Role of Lipoxygenase Metabolites of Arachidonic Acid in Enhanced Pulmonary Artery Contractions of Female Rabbits

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**Abstract**—Pulmonary arterial hypertension is characterized by elevated pulmonary artery pressure and vascular resistance. In women the incidence is 4-fold greater than that in men. Studies suggest that sustained vasoconstriction is a factor in increased vascular resistance. Possible vasoconstrictor mediators include arachidonic acid–derived lipoxygenase (LO) metabolites. Our studies in rabbits showed enhanced endothelium-dependent contractions to arachidonic acid in pulmonary arteries from females compared with males. Because treatment with a nonspecific LO inhibitor reduced contractions in females but not males, the present study identified which LO isoform contributes to sex-specific pulmonary artery vasoconstriction. The 15- and 5- but not 12-LO protein expressions were greater in females. Basal and A23187-stimulated release of 15-, 5-, and 12-hydroxyeicosatetraenoic acids (HETEs) from females and males were measured by liquid chromatography/mass spectrometry. Only 15-HETE synthesis was greater in females compared with males under both basal and stimulated conditions. Vascular contractions to 15-HETE were enhanced in females compared with males (maximal contraction: 44±6% versus 25±3%). The specific 15-LO inhibitor PD146176 (12 μmol/L) decreased arachidonic acid–induced contractions in females (maximal contraction: 93±4% versus 57±10%). If male pulmonary arteries were incubated with estrogen (1 μmol/L, 18 hours), protein expression of 15-LO and 15-HETE production increased. Mechanisms to explain the increased incidence of pulmonary hypertension in women are not known. Results suggest that the 15-LO pathway is different between females and males and is regulated by estrogen. Understanding this novel sex-specific mechanism may provide insight into the increased incidence of pulmonary hypertension in females. (*Hypertension*. 2011;57:825-832.)

**Key Words:** arachidonic acid ■ lipoxygenase ■ vasoconstriction ■ pulmonary artery ■ estrogen

Pulmonary arterial hypertension (PAH) encompasses a group of diseases characterized by high pulmonary artery pressure and pulmonary vascular resistance. One form of PAH presents in an idiopathic form. Although relatively rare, idiopathic PAH is a medically significant disease that occurs more frequently in young women. The disease is usually catastrophic for those afflicted, with a median survival time of 2.8 years postdiagnosis. Mechanisms to explain sex-based differences in PAH have not been well studied.

The increased pulmonary vascular resistance in PAH has been attributed to 2 major factors, vasoconstriction and remodeling of the vascular wall. In severe progressive PAH, the fixed obstructive vascular remodeling, rather than vasoconstriction, is thought to be the major cause of elevated resistance ultimately resulting in right heart failure and death. However, recent studies in animal models of severe occlusive PAH showed that a sustained Rho kinase–mediated vasoconstriction is the primary determinant of increased pulmonary vascular resistance. In patients with PAH that were refractory to other vasodilators, the administration of the Rho-kinase inhibitor fasudil decreased pulmonary arterial pressure. These recent studies are important because they suggest that the role of vasoconstriction in late stages of pulmonary hypertension may be underappreciated. Important to this concept is evidence that pulmonary vascular tone is modulated by factors released from the endothelium. Our previous studies used a rabbit model and demonstrated enhanced endothelium-dependent contractions to arachidonic acid and methacholine in pulmonary arteries from females compared with males. Several other studies suggested that a cyclooxygenase metabolite of arachidonic acid mediates endothelium-dependent contractions of blood vessels. However, in our previous study, pretreatment with the cyclooxygenase inhibitor indomethacin only partially blocked contractions in the females compared with an almost complete attenuation in the males. The combined cyclooxygenase/lipoxygenase (LO) inhibitor BW 755C completely blocked contractions in the females. Therefore, the current study was designed to more completely characterize the role of arachidonic acid metabolites in the sex-related differences.
in vascular responses in males versus females. Studies addressed the specific hypothesis that differences in arachidonic acid metabolism by LO(s) contribute to the increased endothelium-dependent pulmonary vas constrictor in females compared with males. Although not designed to specifically study PAH, the studies lay the fundamental groundwork to further investigate a novel vasoconstrictor signaling pathway that potentially contributes to the disease.

Methods

Animals

Animal protocol was approved by the Medical College of Wisconsin Institutional Animal Care and Use Committee, and procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (1996). Two-month-old female and male New Zealand white rabbits were obtained from New Franken Rabbity (New Franken, WI) or Kuiper Rabbit Ranch (Gary, IN). The animals were housed in the Medical College of Wisconsin Biomedical Research Facilities and maintained on a standard rabbit chow diet and given tap water ad libitum.

Vascular Reactivity

Pulmonary artery tissue was isolated as described previously.6 Briefly, female and male New Zealand white rabbits were euthanized (sodium pentobarbital, 120 mg/kg, IV), the heart and lungs removed as a unit, and they were placed immediately in a Kreb bicarbonate buffer of the following composition (in mmol/L): NaCl 118.0, KCl 4.0, CaCl2 3.3, NaH2PO4 24.0, KMPO4 1.4, MgSO4 1.2, and glucose 11.0 (pH 7.4). The main pulmonary artery was identified at its origin from the right ventricle, and both left and right pulmonary arteries were dissected to their most distal end. The pulmonary artery distal to the first branching of the left or right pulmonary artery was used, and this is referred to as the intrapulmonary artery. After dissection, the tissue was cleaned of adherent lung parenchyma and connective tissue using care not to disturb the endothelial layer. Rings of intrapulmonary artery were obtained (2 to 3 mm) and suspended in 5-mL organ baths containing Kreb bicarbonate buffer (warmed to 37°C) and continuously aerated with a 95% O2-5% CO2 mixture. Isometric tension was measured as described previously.6 Resting tension was adjusted to the length-tension maxima for each rabbit by increasing the length of the rings in a stepwise fashion and measuring tension. The resting tension was 1.0 g and was not different between females and males. Vessels equilibrated at resting tension for 1 hour. Isometric tension was measured as described previously.6 Resting tension was 1.0 g and was not different between females and males.

LC/Electrospray Ionization/Mass Spectrometry

The production of 15-, 12-, and 5-HETE were quantified using LC-electrospray ionization-mass spectrometry (Agilent 1100 LC/MSD, SL model). For these studies, pulmonary arteries (10 to 30 mg) from females and males were incubated for 15 minutes at 37°C in HEPES buffer containing vehicle or arachidonic acid (10−6 mol/L) plus A23187 (20 μmol/L). After incubation, HEPES buffer was removed, internal standard ([15H]5-HETE) was added, and the buffer was acidified to pH 3.0 with glacial acetic acid and extracted over BondElut octadecylsilica extraction columns as described above. The HETEs were eluted with 6 mL of ethyl acetate and then were evaporated to dryness under N2 and stored at −80°C until analysis. Samples were analyzed by LC-electrospray ionization-mass spectrometry using a modification of a method described previously by Nithipatikom et al.14 Briefly, the samples were separated on a RP C18 column (Kromasil, 250×2 mm) using water/acetonitrile with 0.1% acetic acid as a mobile phase at a flow rate of 0.300 mL/min. The mobile phase started at 35% acetonitrile for 1 minute, increased linearly to 68% acetonitrile in 18 minutes, increased to 100% acetonitrile in 15 minutes, and held for 10 minutes. Drying gas flow was 12 L/min, drying gas temperature was 350°C, nebulizer pressure was 35 psi gauge, vaporizer temperature was 325°C, capillary voltage was 3000 V, and fragmentor voltage was 90 V. Detection was made in the negative mode. For quantitative measurements, m/z=319 and 327 ions were used for 15-HETE and [15H]5-HETE, respectively. Standard curves were typically constructed over a range of 5 to 500 pg per injection. The concentrations of 15-, 12-, and 5-HETE in samples were calculated by comparing their ratios of peak areas with the standard curves. The results were normalized to wet tissue weight and expressed as picograms per milligram.

Polyacrylamide Gel Electrophoresis and Western Blotting

Pulmonary artery was obtained from rabbits as described above. Lysates were prepared by homogenizing samples in a buffer containing 20 mmol/L of HEPES, 255 mmol/L of sucrose, 1 mmol/L of EDTA, and 100 μmol/L of phenylmethylsulfonylfluoride (pH 7.4). Protein lysates (30 μg) were analyzed by SDS-PAGE by the method of Laemmli13 using a 4% acrylamide stacking gel and 10% acrylamide resolving gel. Proteins were electrophoretically transferred to nitrocellulose, and membrane was blocked for 2 hour at room temperature with 2% nonfat dry milk in Tris-buffered saline (20 mmol/L of TRIZMA hydrochloride and 500 mmol/L of NaCl [pH 7.5]) with Tween-20 before incubation with a polyclonal 15-LO antibody, a 5-LO antibody, or a 12-LO antibody. Primary antibodies were used at a dilution of 1:1000 (15- and 12-LO) or 1:250 (5-LO) overnight at 4°C. After washing, blots were incubated for 1 hour with dryness under nitrogen, and stored at −40°C until analysis by reverse-phase (RP) high pressure liquid chromatography (LC; HPLC; Beckman Instruments). Recovery of arachidonic acid metabolites by this extraction procedure is >95%. Separation of metabolites of arachidonic acid was accomplished with RP-HPLC and solvent system I, where solvent A was water and solvent B was acetonitrile containing 0.1% glacial acetic acid. The program consisted of a 40-minute linear gradient from 50% B in A to 100% B. RP separations used a Nucleosil C18, column (5 μmol/L, 4.6×250 mm, Phenomenex Inc), and the flow rate was 1 mL/min. Radioactivity of column eluate was collected in 0.2-mL aliquots and measured by liquid scintillation spectrometry. To identify HETE metabolites produced by males and females, fractions 95 to 120 collected from RP-HPLC using solvent system I were pooled, acidified, extracted with cyclohexane/ethyl acetate (50/50), and rechromatographed on normal-phase HPLC using solvent system II. Solvent A was hexane containing 0.1% glacial acetic acid, and solvent B was hexane containing 0.1% glacial acetic acid and 2% isopropanol. The program consisted of a 40-minute linear gradient from 25% solvent B in A to 75% B in A. The flow was 3 mL/min. Radioactivity of column eluate was collected in 0.6-mL aliquots and measured by liquid scintillation spectrometry.

14C-Arachidonic Acid Metabolism

To identify the arachidonic acid metabolites produced by female and male pulmonary arteries, segments of intrapulmonary artery (70 mg of wet weight) were incubated in HEPES buffer (in mmol/L: HEPES 10, NaCl 150, KCl 5, CaCl2 2, MgCl2 1, and glucose 6 [pH 7.4]) containing 14C-arachidonic acid (0.05 μCi, 10−6 mol/L) and the calcium ionophore A23187 (20 μmol/L) for 15 minutes at 37°C. After incubation, HEPES buffer was removed, acidified to pH 2.0 with glacial acetic acid, and extracted over BondElut octadeceylsilica extraction columns as described previously.13 Arachidonic acid metabolites were eluted with 6 mL of ethyl acetate, evaporated to
secondary antibody (horseradish peroxidase–conjugated goat anti-rabbit IgG antibody for 12- and 5-LO or horseradish peroxidase–conjugated donkey antishcific IgG antibody for 15-LO) at a dilution of 1:5000. After again washing with Tris-buffered saline with Tween-20, blots were incubated for 2 minutes with Thermo Scientific Pierce enhanced chemiluminescence Western blotting substrate. Membranes were subsequently exposed to Kodak Biomax MR imaging film and developed. Prestained protein markers (Sigma) were used for molecular mass determination. The anti–15-, 12-, and 5-LO antibodies give immunoreactive bands corresponding to 75-

Role of Estrogen on 15-LO Expression and $^{14}$C-Arachidonic Acid Metabolism

Male pulmonary arteries were isolated as described above. Vessels were incubated in HEPES buffer containing vehicle or 17β-estradiol (1 μmol/L) for 18 hours at 37°C. Lysates were prepared for analysis of 15-LO protein by Western blotting or intact vessel segments were incubated with $^{14}$C-arachidonic acid for identification of 15-HETE by RP-HPLC.

Materials

$^{14}$C-Arachidonic acid was obtained from New England Nuclear. Arachidonic acid, A23187, and 17β-estradiol were from Sigma Chemical Co. 15-, 12-, and 5-LO antibodies, as well as 15-HETE, 5-HETE, and PD146176, were from Cayman Chemical. Arachidonic acid, A23187, PD146176, and HETEs were prepared in ethanol sparged previously with nitrogen. Stock solution and dilutions were made fresh for each experiment and kept on ice and under a nitrogen atmosphere.

Statistics

Data are expressed as the mean±SEM. Statistical analysis of the vascular reactivity data was performed by a 2-way ANOVA followed by Bonferroni posttest. A Student t test was used to analyze the band intensities from immunoblots and the LC-mass spectometry data. Values were considered significant at $P<0.05$.

Results

Arachidonic acid caused endothelium-dependent, concentration-related contractions of the rabbit pulmonary artery from both female and male rabbits (Figure 1), confirming our earlier observations.$^{5}$ The maximum contractions were greater in the female rabbits (maximal response: 101±10% versus 57±11%, female versus male; Figure 1). The EC$_{50}$s for arachidonic acid–induced contractions were 0.26±0.09 and 0.36±0.24 μmol/L for females and males, respectively. The contractions were inhibited by removal of the endothelium to a similar extent in both females and males (data not shown$^{5}$).

Studies next determined whether the protein expressions of LOs were different in pulmonary lysates obtained from females and males. Immunoblots revealed similar 12-LO expressions (Figure 2A), whereas there was a greater expression of both 15-LO (Figure 2B) and 5-LO (Figure 2C) in females compared with males. Segments of pulmonary arteries from female and male rabbits were incubated with $^{14}$C-arachidonic acid, and the metabolites were resolved by RP-HPLC using solvent system I. A representative chromatogram is shown in Figure 3. Female and male rabbit pulmonary arteries synthesized radioactive products that comigrated with the prostaglandins (fractions 10 to 50), dihydroxyeicosatetraenoic acids and dihydroxyeicosatetraenoic acids (fractions 70 to 85), and the HETEs (fractions 90 to 120). In the females, there was increased production of products migrating with the HETEs and dihydroxyeicosatetraenoic acids/dihydroxyeicosatetraenoic acids. Fractions corresponding with HETEs were collected, extracted with cyclohexane/ethyl acetate, and rechromatographed on normal-phase HPLC using solvent system II. A representative chromatogram is shown in Figure 4. The major HETEs produced by the rabbit pulmonary artery comigrated with 12-, 15-, and 5-HETE. The production of all 3 of the HETEs appeared greater in females compared with males. However, a better method for quantifying the HETEs is to use LC-electrospray ionization-mass chromatography, and results from this method are summarized in the Table. The basal production of 15-HETE was greater in females compared with males. No differences in the basal production of 12- and 5-HETE were measured in females and males. A23187 increased the production of all of the measured HETEs in both females and males. The increased production of 15-HETE by A23187 was greater in females compared with males. Although there was a trend for 5-HETE to be greater in female vessels incubated with A23187 compared with males, the increase was not significant. A23187-stimulated 12-HETE production was not different between female and males.

The ability of 5- and 15-HETE to contract pulmonary arteries from female and male rabbits is shown in Figure 5. There was no effect of 5-HETE to elicit contraction in pulmonary arteries (Figure 5, top). In contrast, 15-HETE produced a concentration-dependent vasoconstriction that was significantly greater in females compared with males (Figure 5, bottom). Additional studies tested the hypothesis that 5-HETE may potentiate contractions to 15-HETE. When 15-HETE contractions were repeated in the presence of 5-HETE, no enhancement of contraction was seen when compared with 15-HETE alone (Figure 5, bottom). To further establish 15-HETE as the mediator contributing to enhanced
arachidonic acid–induced contractions in the females compared with males, the effect of the specific 15-LO inhibitor, PD 146176 (12 μmol/L), was tested on vascular reactivity and arachidonic acid metabolism in pulmonary arteries from female rabbits. As shown in Figure 6, PD146176 attenuated arachidonic acid–induced contractions (maximal contraction: 93/11006 4% versus 57/11006 10%, control versus PD146176). The maximal contraction in the presence of the 15-LO inhibitor was similar to that observed in untreated male pulmonary arteries (Figure 1). HPLC analysis showed that this concentration of PD146176 also decreased 15-HETE production (data not shown).

In a final series of experiments, pulmonary arteries obtained from male rabbits were treated for 18 hours with 17β-estradiol (1 μmol/L) and then incubated with 14C-arachidonic acid as described above. Results (Figure 7) clearly showed an increase in LO products in the 17β-estradiol–treated vessels (dotted line) compared with untreated control. Interestingly, there were also other peaks that increased after estrogen treatment and some of these same peaks (dihydroxyeicosatrienoic acids/dihydroxyeicosatetranoic acids) were greater in females compared with males (Figure 3). Furthermore, the protein expression of 15-LO increased after 17β-estradiol treatment (Figure 8).

**Discussion**

Our previous studies suggested that enhanced pulmonary artery contractions to arachidonic acid in females compared with males were mediated by an LO metabolite. The purpose...
of the current study was to systematically characterize the LO pathways in female and male pulmonary arteries. First, it was shown that the protein expression of both 15- and 5-LO was greater in females compared with males. Although pulmonary arteries from both male and females produced the corresponding LO metabolites, 15- and 5-HETE, only the synthesis of 15-HETE was enhanced in females. The increase in 15-HETE was correlated with an increased 15-HETE–mediated pulmonary artery vasoconstriction in females compared with males. These findings are significant because it is the first report that the 15-LO pathway is regulated in a sex-specific way. These results may impact what is known about the sex differences in the incidence of PAH.

The enhanced vasoconstriction in females compared with males could be because of increased production of the vasoconstrictor compound, 15-HETE. Alternative explanations are also possible. The arachidonic acid metabolism studies showed that there are other radioactive peaks migrating with dihydroxyeicosatrienoic acids/dihydroxyeicosatetraenoic acids that are greater in females compared with males. These products were not tested for vascular responses but could contribute to enhanced response in females compared with males. Male rabbits not only produced less 15-HETE than females but also had decreased maximal vasoconstrictor responses, suggesting that another possible explanation for differences in the responses in males and females may relate to a decreased sensitivity of male vessels to 15-HETE.

In women, the incidence of certain forms of PAH has been most recently reported to be 4-fold greater than observed.
in men. Results from the Registry to Evaluate Early and Long-Term Pulmonary Arterial Hypertension Disease Management, which enrolled >2500 patients with PAH from across the United States, found that ≈80% were females. Mechanisms to explain the increased incidence are scarce, but there are suggestions that hormonal changes may contribute to the pathogenesis of the disease. This is based on studies which showed that, in a small percentage of women taking oral contraceptives, pulmonary hypertension developed.\textsuperscript{19,20} There is also evidence that PAH was more prevalent in postmenopausal women receiving hormone replacement therapy.\textsuperscript{21} Others studies have refuted the association of oral contraceptives and PAH.\textsuperscript{22,23} Despite these controversies, it is well known that the sex hormone estrogen modulates vascular reactivity in a variety of different vascular beds (for review, see Reference \textsuperscript{24}). In the isolated perfused rat lung circulation, Farhat and Ramwell\textsuperscript{25} showed that the thromboxane mimetic U46619 produced a greater pressor response in females compared with males. Infusion of 17β-estradiol into the rat lung intensified the pressor response to U46619. Chan et al\textsuperscript{26} also reported a sex difference in isolated rat pulmonary arteries to raloxifene, a selective estrogen receptor modulator. Raloxifene produced greater relaxations in males compared with females. However, other studies in animal models of pulmonary hypertension have shown beneficial effects of estrogen in that females were less likely to develop PAH, and/or removal of estrogen by ovariectomy prevented the development of pulmonary hypertension.\textsuperscript{27–29} Thus, there remains an obvious inconsistency regarding the role of estrogen in pulmonary hypertension.\textsuperscript{30} It should be emphasized that the rabbits for the present study were young unmated males and females. Mean concentrations of 17β-estradiol and progesterone in female rabbits are 29.9±11.4 pg/mL and 17.0±2.05 ng/mL, respectively. These sex hormones are not detected in males. However, when male pulmonary arteries were incubated in vitro with 17β-estradiol, the expression of 15-LO and 15-HETE production increased compared with untreated control vessels. These data are important because they suggest that one mechanism to explain the increased vascular responses in females compared with males involves a regulation of the 15-LO by estrogen. Estrogen also increased other products in the arachidonic acid pathway, and additional studies are needed to address whether these products contribute to pulmonary vascular function.

15-LO is implicated in the pathogenesis of several diseases, including atherosclerosis, asthma, and cancer (for review see Reference \textsuperscript{31}). There is less known about the role of 15-LO in the pulmonary circulation. However, 15-LO is expressed in many cell types found in the human lung, including airway epithelium, eosinophils, reticulocytes, macrophages, and pulmonary artery.\textsuperscript{32–36} Zhu et al\textsuperscript{37} showed that neonatal rabbit pulmonary arteries exposed to chronic hypoxia produced 15-HETE. Other studies in humans indicated that an increase in oxidative stress in severe PAH was associated with an increased production of 5-, 15-, and 12-HETE in lung tissue extracts.\textsuperscript{38} Genetic studies have identified a mutation in the bone morphogenetic protein receptor 2 in most cases of familial PAH.\textsuperscript{39,40} Because only 10% to 20% of individuals with this mutation develop PAH, it is likely that other genes and/or environmental factors are necessary to trigger the disease.\textsuperscript{41} A recent study using heterozygous bone morphogenetic protein receptor 2-mutant mice reported that increased susceptibility to PAH occurred only in mice treated with recombinant adenovirus overexpressing 5-LO.\textsuperscript{42} Interestingly, the female rabbits in the current study also had increased protein expression of 5-LO and a trend for greater concentrations of 5-HETE. Although 5-HETE did not contract pulmonary arteries of either males or females, arachidonic acid is metabolized by 5-LO to the leukotrienes, which are potent inflammatory compounds.

![Figure 7. Representative chromatogram showing the effect of 17β-estradiol (1 μmol/L, 18 hours, 37°C) on the metabolism of 14C-arachidonic acid by male pulmonary arteries. Migration times of known standard eicosanoids are shown. This experiment was repeated 3 times with similar results. Results are presented as counts per minute per milligram of tissue to control for vessel weight. THETA indicates trihydroxyeicosatrienoic acid; HEETA, hydroxyepoxyeicosatrienoic acid.](image)

![Figure 8. 15-LO expression in male pulmonary arteries incubated with 17β-estradiol (1 μmol/L, 18 hours, 37°C). Representative Western immunoblots for 15-LO and β-actin in lysates (30 μg of protein) are shown as an inset for the bar graph. The bar graph shows the 15-LO band density normalized to the β-actin band density. Values represent mean±SEM for n=6. *P<0.05.](image)
involved in allergy and asthma. The cysteinyl-leukotrienes constrict bronchial and pulmonary smooth muscle. There is no evidence that pulmonary artery endothelial cells produce leukotrienes. The present work showed that the combination of 5- and 15-HETE did not produce greater vasoconstriction than that of 15-HETE alone. While speculative at this point, it is still possible that products of 5- and 15-LO may contribute to other aspects related to the development of PAH, like vascular remodeling. This idea is supported by studies showing that both 15-HETE and 5-HETE increase cell proliferation.

Perspective
In summary, PAH is a relatively rare but medically significant disease that occurs more frequently in young women and is usually catastrophic for those afflicted. Mechanisms to explain the predominance in women are not clearly understood. Using a rabbit model, our data indicate that the mechanism of endothelium-dependent pulmonary vasoconstriction is different in females compared with males. The mechanisms contributing to sex-specific differences in vascular function are diverse and complex. Although mammalian LOs have been implicated in the pathogenesis of a number of diseases, including asthma, diabetes mellitus, atherosclerosis, and cancer, there are no known studies that have carefully characterized the LO pathways in a sex-specific manner. Our studies provide the first evidence of a sex-specific difference in 15-LO in pulmonary arteries. Future studies designed to understand the mechanisms regulating this pathway may provide insight into the increased incidence of PAH in females.

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Disclosures
None.

References

Pfister 15-Lipoxygenase and Gender


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