Chronic hypoxia enhances 15-lipoxygenase-mediated vasorelaxation in rabbit arteries

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The endothelial expression of 15-LO-1 and synthesis of THETA and HEETA decreases with maturation in rabbits from neonates to the age of 10 wk (3, 37). Therefore, SKCa channel-mediated relaxations to ACh or AA also decrease with age (3). However, with maturation, nitric oxide (NO) and IKCa channel-mediated relaxation did not change. Thus, with age and maturation, the 15-LO-1-mediated relaxation pathway is silenced in the rabbit arteries.

The endothelial 15-LO-1 expression and THETA and HEETA synthesis are induced by interleukin (IL)-13 (38) or atherosclerosis (18) in rabbit arteries. Moreover, in the arteries of 8-wk-old rabbits, the 15-LO-1 expression and THETA and HEETA synthesis increased with hypercholesterolemia (31). Furthermore, an increase in endothelial 15-LO-1 expression by gene delivery, in vivo or in vitro, in the arteries from rabbits was sufficient to increase the THETA and HEETA synthesis and to increase the relaxations to AA and ACh (2, 4). Thus the intracellular pathways involved in the 15-LO-1-mediated relaxations to agonists are intact in older rabbits. Therefore, a decrease in 15-LO-1-mediated relaxations with age is only due to a decrease in 15-LO-1 protein expression and can be induced upon a stimulation.

Hypoxia increases 15-LO-1 expression and enzymatic activity in the rabbit neonatal pulmonary arteries (43) and in human cerebral microvascular endothelial cells (HRMVECs) (5). Moreover, acute or chronic hypoxia increased relaxations in rat and cat cerebral arteries (12, 14) and in pig and rabbit coronary arteries (23, 28) and also decreased blood pressure in rabbits and rats (35, 40). This enhancement of vascular relaxations and the decrease in blood pressure were independent of NO or prostaglandins (PG) and were due to the enhanced activity of various K+ channels. This increased activity of the K+ channels was also suggested to be due to an EDHF (23). Since THETA and HEETA are EDHFs and increase SKCa channel-mediated relaxations to agonists in the preconstricted arteries (7, 8, 42), we tested the hypothesis that chronic hypoxia enhances 15-LO-1 expression in the endothelium of rabbit arteries and enhances relaxations to agonists by increasing THETA and HEETA synthesis.

METHODS

Rabbit arteries, endothelial cells, and effects of in vitro hypoxia. The animal protocols were approved by the Institutional Animal Care and Use Committee of the Medical College of Wisconsin, and the procedures were performed in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals (

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of Health’s Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85–23, Revised 1996). Eight-week-old male New Zealand White rabbits (Kuiper Rabbit Ranch) were euthanized with a pentobarbital sodium overdose. From the euthanized rabbits, various arteries were removed and maintained at 4°C in N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES) buffer containing (in mM) 10 HEPES, 150 NaCl, 5 KCl, 2 CaCl₂, 1 MgCl₂, and 6 glucose (pH 7.4). To induce hypoxia in vitro, the thoracic aorta was cut into 1- to 2-mm rings, and an equal number of rings were put in 1 ml of HEPES and incubated in the hypoxic chamber (37°C in an atmosphere of N₂ containing 0.7% O₂ and 5% CO₂) for various times. Another set of rings was incubated in the normoxic chamber (37°C in an atmosphere of room air containing 21% O₂ and 5% CO₂) for control.

Endothelial cells (ECs) were harvested from normoxic rabbit thoracic aortas in 75-cm² plastic flasks and cultured in the normoxic chamber in minimum essential medium containing 10% rabbit serum, 10% fetal bovine serum, 1% L-glutamine, 1% antibiotic-antimycotic, and 1% ampicillin. When the cells were 80% confluent, the flasks were transferred to the hypoxic chamber (same as described above for thoracic aorta) for various times.

**Rabbits and effects of in vivo hypoxia.** A group of rabbits was housed in the hypoxic chamber for a maximum of 5 days to induce hypoxia in vivo. Another group of rabbits was housed in the normoxic chamber for control. The hypoxic chamber is supplied with N₂ and continuously monitored to maintain the level of O₂ at 12% (35-40). After 5 days, blood was collected from the rabbit ear arteries while in the hypoxic chamber. Blood was collected in heparinized capillary tubes and capped from both ends. The blood was analyzed by an automatic blood gas analyzer (Roche Diagnostic), and partial pressure of O₂ (Po2), PCO₂, O₂ saturation, O₂ content, and blood pH were recorded. Hematocrit values were measured from the venous blood. Arteries were harvested from these hypoxic or normoxic rabbits for in vitro studies.

**Immunoblotting.** Protein lysates (50 μg protein) were separated by SDS gel electrophoresis and transferred to nitrocellulose membranes as described previously (4). Membranes were exposed to a sheep antibody against rabbit 15-LO-1 (Cayman Chemical) (dilution 1:10,000) or mouse antibody against human platelet EC adhesion molecule (CD62P) (kindly provided by Dr. Peter Newman, Blood Center for Southeastern Wisconsin, Milwaukee, WI) for 1 h at room temperature, rinsed with Tris-buffered saline containing 0.1% Tween-20, and incubated with a 1:5,000 diluted donkey anti-sheep IgG or goat anti-mouse IgG (horseradish peroxidase conjugated, Zymed). Membranes were reprobed with mouse anti-β-actin as a loading control. Immunoreactive bands were identified, and a densitometric analysis was performed.

**Quantitative real-time polymerase chain reaction.** Total nucleotides were isolated from the aortas using TRIzol reagent (Life Technologies). The DNA was digested with amplification-grade DNase-I (Invitrogen). cDNA was synthesized from total RNA by using SuperScript III first-strand synthesis system for quantitative, real-time (qRT)-PCR (Invitrogen). For control, the reaction mixture without reverse transcriptase and one without RNA were made. cDNAs for 15-LO-1 and GAPDH in the reaction mixtures were amplified using Bio-Rad iCycler in a 25 μl mixture containing 2 × 10⁻⁷ g cDNA, 2 × 10⁻⁷ M primers, and 12.5 μl SYBR Green/Fluorescein qPCR Master Mix (Super Array Biosciences Technologies). The primers for 15-LO-1, (reverse) 5’-CCG GCC GGC GAC GTT GAT CCT-3’ and (forward) 5’-TGG CTG CGG CGC TGG TCA TGC-3’, were designed from the cDNA sequence of rabbit 15-LO-1 and were synthesized by Operon (Huntsville, AL). The primers for GAPDH were supplied by Super Array Bioscience Technologies. The program for the iCycler was 94°C for 30 s, 58°C for 1 min, and 72°C for 1.5 min, repeated 40 times, followed by a final extension at 72°C for 7 min. The PCR products obtained after the iCycler amplification were separated by 1% agarose gel electrophoresis and visualized by ethidium bromide staining to confirm the presence of a single amplified product.

**Immunohistochemistry.** Rabbit mesenteric arterial segments were fixed in 4% paraformaldehyde and cut into sections as described previously (4, 38). Mesenteric segments were incubated with mouse anti-human PECAM, sheep anti-rabbit 15-LO-1, or mouse anti-α-actin, diluted 1:100,000 in 0.2% Triton X-100 containing 1% bovine serum albumin. The sections were incubated for 1 h at room temperature, rinsed, and incubated with 1:3,000 anti-mouse Alexa Fluor 594 and anti-rabbit FITC-labeled secondary antibodies (Molecular Probes) for 1 h at 25°C. The sections were mounted with media containing 1% 4,6-diamidino-2-phenylindole (DAPI) and protected by a glass coverslip. Fluorescent images were captured (×200 magnification) using Nikon Eclipse E600 microscope and Spot Advanced software. Arterial images were processed in ImageJ software. The thickness of the media (M) was determined by α-actin staining, whereas total wall thickness (W), internal diameter (ID), and total diameter (TD; 2 × W + ID) were determined from DAPI staining of the same sections. Several radial measurements were taken from the same section. The thickness of the media (M) and the internal diameter (ID) were determined, cleaned, cut into 2- to 3-mm rings, and incubated at 37°C with a cyclooxygenase inhibitor indomethacin (Indo; 10⁻⁵ mol/l) (Sigma) in 5 ml HEPES for 10 min. [¹⁴C]JA (10⁻⁵ mol/l) (Tocris) was added, the incubation was continued for 5 min, and then A-23187 (10⁻⁵ mol/l) (Sigma) was added. After 15 min, the reaction was stopped with ethanol (15% final concentration), and the samples were extracted using Bond Elute octadecylsil columns (Sigma). The extracts were analyzed by reverse-phase high-performance liquid chromatography using a Nucleosil C-18 (5 μm, 4.6 × 250 mm) column. The solvent system consisted of a 50-min linear gradient (flow rate = 1 ml/min) from 50% acetonitrile with 0.1% glacial acetic acid (solvent B) in deionized water (solvent A) to 100% solvent B. Column effluent was collected in 0.2-ml fractions, and the radioactivity was determined. Each experiment was repeated four to five times with the tissue obtained from different rabbits.

**Isometric tension measurements.** Arteries were suspended in a 6-ml tissue bath with Krebs bicarbonate buffer (Krebs) containing (in mM) 118.6 NaCl, 4.7 KCl, 2.5 CaCl₂, 1.17 MgSO₄, 25 NaHCO₃, 1.18 KH₂PO₄, 2 mmol/l L-NAME, 5.5 glucose (at 37°C) and bubbled with 95% O₂/5% CO₂. Isometric tension was measured with force-displacement transducers using wire myographs (Danish Myo Technology). The vessels were gradually adjusted to a resting tension of 1 g and allowed to equilibrate for 30 min. Contractions to KC1 (45 mM) and phenylephrine (Phe; 10⁻⁷–10⁻⁴ M) were determined. The Phe contractions and 1-ethyl-2-benzimidazolinone (1-EBIO) and ACh relaxations were also measured in the endothelium-denuded arteries. The endothelium was removed by rubbing the lumen of the arteries gently with human hair. The activity of the arteries after endothelium denudation was tested by KCl (45 mM) contractions. The absence of the endothelium was confirmed by measuring ACh relaxations in preconstricted arteries. Another set of vessels was treated with Indo (10⁻⁵ M) and nitro-L-arginine (L-NA; 3 × 10⁻³ M), and contractions to Phe, serotonin (5-HT; 10⁻⁷–10⁻⁴ M), or U-46619 (10⁻⁹–10⁻⁶ M) were determined. To measure the relaxations, the vessels were treated with Indo and L-NA for 10 min and then contracted by Phe (10⁻⁷–10⁻⁵ M) to 50–60% of the maximal Phe contraction. Cumulative concentrations of ACh (10⁻⁹–10⁻⁵ M) or AA (10⁻⁹–10⁻⁵ M) were added to the bath, and changes in isometric tension were measured. The contribution of 15-LO-1 to the ACh or AA relaxations was determined by incubating the rings with Indo, L-NA, and either BW-755C (10⁻⁴ M), nordihydroguaiaretic acid (NDGA; 2 × 10⁻⁵ M), Apa (2 × 10⁻⁷ M), or 1-[2-chlorophenyl]diphenylmethyl]-1H-pyrazole (TRAM-34; 10⁻⁷ M) (Tocris). Similarly, the vessels were incubated with Indo and L-NA and contracted by Phe, and the cumulative concentrations of the IKCa channel opener 1-EBIO (10⁻⁸–10⁻⁷ M) (Tocris) were added to the bath to determine changes in the isometric tension. Vasorelaxation was expressed as the percentage of maximum precontraction.
Membrane potential measurements. Membrane potential ($E_m$) was measured in the SMCs of the rabbit mesenteric arteries as published previously (7, 13). Briefly, the arterial rings were cut open laterally, pinned to a silastic layer in a perfusion chamber with the endothelium exposed, and perfused with warmed (37°C) Krebs buffer bubbled with 21% O2-74% N2-5% CO2. After 30 min of equilibration, the $E_m$ of SMCs was measured either in the presence of vehicle, Indo (10$^{-5}$ M), Indo and Phe (10$^{-7}$ M), Indo and Phe plus BW-755C (10$^{-4}$ M), or BW-755C alone. Impalements were done with a glass electrode only in a small section of the artery where the endothelium was removed by a gentle rubbing with a small cotton swab. Glass microelectrodes were filled with 3 M KCl and had an estimated tip size of 0.1 to 0.2 μm, a tip resistance of 30–80 MΩ, and tip potentials of $\pm$3 mV. Electrodes were attached to a high-impedance biological amplifier (Dagan Cell Explorer, Dagan Instruments, Minneapolis, MN). Electrode polarization was eliminated by a Ag/AgCl half cell. The criteria for a successful impalement included 1) an abrupt drop in potential to a new steady-state value, which was maintained for a minimum of 5 s; 2) an $E_m$ value $>\pm$ 20 mV; and 3) an abrupt return to the original baseline when the electrode was retracted from the tissue. AA (10$^{-5}$ M) was added to the arterial segments, and the $E_m$ was measured after 10 min.

Statistical analysis. The experimental data were expressed as means ± SE. The time-dependent effect of hypoxia on the band densities from immunoblots and the change in cycle threshold ($\Delta$Ct) values from RT-PCR in rabbit aortic ECs were analyzed by a repeated-measure one-way ANOVA. The time-dependent change in the band densities and the $\Delta$Ct values from aortas exposed to in vitro hypoxia or normoxia were analyzed by a two-way ANOVA followed by Bonferroni’s posttest. A Student’s t-test was performed to compare values for band densities from immunoblots in various arteries from hypoxic rabbits, blood gas, hematocrit, $E_m$, and vascular dimensions, as repeated-measure two-way ANOVA followed by Bonferroni’s posttest was performed to analyze the relaxations to ACh or AA, the effect of the inhibitors, and the effect of hypoxia on the relaxations. Values were considered significant at $P<0.05$.

RESULTS

Effects of in vitro hypoxia on 15-LO-1 protein and mRNA expression. The effects of in vitro hypoxia on 15-LO-1 protein and mRNA expression in ECs and isolated arteries were determined by immunoblots and RT-PCR, respectively.

For ECs, the immunoblots for 15-LO-1 and β-actin on the lysates from the ECs incubated in the hypoxic chamber for 4, 8, or 12 h are shown in Fig. 1, A and B. 15-LO-1 protein expression (band at 75 kDa) increases with time ($P<0.02$) with a significant maximum increase at 12 h of exposure to hypoxia. The β-actin expression did not change at any time point. 15-LO-1 mRNA was quantified in the ECs by qRT-PCR (Fig. 1, C and D). The Ct of 15-LO-1 CDNA was normalized to the Ct of GAPDH in the same sample to obtain $\Delta$Ct. $\Delta$Ct for 15-LO-1 mRNA expression increased ($P<0.05$) with the time of incubation in the hypoxic chamber with the maximum at 12 h (Fig. 1C), matching the results from the immunoblots. The amplified products from the qRT-PCRs were then separated on the agarose gel (Fig. 1D). A single band of amplified products for 15-LO-1 (326 bp), GAPDH (170 bp) was observed, whereas no band was observed in the absence of reverse transcriptase or CDNA in the reaction mixture.

For isolated arteries, the expression of 15-LO-1, PECAM, and β-actin protein was determined by immunoblots on the lysates from aortic rings incubated for 4, 8, or 12 h in either normoxic or hypoxic conditions (Fig. 2, A and B). The band density for 15-LO-1 decreased with time when the arteries were incubated in the normoxic conditions, whereas it increased when the arteries were incubated in the hypoxic chamber ($P<0.0001$). The expression of β-actin did not change with the time of incubation. PECAM and β-actin expression was determined in these arteries (Fig. 2, C and D). PECAM expression normalized to β-actin did not differ in the arteries incubated in hypoxic or normoxic conditions; however, the expression was reduced after a 12-h incubation in normoxic or hypoxic conditions. 15-LO-1 mRNA expression in these arteries was determined by qRT-PCR (Fig. 2, E and F). $\Delta$Ct of 15-LO-1 decreased with time in the arteries incubated in the normoxic chamber, whereas it increased with time in the arteries incubated in the hypoxic chamber ($P<0.0001$) (Fig. 2E). The agarose gel separation of qRT-PCR products showed a single band for 15-LO-1 mRNA or GAPDH (Fig. 2F).

Effects of in vivo hypoxia. After we determined the effects of in vitro hypoxia on ECs and isolated arteries, the remaining
studies were conducted in arteries from rabbits exposed to hypoxia in vivo. The rabbits were housed in the normoxic or hypoxic chamber for 5 days. Upon gross physical examination, the hypoxic rabbits did not show any signs of stress due to 5 days of housing in the hypoxic chamber. Hematocrit values in the hypoxic rabbits (58 ± 1%) were significantly higher than those in normal rabbits (41 ± 1%) (P < 0.001).

The effect of in vivo hypoxia was studied as follows.

**15-LO-1 expression in the arteries.** 15-LO-1 protein expression was determined in the lysates from thoracic and abdominal aortas as well as renal, femoral, carotid, and mesenteric arteries from normoxic or hypoxic rabbits by immunoblots (Fig. 3). 15-LO-1 expression was higher in all of the arteries, except the femoral arteries, from hypoxic rabbits when compared with normoxic rabbits. β-Actin expression did not change with hypoxia in any of the arteries.

**Metabolism of [14C]AA in the mesenteric arteries.** The enzymatic activity of the 15-LO-1 in mesenteric arteries was determined by analyzing [14C]AA metabolism (Fig. 4). In the presence of Indo, the aortas from normoxic (Fig. 4A) and hypoxic (Fig. 4B) rabbits metabolized [14C]AA to [14C]THETA, [14C]HEETA, and [14C]15-HETE. A synthesis of [14C]THETA, [14C]HEETA, and [14C]HETEs was increased in arteries from hypoxic rabbits compared with normoxic rabbits. The maximum increase in THETA and HEETA synthesis was observed after 5 days.

**15-LO-1 expression and intima thickening by immunohistochemistry.** Immunofluorescence was performed to determine the cellular expression of 15-LO-1 in freshly harvested mesenteric arteries from normoxic and hypoxic rabbits (Fig. 5A). The fluorescence signal from arteries treated with vehicle or only secondary antibodies was negligible (data not shown). A weak fluorescence signal for 15-LO-1 was observed in the endothelium of mesenteric arteries from normoxic rabbits (Fig. 5A, a). The fluorescence signal for 15-LO-1 in the mesenteric arteries from hypoxic rabbits was stronger (Fig. 5A, b).

Fig. 2. 15-LO-1 and platelet endothelial cell adhesion molecule (PECAM) expression in isolated rabbit aortas. Aortic rings incubated in normoxic (N) or hypoxic (H) conditions for various times (in hours). Lysates were prepared and separated on 10% SDS gel. A: Western immunoblot of 15-LO-1 and β-actin in lysates (40 μg protein). B: 15-LO-1 band density was normalized to β-actin band density. C: Western immunoblot of PECAM and β-actin in lysates (40 μg protein). D: PECAM band density was normalized to the β-actin band density. E: 15-LO-1 mRNA expression in aortic rings incubated in normoxic or hypoxic conditions and quantified by qPCR. Ct values for 15-LO-1 were normalized to Ct values for GAPDH to obtain ΔΔCt. F: agarose gel separation of the amplified products obtained from qPCR. Values represent means ± SE. **P < 0.01 and *P < 0.05 compared with the corresponding values in normoxic arteries.

Fig. 3. 15-LO-1 protein and mRNA expression in arteries from normoxic or hypoxic rabbits. A: Western immunoblot of 15-LO-1 and β-actin in lysates (40 μg protein). Lysates were prepared from various arteries from normoxic or hypoxic rabbits and separated on 10% SDS gel. B: 15-LO-1 band density was normalized to the β-actin band density. Values represent means ± SE. ***P < 0.001, **P < 0.01, and *P < 0.05 compared with the corresponding values in normoxic arteries.
arterial endothelium of hypoxic rabbits was greater than that in arteries from normoxic rabbits (Fig. 5A, b). When stained for 15-LO-1, no inflammatory cells were observed in the wall of the arteries from either normoxic or hypoxic rabbits. The presence of intact endothelium in the arteries was confirmed by staining with the endothelial marker protein PECAM (Fig. 5A, c and d). An adjacent layer of cells was shown by nuclear staining with DAPI and did not express 15-LO-1 (Fig. 5A, e and f).

The medial thickening in the arteries due to hypoxia was determined by staining for smooth muscle α-actin in the mesenteric arteries (Fig. 5B). The α-actin expression in the arteries from normoxic or hypoxic rabbits was not different (Fig. 5B, a and b). The total wall thickening was determined by staining for the nucleus by DAPI (Fig. 5B, c and d). The external diameter of the hypoxic or normoxic arteries ranged between 350–450 μm. Media thickness (M) was determined from the α-actin staining, and wall thickness (W), internal diameter (ID) and total diameter (TD: 2 × W + ID) were determined from the DAPI staining as shown in B,c and B,d. Several radial measurements (C) [M-to-W (M/W), M-to-ID (M/ID), and M-to-TD (M/TD) ratios] were calculated. Values represent means ± SE.

Em in the SMCs of the arteries. The Em of the SMCs in an intact artery from normoxic or hypoxic rabbits was measured (Fig. 6). The resting Em in the SMCs from the normoxic rabbits is −55.81 ± 2.8 mV and from the hypoxic rabbits is −54.52 ± 2.8 mV. The presence of Indo did not alter the resting Em.
similar to the contractions to Phe in the absence of the endothelium in both hypoxic and normoxic arteries, respectively, indicating that the endothelium did not modify the contractions.

The contractions to Phe (10⁻⁹–10⁻⁴ M), 5-HT (10⁻⁹–10⁻⁴ M), and U-46619 (10⁻⁹–10⁻⁴ M) were also determined in the endothelium-intact arteries in the presence of Indo and L-NA (Fig. 7, C–E). The maximum contraction to Phe and 5-HT in arteries from normoxic rabbits was 3.8 ± 0.2 and 0.23 ± 0.05 g, whereas in arteries from hypoxic rabbits, it was 2.7 ± 0.1 and 0.08 ± 0.05 g, respectively. The maximum contractions to U-46619 (1.3 ± 0.1 g) did not differ; however, the concentration-response curve to U-46619 was shifted rightward in hypoxic arteries.

The PG- and NO-independent relaxations to Ach or AA were measured in mesenteric arteries from hypoxic and normoxic rabbits (Fig. 8). Ach or AA caused concentration-dependent relaxation with maximum relaxations at 3 × 10⁻⁷ M or 3 × 10⁻⁶ M, respectively. Arteries from hypoxic rabbits had greater Ach relaxations (maximum, 79.7 ± 2.2%) compared with arteries from normoxic rabbits (maximum, 49.7 ± 6.9%) (Fig. 8A). Relaxations to AA were also greater (maximum, 58.3 ± 4.5%) with arteries from hypoxic rabbits compared with arteries from normoxic rabbits (maximum, 19.9 ± 2.0%) (Fig. 8B). To determine the importance of the endothelium and to confirm that 15-LO-1 expression was limited to the endothelium, Ach relaxations were measured in endothelium-denuded hypoxic arteries. In the absence of the endothelium, Ach caused contractions instead of relaxations in the hypoxic arteries (data not shown).

The contribution of AA metabolites from 15-LO-1 to the ACh relaxations was determined in the presence of Indo and L-NA (Fig. 9). In the arteries from normoxic rabbits, NO- and PG-independent maximum Ach relaxations (54.8 ± 6.2%) were not changed by BW-755C (maximum, 56.0 ± 10.8%) or NDGA (maximum, 59.1 ± 7.9%) (Fig. 9A). In the arteries from hypoxic rabbits, the maximum ACh relaxations (76.7 ± 2.7%) were reduced by BW-755C to 54.6 ± 6.2% and by NDGA to 56.7 ± 4.4% (Fig. 9B). In the absence of PGs and NO, ACh relaxed rabbit arteries due to THETA and HEETA that open SKCa channels (42). Therefore, ACh relaxations were investigated in the presence of the SKCa channel inhibitor Apa. In the arteries from normoxic rabbits, the absence or presence of Apa did not change the maximum ACh relaxations (56.3 ± 6.2% and 47.4 ± 6.2%, respectively) (Fig. 9C). However, in the arteries from hypoxic rabbits, Apa reduced the maximum ACh relaxations from 81.7 ± 4.0% to 52.2 ± 4.3% (Fig. 9D). This reduction was comparable with the reductions by 15-LO inhibitors.

To determine the effect of hypoxia on the activity of IKCa channels, ACh or I-EBIO relaxations were measured in the presence of Indo and L-NA in mesenteric arteries (Fig. 10). TRAM-34, a specific inhibitor of IKCa channels, reduced the maximum ACh relaxations from 59.7 ± 2% to 37.1 ± 4% in the arteries from normoxic rabbits (Fig. 10A) and from 88.4 ± 4% to 65.3 ± 4% in the arteries from hypoxic rabbits (Fig. 10B). The reduction by TRAM-34 in hypoxic or normoxic arteries occurred to the same extent, which suggests that IKCa channel-mediated ACh relaxations did not differ with hypoxia. Additionally, after TRAM-34 inhibition, the remaining relaxations to ACh in the hypoxic arteries were further reduced by
BW-755C to a maximum of 23.7 ± 2% and, in normoxic arteries, to a maximum of 18.9 ± 2%. To confirm the effect of hypoxia on the IKCa channels, the relaxations to IKCa channel opener 1-EBIO were measured. In the absence of the endothelium, 1-EBIO did not relax the hypoxic arteries (data not shown), indicating that IKCa channels were localized to the endothelium in these arteries. In the presence of Indo and l-NA, 1-EBIO relaxation in the endothelium intact arteries from normoxic (maximum, 73 ± 2%) and hypoxic (maximum, 64 ± 8%) rabbits were similar (Fig. 10).

**DISCUSSION**

Metabolites of AA from the 15-LO-1 pathway THETA and HEETA are EDHFs and relax rabbit arteries (7, 8, 42). Other metabolites of AA from LO pathways 15-HETE, 12-HETE, and 15(S)-hydroperoxyeicosatetraenoic acid are inactive in the rabbit arteries (10, 25, 31). We have previously shown that ACh or AA relaxations were higher in the arteries from younger rabbits (neonates to 4 wk old) compared with the arteries from their older counterparts (8 or 16 wk old) (3, 37). This difference was independent of the NO or PG effect but was due to lower 15-LO-1 expression and decreased THETA and HEETA synthesis in the endothelium of the arteries from 8- and 16-wk-old rabbits. The 15-LO-1 expression and THETA and HEETA synthesis were induced by IL-13 (38) and hypercholesterolemia (18, 30) in rabbit aortas and by hypoxia in rabbit neonatal pulmonary arteries (47) and HRMVECs (5).

An increased expression of endothelial 15-LO-1 is also sufficient to enhance the relaxations to ACh or AA in rabbit arteries (2, 4). Therefore, we speculated that hypoxia may enhance 15-LO-1 expression, THETA and HEETA synthesis, and vascular relaxations to agonist in the arteries from 8-wk-old rabbits.

We first determined the effect of hypoxia on 15-LO-1 expression in vitro. The incubation of HRMVECs at 1% O2 for 12 h increased the expression of 15-LO-1 (5). We observed an enhanced 15-LO-1 protein and mRNA expression in rabbit aortic ECs and isolated arteries incubated at 0.7% O2 in 8 h. This also implies that the effect of hypoxia is intracellular and not due to the influence of any in vivo mechanisms such as hypoxia-mediated increased amounts of circulating cytokines (11) that may increase the expression of 15-LO-1 (21, 38). Furthermore, PECAM expression in isolated arteries did not differ in normoxic or hypoxic conditions at any time point, so the hypoxia-mediated increase in 15-LO-1 expression was not due to the increase in the number of ECs.

After observing the effects of in vitro hypoxia on 15-LO-1 expression, we studied the vascular changes in rabbits exposed to hypoxia in vivo. To study various effects of hypoxia, humans, rabbits, and rats have been subjected to 12% O2 for various times (hours or days) to decrease Po2 by ~40% without any signs of distress or discomfort (11, 17, 24, 35, 40, 43). We also exposed the rabbits to chronic/subacute hypoxia...
in a chamber with 12% O2 for a maximum period of 5 days and did not see any signs of discomfort. 15-LO-1 expression increases to different degrees in various arteries of these rabbits. The metabolism of AA was determined in the aortas and mesenteric arteries of the hypoxic rabbits to test the enzymatic activity of 15-LO-1. In the mesenteric arteries and aortas, the maximum increase in THETA and HEETA synthesis was observed after 5 days. The exact mechanism by which hypoxia induces the 15-LO-1 expression in the arteries is not known. However, based on the effect of hypoxia on transcription factors, chromatin remodeling, and hypoxia-inducible factor-1α (HIF-1α), certain speculation can be made. For example, when cultured rat vascular SMCs were exposed to hypoxia, there is an increase in the phosphorylation of protein kinase B (Akt), p38, and STAT-3 (6). The activation of STAT-3 was also higher than that of other kinases, presumably due to the increased activity of AKT. The phosphorylation of STAT-3 was completely inhibited by AG-490, a JAK/STAT inhibitor (6). Since the STAT-6, STAT-4, STAT-3, JAK2, p38, and TYK2 have been implicated in the regulation of 15-LO-1 expression (22, 34, 41), the hypoxia-mediated 15-LO-1 expression may be due to an increased activity of JAK/STAT pathway in the endothelium (6). Moreover, because of the increased activity of several transcription factors in hypoxia, the overexpression of several growth factors and inflammatory cytokines were also reported (19). Cytokines such as IL-4 and -13 increase the expression of 15-LO-1 through the JAK/STAT pathway (22). Therefore, it can be speculated that such an increase in cytokine levels may increase 15-LO-1 expression. The effect of hypoxia on the gene regulation can also be explained by the classical pathway of HIF-1α/β. Under conditions of reduced O2 availability, HIF-1α accumulates, dimerizes with HIF-1β, binds to cis-acting hypoxia response elements in target genes, and leads to increased transcription (19). However, hypoxia response elements (5'-RCGTG-3') are not present in the promoter region of 15-LO, suggesting that HIF may act indirectly to increase 15-LO.

Small resistance arteries contribute more toward the regulation of blood pressure and have a greater component of EDHFs-mediated relaxations (26). Therefore, we focused the rest of the study on the mesenteric arteries that have an external diameter ranging between 350 and 450 μm. A histological study of the mesenteric arteries from hypoxic rabbits confirmed that 1) the expression of 15-LO-1 was limited to the endothelium; 2) circulating cells such as macrophages or polymorphonuclear leukocytes, which may express 15-LO-1, were not attached to the endothelium; 3) PECAM expression did not change with hypoxia, suggesting that the proportion of ECs was unchanged; and 4) there was no vascular remodeling due to hypoxia.

THETA and HEETA are EDHFs that open SKCa channels, cause an efflux of K+, and hyperpolarize SMCs and thus relax the arteries (7, 8, 42). The AA-mediated repolarization of the SMCs was greater in arteries from hypoxic rabbits compared with the arteries from the normoxic rabbits. This repolarization was also inhibited by BW-755C, indicating that the hyperpolarization was due to the increased synthesis of THETA and HEETA from AA.

Contractile and relaxation responses were determined in the mesenteric arteries from rabbits. In the present study, the contractile responses to various agonists were less in the mesenteric arteries from hypoxic rabbits. Hypoxia-mediated decreased con-
In rats, chronic hypoxia increased the expression of cytochrome P-450 2C9 (CYP2C9) in rat mesenteric arteries that decreased the Phe-induced contractions (9). The reduced contractions to Phe were due to increase in the synthesis of epoxyeicosatrienoic acids (EETs) that dilate the vessels (9). However, in our experiments, we did not see a synthesis of EETs in normoxic or hypoxic rabbit arteries. CYP2C9, CYP2C8, and CYP2C11 do not contribute to the synthesis of THETA or HEETA in rabbit arteries (33). Thus the LO pathway does not contribute to reduced Phe contractions in hypoxic rabbit arteries. In rat arteries, hypoxia enhances the expression of heme oxygenase that increases metabolism of heme to carbon monoxide (CO) and thus cause reduced Phe contractions (15). Although we did not confirm the role of heme oxygenase here, it can be inferred that the hypoxia-mediated decrease in contractile responses could be due to one or more of several mechanisms, including increased EDHF activity.

We have previously shown that 15-LO-1 expression and an increase in THETA and HEETA synthesis are sufficient to increase ACh and AA relaxations in rabbit arteries (2, 4). We determined the NO- and PG-independent ACh and AA relaxations in the mesenteric arteries. PG-independent AA-induced relaxations increased in arteries from hypoxic rabbits compared with arteries from normoxic rabbits, confirming the contribution of the AA metabolites. NO- and PG-independent ACh relaxations were also increased in arteries from hypoxic rabbits, and the increased relaxations were reduced by the LO inhibitors. Additionally, the inhibitors of 15-LO and SKCa channels reduced the ACh relaxations to a greater extent in hypoxic rabbit arteries compared with normoxic rabbit arteries. This is consistent with the higher expression of 15-LO-1 and the greater synthesis of THETA and HEETA in the arteries from hypoxic rabbits. ACh did not cause relaxations in the endothelin-denuded hypoxic arteries, confirming that the relaxations were endothelium dependent and that the 15-LO-1 expression was localized to the endothelium only. ACh-induced relaxations in the rabbit arteries are mediated by the combined action of SKCa and IKCa channels (42). In 8-wk-old rabbits, in presence of Indo and L-NA, the IKCa channel pathway contributes more toward the ACh relaxations, whereas an SKCa channel pathway has a very little contribution (2). In the present studies, the IKCa channel-mediated ACh or 1-EBIO relaxations did not differ in the hypoxic and normoxic arteries. Therefore, the activity of IKCa channels was not altered with hypoxia. Additionally, 1-EBIO did not relax the hypoxic arteries with the endothelium removed. This suggests that the IKCa channels were localized in the endothelium in the hypoxic arteries. The inhibition of SKCa channels by Apa also reduced the ACh relaxations to a greater extent in hypoxic rabbit arteries compared with normoxic rabbit arteries, suggesting the enhanced opening of SKCa channels by THETA and HEETA.
Thus hypoxia increased the 15-LO-1 expression and the synthesis of THETAs and HEETAs in the endothelium. Increased amounts of THETA and HEETA enhance the opening of Apa-sensitive SKCa channels that hyperpolarize the SMCs and relax the arteries. Thus HEETAs and THETAs mediate the increased relaxations to agonists in arteries from hypoxic rabbits.

Perspective. 15-LO-1 is expressed in the vascular endothelium of several species including humans (20, 27). We have observed THETA and HEETA synthesis in rabbit, human, and mouse arteries. The endothelial expression of 15-LO-1 increases with atherosclerosis and cytokines treatment (18, 38). The present study shows that hypoxia also induces endothelial 15-LO-1 expression, THETA and HEETA synthesis, and thus relaxations to an agonist. Therefore, it appears that 15-LO-1 is an inducible pathway that enhances endothelium-dependent relaxations.

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