solution 1 (1.4 mg/mL collagenase I and 0.5 mg/mL elastase VI) for 10 min at 37 °C. In the second step, media tissue pieces were further digested in enzyme solution 2 (2 mg/mL collagenase I and 0.5 mg/mL elastase VI) by incubating for another 90 min. Dulbecco's modified Eagle's medium (DMEM) containing 10% (*v*/v) FBS was added immediately after all tissue was digested, then the mixture was centrifuged at 500 *g* for 5 min at 4 °C to pellet the cells. The cells were resuspended in culture medium, counted and plated in a final volume of 2.5 mL at 0.4–0.6 × 10^6 cells per 25-mm^2 flask. Cell types and purity were confirmed by SMC-specific α-actin antibody immunofluorescent staining.

2.7. VMSC proliferation and migration

For proliferation assay, rat VSMCs were seeded in 96-well plates (5000 cells/well) and cultured for 24 h in DMEM/F-12 containing 10% FBS. Cells were serum-starved (0.1% FBS) for the following 24 h, then stimulated with 10% FBS containing medium in the presence and absence of L-NAME (10 μM) alone or combined with LTB4 (100 nM) or CysLTs (100 nM) for another 72 h. BrdU was then added into the medium, and the amount of incorporated BrdU was determined colorimetrically by using the Cell Proliferation ELISA BrdU colorimetric kit according to the manufacturer's instructions [20]. For VSMC migration assay, VSMCs (10^5 cells) were seeded into the upper chambers of transwell chambers (8 μm pore size; Millipore, USA) in 200 μl fresh medium without FBS, then 500 μl medium was added with 20% FBS with or without L-NAME (10 μM) alone or with LTB4 (100 nM) or CysLTs (100 nM) into the lower chamber [20]. After 6 h, cells remaining in the top wells were scraped off by using cotton swabs and cells that had migrated to the lower membrane surface were fixed with methanol for 5 min, then stained with DAPI. Six replicates were used for cell counting with blinding. Five fields of vision (20 × objective) per well were randomly chosen and cell counts were performed manually. Data were normalized to the control VSMC migration levels.

2.8. VMSC phenotype modulation

Rat VSMCs were cultured on laminin-coated plates in DMEM/F12 (supplemented with 0.2% fatty acid–free BSA) with IGF-I (2 ng/mL) for 1 day. The medium was changed to DMEM containing L-NAME (10 μM), LTB4 (100 nM) or CysLTs (100 nM) alone or L-NAME with LTB4 or CysLTs for another 12 h. Then total RNA was extracted by using TRIzol reagent and reverse transcribed to cDNA, which was used for quantitative real-time PCR (qPCR) assay. The qPCR reactions system involved SYBR Green Supermix, target cDNA and phenotype modulation marker gene primers. DNA targets were amplified with primers (Table 1). The

Table 1
Sequences of the oligonucleotides used for real-time PCR amplification in mouse aortic tissues and cultured rat VSMCs.

Primer name	Sequence
GAPDH	
Forward	5'-CTGCACCACCAACTGCTTAG-3'
Reverse	5'-GGGCCATCCACAGTCTTCT-3'
Smoothelin B	
Forward	5'-CCACAGAGCCCTCTGATACC-3'
Reverse	5'-ACAGACAGGGAGCGTTGG-3'
Telokin	
Forward	5′-AGGAGGAGGAGGAGTGAAGC-3′
Reverse	5′-ATCTACCACACCAGGTGCC-3′
SMA	
Forward	5'-AGAGCAAGAGAGGGATCCTGA-3'
Reverse	5'-GTCGTCCCAGTTGGTGATGAT-3'
SM22	
Forward	5'-TGAAGAAAGCCCAGGAGCAT-3'
Reverse	5'-TGCTTCCCCTCCTGCAGTT-3'
CN	
Forward	5'-ACCAAGCGGCAGATCTTTGA-3'
Reverse	5'-CATCTGCAAGCTGACGTTGA-3'
CALD	
Forward	5'-GGAGGCTGATCGAAAAGCTA-3'
Reverse	5'-AGCTTCTGCCCTTCTCCTTT-3'

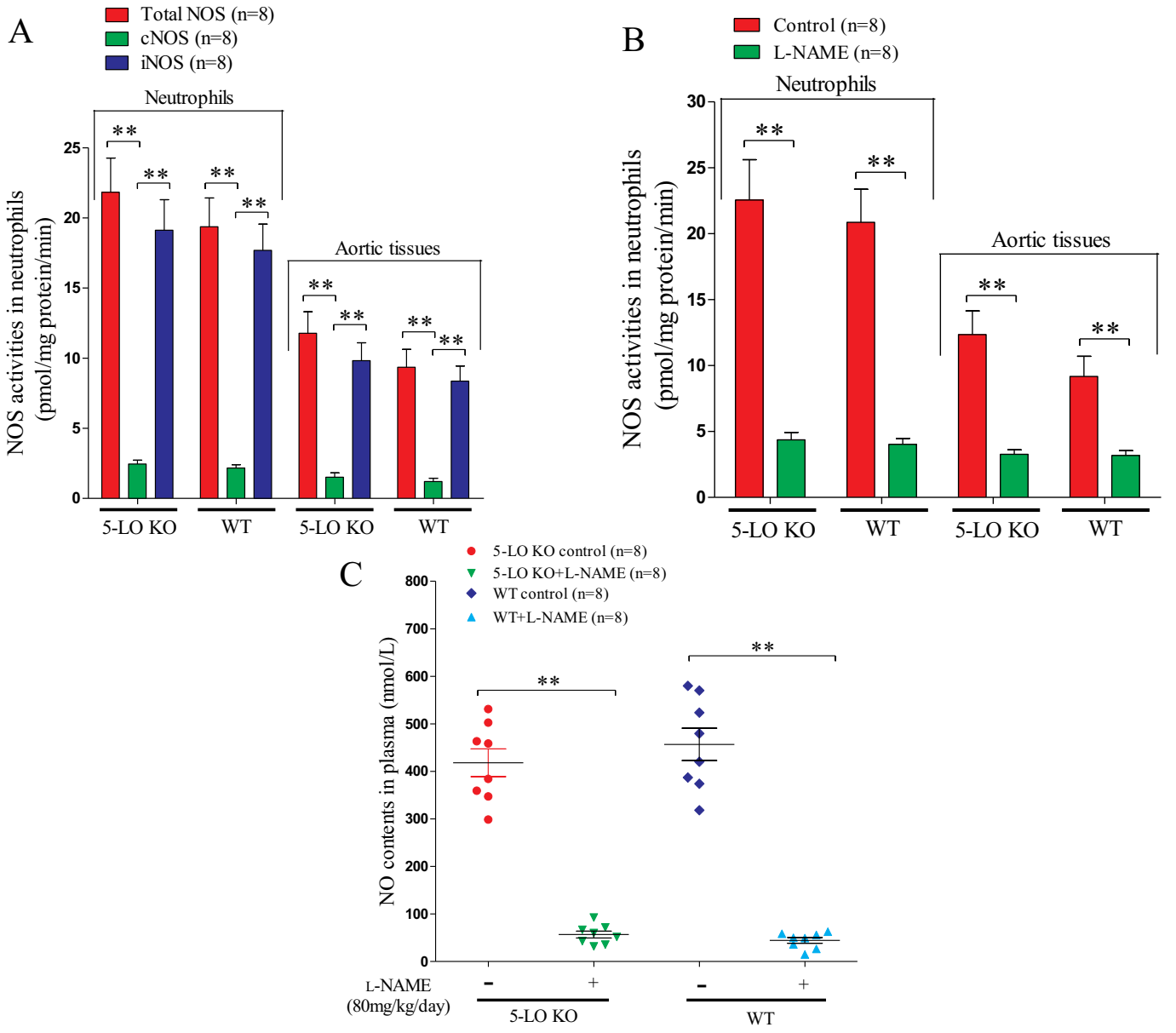

Fig. 1. L-NAME treatment inhibited NOS activities in mouse neutrophils and aortic tissues and decreased NO content in plasma.
A. Calcium-dependent (constitutive) and -independent (inducible) NOS activities in neuthophils and aortic tissues of WT and 5-LO–KO mice (n = 8/group).
B. NOS activities in neutrophils and aortic tissues of WT and 5-LO–KO mice (n = 8/group).
C. Nitrate and nitrite (NOx) contents in plasma of WT and 5-LO–KO mice (n = 8/group).
Data are mean ± SEM. $^{**}P < 0.01$ by one-way ANOVA. WT, wild-type mice; 5-LO–KO, 5-lipoxygenase-knockout mice; NOS, nitric oxide synthase; cNOS, calcium-dependent (constitutive) NOS; iNOS, calcium-independent (inducible) NOS; L-MANE, N(ω)-nitro-L-arginine methyl ester.

quantified expression of genes was recorded and calculated with a Chromo4 Real-Time PCR Detection System (Bio-Rad Life Sciences, CA, USA) [21].

2.9. Primary neutrophil isolation

Neutrophils were purified from buffy coats as described [22] with some modification. Mice were anesthetized by intraperitoneal injection of 45 mg/kg pentobarbital, and then blood was obtained from the abdominal aorta after anticoagulation with an intraperitoneal injection of 800 U/kg heparin. Buffy coats of blood were mixed with an equal volume of 1% Dextran T-500 for sedimentation to remove erythrocytes, then the neutrophils were separated over Histopaque-1083 and -1119 gradients and collected after centrifugation (300*g*, 30 min). Cells were washed and suspended in Hank's balanced salt solution (HBSS) without Ca^{2+} or Mg^{2+}. Purity (> 90%) of isolated PMNs was evaluated with a BD FACSAria I flow cytometer (BD, USA) by calculating the ratio of both CD11b- and CD45-positive cells to total cells after staining with anti-CD11b FITC and anti-CD45 PerCP antibodies.

2.10. Neutrophil stimulation evaluated by immunofluorescent microscopy

Aliquots of 1.0×10^5 isolated neutrophils were suspended in 300 µl RPMI 1640 medium (phenol red free) with 1.5 mM Ca^{2+} and Mg^{2+} in each well in a 24-well plate. Neutrophils were challenged with L-NAME (10 µM), the NO donor SNOG (1 mM) or LPS (1 µg/mL) for 60 min at 37 °C, 5% CO_2, then centrifuged to obtain the neutrophil pellets, which were re-suspended in distilled deionized water and freeze-thawed twice

Table 2
Characteristics of wild-type and ALox5-knockout (5-LO–KO) mice with and without L-NAME (80 mg/kg/day) treatment.

	Wild type		5-LO–KO	
	Control	L-NAME	Control	L-NAME
Body weight (g)				
Baseline	21.75 ± 0.57	22.31 ± 0.72	21.96 ± 0.63	22.17 ± 0.77
6-week L-NAME	26.22 ± 1.21	26.84 ± 0.99	25.71 ± 1.35	26.255 ± 1.27
Heart weight/ body weight (mg/g)	4.71 ± 0.74	4.94 ± 0.96	4.47 ± 0.85	4.78 ± 0.88

Data are mean ± SEM (n = 8/group).

to lyse neutrophil membrane [23]. The nuclei were further obtained by centrifugation and then suspended and attached quickly on slides, which were fixed in anhydrous acetone for 15 min and allowed to dry for 10 min. The neutrophil nuclei were treated with sodium azide (0.1%) and hydrogen peroxide (0.3%) for 30 min to irreversibly inhibit endogenous peroxidase. Nonspecific background staining was minimized by blocking with BSA (1%, w/v) in DMEM. To recognize the activity and nuclear membrane translocation of the 5-LO pathway in neutrophils, immunofluorescent double staining was performed by incubating neutrophil nuclei with primary antibodies for both 5-LO (1:50 dilution) and FLAP (1:50 dilution) overnight (4 °C). After washes to remove primary antibodies, the neutrophil nuclei were further incubated with both Alexa Fluor 488- and Alexa Fluor 594-conjugated secondary antibodies (1:1000 dilution) for 1 h at room temperature to identify positive staining of 5-LO (green) and FLAP (red), respectively. Immunofluorescent photos were taken under a Zeiss laser scanning confocal microscope (Zeiss, Germany) to observe the colocalization position of 5-LO and FLAP (yellow). As reported previously [14,24], the colocalization of 5-LO and FLAP proteins on the nuclear envelope was identified as a 5-LO and FLAP complex and activation of the 5-LO pathway. The amount of colocalization dots (yellow) of fluorescent-labeled 5-LO and FLAP proteins in the nuclear envelope was analyzed from at least 50 random neutrophil nuclei and presented as dots per neutrophils.

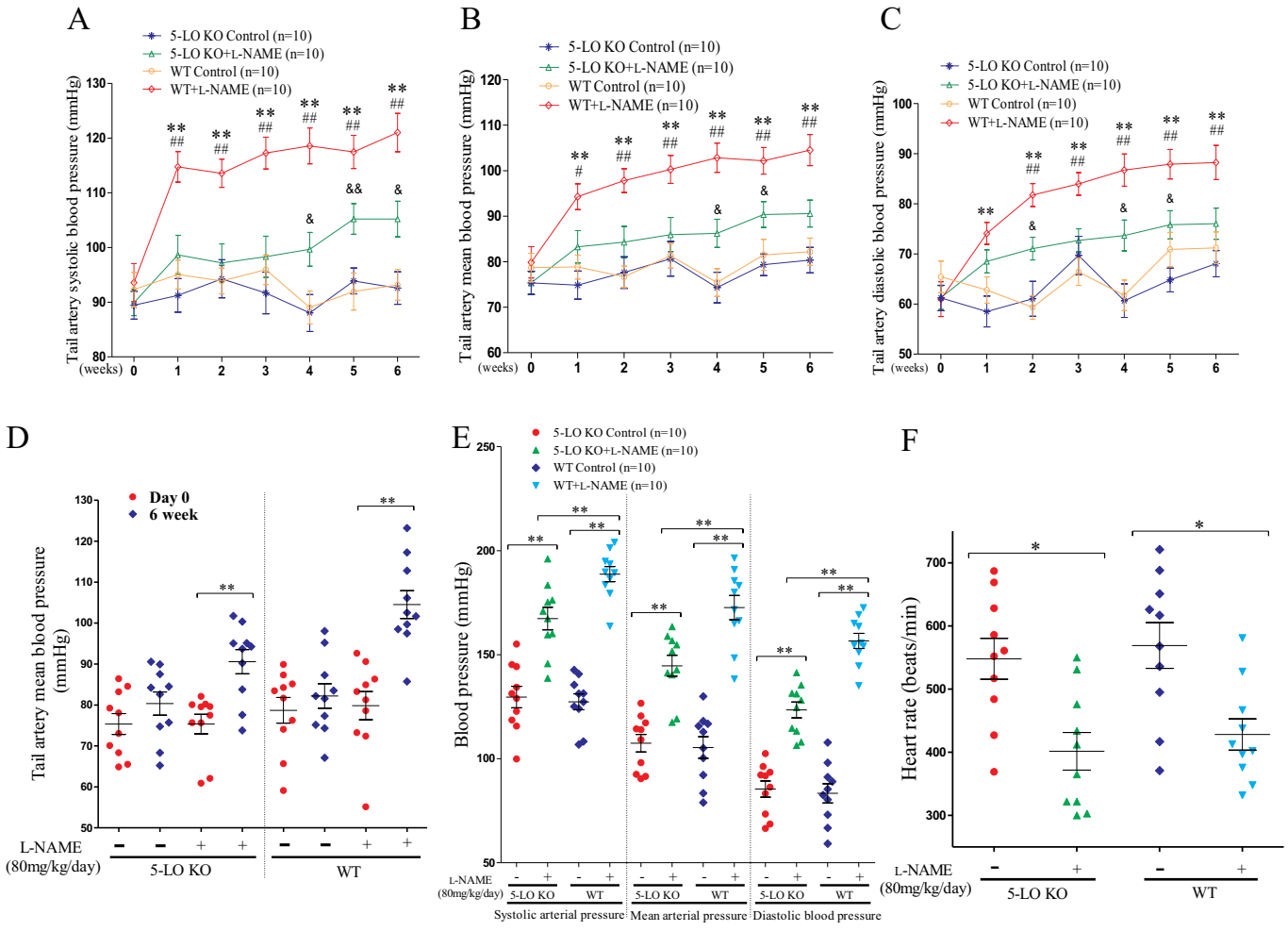

Fig. 2. Blood pressure and heart rates in hypertensive mice exposed to L-NAME.
A-C. L-NAME (80 mg/kg/day) treatment results for caudal artery blood pressure in WT mice and 5-LO–KO mice (n = 10/group). L-NAME treatment results for systolic blood pressure (A), mean arterial blood pressure (B) and diastolic blood pressure (C) of the caudal artery in WT and 5-LO–KO mice. **P < 0.01 vs the WT control mice; & p < 0.05 and && P < 0.01 vs 5-LO–KO control mice; # p < 0.05 and ## P < 0.01 vs L-NAME–treated 5-LO–KO group.
D. Mean arterial blood pressure of caudal artery before and after 6-week L-NAME treatment (n = 10/group).
E. Effect of 5-LO gene deficiency on SBP, MBP and DSP in common carotid artery after 6-week L-NAME (80 mg/kg/day) exposure (n = 10/group).
F. Effect of L-NAME exposure on mouse heart rate (n = 10/group).
Data are mean ± SEM. Data in D were analyzed by paired t-test. Data in A, B, C, E and F were analyzed by one-way ANOVA, *p < 0.05 and **P < 0.01. WT, wild-type mice; 5-LO–KO, 5-lipoxygenase-knockout mice; L-MANE, N(ω)-nitro-L-arginine methyl ester.

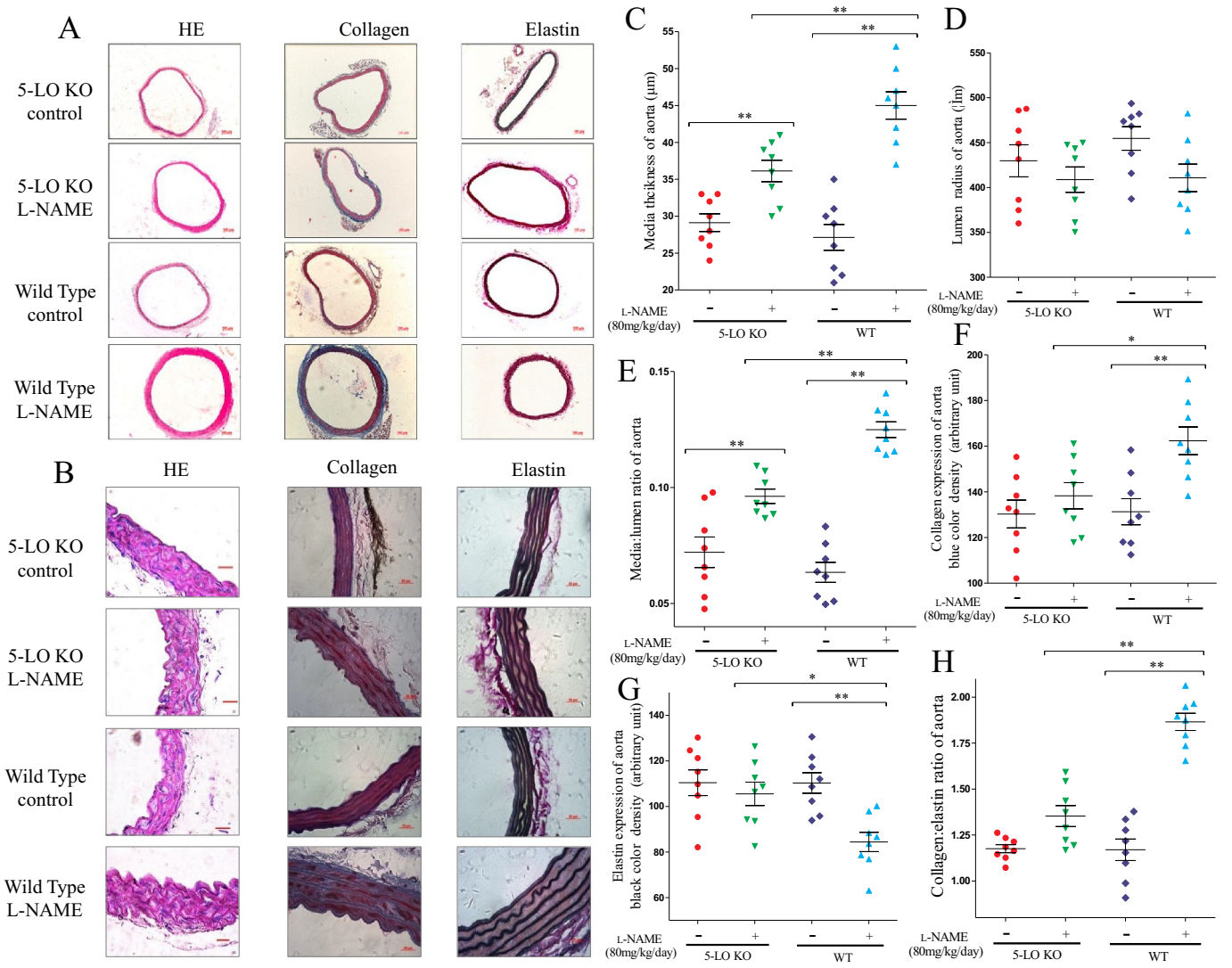

Fig. 3. 5-LO deficiency ameliorated aorta remodeling in mice with hypertension induced by L-NAME exposure.
A and B. Vascular remodeling analyzed in thoracic aorta sections from 5-LO–KO and WT mice treated with or without 6-week 80 mg/kg/day L-NAME (n = 8/group). Representative images of vessel sections stained with HE, Masson trichrome blue staining (blue represents collagen), or Van Gieson staining (blackish-brown represents elastin). Magnification 100×, scale bar = 100 μm in A, and magnification 400×, scale bar = 20 μm in B.
C. media thickness of aortas (n = 8/group).
D. Lumen radius of aortas (n = 8/group).
E. Media-to-lumen ratio of aortas (n = 8/group).
F. Density of collagen and G. elastin staining in aortic smooth muscle wall (n = 8/group).
Data are mean ± SEM. *p < 0.05 and **P < 0.01 by one-way ANOVA. WT, wild-type mice; 5-LO–KO, 5-lipoxygenase-knockout mice; L-MANE, N(ω)-nitro-L-arginine methyl ester.

2.11. Measurement of LTs in culture supernatant and plasma

Neutrophils (2.5×10^5 cells) were suspended in 300 μl phenol red–free RPMI 1640 containing 1.5 mM Ca^{2+} and Mg^{2+}, then challenged with L-NAME (10 μM), SNOG (1 mM), or LPS (1 μg/mL) for 12 h at 37 °C, 5% CO_2. The culture supernatants were collected by centrifugation (10, 000*g*, 15 min). A specific ELISA assay was used to detect LTB4 and CysLT (including the total CysLTs, LTC4, LTD4 and LTE4, respectively) secretion in cell-free supernatants and plasma samples following the manufacturer's instructions [14]. Optical density was measured at 450 nm by a Synergy Muti-Mode Microplate reader (Biotek, VT, USA).

2.12. Assays for nitric oxide content in plasma and cell culture supernatant and NOS activity in neutrophils and aortic tissues

NO content in mouse plasma and neutrophil culture supernatants was assayed by use of a Griess Reagent kit according to the manufacturer's instructions [25], and NOS activity in neutrophils and aortic tissues was detected by the conversion of L-[^{3}H]arginine to L-[^{3}H] citrulline as described [26]. Briefly, aliquots of 1.0×10^6 isolated neutrophils were seeded per well in 6-well plates. After exposure with L-NAME (10 μM), SNOG (1 mM) or LPS (1 μg/mL) for 30 min, the cell culture supernatant was collected for NO content assay, and cells were lysed for NOS activity detection. Fresh aortas from hypertensive mice

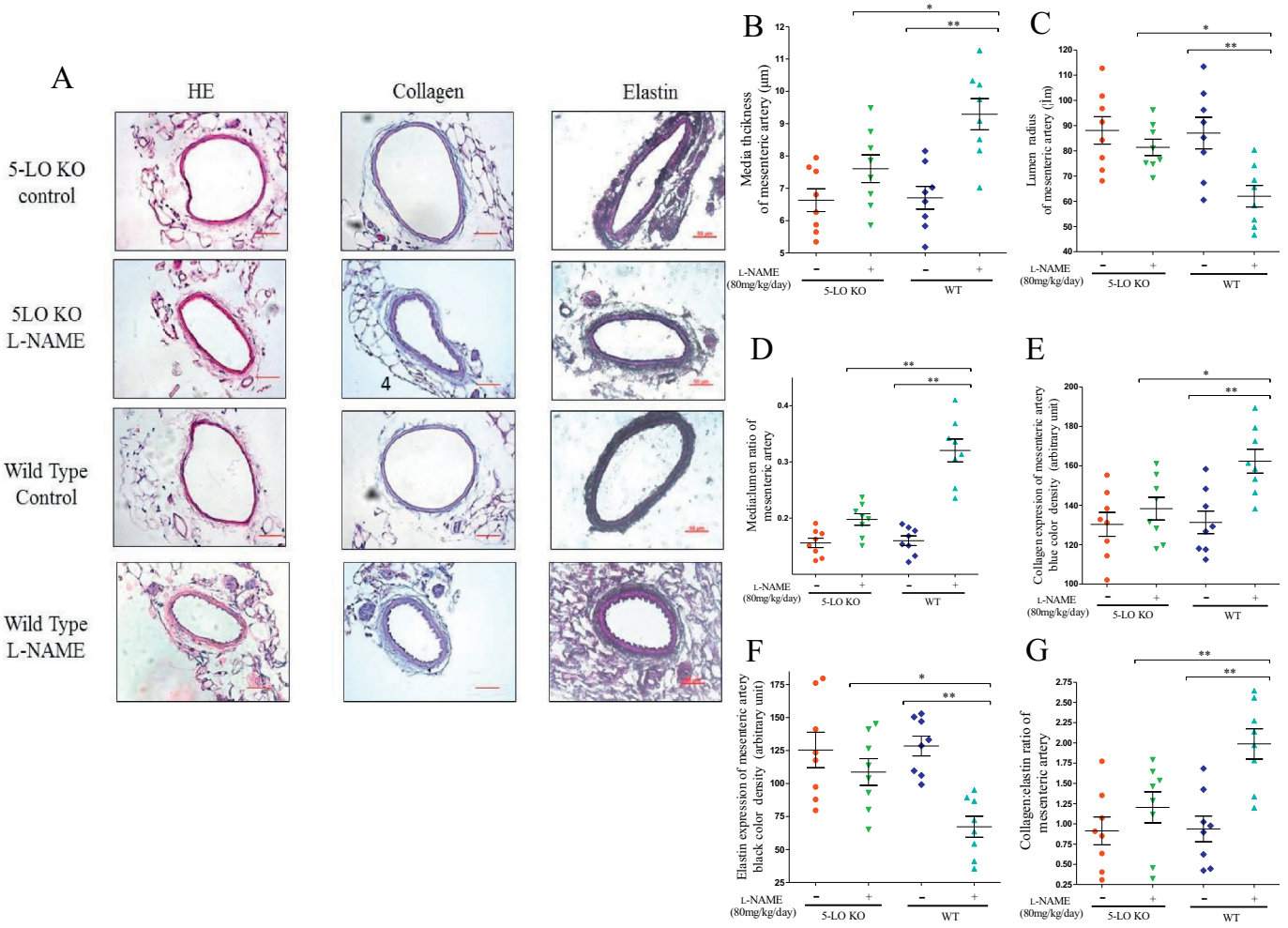

Fig. 4. 5-LO deficiency ameliorated mesenteric aorta remodeling in mice with hypertension induced by L-NAME exposure.
A. Vascular remodeling was analyzed in mesenteric aorta sections from 5-LO–KO and WT mice treated with or without 6-week 80 mg/kg/day L-NAME (n = 8/group). Representative images of vessel sections stained with HE, Masson trichrome blue (blue represents collagen), or Van Gieson staining (blackish-brown represents elastin). Magnification 200×, scale bar = 50 μm.
B. media thickness of mesenteric aortas (n = 8/group).
C. Lumen radius of mesenteric aortas (n = 8/group).
D. Media-to-lumen ratio of mesenteric aortas (n = 8/group).
E. Media-to-lumen ratio of mesenteric aortas (n = 8/group).
F. Density of collagen and G. elastin staining in mesenteric aortas (n = 8/group).
Data are mean ± SEM. *$p < 0.05$ and **$P < 0.01$ by one-way ANOVA. WT, wild-type mice; 5-LO–KO, 5-lipoxygenase-knockout mice; L-MANE, N(ω)-nitro-L-arginine methyl ester.

were isolated and placed in ice-cold PBS (pH 7.4). The aortas were cleared of adventitial tissue and cut into segments, which were further homogenized by using a Brinkmann Polytron homogenizer. The protein content of neutrophil lysates and aortic homogenates was determined by the Lowry method [26]. Lysates/homogenates (100 μg protein in 100 μL) were added into a 37 °C-prewarmed reaction buffer (1 mM NADPH, 10 mM FAD, 10 mM tetrahydrobiopterin, 60 mM L-valine, 50 mM potassium phosphate, 1.2 mM $MgCl_2$, 1 μg/mL bovine serum albumin, pH 7.2) containing L-[^{3}H]arginine (150–200 cpm/pmol). Furthermore, 1 mM $CaCl_2$ and 1 mM calmodulin were added into the reaction buffer to detect total NOS activity, or 1.2 mM EDTA and 1 mM EGTA were added into the reaction buffer to determine calcium-independent NOS (iNOS) activity. In addition, calcium-dependent NOS (cNOS) activity was calculated from the L-[^{3}H]citrulline difference between the samples treated with calcium/calmodulin and EDTA/EGTA. After incubation at 37 °C for 30 min, the ice-cold stop buffer (100 mM HEPES, 12 mM EDTA, pH 5.5) was added to stop the reaction, and the excess L-[^{3}H]arginine was removed, then the supernatant was collected by centrifuging and the radioactivity of L-[^{3}H]citrulline was measured by using a β-scintillation counter. NO content is presented as μmol per liter, and NOS activity is expressed as pmol of L-[^{3}H]citrulline produced per minute per milligram of protein.

2.13. Statistical analysis

Data are shown as mean ± SEM from at least 3 independent experiments. Statistical analyses involved using GraphPad Prism 6 (Graphpad Software). Unpaired Student's *t*-test (two tailed) was used for analyzing two groups and one-way ANOVA followed by Tukey multiple comparison tests for 3 or more groups. $P < 0.05$ was considered statistically significant.

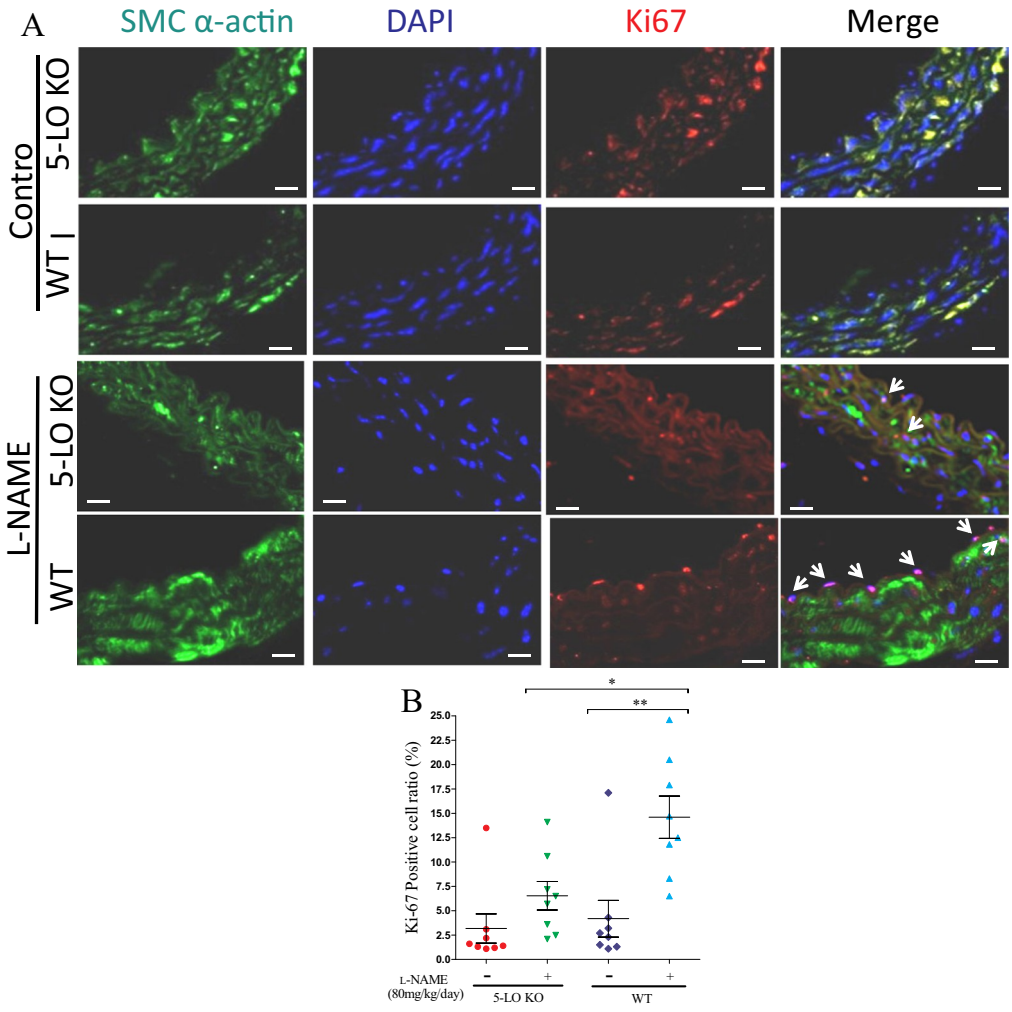

Fig. 5. 5-LO gene deficiency reduced VSMC proliferation in hypertensive mouse aorta induced by L-NAME exposure.
A. Representative images of Ki67 immunostaining (red) on aorta sections. VSMCs were identified by α-smooth muscle actin staining (green). The nuclei were counterstained with DAPI (blue). White arrows indicate Ki-67–positive VSMCs (amaranth color). Magnification 200×, scale bar = 50 μm.
B. Quantification of percentage of Ki-67–positive VSMC nuclei to total VSMC nuclei in the aortic media (n = 8/group).
Data are mean ± SEM. **P < 0.01 by one-way ANOVA. WT, wild-type mice; 5-LO–KO, 5-lipoxygenase-knockout mice; L-MANE, N(ω)-nitro-L-arginine methyl ester.

2.14. Key resources table

Resource	Source	Identifier
Antibodies		
Alexa Fluor 488-, Alexa Fluor 594–conjugated secondary antibodies		
anti-CD PerCP		
anti-CD11b FITC		
anti-CD45 PerCP		
anti-FLAP		
anti-Ki-67		
anti–lipoxygenase		
anti-mouse Ki-67 primary		
anti-SMC a-actin		
FITC-conjugated secondary		
SMC-specific a-actin		
Chemical		
acetone		
actinomycin D		N/A
arginine		
BrdU		
$CaCl_2$		
calcium		
calmodulin		N/A
citrulline		
CO_2		
CysLTs	N/A	N/A
DAF-FM		
DAPI		
dextran T-500		
Dulbecco's modified Eagle's medium		N/A
EDTA		
EGTA		
fatty acid		
fetal bovine serum	N/A	N/A
Hank's Balance Salt Solution	N/A	N/A
hemotoxylin-eosin		
HEPES		
histopaque		
hydrogen peroxide		
insulin-like growth factor 1		N/A
isoflurane		
L-NAME		
LPS	N/A	N/A
LTB4		
LTC4		
LTD4		
LTE4		
L-valine		
methanol		
Nitric Oxide		
pentobarbital		
phenol red		
potassium phosphate		
RPMI 1640 medium	N/A	N/A
S-nitrosoglutathione		
SNOG		
sodium azide	N/A	N/A
tetrahydrobiopterin		
TRIzol reagent	N/A	N/A
5-LO		
collagenase I		N/A
elastase VI		

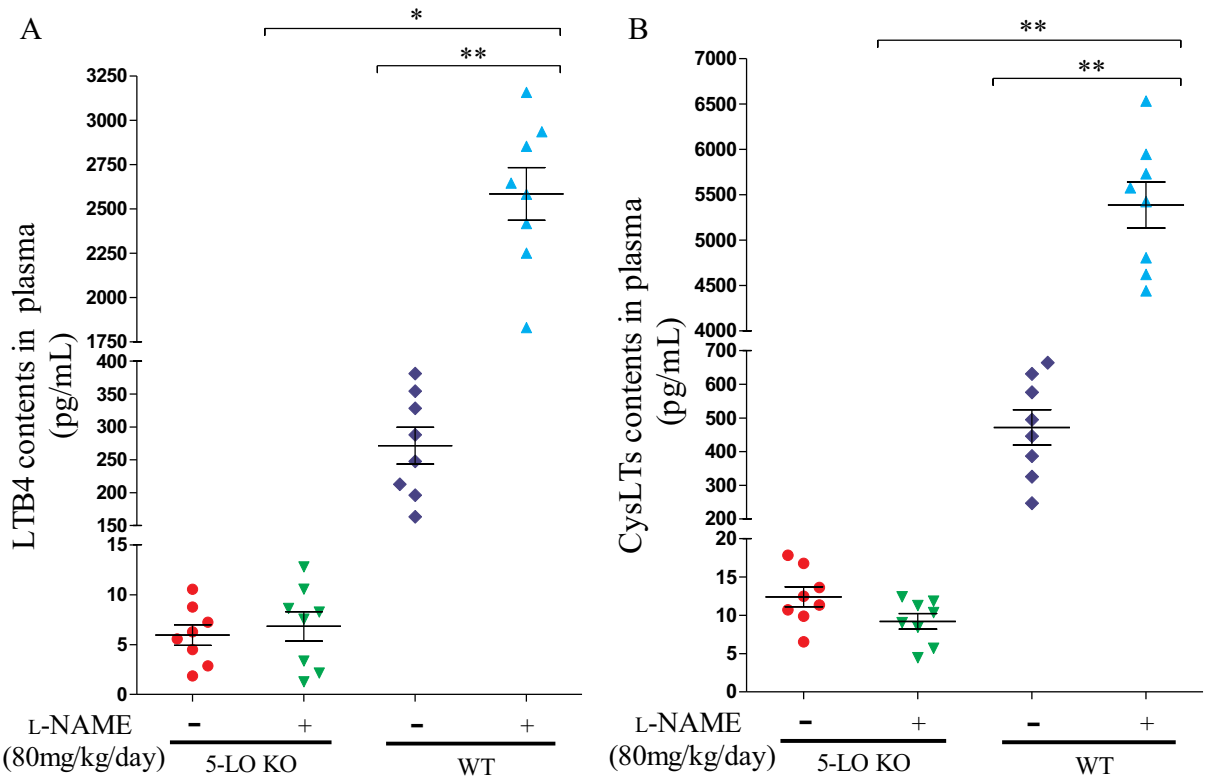

Fig. 6. Leukotriene (LT) content in mouse plasma.
A. LTB4 and B. cysteinyl LT (CysLT) content in mouse plasma with or without L-NAME exposure (n = 8/group).
Data are mean ± SEM. **P < 0.01 by one-way ANOVA. WT, wild-type mice; 5-LO–KO, 5-lipoxygenase-knockout mice; L-MANE, N(ω)-nitro-L-arginine methyl ester.

3. Results

3.1. 5-LO gene deletion suppressed increased blood pressure in L-NAME–induced hypertensive mice

Mice were treated with L-NAME at 80 mg/kg/day for 6 weeks, then NOS activity in neutrophils and aortic tissues and nitrite content in plasma were detected. In neutrophils and aortic tissues of both 5-LO–KO and WT mice, the calcium-independent NOS (iNOS) activity represented > 90% of the total NOS activity, and the calcium-dependent NOS (cNOS) activity was every low (< 10% of the total NOS activity) (Fig. 1A). 5-LO–KO and WT mice did not differ in baseline NOS activity (including total NOS, iNOS and cNOS activity, Fig. 1A and B) in neutrophils and aortas as well as plasma NO level (Fig. 1C). Six-week L-NAME (80 mg/kg/day) exposure significantly decreased NOS activity in neutrophils and aortas as well as NO content in plasma in both 5-LO–KO and WT mice as compared with controls (Fig. 1A–C). Furthermore, the ratio of heart weight to body weight did not significantly differ between control WT and 5-LO–KO mice and were not further affected by 6-week L-NAME exposure (Table 2).

The regulatory role of 5-LO in basal pressure maintenance and L-NAME–induced hypertension were studied by blood pressure monitoring. The noninvasive tail-cuff method was used for weekly detection of caudal arterial pressure. At baseline and during 6-week normal water treatment, 5-LO–KO and WT mice did not differ in SBP, MBP or DBP (Fig. 2A–D). L-NAME exposure rapidly increased caudal arterial pressure in WT mice at 1 week, which gradually peaked at 6 weeks (Fig. 2A-2D). L-NAME exposure significantly increased SBP at 4, 5 and 6 weeks, MBP at 4 and 5 weeks, and DBP at 2, 4 and 5 weeks in 5-LO–KO mice as compared with controls. L-NAME–treated 5-LO–KO mice showed a significant decrease in the caudal artery pressure as compared with L-NAME–treated WT mice (Fig. 2A-2D), which suggests that 5-LO gene deficiency significantly attenuated the L-NAME–increased caudal arterial pressure.

To examine arterial blood pressure more precisely, at the end of L-NAME treatment, we further detected mouse carotid artery pressure by using the cannulation method and found no significant difference in baseline SBP, MBP and DBP of the carotid artery between WT control (127.4 ± 4.3, 105.4 ± 5.8 and 83.4 ± 5.1 mmHg, respectively) and 5-LO–KO control mice (129.6 ± 5.8, 107.5 ± 4.7 and 85.4 ± 4.3 mmHg). L-NAME exposure significantly increased arterial blood pressure in both WT and 5-LO–KO mice. However, SBP, MBP and DBP of the carotid artery was lower in L-NAME–treated 5-LO–KO than WT mice (Fig. 2E). In addition, L-NAME exposure significantly slowed the heart rate in both WT and 5-LO–KO mice as compared with controls. L-NAME–treated 5-LO–KO and WT mice did not differ in heart rate (Fig. 2F).

3.2. 5-LO gene deletion attenuated L-NAME-induced aorta and mesenteric artery remodeling

On analyzing the histological sections of aortas, L-NAME-induced aortic remodeling in WT mice was characterized by a significant increase in aorta media thickness, media-to-lumen ratio, collagen deposition, and collagen-to-elastin ratio but a significant decrease in elastin expression in the aortic media (Fig. 3A–H). In 5-LO–KO mice, L-NAME exposure significantly increased aorta media thickness and media-to-lumen ratio (Fig. 3A–H). However, L-NAME exposure caused a lesser increase in aortic media thickness, media-to-lumen ratio, collagen deposition, and ratio of collagen to elastin as well as a much lesser decrease in elastin expression in aortic media in 5-LO–KO than WT mice (Fig. 3A–H). The lumen radius was not changed in L-NAME–treated 5-LO–KO and WT mice or their controls (Fig. 3D).

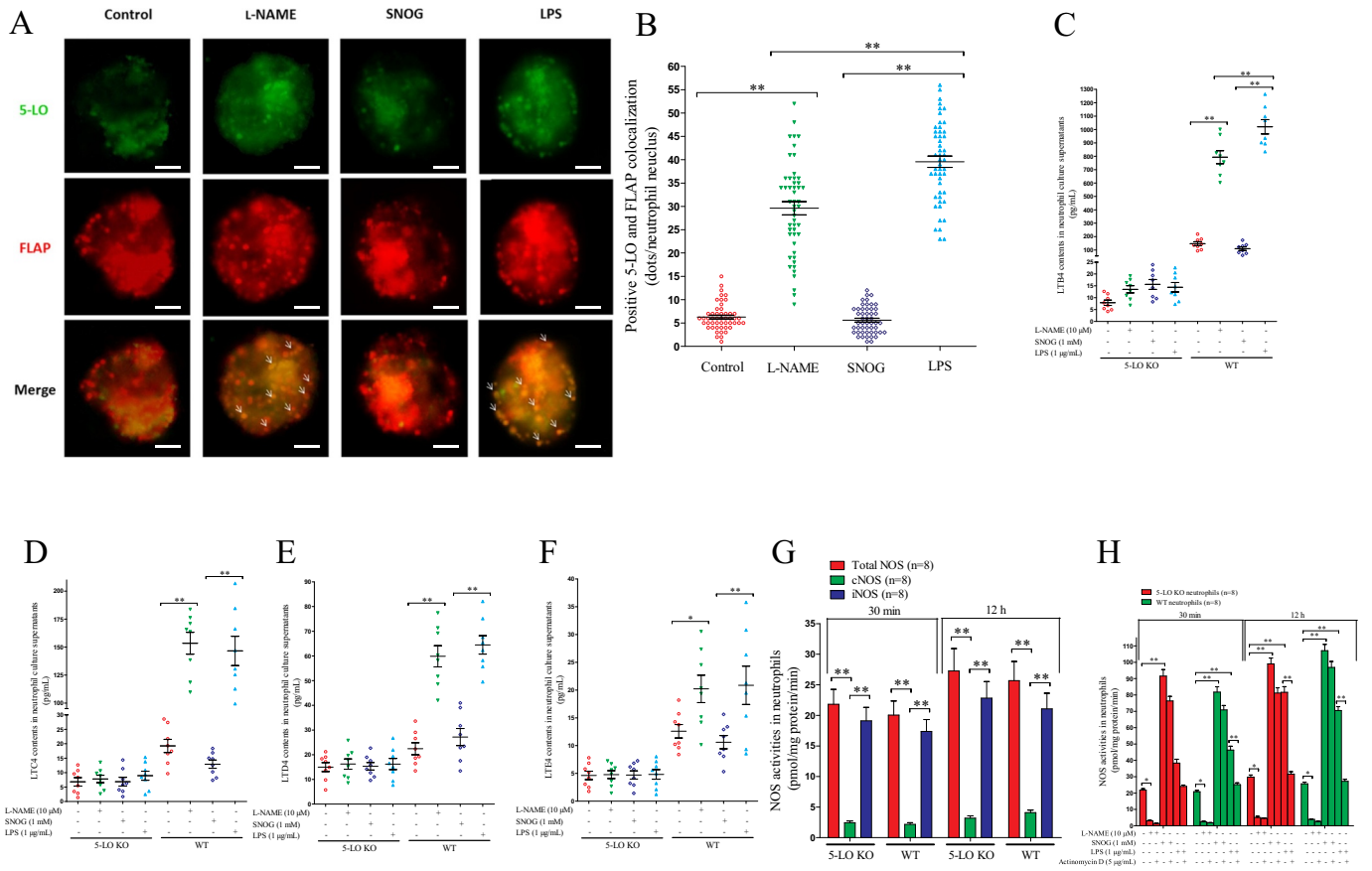

Fig. 7. L-NAME treatment activated 5-LO/LT pathway in the isolated mouse neutrophils.

A. Representative images of neutrophils showing that L-NAME (10 μM) exposure increased the colocalization (yellow color) of 5-LO (green color) with FLAP (red color) on the nuclear envelope similar to an accepted neutrophil stimulator, LPS (1 μg/mL). SNOG (1 mM), an NO donor, did not affect the activation of 5-LO. White arrows indicate colocalization (yellow color) of 5-LO and FLAP on the nuclear envelope. Magnification 400×, scale bar =50 μm.

B. Quantification of colocalization (yellow color) of fluorescent-labeled 5-LO (green) and FLAP (red) on the neutrophil nuclear envelope (n = 50/group).

C. LTB4 and D. LTC4, E. LTD4 and F. LTE4 content in culture supernatants of neutrophils of WT and 5-LO–KO mice with or without L-NAME, SNOG or LPS (n = 8/group).

G. Calcium-dependent (constitutive) and -independent (inducible) NOS activity in neuthophils of WT and 5-LO–KO mice (n = 8/group). Neuthophils was incubated in vitro for 30 min or 12 h before NOS activity measurement (n = 8/group).

H. NOS activity in neutrophils of WT and 5-LO–KO mice receiving L-NAME (10 μM), SNOG (1 mM) or LPS (1 μg/mL) alone or with actinomycin D (a specific inhibitor of protein synthesis, 5 μg/mL) for 30 min and 12 h (n = 8/group).

Data are mean ± SEM. *p < 0.05 and **P < 0.01 by one-way ANOVA. WT, wild-type mice; 5-LO–KO, 5-lipoxygenase-knockout mice; LPS, lipopolysaccharide; SNOG, S-nitroso-glutathione; L-MANE, N(ω)-nitro-L-arginine methyl ester. NOS, nitric oxide synthase.

In a much smaller artery, the mesenteric artery, L-NAME–induced vascular remodeling in WT mice was characterized by a significant increase in vascular media thickness, media-to-lumen ratio, collagen deposition, and collagen-to-elastin ratio but a significant decrease in lumen radius and elastin expression in media (Fig. 4A–G). In 5-LO–KO mice, L-NAME exposure had no significant effect on mesenteric artery media thickness, collagen deposition, lumen radius, elastin expression or collagen-to-elastin ratio. However, as compared with L-NAME-treated WT mice, L-NAME-exposed 5-LO–KO mice showed less vascular media thickness, media-to-lumen ratio and collagen-to-elastin ratio as well as a larger lumen radius and higher elastin expression in mesenteric arteries.

3.3. 5-LO gene deletion decreased L-NAME-induced VSMC proliferation in the aorta

Nuclear Ki-67 immunoreactivity is often used as an index of cellular proliferation [19]. The number of Ki-67–positive VSMC nuclei in the aortic media was counted and expressed as a percentage of total number of VSMC nuclei in the media. As compared with control WT mice without any inducer, L-NAME induced a significant increase in ki-67–positive VSMC nuclei in the WT mouse aortic media by approximately 3.4-fold (Fig. 5A and B). In the 5-LO–KO mouse aortic media, L-NAME treatment had no effect on ki-67–positive VSMC nuclei (p > 0.05, Fig. 5A and B). L-NAME exposure induced a greater increase in Ki-67–positive VSMC nuclei ratio in the WT mouse aortic media than the 5-LO–KO mouse aorta, by 1.2-fold. WT and 5-LO–KO control mice showed no significant difference in Ki-67–positive VSMC nuclei ratio in the aortic media.

3.4. L-NAME exposure increased LTB4 and CysLT contents in plasma of WT mice

LTs are important lipid mediators of infection and inflammation derived from the 5-LO metabolism of arachidonate [5,6]. LTB4 and CysLT (mixture of LTC4, LTD4, and LTE4) levels in plasma were measured by using ELISA kits [14]. L-NAME exposure significantly increased plasma LTB4 and CysLT contents in WT mice by 8.5- and 10.4-fold, respectively (Fig. 6A and B). However, in 5-LO–KO mice, the baseline levels of LTB4 and CysLTs were under the detection limit, and L-NAME exposure had no significant effect on plasma LT concentrations in 5-LO–KO mice.

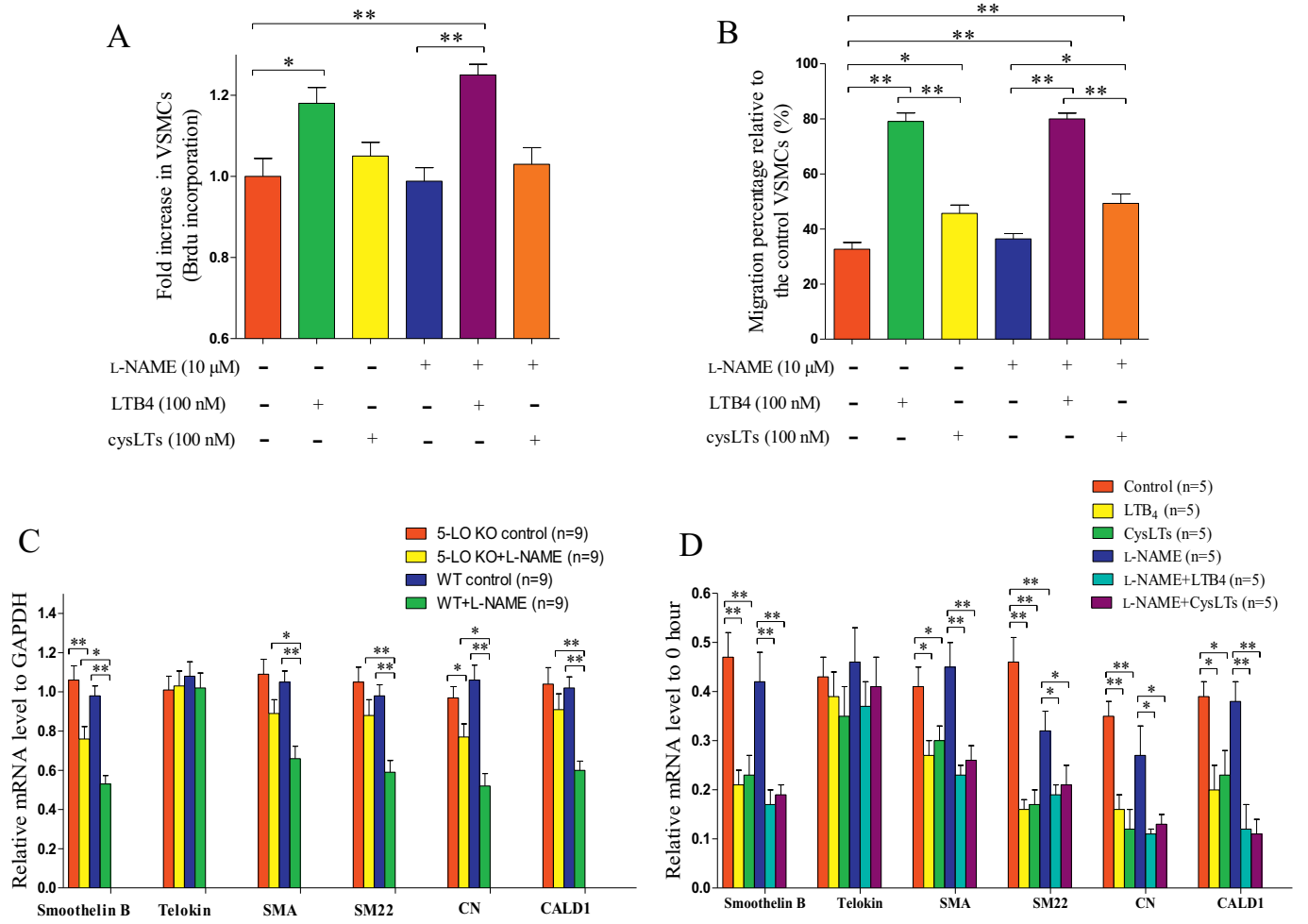

Fig. 8. LTs aggravated aortic/VSMC remodeling induced by L-NAME exposure.

A. VSMC proliferation in cultured primary rat VSMCs with LTB4 and CysLT treatment with or without L-NAME co-incubation on BrdU incorporation assay (n = 5/group).

B. LTs induced VSMC migration in cultured primary rat VSMCs with LTB4 and CysLT treatment with or without L-NAME exposure in a transwell migration assay (n = 5/group).

C. 5-LO–KO attenuated the decrease in mRNA expression of VSMC-specific contractile proteins, including smoothelin B, telokin, SMA, SM22, CN and CALD1, induced by 6-week L-NAME exposure (n = 9/group).

D. LTs decreased VSMC-specific contractile-protein mRNA expression in L-NAME–induced VSMC phenotypic switching (n = 5/group). RNA of each group was extracted at 0 or 6 h after VSMCs were isolated and cultured in media supplemented with insulin-like growth factor (IGF) (2 ng/mL) on a lamina-covered plate. Real-time PCR was used to measure the expression of the marker genes smoothelin B, telokin, SMA, SM22, CN and CALD1. The relative mRNA level for the four marker genes was the ratio of 6 h to 0 h mRNA level with LT exposure with or without L-NAME (10 μM) co-incubation.

Data are mean ± SEM. *p < 0.05, **p < 0.01 by one-way ANOVA. WT, wild-type mice; 5-LO–KO, 5-lipoxygenase-knockout mice; LTs, leukotrienes; LTB4, leukotriene B4; CysLTs, cysteinyl leukotrienes; L-MANE, N(ω)-nitro-L-arginine methyl ester; SMA, SM α-actin; SM22, smooth muscle 22 alpha; CN, calponin; CALD1, caldesmon 1.

3.5. L-NAME exposure increased the colocalization of 5-LO and FLAP and promoted the production of LTs in cultured mouse neutrophils

5-LO translocates from the cytosolic to the nuclear membrane upon activation and further associates with its scaffold protein, FLAP, to form the core of the multiprotein LT synthetic complex, which initiates LT formation [5,6]. Neutrophils, macrophages, eosinophils, and mast cells can synthesize and release LTs via a 5-LO-FLAP–dependent pathway [6]. To reveal why L-NAME elevated LT levels in plasma, we isolated peripheral blood neutrophils, the most abundant leukocytes in the blood, from WT mice. After 30-min treatment with L-NAME (10 μM), SNOG (a NO donor, 1 mM), or LPS (a positive control, 1 μg/mL), neutrophil nuclei were extracted and stained to present the 5-LO and FLAP complex in the nuclear membrane for activation of the 5-LO pathway. 5-LO and FLAP colocalization showed a nuclear envelope patterning (Fig. 7A) as previously reported for 5-LO and FLAP in activated bone marrow-derived neutrophils [27]. In the control neutrophil nuclei without any inducer, little 5-LO– and FLAP–positive staining was present in the nuclear membrane, and almost no 5-LO and FLAP complex was observed (Fig. 7A and B). L-NAME or LPS exposure significantly increased both 5-LO– and FLAP-positive stains, accompanied by a significant increase in colocalization of 5-LO and FLAP proteins in the nuclear membrane, by 4.8- or 6.9-fold, respectively, as compared with control neutrophils (Fig. 7A and B). In addition, treatment with the NO donor SNOG had no obvious effect on 5-LO– or FLAP-positive stains or their colocalization in the nuclear membrane (Fig. 7A and B).

LT content in neutrophil culture supernatants was further detected by using ELISA kits [14]. After L-NAME and LPS exposure for 12 h, LTB4, LTC4, LTD4 and LTE4 contents were increased significantly, by 4.4- and 8.3-fold, 7.0- and 10.3-fold, 1.7- and 1.4-fold and 60.5% and 96.5%, respectively, as compared with culture supernatants of control neutrophils (Fig. 7C–F). However, all LTB4, LTC4, LTD4 and LTE4 contents in culture

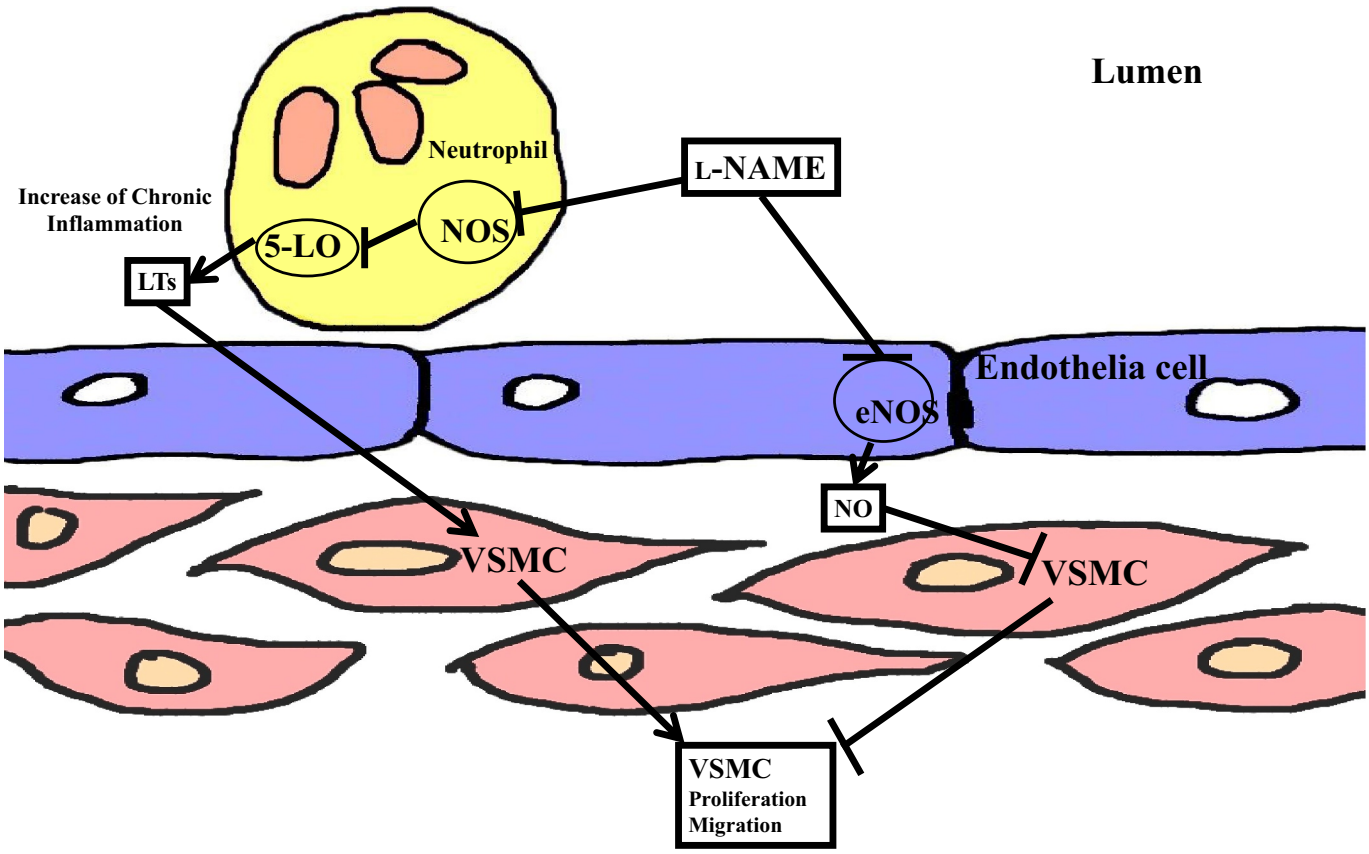

Fig. 9. Schematic diagram summarizing the molecular mechanisms underlying 5-LO/LT-mediated promotion of vascular remodeling in hypertension induced by L-NAME exposure. In response to L-NAME insult, hypertension develops by eNOS inhibition in vascular endothelium. However, L-NAME–induced NOS suppression activates the 5-LO/LT pathway in blood immunocytes such as neutrophils and further increases 5-LO metabolites in blood, which aggravates the inflammatory reaction and vascular remodeling in hypertension. 5-LO, 5-lipoxygenase; LTs, leukotrienes; L-NAME, L-NG-nitroarginine methyl ester; NOS, nitric oxide synthase; NO, nitric oxide.

supernatants from neutrophils of 5-LO–KO mice were under the detection limit, and L-NAME, LPS, or SNOG exposure had no effect on LT content in culture supernatant of neutrophils with 5-LO deficiency (Fig. 7C–F).

NOS activity in neutrophils was further detected after exposure to L-NAME, SNOG, or LPS. In cultured control neutrophils incubated without any inducers for 30 min and 12 h, calcium-independent NOS (iNOS) activity was observed to consist of > 90% of the total NOS activity, whereas calcium-dependent NOS (cNOS) activity was every low and < 10% of the total NOS activity (Fig. 7G). As compared with control counterparts, L-NAME exposure significantly decreased and SNOG treatment significantly increased total NOS activity at 30 min and 12 h after incubation, and LPS insult significantly increased total NOS activity at 30 min in WT neutrophils and at 12 h in both 5-LO–KO and WT neutrophils (Fig. 7H). A specific inhibitor of protein synthesis, actinomycin D (5 μg/mL), was further supplemented to evaluate the contribution of NOS protein synthesis to total NOS activity. Co-incubation with actinomycin D almost abrogated the induction of total NOS activity by LPS exposure for 30 min in WT neutrophils and 12 h in both 5-LO–KO and WT neutrophils, with no significant effect on total NOS activity of both 5-LO–KO and WT neutrophils incubated with L-NAME or SNOG for 30 min and 12 h, respectively (Fig. 7H).

3.6. LTB4 and CysLT exposure promoted VSMC proliferation and migration in vitro

LTs and peptido-LTs are 5-LO metabolites of AA that appear to have unique effects on VSMCs [6,7]. We investigated the induction of LTB4 or CysLTs on rat VSMC proliferation by BrdU incorporation assay [20]. As compared with control VSMCs without any inducer, 72-h LTB4 (100 nM) treatment alone increased VSMC proliferative ability by 14.0%, whereas 72-h L-NAME (10 μM) or CysLT (100 nM) exposure alone had no effect on VSMC proliferation (Fig. 8A). As compared with L-NAME treatment alone, 72-h LTB4 but not CysLT supplementation increased VSMC proliferative ability upon co-incubation with L-NAME, by 20.4%.

The induction of LTB4 or CysLTs on rat VSMC migration was further evaluated by using a modified Boyden Chamber assay with LTB4 or CysLTs in the lower chamber with or without L-NAME co-incubation for 6 h. As compared with control VSMCs, LTB4 (100 nM) or CysLT (100 nM) exposure alone increased VSMC migration ability by 1.4-fold and 16.4%, respectively (Fig. 8B). L-NAME (10 μM) alone had no significant effect on VSMC migration. As compared with L-NAME treatment alone, both LTB4 and CysLT insult significantly increased VSMC migration on co-incubation with L-NAME, by 1.2-fold and 29.9%, respectively (Fig. 8B).

3.7. 5-LO metabolites suppress VSMC-specific contractile protein gene expression in vitro and vivo

The VSMC phenotype usually switches from a contractile to a synthetic state during vascular remodeling [21]. To clarify whether LTs modulated the VSMC phenotype, we detected the mRNA expression of VSMC-specific differentiation genes, including smoothelin B, telokin, SMA, smooth muscle protein of 22 kDa (SM22), calponin (CN) and caldesmon 1 (CALD1), by using a real-time PCR assay in mouse aortic tissues and primary cultured rat VSMCs within the first passage. As compared with control counterparts, L-NAME exposure significantly decreased the mRNA levels of smoothelin B and CN in the 5-LO–KO mouse aorta as well as SMA, SM22, CN and CALD1 in WT aortic tissues (Fig. 8C). In addition, the mRNA levels of all five differentiation marker

genes were higher in L-NAME–treated 5-LO–KO than WT aortic tissues. As compared with control VSMCs without any inducer, LTB4 (100 nM) or CysLT (100 nM) treatment significantly decreased the mRNA expression of the VSMC-specific differentiation genes, including smoothelin B, SMA, SM22, CN and CALD1 (Fig. 8D). As compared with L-NAME treatment alone, LTB4 (100 nM) or CysLT (100 nM) treatment significant decreased the mRNA expression of the five differentiation marker genes in L-NAME–co-incubated VSMCs (Fig. 8D). However, the mRNA expression of a 17-kDa smooth muscle-specific protein, telokin, was not significantly changed in aortic tissues and VSMCs with different treatments (Fig. 8C and D).

4. Discussion

In this study, we have demonstrated that (1) 5-LO deficiency significantly decreased levels of 5-LO pro-inflammatory products, LTB4 and cysLTs, in plasma, and alleviated the high blood pressure and vascular remodeling in a hypertensive mouse model with an impaired NO/NOS system induced by L-NAME exposure; (2) L-NAME exposure activated the 5-LO pathway in peripheral blood neutrophils to promote LT synthesis, which are the major source of proinflammatory LTs in plasma; and (3) 5-LO products, LTs, including LTB4 and cysLTs, aggravated vascular remodeling phenotypes in VSMCs by inducing proliferation and migration and phenotype modulation.

Vascular remodeling refers to alterations in the structure of resistance vessels contributing to elevated systemic vascular resistance in hypertension, associated with changes in the growth and migration of VSMCs, endothelial dysfunction, inflammatory processes, and the synthesis or degradation of extracellular matrix components [1,3]. NO is an important regulator of vascular function and blood pressure [9]. The chronic administration of a selective inhibitor of NOS, L-NAME, provides a reliable model of hypertension with pronounced target organ damage [10]. L-NAME–induced pressure overload is associated with cardiac and vascular remodeling, whereas left ventricle (LV) remodeling is characterized by no increase in LV mass [28]. In our WT mice, 6-week L-NAME exposure markedly decreased aortic NOS activity, plasma nitrite content and heart rate and significantly increased blood pressure. Thus, NOS inhibition resulted in a well-established animal model for hypertension. In hypertension, aortic remodeling usually undergoes outward hypertrophic remodeling with increased aortic diameter and stiffness, whereas small artery remodeling involves inward eutrophic remodeling and hypertrophic remodeling, which are characterized by a decrease in vessel diameter and an increase in media-to-lumen ratio [1–3]. In our WT mice, L-NAME exposure induced high media-to-lumen ratio and more collagen deposition and Ki-67–positive VSMCs but less elastin expression and elastin-to-collagen ratio in both aortas and mesenteric arteries. The lumen radius did not significantly change in aortas but remarkably decreased in mesenteric arteries. Therefore, L-NAME treatment caused a typical vascular remodeling phenotype, including VSMC proliferation, extensive collagen deposition, and elastin degradation (collagen/elastin switch).

Inflammation is important for the pathogenesis of hypertension [4]. We have strong evidence of a direct correlation between blood pressure and levels of plasma intercellular adhesion molecule-1, C-reactive protein, or interleukin 6 [4,8], which suggests that low-grade systemic inflammation occurs in hypertension [4]. 5-LO is the key enzyme in LT biosynthesis, and LTs are potent mediators of inflammatory and allergic reactions [5,6]. The 5-LO/LT pathway is involved in the development of fibrosis and airway remodeling in lung injury and allergic inflammation and participates in atherogenesis and arterial wall remodeling in atherosclerosis [5,6]. In our NO/NOS dysfunction-induced hypertension model, as compared with L-NAME–exposed WT mice, L-NAME–treated 5-LO–KO mice showed lower blood pressure and media-to-lumen ratio, less collagen deposition and fewer Ki67-positive VSMCs but more elastin expression and higher elastin-to-collagen ratio in both aortas and mesenteric arteries. These results indicate that 5-LO gene deletion protected against L-NAME–induced hypertension and vascular remodeling by inhibiting VSMC proliferation and extensive collagen deposition as well as increasing elastin expression. Our results are consistent with those from Stanke-Labesque et al. [29], finding that an LT biosynthesis inhibitor, MK-886, prevented L-NAME–increased arterial blood pressure in rat. LTs derived from 5-LO activity have potent actions on smooth muscle [5,6]. However, little is known about the regulatory role of 5-LO/LTs in blood pressure control, VSMC functions, and vascular compensatory changes under high blood pressure [5–7].

5-LO metabolites such as LTB4 and CysLTs have been implicated in the pathogenesis of inflammation in respiratory diseases such as bronchial asthma and also in cardiac, hemodynamic, and microcirculatory pathophysiology [6,7]. The LTB4 receptors BLT1 and BLT2 and the CysLT receptors CysLT1 and CysLT2 are expressed in endothelial cells and VSMCs, so these LTs are closely related to the regulation of vascular functions [5,6]. Both LTB4 and CysLTs induce vasoconstriction and promote VSMC proliferation and migration [6,7], which are important components of vascular remodeling in hypertension [1,3]. We observed that LTB4 remarkably promoted VSMC proliferation, and both LTB4 and CysLTs induced VSMC migration with and without L-NAME co-incubation. Therefore, LTB4 and CysLTs are involved in vascular remodeling by promoting VSMC proliferation and migration, which is comparable to findings by Moraes et al. [30] and Kaetsu et al. [31], showing that LTs mediated VSMC proliferation and migration.

In response to vascular remodeling, differentiated VSMCs undergo a unique process known as “phenotype modulation,” transitioning from a quiescent, “contractile” phenotype to a proliferative, “synthetic” state [32]. We further evaluated the change in expression of 6 classical markers of VSMC phenotype modulation — smoothelin B, telokin, SMA, SM22, CN and CALD1 — in L-NAME–treated mouse aortas and LT-exposed VSMCs [21]. Excluding telokin, 5-LO–KO effectively attenuated the decreased mRNA expression of the other five specific VSMC phenotypic markers in the L-NAME–treated mouse aorta. However, both LTB4 and CysLT exposure significantly decreased the mRNA expression of these five genes with and without L-NAME treatment. Thus, LTs promoted NOS inhibition-induced changes in VSMCs from a contractile to a proliferative/synthetic remodeling phenotype, which was attenuated by 5-LO deletion.

We further detected LT contents in plasma and observed that in WT mice, L-NAME exposure markedly increased both LTB4 and CysLT levels. However, in 5-LO–KO mice with or without L-NAME exposure, the LT levels in plasma were near the detection limit. Hence, L-NAME induced hypertensive vascular remodeling in part by inducing proinflammatory mediators, including LTB4 and CysLTs. The genetic loss of 5-LO reduced levels of proinflammatory mediators, which might contribute to the attenuation of hypertensive vascular remodeling. At present, little is known about the role of 5-LO in hypertension induced by NOS/NO deficiency [6,7].

NO signaling pathways and lipid oxidation reactions are of central importance in both maintaining vascular homeostasis and the progression of vascular diseases [10,11]. NO appears to mediate active vasodilation, whereas 5-LO–derived products induce vasoconstriction [5,10,11]. Neutrophils have all NOS isoforms: eNOS, inducible NOS (iNOS), and neuronal NOS [33]. Bellot et al. [34] observed that nordihydroguaiaretic acid, a 5-LO inhibitor, improved the anti-inflammatory activity of L-NAME in endotoxin-induced uveitis. Gilchrist et al. [14] reported that eNOS was activated in human mast cells and translocated to the nucleus and colocalized with 5-LO; L-NAME potentiated the LT release from mast cells dose-dependently. This evidence shows an interesting negative regulation between NOS and 5-LO [11,14]. By using immunofluorescent confocal microscopy, we monitored 5-LO translocation and its colocalization with FLAP into neutrophil nuclear envelopes. L-NAME insult activated the 5-LO pathway by inducing striking 5-LO translocation into the nuclear envelope as well as 5-LO colocalization with FLAP, in a similar manner to an accepted 5-LO activator, LPS. However, SNOG, a NO donor, had no

obvious effect on 5-LO translocation and its colocalization with FLAP in the neutrophil nuclear envelope. These results were consistent with LT content in culture supernatants. In WT neutrophils, L-NAME or LPS treatment significantly increased both LTB4 and CysLT contents, which were significantly decreased by SNOG exposure. In neutrophils from 5-LO–KO mice with and without L-NAME, SNOG or LPS treatment, the LT levels in culture supernatants were near the detection limit. These results were comparable to those in mast cells [14] and indicated that L-NAME exposure induced the activation of 5-LO-FLAP complex in neutrophils, then promoted the production of 5-LO metabolites. 5-LO deficiency induced insufficiency in LT inflammatory mediators. The mechanisms involved in the NO modulation of LT release from neutrophils are complex. Coffey et al. [35] observed that NO derived from iNOS or NO donors inhibited LT activity by nitrosylation of 5-LO in macrophages. Further studies are needed to confirm the mechanisms underlying the interaction between 5-LO and NOS/NO in neutrophils.

Our study reveals the unique mechanism of 5-LO deletion/inhibition protecting against NO/NOS dysfunction-induced hypertension and vascular remodeling by reducing the production of detrimental 5-LO metabolites, including LTB4 and CysLTs, which have proinflammatory and vasoactive actions and induce vascular remodeling (Fig. 9). These findings suggest that 5-LO and its metabolites are critically important for the development of hypertension and vascular remodeling in NO-deficient hypertension, which may be used to develop new therapeutic approaches to ameliorate hypertensive vascular diseases.

Transparency document

The Transparency document associated with this article can be found, in online version.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (grant nos. 81870221, 81670249, 31271226 and 3071001 to Dr. Wei Jiang, no. 81700229 to Dr. Xiao-Xiao Wang, and no. 81300108 to Dr. Lu Gan). Special thanks for Laura Smales for her help in editing and revising the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbadis.2019.05.021.

References

[1] E.L. Schiffrin, Vascular remodeling in hypertension: mechanisms and treatment, Hypertension 59 (2012) 367–374.
[2] J.E. Hall, J.P. Granger, J.M. do Carmo, A.A. da Silva, J. Dubinion, E. George, S. Hamza, J. Speed, M.E. Hall, Hypertension: physiology and pathophysiology, Compr. Physiol. 2 (2012) 2393–2442.
[3] N.F. Renna, N. de Las Heras, R.M. Miatello, Pathophysiology of vascular remodeling in hypertension, Int. J. Hypertens. 2013 (2013) 808353.
[4] F. Montecucco, A. Pende, A. Quercioli, F. Mach, Inflammation in the pathophysiology of essential hypertension, J. Nephrol. 24 (2011) 23–34.
[5] C.D. Funk, Prostaglandins and leukotrienes: advances in eicosanoid biology, Science 294 (2001) 1871–1875.
[6] C.D. Funk, Leukotriene modifiers as potential therapeutics for cardiovascular disease, Nat. Rev. Drug Discov. 4 (2005) 664–672.
[7] D. Poeckel, C.D. Funk, The 5-lipoxygenase/leukotriene pathway in preclinical models of cardiovascular disease, Cardiovasc. Res. 86 (2010) 243–253.
[8] H.D. Intengan, E.L. Schiffrin, Vascular remodeling in hypertension: roles of apoptosis, inflammation, and fibrosis, Hypertension 38 (2001) 581–587.
[9] M. Hermann, A. Flammer, T.F. Lüscher, Nitric oxide in hypertension, J. Clin. Hypertens. (Greenwich) 8 (2006) 17–29.
[10] J. Török, Participation of nitric oxide in different models of experimental hypertension, Physiol. Res. 57 (2008) 813–825.
[11] V.B. O'Donnell, B.A. Freeman, Interactions between nitric oxide and lipid oxidation pathways: implications for vascular disease, Circ. Res. 88 (2001) 12–21.
[12] F. László, B.J. Whittle, Colonic microvascular integrity in acute endotoxaemia: interactions between constitutive nitric oxide and 5-lipoxygenase products, Eur. J. Pharmacol. 277 (1995) R1–R3.
[13] T.V. Arutyunyan, A.F. Korystova, L.N. Kublik, M.K. Levitman, V.V. Shaposhnikova, Y.N. Korystov, Effects of taxifolin on the activity of angiotensin-converting enzyme and reactive oxygen and nitrogen species in the aorta of aging rats and rats treated with the nitric oxide synthase inhibitor and dexamethasone, Age (Dordr.) 35 (2013) 2089–2097.
[14] M. Gilchrist, S.D. McCauley, A.D. Befus, Expression, localization, and regulation of NOS in human mast cell lines: effects on leukotriene production, Blood 104 (2004) 462–469.
[15] A.E. Boe, M. Eren, S.B. Murphy, C.E. Kamide, A. Ichimura, D. Terry, D. McAnally, L.H. Smith, T. Miyata, D.E. Vaughan, Plasminogen activator inhibitor-1 antagonist TM5441 attenuates Nω-nitro-L-arginine methyl ester-induced hypertension and vascular senescence, Circulation 128 (2013) 2318–2324.
[16] H.Y. Zhang, W. Jiang, J.Y. Liu, Y. Li, C.L. Chen, H.B. Xin, D.J. Huang, Intermedin is upregulated and has protective roles in a mouse ischemia/reperfusion model, Hypertens. Res. 32 (2009) 861–868.
[17] L.E. Calderon, S. Liu, W. Su, Z. Xie, Z. Guo, W. Eberhard, M.C. Gong, iPLA2β overexpression in smooth muscle exacerbates angiotensin II-induced hypertension and vascular remodeling, PLoS One 7 (2012) e31850.
[18] P. Basu, U. Sen, N. Tyagi, S.C. Tyagi, Blood flow interplays with elastin: collagen and MMP: TIMP ratios to maintain healthy vascular structure and function, Vasc. Health Risk Manag. 6 (2010) 215–228.
[19] H. Louis, A. Kakou, V. Regnault, C. Labat, A. Bressenot, J. Gao-Li, H. Gardner, S.N. Thornton, P. Challande, Z. Li, P. Lacolley, Role of alpha1beta1-integrin in arterial stiffness and angiotensin-induced arterial wall hypertrophy in mice, Am. J. Physiol. Heart Circ. Physiol. 293 (2007) H2597–H2604.
[20] H. Matsumae, Y. Yoshida, K. Ono, K. Togi, K. Inoue, Y. Furukawa, Y. Nakashima, Y. Kojima, M. Nobuyoshi, T. Kita, M. Tanaka, CCN1 knockdown suppresses neointimal hyperplasia in a rat artery balloon injury model, Arterioscler. Thromb. Vasc. Biol. 28 (2008) 1077–1083.
[21] H. Guo, N. Makarova, Y. Cheng, E. S, R.R. Ji, C. Zhang, P. Farrar, G. Tigyi, The early- and late stages in phenotypic modulation of vascular smooth muscle cells: differential roles for lysophosphatidic acid, Biochim. Biophys. Acta 1781 (2008) 571–581.
[22] Y. Hu, Isolation of human and mouse neutrophils ex vivo and in vitro, Methods Mol. Biol. 844 (2012) 101–113.
[23] Y. Drastini, F.S. Kibenge, P.K. McKenna, A. Lopez, Comparison of eight different procedures for harvesting avian reoviruses grown in Vero cells, J. Virol. Methods 39 (1992) 269–278.
[24] A.S. Cowburn, S.T. Holgate, A.P. Sampson, IL-5 increases expression of 5-lipoxygenase-activating protein and translocates 5-lipoxygenase to the nucleus in human blood eosinophils, J. Immunol. 163 (1999) 456–465.
[25] J. Delgado Alves, L.J. Mason, P.R. Ames, P.P. Chen, J. Rauch, J.S. Levine, R. Subang, D.A. Isenberg, Antiphospholipid antibodies are associated with enhanced oxidative stress, decreased plasma nitric oxide and paraoxonase activity in an experimental mouse model, *Rheumatology* (Oxford) 44 (2005) 1238–1244.
[26] L. Lin, W.H. Ding, W. Jiang, Y.G. Zhang, Y.F. Qi, W.J. Yuan, C.S. Tang, Urotensin-II activates L-arginine/nitric oxide pathway in isolated rat aortic adventitia, Peptides 25 (2004) 1977–1984.
[27] J.W. Woods, J.F. Evans, D. Ethier, S. Scott, P.J. Vickers, L. Hearn, J.A. Heibein, S. Charleson, I.I. Singer, 5-lipoxygenase and 5-lipoxygenase-activating protein are localized in the nuclear envelope of activated human leukocytes, J. Exp. Med. 178 (2004) 1935–1946.
[28] J. Bartunek, E.O. Weinberg, M. Tajima, S. Rohrbach, S.E. Katz, P.S. Douglas, B.H. Lorell, Chronic N(G)-nitro-L-arginine methyl ester-induced hypertension: novel molecular adaptation to systolic load in absence of hypertrophy, Circulation 101 (2000) 423–429.
[29] F. Stanke-Labesque, G. Hardy, F. Caron, J.L. Cracowski, G. Bessard, Inhibition of leukotriene synthesis with MK-886 prevents a rise in blood pressure and reduces noradrenaline-evoked contraction in L-NAME-treated rats, Br. J. Pharmacol. 140 (2003) 186–194.
[30] J. Moraes, J. Assreuy, C. Canetti, C. Barja-Fidalgo, Leukotriene B4 mediates vascular smooth muscle cell migration through αvβ3 integrin transactivation, Atherosclerosis 212 (2010) 406–413.
[31] Y. Kaetsu, Y. Yamamoto, S. Sugihara, T. Matsuura, G. Igawa, K. Matsubara, O. Igawa, C. Shigemasa, I. Hisatome, Role of cysteinyl leukotrienes in the proliferation and the migration of murine vascular smooth muscle cells in vivo and in vitro, Cardiovasc. Res. 76 (2007) 160–166.
[32] N.A. Neuman, S. Ma, G.R. Schnitzler, Y. Zhu, G. Lagna, A. Hata, The four-and-a-half LIM domain protein 2 regulates vascular smooth muscle phenotype and vascular tone, J. Biol. Chem. 284 (2009) 13202–13212.
[33] S. Kumar, A. Jyoti, R.S. Keshari, M. Singh, M.K. Barthwal, M. Dikshit, Functional and molecular characterization of NOS isoforms in rat neutrophil precursor cells, Cytometry A 77 (2010) 467–477.
[34] J.L. Bellot, M. Palmero, N. Alcoriza, A. Blanco, C. García-Cabanes, C. Hariton, A. Orts, Concomitant treatment with a 5-lipoxygenase inhibitor improves the anti-inflammatory effect of the inhibition of nitric oxide synthase during the early phase of endotoxin-induced uveitis in the rabbit, Ophthalmic Res. 29 (1997) 227–236.
[35] M.J. Coffey, S.M. Phare, M. Peters-Golden, Interaction between nitric oxide, reactive oxygen intermediates, and peroxynitrite in the regulation of 5-lipoxygenase metabolism, Biochim. Biophys. Acta 1584 (2002) 81–90.