Enhanced endothelin-1 and endothelin receptor gene expression in chronic hypoxia

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Li, Huaibin, Shi-Juan Chen, Yiu-Fai Chen, Qing Cheng Meng, Joan Durand, Suzanne Oparil, and Terry S. Elton. Enhanced endothelin-1 and endothelin receptor gene expression in chronic hypoxia. *J. Appl. Physiol.* 77(3): 1451–1459, 1994.—To test the hypothesis that endothelin (ET)-1 synthesis and ET receptor levels are increased selectively in the lung of rats with chronic hypoxic pulmonary hypertension, the current study examined the effects of exposure to chronic hypoxia (10% O_2, 1 atm, 4 wk) on pulmonary arterial pressure, ET-1 levels in plasma and lung, and ET-1 and ET_A and ET_B receptor mRNA levels in lung, heart, pulmonary artery, aorta, kidney, spleen, and liver. Hypoxic exposure was associated with increases in pulmonary arterial pressure, plasma ET-1 levels, ET-1 mRNA in lung and pulmonary artery, and ET-1 stores and ET_A and ET_B receptor mRNA levels in lung. In thoracic aorta and the four heart chambers, ET_A and ET_B receptor mRNA levels were increased, but ET-1 mRNA levels were unchanged from air control levels. No change in ET-1 or ET receptor mRNA levels was seen in organs perfused by the systemic vascular bed, except in liver, where ET_A receptor mRNA levels were decreased. The findings of concomitant increases in gene transcript levels for ET-1 and the ET_A and ET_B receptors in lung, but not in the great vessels or any other organ examined, are consistent with the hypothesis that increased ET-1 synthesis in the lung contributes to pulmonary vascular remodeling and the maintenance of chronic hypoxic pulmonary hypertension.

endothelin; endothelin receptor; pulmonary hypertension; vasoconstriction; oxygen tension; messenger ribonucleic acid

PULMONARY HYPERTENSION develops in many species, including humans, during chronic exposure to normobaric or hypobaric hypoxia. In the rat, exposure to hypoxia results in an acute increase in pulmonary arterial pressure as a result of vasoconstriction, followed by sustained pulmonary hypertension resulting from a combination of polycythemia and morphological alteration of the pulmonary vascular bed (25). Chronic hypoxia-induced remodeling of the pulmonary vasculature is characterized by extension of vascular smooth muscle into smaller and more peripheral arteries than normal ("neomuscularization"), an increase in the thickness of the medial muscular coat in the normally muscular arteries, and a reduction in the number of peripheral arteries (23). The extent of structural adaptation is correlated with the pulmonary arterial pressure. Systemic arterial pressure is unchanged in hypoxia-adapted animals.

The mechanism(s) of acute and chronic hypoxia-induced pulmonary hypertension is incompletely understood. A number of vasoactive agents, most of which are endothelium- or platelet-derived vasoconstrictor factors, such as endothelin (ET)-1, platelet-activating factor, or arachidonic acid metabolites such as leukotriene C_4, have been adduced as possible mediators of hypoxia-induced pulmonary vasoconstriction and hypertension (5, 7, 12, 22). ET-1 and ET-3 isoforms of ET, as well as both major subtypes of ET receptor, ET_A and ET_B, are expressed in normal rat lung (13). The ET_A receptor subtype is found in pulmonary vascular and bronchial smooth muscle and in mycardium and is relatively selective for ET-1. The ET_A receptor mediates the vasoconstrictor, bronchoconstrictor, and positive inotropic effects of ET in these tissues. The ET_B receptor, which has equal affinity for the three ET isopeptides, is distributed in multiple cell types, including endothelial cells and vascular smooth muscle. The dominant effect of activating vascular ET_B receptors is vasorelaxation via release of prostacyclin and nitric oxide from endothelial cells (29).

The synthesis of ET-1 in blood vessels has been shown to be stimulated by hypoxia (24). ET-1 elicits several biological effects, including vascular smooth muscle cell contraction (35) and proliferation (30), that are compatible with a role in hypoxia-induced pulmonary vasoconstriction and vascular remodeling. The mitogenic effect of ET-1 is related to its ability to stimulate the phosphatidylinositol signaling pathway and the expression of the early-response genes *fos, jun,* and *myc* (14, 30). In addition, ET-1 can induce the expression of other growth factors, such as platelet-derived growth factor (PDGF)-A and transforming growth factor-β, in cultured vascular smooth muscle cells (10), suggesting that ET-1 can contribute to the remodeling of surrounding (vascular) tissue by paracrine/autocrine regulatory mechanisms (14).

Previous studies from our laboratory showed that acute (48-h) exposure to normobaric hypoxia increases pulmonary arterial pressure and plasma ET-1 levels and selectively enhances ET-1 gene expression in rat lung and main pulmonary artery (7). Furthermore preliminary observations in our laboratory suggest that the acute pulmonary vasoconstrictor response to hypoxia can be blocked by administration of the selective ET_A receptor antagonist BQ-123 (cyclo-[D-Trp-D-Asp-Pro-D-Val-Leu]) (2). The current study tested the hypothesis that increased circulating ET-1 levels and selective enhancement in ET-1 and ET receptor gene expression in lung persist during chronic (4-wk) hypoxic exposures. We hypothesized that selective enhancement in ET-1 expression would persist in lung but would not be found in organs supplied by the systemic circulation of rats exposed to chronic hypoxia. Furthermore we hypothesized that expression of ET_A and ET_B receptor genes would be increased or at least would not be reduced in the lung but not in other organs of hypoxia-adapted rats, reflecting a

role for ET-1 as a sustained growth stimulus. We reasoned that the combination of increased local synthesis of ET-1 and increased (or at least not decreased) levels of ET receptors in lung of hypoxia-adapted rats would result in pulmonary vascular hypertrophy and remodeling via a paracrine mechanism. We further hypothesized that this combination of increased agonist and increased receptor levels would not occur in other organs and therefore would not contribute to generalized vascular remodeling in the presence of hypoxia. Our data showed significantly increased pulmonary arterial pressures, plasma ET-1 concentrations, and ET-1 and ET_A and ET_B receptor gene transcript levels in lung of rats exposed to chronic hypoxia compared with air control rats. ET_A and ET_B receptor steady-state mRNA levels were also increased in heart and thoracic aorta of hypoxia-exposed rats compared with air controls. ET-1 and ET_A and ET_B receptor mRNA levels in organs supplied by the systemic vascular bed (liver, spleen, and kidney) were largely unaffected by hypoxic adaptation. These findings are consistent with a role for ET-1 in the maintenance of hypoxic pulmonary hypertension.

METHODS

Male Sprague-Dawley rats were obtained from Charles River Breeding Laboratories (Wilmington, MA) at 7 wk of age. Rats were maintained in 10% O_2 (hypoxia) or room air (normoxic controls) for 28 days, as previously described (15). Rats were exposed to hypoxia in a 330-liter Plexiglas glove box (Manostat, Brooklyn, NY). Hypoxic exposures (range 10.0 ± 0.5% O_2) were accomplished by intermittent addition of N_2 (Southern Welding, Birmingham, AL) to the chamber from a liquid N_2 reservoir, the gas outflow of which was regulated by a solenoid valve controlled by the recorder output of an S3-A O_2 analyzer (Applied Electrochemistry, Sunnyvale, CA) through a control circuit (model 371-K, LFE, Clinton, MA). A Baralyme CO_2 scrubber (Allied Health Care Products, St. Louis, MO) kept the CO_2 concentration at <0.2%. Relative humidity within the chamber was kept at <70% with anhydrous $CaSO_4$. Boric acid was used to keep NH_3 levels within the chamber at a minimum. The animals were allowed standard laboratory chow and tap water ad libitum. Daily animal maintenance was carried out without interruption of the exposures through double ports in the chamber. Control animals were caged similarly and were exposed to filtered room air for identical periods.

After 24–26 days of hypoxia or air control exposures, the pulmonary artery was cannulated in situ by a closed-chest technique under pentobarbital sodium anesthesia (15). A small transverse cut was made in the proximal right external jugular vein, through which an introducer and a silicone rubber cannula (0.32 mm ID, 0.64 mm OD) were passed. The introducer was a blunted 7.5-cm 19-gauge needle with the tip turned up 30°. The silicone rubber cannula filled with heparin-saline solution was passed through the introducer and attached by a 25-gauge blunted needle to a pressure transducer (model CP-01, Century Technology, Inglewood, CA) coupled to a polygraph (model 7, Grass Instruments, Quincy, MA). After the introducer was placed in the right ventricular cavity, the tip was directed anteriorly. The cannula was then advanced into the pulmonary artery. Cannula position was identified by the pressure tracing. The introducer was slipped out over the cannula and removed after a typical pulmonary arterial pressure tracing was recorded. The cannula was affixed to the vein and to the surrounding tissue distally by basket-weave sutures and connected to polyethylene tubing (PE-10 fused to PE-20) with a loop. The PE-20 tubing was exteriorized at the back of the neck by a stainless steel wire tunneled subcutaneously. The pulmonary artery cannulas were exteriorized via a small hole in the wall of the chamber so that pressure measurements could be performed without interruption of the hypoxic exposure. At 48 h after catheterization, mean pulmonary arterial pressure was recorded.

Blood (2 ml) was removed from the femoral arterial cannula for determination of plasma ET-1 levels by radioimmunoassay (RIA) (7). Rats were then killed by decapitation without prior anesthesia, and the lungs, main trunk of the pulmonary artery, thoracic aorta, kidneys, spleen, liver, and heart were removed quickly and frozen in liquid N_2. Hearts were dissected into right and left atrium, right ventricle, and left ventricle plus septum before freezing. Plasma and tissues were stored at −80°C until RIA for ET-1 and extraction of RNA, respectively.

ET-1 RIA. Plasma and tissue ET-1 concentrations were measured by RIA with kits from Peninsula Laboratories (Belmont, CA) (7). Blood samples were collected in chilled syringes and transferred to polypropylene tubes containing EDTA (1 mg/ml of blood) and aprotinin (500 KIU/ml of blood) at 4°C. Blood was centrifuged at 2,000 *g* for 15 min at 4°C, and the plasma was stored at −80°C until assay. Plasma was acidified with 0.1% trifluoroacetic acid (TFA; high-performance liquid chromatography grade), centrifuged at 17,000 *g* for 20 min at 4°C, and applied to a Sep-Pak C_{18} column (Waters Associates, Milford, MA) that had been activated by washing with 60% acetonitrile (high-performance liquid chromatography grade) in 0.1% TFA (4 × 1 ml) followed by 0.1% TFA (4 × 5 ml). The plasma was applied to the column, and the column was washed with 50 ml of 0.1% TFA. ET-1 was then eluted from the column with 60% acetonitrile in 0.1% TFA (3 × 1 ml) into a polypropylene tube and evaporated to dryness in a lyophilizer. Lung tissue was homogenized in 10 vol of 1 M acetic acid with 0.1% Triton X-100 by use of a Polytron homogenizer for 1 min and immediately boiled for 7 min to inactivate proteases. After the homogenate was chilled, it was centrifuged at 30,000 *g* for 30 min at 4°C, and aliquots of the supernatants were applied to Sep-Pak C_{18} columns and processed as described for plasma.

Samples were reconstituted in RIA buffer and subjected to RIA with use of a rabbit anti-ET-1 antiserum. The anti-ET-1 antiserum was used at a titer of 1:18,000 and had 100% cross-reactivity with human ET-2, 70% cross-reactivity with human and rat ET-3, and 100% cross-reactivity with human and porcine big ET (7). There was no cross-reactivity with human big ET-(22—38), mouse ET-β, sarafotoxin S6b, α-atrial natriuretic peptide-(1—28), brain natriuretic peptide, angiotensin I and II, and arginine vasopressin. Recovery from the Sep-Pak C_{18} columns averaged 90%, and the sensitivity of the assay for ET-1 was 1.5–2.0 pg.

ET-1 cDNA probe. A 0.5-kb rat preproET-1 cDNA probe contains most of the coding sequence of preproET-1 cDNA and does not cross hybridize with ET-2 or ET-3 mRNA. The specificity of the ET-1 cDNA probe has been previously confirmed in our laboratory (7). The cDNA probes were radiolabeled to a specific activity of 10^8–10^9 counts · min^{-1} · μg^{-1} by utilization of a random hexamer primer-labeling system (8) (Prime-a-Gene Labeling System, Promega, Madison, WI). The radiolabeled ET-1 probe was separated from unincorporated nucleotides by use of Quick Spin columns containing G-50 Sephadex (Boehringer Mannheim, Indianapolis, IN).

ET_A and ET_B receptor cDNA probes. ET_A- and ET_B-specific cDNA probes were prepared utilizing standard polymerase chain reaction (PCR) procedures and rat genomic DNA template. An ET_A sense primer (5′-TGTTGCTGTTGTCACCAGTCC-3′) corresponding to nucleotides 1153–1173 of the published ET_A receptor cDNA sequence and an antisense primer (5′-GAGCGCAGCTGCTGCGTGACCG-3′) corresponding

TABLE 1. *Effects of 4-wk exposure to hypoxia on MSAP, MPAP, plasma ET-1, and RV- and (LV+S)-to-BW ratios*

	MSAP, mmHg	MPAP, mmHg	ET-1, pg/ml	RV/BW, mg/g	(LV+S)/BW, mg/g
Air control	117±3	18±1	3.6±1.5	0.49±0.03	1.80±0.11
Hypoxia	116±3	37±1*	12.4±4.1*	1.44±0.06*	1.99±0.12

Values are means ± SE; $n = 8$. MSAP, mean systemic arterial pressure; MPAP, mean pulmonary arterial pressure; ET-1, endothelin-1; RV, right ventricle; BW, body weight; LV, left ventricle; S, septum. * $P < 0.05$ compared with air control.

to nucleotides 1351–1330 of the published sequence were designed on the basis of the published sequence (17). Rat genomic DNA template (Promega) was amplified with the primers by use of GeneAMP PCR Kits (Perkin-Elmer Cetus, Norwalk, CT). A single PCR product of expected size (199 bp) was obtained and subcloned into pCR II vector with use of a TA cloning kit (Invitrogen, San Diego, CA). Sequence analysis demonstrated that the PCR product was authentic. The construct pCR-ET_A-199 was amplified, and the insert was utilized as a hybridization probe.

The ET_B receptor cDNA probe was prepared in a similar way. PCR primers spanning the nonhomologous region in the 3′ flanking region of the rat ET_B receptor (26) sequence were utilized. The sense primer (5′-CTGCTGGTGCCAAACGTTT-GAG-3′) corresponded to nucleotides 1203–1224 of the published sequence and the antisense primer (5′-CCATGGCTT-TCTTAGGTTGTA-3) to nucleotides 1525–1505. Rat genomic DNA was amplified with the primers, and a single PCR product of 323 bp was obtained and subcloned into the pCR II vector (pCR-ET_B-323), as described above.

RNA isolation and Northern and slot-blot analyses. Total tissue RNA was extracted from the organs mentioned above by the method of Chomczynski and Sacchi (3), as previously described (7). Northern and slot-blot analyses were used to identify and quantitate ET_A and ET_B receptor mRNA. To generate a Northern blot for characterization of ET_A and ET_B receptor mRNA, 40 μg of total RNA were extracted from lungs of air control rats and denatured at 65°C for 5 min in 50% formamide and 6% formaldehyde [in 22.5 mM 3-(*N*-morpholino)-propanesulfonic acid with 1.2 mM EDTA], size fractionated by electrophoresis through 1.5% agrose-3% formaldehyde gels in 20 mM 3-(*N*-morpholino)propanesulfonic acid-5 mM sodium acetate-1 mM EDTA (pH 7.0), and blotted onto Nytran membrane (0.45, Schleicher and Schuell, Keene, NH) in 20× SSC (1× SSC = 0.15 M NaCl-15 mM sodium citrate, pH 7.0) according to a modification of the technique of Thomas (31). For slot-blot analysis, 15 μg of total RNA were denatured in 6% formaldehyde in 8.3× SSC, incubated at 60°C for 20 min, and applied directly onto Nytran membrane with use of a Schleicher and Schuell slot-blot apparatus. All Northern and slot blots were cross-linked by ultraviolet radiation for a total energy of 125 mJ in a GS Gene Linker (Bio-Rad, Richmond, CA).

The blots were prehybridized in QuickHyb hybridization buffer (Stratagene, La Jolla, CA) for 15 min and hybridized in the same buffer with the random primer ^{32}P-labeled probes (10^6 counts · min^{-1} · ml^{-1}) at 68°C for 1 h. After hybridization, blots were washed twice in 500 ml of 2× SSC-0.1% sodium dodecyl sulfate at room temperature for 15 min and twice in 0.1× SSC-0.1% sodium dodecyl sulfate at 60°C for 20 min. Blots were slightly dried and exposed to X-ray film (Kodak X-OMAT film, Sigma Chemical, St. Louis, MO). All blots were probed with ^{32}P-labeled ET_A and ET_B receptor cDNA probes sequentially. RNA blots of lung, main pulmonary artery, and thoracic aorta were further probed with the ET-1 cDNA probe. Between each reprobing, ^{32}P-labeled cDNA was stripped off the membrane by washing in 5 mM tris(hydroxymethyl)aminomethane · HCl (pH 8.0), 0.2 mM Na_2EDTA, 0.05% sodium pyrophosphate, 0.002% polyvinyl-pyrrolidone, 0.002% bovine serum albumin, and 0.002% Ficoll at 70°C for 3 h.

To quantitate total RNA loaded onto slot blots, the blots were stripped as described above and rehybridized with a ^{32}P-labeled α-tubulin probe or a ^{32}P-labeled 18S rRNA-specific oligonucleotide probe (5′-ACGGTATCTGATCGTCTTCGA-ACC-3′) under the conditions specified above. The control probe was utilized so that the densitometric data could be normalized to account for variations in RNA loading. Density of autoradiographic signals was quantitated with an optical densitometer (model GS-670 Imaging Densitometer, Bio-Rad). To estimate tissue ET-1, ET_A, and ET_B receptor mRNA steady-state levels, the ET-1, ET_A, and ET_B receptor mRNA-to-18S rRNA ratios were determined by dividing the absorbance corresponding to ET-1, ET_A, or ET_B receptor cDNA probe hybridization by the absorbance corresponding to α-tubulin or 18S rRNA probe hybridization. This allows for correction for any variation in the amount of RNA loaded.

Statistical analysis. Results are expressed as means ± SE. Data were analyzed using the CRUNCH statistical package on an IBM PC/AT computer. Statistical comparisons of plasma and tissue ET-1 and tissue ET-1, ET_A, and ET_B mRNA levels between hypoxic and air control groups were performed using Student's unpaired *t* test. Differences were reported as significant at $P < 0.05$.

RESULTS

Rats exposed to chronic normobaric hypoxia (10% O_2, 4 wk) exhibited significantly increased pulmonary arterial pressure and right ventricle-to-body weight ratios compared with air controls (Table 1). Systemic arterial pressure and left ventricle-to-body weight ratios were not altered by chronic hypoxia. Circulating ET-1 levels in arterial plasma were significantly elevated in hypoxia-adapted rats compared with air controls.

To demonstrate the specificity of the rat ET_A and ET_B receptor cDNA probes described above, a Northern analysis of total RNA extracted from lungs of air-exposed rats was performed. The ET_A receptor cDNA probe detected two bands of ~5.2 and ~4.2 kb (Fig. 1*A*). The sizes of these bands were identical to those of authentic ET_A receptor mRNA previously described (13, 17). The ET_B receptor-specific probe hybridized with a single band of ~5.0 kb (Fig. 1*B*), again corresponding to published data for authentic ET_B receptor mRNA (13, 17). ET-1 and ET_A and ET_B receptor mRNA in lung, main trunk of pulmonary artery, right and left atria, right and left ventricles, kidney, spleen, liver, and thoracic aorta was quantitated by slot-blot analysis with use of the probes described above. Chronic hypoxic exposure was associated with a significant increase in ET-1 mRNA in lung (Fig. 2, Table 2). Lung ET-1 peptide content, determined by RIA, was also significantly increased in rats adapted to chronic hypoxia compared with air controls (Fig. 2). ET_A and ET_B receptor mRNA levels were significantly increased in hypoxia-adapted lungs compared with air controls (Fig. 2, Table 2).

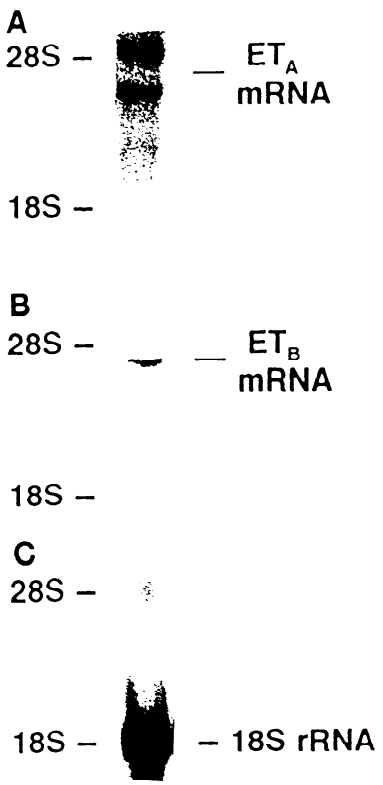

FIG. 1. Northern blot probed with radiolabeled endothelin receptor subtypes ET_A and ET_B cDNA probes. Each lane contained 40 μg of total RNA isolated from lung of air control rats. Two bands (5.2 and 4.2 kb) were detected by ET_A cDNA probe (*A*). ET_B cDNA probe detected a single 5.0-kb band (*B*). An oligonucleotide probe (24 bases) specific for 18S rRNA was used to measure amount of total RNA loaded (*C*).

Figure 3 contrasts ET-1 and ET_A and ET_B receptor mRNA levels in main pulmonary artery and thoracic aorta of chronic hypoxia-exposed and air control rats. Similar to lung, ET-1 mRNA levels were significantly elevated during chronic hypoxic exposure in pulmonary artery. In association with this increase in ET-1 gene expression, a significant decrease in ET_A receptor mRNA levels was observed in main pulmonary artery of hypoxia-exposed rats compared with air controls. Pulmonary arterial ET_B receptor mRNA levels were unaffected by chronic hypoxic exposure. Similar to organs (liver, kidney, spleen) perfused by the systemic vascular bed, ET-1 mRNA levels in thoracic aorta were unaffected by hypoxic exposure (Fig. 3). ET_A and ET_B receptor gene transcript levels were increased in thoracic aorta of hypoxia-adapted rats compared with air controls.

ET-1 mRNA levels were not significantly altered by hypoxic exposure in any heart chamber (Fig. 4, Table 2). ET_A receptor gene transcript levels were significantly increased in right atrium and both ventricles of hypoxia-adapted rats; ET_B receptor mRNA levels were significantly increased in all four heart chambers of hypoxia-adapted rats compared with air controls.

ET-1 and ET_B receptor mRNA levels were unchanged in organs perfused by the systemic circulation (liver, spleen, and kidney) of hypoxia-adapted rats (Table 2). ET_A receptor gene transcript levels were unchanged in kidney and spleen but were significantly decreased in liver in response to chronic hypoxic exposure.

DISCUSSION

This study demonstrated that exposure of rats to chronic normobaric hypoxia (10% O_2 at normal atmospheric pressure for 28 days) was associated with increased ET-1 gene transcript levels in lung and main pulmonary artery and with increased circulating ET-1 levels but had no effect on steady-state ET-1 mRNA levels in systemic vessels (thoracic aorta) or in organs supplied by the systemic vascular bed. Thus the selective increase in pulmonary ET-1 gene transcript levels that we previously demonstrated in rats exposed to acute (48-h) hypoxia was sustained during chronic hypoxic exposure. The magnitude (3.4-fold) and selective pattern of enhancement of ET-1 gene expression were similar to those observed in our previous study, where ET-1 gene expression was stimulated only in lung (2- to 2.5-fold) and right atrium in response to acute hypoxia. Lung ET-1 peptide content was also increased (~2-fold) after chronic hypoxic exposure, indicating that the hypoxia-induced increase in steady-state gene transcript levels was reflected in increased levels of gene product. ET_A and ET_B receptor mRNA levels were elevated in lung, heart, and thoracic aorta but unaltered in organs supplied by the systemic vascular bed (liver, kidney, spleen) in response to chronic hypoxic exposure. These findings are consistent with the hypothesis that a selective increase in synthesis and release of ET-1 from the main pulmonary artery and more distal sites in lung could account for selective hypoxic pulmonary vasoconstriction, pulmonary vascular remodeling, and the maintenance of hypoxia-induced pulmonary hypertension via paracrine and possibly autocrine effects on pulmonary vascular smooth muscle cells. The combination of increased local ET-1 synthesis and increased ET receptors was not found in heart, great vessels, or any other organ studied, thus accounting for the absence of vascular remodeling in extrapulmonary sites during hypoxic exposure.

The current study confirmed the selectivity of hypoxia-induced enhancement in ET-1 gene expression for the lung and pulmonary circulation. The finding of concomitant increases in ET_A and ET_B receptor mRNA levels in lung suggests that increased levels of ET-1 synthesized by pulmonary vascular endothelial cells may act locally on smooth muscle cells in the pulmonary arterial tree to produce chronic vasoconstriction, vascular remodeling, and chronic hypoxic pulmonary hypertension. The recent finding of high levels of ET-1-like immunoreactivity and ET-1 mRNA in endothelial cells of pulmonary arteries in patients with plexogenic pulmonary arteriopathy, many of whom had clinical findings characteristic of primary pulmonary hypertension, tends to support this interpretation (9). In this study, very low levels of ET-1 were detected by immunocytochemical analysis and very low levels of ET-1 mRNA were detected by in situ hybridization in vascular endothelial cells in control human lungs. ET-1 immunostaining and ET-1 mRNA signals tended to colocalize in endothelial cells of thick-

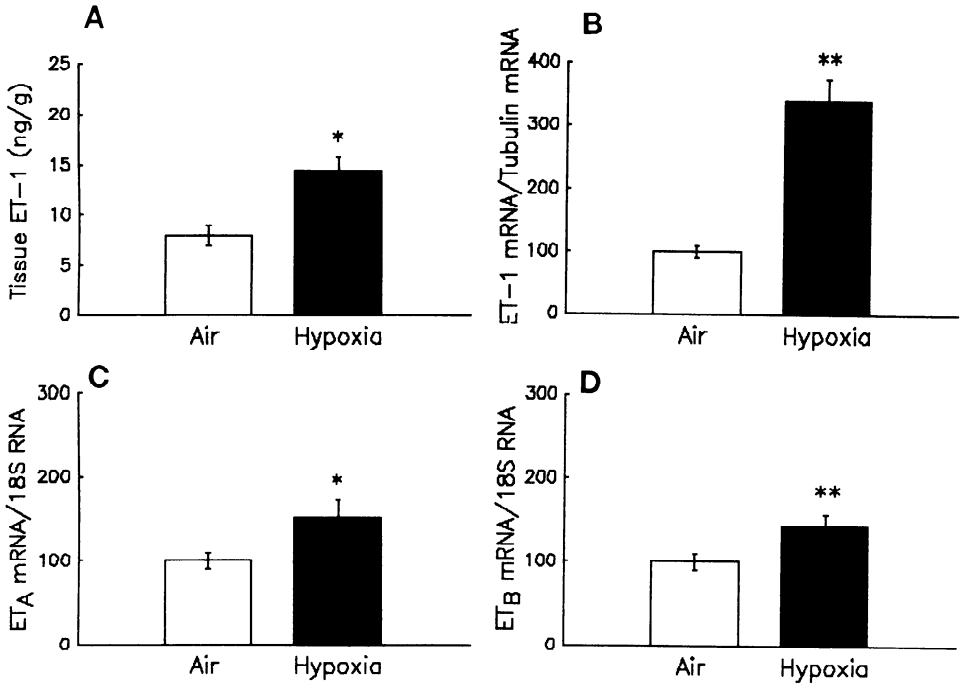

FIG. 2. Effects of chronic hypoxic exposure on endothelin-1 (ET-1) peptide content (*A*), ET-1 mRNA levels (*B*), and ET_A (*C*) and ET_B (*D*) receptor mRNA levels in rat lung. ET-1 peptide content was quantitated by radioimmunoassay. Slot-blot analysis was carried out with 15 μg of total RNA extracted from lung and probed with radiolabeled cDNA probes. Data were normalized to allow for variations in RNA loading with use of ^{32}P-labeled α-tubulin mRNA or 18S rRNA probes. mRNA from each animal was quantitated individually, then means ± SE for each group (n = 18 for air control, n = 22 for hypoxia) were determined. * $P<0.05$, ** $P<0.01$ vs. air control.

ened and fibrotic elastic and muscular pulmonary arteries in patients with plexogenic pulmonary arteriopathy. In contrast, no staining was found in systemic vessels in the heart and kidney of these patients. There was a strong positive correlation between the intensity of ET-1-like immunoreactivity and pulmonary vascular resistance in the patients with plexogenic pulmonary arteriopathy, suggesting that local production of ET-1 may contribute to the vascular abnormalities associated with this disorder. Detectable immunostaining for ET-1 was also found in neuroendocrine cells, pulmonary arterial smooth muscle cells, and type II alveolar pneumocytes in patients with pulmonary hypertension. Alveolar epithelial cells expressed ET-1 in areas of fibrous replacement of alveolar structures, suggesting a role for ET-1 in the pathogenesis of pulmonary fibrosis (9).

In constrast, the cellular location of ET_A and ET_B receptors has not been clearly established in normal or hypoxia-adapted lung. In situ hybridization and Northern analyses showed high levels of expression of ET_A mRNA in the smooth muscle cells of the pulmonary vasculature and bronchi of the rat (13). Hybridization signals for ET_A and ET_B mRNAs have been observed in a patchy distribution throughout the lung parenchyma. A similar pattern of distribution has been described for ET-1 mRNA, suggesting that ET-1 synthesized in lung acts by a paracrine mechanism on receptors close to its site of synthesis (18). Because of the technical limitations of the in situ hybridization method, the cell types expressing ET_A and ET_B mRNA have not yet been identified by light microscopy, however (13). Specific binding sites for ET-1 on bronchial smooth muscle have been identified, and increased levels of ET-1 have been found in the bronchial epithelium, glandular epithelium, vascular endothelium, and bronchoalveolar lavage fluid of asthmatic patients compared with normal subjects (28). ET-1 is a potent bronchoconstrictor (32) and mitogen for airway smooth muscle cells (21). Thus there is a functional endogenous ET-1–ET receptor system in the airways similar to that described in the pulmonary vasculature, and it has been

TABLE 2. *Effects of 4-wk exposure to hypoxia (1 atm, 10% O_2) on tissue ET-1 and ET_A and ET_B receptor mRNA levels*

	ET-1		ET_A		ET_B	
	Chronic hypoxia (n = 22)	Air control (n = 18)	Chronic hypoxia (n = 22)	Air control (n = 18)	Chronic hypoxia (n = 22)	Air control (n = 18)
Lung	342±34†	100±9	152±21*	100±10	145±13†	100±10
Right atrium	118±5	100±12	167±30*	100±12	242±48†	100±9
Right ventricle	115±12	100±12	185±23*	100±6	340±28†	100±7
Left atrium	73±8	100±16	113±11	100±6	178±17†	100±10
Left ventricle	134±30	100±20	159±25*	100±17	142±12†	100±5
Pulmonary artery	313±82*	100±11	55±14*	100±13	141±43	100±13
Thoracic aorta	112±22	100±15	319±59†	100±11	252±33†	100±11
Liver	113±18	100±12	62±5†	100±11	83±27	100±11
Kidney	126±14	100±25	166±41	100±12	81±10	100±9
Spleen	71±5	100±22	87±10	100±12	104±23	100±13

Values are means ± SE. Levels of α-tubulin mRNA and 18S rRNA were used as internal controls to quantitate total RNA loaded. ET-1 mRNA-to-α-tubulin mRNA and ET_A and ET_B receptor mRNA-to-18S rRNA ratios are expressed as percentage of air control. * $P < 0.05$, † $P < 0.01$ vs. air control.

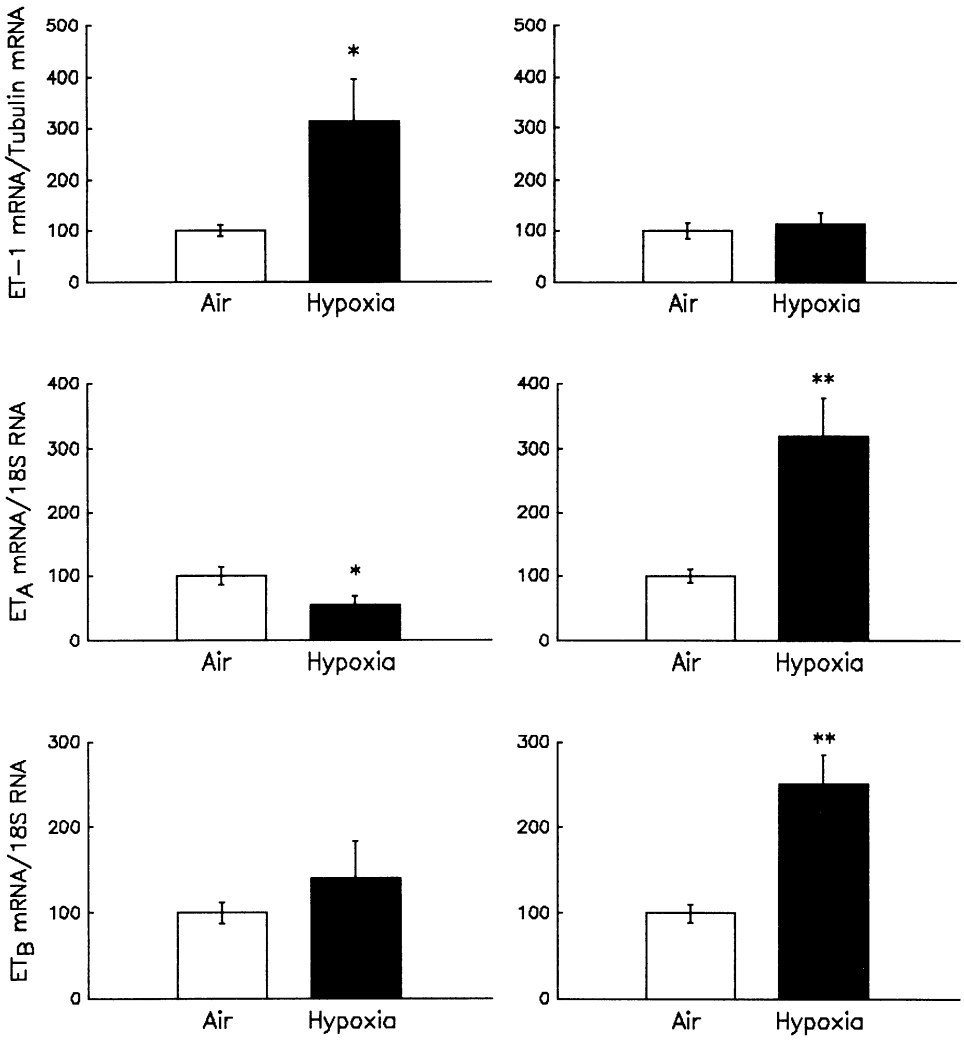

FIG. 3. Effects of hypoxic exposure on ET-1 and ET_A and ET_B receptor mRNA levels in pulmonary artery (*left*) and thoracic aorta (*right*) of rat. Slot-blot analysis was carried out with 15 μg of total RNA extracted from each vessel and probed with radiolabeled cDNA probes. Data were normalized to allow for variations in RNA loading with use of ^{32}P-labeled α-tubulin mRNA or 18S rRNA probes. mRNA from each animal was quantitated individually, then means ± SE for each group (n = 18 for air control, n = 22 for hypoxia) were determined. * $P < 0.05$, ** $P < 0.01$ vs. air control.

suggested that ET-1 may contribute to the increase in smooth muscle mass of the airways in asthmatic patients via its mitogenic effect on airway smooth muscle cells (21). Whether the airway ET-1–ET receptor system is stimulated by chronic hypoxic exposures and contributes to the structural and functional alterations in the hypoxia-adapted lung remains to be determined. Further studies utilizing cultured cells and in situ hybridization analyses of intact lungs of hypoxia-adapted animals are needed to determine the cellular sites of origin and action of ET-1 in lungs from subjects with chronic hypoxic pulmonary hypertension.

Expression of the genes for the ET receptors did not decrease appropriately in lung in response to increased local (lung parenchyma and pulmonary artery) and circulating ET-1 concentrations in the current study. Down-regulation of the ET receptors in response to increased concentrations of their ligand in the extracellular space, as previously described for other G protein-coupled receptors, would be expected in the lung of hypoxia-adapted rats. The observation of decreased binding of ^{125}I-labeled ET to rat vascular smooth muscle cells in culture in response to addition of exogenous ET to the media (11) was the first demonstration that ET-1 modulates the number of ET binding sites on its target cells. More recently, it has been shown that maximal binding of ^{125}I-labeled ET-1 to endothelial cells derived from human umbilical vein and rat aorta and to rat mesangial cells is greatly increased by blocking the generation of endogenous ET-1 from big ET-1 with phosphoramidon (4). Whether similar mechanisms of ET receptor regulation operate in vivo is a matter for further study. The methods employed in the current study do not permit us to determine whether the increases in steady-state ET_A and ET_B receptor mRNA levels in lung of rats adapted to chronic hypoxia simply reflect the major increase in vascular smooth muscle mass in the hypoxic lung or whether they represent disordered regulation of ET receptor gene expression in response to hypoxia or to hypoxia-induced alterations in other, as yet unidentified, growth factors and mediators. Further additional study is needed to determine whether the hypoxia-induced alterations in ET receptor steady-state gene transcript levels are translated into alterations in receptor density, as assessed by radioligand techniques.

The ET_A receptor has been most often implicated in the pulmonary vasoconstrictor function of ET-1. Our observation of increased ET_A receptor mRNA levels in lung of rats adapted to chronic hypoxia is consistent with an enhanced pulmonary vasoconstrictor role for endoge-

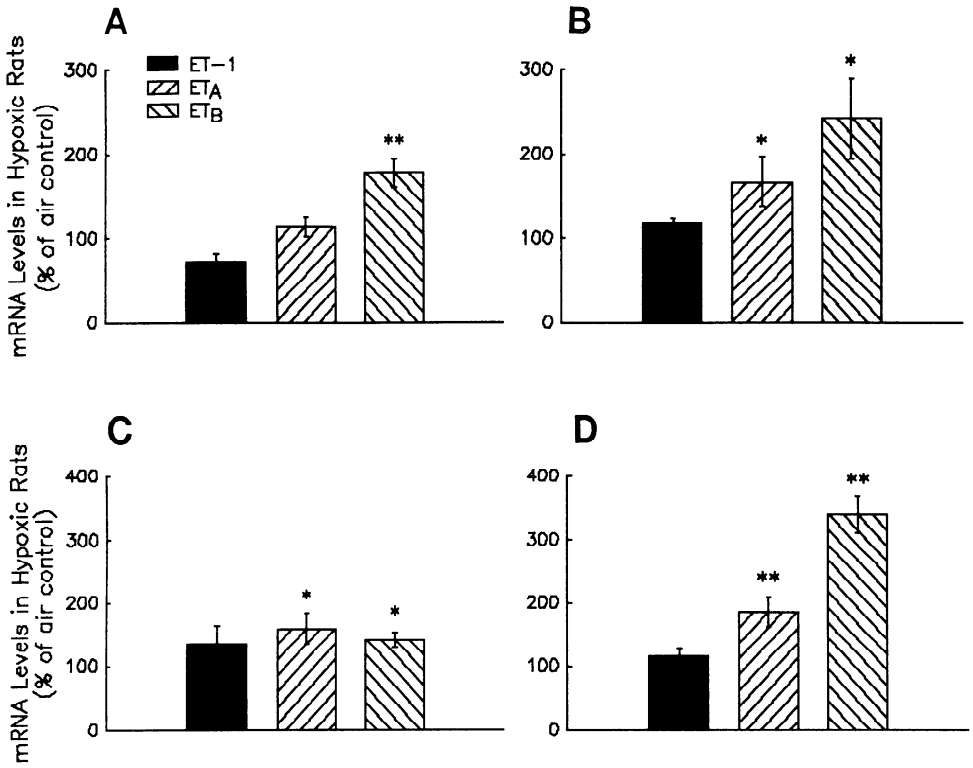

FIG. 4. Effects of hypoxic exposure on ET_A and ET_B receptor mRNA levels in left (*A*) and right (*B*) atria and left (*C*) and right (*D*) ventricles. Slot-blot analysis was carried out with 15 μg of total RNA extracted from each chamber and probed with radiolabeled cDNA probes. Data were normalized to allow for variations in RNA loading with use of ^{32}P-labeled α-tubulin mRNA or 18S rRNA probes and expressed as percentage of air control group (control = 100%; data not shown). mRNA from each animal was quantitated individually, then means ± SE for each group (n = 18–22) were determined. * $P < 0.05$, ** $P < 0.01$ vs. air control.

nous ET-1 in chronic hypoxic pulmonary hypertension. Furthermore the current data provide a molecular basis for the previous observations that exogenous ET-1 produces ET_A receptor-mediated constriction of the pulmonary vasculature in the rat and that this effect is amplified by exposure to chronic hypoxia (1, 6). Injection of exogenous ET-1 into the pulmonary artery of conscious rats exposed to chronic hypoxia has been shown to increase pulmonary vascular resistance without altering pulmonary arterial pressure; ET-1 did not alter pulmonary vascular resistance in air control rats (6). Enhancement of the vasoconstrictor response to ET-1 in the chronic hypoxia-adapted lung is likely related to a combination of increased ET receptor numbers in the small intrapulmonary arteries, increased pulmonary arterial muscle mass, and decreased endothelium-dependent relaxation (endothelium-derived relaxing factor release). Whether the hypoxia-enhanced ET-1 pulmonary vasoconstrictor response is transduced entirely through ET_A receptors is uncertain. Whereas selective ET_A receptor antagonists have been shown to block ET-1-induced pulmonary vasoconstriction in the isolated perfused rat lung studied under normoxic conditions (1) and the acute hypoxic response in intact conscious rats (2), the effects of these agents on the maintenance of chronic hypoxic pulmonary hypertension have not been examined.

The role of the ET_B receptors in maintaining chronic hypoxic pulmonary hypertension has not been studied. ET_B and ET_A receptor gene transcript levels appear to be increased to the same extent in hypoxia-adapted lung. Although it was originally believed that ET_A receptors mediate the contractile effects of ET and ET_B receptors, its vasodilator effects, it is now clear that both receptor subtypes can mediate vasoconstriction (34). ET_A and ET_B receptors have been described on smooth muscle cells in a variety of vascular beds, including rat aorta, rabbit pulmonary artery, porcine pulmonary and coronary arteries, and saphenous veins of dog, monkey, and rabbit (34). Furthermore ET_B receptors mediate constriction of the rabbit pulmonary artery and rabbit and dog saphenous vein (34). ET_B receptors in rat aortic smooth muscle cells have been shown to be coupled to mobilization of Ca^{2+} from an intracellular pool, suggesting a contractile function. We hypothesize that, because the smooth muscle cell mass in the pulmonary vasculature of chronic hypoxia-adapted rats is greatly increased, the increased ET_B receptor gene expression that we observed in the lung and pulmonary artery of these animals may localize mainly in vascular smooth muscle. We further hypothesize that these ET_B receptors may have a vasoconstrictor rather than a vasodilator function. Alternatively, ET_B receptors could be increased in the pulmonary vascular endothelium of hypoxic animals to promote vasodilation and counteract the effects of pulmonary hypertension. In situ hybridization analysis for cellular localization of ET_B receptor mRNA and studies of the effects of selective ET_B receptor agonists and antagonists on pulmonary vascular tone in rats adapted to chronic hypoxia are needed to test these hypotheses.

Both ET receptor subtypes have been identified in heart by use of radioligand-binding techniques (20). Furthermore ET has important effects in the heart, including stimulation of myocyte hypertrophy and atrial natriuretic peptide secretion from myocytes (27). In the current study, steady-state mRNA levels for ET_A and ET_B receptors were increased in thoracic aorta and heart of hypoxia-adapted rats compared with air controls, but mRNA levels for ET-1 in heart were unchanged from air controls. Whether these alterations in ET receptor gene expression were a direct consequence of hypoxia per se or

of other, as yet unidentified, hypoxia-induced mediators or growth factors or were secondary to hypoxia-induced cardiovascular hypertrophy is unclear from the current data. Further additional experimentation is needed to determine whether ET contributes to the increased atrial natriuretic peptide synthesis and release and the right ventricular hypertrophy in chronic hypoxia.

The stimulus for increased ET-1 gene expression in hypoxia appears to be direct hypoxia-mediated enhancement in ET-1 gene transcription, as first described by Kourembanas et al. (16) in human umbilical vein endothelial cells in culture. In contast, it is unclear whether the responses of ET_A and ET_B receptor gene expression in lung to chronic hypoxia are mediated directly by hypoxia or indirectly by alterations in tissue levels of ET-1 or of ET-1-sensitive mediators or whether they result from the extensive pulmonary vascular remodeling process. Hypoxia-induced enhancement in ET-1 gene expression in the lung of the intact rat has been shown to be a graded response that is proportional to the Po_2 in the blood supply (7). Hypoxia-mediated enhancement in gene transcription has also been reported for PDGF-B and for transforming growth factor but not for other endothelial cell-derived growth factors, including PDGF-A, granulocyte-macrophage colony-stimulating factor, basic fibroblast growth factor, von Willebrand factor, Na-K-adenosinetriphosphatase, and β-actin (16). The molecular mechanism by which hypoxia stimulates ET-1 gene transcription is unknown. The ET-1 gene does not share sequence homology with the hypoxia-responsive transcriptional enhancer that has recently been identified in the human erythropoietin gene (19) or with the DNA binding site for the recently characterized hypoxia-inducible nuclear protein that activates transcription of the human erythropoietin gene (33). Further study is needed to identify hypoxia-responsive transcriptional enhancers and to isolate genes encoding protein factors that direct the transcriptional response of the ET-1 gene to hypoxia. Characterization of these *cis*- and *trans*-activating factors for the ET-1 gene would enhance our understanding of oxygen sensors and oxygen-related gene expression in general.

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