Chronic Hypoxia Activates Lung 15-Lipoxygenase, Which Catalyzes Production of 15-HETE and Enhances Constriction in Neonatal Rabbit Pulmonary Arteries

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Abstract—Hypoxia causes localized pulmonary arterial (PA) constriction to divert blood flow to optimally ventilated regions of the lung. The biochemical mechanisms for this have remained elusive, especially during prolonged exposures to reduced $P_{O_2}$. We have evidence that subacute hypoxia activates 15-lipoxygenase (15-LO) in small PAs of neonatal rabbits maintained for 9 days in hypoxic environments ($F_{O_2}=0.12$ U) compared with siblings raised under normoxia. PA microsomal products of 15-LO, 15-hydroxyeicosatetraenoic acid (HETE), 11,14,15-trihydroxyeicosatrienoic acid (THETA), and 11,12,15-THETA were identified by gas chromatography/mass spectrometry. Increased amounts of these products are synthesized in vivo and in vitro by the lungs of animal raised in hypoxic versus normoxic environments. 15-HETE formation is attenuated by lipoxygenase, but not cytochrome P450 or cyclooxygenase inhibitors. Activation of 15-LO is associated with translocation of the enzyme from the cytosol to membrane as seen by Western immunoblotting. Immunohistochemical analysis demonstrates that 15-LO expression is clearly localized in vascular cells in lungs from normoxic and hypoxic kits. 15-HETE causes concentration-dependent constriction of PA rings from animals exposed to hypoxic but not normoxic environments. In addition, lipoxygenase inhibitors reduce phenylephrine-induced constriction of PA rings. Therefore, subacute hypoxia increases expression of and activates 15-LO, and enhances sensitivity of pulmonary arteries to its product, 15-HETE. Because 15-HETE is a constrictor in this vascular bed, it may play an important role in hypoxia-induced pulmonary vasoconstriction in rabbit kits. Although a clear causal relationship remains to be demonstrated, these data suggest a previously unrecognized role for 15-LO in hypoxic vasoconstriction in neonatal mammals. (Circ Res. 2003;92:lll-lll.)

Key Words: hypoxia • 15-lipoxygenase • 15-hydroxyeicosatetraenoic acid • hypoxic pulmonary vasoconstriction • trihydroxyeicosatrienoic acid • eicosanoids

Hypoxic vasoconstriction (HPV) is an essential mechanism to balance perfusion with ventilation and is unique to the pulmonary circulation. This action regulates one of the most important physiological parameters in mammals, arterial oxygen tension. The acute response is biphasic in isolated perfused vessels, whereas sustained hypoxia in vivo can lead to structural remodeling and matrix deposition in pulmonary arteries, which results in increased arterial tone.1 The biochemical mechanisms that have been hypothesized to underlie this response are varied,1-3 except for the consensus that HPV is a “multifactorial manifestation, with elements of energy, oxygen and lipid metabolism.”1

Recently, specific candidates that mediate HPV have been carefully examined (eg, Archer et al5). Nitric oxide (NO), cytochrome P450 metabolites, leukotrienes, or direct effects of oxygen on ion channels in vascular smooth muscle cells have been proposed as modulators/mediators of acute hypoxic responses of the pulmonary vasculature, but none appears to account for all the features of hypoxic vasoconstriction.2 Our overriding goal was to understand adaptation of pulmonary arteries (PAs) to chronic hypoxia. Arachidonic acid (AA) metabolites modulate vasoactivity, and oxygen is a substrate in eicosanoid synthesis, which makes these products ideal candidates to contribute to HPV. 5-hydroxyeicosatetraenoic acid (HETE), the major metabolites of the 5-lipoxygenase pathway, is increased in macrophages exposed to hypoxia.4 However, hypoxic pulmonary vasoconstriction is not affected by the 5-lipoxygenase inhibitor MK886 in perfused rabbit lungs.5

To examine the effects of subacute hypoxia on pulmonary metabolism of AA and the reactivity of PAs to these metabolites, we used an animal model in which rabbit kits were exposed to low $F_{O_2}$ (0.12) for 9 days after birth.6 We selected this model because, although we were interested in mediators of acute increases in pulmonary vasomotor tone, continued exposure to hypoxia in this model results in...
increased right ventricular mass by 50 to 60 days of sustained hypoxia.6,7 Lung tissue from animals exposed to subacute hypoxia relative to those maintained in a normoxic environment exhibits enhanced capacity to synthesize a number of products from AA. Striking increases were observed in the synthesis of 15-lipoxygenase (15-LO) products 15-HETE, as well as trihydroxyeicosatetraenoic acids (THETAs) that are also products of 15-LO.8 Fluorescent assays8,10 of endogenously formed eicosanoids confirmed enhanced synthesis of 15-HETE in pulmonary arteries from hypoxic relative to normoxic kits. Chronic hypoxia induced translocation (and subsequent activation) of 15-LO from the cytosol to the microsomal fraction, and in addition, it also increased activity of PAs to 15-HETE. 15-HETE has recognized vasoactive properties in systemic and pulmonary vascular beds, so is well suited to be a participant in HPV. We suggest that activation of 15-LO as demonstrated at the protein and product levels and increased sensitivity to constriction of PAs by 15-HETE may contribute to pulmonary HPV in neonatal rabbits.

Materials and Methods

Reagents
Arachidonic acid was purchased from Cayman. [1-14C]-arachidonic acid (50 mCi/mmol) was purchased from New England Nuclear. Primary antibody for 15-lipoxygenase raised in sheep (Cayman) and 12-lipoxygenase (Cayman) were used for Western blotting. Anti CD-11a was purchased from Pharmingen. Secondary antibodies were purchased from Sigma. All other reagents were from common commercial sources.

Animals Model
Rabbits were housed in the Animal Resource Center, which is fully accredited by the AALAC. Neonatal rabbits (kits) were born to pregnant New Zealand White females in a normoxic environment and immediately after their first feeding transferred to an environmental chamber in which the FIO 2 was either reduced to 0.12 (hypoxia) or 0.21 (normoxia).7 Kits from both groups were returned to their mother (in a normoxic environment) for 20 minutes once a day for feeding and then replaced in the environmental chambers. After 9 days, kits were anesthetized with 4% halothane, and the chest was opened for removal of the heart and lungs en bloc.

Histochemical Staining of Lung Sections
Lungs were perfused in vivo with sucrose (0.25 mol/L) until free of blood, then removed and frozen on dry ice. Tissue was sectioned (8 μm in thickness) in a cryostat, mounted on slides, fixed with 4% paraformaldehyde, and reacted with pentachrome stain. The sections were viewed and photographed with a Eclipse 600 Nikon microscope with an attached digital camera.

Harvesting of PAs and Peripheral Lung for Biochemical Studies and Cell Culture
After sucrose perfusion and removal en bloc from the chest, peripheral lung tissue dissected free of large airways and vessels was homogenized and microsomal and cytosolic fractions were prepared by differential centrifugation at 9000g for 30 minutes followed by 100 000g for 90 minutes11 for 14C-AA conversion studies. Proteins were quantitated according to the method of Bradford.12 In some experiments, PAs of 1.5- to 0.15-mm diameter were microdissected free of surrounding tissue and adventitia for fluorescent assays or for isolation of endothelial or vascular smooth muscle cells.

Metabolism of AA In Vitro
Lung microsomes (prepared as described above) were incubated at a final protein concentration of 200 mg/mL (200 microliters final volume) for 30 minutes at 37°C with [1-14C] arachidonic acid (2 μmol/L), 1 mmol/L NADPH, and an NADPH regenerating system containing 10 mmol/L isocitrate and 0.1 U/mL isocitrate dehydrogenase. Reactions were terminated by acidification and extracted for resolution by HPLC.13 Where indicated, inhibitors were preincubated with PA microsomes at room temperature for 30 minutes.

High-Pressure Liquid Chromatography (HPLC) and Gas Chromatography/Mass Spectrometry (GC/MS)
Extracted eicosanoid products of 14C-AA were resolved by rpHPLC as described.6,8,13 In System I, solvent A was water, and solvent B was acetonitrile containing 0.1% glacial acetic acid. The program was a 40-minute linear gradient from 50% solvent B in A to 100% solvent B. Radioactive fractions were either rechromatographed on rpHPLC with the use of System II for the THETA metabolites8 or normal phase (np) HPLC with Method 313 for 15-HETE. Solvent A in System II was water containing 0.1% glacial acetic acid, and solvent B was acetonitrile. The program consisted of a 5 minute isocratic phase with 35% solvent B in A followed by a 35 minute linear gradient to 85% solvent B in A. For Method 3, solvent A was hexane containing 0.1% glacial acetic acid and solvent B was hexane containing 2% isopropanol and 0.1% glacial acetic acid. HETEs were eluted from a Nucleosil silica column over 40 minutes with a linear gradient of 25% B in A to 75% B in A.

Radioactive peaks for both THETAs and 15-HETE peaks were separately collected, derivatized to their methyl esters trimethylsilyl (TMS) ether, and analyzed by gas chromatography mass spectrometry in positive ions chemical ionization (PCI) mode.8

Measurement of Endogenous 15-HETE in Cultured Cells and PAs
Endothelial cell cultures from microdissected PAs were obtained by methods described previously by us14 and used before passage 3. Smooth muscle cells were isolated and cultured from dissected PAs by the protocol described13 and also used before passage 3. Endogenous lipids were extracted from cells and PAs, tagged with fluorescent label, and identified by rp-HPLC using methods we developed for this purpose.10,16

Immunohistochemical Staining of 15-LO in Lung
Sections of lung were stained with antibody specific to 15-lipoxygenase as described,14 and the staining was quantitated with the software package, Metamorph.

Immunospecific Protein Identification
Cytosolic and microsomal suspensions from lung homogenates were used for Western blotting with specific antibody for 15-LO and 12-LO (see Reagents) as described.11

Tension Studies of Pulmonary Arterial Rings
Microdissected PAs were cut into rings ~0.5 to 1.5 mm diameter and examined for isometric contractile responses as described.16 Rings were attached to tension-measuring devices by tungsten wire hooks (0.005 inch diameter) initially loaded with 0.3 g resting tension. Endothelial cell function was assessed in randomly selected rings based on transient relaxation to acetylcholine. 15-HETE, U46619, or vehicle were added from a stock solution in ethanol (vehicle) to final concentrations to determine a concentration-response relationship. In separate experiments, the concentration-response to incremental amounts of phenylephrine from 10^-8 to 10^-7 mol/L was determined. After the phenylephrine was washed out, and the baseline tension of the rings recovered, rings were incubated with 5 μmol/L cinnamyl-3, 4 dihydroxy-cyanoanamine (CDC) or 30 μmol/L nordihydroguaiaretic acid (NDGA) for 30 minutes and phenylephrine concentration responses repeated.
Statistical Methods
Comparisons of data such as rates of conversion of substrate to 15-HETE in normoxia- or hypoxia-exposed tissues were accomplished by unpaired Student’s t tests. When more than 2 conditions were studied (eg, ring tensions in resting state, after exposure to 2 concentrations of 15-HETE, etc.), we used analysis of variance with post hoc tests when permitted to detect differences between groups.

Results
Morphology of the Lungs Is Not Altered by Exposure of Kits to Subacute Hypoxia
Ratios for lung weight to body weight (11.6±0.3 versus 11.1±0.6; n=8 each), wet weight to dry lung weight (5.3±0.3 versus 5.1±0.2; n=8 each), and PA wall to lumen areas (0.76±0.04 versus 0.78±0.04; n=30 each) in kits exposed to normoxic or hypoxic environments were not significantly different. Histology of lungs from normoxic and hypoxic kits was indistinguishable, with no evidence of edema, cellular infiltrates, or alveolar filling in hypoxia-exposed lungs. Pulmonary artery measurements, from kits exposed to hypoxia or normoxia, were processed with the Image Tool Program (http://ddsdx.uthscsa.edu/dig/itdesc.html) and showed similar structure.

Increased Synthesis of Derivatives of AA in Microsomes Isolated From Lungs Exposed to Hypoxia
Lungs from kits exposed to hypoxia (Hyp-L) and controls raised under normoxia (Nor-L) were used to prepare lung microsomes, which were incubated with [1-3H] AA. Derivatives from these reactions were resolved by rpHPLC,11 which showed two major peaks that were increased by chronic exposure to hypoxia (Figures 1A and 1B). The first peak migrated with standard 11,12,15- and 11,14,15-THETAs and the second with authentic 15-HETE, which was the major product and was increased greater than 3-fold in Hyp-L samples (13.35±1.03 pmol/mg protein per min, n=7) versus Nor-L (3.98±0.55 pmol/mg protein per min, n=22) (Figure 1C). We did not see differences in the activity of soluble (cytosolic) 15-LO (an enzyme that catalyzes formation of 15-HETE) between Nor-Ls and Hyp-L (Figure 1D).

Confirmation by GC/MS of THETAs and 15-HETE Synthesized by Microsomes From Hyp-Ls
To identify these products by GC/MS, microsomes incubated with [1-14C] AA from Hyp-Ls were first extracted and resolved by rpHPLC System I (Figure 2A, top), and peak fractions (27–35) were rechromatographed by rpHPLC System II. A standard positional isomer for 11,14,15-THETA comigrated with fraction No. 1 (Figure 2A, bottom). This fraction contained two major products that were collected and analyzed by GC/MS. The fragmentation patterns were consistent with the two products being 11,12,15- and 11,14,15-THETAs (see Figure 2B).

To confirm the dominant peak synthesized by Hyp-Ls was 15-HETE, fractions 100 to 125 from rpHPLC System I (Figure 3A, top) were collected, extracted, and rechromatographed on npHPLC by Method 3 (Figure 3A, bottom). The major radioactive peak was collected, and the product derivatized and analyzed by GC/MS. Fragments with the expected mass-to-charge ratios consistent with the methyl ester TMS-ether derivative of 15-HETE were observed, confirming the rpHPLC result (see Figure 3B).

15-HETE Is Synthesized by 15-LO
Microsomes from hypoxia-treated rabbit kits were preincubated with inhibitors for enzymes known to metabolize AA and assayed for production of 15-HETE. The synthesis of 15-HETE was reduced by inclusion of the lipoxigenase inhibitor, 5 μmol/L CDC (Figure 4A).17 Cytochrome P450 inhibitors, including 200 μmol/L secobarbital (specific for 2B isoforms),18 200 μmol/L disulfiram (specific for 2E isoforms),19 200 μmol/L α-naphthoflavone (specific for 1A1 isoforms),20 and 10 μmol/L 17-ODYA (specific for omega hydroxylase and epoxygenases)21 did not block the formation of 15-HETE in Hyp-L microsomes. In addition, inhibition of cyclooxygenase with 100 μmol/L indomethacin22 had no effect on conversion of AA to 15-HETE (Figure 4A). We also assayed for presence of polymorphonuclear leukocytes (PMNs) in the microsomal preparations because these cells contain 15-LO.23 A primary antibody directed against the PMN-specific marker CD11a did not detect signal in the microsomes (Figure 4B) from either Hyp-Ls or Nor-Ls.

Hypoxia-Induced Increases in 15-LO Are Localized to PAs
To localize 15-LO, cryosectioned lung samples were reacted with specific antibody for 15-LO. 15-LO uptake was detected in airways as well as pulmonary arteries (Figure 5), in both the endothelium and vascular smooth muscle cells. This signal is enhanced in sections derived from Hyp-Ls over that of Nor-Ls (relative densitometric values in 11 PAs from hypoxic kits compared with 16 normoxic kit lungs 6.1±0.6 versus 3.3±0.4; P<0.01), and in both conditions appears to be stronger in endothelial than vascular smooth muscle cells.

Endogenous 15-HETE Is Higher in Hyp-PAs
In order to verify the vascular localization demonstrated by our immunohistochemical studies and hypoxic-induced activation of 15-LO, we measured endogenous levels of 15-HETE in PAs from Hyp-Ls and Nor-Ls by the fluorescent-tagged HPLC method.9,10 Our results show an increase in endogenous 15-HETE in PAs of kits raised in a hypoxic environment compared with their siblings raised in normoxia (Figure 6A). To begin to examine the cellular sources of 15-HETE production within the pulmonary arteries, we performed additional fluorescent assays with primarily isolated, passage 1 or 2 endothelial cells or vascular smooth muscle cells (both cultured in normoxic conditions) and observed greater concentrations of 15-HETE in the endothelium as compared with the smooth muscle (Figure 6B).

Chronic Hypoxia Induces Translocation of 15-LO From Cytosol to Membranes
15-LO (expected molecular size ≈74 kDa) was increased in the microsomal fraction of Hyp-Ls compared with those of Nor-Ls (Figure 7A) as determined by Western Analysis. The relative amounts of this enzyme partitioned between cytosol
and microsomal fractions in Nor-Ls and Hyp-Ls are shown in Figures 7B and 7C. In Hyp-Ls, 15-LO was concentrated in the microsomes (Figure 7B), whereas in Nor-Ls, 15-LO was localized in the cytosol (Figure 7C). The ratios of cytosolic to microsomal 15-LO were 1.61 ± 0.24 in Nor-Ls versus 0.78 ± 0.15 in Hyp-Ls. In contrast, we did not see changes in the distribution of 12-LO between the cytosol or membrane associated fractions in Nor-Ls versus Hyp-Ls (Figures 7D and 7E).

**Reactivity of PAs to 15-HETE Is Increased by Hypoxia**

15-HETE in concentrations ≥10⁻⁷ mol/L increases tension of PAs at basal tone or after preconstriction with phenylephrine.
Figure 2. A, Top, rpHPLC fractionation of AA-metabolites from lung microsomes of rabbit kits exposed to hypoxia. Bottom, Rechromatographed products from the first major peak shown in the top. Peak labeled #1 was collected and analyzed by GC/MS. B, Two major products separated from Fraction #1 were analyzed by positive ion chemical ionization (PCI) GC/MS. Top and bottom, Fragmentation pattern of the methyl ester, TMS ether of 11,12,15-THETA, and 11,14,15-THETA, respectively. Major ions for both are (M+15584). 569 = M+1-15; loss of CH3 = 301 = M+283; loss of ((CH3)3)SiO)-CH(CH3)3-COOCH3. 283 = M+301; loss of CH3 = 173 = M+411; loss of ((CH3)3)SiO)-CH(CH3)3-COOCH3. Intensity of 283 m/z exceeds 173 m/z for 11,12,15-THETA, whereas the opposite is observed for 11,14,15-THETA.
over that of vehicle. Concentration-dependent constriction to 15-HETE is significantly greater in PAs from Hyp-Ls (Figure 8A). In contrast to these findings, the thromboxane mimetic U46619 shows reduced constriction in Hyp-PAs relative to Nor-PAs (Figure 8B). The substrate for 15-LO, arachidonic acid, caused modest constriction in PAs from both normoxic and hypoxic kits (data not shown).

Figure 3. A, Top, rpHPLC fractionation of AA-metabolites from lung microsomes of rabbit kits exposed to hypoxia. Bottom, Rechromatographed (by npHPLC) products from the fractions indicated by the bar in the upper panel. The 15-HETE peak was collected for analysis by GC/MS. B, GC/MS (PCI) profile representing the major ions for the methyl ester, TMS ether derivative of 15-HETE; 407 (M$^+$+1), 391 = M-CH$_3$; 317 = M-(CH$_3$)$_2$SiOH; 225 = M-CH-CH=CH-CH=CH-CH-(CH$_3$)$_2$-COOCH$_3$; 173 = (CH$_3$)$_2$SiO-CH-(CH$_2$)$_4$-CH$_3$. 15-HETE was also derivatized and comigrated with the product on GC and yielded a similar fragmentation pattern (not shown).

Figure 4. A, Inhibitors for CYP2B (secobarbital), CYP2E (disulfiram), CYP 1A1 (a-naphthoflavone), CYP450 ω-hydroxylase and epoxigenase (17-ODYA), cyclooxygenase 1 and 2 (indomethacin), and lipoxygenase (CDC) had no effect on conversion of AA into 15-HETE. Only lipoxygenase inhibitors blocked formation of 15-HETE. B, Western blot showing absence of PMN leukocytes in lung microsomes. Protein from the microsomes (50 μg) were analyzed for presence of the neutrophil marker CD11a. Lysates from blood cells (50 μg) demonstrated a distinct band corresponding to CD11, which was not present in microsomes from normoxic or hypoxic lung microsomes.
To examine the role of endogenously formed 15-HETE on PA tone, PA rings from hypoxia-treated rabbits were preincubated with vehicle, 5 μmol/L CDC (Figure 8C), or 30 μmol/L NDGA24,25 (Figure 8D) inhibitors for lipoxygenases. Phenylephrine-induced concentration-related constriction (with ED50 of 5×10⁻⁸ mol/L) is shifted to the right, and the maximal response is reduced (Figure 8D) by blocking lipoxygenase activity with NDGA. The decrease in response to PE in PAs treated with CDC is greater in arteries harvested from hypoxic kits compared with normoxic kits.

Discussion

15-HETE as a Mediator of HPV

Hypoxic pulmonary constriction was first described nearly a century ago, and a number of mechanisms have been proposed to explain it. Leukotrienes have been considered as potential mediators, but leukotriene C4, along with inhibitors that block its synthesis, does not effect HPV in rats.26 Cytochrome P450 pathways have been proposed as mediators responsible for triggering HPV because agents that bind to the CYP450 proteins (eg, CO and metapyrone) block HPV.27 However, in intact rat lungs, cytochrome P450 enzymes do not appear to be required for HPV.28 Another hypothesis is that the level of O₂ may have a direct effect on the K⁺ and Ca²⁺ channels of the vascular smooth muscle to elicit vasoconstriction.3 Inhibition of PASMC Kv channels by hypoxia may result in cellular depolarization triggering L-type Ca²⁺ channels to induce vasoconstriction. However, reactive oxygen species, which proliferate in hypoxic environments, can also effect a number of cellular processes that modulate channel activity. Therefore, no single hypothesis has yet proved conclusive or inclusive in explaining HPV. Our data show that chronic hypoxia in neonatal rabbits activates the 15-lipoxygenase-15-HETE constrictor response of lung PAs.

Figure 5. Arrows depict 15-LO signal in PAECs and PAVSM, which was evident in both Nor-Ls and Hyp-Ls, although more prominent in samples from hypoxic kits.

Figure 6. Endogenous levels of 15-HETE as detected by fluorescent assays were higher in PAs from hypoxic than normoxic kits (A). B, 15-HETE was detected in passage 1 or 2 pulmonary artery endothelial or vascular smooth muscle cells grown in normoxic environments and was higher in endothelial than smooth muscle cells.
at multiple levels. First, the enzyme 15-LO is activated by subacute hypoxia as measured by an in vitro assay. Using a combination of HPLC and GC/MS, the synthesis of 15-HETE was shown to be 3-fold greater in microsomes from Hyp-Ls as compared with Nor-Ls, and the protein is preferentially translocated to the microsomes in Hyp-Ls. In contrast, the distribution of 12-LO does not appear to be changed by hypoxic treatment (D and E).

Figure 7. A, 15-LO in the microsomes from Hyp-L and Nor L (10 μg protein each). B and C, Distribution of 15-LO between the soluble vs membrane fractions of Hyp-L and Nor-L, respectively. There is more 15-LO in microsomes from Hyp-Ls as compared with Nor-Ls, and the protein is preferentially translocated to the microsomes in Hyp-Ls. In contrast, the distribution of 12-LO does not appear to be changed by hypoxic treatment (D and E).

The physiological significance of this result was tested by determining if 15-HETE was able to constrict PAs. 15-HETE constricted vessels from hypoxic kits more potently than those from animals exposed to normal PO₂. In addition, inhibition of lipoxygenase with CDC or NDGA blunted phenylephrine-induced constriction of PAs from hypoxic over normoxic kits (Figure 8). Three pieces of information localize hypoxic-induced upregulation and activation of 15-LO to the pulmonary vasculature. First, immunohistochemistry demonstrates clear expression of 15-LO in pulmonary artery endothelial and vascular smooth muscle cells, particularly in PAECs from hypoxic animals. Second, primary isolations of PA endothelial and, to a lesser extent, VSM cells contain 15-HETE as demonstrated by fluorescent assays of endogenously formed lipids. Third, PAs harvested from hypoxic kit lungs exhibited increased 15-HETE concentrations over those of normoxic controls. Although experiments in this study do not definitively link 15-HETE formation in PAs with HPV in vivo, they are a critical first steps in defining 15-HETE as a candidate for this response in rabbit kits.

15-HETE Is Synthesized by 15-LO
Arachidonic acid is metabolized to 15-HETE by cytochrome P450 enzymes, cyclooxygenase (COX), and 15-LO. CYP 450 and COX inhibitors had no significant effect on formation of 15-HETE from AA in Hyp-Ls (Figure 4A), suggesting they were not responsible for the increased 15-HETE production. However, CDC, a lipoxygenase inhibitor, attenuated the production of 15-HETE by Hyp-Ls consistent with the hypothesis that the synthesis of 15-HETE in lungs exposed to hypoxia is mediated by 15-LO.

Increased 15-LO Activity Is Not Caused by Infiltration of PMNs and Is Not Generalized to 12-LO
15-LO is expressed in a variety of mammalian cells and tissues including rabbit and human reticulocytes, airway epithelial cells, leukocytes, alveolar macrophages, etc (see
review by Kuhn and Bohngraber\textsuperscript{(32)}. Although leukocytes have a well-recognized capacity to synthesize 15-HETE, we do not believe that PMNs are a major source of augmented 15-HETE production in our model for two reasons: (1) histological studies do not support obvious PMN sequestration in lungs of hypoxic kits (result not shown), and (2) Western analysis of microsomal proteins did not show significant levels of the PMN marker, CD11a. Furthermore, all lipoxygenases are not globally increased by hypoxia in that 12-LO levels appear to be comparable in Hyp-Ls and Nor-Ls.

**Activation of 15-LO by Translocation**

15-LO is commonly regarded as a cytosolic protein\textsuperscript{(33)} and becomes membrane-bound by a calcium-dependent mechanism, leading to an increase in the oxygenase activity of the enzyme.\textsuperscript{(34)} For maximal intracellular activity translocation is critical. In our experiments long-term hypoxia triggered translocation of 15-LO from cytosol to membranes (Figure 7), which may be the key step for the increased reactivity observed with Hyp-L. Hypoxia also causes release of calcium from intracellular stores,\textsuperscript{(35)} and this could be an explanation for the increased reactivity of 15-LO in Hyp-Ls.

**Figure 8.** Constriction of PA rings from Nor-Ls and Hyp-Ls to (A) 15-HETE or (B) U46619. Each value represents the mean±SEM. Note the differential response to the two agents. Effects of lipoxygenase inhibitors CDC (C) and NDGA (D) on phenylephrine-induced vaso-constriction in PAs from normoxic and hypoxic lungs. Each value represents the mean±SEM. Both CDC and NDGA blunt PE-associated increases in PA tension in arteries from hypoxic as compared with normoxic kits. CDC decreases PE-induced tension of PAs from hypoxic kits more effectively than those from normoxic kits.
for enhanced membrane translocation and in vivo activation of 15-LO in Hyp-L.

**Effect of 15-HETE on Constriction of PAs Exposed to Chronic Subacute Hypoxia**

Our data show that 15-HETE increases the tension of pulmonary arteries from hypoxic kits in a concentration-dependent manner. Other investigators have shown that contraction of rabbit aortic rings caused by 15-HETE is inhibited by the prostaglandin H2 (PGH2)/thromboxane A2 (TXA2) receptor blocker SQ29584, suggesting that the mechanism of 15-HETE vasoconstriction involves interaction with PGH2/TXA2 receptors. It is not known if 15-HETE uses these same receptors in PAs. We observed that U46619, a thromboxane A2 mimetic, elicits 3-fold greater contraction of PAs from Nor-Ls as compared with PAs from Hyp-Ls (Figure 8B), perhaps consistent with an alteration in receptor sensitivity or number after exposure to hypoxia. However, because the effects of 15-HETE are opposite to those of U46619 between Nor-L-PAs and Hyp-L-PAs, both agents do not appear to bind to the same PGH2/TXA2 receptors in our model. Although we cannot exclude hypoxic-induced changes in thromboxane synthesis or receptors in vivo, thromboxane metabolites from in vitro assays were not different in the two groups.

Others have reported pulmonary vasoactive effects of 15-HETE. In isolated perfused guinea pig lungs, 15-HETE increased PA pressure to a greater extent than 5- or 12-HETE. Human PA rings precontracted with PGF2α relaxed in a concentration- and cyclooxygenase-dependent but endothelium-independent manner to 15-HETE. In contrast, guinea pig PA rings that were precontracted with noradrenaline relaxed to low concentrations of 15-HETE, but were constricted by high concentrations.

**Production of 11,14,15- and 11,12,15-THETA in Hyp-Ls**

Identification of the AA metabolites 11,14,15- and 11,12,15-THETAs from rabbit lung microsomes incubated with [1-14C]AA (Figures 2A and 2B) was based on retention times similar with [U-14C]-AA. The THETAs are synthesized by 15-lipoxygenase reported previously in lysates of rabbit aorta that were incubated by chronic exposure to hypoxia. These 2 THETAs have been identified as 283 fragment as shown in the Figures 2A, which is more of the 173 fragment as seen in the structures. The vicinal diol in the 11,14,15-THETA is easily broken to yield the abundant 173 fragment as seen in the structures. The 11,14,15-THETA from rabbit lung microsomes incubated with [1-14C]AA was based on retention times similar with [U-14C]-AA. The THETAs are synthesized by 15-lipoxygenase from 15-hydroperoxyeicosatetraenoic acid (15-HPETE). Hydrolysis of the epoxy group results in the formation of 11,12,15- and 11,14,15-THETA. Rabbit lung has previously been reported to convert the 12-LO product, 12-HPETE, to both 8-H-11,12-EETA and 10-H-11,12-EETA. These compounds are converted into the corresponding 8,11,12-THETA and 10,11,12-THETA which potentiate the contractile response to norepinephrine. We did not detect synthesis of 8,11,12- or 10,11,12-THETAs but did note that a small amount of 12-HETE was produced. The action of the 11,14,15 and 11,12,15-THETAs on lung vasculature has yet to be determined.

In summary, this study examines the effect of in vivo exposure to subacute/chronic hypoxia on changes in activity of enzymes that metabolize AA in the lung. This report is the first to describe a mechanism for hypoxia-induced activation of the 15-LO enzyme and also shows its product, 15-HETE, to function on PAs in neonatal mammals, suggesting a potential role for 15-HETE in mediating HPV. In addition this is the first report of the presence of THETAs in rabbit lung as well as increased synthesis by microsomes from Hyp-Ls. The study therefore expands the list of existing candidates for HPV. We do not know if chronic hypoxia activates 15-LO in lungs of other species. Moreover, the causal relationship between 15-LO activation and enhanced vasoconstriction of PAs from hypoxic subjects remains to be established.

**Acknowledgments**

Financial support for this study from NIH/HLBI for R01 HL-49294, R01 HL-57075, P01 59996, and HL-37981 and AHA-01602692 is gratefully acknowledged. The excellent assistance of Ying Gao and Ryan McAndrew made this work possible.

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Circ Res. published online April 10, 2003;

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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