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Elevated Endothelial Nitric Oxide Bioactivity and Resistance to Angiotensin-Dependent Hypertension in 12/15-Lipoxygenase Knockout Mice

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12/15-Lipoxygenase (12/15-LOX) plays a pathogenic role in atherosclerosis. To characterize whether 12/15-LOX also contributes to endothelial dysfunction and hypertension, regulation of vessel tone and angiotensin II (ang II) responses were characterized in mice deficient in 12/15-LOX. There was a twofold increase in the magnitude of L-nitroarginine-methyl ester-inhibitable, acetylcholine-dependent relaxation or phenylephrine-dependent constriction in aortic rings isolated from 12/15-LOX^{-/-} mice. Plasma NO metabolites and aortic endothelial NO synthase (eNOS) expression were also elevated twofold. Angiotensin II failed to vasoconstrict 12/15-LOX^{-/-} aortic rings in the absence of L-nitroarginine-methyl ester, and ang II impaired acetylcholine-induced relaxation in wild-type, but not 12/15-LOX^{-/-} rings. *In vivo*, 12/15-LOX^{-/-} mice had similar basal systolic blood pressure measurements to wild type, however, blood pressure elevations in response to ang II infusion (1.1 mg/kg/day) were significantly attenuated (maximal pressure, 143.4 ± 4 mmHg versus 122.1 ± 5.3 mmHg for wild type and 12/15-LOX^{-/-}, respectively). In contrast, vascular hypertrophic responses to ang II, and ang II type 1 receptor (AT1-R) expression were similar in both strains. This study shows that 12/15-LOX^{-/-} mice have increased NO bio-synthesis and impaired ang II-dependent vascular responses *in vitro* and *in vivo*, suggesting that 12/15-LOX signaling contributes to impaired NO bioactivity in vascular disease *in vivo*. (Am J Pathol 2005, 166:653–662)

Lipoxygenases (LOXs) are a family of enzymes that play central roles in human and animal vascular disease. Their expression in vascular tissue is highly localized, with sources including platelets (12-LOX), monocytes/macrophages (12/15-LOX), and neutrophils (5-LOX). LOXs catalyze the oxidation of unsaturated fatty acids to hydroperoxides and other bioactive metabolites using a nonheme iron active site.^{1,2} The murine 12/15-LOX isoform is considered the functional equivalent of the human 15-LOX isoform and is constitutively expressed in peritoneal macrophages. There is experimental evidence for involvement of 12/15-LOX isoforms in inflammatory vascular disease. 15-LOX mRNA, protein, and lipid products are found in human atheroma, and 15-LOX inhibition prevents diet-induced atherosclerosis in rabbits.^{3–7} Also, 12/15-LOX knockout mice are less susceptible to diabetes or atherosclerosis when crossed with Apo E- or low density lipoprotein-receptor-deficient mice.^{8–10}

Endothelial dysfunction resulting in reduced NO bio-availability is a hallmark of inflammatory vascular disease, including angiotensin II (ang II)-dependent hypertension.^{11–13} It is widely accepted that NO deficiency is a primary event in initiation and progression of vascular disease, via loss of its vascular protective and homeostatic functions. In some studies, a role for superoxide (O₂⁻) reacting with NO to form peroxynitrite (ONOO⁻) has been found, however this only accounts for up to 50% of the impaired NO signaling because it is incompletely restored by O₂⁻ scavengers.¹¹ Additional mechanisms may include NO scavenging by alternative radical species, altered expression or activity of eNOS and impaired cGMP generation/responses.^{14–16}

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LOX and NO signaling pathways interact at several levels *in vitro* that could potentially impact on development of vascular disease and impairment of NO signaling.^{17–19} For example, NO inhibits LOX turnover at micromolar concentrations through formation of a nitrosyl complex, or reaction with an enzyme-substrate intermediate.^{18,19} Also, catalytic scavenging of NO by LOXs can attenuate NO signaling in isolated porcine monocytes.¹⁷ Therefore, to explore the potential role of 12/15-LOX in regulating NO bioavailability, control of vascular tone was examined in 12/15-LOX^{−/−} mice *in vitro* and *in vivo* after infusion of a pressor dose of ang II. We observed that 12/15-LOX^{−/−} aortic rings showed twofold elevations in NO bioactivity and endothelial nitric NO synthase (eNOS) expression, and lacked ang II responses, including vasoconstriction and impairment of ACh-induced dilatation. Inhibition of NO synthesis restored ang II signaling *in vitro*. *In vivo*, plasma NO metabolites were approximately twofold elevated at baseline and development of hypertension after ang II infusion was significantly impaired.

Materials and Methods

Materials

Rabbit anti-human AT1-R and anti-eNOS were from Santa Cruz Biotechnology (Santa Cruz, CA), goat anti-rabbit IgG-Alexa 568 was from Molecular Probes (Eugene, OR). 1400W, DETA NONOate, and *N*^ω-propyl-L-arginine were from Cayman. Unless otherwise stated, all compounds were from Sigma (Poole, UK).

Animal Studies

All animal experiments were performed in accordance with the United Kingdom Home Office Animals (Scientific Procedures) Act of 1986. 12/15-LOX knockout mice generated as described previously and wild-type male C57BL/6 mice (25 to 30 g) from Harlan, UK, were kept in constant temperature cages (20 to 22°C) and given free access to water and standard chow.⁹

Isometric Tension Functional Studies

Male wild-type mice or 12/15-LOX^{−/−} mice (25 to 30 g) were sacrificed by cervical dislocation. The thoracic aorta was removed and placed in Krebs-Henseleit buffer (120 mmol/L NaCl, 4.7 mmol/L KCl, 1.2 mmol/L MgSO₄·7H₂O, 24 mmol/L NaHCO₃, 1.1 mmol/L KH₂PO₄, 10 mmol/L glucose, 2.5 mmol/L CaCl₂·2H₂O). The aorta was dissected of adipose tissue, cut into rings (2 to 3 mm) and suspended in an isometric tension myograph (model 610; DMT, Aarhus, Denmark) containing Krebs buffer at 37°C and gassed with 5% CO₂/95% O₂. A resting tension of 3 mN was maintained, and changes in isometric tension recorded via Myodaq software (DMT).

After a 60-minute equilibration period, vessels were primed with 48 mmol/L KCl before a concentration (0.1 μmol/L) of phenylephrine producing ~75% contraction was added. Once the response stabilized, 1 μmol/L ACh

was added to assess endothelial integrity. Any rings that did not maintain contraction to PE, or relaxed <50% of the phenylephrine-induced tone after addition of ACh (1 μmol/L) were discarded. No differences in the number of rings discarded between wild-type and 12/15-LOX^{−/−} vessels were observed. Rings were washed for 30 minutes after which a cumulative concentration-response curve to phenylephrine was constructed (1 nmol/L to 1 μmol/L) to assess vasoconstrictor activity. The tissues were then washed for 60 minutes to restore basal tone before contracting to ~80% of the 1 μmol/L phenylephrine-induced response. Once a stable response to phenylephrine was observed, cumulative concentration-response curves were constructed to 1 nmol/L to 10 μmol/L ACh, and then after a 30-minute wash, curves were constructed to either 1 nmol/L to 10 μmol/L sodium nitropruside (SNP) or 0.1 nmol/L to 100 μmol/L DETA-NONOate to assess endothelium-dependent and -independent relaxations, respectively. Experiments were repeated in the presence of either 300 μmol/L L-nitroarginine-methyl ester (L-NAME), 10 μmol/L 1400W, or 1 μmol/L *N*^ω-propyl-L-arginine. In some experiments, concentration-constriction curves were constructed to ang II (1 nmol/L to 10 μmol/L). In other experiments, ang II (0.1 μmol/L) was added for the duration of the 30-minute wash period immediately after assessment of endothelial integrity. Constriction and dilation curves were then constructed as described previously. Responses were expressed as a percentage of either baseline tension (vasoconstriction) or contracted tension (vasodilation). Responses from patent rings of each animal (three to four rings) were combined to produce an average for each sample (*n*).

Nitric Oxide Metabolite Measurements

The NO metabolites nitrate and nitrite (NOx) were determined using the Griess reaction.²⁰ Whole blood was centrifuged to recover plasma, which was then centrifuged through a 10-kd filter (Microcon; Millipore) for 30 minutes. Filtrate was analyzed for NOx by addition of sulfanilamide/HCl (1 mmol/L, 0.6 mol/L, respectively) and *N*-(1-naphthyl)-ethylenediamine (NEDA, 1 mmol/L), either with or without previous reduction using nitrate reductase (Aspergillus; Boehringer Mannheim) for 2 hours, 37°C. Absorbances were measured at 530 nm and compared with standard curves generated using sodium nitrate or nitrite.

Extraction of Oxidized Arachidonate Products from Wild-Type and 12/15-LOX^{−/−} Lavage and Aortae

Peritoneal lavage was obtained in phosphate-buffered saline (PBS), then immediately placed on ice and supplemented with 0.1 mmol/L butylated hydroxytoluene, 0.1 mmol/L diethylenetriamine pentaacetic acid, and 0.1% (w/v) SnCl₂ in screw-cap cryovials. Specimens were purged with argon and then snap-frozen in liquid N₂ and stored at −80°C until analysis. Thoracic aortae, dissected of adipose tissue, were placed into cryovials con-

taining 0.5 ml of PBS supplemented with 0.1 mmol/L butylated hydroxytoluene, 0.1 mmol/L DTPA, 0.1% (w/v) SnCl₂, purged with argon, and snap-frozen in liquid N₂ until lipid extraction. At time of analysis, aortic specimens were thawed, then homogenized on ice, with argon gas stream directed to top of homogenizer. During lipid extraction, 10 ng each of deuterated internal standards 12(S)hydroxy-5,8,10,14-eicosatetraenoic-5,6,8,9,11,12,14,15-d₈ acid (12-HETE-d₈) and prostaglandin F_{2α} (PGF_{2α}-d₄) (Cayman Chemical Co.) were added. Lipids were extracted in glass PTFE-lined screw-cap hydrolysis tubes by adding 1 mol/L acetic acid/2-isopropanol/hexane (2:20:30, v/v/v) to the sample at a ratio of 2.5 ml:1 ml (solvent:sample), layering with argon to purge headspace air, vortexing, and then adding 2.5 ml of hexane. After vortexing and centrifugation, lipids were recovered in the upper hexane layer. Samples were immediately re-extracted by an equal volume of hexane. The combined extracts were dried under argon. For saponification, lipids were resuspended in 1.5 ml of 2-isopropanol, and then 1.5 ml of 0.2 mol/L NaOH added at 60°C for 30 minutes under argon atmosphere. Hydrolyzed samples were acidified to pH 3.0 with 0.5 mol/L HCl, and then fatty acids extracted twice with 4 ml of hexane. The combined hexane layers were dried under N₂, resuspended in 100 μl of methanol, and stored under argon at -80°C until analysis (typically within hours) by reverse phase high performance liquid chromatography (HPLC) with on-line electrospray ionization tandem mass spectrometry (LC/ESI/MS/MS), as described below.

Lipid Analyses by LC/ESI/MS/MS

LC/ESI/MS/MS was used to quantify the multiple distinct oxidation products of arachidonic acid and linoleic acid, including individual hydroxyeicosatetraenoic acids (HETEs), F₂-isoprostanes, and hydroxyoctadecadienoic acids (HODEs) as previously described.²¹ Analyses were performed using electrospray ionization in negative ion mode with multiple reaction monitoring of parent and characteristic daughter ions specific for each isomer monitored. The transitions monitored were mass-to-charge ratio (*m/z*): *m/z* 295 → 171 for 9-HODE; *m/z* 295 → 195 for 13-HODE; *m/z* 279 → 261 for linoleic acid; *m/z* 319 → 115 for 5-HETE; *m/z* 319 → 155 for 8-HETE; *m/z* 319 → 151 for 9-HETE; *m/z* 319 → 167 for 11-HETE; *m/z* 319 → 179 for 12-HETE; *m/z* 319 → 175 for 15-HETE; *m/z* 303 → 259 for arachidonic acid; *m/z* 327 → 184 for 12-HETE-d₈; *m/z* 353 → 309 for F₂-isoprostanes; and *m/z* 357 → 313 for PGF_{2α}-d₄. The internal standard 12-HETE-d₈ was used for quantification of HETEs as well as to calculate extraction efficiencies of HODEs and HETEs (which were >85%). The internal standard PGF_{2α}-d₄ was used to for quantification of F₂-isoprostanes.

Hypertension Studies

Male 10- to 14-week-old wild-type and 12/15-LOX^{-/-} mice were anesthetized via inhalation of 2% isoflurane (98% oxygen). Osmotic minipumps (Alzet model 1002;

Durect Corp., Cupertino, CA) containing angiotensin II (infusion rate, 1.1 mg/kg/day) or vehicle were implanted subcutaneously in the midscapular area. In a separate group of animals, no minipumps were used, but L-NAME (100 mg.kg⁻¹.day⁻¹) was added to the drinking water. Systolic blood pressure was monitored daily for 2 days before implantation (training) and 7 days after implantation or after L-NAME administration via tail cuff plethysmography (World Precision Instruments, UK) in conscious mice.

Assessment of Heart: Body Weight Area, Aortic Medial Area, and Immunohistochemistry

Seven days after implantation, mice were sacrificed by cervical dislocation and heart and body weights were recorded. The descending thoracic aorta was removed, cleaned of adipose tissue, and fixed in paraffin wax. For medial area, 15-μm sections were taken using a microtome, placed on glass slides, fixed using acetone, then stained with hematoxylin. Images were acquired using a ×5 air lens, with a Axiovert S100TV microscope and Hamamatsu Orca digital camera, and medial area calculated using Scion Image for Windows (Scion Corp., USA). Media was outlined manually for each aorta and area computed using the measure command with three separate sections analyzed and averaged per aorta. For immunohistochemistry, 10-μm sections were methanol-fixed on glass slides, permeabilized using 0.1% (w/v) Triton X-100/PBS, blocked using 1% (w/v) bovine serum albumin/PBS. Angiotensin II type 1 receptor (AT1-R) or eNOS expression was visualized using either rabbit anti-human AT1-R or rabbit anti-eNOS, with goat anti-rabbit IgG-Alexa 568 as secondary. Negative controls used equivalent concentrations of isotype rabbit IgG antibody. Imaging was performed on an Axiovert 100 inverted microscope connected to a Bio-Rad MRC 1024ES laser scanning system (Bio-Rad Microscience, Hemel Hempstead, UK) using standard analysis software (Lasershar 2000, Bio-Rad Microscience). Images were acquired using a ×10 air lens, with excitation at 568 nm and emission 595/35 nm. Postacquisition processing used Adobe Photoshop, with average fluorescence intensity of specific stained regions (arbitrary units) of unprocessed images determined using Lasershar 2000. For each aorta, three to four separate sections were imaged and pixel intensity calculated at three to four separate areas of each section. Therefore for each aorta (*n*) an average of 12 separate pixel intensity determinations were made.

Results

Generation of 12-HETE by Aortic Tissue of Wild-Type and 12/15-LOX^{-/-} Mice

To examine for 12/15-LOX activity in aortae of wild-type and 12/15-LOX^{-/-} mice, hydroxylipid content was determined by LC/ESI/MS/MS after reduction and saponification. As positive control, peritoneal lavage from the same

Figure 1. Detection of 12/15-LOX products in peritoneal lavage and aortae of 12/15-LOX^{-/-} and wild-type mice. Arachidonate, linoleate, and their oxidized products were analyzed in peritoneal lavage and aorta from 12/15-LOX^{-/-} and wild-type mice using LC/ESI/MS/MS after reduction, extraction, and saponification, as described in Materials and Methods. Results are expressed as ratio of oxidized lipid product:precursor fatty acid. HETE, hydroxyeicosatetraenoic acid; HODE, hydroxyoctadecadienoic acid.

animals, which contains 12/15-LOX-expressing macrophages was also examined. Peritoneal extracts from wild-type mice contained a number of HODE and HETE isomers; however, by far the most predominant were the specific 12/15-LOX products, 13-HODE and 12-HETE (Figure 1). Similarly, aortic extracts contained 13-HODE and 12-HETE, but levels were ~10-fold lower than peritoneal lavage. In contrast, tissue from 12/15-LOX^{-/-} animals contained virtually undetectable HODEs or HETEs (Figure 1). Specific detection of 12-HETE and 13-HODE versus other isomers in wild-type, and its virtual absence in 12/15-LOX^{-/-} mice, is consistent with formation of these regio-specific products from 12/15-LOX turnover in the vessel wall *in vivo*.

In Vitro Studies on Vascular Reactivity of Wild-Type and 12/15-LOX^{-/-} Aortic Rings

To examine NO-dependent control of vascular tone in wild-type and 12/15-LOX^{-/-} mice, the constriction/relaxation responses of thoracic aortic rings were characterized, in the presence and absence of L-NAME. In initial studies, phenylephrine caused a concentration-dependent constriction in wild-type rings, which was significantly attenuated (~40%) in 12/15-LOX^{-/-} rings (Figure 2A). No differences in the time to develop force or ability to maintain constriction were observed between wild-type and 12/15-LOX^{-/-} rings. Endothelium-dependent vasodilation was also reduced in 12/15-LOX^{-/-} rings, but to a far lesser extent (~20%) (Figure 2B). No differences in SNP or DETA NONOate-dependent dilatation were found between wild-type and 12/15-LOX^{-/-} rings (Figure 2, C and D). Identical relaxation dose-response curves to these compounds indicate intact smooth muscle responses to NO in 12/15-LOX^{-/-} vessels.

Inclusion of 300 μ M L-NAME had no effect on basal tone, but enhanced phenylephrine-dependent constriction in both wild-type and 12/15-LOX^{-/-} rings. However effects of L-NAME were approximately twofold greater for 12/15-LOX^{-/-} (Figure 3A). This indicates that endoge-

nous generation of NO continuously antagonizes the constrictive effects of phenylephrine in both strains, with a significantly greater contribution in 12/15-LOX^{-/-}. Inclusion of L-NAME inhibited ACh-dependent dilatation ~80% in wild-type rings, however in 12/15-LOX^{-/-} rings L-NAME reversed dilatation to such an extent that ACh became somewhat vasoconstrictive, with the inhibitory effect of L-NAME being more than twofold greater in 12/15-LOX^{-/-} than wild type (Figure 3B). To examine which NOS isoform was responsible for the elevated NO in these aortae, effects of isoform-selective inhibitors on constriction of 12/15-LOX^{-/-} rings were examined. The data shows that inclusion of an nNOS (*N*^ω-propyl-L-arginine) or iNOS (1400W)-selective inhibitor did not significantly enhance constriction, in contrast to L-NAME (Figure 3; A, C, and D). These data collectively suggest that the bioactivity of NO is greater in 12/15-LOX^{-/-} than wild type and that the likely source of this NO is eNOS.

Expression and Activity of eNOS in Vivo

Aortic expression of eNOS was examined using immunohistochemistry and after quantification of pixel intensity, was found to be approximately twofold higher in 12/15-LOX^{-/-} than wild type (Figure 4; A to G). Similarly, plasma total NOx concentration determined using the Griess reaction was found to be elevated twofold (Figure 4H). These data indicate increased NO biosynthesis in 12/15-LOX^{-/-} mice that may result from elevated eNOS expression.

12/15-LOX^{-/-} Aortic Rings Do Not Constrict in Response to Ang II

In wild-type aortic rings, ang II caused a small but significant vasoconstriction, which was absent in 12/15-LOX^{-/-} (Figure 5A). In the presence of L-NAME, there was a small but not significant effect on ang II constriction in wild-type rings. However, ang II-dependent constriction 12/15-LOX^{-/-} rings was fully restored to wild-type levels by L-NAME addition, in fact at higher ang II concentrations constriction was significantly greater than for wild type (Figure 5B). This indicates that elevated NO bioactivity antagonizes the vasoconstrictive actions of ang II in 12/15-LOX^{-/-}.

Ang II Preincubation Impairs ACh Relaxation in Wild-Type but Not 12/15-LOX^{-/-} Rings

Incubation of wild-type rings in 0.1 μ M/L ang II for 30 minutes caused a significant right-shift in the relaxation dose-response to lower concentrations of ACh, indicating acute vascular dysfunction (Figure 5C). This response was absent in 12/15-LOX^{-/-} rings, again demonstrating a requirement for 12/15-LOX^{-/-} in acute ang II-dependent vascular responses *in vitro* (Figure 5D).

Figure 2. 12/15-LOX^{-/-} aortic rings show alterations in phenylephrine constriction and ACh relaxation. Aortic ring functional responses were determined as described in Materials and Methods. **A:** Constriction dose response to phenylephrine in wild-type or 12/15-LOX^{-/-} rings ($n = 8$ to 9). **B:** ACh relaxation dose-response curves in wild-type or 12/15-LOX^{-/-} rings ($n = 8$ to 9). **C:** Dose-response curves to SNP in wild-type or 12/15-LOX^{-/-} rings ($n = 8$ to 9). **D:** Dose-response curves to DETA NONOate in wild-type or 12/15-LOX^{-/-} rings ($n = 3$). $\star$, $P < 0.05$ compared to wild-type group, using two-way analysis of variance to isolate differences between groups. Data are expressed as mean $\pm$ SEM.

Deletion of 12/15-LOX Attenuates Ang II Elevation of Systolic Blood Pressure

Basal blood pressure of wild-type and 12/15-LOX^{-/-} mice were not significantly different (107.2 ± 2.8 mmHg versus 107.9 ± 3.2 mmHg, respectively). Subcutaneous infusion of ang II caused significant increases in systolic blood pressure in wild-type mice (Figure 6A; maximal pressure, 143.4 ± 4 mmHg at 4 days after infusion, $P < 0.01$ compared with day 0, using analysis of variance with Dunnett's posthoc test to isolate differences). However, in 12/15-LOX^{-/-} mice ang II caused a small increase in pressure, which was not significantly different from baseline (maximum pressure, 122.1 ± 5.3 mmHg at 4 days), but was significantly lower than wild-type ang II-infused mice. Infusion of vehicle (saline) had no effect on systolic blood pressure in either wild-type or 12/15-LOX^{-/-} mice (maximum pressure, 107.7 ± 3.9 mmHg and 104 ± 3.2 mmHg for wild type and 12/15-LOX^{-/-}, respectively). These data demonstrate that although not required for maintenance of basal blood pressure, 12/15-LOX is required for induction of ang II-induced systolic hypertension.

Ang II Induces Similar Cardiac and Aortic Hypertrophy in Wild-Type and 12/15-LOX^{-/-} Mice

Before ang II infusion, heart:body weight ratios and aortic medial areas were similar for wild-type and 12/15-LOX^{-/-} mice (Figure 6, B and C). Also, subcutaneous infusion of ang II caused similar increases in heart:body weight ratio and aortic medial area in both strains (Figure 6, B and C). These data demonstrate that 12/15-LOX is not required for normal heart or vascular thickness, or ang II-induced vascular and cardiac hypertrophy *in vivo*. Finally, after angiotensin II infusion, total body weights were 28.63 ± 0.79 g versus 27.4 ± 0.8 g, for wild type or 12/15-LOX^{-/-}, respectively, and were not significantly different.

Infusion of L-NAME Causes More Sustained Elevation of Blood Pressure in 12/15-LOX^{-/-} Mice

Administration of L-NAME to both wild-type and 12/15-LOX^{-/-} mice acutely and significantly elevated blood pressure (Figure 7). Although the initial maximum increase in blood pressure did not differ between strains, 12/15-LOX^{-/-} remained significantly raised for 3 days longer. This indicates that inhibition of NO generation causes a more sustained elevation in blood pressure in 12/15-LOX^{-/-} than wild-type mice. These data suggest that elevated NO in 12/15-LOX-deficient mice influences basal blood pressure, however this process may play a more significant role in control of tone under conditions of vascular inflammation, for example ang II signaling which is known to involve accelerated scavenging of NO by oxidative pathways.

Figure 4. 12/15-LOX^{-/-} mice have elevated eNOS and plasma NOx *in vivo*. Aortic sections from wild-type and 12/15-LOX^{-/-} mice were sectioned and stained for eNOS, as described in Materials and Methods. Representative sections are shown for each condition. **A** and **B**: Wild type; **C** and **D**: 12/15-LOX^{-/-}; **E** and **F**: isotype control antibody. **A**, **C**, and **E**: eNOS fluorescence; **B**, **D**, and **F**: corresponding phase contrast images. **G**: Pixel intensity was determined after fluorescence staining of aortic sections as described in Materials and Methods ($n = 3$ separate aortae; *, $P < 0.05$, unpaired Student's *t*-test). **H**: NOx levels in plasma from wild-type or 12/15-LOX^{-/-} mice were determined as described in Materials and Methods ($n = 6$; *, $P < 0.05$, unpaired Student's *t*-test).

Figure 3. NO bioactivity is higher in 12/15-LOX^{-/-} than wild-type aortic rings, but is not modulated by selective iNOS or nNOS inhibitors. Aortic ring functional responses were determined as described in Materials and Methods. **A**: Constriction dose response to phenylephrine in wild-type or 12/15-LOX^{-/-} rings in the presence or absence of 300 μmol/L L-NAME ($n = 6$ to 9). **B**: Relaxation dose response to ACh in phenylephrine-precontracted wild-type or 12/15-LOX^{-/-} rings in the presence or absence of 300 μmol/L L-NAME ($n = 6$ to 9). **C**: Effect of selective nNOS (1 μmol/L, N^ω-propyl-L-arginine, NPA) or iNOS (10 μmol/L, 1400W) inhibition on PE constriction of 12/15-LOX^{-/-} aortic rings. **D**: Effect of selective nNOS (1 μmol/L, NPA) or iNOS (10 μmol/L, 1400W) inhibition on ACh relaxation of 12/15-LOX^{-/-} aortic rings. *, $P < 0.05$ compared to wild-type group; †, $P < 0.05$ compared to 12/15-LOX^{-/-} group using two-way analysis of variance to isolate differences between groups; ‡, $P < 0.05$ compared to L-NAME-treated wild-type group using two-way analysis of variance with Bonferroni's posttest to isolate differences between concentrations. Data are expressed as mean ± SEM.

Expression of AT₁-R Is Similar in Wild-Type and 12/15-LOX^{-/-} Aortae

Ang II-dependent elevations in blood pressure are mediated via AT₁-R. Using immunohistochemistry, both wild-type and 12/15-LOX^{-/-} thoracic aortic rings demonstrated clear expression of AT₁-R (Figure 8). Also, for both wild type and 12/15-LOX^{-/-}, AT₁-R was ~50% down-regulated after ang II infusion (Figure 8). Down-regulation of AT₁-R by ang II was previously reported *in vitro*, and has been proposed to involve AT₂-R signaling.²²⁻²⁴ These data demonstrate that the lack of ang II-induced hypertension in 12/15-LOX^{-/-} is not because of lack of AT₁-R expression, and that ang II-dependent down-regulation of AT₁-R is not dependent on 12/15-LOX.

Discussion

In vitro, LOX- and NO-signaling pathways interact in several ways that could impact on vascular disease progression and control of vessel tone. Herein, using knockout mice deficient in 12/15-LOX, *in vitro* vasoreactivity of isolated aortic rings, and *in vivo* development of ang II-dependent hypertension was examined to characterize the control of vascular NO signaling by 12/15-LOX.

Initial studies showed alterations in constriction/relaxation dose responses to phenylephrine and acetylcholine in 12/15-LOX^{-/-} versus wild-type isolated aortic rings (Figure 2). In large vessels such as aorta, vascular tone is regulated by the balance between several constrictive and relaxant stimuli (eg, noradrenaline, endothelin, prostacyclin, NO). Therefore, to reveal the contributions of NO signaling alone, dose-response curves were constructed in the presence or absence of the nonspecific NOS inhibitor, L-NAME. In both wild-type and 12/15-LOX^{-/-} rings, inclusion of L-NAME increased PE-dependent vasoconstriction. However, this increase was approximately twofold greater in 12/15-LOX^{-/-} than wild type, suggesting that basal NO levels exert a much greater effect on control of tone in this strain (Figure 3A). Furthermore, L-NAME restored ang II-dependent constriction, which was absent in 12/15-LOX^{-/-} rings, to greater than wild-type levels (Figure 5B). This indicated that the lack of ang II constriction is primarily because of an excess of NO. Inclusion of L-NAME in relaxation experiments inhibited ACh-dependent relaxation in both strains. Similar to constriction experiments, there was a twofold greater effect of L-NAME in 12/15-LOX^{-/-} than wild type. Indeed, there was a small constriction observed in rings from this strain

Figure 5. 12/15-LOX^{-/-} aortic rings lack functional responses to ang II. **A:** Ang II constrictor dose-response curves were constructed using aortic rings from wild-type and 12/15-LOX^{-/-} mice ($n = 5$ to 8). **B:** Ang II constrictor dose-response curves using aortic rings from wild-type and 12/15-LOX^{-/-} mice were repeated in the presence of 300 $\mu\text{mol/L}$ L-NAME ($n = 5$ to 8). $\star$, $P < 0.05$ compared to 12/15-LOX^{-/-} group; unpaired Student's *t*-test. **C:** ACh dose-response curves were constructed for wild-type aortic rings, with or without preincubation in 0.1 $\mu\text{mol/L}$ ang II for 30 minutes ($n = 5$ to 9). **D:** ACh dose-response curves were constructed for 12/15-LOX^{-/-} aortic rings, with or without preincubation in 0.1 $\mu\text{mol/L}$ ang II for 30 minutes ($n = 5$ to 9). $\star$, $P < 0.05$ compared to wild-type group using two-way analysis of variance to isolate differences. Data are expressed as mean $\pm$ SEM.

Figure 6. Ang II-dependent elevations in systolic blood pressure are attenuated in 12/15-LOX^{-/-} mice, but vascular hypertrophy is unaltered. Ang II (1.1 mg/kg/day) was infused into male 10- to 12-week-old wild-type and 12/15-LOX^{-/-} mice by osmotic minipump as described in Materials and Methods. **A:** Systolic blood pressure (blood pressure) was monitored daily for 2 days before implantation and 7 days after implantation via tail cuff plethysmography. ○, Ang II-infused 12/15-LOX^{-/-}; □, ang II-infused wild-type mice ($n = 5$ to 9 animals per group, mean $\pm$ SEM; *, $P < 0.01$ compared to day 0; using analysis of variance test with Dunnett's posthoc test to isolate differences). **B:** Heart and body weight was recorded and compared before and after ang II infusion ($n = 6$ to 11, mean $\pm$ SEM; *, $P < 0.05$, compared to wild-type group; *, $P < 0.05$ compared to 12/15-LOX^{-/-} group; unpaired Student's *t*-test). **C:** Medial area was determined as described in Materials and Methods. For each aorta, three sections were analyzed and averaged ($n = 5$, mean $\pm$ SEM; *, $P < 0.05$, compared to wild-type group; *, $P < 0.05$ compared to 12/15-LOX^{-/-} group; unpaired Student's *t*-test).

Figure 7. L-NAME induced hypertension in wild-type and 12/15-LOX^{-/-} mice. L-NAME (100 mg/kg⁻¹·day⁻¹) was administered in drinking water to male 10- to 12-week-old wild-type and 12/15-LOX^{-/-} mice, and blood pressure was monitored daily using tail-cuff plethysmography, as described in Materials and Methods ($n = 5$ animals per group, mean $\pm$ SEM; *, $P < 0.01$ compared to baseline; using analysis of variance test with Dunnett's posthoc test to isolate differences).

(Figure 3B). This may indicate an enhanced contractile state in the 12/15-LOX^{-/-}, induced as a response to elevated NO to effectively maintain normal blood pressure and tone. *In vivo* infusion of L-NAME partially supported this concept, with a more sustained elevation of blood pressure in 12/15-LOX^{-/-} versus wild-type mice (Figure 7). Finally, eNOS expression and plasma NOx were elevated twofold in 12/15-LOX^{-/-}, indicating up-regulation of NO generation *in vivo*, and selective inhibitors of nNOS (*N*^ω-propyl-L-arginine) or iNOS (1400W) were without significant effect on constriction or relaxation (Figures 3 and 4). These studies clearly demonstrate up-regulation of eNOS expression and activity is associated with 12/15-LOX deletion in the vasculature.

Previous studies using the nonspecific inhibitors bacilein, phenidone, or cinnamyl-3,4-dihydroxycyano cinnamate in spontaneously hypertensive rats suggested a role for LOX isoforms in ang II-dependent hypertension.^{25–29} However, these inhibitors are not specific for LOXs, and furthermore do not distinguish which isoform is involved. In our study, basal blood pressures were similar, indicating that 12/15-LOX is not required for maintenance of physiological blood pressure, however 12/15-LOX^{-/-} mice were significantly protected against ang II-dependent hypertension *in vivo* and lacked *in vitro* ang II responses (Figures 5 and 6). Use of mice genetically deficient in 12/15-LOX conclusively establishes a role for this particular isoform in ang II signaling and indicates it as a potential target for anti-hypertensive therapy.

Previous studies have suggested that ang II-induced vascular dysfunction and hypertension is mediated at least in part by up-regulated ROS generation that decreases NO bioactivity via radical termination.^{5,30–34} Our studies show that elevated NO bioactivity in 12/15-LOX^{-/-} mice can effectively prevent ang II signaling. Although this may primarily result from elevated expression and activity of eNOS, 12/15-LOX could also decrease NO through catalyzing ROS-dependent decay, and/or direct signaling by 12/15-LOX products. However, the lack of difference in vascular responses to exogenous NO (DETA NONOate) suggest that accelerated scavenging of NO is unlikely, at least under these conditions

Figure 8. AT1-R is down-regulated after ang II infusion in both wild-type and 12/15-LOX^{-/-} mice. Aortic sections from wild-type and 12/15-LOX^{-/-} mice before or after ang II infusion were sectioned and stained for AT1-R, as described in Materials and Methods. Representative sections are shown for each condition. **A:** Wild type; **B:** 12/15-LOX^{-/-}; **C:** wild type + ang II; **D:** 12/15-LOX^{-/-} + ang II; **E:** isotype control antibody. **F–J:** Phase-contrast images for **A–E**, respectively. **K:** Pixel intensity was determined after fluorescence staining of aortic sections as described in Materials and Methods ($n = 4$ separate animals, with each three separate sections per aorta analyzed in triplicate for each section; $\star, P < 0.05$, unpaired Student's *t*-test).

(Figure 2D). Additionally, although 12/15-LOX products may directly influence vascular tone, this requires $\mu\text{mol/L}$ concentrations and might also be considered unlikely.^{8,35,36} Using LC/ESI/MS/MS, specific 12/15-LOX prod-

ucts were found in the aortic tissue of wild-type but not 12/15-LOX^{-/-} mice, confirming the presence of 12/15-LOX in the aorta of wild-type mice. However it was not possible to determine exact concentrations of 12-HETE in specific cellular locations within the vessel wall (Figure 1).

Lack of *in vivo* and *in vitro* ang II responses is also observed in NADPH oxidase-deficient mice.^{37–40} This indicates that both NADPH oxidase and 12/15-LOX are required for ang II signaling, and suggests that there may be a functional interplay between these pathways in vascular regulation. The data also indicates that vascular hypertrophy at the pressor concentrations of ang II used herein can occur independently of blood pressure elevation. It is likely that more than one mechanism may be involved in generation of hypertrophy in this model (ie, pressure-dependent and -independent). In support, a subpressor dose of ang II was previously found to induce hypertrophy, with no increase in systolic pressure.³⁹ Finally, several *in vitro* studies have suggested an involvement of 12/15-LOX and its products in ang II-dependent proliferation and cellular hypertrophy.^{5,41–43} Our *in vivo* experiments do not support this idea, however alterations in other hypertrophic pathways, such as NADPH oxidase cannot be excluded.

In addition to attenuated hypertension as demonstrated herein, 12/15-LOX^{-/-} mice are resistant to atherosclerosis and diabetes.^{8,9} Because decreased NO bioactivity *in vivo* is a hallmark of these pathophysiological conditions, the elevated intravascular NO found in 12/15-LOX^{-/-} mice may also contribute to disease resistance in those models. In summary, our data demonstrates that 12/15-LOX serves as a regulator of endothelial NO signaling and ang II-dependent dysfunction in the vasculature, both *in vivo* and *in vitro*, and suggests that specific inhibition of this isoform may attenuate hypertension *in vivo*.

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