Adenoviral expression of 15-lipoxygenase-1 in rabbit aortic endothelium: role in arachidonic acid-induced relaxation

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Aggarwal NT, Holmes BB, Cui L, Viita H, Yla-Herttuala S, Campbell WB. Adenoviral expression of 15-lipoxygenase-1 in rabbit aortic endothelium: role in arachidonic acid-induced relaxation. Am J Physiol Heart Circ Physiol 292: H1033–H1041, 2007. First published October 13, 2006; doi:10.1152/ajpheart.00624.2006.—Endothelium-dependent vasorelaxation of the rabbit aorta is mediated by either nitric oxide (NO) or arachidonic acid (AA) metabolites from cyclooxygenase (COX) and 15-lipoxygenase (15-LO) pathways. 15-LO metabolites of AA, 11,12,15-trihydroxyeicosatrienoic acid (THETA), and 11,12-epoxyeicosatrienoic acid (HEETA) cause concentration-dependent relaxation. We tested the hypothesis that in the 15-LO pathway of AA metabolism, 15-LO-1 is sufficient and is the rate-limiting step in inducing relaxations in rabbit aorta. Aorta and rabbit aortic endothelial cells were treated with adenoviruses containing human 15-LO-1 cDNA (Ad-15-LO-1) or β-galactosidase (Ad-β-Gal). Ad-15-LO-1-transduction increased the expression of a 75-kDa protein corresponding to 15-LO-1, detected by immunoblotting with an anti-human15-LO-1 antibody, and increased the production of HEETA and THETA from [14C]AA. Immunohistochemical studies on Ad-15-LO-1-transduced rabbit aorta showed the presence of 15-LO-1 in endothelial cells. Ad-15-LO-1-treated aortic rings showed enhanced relaxation to AA (max 31.7 ± 2.8% compared with Ad-β-Gal-treated (max 12.7 ± 3.2%) or control nontreated rings (max 13.1 ± 1.6%) (P < 0.01). The relaxations in Ad-15-LO-1-treated aorta were blocked by the 15-LO inhibitor cinnamyl-3,4-dihydroxy-a-cyanoaminacetaldehyde. Overexpression of 15-LO-1 in the rabbit aortic endothelium is sufficient to increase the production of the vasodilatory HEETA and THETA and enhance the relaxations to AA. This confirms the role of HEETA and THETA as endothelium-derived relaxing factors.

Endothelial cells (ECs) regulate the vascular tone through the release of soluble mediators that cause vasodilation (10, 15, 27). These mediators include prostaglandin (PG) I2, nitric oxide (NO), and endothelium-derived hyperpolarizing factors (EDHFs). The vascular endothelium metabolizes arachidonic acid (AA) by the cyclooxygenase (COX), lipoxigenase (LO), and cytochrome P-450 (CYP) pathways (36, 37). Metabolites of these pathways mediate the endothelium-dependent relaxation to AA and other agonists such as acetylcholine (ACH), bradykinin, and shear stress (13, 16, 30, 32, 44, 53). Metabolites of the 15-LO and the CYP pathways function as EDHFs in the rabbit aorta and bovine and porcine coronary arteries, respectively (7, 9, 12, 17, 51, 52). Rabbit aorta relaxes to ACh and AA in the presence of COX and endothelial NO synthase (eNOS) inhibitors (30, 53). This indicates that besides PGs and NO, there are other metabolites of AA that mediate aortic relaxations. These non-PGs, non-NO-mediated relaxations to ACh and AA are blocked by LO inhibitors such as nordihydroguaiaretic acid, cinnamyl-3,4-dihydroxy-a-cyanoaminacetaldehyde (CDC), and ebselen (9, 32). Thus a LO metabolite of AA mediates these relaxations.

15-LO-1 was first identified and cloned from rabbit reticulocytes and subsequently from human leukocytes and reticulocytes (41, 42, 48). The 15-LO-1 is predominantly localized in the cytosol (5) and is implicated in many physiological and pathological conditions. 15-LO-1 is also present in vascular endothelium and selectively metabolizes free fatty acids and prefers AA (3, 4, 45). In the vascular endothelium, 15-LO-1 metabolizes AA to 15-hydroperoxyeicosatetraenoic acid (15-HPETE) and to a lesser extent 12-HPETE (33, 37). 15-HPETE and 12-HPETE are further reduced to 15-hydroxyeicosatetraenoic acid (15-HETE) and 12-HETE via glutathione peroxidase. 15-HPETE is also converted to 11-hydroxy-14,15-epoxyeicosatrienoic acid and 15-hydroxy-11,12-epoxyeicosatrienoic acid (collectively called as HEETAs) via a hydroperoxide isomerase. HEETAs are further converted to 11,14,15-trihydroxyeicosatrienoic acid and 11,12,15-trihydroxyeicosatrienoic acid (collectively called as THETA) by epoxide hydrase (31, 33). Although HETEs are the major products of AA metabolism by 15-LO-1, neither 15-HETE nor 12-HETE have relaxing effects on the preconstricted rabbit aorta (14, 31, 32). Similarly, 12-HETE failed to relax U-46619-constricted canine and porcine epicardial coronary arteries (38, 55). On the other hand, the HEETAs and THETAs relax preconstricted rabbit aorta by activating calcium-dependent potassium channels (KCa), thereby hyperpolarizing the smooth muscles cells (17, 33). Of the two THETA regioisomers synthesized by the rabbit aorta, only 11,12,15-THETA causes relaxation (9, 33). Thus modulation of the 15-LO-1 pathway of AA metabolism and the synthesis of vasoactive LO metabolites in aortic endothelium might represent a mechanism for regulating vascular activity. Expression of 15-LO-1 is regulated by both transcription and translation (22). For example, cytokines such as interleukins-4 and -13 (IL-4 and -13) induce the expression of 15-LO-1 (6, 11, 28). However, cytokines also have effects on the other enzymes such as COX, eNOS, and glutathione peroxidase (2, 18, 39, 40).

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By specifically overexpressing 15-LO-1, we tested the hypothesis that 15-LO-1 is sufficient to increase the aortic relaxation response to AA and is the rate-limiting step in production of vasoactive metabolites. 15-LO is expressed in various animals and in human tissues including the vascular endothelium (22, 41, 43, 46), under normal physiological conditions. Thus AA metabolites of the LO pathway may play a significant role in regulating the vascular tone. Alterations in 15-LO occur in early stages of atherosclerosis (21, 50), in hypercholesterolemia (31), or with exposure to cytokines (6, 11, 28) and may enhance the contributions of this pathway to the regulation of vascular tone. We used rabbit aortic endothelial cells (RAECs) and rabbit aorta as a model to test whether enhanced expression of 15-LO-1 increases production of vasodilatory eicosanoids and enhanced relaxations to AA. Expression of 15-LO-1 was enhanced by treatment with an adenovirus containing the human 15-LO-1 cDNA (Ad-15-LO-1). Overexpression of 15-LO-1 in RAEC and rabbit aorta increases the production of 15-HETE, THETA, and HEETA. These metabolites increase AA-mediated relaxation. AA-induced relaxations in Ad-15-LO-1-transduced aorta were blocked by inhibition of 15-LO-1.

The sections were fixed again in 4% paraformaldehyde for 30 min and permeabilized by incubating the sections with 0.2% Triton X-100 for 15 min (47, 53). Sections were incubated with either anti-α-actin (Sigma), anti-platelet endothelial cell adhesion molecule-1 (PECAM, kindly provided by Dr. Peter Newman, Blood Center for Southeastern Wisconsin, Milwaukee, WI), or anti-human 15-LO-diluted 1:500 in 0.2% Triton X-100 containing 1% normal goat serum. For the anti-human 15-LO antibody, a 15-LO-specific peptide (YLRLPSRVENSVAI) was synthesized and coupled to keyhole limpet hemocyanin. The conjugate (0.25 mg) was mixed in Freund’s adjuvant and injected at multiple intradermal sites in rabbits, and then blood was collected 7 days later. The serum was tested by ELISA using the peptide conjugated to bovine serum albumin and immunoblotting using the purified 15-LO-1 (Caymen Chemical). The sections were incubated 1 h at room temperature, rinsed, and incubated with 1:500 anti-mouse or anti-rabbit Alexa-Fluor 594-labeled secondary antibodies (Molecular Probes) for 1 h at 25°C. The slides were then rinsed and incubated for 30 min with mounting media containing 1% 4,6-diamidino-2-phenylindole and protected by a glass coverslip. Fluorescent images were captured using a cooled (×200 magnified) charged couple device camera or with a Nikon Eclipse E600 microscope and Spot Advanced software.

Metabolism of [14C]AA. Control and treated RAECs and aortic rings were placed into 5 ml of HEPES buffer (in mM): 10 HEPES, 150 NaCl, 5 KCl, 2 CaCl2, 1 MgCl2, and 6 glucose; pH = 7.4. RAECs and aortic rings were incubated in this buffer at 37°C with indomethacin (10−5 M) for 10 min, and then [14C]AA (0.5 μCi, 10−5 M) was added. After 5 min, A23187 (10−5 M) was added. After 10 min, the reaction was stopped with ethanol (15% final concentration), and the samples were extracted using Bond Elute octadecylsilyl columns (33, 35). The extracts were analyzed by reverse-phase high-pressure liquid chromatography (HPLC) using solvent system I and a Nucleosil C-18 (5 μm, 4.6 × 250 mm) column (33). 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. The column effluent was collected in 0.2-ml fractions, and the radioactivity was determined. The fractions corresponding to THETAs (2–10 min) were collected, acidified, extracted with cyclohexane-ethyl acetate (50:50, vol/vol), and analyzed with LC-tandem mass spectrometry (LC-MS/MS) using a Waters-MicroMass Quattro tandem quadrupole mass analyzer. The pooled fraction was chromatographed using solvent system II and a Kromasil C-18 (5 μm, 4.6 × 250 mm) column (29). System II consisted of a 15-min linear gradient (flow rate = 0.2 ml/min) from 40% solvent B (acetonitrile with 0.005% glacial acetic acid) in solvent A (deionized water with 0.005% glacial acetic acid) to 65% B in A followed by a linear gradient over 10 min to 100% solvent B. The capillary voltage was 3.5 kV, cone voltage 25 V, source temperature 130°C, desolvation temperature 350°C, and gas flow 50 l/h. Ions were monitored in the negative ion mode.

Western immunoblotting assay. Aortic rings were carefully cleaned of adherent connective tissue, frozen in liquid N2, and crushed into powder with mortar and pestle maintained at liquid N2 temperature. The powdered aorta or RAECs were homogenized in the lysis buffer (in mM): 50 HEPES, 150 NaCl, 1.5 MgCl2, and 10% glycerol, 1% Triton X-100, and protease inhibitor cocktail (Roche Molecular Biochemicals, Germany). These homogenates were further incubated on ice in the flasks for 10 min in lysis buffer. The homogenates were vortexed and sonicated five times for 30 s each. The homogenates were then centrifuged for 10 min at 2,000 g. The supernatant was stored at −80°C. Equal amounts of supernatant protein (30 × 10−6 g) were loaded in each lane and separated by SDS-PAGE electrophoresis using a 10% resolving gel and 4% stacking gel. Protein was then transferred to nitrocellulose membranes. Nonspecific binding was blocked with Tris-buffered saline (TBS) buffer containing 20 mM Tris base, 150 mM NaCl, 0.1% sodium azide, and 3% bovine serum albumin overnight at 4°C. Rabbit antibody against human 15-LO was used as primary antibody (see
above). The membranes were exposed to primary antibody (dilution 1:2,000) in blocking buffer for 1 h at room temperature and rinsed with TBS buffer containing 0.1% Tween-20. The membrane was then incubated with 1:5,000 horseradish peroxidase-conjugated goat anti-rabbit IgG for 1 h at room temperature and washed with TBS buffer. Immunoreactive bands were identified using the Renaissance chemiluminescence detection kit and Kodak BioMax ML film. To determine the loading control, the membrane was washed with a solution of 100 mM 2-mercaptoethanol, 2% SDS, and 62.5 mM Tris-HCl. The protein bands were reprobed with mouse anti-β-actin antibody (dilution 1:2,000) as primary antibody and horseradish peroxidase-conjugated goat anti-mouse IgG (dilution 1:2,000) as secondary antibody.

Isometric tension in aortic rings. Aortic rings from the Ad-15-LO-1, Ad-β-Gal, or vehicle-treated groups were suspended in a 6-ml tissue bath with PSS at 37°C and bubbled with 95% O2–5% CO2 (33). Isometric tension was measured with force-displacement transducers (Danish MyoGraph) and recorded with a Macintosh computer and MacLab software (46). The vessels were gradually adjusted to 2-g resting tension and allowed to equilibrate for 1 h. The vessels were then tested for the maximum response with KCl as described previously (9). The aortic rings were treated with indomethacin (10⁻⁵ M) and nitro-L-arginine (3 × 10⁻⁴ M) for 10 min and then contracted by phenylephrine (10⁻⁷ M) to 50–60% of the maximal KCl contraction. In some studies, the aortic rings were pretreated with CDC (20 × 10⁻⁶ M). Cumulative concentrations of AA (10⁻⁷–10⁻⁴ M) were added to the bath, and changes in isometric tension were measured. Vasorelaxation was expressed as percentage of maximum precontraction. Statistical comparison of the data obtained from treated and control groups was performed using an ANOVA with P < 0.05 considered as statistically significant.

RESULTS

15-LO-1 overexpression by Ad-15-LO-1. 15-LO-1 expression in RAECs and aorta were determined by Western immunoblotting. A 75-kDa band corresponding to human 15-LO-1 was detected with an anti-human 15-LO-1 antibody (Fig. 1A). 15-LO-1 was not detected in untreated and Ad-β-Gal-treated cells under these conditions. The expression of 15-LO-1 increased with increasing amounts of Ad-15-LO-1 (1.2–3.6 × 10⁸ pfu/1.2 × 10⁶ cells in 10 ml) (Fig. 1B). When the aortic lumen was infected with either Ad-15-LO-1, Ad-β-Gal, or vehicle control for 12 h, the expression of human 15-LO-1 was increased in Ad-15-LO-1-treated aortas but was unaffected by vehicle treatment or Ad-β-Gal treatment (Fig. 1C). 15-LO expression was increased by 158 ± 5% when the aorta was transduced with 100 µl of Ad-15-LO-1 (2 × 10⁸ pfu/µl) of aorta (Fig. 1D).

Immunohistochemical localization of aortic 15-LO. Immunofluorescence was performed on rabbit aortas treated with vehicle or Ad-15-LO-1. Figure 2 represents three separate experiments, and only a portion of the aortic ring is shown to accommodate the ×200 magnification. The fluorescence microscope settings for magnification, exposure time, and gain were kept constant so fluorescence intensity could be compared. No fluorescence signal was observed for the endogenous 15-LO-1 expression in the nontransduced aortic sections (Fig. 2, top). Lack of fluorescence for 15-LO-1 in the rabbit aorta is in contrast with our immunoblots (Fig. 1C) and published results (46, 47). This is due to the low camera time exposure and gain that captures the overexpressed 15-LO-1 but does not reveal the endogenous levels of 15-LO-1. The vascular endothelium and smooth muscle cells were well preserved by fixation even after 12 h of incubations as indicated by PECAM-1 and α-actin immunostaining, respectively. No attached or resident blood or inflammatory cells were detected in the inner vascular wall. In Ad-15-LO-1–treated samples, 15-LO immunofluorescence was strongly associated with the vascular endothelium. Only weak autofluorescence of the tissue was observed when the primary anti-15-LO antibody was omitted.

AD-15-LO-1 transduction on AA metabolism. RAECs (1.2 × 10⁶) were incubated with vehicle control, Ad-β-Gal

![Fig. 1. Western immunoblot for human 15-lipoxygenase (15-LO-1) of cell lysates from rabbit aortic endothelial cells (RAECs) transduced either with adenovirus containing β-galactosidase (Ad-β-Gal) or Ad-15-LO-1 for 12 h. The concentration [plaque-forming units (pfu)/ml] of Ad-15-LO-1 and Ad-β-Gal was varied (A). 15-LO expression was corrected for α-actin (B). Western immunoblot for human 15-LO expression in tissue homogenate from rabbit aorta transduced with either Ad-β-Gal, Ad-15-LO-1, or buffer alone (control) (C). Approximately 100 µl of 2 × 10⁸ pfu/µl of virus/inch of aorta were used. 15-LO expression was normalized to β-actin (D).](http://ajpheart.physiology.org/)

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(2.4 × 10^8 pfu/10 ml), or Ad-15-LO-1 (2.4 × 10^8 pfu/10 ml) for 12 h, treated with indomethacin, and incubated with [14C]AA. Only small amounts of polar metabolites were observed when cells were incubated with vehicle or Ad-β-Gal (Fig. 3, A and B). The Ad-15-LO-1-transduced cells metabolized AA to metabolites that comigrated with THETA, HEETA, 15-HETE, and 12-HETE (Fig. 3C). Similarly, aorta metabolized AA to THETAs, HEETAs, 15-HETE, and 12-HETE (Fig. 4A). In Ad-15-LO-1-transduced aorta, there was an increase in peaks comigrating with THETA, HEETA, 15-HETE, and 12-HETE (Fig. 4B) compared with vehicle-treated aorta. Overall, the metabolism of AA in Ad-15-LO-1-transduced ECs is less than Ad-15-LO-1-transduced rabbit aorta. After 12 h of Ad-15-LO-1 infection, the aorta was denuded of the endothelium. In the absence of the endothelium, there was no THETA, HEETA, 15-HETE, or 12-HETE production (Fig. 4C). These data indicate that 15-LO was exclusively overexpressed in the endothelium and the endothelium is needed for AA metabolism.

The column fractions containing the THETAs were collected from the incubations with Ad-15-LO-1-transduced rabbit aorta. The column fractions representing THETA (2–10 min) were analyzed by LC-MS/MS using the system II (Table 1). Two major peaks were detected eluting at 10.2 and 10.8 min. The mass spectra of both peaks were similar with a major (M-H) ion of 353 m/z. The 10.2-min peak comigrated with 11,14,15-THETA and the 10.8-min peak with 11,12,15-THETA. The 353 ions for these two peaks were subjected to collision-induced dissociation and daughter ions analyzed by MS/MS (Fig. 5). The daughter ion spectrum of the 10.2-min peak was identical to synthetic 11,14,15-THETA with major ions as the following: 353 [M-H], 335, 317, 309, 289, 235, 197, 167, 99, 85, and 71 m/z (Fig. 5A). The daughter ion spectrum of the 10.8-min peak was identical to 11,12,15-THETA and had major ions of 353 [M-H], 335 [M-H-H2O], 197, 179, 167, 157, 139, 121, 85, and 71 m/z (Fig. 5B). The difference in the MS/MS spectra of the 353 ion is due to the favored cleavage between 14,15 (235 and 99 m/z) and 11,12 (197 and 139 m/z) position containing vic-diol groups. These data are consistent with the synthesis of 11,12,15- and 11,14,15-THETA by Ad-15-LO-1-transduced aorta.

**AA-induced vasorelaxation.** Because HEETA and THETA, but not HETEs (14, 31, 33, 38, 55), are involved in the regulation of vascular tone in the aorta, enhanced production of HEETAs and THETAs may increase vascular relaxation. Contractions to phenylephrine and KCl were not different among control, Ad-15-LO-1-transduced, and Ad-β-Gal-transduced aortas. AA caused a concentration-related relaxation in Ad-15-LO-1-transduced, Ad-β-Gal-transduced, and control vessels (Fig. 6A). AA-mediated relaxation was greatest in Ad-15-LO-1-treated rings. There was a significant (P < 0.0001) increase in the relaxations to AA in the Ad-15-LO-1 group compared with control.
with those in the Ad-β-Gal and control groups. The maximum relaxation in response to $3 \times 10^{-5}$ M AA with the Ad-15-LO-1-transduced aortic rings ($n = 9$) was $31.7 \pm 3.2\%$, which is significantly different ($P < 0.05$) from either control rings ($n = 9$) ($13.1 \pm 1.6\%$) or Ad-β-Gal-transduced rings ($n = 8$) ($12.7 \pm 3.2\%$) (Fig. 6A). Furthermore, the enhanced relaxations in Ad-15-LO-1-transduced aortic rings ($n = 11$) ($35.1 \pm 8.7\%$) were blocked by the LO inhibitor CDC ($n = 13$) ($-0.8 \pm 3.3\%$) (Fig. 6B). These data indicate that 15-LO is responsible for the AA-dependent relaxations in rabbit aorta.

Fig. 3. Effects of 15-LO-1 on $[^{14}C]$arachidonic acid (AA) metabolism in RAECs. ECs were incubated with either vehicle control (A), Ad-β-Gal (B), or Ad-15-LO-1 (C). After 12 h, ECs were incubated with $[^{14}C]$AA in the presence of indomethacin ($10^{-5}$ M). Media were removed and extracted, and the eicosanoids were resolved by HPLC (system I). Migration times of known standards are indicated above each chromatogram. These data are representative of 3 separate experiments on 3 different sets of cells. CPM, counts per minute; THETA, 11,12,15-trihydroxyeicosatrienoic acid; HEETA, 15-hydroxy-11,12-epoxyeicosatrienoic acid; 12- and 15-HETE, 12- and 15-hydroxyeicosatetraenoic acid, respectively.

Fig. 4. Effects of 15-LO-1 on $[^{14}C]$AA metabolism in rabbit aorta. Aortas were incubated for 12 h with either vehicle (A) or Ad-15-LO-1. In the Ad-15-LO-1 group, the endothelium was either left intact (B) or removed (C). Aortic segments were incubated with $[^{14}C]$AA in the presence of indomethacin ($10^{-5}$ M). The media were removed and extracted, and the metabolites were resolved by HPLC (system I). Migration times of known standards are indicated above each chromatogram. These data are representative of 3 sets of experiments on aortas from different groups of rabbits.
through the metabolism of AA to vasoactive HEETA and THETA.

DISCUSSION

Endothelial 15-LO metabolites of AA are important regulators of vascular tone (9, 33, 34, 45). In the rabbit aorta, the relaxations to ACh are reduced, but not blocked, by the combination of the COX inhibitor indomethacin and the eNOS inhibitor nitro-L-arginine. Thus NO and another factor contribute to the relaxations. These non-NO, non-PG mediated relaxations to ACh are inhibited by LO inhibitors nordihydroguaiaretic acid, CDC, and ebselen (9, 32). These data indicated that AA metabolites of LO pathway contribute to the non-PG, non-NO relaxations to ACh in rabbit aorta. These LO metabolites are products of 15-LO metabolism of AA in the rabbit aortic endothelium. AA is metabolized to 15-HPETE by 15-LO, which is then either reduced to 15-HETE by a peroxidase or converted to 11,12,15-HEETA and 11,14,15-HEETA by hydroperoxide isomerase (33, 34). HEETAs are then converted to THETAs by epoxide hydrolase. 15-LO-1 also synthesizes 12-HETE in fewer amounts than 15-HETE. Although 15-HETE constricts rat and rabbit pulmonary artery and canine sephenous vein (8, 25, 49), it is without effect on the rabbit aorta (14, 31, 32). Similarly, 12-HETE and 12-HPETE also have no effect on norepinephrine-preconstricted rabbit aortas (31). 12-HETE also failed to produce any relaxation in U-46619-constricted canine epicardial coronary arteries (38) and did not significantly relax or hyperpolarize porcine epicardial coronary arteries (55). Noticably, 12-HETE may contribute to the vasodilation to AA in some coronary and mesenteric

Table 1. Analysis of THETA fraction by LC/MS/MS identification of THETAs produced by Ad-15-LO-1-transduced rabbit aortas

<table>
<thead>
<tr>
<th>Elution Time, min</th>
<th>MS1</th>
<th>MS2</th>
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<tbody>
<tr>
<td>10.2</td>
<td>353 (M-H)</td>
<td>335, 317, 309, 289, 235, 197, 167, 99, 85, 71</td>
</tr>
<tr>
<td>10.8</td>
<td>353 (M-H)</td>
<td>335, 197, 179, 167, 157, 139, 121, 85, 71</td>
</tr>
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The THETA fraction (2–10 min) from Fig. 4B was collected and analyzed by LC-MS/MS using solvent system II. Metabolites eluting at 10.2 and 10.8 min were analyzed by MS/MS. The daughter ion spectra (MS2) of 353 m/z ions are listed. See text for definitions of abbreviations.

Fig. 5. LC/MS/MS analysis of THETAs. Collision-induced dissociation of 353 m/z ions and daughter ions analysis by MS/MS. The THETA fractions (2–10 min from Fig. 4B) produced by Ad-15-LO-1-transduced rabbit aortas were collected and analyzed by LC/MS/MS using solvent system II. Metabolites eluting at 10.2 min (A) and 10.8 min (B) were analyzed by MS/MS.

Fig. 6. Effects of 15-LO-1 overexpression on the vascular activity of rabbit aortas. Aortic rings were treated with either vehicle or transduced with Ad-β-gal or Ad-15-LO-1 for 12 h. Vessels were pretreated with indomethacin (10^{-5} M) and nitro-L-arginine (3 × 10^{-5} M) (A) and cinnamyl-3,4-dihydroxy-a-cyanocinnamate (CDC, 2 × 10^{-5} M) when indicated, precontracted with phenylephrine (10^{-7} to 10^{-6} M) and relaxations to AA determined (B). Data are expressed as percent relaxations, and each value represents mean ± SE. ***P < 0.001, **P < 0.01.
arteries (26, 54, 55). The mechanisms responsible for the disparate effects of 12-HETE are unclear. Among the AA metabolites of 15-LO, 15-H-11,12-EETA, and 11,12,15-THETA are the vasoactive metabolites that relax the rabbit aorta (9, 32, 33). They relax arteries by activating apamin-sensitive K⁺ channels and thus hyperpolarizing smooth muscle cells (16). In addition, these metabolites act as EDHFs in rabbit arteries (9).

15-LO expression increases in pathological conditions such as in early stages of atherosclerosis (21, 50) and hypercholesterolemia (31). Also, cytokines such as IL-4 and IL-13 increase 15-LO expression in human monocytes, macrophages, and human lung cancer cells (6, 11, 28). In cultured human ECs, IL-4 treatment induces 15-LO mRNA expression; however, no active 15-LO protein was expressed and thus AA metabolism was unaltered (24). Therefore, we wondered if changing the expression of the first enzyme in this pathway, i.e., 15-LO, is sufficient to alter the relaxation responses via altering the formation of the vasoactive eicosanoids. This will also provide insight into the rate-limiting step in the 15-LO pathway of AA metabolism in endothelium. IL-4 and IL-13 can be used to increase 15-LO expression in the aortic tissue; however, these cytokines may alter the expression of a variety of proteins, which may affect AA metabolism and thus the vasodilatory effects of agonists. For example, IL-13 decreases the expression of phospholipid hydroperoxide glutathione peroxidase while increasing 15-LO expression in A549 cells (39, 40). Since this glutathione peroxidase is involved in the reduction of 15-HPETE to 15-HETE, a change in its expression may influence AA metabolism.

Previous studies indicate that arteries transduced with an adenovirus express the adenoviral protein(s) exclusively in the endothelium (20). As a result, we used an adenovirus containing the human 15-LO-1 cDNA to overexpress human 15-LO-1 in RAECs and endothelium of rabbit aorta. Transduction with the Ad-15-LO-1 increased the expression of 15-LO-1 that was related to the amount of adenovirus added. The maximum expression of 15-LO-1 protein was observed after 12 h as detected by immunoblotting with a human 15-LO-1 antibody. In histological sections of the rabbit aorta, there was no signal for the endogenous 15-LO-1 in the endothelium due to the camera settings; however, endogenous 15-LO-1 was detected by immunoblotting. In our previous studies with untreated aorta, 15-LO-1 expression was found to be associated with the endothelium and smooth muscle cells adjacent to the endothelial layer. In Ad-15-LO-1-transduced aortic sections, the human 15-LO-1 antibody indicated the presence of the enzyme exclusively in the endothelium. The expressed 15-LO-1 was enzymatically active since the metabolism of AA to THETAs, THETAs, and 15-HETE was increased in Ad-15-LO-1, but not Ad-β-Gal-transduced, ECs and aortic segments. We observed a greater synthesis of 15-HETE and 12-HETE from Ad-15-LO-1-transduced aorta than from Ad-15-LO-1-transduced ECs. This may be due to the culture conditions or number of passages that influence the metabolism of AA in ECs. These studies indicate that overexpression of 15-LO-1 in ECs and aortic rings results in an active enzyme that metabolizes AA. Also, when the endothelium was removed from the 15-LO-1-transduced aortic segments, AA metabolism was abolished. This indicates that the endothelium is necessary and that 15-LO-1 overexpression was limited to the endothelium and not to the smooth muscle cells. In addition, the THETA from Ad-15-LO-1-transduced aortas comigrated with the 11,12,15-THETA standard in two HPLC systems. It had a molecular weight of 354 and a mass spectrum and fragmentation pattern consistent with 11,12,15-THETA. Thus the vaso-dilatory 11,12,15-THETA is synthesized in Ad-15-LO-1-transduced aortas.

The synthesis of THETAs and HEETAs requires the sequential metabolism of AA by 15-LO-1 and a hydroperoxide isomerase possibly by a CYP (32, 34). The finding that THETA and HEETA synthesis is increased with the overexpression of 15-LO-1 suggests that 15-LO-1 is a rate-limiting step in the endothelium. Therefore, variations in 15-LO-1 regulate the function of this pathway. As a result, discovery of endogenous compounds such as IL-4 or IL-13, which increase the expression of 15-LO-1 by the endothelium, may represent an important mechanism for enhancing this dilatory pathway. Alternatively, pathological conditions such as early stages of atherosclerosis (21, 50) that alter the expression of 15-LO-1 may contribute to changes in endothelium-dependent dilation and endothelial dysfunction.

AA is not a physiological stimulus but is used to activate the endothelial 15-LO pathway and cause vascular relaxation by a receptor-independent mechanism. In this regard, AA causes a concentration-related relaxation of the rabbit aorta in the presence of indomethacin that is inhibited by LO inhibitors (9). In addition, AA caused endothelium-dependent hyperpolarization of aortic smooth muscle cells (9). The relaxations to AA are inhibited by the inhibitor of small conductance K⁺ channels (Figs. 3 and 4), and the synthesis of HEETAs and THETAs were detected. AA relaxed the aortic rings; however, the maximum relaxations to AA were observed at a higher concentration (10⁻⁷ M AA). The active concentration of AA in the endothelial cells and the concentration of THETA and HEETA in the myoendothelial space are not known. Exogenous AA must cross the endothelial cell membrane and in the cell has several fates including metabolism by 15-LO, esterification into phospholipids, or β-oxidation. In the present studies, the aortic segments were incubated for 12 h after the Ad-15-LO-1 or Ad-β-Gal transduction or vehicle treatment to achieve maximum 15-LO-1 or β-Gal expression. The 12-h incubation did affect the tension response of aortic rings. Acetylcholine contracted, rather than relaxed, the rings under all treatment conditions. However, the aortic rings treated with vehicle or transduced with Ad-β-Gal relaxed to AA. The relaxations to AA were enhanced in Ad-15-LO-1-transduced aortic rings, and these relaxations were blocked by the 15-LO inhibitor CDC. These findings show that the enhanced relaxations with Ad-15-LO-1 were due to increased synthesis of 15-LO-1 metabolites of AA. The vasodilatory metabolites of 15-LO must mediate the relaxations to AA and that 15-LO overexpression increases endothelium-dependent relaxations. 15-LO expression alone is sufficient to enhance the relaxations to AA in the aorta.

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15-LIPOXYGENASE-MEDIATED RELAXATION

REFERENCES


