Endothelial 15-Lipoxygenase-1 Overexpression Increases Acetylcholine-Induced Hypotension and Vasorelaxation in Rabbits

Nitin T. Aggarwal, Yuttanna Chawengsub, Kathryn M. Gauthier, Helena Viita, Seppo Yla-Herttuala, William B. Campbell

Abstract—Arachidonic acid is metabolized by the 15-lipoxygenase-1 pathway to the vasodilatory eicosanoids hydroxyepoxyeicosatrienic and trihydroxyeicosatrienic acid. We determined the in vitro and in vivo effects of the 15-lipoxygenase-1 metabolites in rabbits infected with adenovirus containing cDNA for human 15-lipoxygenase-1 (Ad-15-LO-1). Forty hours after intravenous adenoviral injection, 15-lipoxygenase-1 expression increased in liver and mesenteric arteries 10-fold and 3-fold, respectively. Expression of 15-LO-1 was limited to the endothelium of mesenteric arteries. Overexpression did not occur in tissues from rabbits infected with a β-galactosidase containing adenovirus. Trihydroxyeicosatrienic acid and hydroxyepoxyeicosatrienic acid synthesis per milligram of tissue increased by 2.1- and 1.5-fold, respectively, in mesenteric arteries from Ad-15-LO-1–infected rabbits compared with normal rabbits. Pretreatment with a 15-lipoxygenase inhibitor BW755C inhibited the synthesis. NO and prostaglandin-independent, maximal relaxations to acetylcholine were greater in mesenteric arteries from Ad-15-LO-1–infected rabbits (98.3 ± 1.7%) compared with normal (60.93 ± 10.5%) and β-galactosidase containing adenovirus–infected rabbits (68.3 ± 7.7%). Pretreatment with BW755C decreased these relaxations. Mean arterial pressure and heart rate did not differ in Ad-15-LO-1–infected rabbits compared with normal or β-galactosidase containing adenovirus–infected rabbits. The hypotensive responses to acetylcholine in the presence and absence of inhibition of NO and prostaglandins were greater in Ad-15-LO-1–infected rabbits (−52 ± 2% and −47 ± 2%) compared with normal (−31 ± 3% and −25 ± 5%) or β-galactosidase containing adenovirus–infected rabbits (−38 ± 2% and −30 ± 3%). Therefore, increased expression of 15-LO-1 increases acetylcholine relaxation in arteries and hypotensive responses in rabbits because of increased hydroxyepoxyeicosatrienic acid and trihydroxyeicosatrienic acid synthesis. (Hypertension. 2008;51:246-251.)

Key Words: endothelium-derived hyperpolarizing factors • endothelium • adenovirus • hypotension • mean arterial pressure

15-Lipoxygenase-1 (15-LO-1) is expressed in the vascular endothelium.1 It metabolizes arachidonic acid (AA) to 15-hydroperoxyeicosatetraenoic acid. 15-Hydroperoxyeicosatetraenoic acid is reduced to 15-hydroxyicosatetraenoic acid and rearranged to 15-hydroxy-11,12-epoxyeicosatrienoic acid and 11-hydroxy-14,15-epoxyeicosatrienoic acid. Hydroxyeicosatrienoic acids (HEETAs) are further hydrolyzed to 11,12,15-trihydroxyicosatrienoic acid and 11,14,15-trihydroxyeicosatrienoic acid.2 Trihydroxyeicosatrienoic acid (THETA) and HEETA relax rabbit arteries.2,3 In the presence of NO synthase and cyclooxygenase (COX) inhibition, acetylcholine (ACH) and AA relaxed preconstricted rabbit arteries. These relaxations were inhibited by 15-lipoxygenase inhibitors.3,4 Thus, 15-LO-1 metabolites mediate relaxations to ACH and AA. 15-Hydroxyeicosatetraenoic acid is inactive in rabbit aorta.5,6 However, 11,12,15-trihydroxyeicosatrienoic acid hyperpolarizes smooth muscle cells and relaxes rabbit aorta and mesenteric arteries (MAs).3,4,7 Relaxations induced by 11,12,15-trihydroxyeicosatrienoic acid are inhibited by high K+ and calcium-dependent potassium–channel inhibitors. Therefore, 11,12,15-trihydroxyeicosatrienoic acid is an endothelium-derived hyperpolarizing factor (EDHF). Recently, we reported that 15-LO-1 is necessary8 and sufficient9 to increase ACH-induced relaxations in preconstricted rabbit aorta in the presence of NO synthase and COX inhibition. The activity of eicosanoids varies with species and vascular bed. For example, THETA is more potent in small MAs than in large conduit arteries, such as the aorta. The vasodilatory activity of THETA in other arteries is unknown. 15-Hydroxyeicosatetraenoic acid is inactive in the aorta but...
contracts rabbit pulmonary arteries. 10 15-Hydroperoxycicosatetraenoic acid evokes relaxations at lower concentrations but contractions at higher concentrations in aorta and pulmonary arteries. 11,12 The vascular activity of HEETA requires further investigation. Therefore, vascular activity of the AA metabolites from 15-LO-1 cannot be extrapolated to all arteries.

Because 15-LO-1 is expressed in the endothelium and 15-LO-1 metabolites relax arteries, the effect of modulating 15-LO-1 expression and the resulting alteration of blood pressure regulation are of interest. Therefore, we overexpressed 15-LO-1 in rabbits in vivo and analyzed its effect on basal hemodynamics, as well as responses to ACH both in vivo and in vitro.

Methods

Rabbits and Arterial Tissue

Animal protocols were approved by the animal care committee of the Medical College of Wisconsin, and procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (1996). Age (8 weeks) -matched New Zealand White rabbits (Kuiper Rabbit Ranch) maintained on normal chow were injected with 2 x 10⁶ plaque-forming units of adenovirus in 0.15 mL of 0.9% NaCl. Adenovirus contained either cDNA for human 15-LO-1 (Ad-15-LO-1) or β-galactosidase (Ad-β-Gal). 9,13,14 After adenoviral injection, rabbits were kept in a biosafety area for a period of 40 hours, used for the in vivo experiments, and then euthanized with a pentobarbital overdose. Tissues were removed for in vitro experiments.

Immunoblotting, Immunohistochemistry, Metabolism of 14C-AA, and Isometric Tension

The methods for immunoblotting, 9 immunohistochemistry, 15,16 14C-AA metabolism studies, 2 and isometric tension recording 9 have been published previously. For details see the Methods section of the online data supplement at http://hyper.ahajournals.org.

Mean Arterial Pressure and Measurement of ACH Responses

Age matched (weight: 1.8±0.1 kg) adenoviral-infected or normal rabbits were anesthetized with 20 mg/kg of pentobarbital IV through a marginal ear vein. Anesthesia was immediately followed by endotracheal intubation and artificial ventilation. A catheter was placed into the right carotid artery and connected to a pressure transducer (model MS-01699, ADInstruments). Mean arterial pressure (MAP) was recorded using Mac-Laboratory Chart 7.0 software with a PowerLab amplifier and A/D converter. A catheter was placed in the right jugular vein for drug administration. After these procedures, rabbits were stabilized for 30 minutes. ACH (0.4 to 4000.0 ng/kg, IV) was administered, and change in MAP was recorded. For prostaglandin- and NO-independent ACH responses, rabbits were given indomethacin (INDO; 6 mg/kg, IP) and Nω-nitro-l-arginine methyl ester (l-NNAME; 20 and 5 mg/kg per hour, IV) and equilibrated for 30 minutes. All of the drugs except INDO were dissolved in normal saline and injected in a volume of 0.1 mL followed by 0.1 mL of saline. INDO was suspended in normal saline for intraperitoneal injection.

Statistical Analysis

The experimental data are expressed as means±SEMs. Values in all of the groups were analyzed by a repeated-measure 2-way ANOVA that was performed to compare band densities for immunoblots, percentage of counts per minute (CPM) per milligram of tissue for enzymatic assays, and ACH responses in vivo and vitro. Bonferroni posttest was performed to compare ACH responses between 2 groups. MAP and heart rate (HR) values were analyzed using unpaired Student t test. Values were considered significant at P<0.05 or smaller.

Results

Immunoblots

Expression of 15-LO-1 was analyzed by Western blots in aorta, MA, and liver from the untreated (normal), Ad-β-Gal–infected, and Ad-15-LO-1–infected rabbits (Figure 1). A 75-kDa immunoreactive band comigrating with standard rabbit reticulocyte 15-1ipoxygenase was observed only in the liver and MA lysates from Ad-15-LO-1–infected rabbits but not in lysates from normal (Figure 1A) or Ad-β-Gal–infected rabbits (Figure 1B). However, increasing the exposure time of the photographic film to the gel revealed a 75-kD band in aorta and MA lysates from normal and Ad-β-Gal–infected rabbits (Figure S1, available online at http://hyper.ahajournals.org). Expression of 15-LO-1 in the MA and liver of Ad-15-LO-1–infected rabbits increased 3.7- and 10.1-fold compared with MA and liver from normal rabbits and 6.7- and 18.2-fold compared with MA and liver from Ad-β-Gal–infected rabbits, respectively (Figure 1C). Ad-β-Gal infection did not significantly alter 15-LO-1 expression in aorta or MAs compared with normal rabbits.

Immunohistochemistry

Immunofluorescence was performed to determine the location of 15-LO-1 in MAs (Figure 2) from normal, as well as Ad-15-LO-1–infected, rabbits. General appearance of the tunica intima, media, and adventitia were unaltered in the arteries obtained from adenovirus-infected rabbits. Fluorescence from MA sections incubated without antibodies or with secondary antibodies only was negligible (Figure 2A and 2E). The fluorescence signal for 15-LO-1 was observed in the endothelium of MAs (Figure 2B and 2F). The presence of endothelium in MAs was confirmed by platelet endothelial cell adhesion molecule (PECAM) staining (Figure 2C and 2G). The fluorescence signal for 15-LO-1 in the endothelium of normal MAs (Figure 2B) was weaker than Ad-15-LO-1–
infected MAs (Figure 2F). This expression is also limited to the endothelium only, as confirmed by PECAM staining. Adjacent layers of cells, shown by nuclear staining with 4',6-diamidino-2-phenylindole (Figure 2D and 2H), did not express 15-LO-1.

Similarly, 15-LO-1 and PECAM were stained in aortic sections from Ad-15-LO-1–infected or normal rabbits (Figure S2). A weak 15-LO-1 fluorescence signal was observed in the aorta from normal (Figure S2B) and from Ad-15-LO-1–infected (Figure S2F) rabbits. PECAM expression demonstrated the presence of an intact endothelium in normal (Figure S2C) and in Ad-15-LO-1–infected rabbit aorta (Figure S2G). The layers of cells adjacent to endothelium (Figure S2D and S2H) were devoid of 15-LO-1 expression.

**Metabolism of 14C-AA**

Enzymatic activity of the 15-LO-1 in aorta and MA of adenovirus-infected or normal rabbits was determined by analyzing 14C-AA metabolism. In the presence of INDO, MA from normal (Figure 3A) and Ad-15-LO-1 (Figure 3B)–infected rabbits metabolized 14C-AA to 14C-THETA, 14C-HEETA, and 14C-hydroxyeicosatetraenoic acid (HETE) produced per milligram of MA is shown in Figure 3D. 14C-THETA synthesis was similar in MAs from normal and Ad-β-Gal–infected rabbits. However, 14C-THETA synthesis increased 2.1-fold in MAs from Ad-15-LO-1–infected rabbits compared with MAs from normal rabbits. Similarly, 14C-HEETA synthesis increased 1.5-fold in MAs from Ad-15-LO-1–infected rabbits (2.2±0.4%) compared with MAs from normal (1.4±0.1%) or Ad-β-Gal–infected (1.2%) rabbits. 14C-HETE synthesis increased 2.7-fold in MA from Ad-15-LO-1–infected rabbits compared with MAs from normal rabbits.

BW755C significantly reduced 14C-THETA, 14C-HEETA, and 14C-HETE synthesis. Because tissues for these studies were obtained from the animals that had undergone the surgical processes, a surgical control was also prepared and consisted of MAs from rabbits that had undergone the same surgical process but were not infected with adenovirus. This group of rabbits was called the “surgical control.” In this surgical control, 14C-THETA, 14C-HEETA, and 14C-HETE synthesis were similar to MAs from normal rabbits (data not shown).

14C-AA metabolism in aorta from normal, Ad-15-LO-1–infected, Ad-β-Gal–infected, and surgical control rabbits was also analyzed (Figure S3). Aorta from normal (Figure S3A) and Ad-15-LO-1–infected rabbits (Figure S3B) synthesized small amounts of 14C-THETA, 14C-HEETA, and 14C-HETE from 14C-AA. BW755C further reduced the synthesis of these metabolites (Figure S3C). Metabolite synthesis was similar in aortas from all of the rabbit groups (Figure S3D).

**Isometric Tension Recording in Arteries**

Prostaglandin (PG) and NO-independent relaxations to ACH in MAs or aorta from the adenoviral-infected or normal rabbits were determined. In the presence of INDO and L-NAME, ACH caused concentration-dependent relaxations in MAs and aorta from adenovirus-infected or noninfected rabbits (Figure 4). In MAs from Ad-15-LO-1–infected rabbits, ACH (3×10⁻⁷ mol/L) caused greater maximal relaxations (97.0±1.7%) compared with MAs from normal (69.3±10.5%; Figure 4A), from Ad-β-Gal–infected (68.3±7.7%; Figure 4B), or from surgical control rabbits (54.8±12.8%). The ACH relaxations in MAs from Ad-15-LO-1–infected rabbits were eliminated by the endothelium.
LO-1– or Ad-15-LO-1–infected rabbits. Basal MAP and HR were similar in Ad-15-LO-1–infected rabbits. BW755C-treated MAs from Ad-15-LO-1–infected rabbits relaxations were determined in the presence of BW755C (5\(\times\)10\(^{-7}\) mol/L) and relaxations were determined in the presence of BW755C (5\(\times\)10\(^{-7}\) mol/L). D, Relaxations were determined in aortas from normal or Ad-15-LO-1–infected rabbits. Data are expressed as percentage of relaxation. Each value represents mean\(\pm\)SEM (**P<0.0001; ***P<0.0001). n indicates the number of arterial rings tested. Normal (○), Ad-15-LO-1–infected (□), Ad-β-Gal–infected (●), endothelium-denuded MA from Ad-15-LO-1–infected (□), and BW755C-treated MA from Ad-15-LO-1–infected (▲) rabbits.

Figure 4. Effects of 15-LO-1 overexpression on vascular activity of rabbit arteries. MAs or aortic rings were pretreated with indomethacin (10\(^{-6}\) mol/L) and L-nitroarginine (3\(\times\)10\(^{-7}\) mol/L), precontracted with phenylephrine (10\(^{-4}\) to 10\(^{-5}\) mol/L), and relaxation to cumulative concentrations of ACH were determined. Relaxations were determined in MAs from normal and Ad-15-LO-1–infected rabbits. A, MAs from Ad-15-LO-1–infected and Ad-β-Gal–infected rabbits. B, Endothelium-denuded MAs from Ad-15-LO-1–infected rabbits. C, In MAs from Ad-15-LO-1–infected rabbits, relaxations were determined in the presence of BW755C (5\(\times\)10\(^{-7}\) mol/L). D, Relaxations were determined in aorta from normal or Ad-15-LO-1–infected rabbits. Data are expressed as percentage of relaxation. Each value represents mean\(\pm\)SEM (**P<0.0001; ***P<0.0001). n indicates the number of arterial rings tested. Normal (○), Ad-15-LO-1–infected (□), Ad-β-Gal–infected (●), endothelium-denuded MA from Ad-15-LO-1–infected (□), and BW755C-treated MA from Ad-15-LO-1–infected (▲) rabbits.

Basal Hemodynamics and ACH Responses In Vivo

After the adenoviral treatment, no hemodynamic changes were observed in either of the Ad-15-LO-1– or Ad-β-Gal–infected rabbits. Basal MAP and HR were similar in Ad-15-LO-1– or Ad-β-Gal–infected and normal rabbits (Table). ACH decreased MAP transiently in a dose-dependent manner with the maximum decrease at 4000 ng/kg without affecting the basal HR (Figure 5 and Table) in normal, Ad-15-LO-1–infected, or Ad-β-Gal–infected rabbits. Maximal MAP decrease to ACH was −31±3% and −38±2% in normal and Ad-β-Gal–infected rabbits, respectively, and −52±2% in Ad-15-LO-1–infected rabbits (P<0.001; Figure 5A). INDO and l-NAME treatment did not alter MAP and HR from the basal values in any rabbit group after 30 minutes of equilibration time (Table). ACH produced a maximum MAP decrease at 4000 ng/kg without affecting the HR in these rabbits. The maximum MAP decrease in the presence of INDO and l-NAME in normal rabbits was −25±5%, −30±3% in Ad-β-Gal–infected rabbits, and −47±2% in Ad-15-LO-1–infected rabbits (P<0.001; Figure 5B).

Discussion

Previously, we reported that 15-LO-1 can be overexpressed in vitro by adenoviral infection in rabbit aorta.9 This overexpressed 15-LO-1 was functionally active and enhanced AA metabolites on the vasculature and blood pressure regulation.15-LO-1 and determined the integrated effect of 15-LO-1 expression to each dose of ACH was determined. B, Rabbits were treated with l-NAME (20 mg/kg and 5 mg/kg per hour) and INDO (6 mg/kg), and ACH responses were repeated. Decreases in MAP are expressed as the percentage decrease from baseline. Each value represents mean\(\pm\)SEM. MAP changes to ACH in Ad-15-LO-1–infected rabbits are significantly different from normal or Ad-β-Gal–infected rabbits (P<0.05, **P<0.001, ***P<0.0001). Normal (○), Ad-15-LO-1–infected (□), and Ad-β-Gal–infected (●) rabbits.

Figure 5. Effect of ACH on MAP in rabbits. Rabbits were anesthetized, and doses of ACH were injected intravenously in increasing concentrations. A, The percentage decrease in MAP to each dose of ACH was determined. B, Rabbits were treated with l-NAME (20 mg/kg and 5 mg/kg per hour) and INDO (6 mg/kg), and ACH responses were repeated. Decreases in MAP are expressed as the percentage decrease from baseline. Each value represents mean\(\pm\)SEM. MAP changes to ACH in Ad-15-LO-1–infected rabbits are significantly different from normal or Ad-β-Gal–infected rabbits (P<0.05, **P<0.001, ***P<0.0001). Normal (○), Ad-15-LO-1–infected (□), and Ad-β-Gal–infected (●) rabbits.

Table. MAP and HR in Rabbits: Effects of l-NAME, INDO, and ACH

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Normal</th>
<th>Ad-15-LO-1 Infected</th>
<th>Ad-β-Gal Infected</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAP, mm Hg</td>
<td>83±2</td>
<td>87±3</td>
<td>85±3</td>
</tr>
<tr>
<td>HR, bpm</td>
<td>247±23</td>
<td>238±18</td>
<td>235±17</td>
</tr>
<tr>
<td>HR at 4000 ng/kg of ACH, bpm</td>
<td>243±16</td>
<td>248±23</td>
<td>233±27</td>
</tr>
</tbody>
</table>

Each value represents mean\(\pm\)SEM.
dose (2×10⁶ plaque-forming units) was delivered by intra-coronary infusion into rabbits without evidence of an inflammatory response or coronary arterial necrosis. At 40 hours after injection, the MA, but not aorta, showed higher expression of 15-LO-1 compared with the arteries from normal rabbits. Liver from Ad-15-LO-1–infected rabbits also expressed high amounts of 15-LO-1, because it has sinusoids and small capillaries, which absorbed Ad-15-LO-1. Ad-β-Gal infection did not alter the expression of 15-LO-1 in aorta, MA, or liver, confirming that the increase in expression was not because of only viral infection. Similarly, the immunofluorescence signal was observed in MA but not in aorta from Ad-15-LO-1–infected rabbits. In addition, the fluorescence signal for 15-LO-1 was higher in the MA from Ad-15-LO-1–infected rabbits compared with MA from normal rabbits. Ad-15-LO-1 expressed 15-LO-1 exclusively in the endothelium of the arteries. This is consistent with the report by Gruchala et al., where an adenovirus transduced exclusively endothelial cells in rabbit arteries. Unlike MA, the 15-LO-1 expression in aorta of Ad-15-LO-1–infected rabbits was not changed. The unsuccessful adenoviral infection in aorta was not because of damage to the endothelium, because PECAM staining confirmed the presence of intact endothelium. Successful adenoviral infection depends on the presence of adenovirus receptors, secondary receptors, nuclear transport, and decay of the virus in the endosomes. Any differences in these aspects between mesenteric and aortic endothelium might cause differential infection.

Rabbit MA synthesized THETA, HEETA, and HETE; however, the synthesis increased significantly in MA from Ad-15-LO-1–infected rabbits. This validates that overexpressed 15-LO-1 increases the metabolism of AA. On the other hand, only small amounts of THETA, HEETA, or HETE were synthesized in aorta from Ad-15-LO-1–infected rabbits, which correlates with the failure of Ad-15-LO-1 treatment to increase 15-LO-1 expression. These enzymatic assays confirm that the synthesis of THETA, HEETA, and HETE is increased only in tissue where the expression of 15-LO-1 was increased and is consistent with our previous in vitro results. Furthermore, the lipoxygenase inhibitor, BW755C, inhibited the synthesis of THETA, HEETA, and HETE in the MA from Ad-15-LO-1–infected rabbits confirming the role of 15-LO-1 in the synthesis of these metabolites.

Vasoactivity of the arteries was determined by measuring the NO- and PG-independent relaxations to ACH in preconstricted MA and aorta from infected or noninfected rabbits. The NO- and PG-independent ACH relaxations were greater in the MA from Ad-15-LO-1–infected rabbits compared with the MA from normal or Ad-β-Gal–infected rabbits. On the other hand, NO- and PG-independent ACH relaxation was not increased in aorta from Ad-15-LO-1–infected rabbits, because the 15-LO-1 expression was not increased with Ad-15-LO-1 infection. It is noteworthy that the contractile responses to KCl or phenylephrine in MA or aortas from normal, Ad-15-LO-1–infected, or Ad-β-Gal–infected rabbits were not different. These results substantiate that 15-LO-1 is sufficient to increase the vasorelaxations to ACH. These NO- and PG-independent ACH relaxations were mediated by 15-LO-1 metabolites, because BW755C reduced the relaxation in MA from Ad-15-LO-1–infected rabbits.

The integrated effect of 15-LO-1 overexpression in the vasculature was determined by measuring the MAP and HR in infected or noninfected rabbits. Despite the synthesis of higher quantities of the dilator THETA in arteries of Ad-15-LO-1–infected rabbits, basal MAP in Ad-15-LO-1–infected rabbits was not different than MAP of normal or Ad-β-Gal–infected rabbits. This may be because other regulatory mechanisms maintain the MAP at the basal levels. Although the doses of INDO and l-NAME used in the present study have been shown to produce maximal inhibition of COX and endothelial NO synthase, respectively, they did not alter basal MAP in normal, Ad-β-Gal–, or Ad-15-LO-1–infected rabbits after 30 minutes of equilibration time. We did observe a transient increase in MAP with INDO and l-NAME that returned to the basal levels in 6 to 10 minutes. These results are consistent with observations by Rajapakse et al., who reported a basal MAP of 74 ± 1 mm Hg in rabbits. They stated that with vehicle or nitro-arginine treatment, "there were no systemic differences in these levels." Moreover, Oliver et al. reported that COX inhibition by ibuprofen increased the MAP only transiently for 10 minutes. This finding indicates that, after COX and endothelial NO synthase inhibition, a compensatory mechanism brings the raised MAP to basal levels in 6 to 10 minutes. Because EDHFs are released by the endothelium on stimulation, ACH caused hypotensive responses in rabbits, rats, and humans in the presence of INDO and l-NAME. These responses were sensitive to the calcium-dependent potassium channel inhibitors apamin and charybdotoxin. Therefore, these responses were because of an EDHF. THETAs are also EDHFs that are released on ACH stimulation, activate the small conductance calcium-dependent potassium channels, and hyperpolarize smooth muscle cells causing relaxations. The maximum ACH-induced decrease in MAP was greater in Ad-15-LO-1–infected rabbits than normal or Ad-β-Gal–infected rabbits. This indicates that 15-LO-1 overexpression contributed to ACH-induced hypotension, and this effect was not because of the viral infection. NO- and PG-independent ACH-induced hypotension was greater in Ad-15-LO-1–infected rabbits than normal or Ad-β-Gal–infected rabbits. Thus, these NO- and PG-independent ACH responses were because of 15-LO-1 overexpression in the vascular endothelium.

In summary, overexpression of 15-LO-1 increases the synthesis of THETA, HEETA, and HETE; increases the ACH relaxations in vitro; and enhances ACH-induced hypotensive responses in vivo in rabbits. These findings demonstrate the integrated effect of 15-LO-1 metabolites of AA in regulating vasoactivity in rabbits.

**Perspectives**

THETA and HEETA are EDHFs that relax rabbit arteries. They are also synthesized in rat, mouse, guinea pig, and human arteries (unpublished data). Although direct activity of biological THETA has only been demonstrated in rabbit arteries, overexpression of 15-LO-1 demonstrated the role of THETA in regulating vasorelaxations. Our in vivo findings are important during conditions where 15-LO-1 expression increases in arteries. Indeed, hypercholesterolemia increases 15-LO-1 expression in the atherosclerotic lesions.
more, hypercholesterolemia without atherosclerosis5 and treatment with interleukin-4 or interleukin-13 increased THETA and HEETA synthesis.15,28 Alternatively, overexpression of 15-LO-1 could reverse endothelial dysfunction. Thus, 15-LO-1 may play an indirect role in regulating vascular relaxations and blood pressure through synthesis of THETA and HEETA from AA under such pathological conditions.

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Disclosures
None.

References
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Methods

Immunoblotting

Protein lysates (30 µg protein) were loaded in each lane and separated by SDS gel electrophoresis and transferred to nitrocellulose membranes as described previously (1). Membranes were exposed to a rabbit antibody against human 15-LO-1 (1) (dilution 1:5000 in TBS) for 1 h at room temperature, rinsed with TBS buffer containing 0.1% Tween-20 and incubated with a 1:5000 diluted goat anti-rabbit IgG (horseradish peroxidase conjugated, Zymed). Membranes were re-probed with mouse anti-β-actin as a loading control. Immunoreactive bands were identified and densitometric analysis was performed.

Immunohistochemistry

Rabbit aortic or mesenteric arterial segments were fixed in 4% paraformaldehyde and cut into sections as described previously (2, 3). Sections were incubated with mouse anti-human platelet endothelial cell adhesion molecule (PECAM) (kindly provided by Dr. Peter Newman, Blood Center for Southeastern Wisconsin, Milwaukee, WI) and rabbit anti-human 15-LO-1 diluted 1:500 in 0.2% Triton X-100 containing 1% normal goat serum (1). Sections were incubated for 1 h at room temperature, rinsed, and incubated with 1:500 anti-mouse Alexa-Fluor 594 and anti-rabbit FITC labeled secondary antibodies (Molecular Probes) for 1 h at 25°C. Sections were mounted with media
containing 1% 4,6-diamidino-2-phenylindole (DAPI) and protected by a glass coverslip. Fluorescent images were captured (200X magnification) using Nikon Eclipse E600 microscope and Spot Advanced software.

**Metabolism of $^{14}$C-AA**

Arteries were dissected, cleaned and cut into 2-3 mm rings in N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES) buffer (mmol/L): 10 HEPES, 150 NaCl, 5 KCl, 2 CaCl$_2$, 1 MgCl$_2$, 6 glucose, pH 7.4. Rings were incubated at 37°C with indomethacin (INDO) (10$^{-5}$ mol/L) (Sigma, MO) or with INDO and BW755C (5 X 10$^{-5}$ mol/L) (Burroughs Wellcome, Sandwich, England) in 5 ml HEPES for 10 min and then [14C]-AA (0.5 µCi, 10$^{-7}$ mol/L) was added. After 5 min, A23187 (10$^{-5}$ mol/L) (Sigma, MO) was added. After 15 min, the reaction was stopped with ethanol (15% final concentration), and the samples were extracted using Bond Elute octadecylsilyl columns (4). The extracts were analyzed by reverse phase high-pressure liquid chromatography (HPLC) using solvent system I and a Nucleosil C-18 (5µ, 4.6 x 250 mm) column. System I consisted of a 40 min linear gradient (flow rate=1 ml/min) from 50% solvent B (acetonitrile with 0.1% glacial acetic acid) in solvent A (deionized water) to 100% solvent B. Column effluent was collected in 0.2 ml fractions and the radioactivity was determined.

**Isometric tension in vessels**

Arterial rings were suspended in a 6 ml tissue bath with Krebs bicarbonate buffer (concentration in mmol/L, 119 NaCl, 4.7 KCl, 2.5 CaCl$_2$, 1.17 MgSO$_4$, 25 NaHCO$_3$, 1.18
KH$_2$PO$_4$, 0.027 EDTA, 5.5 glucose, at 37°C and bubbled with 95% O$_2$ and 5% CO$_2$. Isometric tension was measured with force-displacement transducers (Danish Myo Technology). The vessels were gradually adjusted to resting tension (aorta; 2 gm and mesenteric artery; 0.15 gm), allowed to equilibrate for 30 min and tested for the maximum response with 60 mmol/L KCl as described previously (5). The vessels were treated with INDO (10$^{-5}$ mol/L) and L-nitro-arginine (LNA) (Sigma, MO) (3 x 10$^{-5}$ mol/L) and BW755C (5 X 10$^{-5}$ mol/L) for 10 min and then contracted by phenylephrine (approx. 10$^{-7}$ mol/L) to 50-60% of the maximal KCl contraction. Cumulative concentrations of ACH (Sigma, MO) (10$^{-9}$-10$^{-5}$ mol/L) were added to the bath and changes in isomeric tension were measured. Vasorelaxation was expressed as percentage of maximum precontraction.
References to online supplement


Legends to Supplemental Figures

**Figure S1:** Western immunoblot for 15-LO-1 expression in homogenates from mesenteric arteries (A) and aortas (B) from normal, Ad-β-Gal infected, and Ad-15-LO-1 infected rabbits. β-actin expression is also shown as loading control. Lanes: 1; Control lysates, 2; Ad--β-Gal infected lysates, 3; Ad-15-LO-1 infected lysates.

**Figure S2:** Immunohistochemical localization of 15-LO-1 in aortas from normal or Ad-15-LO-1 infected rabbits. Histological sections were labeled with a human 15-LO-1 antibody. Endothelial cells and nuclei were identified by labeling with PECAM antibody and DAPI, respectively. 15-LO-1 and PECAM in the same section at 200X magnification is shown. Tissue sections incubated with vehicle only or with secondary antibodies only are also shown. The data shown is representative of samples taken from at least 3 different non-infected or infected rabbits.

**Figure S3:** Effects of 15-LO-1 on [14C]-AA metabolism in aorta from rabbits. Aortic rings dissected from normal (A) or Ad-15-LO-1 infected rabbits (B) were incubated with [14C]-AA in the presence of indomethacin (10⁻⁵ M). Aortas from Ad-15-LO-1 infected rabbits were incubated with [14C]-AA in the presence of indomethacin and BW755C (5 X 10⁻⁵ M) (C). The media was removed, extracted and the metabolites resolved by HPLC (system I). Migration times of known standards are indicated above the chromatogram in panel A. Percentage CPM of the metabolites synthesized per mg of tissue was measured (D) and expressed as mean±SEM.
Mesenteric arteries

1  2  3
75kDa

β-actin

Aortas

1  2  3
75kDa

β-actin

Figure S1
Figure S2
Figure S3