Published in final edited form as:

Prostaglandins Other Lipid Mediat. 2013 October ; 0: . doi:10.1016/j.prostaglandins.2013.07.002.

EFFECT OF HUMAN 15-LIPOXYGENASE-1 METABOLITES ON VASCULAR FUNCTION IN MOUSE MESENTERIC ARTERIES AND HEARTS

Tamas Kriska¹, Cody Cepura¹, Lawan Siangjong¹, Tina C. Wan^{1,2}, John A. Auchampach^{1,2}, Aviv Shaish³, Dror Haratz^{3,4}, Ganesh Kumar⁵, John R. Falck⁵, Kathryn M. Gauthier¹, and William B. Campbell¹

¹Department of Pharmacology and Toxicology, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, WI 53226

²Cardiovascular Center, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, WI 53226

³The Bert W. Strassburger Lipid Center, Tel Aviv University, 52621 Hashomer Tel, Israel

⁴Sheba Medical Center and Sackler School of Medicine, Tel Aviv University, 52621 Hashomer Tel, Israel

⁵Department of Biochemistry, University of Texas Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas, TX 75390

Abstract

Lipoxygenases regulate vascular function by metabolizing arachidonic acid (AA) to dilator eicosanoids. Previously, we showed that endothelium-targeted adenoviral vector-mediated gene transfer of the human 15-lipoxygenase-1 (h15-LO-1) enhances arterial relaxation through the production of vasodilatory hydroxyepoxyeicosatrienoic acid (HEETA) and trihydroxyeicosatrienoic acid (THETA) metabolites. To further define this function, a transgenic (Tg) mouse line that overexpresses h15-LO-1 was studied. Western blot, immunohistochemistry and RT-PCR results confirmed expression of 15-LO-1 transgene in tissues, especially high quantity in coronary arterial wall, of Tg mice. Reverse-phase HPLC analysis of [¹⁴C]-AA metabolites in heart tissues revealed enhanced 15-HETE synthesis in Tg vs. WT mice. Among the 15-LO-1 metabolites, 15-HETE, erythro-13-H-14,15-EETA, and 11(R),12(S),15(S)-THETA relaxed the mouse mesenteric arteries to the greatest extent. The presence of h15-LO-1 increased acetylcholine- and AA-mediated relaxation in mesenteric arteries of Tg mice compared to WT mice. 15-LO-1 expression was most abundant in heart; therefore, we used the Langendorff heart model to test the hypothesis that elevated 15-LO-1 levels would increase coronary flow following a short ischemia episode. Both peak flow and excess flow of reperfused hearts were significantly elevated in hearts from Tg compared to WT mice being 2.03 and 3.22 times greater, respectively. These results indicate that h15-LO-1-derived metabolites are highly vasoactive and may play a critical role in regulating coronary blood flow.

© 2013 Elsevier Inc. All rights reserved.

Address correspondence to: Tamas Kriska, Department of Pharmacology and Toxicology, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, WI 53226. Phone: 414.955.8045, Fax: 414.955.6545, tkrisk@mcw.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Keywords

15-lipoxygenase; eicosanoids; vasodilation; coronary flow; ischemia/reperfusion; reactive hyperemia; endothelium-derived hyperpolarizing factor

Introduction

Lipoxygenases (LOs) are a family of dioxygenase enzymes that play a central role in physiological and pathophysiological processes of the cardiovascular system, such as vasodilation, blood pressure regulation and atherosclerosis. In rabbit arteries, endothelial 15-LO-1 converts arachidonic acid (AA) to monohydroperoxyeicosatetraenoic acids (HPETEs) that are further metabolized by a hydroperoxide isomerase and soluble epoxide hydrolase (sEH) to vasoactive hydroxyl-epoxyeicosatrienoic acids (HEETAs) and trihydroxyeicosatrienoic acid (THETA), respectively ^{1, 2}. These metabolites function as endothelium-derived hyperpolarizing factors (EDHFs), mediating the non-nitric oxide (NO) and non-prostaglandin (PG) portion of the endothelium-dependent relaxations to acetylcholine (ACh) and AA ³⁻⁵. This pathway functions as an inducible EDHF ⁶. HEETA's and THETA exert their activity through activation of potassium channels localized to the smooth muscle cell membrane causing membrane hyperpolarization and vascular relaxation ^{4, 7, 8}. Among these metabolites, the 11(R),12(S),15(S)-THETA stereoisomer was identified as a potent mediator of vascular relaxation ⁸.

In the mouse, several LOs produce 12- and 15-HPETE ⁹⁻¹². Among these are 8-LO (Alox8), platelet-type 12-LO (Alox12), and leukocyte-type 12/15-LO (Alox15). While 15-LO-1 plays an important role in endothelium-dependent relaxation in rabbit arteries ², the LO that contributes to vascular relaxations in the mouse has not been identified. In a recent study ¹³ we found no aortic expression of Alox15, the mouse homologue of rabbit and human 15-LO-1 ^{11, 14, 15}. As a consequence, there was no difference between vascular relaxations to ACh in aortas from wild type (WT) and Alox15 knockout mice.

As a different approach, 15-LO transgenic (h15-LO-1 Tg) mouse model was developed using human 15-LO-1 cDNA. This transgene is under the control of a murine preproendothelin-1 promoter region to achieve endothelial expression ¹⁶. These mice have been used to define the biological role of 15-LO-1 in pathological conditions such as atherosclerosis ¹⁷, thymocyte apoptosis ¹⁸, and carcinogenesis ¹⁹. In this study, we utilized the h15-LO-1 Tg mouse model to determine the effect of 15-LO-1 metabolites of AA on vascular relaxations in the mesenteric and coronary circulation.

Experimental Procedures

Chemicals

Acetylcholine (ACh), nitro-L-arginine (L-NA), G-nitro-L-Arginine-methyl ester (L-NAME), indomethacin (Indo), nordihydroguaiaretic acid (NDGA), Triton X-100, KCl, and the calcium ionophore A23187 were purchased from Sigma (St. Louis, MO). AA was purchased from Nu-Chek Prep (Elysian, MN), [¹⁴C]-AA was from PerkinElmer Life Sciences (Boston, MA). Both AA and [¹⁴C]-AA were further purified by HPLC before use. The thromboxane mimetic U46619 was obtained from Cayman Chemical Company (Ann Arbor, MI). All solvents were HPLC grade and purchased from Burdick and Jackson or Sigma.

Animals

Male or female C57BL6 (WT) mice (20–25g) were obtained from The Jackson Laboratory (Bar Harbor, Maine). Human 15-LO-1 transgenic mice on a C57BL6 background were developed and provided by Dr. Dror Harats at the Bert W. Strassburger Lipid Center (Sheba Medical Center, Tel-Hashomer, Israel). Since our preliminary results showed no difference between male and female mice, all subsequent experiments reported in this paper were performed exclusively on male mice. Animal protocols were approved by the animal care committee of the Medical College of Wisconsin and procedures were performed in accordance with the National Institute of Health Guide for the Use of Laboratory Animals (2011).

Tissue preparation

Arteries were dissected and cleaned of connective tissue in ice-cold HEPES buffer (10 mM HEPES, 150 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 6 mM glucose, pH=7.4). Heart, lung and trachea were used immediately or snap-frozen in liquid nitrogen immediately after dissection and stored at –80°C until further use.

Western immunoblotting and immunohistochemistry

Tissues frozen in liquid nitrogen were pulverized and homogenized in lysis buffer (10 mM HEPES, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM sodium bisulfite, pH 7.5) containing Complete™ Mini cocktail protease inhibitor (Roche Diagnostics) and 0.5% Triton X-100. Homogenates were further incubated on ice for 30 min with occasional vortexing. The homogenates were centrifuged at 12,000 g for 20 min, and the supernatants were used for analysis. 50 mg of total protein per lane was separated on 7.5% SDS-PAGE (Criterion Precast Gel, BioRad) and transblotted onto nitrocellulose membrane. After blocking with 5% non-fat dry milk (BioRad) in TBS-T buffer (20 mM Tris base, 150 mM NaCl, 0.1% sodium azide, 3% BSA and 0.1% Tween 20), the membranes were probed with 15-LO-1 primary antibody (1:1000 dilution) provided by Dr. Hartmut Kuhn (Institute of Biochemistry at Humboldt University, Berlin, Germany)²⁰. Horseradish peroxidase (HRP)-conjugated anti-guinea-pig IgG (1:10,000 dilution, GE Healthcare) was used as secondary antibody. -Actin was the loading control detected by a mouse monoclonal anti-actin primary antibody (1:10000 dilution, Santa Cruz Biotechnology).

Immunoreactive bands were visualized using SuperSignal West Pico or West Femto chemiluminescence reagents (Thermo Scientific). Immunohistochemistry was performed on deparaffinized cryostat sections permeabilized with 0.1% Tween-20. After 30 min of blocking in a 1% BSA solution, slides were incubated overnight with human 15-LO-1 primary antibody, followed by a one-hour incubation with goat (Alexa Fluor 594)-conjugated anti-guinea-pig IgG (1:1000 dilution) as secondary antibody. Negative controls were incubated with only secondary antibody.

RT-PCR

Total RNA was isolated from aorta, lung, heart and trachea using RNeasy Mini Kit (Qiagen) and subjected to reverse transcription with SuperScript III First-Strand cDNA Synthesis System (Invitrogen). Samples without reverse transcriptase or RNA were prepared as controls. cDNA samples were analyzed by qPCR (iCycler, BioRad) using iQ™ SYBR® Green Supermix (BioRad) reagent. Human 15-LO-1 specific primers synthesized by Eurofins MWG Operon (Huntsville, AL) were as follows: forward 5'-TTG GTT ATT TCA GCC CCC ATC -3', reverse 5'-TGT GTT CAC TGG GTG CAG AGA -3'. Expression of the 18S gene was used as loading control. Primers for mouse 18S were as follows: forward 5'-AAA TCA GTT ATG GTT CCT TTG GTC -3', reverse 5'-GCT CTA GAA TTA CCA

CAG TTA TCC AA -3 . The PCR program consisted of 35 cycles of 94°C for 45 s, 60°C for 45 min, and 72°C for 2 min. Amplified PCR products were separated by 1.5% agarose gel electrophoresis and visualized with ethidium bromide staining.

[¹⁴C]-Arachidonic acid metabolism and RP-HPLC analysis

Cleaned aortas or hearts were cut into 3–5 mm pieces and incubated for 10 min at 37°C in 5 ml HEPES buffer containing Indo (10 μM). Tissues were then incubated with AA (0.1 μM) plus [¹⁴C]-AA (0.05 μCi). After 10 min, calcium ionophore A23187 (20 μM) was added and the incubation continued for another 15 min. Reactions were stopped by adding ice-cold ethanol to a final concentration of 15%. The buffer was acidified (pH < 3.5) with glacial acetic acid and extracted on Bond Elute ODS solid-phase extraction columns (Agilent Technology) as previously described¹. Extracted metabolites were dried under a stream of nitrogen and stored at –40°C until analysis.

[¹⁴C]-AA metabolites were resolved by reverse phase HPLC using a Nucleosil-C18 column (5 μ, 4.6 × 250 mm). The following solvent system was used: solvent A was water and solvent B was acetonitrile (MeCN) containing 0.1% glacial acetic acid. The program consisted of a 40 min linear gradient from 50% solvent B in A to 100% solvent B. Flow rate was 1 ml/min. Column eluate was collected in 0.2 ml fractions¹. Absorbance was monitored at 205 nm, and column fraction radioactivity was determined by liquid scintillation spectrometry.

Isometric tension in mesenteric arteries

Arterial sections (~1.5 mm in length) from WT or h15-LO-1 Tg mice were mounted in a 4-chamber wire myograph (model 610M, Danish Myo Technology A/S) and maintained at 37°C in physiological saline solution (PSS): 119 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl₂, 1.17 mM MgSO₄, 24 mM NaHCO₃, 1.18 mM KH₂PO₄, 0.026 mM EDTA, and 5.5 mM glucose, gassed with O₂ containing 5% CO₂ as previously described^{21, 22}. Primary and major secondary branches of the mesenteric artery with relative diameters of 150 to 300 μm were stretched to a resting tension of 0.25 millinewtons (mN). The arteries were stimulated 3 times with 60 mM KCl plus 100 nM U46619 for 8–10 min at 10 min intervals. Arteries were contracted with submaximal concentrations of U46619 (50–300 nM) to 50–90% of their maximum KCl/U46619 challenge. Cumulative concentrations of ACh (0.1 nM – 10 mM) or AA (100 nM–450 μM) were added, or cumulative concentrations of 15-LO derived metabolites (1 nM–450 μM) of AA including 15(S)-HETE, cis-15-H-11,12-EETA, trans-15-H-11,12-EETA, erythro-13-H-14,15-EETA, threo-13-H-14,15-EETA, and 11(R),12(S), 15(S)-THETA. For ACh and AA responses, the arteries were pretreated with the NOS inhibitor, L-NA (30 μM) and the COX inhibitor, Indo (10 μM). Results are expressed as % relaxation of the U46619-precontracted arteries with 100% representing basal tension.

Blood pressure measurement

Systolic blood pressure of male and female WT and h15-LO-1 Tg mice was determined using tail cuff method with IITC Life Sciences Blood Pressure System (Woodland Hills, CA). The same method was used to monitor the systolic blood pressure of mice during the induced hypertension experiments. Mice were acclimated to the blood pressure chamber twice a week starting from age of 6 weeks. Recordings were started after 5–10 minutes of acclimation. Three successful measurements were averaged as a single data point.

Experimental hypertension models

In the L-NAME induced hypertension, model mice received 100 mg/kg/day of G-nitro-L-Arginine-methyl ester in regular drinking water for five days. The water intake was

monitored during this time period to exclude any possible differences between experimental groups. Blood pressure was monitored with the tail cuff method (see above).

Langendorff heart

Hearts from sodium pentobarbital anaesthetized WT and h15-LO-1 Tg mice were isolated and perfused in the Langendorff mode ²³ using the Krebs-Henseleit buffer containing (in mmol/L): NaCl 120, KCl 5.9, MgSO₄·7H₂O 1.2, CaCl₂·2H₂O 1.75, NaHCO₃ 25, and glucose 11. The buffer was aerated with 95% O₂:5% CO₂ to give a pH of 7.4 at 37°C and perfused at a constant pressure of 100 cm H₂O. Hearts were perfused for 15 min to allow for stabilization and then for additional 10 min while pacing at 420 beats/min. Coronary flow was monitored by an in-line flow probe connected to a flowmeter (model T206, Transonics Systems Inc.). The left ventricular pressure signals (heart rate, LVDP, dP/dt) were acquired continuously by a water-filled balloon inserted into the left ventricle and connected to a MacLab/2e data acquisition system (AD Instruments Inc.). After a 25 min stabilization period, 2–3 occlusion periods (40 s) were initiated separated by 10-minute intervals to allow coronary flow to recover to the pre-occlusion level. For each occlusion periods, the hyperemic peak flow and excess flow (defined as max flow or total excess flow over the baseline) were determined and normalized to the wet heart weight as ml/min/g. Heart weights and heart weight-to-body weight ratios (HW:BW) did not differ between WT and Tg mice. Male WT heart weights were 0.18±0.01 g (HW: BW 4.2±0.4×10⁻³), and male Tg hearts were 0.17±0.01 g (HW:BW 4.4±0.3×10⁻³).

Statistics

Data are presented as means±SEM. The two-tailed Student's *t* test or ANOVA was used for determining the significance of observed differences between experimental values, with *p*<0.05 considered statistically significant.

Results

h15-LO-1 protein and mRNA expression in mouse tissues

h15-LO-1 protein content was determined by immunoblotting of aorta, lung, trachea, and heart lysates using an anti-15-LO-1 primary antibody. All tissues from transgenic animals showed elevated h15-LO-1 protein levels as compared to the WT counterparts (Figure 1A). The expression of h15-LO-1 was highest in heart and trachea, moderate in lung and small in aorta of the Tg animals (ratios of densitometric values were 0.57, 1, 0.08, and 0.005, respectively). It is interesting to note that in the WT lung and trachea a very strong immunoreactive 75 kDa band co-migrated with the h15-LO-1 bands of the Tg organs.

RT-PCR confirmed the presence of h15-LO-1 mRNA in tissues of Tg but not WT mice. mRNA expression was detected in aorta, lung, trachea and heart from Tg mice. In contrast, no expression was detected in organs of WT mice (Figure 1B). Intensities of the amplified products exhibited good correlation with the immunoblot data. The highest mRNA expression was found in heart, while aorta was the lowest. Murine 18S was used as a loading control. This experiment also confirmed the absence of h15-LO-1 expression in organs of WT mice, suggesting a cross-reaction between the 15-LO-1 antibody and a murine LO in the immunoblot experiment.

Tissue specific localization of h15-LO-1 protein in Tg mice

Localization of the h15-LO-1 protein in the heart and aorta of Tg vs. WT mice was determined by immunohistochemistry. As can be seen in Figure 1C (upper panels), there were no differences in h15-LO-1 protein expression between aortic segments from WT and Tg mice, or from segments stained with only secondary antibody. The visible signal was a

result of the characteristic auto-fluorescence of the elastic lamina. In contrast, heart sections showed significant differences in staining. While the fluorescence of WT heart samples did not exceed the fluorescence of samples prepared with only secondary antibody, the h15-LO-1 Tg heart sections showed significant increase in fluorescent staining. Some of the staining was localized in cardiomyocytes, although the detection is complicated by autofluorescence caused by chemical fixation of the tissue. On the contrary, the strong signal detected in the coronary arterial wall of the h15-LO-1 Tg hearts was completely absent in WT samples or Tg samples with only secondary antibody. (Fig 1C, lower panels).

AA metabolism by mouse aorta and heart

Hearts and aortas from WT and h15-LO-1 Tg mice were incubated with [14 C]-AA in the presence of indomethacin and calcium ionophore, A23187. Using reverse-phase HPLC, major [14 C]-AA metabolites were identified by comparing the retention times of radioactive metabolites with known standards for THETAs, HEETAs and HETEs. The WT heart and aortic segments produced similar profiles of metabolites (Figure 2A and 2C). The predominant metabolite was 12-HETE in both cases. No synthesis of 15-HETE was observed. Incubations from hearts and aortic segments from h15-LO-1 Tg mice resulted in different metabolic profiles (Figure 2B and 2D). In the Tg heart, a prominent 15-HETE peak appeared, while it remained under the detection limit in Tg aorta. These results are in agreement with the low h15-LO-1 protein and mRNA levels detected in Tg aorta. It is interesting to note that WT aorta produce endogenous THETAs and HEETAs while the heart tissue does not. In murine heart tissue, THETAs and HEETAs are produced only in the presence of the h15-LO-1 transgene. The production of THETAs was also elevated in the Tg hearts.

Vasorelaxation to 15-LO-1 derived AA metabolites in WT mouse mesenteric arteries

We tested the ability of 15(S)-HETE, 11(R),12(S),15(S)-THETA, and diastereomers of 15(S)-H-11,12-EETA and 13-H-14,15-EETA to relax U46619 pre-constricted arterial rings. All tested metabolites caused concentration-dependent relaxations (1×10^{-9} - 5×10^{-4} M). As can be seen in Figure 3A, the most potent relaxing compound was the erythro-13-H-14,15-EETA (Er-HEETA). The threo-13-H-14,15-EETA (Th-HEETA) was much less potent, and its relaxing potency was very close to those of cis and trans 15(S)-H-11,12-EETA (Figure 3B). The relaxing potency of the 15(S)-HETE, the major 15-LO metabolite was almost identical to the 11(R),12(S),15(S)-THETA (Figure 3C). These data indicate that compounds from all three major 15-LO metabolite groups are capable of vasorelaxation in mouse mesenteric arteries.

Vasorelaxation to ACh and AA in WT vs. h15-LO-1 Tg mouse mesenteric arteries

In U46619 pre-constricted mesenteric arterial rings that were pretreated with Indo and L-NA, ACh (10^{-10} and 10^{-5} M) caused concentration-dependent relaxations. At 10^{-7} M, ACh induced a small, but significantly greater, relaxation in h15-LO-1 Tg arteries as compared to arteries from WT mice (Figure 4A). Similar patterns were observed with AA-induced relaxations. AA (10^{-7} to 5×10^{-4} M) caused concentration-related relaxations of WT and Tg pre-constricted mesenteric arteries in the presence of Indo and L-NA. Arteries from h15-LO-1 Tg mice relaxed to the significantly greater extent at 10^{-5} M AA as compared to arteries from WT mice (Figure 4B). These data indicate that h15-LO-1 metabolites of AA contribute to ACh and AA-mediated relaxations of h15-LO-1 Tg mouse mesenteric arteries; however, this contribution is only slightly greater when compared to the relaxations in arteries from WT mice.

Systolic blood pressure of WT vs. h15-LO-1 Tg mice under normal or induced hypertension conditions

Systolic blood pressure of WT and h15-LO-1 Tg mice (Figure 4C) was monitored using the tail-cuff method. Basal blood pressures were similar between the WT and h15-LO-1 Tg mice and ranged from 98 to 112 mmHg. When experimental hypertension was induced by L-NAME treatment, systolic blood pressure increased to the same extent in both groups. These data indicate that h15-LO-1 overexpression did not alter neither basal blood pressure nor experimental hypertension.

Reactive hyperemia in WT vs. h15-LO-1 Tg murine hearts

The effect of h15-LO-1 overexpression was tested on Langendorff-perfused hearts. The baseline coronary flow was approximately 2.4 ml/min in both WT and h15-LO-1 Tg hearts. Both genotypes produced reactive hyperemic response to an ischemic stimulus. However, in Tg hearts, the hyperemic effect was significantly greater as compared to WT hearts. Representative traces from a control and Tg hearts are shown in Figure 5A. Peak hyperemic flow increased by 1.97 ± 0.59 ml/min in Tg hearts, while only by 0.97 ± 0.16 ml/min in WT hearts after 40 seconds of occlusion (Figure 5B). The same ischemic episode produced total excess flow increase of 1.16 ± 0.39 ml and 0.36 ± 0.12 ml for WT and h15-LO-1 Tg hearts, respectively (Figure 5C). To exclude any direct effect of the transgene on the coronary flow through changes in cardiac function, left ventricular developed pressure (LVDP) and maximum or minimum rate of change in left ventricular developed pressure (dP/dt max and dP/dt min) were continuously monitored during the reactive hyperemia cycles. The results are summarized on panel D of Figure 5. No significant differences were detected between the cardiac functions of WT and Tg hearts during pre- or post-ischemic phases of reactive hyperemia indicating that 15-LO metabolites are responsible for the observed effect. In contrast to reactive hyperemia, both ACh and bradykinin had dramatic effect on cardiac contractility so their effects on flow could not be assessed in WT and Tg hearts (data not shown).

Discussion

EDHFs contribute to the regulation of vascular tone by mediating a portion of the endothelium-dependent relaxations to ACh, bradykinin and shear stress^{5, 7}. Initially, EETs, H₂O₂ and C-type natriuretic peptide were described to function as EDHFs^{24–26} in mice. As we recently demonstrated, LO metabolites of AA also mediate EDHF activity in murine resistance arteries^{13, 22}. The EDHF activity in these arteries is inhibited by LO and hydroperoxide isomerase inhibitors. The composition of AA metabolites identified as LO products were very similar between rabbit and mouse arteries including 12-HETE, HEETA, 11,14,15-THETA and 11,12,15-THETA^{21, 22}. Among THETAs, 11(R),12(S),15(S)-THETA was the biologically active stereoisomer causing vascular relaxation through activation of smooth muscle apamin-sensitive K⁺ channels^{2–4, 8} in both species. Here, we presented further evidence that 15-LO products, such as diastereomers of 11,12,15-HEETA and 13-H-14,15-EETA are also highly vasoactive. Erythro 13-H-14,15-EETA was a particularly powerful vasodilator. Despite the similarities between rabbit and mouse aortas, the Alox15, which is considered the vascular LO analog of rabbit 15-LO-1^{22, 27, 28}, was not found in murine arteries¹³. Thus, the functional murine LO isozyme responsible for synthesis of vasoactive AA metabolites is still unknown.

Previously, we found that adenoviral transduction of the human 15-LO-1 increased the production of THETA and HEETA and subsequently the ACh induced vasorelaxation in rabbit arteries²⁹. In the present study, we used a transgenic murine model that expresses h15-LO-1 to study the effect of 15-LO-1 metabolites on the murine vasculature. These data

can help identify the endogenous murine vascular LO and yield information on the functional properties of endogenous 15-LO-1 metabolites.

Although the h15-LO-1 transgene is under the control of the murine preproendothelin-1 promoter,³⁰ very little LO expression was found in arteries as assessed by western immunoblot, RT-PCR, and enzymatic assay techniques. As a consequence, expression of the transgene exerted only a small effect on ACh- and AA-mediated vasorelaxations. Small, but significant, differences were detected near the midpoint of the concentration-relaxation curves (Figure 4A). In addition, the systolic blood pressures of both WT and h15-LO-1 Tg mice were elevated to the same extent in an L-NAME model of hypertension. Thus, this model has failed to prove our initial hypothesis that 15-LO-1 metabolites would attenuate experimental hypertension by enhanced relaxation of the resistance arteries.

In a model originally designed to express the 15-LO-1 transgene in arteries, h15-LO-1 protein was abundantly expressed in heart tissue. This finding can be explained by high cardiac expression of preproendothelin-1^{31,32}. Comparison of AA metabolite profiles between aorta and heart tissues of WT and h15-LO-1 Tg mice revealed an interesting difference. While there were endogenous THETAs and HEETAs in WT murine aorta, only hearts expressing the h15-LO-1 transgene produced these metabolites (Figure 2). Our immunohistochemical analyses showed localization for the transgenic protein in cardiomyocytes, confirming the results of recently published reports^{33,34}. Additionally, the major expression was found in coronary arterial wall as was originally reported³⁰. These findings open the possibility that 15-LO-1 regulates heart function during short ischemia episodes.

The role of 15-LOs in ischemia-reperfusion injury is not understood. In mouse models, the effect of overexpression or knockout of the leukocyte-type 12/15-LO-1 on reperfusion injury was extensively studied. Most reports point to the adverse effect of this LO³⁵⁻³⁷. Murphy et al³⁸ showed impaired ischemic preconditioning-induced cardioprotection in 12/15-LO knockout animals. The negative effects were usually accompanied with high expression of proinflammatory markers implicating oxidative stress. The recent finding that cardiac expression of 15-lipoxygenase-1 is upregulated in ischemic tissues of human patients³³ makes our model of additional relevance.

The isolated, buffer-perfused mouse heart model has been developed to facilitate analysis of genetic manipulations³⁹, cardioprotection during ischemia-reperfusion³⁸, and coronary vascular function⁴⁰. In the latter case, the perfused heart was used to characterize reactive hyperemia parameters of peak and excess flow following a brief periods of ischemia. The exact mechanism of reactive hyperemia is unclear, but EDHF involvement is likely⁴¹ especially in early phases dominated by peak flow. Our primary goal was to assess the vascular effect of h15-LO-1 metabolites in reactive hyperemia in perfused hearts. Since 15-LO metabolites are potent vasodilators, we expected an increase in the reactive hyperemic response in hearts from the h15-LO-1 Tg mice. Both the peak flow and excess flow of the perfused h15-LO-1 Tg hearts were significantly elevated compared to WT hearts. Contribution of LO metabolites to coronary dilation has been documented previously⁴¹. Pharmacological inhibitors of LOs⁴² inhibited histamine-induced dilation and release of vasorelaxing endothelial metabolites.

Conclusion

Preproendothelin-1-controlled overexpression of the h15-LO protein in mice produced small but significant effects on endothelium-dependent vascular relaxations to ACh and AA. Endothelial expression of h15-LO-1 was too low to influence the basal blood pressure or attenuate L-NAME-induced hypertension. Although RT-PCR analysis revealed transcription

of 15-LO-1 mRNA in aortas of h15-LO-1 Tg mice, most of the h15-LO transgene expression was found in the heart tissue. This provided an opportunity to study the effect of 15-LO metabolites of AA on vascular function in the intact coronary circulation. In perfused hearts, the reactive hyperemic response was increased in h15-LO-1 Tg hearts compared to WT hearts. This finding is consistent with the presence of vasoactive metabolites of h15-LO-1 mediating coronary hyperemia.

Acknowledgments

The authors thank Ms. Devora Magier and Ms. Chuely Vang for their technical assistance and Ms. Gretchen Barg for her secretarial assistance. These studies were supported by grants from the National Heart, Lung and Blood Institute (HL-37981 and HL-103673), the Robert A. Welch Foundation (GL625910), and National Institute of General Medical Sciences (GM-31278).

The abbreviations used are

LOs	lipoxygenases
AA	arachidonic acid
ACh	acetylcholine
HPETEs	monohydroperoxyeicosatetraenoic acids
HETEs	hydroperoxyeicosatetraenoic acids
HEETAs	hydroxy-epoxyeicosatrienoic acids
THETAs	trihydroxyeicosatrienoic acids
sEH	soluble epoxide hydrolase
EDHFs	endothelium-derived hyperpolarizing factors
L-NA	nitro-L-arginine
Indo	indomethacin
NDGA	nordihydroguaiaretic acid

References

1. Pfister SL, Spitzbarth N, Nithipatikom K, Edgmond WS, Falck JR, Campbell WB. Identification of 11,14,15- and 11,12,15-trihydroxyeicosatrienoic acids as endothelium-derived relaxing factors of rabbit aorta. *J Biol Chem.* 1998; 273:30879–87. [PubMed: 9812980]
2. Tang X, Holmes BB, Nithipatikom K, Hillard CJ, Kuhn H, Campbell WB. Reticulocyte 15-lipoxygenase-1 is important in acetylcholine-induced endothelium-dependent vasorelaxation in rabbit aorta. *Arterioscler Thromb Vasc Biol.* 2006; 26:78–84. [PubMed: 16239596]
3. Campbell WB, Spitzbarth N, Gauthier KM, Pfister SL. 11,12,15-Trihydroxyeicosatrienoic acid mediates acetylcholine-induced relaxations in the rabbit aorta. *Am J Physiol.* 2003; 285:H2648–H56.
4. Gauthier KM, Spitzbarth N, Edwards EM, Campbell WB. Apamin-sensitive K⁺ currents mediate arachidonic acid-induced relaxations of rabbit aorta. *Hypertension.* 2004; 43:413–9. [PubMed: 14691199]
5. Chawengsub Y, Gauthier KM, Campbell WB. Role of arachidonic acid lipoxygenase metabolites in the regulation of vascular tone. *Am J Physiol.* 2009; 297:H495–H507.
6. Campbell WB, Gauthier KM. Inducible endothelium-derived hyperpolarizing factor (iEDHF): Role of the 15-lipoxygenase-EDHF pathway. *J Cardiovasc Pharmacol* 2013. 2012 Dec 17. Epub.
7. Campbell WB, Falck JR. Arachidonic acid metabolites as endothelium-derived hyperpolarizing factors. *Hypertension.* 2007; 49:590–6. [PubMed: 17200437]

8. Gauthier KM, Chawengsub Y, Goldman DM, Conrow RE, Anjaiah S, Falck JR, Campbell WB. 11(R),12(S),15(S)-Trihydroxyeicosatrienoic acid: An endothelium-derived 15-lipoxygenase metabolite that relaxes rabbit aorta. *Am J Physiol.* 2008; 294:H1467–H72.
9. Bryant RW, Bailey JM. Rearrangement of 15-hydroperoxy-eicosatetraenoic acid (15-HPETE) during incubations with hemoglobin: a model for platelet lipoxygenase metabolism. *Prog Lipid Res.* 1981; 20:279–81. [PubMed: 6804964]
10. Serhan, CN.; Hamberg, M.; Samuelson, B. Lipoxins: a new series of biologically active compounds. In: Bailey, JM., editor. *Prostaglandins, leukotriens and lipoxins*. New York: Plenum Press; 1985. p. 3-16.
11. Funk CD. The molecular biology of mammalian lipoxygenases and the quest for eicosanoid function using lipoxygenase-deficient mice. *Biochim Biophys Acta.* 1996; 304:65–84.
12. Brash AR. Lipoxygenases: Occurrences, function, catalysis, and acquisition of substrate. *J Biol Chem.* 1999; 274:23679–82. [PubMed: 10446122]
13. Kriska T, Cepura C, Magier D, Siangjong L, Gauthier KM, Campbell WB. Mice lacking macrophage 12/15-lipoxygenase are resistant to experimental hypertension. *Am J Physiol Heart Circ Physiol.* 2012; 302:H2428–38. [PubMed: 22467300]
14. De Marzo N, Sloane DL, Dicharry S, Highland E, Sigal E. Cloning and expression of an airway epithelial 12-Lipoxygenase. *Am J Physiology.* 1992; 262:L198–L207.
15. Shappell SB, Olson SJ, Hannah SE, Manning S, Roberts RL, Masumori N, Jisaka M, Boeglin WE, Vader V, Dave DS, Shook MF, Thomas TZ, Funk CD, Brash AR, Matusik RJ. Elevated expression of 12/15-lipoxygenase and cyclooxygenase-2 in a transgenic mouse model of prostate carcinoma. *Cancer Research.* 2003; 63:2256–67. [PubMed: 12727848]
16. Harats D, Kurihara H, Belloni P, Oakley H, Ziober A, Ackley D, Cain G, Kurihara Y, Lawn R, Sigal E. Targeting gene expression to the vascular wall in transgenic mice using the murine preendothelin-1 promoter. *J Clin Invest.* 1995; 95(3):1335–44. [PubMed: 7883980]
17. Harats D, Shaish A, George J, Mulkins M, Kurihara H, Levkovitz H, Sigal E. Overexpression of 15-lipoxygenase in vascular endothelium accelerates early atherosclerosis in LDL receptor-deficient mice. *Arterio Thromb Vasc Biol.* 2000; 20:2100–5.
18. Afek A, Bar-Dayana Y, Polak-Charcon S, Goldberg I, Deutsch M, Kopolovich J, Keren G, Harats D, George J. Overexpression of 15-lipoxygenase in the vascular endothelium is associated with increased thymic apoptosis in LDL receptor-deficient mice. *Pathobiology.* 2004; 71:261–6. [PubMed: 15459485]
19. Harats D, Ben-Shushan D, Cohen H, Gonen A, Barshack I, Goldberg I, Greenberger S, Hodish I, Harari A, Varda-Bloom N, Levanon K, Grossman E, Chaitidis P, Kuhn H, Shaish A. Inhibition of carcinogenesis in transgenic mouse models overexpressing 15-lipoxygenase in the vascular wall under the control of murine preendothelin-1 promoter. *Cancer Lett.* 2005; 229(1):127–34. [PubMed: 16157225]
20. Brinckman R, Schnurr K, Heydeck D, Rosenbach T, Kuhn H. Membrane translocation of 15-lipoxygenase in hematopoietic cells is calcium-dependent and activates the oxygenase activity of the enzyme. *Blood.* 1998; 91:64–74. [PubMed: 9414270]
21. Zhang DX, Gauthier KM, Chawengsub Y, Campbell WB. ACh-induced relaxations of rabbit small mesenteric arteries: Role of arachidonic acid metabolites and K. *Am J Physiol.* 2007; 293:H152–H9.
22. Gauthier KM, Goldman DH, Aggarwal NT, Chawengsub Y, Falck JR, Campbell WB. Role of arachidonic acid lipoxygenase metabolites in acetylcholine relaxations of mouse arteries. *Am J Physiol.* 2011 In press.
23. Cross HR, Lu L, Steenbergen C, Philipson KD, Murphy E. Overexpression of the cardiac Na⁺/Ca²⁺ exchanger increases susceptibility to ischemia/reperfusion injury in male, but not female, transgenic mice. *Circ Res.* 1998; 83:1215–23. [PubMed: 9851938]
24. Matoba T, Shimokawa H, Nakashima M, Hirakawa Y, Mukai Y, Hirano K, Kandaide H, Takeshita A. Hydrogen peroxide is an endothelium-derived hyperpolarizing factor in mice. *J Clin Invest.* 2000; 106:1521–30. [PubMed: 11120759]

25. Chauhan SD, Nilsson H, Ahluwalia A, Hobbs AJ. Release of C-type natriuretic peptide accounts for the biological activity of endothelium-derived hyperpolarizing factor. *Proc Natl Acad Sci USA*. 2003; 100:1426–31. [PubMed: 12552127]
26. Hercule HC, Schunck W-H, Gross V, Seringer J, Leung FP, Weldon SM, Goncalves A, Huang Y, Luft FC, Gollasch M. Interaction between P450 eicosanoids and nitric oxide in the control of arterial tone in mice. *Arterioscler Thromb Vasc Biol*. 2009; 29:54–60. [PubMed: 18927469]
27. Sun D, Funk CD. Disruption of 12/15-lipoxygenase expression in peritoneal macrophages. *J Biol Chem*. 1996; 271:24055–62. [PubMed: 8798642]
28. Kuhn H, Thiele BJ. Arachidonate 15-lipoxygenase. *J Lipid Mediat Cell Signal*. 1995; 12:157–70. [PubMed: 8777563]
29. Aggarwal N, 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*. 2007; 292:H1033–H41.
30. Harats D, Kurihara H, Belloni P, Oakley H, Zlober A, Ackley D, Cain G, Kurihara Y, Lawn R, Sigal E. Targeting gene expression to the vascular wall in transgenic mice using the murine preproendothelin-1 promoter. *J Clin Invest*. 1995; 95:1335–44. [PubMed: 7883980]
31. Sakurai T, Yanagisawa M, Inoue A, Ryan US, Kimura S, Mitsui Y, Goto K, Masaki T. cDNA cloning, sequence analysis and tissue distribution of rat preproendothelin-1 mRNA. *Biochem Biophys Res Comm*. 1991; 175(1):44–7.
32. Tonnessen T, Liunde PK, Giaid A, Sejersted OM, GC. Pulmonary and cardiac expression of preproendothelin-1 mRNA are increased in heart failure after myocardial infarction in rats. Localization of preproendothelin-1 mRNA and endothelin peptide. *Cardiovas Res*. 1998; 39(3): 633–43.
33. Magnusson LU, Lundqvist A, Asp J, Synnergren J, Johansson CT, Palmqvist L, Jeppsson A, Mattsson Hulten L. High expression of arachidonate 15-lipoxygenase and proinflammatory markers in human ischemic heart tissue. *Biochem Biophys Res Comm*. 2012; 424(2):327–30.
34. Asp J, Synnergren J, Jonsson M, Dellgren G, Jeppsson A. Comparison of human cardiac gene expression profiles in paired samples of right atrium and left ventricle collected in vivo. *Physiol Genomics*. 2012; 44:89–98. [PubMed: 22085905]
35. Kayama Y, Minamino T, Toko H, Sakamoto M, Shimizu I, Takahashi H, Okada S, Tateno K, Moriya J, Yokoyama M, Nojima A, Yoshimura M, Egashira K, Aburatani H, Komuro I. Cardiac 12/15 lipoxygenase-induced inflammation is involved in heart failure. *J Experimental Medicine*. 2009; 206(7):1565–74.
36. Yigitkanli K, Pekcec A, Karatas H, Pallast S, Mandeville E, Joshi N, Smirnova N, Gazaryan I, Ratan RR, Witztum JL, Montaner J, Holman TR, Lo EH, van Leyen K. Inhibition of 12/15-lipoxygenase as therapeutic strategy to treat stroke. *Annals Neurology*. 2013; 73(1):129–35.
37. Jin G, Arai K, Murata Y, Wang S, Stins MF, Lo EH, van Leyen K. Protecting against cerebrovascular injury: contribution of 12/15-lipoxygenase to edema formation after transient focal ischemia. *Stroke*. 2008; 39(9):2538–43. [PubMed: 18635843]
38. Gabel SA, London RE, Funk CD, Steenbergen C, Murphy E. Leukocyte-type 12-lipoxygenase-deficient mice show impaired ischemic preconditioning-induced cardioprotection. *Am J Physiol Heart Circ Physiol*. 2001; 280:H1963–H9. [PubMed: 11299195]
39. Seubert J, Yang B, Bradbury JA, Graves J, Degraff LM, Gabel S, Gooch R, Foley J, Newman JW, Mao L, Rockman HA, Hammock BD, Murphy E, Zeldin DC. Enhanced postischemic functional recovery in CYP2J2 transgenic hearts involves mitochondrial ATP-sensitive K⁺ channels and p42/p44 MAPK pathway. *Circ Res*. 2004; 95:506–14. [PubMed: 15256482]
40. Heintz A, Damm M, Brand M, Koch T, Deussen A. Coronary flow regulation in mouse heart during hypercapnic acidosis: role of NO and its compensation during eNOS impairment. *Cardiovas Res*. 2008; 77:188–96.
41. Zatta AJ, Headrick JP. Mediators of coronary reactive hyperemia in isolated mouse heart. *Brit J Pharmacol*. 2005; 144:576–87. [PubMed: 15655499]
42. Toda N. Mechanism of histamine actions in human coronary arteries. *Circ Res*. 1987; 61:280–6. [PubMed: 3113761]

Highlights

- Arachidonic acid metabolites of human 15-LO enzyme are vasoactive.
- Preproendothelin-1 controlled human 15-LO transgene is highly expressed in heart.
- Human 15-LO transgene enhances coronary flow following an ischemia.
- 15-LO metabolites contribute to coronary reactive hyperemia.

Figure 1.

Analysis of h15-LO-1 expression in tissues from WT and h15-LO-1 Tg mice. Panel A shows h15-LO-1 protein expression in aorta, lung, trachea, and heart tissues of WT and h15-LO-1 Tg mice as measured by western immunoblot. 50 μ g total protein were loaded per lane. -Actin was used as a loading control. Representative blots of 3 experiments are shown. Panel B shows h15-LO-1 mRNA expression in aorta, lung, trachea, and heart tissues of WT and h15-LO-1 Tg mice as measured by RT-PCR. Murine s18 was used as a reference gene. Representative gels of three experiments are shown. Panel C shows the expression pattern of h15-LO-1 protein in aorta and heart samples of WT and h15-LO-1 Tg mice that were subjected to immunohistochemical staining with an anti-h15-LO-1 antibody. Control samples were stained with only secondary (goat anti-guinea-pig IgG) antibody. Asterisks are marking the lumens of coronary arteries. Representative areas of 5 slides are shown.

Figure 2. Metabolism of [^{14}C]-arachidonic acid (AA) by aorta and heart tissues of WT and h15-LO-1 Tg mice. Aortic rings from WT (A) or h15-LO-1 Tg (B) mice and myocardial tissue from WT (C) or h15-LO-1 Tg (D) mice were incubated with [^{14}C]-AA in the presence of 10 μM Indo, 30 μM L-NA and 20 μM of A23187. Metabolites were extracted and resolved by RP-HPLC. Migration times of known standards are indicated in each panel. Traces are representative of at least 3 experiments.

Figure 3.

Relaxations of U46619-precontracted mesenteric arteries from WT mice by 15-LO metabolites of arachidonic acid. Panel A: relaxation to erythro- or threo-13-H-14,15-EETA. Panel B: relaxation to trans- or cis-15(S)-H-11,12-EETA. Panel C: relaxation to 11(R), 12(S),15(S)-THETA or 15(S)-HETE. Arteries were pretreated with 30 μ M L-NA and 10 μ M Indo to block the NO and COX pathways. Each value represents the mean $\pm$ SEM, n=8.

Figure 4.

Effect of h15-LO-1 overexpression on vascular relaxation and susceptibility to experimental hypertension. (A) acetylcholine (ACh) and (B) arachidonic acid (AA) relaxations of mouse mesenteric arteries from WT and h15-LO-1 Tg mice. The arteries were pretreated with 30 μ M L-NA and 10 μ M Indo to block the NO and COX pathways and preconstricted with U46619. Systolic blood pressures of WT and h15-LO-1 Tg male (C) and female (D) mice under basal conditions or L-NAME-induced hypertension. Each value represents the mean $\pm$ SEM, n=6–8. * $p<0.05$, ** $p<0.01$, compared to control.

Figure 5.

Reactive hyperemic responses of WT and h15-LO-1 Tg hearts as measured in Langendorff perfusion model. (A) Representative traces of coronary flow are shown with a 40 second period of ischemia. Average peak flow (B) and excess flow (C) values were used for quantitative analysis. (D) Summary of cardiac function (left ventricular developed pressure (LVDP) or maximum and minimum rate of change in left ventricular developed pressure (dP/dt max and dP/dt min)) during pre- and post-ischemic phases of reactive hyperemia. Values represent mean ± SEM, n=11. * p<0.01.