ET-1 receptor gene expression and distribution in L1 and L2 cells from hypertensive sheep pulmonary artery

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Balyakina, Elena V., Daohong Chen, Mayme L. Lawrence, Suzanne Manning, Richard E. Parker, Scott B. Shappell, and Barbara Meyrick. ET-1 receptor gene expression and distribution in L1 and L2 cells from hypertensive sheep pulmonary artery. Am J Physiol Lung Cell Mol Physiol 283: L42–L51, 2002. First published February 8, 2002; 10.1152/ajplung.00337.2001.—We examined gene and surface expression and activity of the endothelin (ET)-1 receptors (ETA and ETB) in subendothelial (L1) and inner medial (L2) cells from the main pulmonary artery of sheep with continuous air embolization (CAE)-induced chronic pulmonary hypertension (CPH). According to quantitative real-time RT-PCR, basal gene expression of both receptors was significantly higher in L2 than L1 cells, and hypertensive L2 cells showed significantly higher gene expression of ETB than controls. Expression of both genes in hypertensive L1 cells was similar to controls. Fluorescence-activated cell sorter analysis confirmed the increased distribution of ETB in hypertensive L2 cells. Although only the ETA receptors in control L2 cells showed significant binding of 125I-labeled ET-1 at 1 h, both receptors bound ET-1 to hypertensive cells. Exposure to exogenous ET-1 for 18 h revealed that only the L2 cells internalized ET-1, and internalization by hypertensive L2 cells was significantly reduced when compared with controls. Treatment with ETA (BQ-610) and ETB (BQ-788) receptor antagonists demonstrated that both receptors contributed to internalization of ET-1 in control L2 cells, whereas in hypertensive cells only when both receptor antagonists were used in combination was significant suppression of ET-1 internalization found. We conclude that in sheep receiving CAE, alterations in ETB receptors in cells of the L2 layer may contribute to the maintenance of CPH via alterations in their expression, distribution, and activity.

ETA receptor; ETB receptor; smooth muscle cells; pulmonary hypertension; endothelin

Since its discovery 12 years ago, endothelin (ET) has been shown to contribute to a wide variety of physiological and pathophysi-ological processes in various systems (22). The ET family of 21 amino acid peptides exists in three distinct isoforms: ET-1, ET-2, and ET-3. It is an extremely potent vasoconstrictor resulting in slowly developing and sustained contraction. Although the endothelial cell was originally described as the source of ET-1, it is now known that several other cell types, including pulmonary vascular smooth muscle cells, synthesize this peptide (33, 36).

The biological effects of ET-1 are mediated by two distinct ET receptor subtypes, ETA and ETB (1, 35). The affinity of the ETA receptor for ET-1 has been shown to be ~100 times that for ET-3 (ET-1 > ET-2 > ET-3), whereas the affinity of the ETB receptor is equipotent for all three isoforms of ET (34). Early reports indicated that the ETA receptor on smooth muscle was responsible for the vasoconstrictor effect of ET-1, whereas the ETB receptor on endothelial cells mediated ET-1-induced vasodilation. However, recent pharmacological studies suggest that there are two classes of ETB with cell specific effects, ETB1 on endothelial cells mediating vasodilation and ETB2 on smooth muscle cells mediating vasoconstriction (9). The ETB receptor also functions as a “clearance receptor” and is particularly important in the lung, where 50% of ET-1 is retained (22, 29).

ET-1 has been linked to the development of chronic pulmonary hypertension (CPH). Increased expression of the peptide has been demonstrated in endothelial cells of pulmonary arteries from patients with idiopathic and secondary forms of pulmonary hypertension (15), and arterial-to-venous ratios of ET-1 protein were found to be elevated above the normal range in patients with pulmonary hypertension (40). Several studies have linked increased expression of ET-1 to the development of hypoxia-induced CPH in rats (5, 24), and use of ET-1 receptor antagonists has been shown both to inhibit progression of the disease and to promote recovery (4, 10, 43).

Although these studies support a central role for ET-1 in the pathogenesis of CPH, the picture is likely more complicated than first suspected, in that prepro-ET-1 (ppET-1) mRNA is decreased in the monocrotaline model of CPH, and in addition, we recently reported regional variability in ppET-1 gene expression in the pulmonary artery of normal sheep. Furthermore, we found in a model of CPH, sheep receiving
continuous air embolization (CAE) into the main pulmonary artery, that ppET-1 gene expression in the main pulmonary artery and peripheral lung gradually decreased with the onset of pulmonary hypertension while the midregion artery (9th–10th generation) showed a gradual increase (42).

At least four cell types have been identified in the bovine main pulmonary artery: subendothelial L1 cells, inner medial L2 cells, and outer medial L3S and L3R cells (12, 13). We recently showed that L1 cells (a cell with characteristics of both endothelial and smooth muscle cells) cultured from control sheep produce strikingly more ET-1 than L2 cells (mature smooth muscle cells) under basal conditions, but when exposed to exogenous ET-1, only the L2 cells internalize exogenous ET-1 (41). The present study examines whether ET A and ET B gene and surface expression is altered in L1 and L2 cells from control and hypertensive sheep. This study also examines whether ET-1 receptor distribution and activity are altered in L1 and L2 cells cultured from hypertensive sheep induced by 12 days of CAE.

METHODS

Sheep model of CPH. The protocol for delivery of CAE into the sheep pulmonary artery has been described previously, and the physiological and structural changes for this model of CPH have been reported in detail (16, 32). Briefly, a left thoracotomy was performed on yearling mixed-breed sheep under general anesthesia, and pulmonary artery and left atrial catheters were inserted. A jugular venous introducer and carotid artery catheter were placed through a neck incision. After a recovery period of 3–5 days, a Swan-Ganz catheter was positioned in the pulmonary artery through the jugular vein introducer. Baseline measurements of pulmonary arterial pressure (Ppa), cardiac output (CO), and left atrial pressure (PLa) were made, and pulmonary vascular resistance (PVR) was calculated using the formula: PVR = (Ppa – Ppa/CO). After baseline measurements, the sheep received CAE (n = 3) into the pulmonary artery by way of the proximal port (lying in the right heart) of the Swan-Ganz catheter over a 12-day period. The rate of air embolization proximal port (lying in the right heart) of the Swan-Ganz resistance (PVR) was calculated using the formula: PVR = (Ppa – Ppa/CO).

Cell culture. At the end of the experiment, the animals were killed with an overdose of sodium barbiturate (pentobarbital sodium 325 mg \text{ ml}^{-1} \cdot 5 \text{ kg}^{-1} \text{ body wt}, Euthanazia-5 solution; Henry Schein, Port Washington, NY), and the lungs were removed. The main pulmonary artery was dissected from a point 1 cm from its exit from the right ventricle to its point of entry into the right and left lungs, and a section was taken for routine light microscopy and for 1-μm sections of epoxy-embedded tissue. Cells were isolated from the subendothelial (L1) and inner medial (L2) layers of the main pulmonary artery from control and hypertensive sheep as previously described (41, 42). Briefly, the artery was opened, and the endothelial layer was removed by gentle scraping; the subendothelial L1 cells were isolated from pulmonary artery segments by deep scraping. The inner media L2 cells were isolated either by a modified explant technique or by enzymatic dissociation. Using phase contrast microscopy, we identified L1 and L2 cell colonies by their morphology, trypsinized them in “cloning rings” and transferred them into culture dishes. Both cell types were seeded and cultured in RPMI-1640 containing 100 U/ml penicillin, 100 μg/ml streptomycin, 40 μg/ml gentamicin, 2 μmol/l t-glutamine (GIBCO-BRL, Grand Island, NY), and 15% heat-inactivated calf serum (Atlanta Biologicals, Norcross, GA). Confluent cells (passages 3–6) were used for all experiments; higher passage numbers have been associated with a substantial increase in ET B receptor density (27). Cell lines cultured from each of three different control and three different hypertensive animals were used for each assay.

Experimental protocols. All experiments were carried out in RPMI-1640 containing 0.1% BSA. Experiments were initiated after overnight incubation of the cells in that medium. For real-time RT-PCR analysis of ET A and ET B gene expression, cells were harvested 90 min after addition of 0.1% BSA in RPMI-1640. For dose-response studies of ET-1 internalization, intracellular levels of ET-1 were measured after 18-h exposure to exogenous ET-1 at concentrations from 0.01 to 1,000 nM. Experiments with ET A (BQ-610) and ET B (BQ-788) antagonists included a 2-h preincubation period before addition of exogenous ET-1 (10 nM) for 18 h. The dose of each antagonist used in these experiments was based on dose-response curves. The concentrations of BQ-610 tested were from 1 to 100 μM, and those for BQ-788 were from 0.01 to 100 μM. The dose chosen was that dose that gave a 50% reduction in uptake of exogenous ET-1 by control L2 cells. For ET-1 binding assays, cells in two P150 dishes were pretreated with either BQ-610 or BQ-788 for 2 h before isolation of cell membranes. Untreated L1 and L2 cells and cells treated with each antagonist alone served as controls.

Quantitative real-time RT-PCR for ET A and ET B. Total RNA was isolated with RNA STAT-60 reagent (TEL-TEST “B”, Friendswood, TX). mRNA copy numbers of ET A and ET B in L1 and L2 cultured cells from control and hypertensive sheep were determined by real-time quantitative RT-PCR using a Lightcycler fluorescence temperature rapid-air cycler (Roche Molecular Biochemicals, Indianapolis, IN) with cDNA (ET A) or RNA (ET B) standard curves and the double-stranded DNA binding fluorescent probe SYBR green (38). Amplifications were done in glass capillary tubes with a 20-μl reaction of 200 ng for L2 and L1 total RNA samples, 6 mM magnesium chloride, 2.0 μl 1 × SYBR green RNA master-mix (Roche Molecular Biochemicals), 0.4 μl RT enzyme (Roche Molecular Biochemicals), and 0.2 μM each primer.

The cDNA template for ET A standard curves was derived by reverse transcription of total RNA from control L2 cells (17). Total RNA from L2 cells was used as a template for standard curves for the RT-PCR assay for ET B. Copy number for ET A and ET B standard templates was assigned based on the concentration of the total cDNA or RNA and the known number of base pairs (bp) in the full-length cDNAs or RNA of ET A and ET B, respectively. The primers for ET A (based on the human sequence) were 5′ AGC TTC CTG GTT ACC ACT CAT CAA 3′ (forward) and 5′ TCA ACA TCT CAC AAG TCA TGA G 3′ (reverse), which give a 714-bp product (corresponding to nucleotides 136–850) (45). Primers for ET B were derived from a newly generated sheep cDNA sequence, because our first attempts at real-time RTPCR using primers generated from the human ET B cDNA sequence revealed several nontypical products. Briefly, RT-PCR amplimers for sheep ET B were cloned into pGEM-T Easy Vector System (Promega, Madison, WI) and transformed in DH5α-competent cells. Positive colonies were selected by color screening, and the plasmid DNA was iso-
lulated using a QIAprep Spin Miniprep Kit (QIAGEN). Digestion of the plasmid DNA with EcoRI gave inserts of the expected size (701 bp). Comparison of the sequence of these clones with the human sequence demonstrated 89–90% homology. New ETα primers were designed to amplify a 297-bp fragment from this sequence using DNA-STAR software. The utilized ETα primers were: 5'-TTT GGC CAC TTC CGC TCT CTG TTT 3' (forward) and 5' ATT TGG GTG GTC TTC GGT GTT CGT 3' (reverse).

The one-step real-time RT-PCR reactions consisted of the following steps: reverse transcription at 55°C for 15 min, denaturation at 95°C for 1 min, amplification for 55 cycles, and melting curve analysis from 95 to 65°C at a rate of 0.1°C/s under continuous fluorescence monitoring. The amplification program for ETα consisted of heating at 20°C/s to 95°C, cooling at 20°C/s to 57°C, annealing at 57°C for 5 s, heating at 20°C/s to 72°C, elongation at 72°C for 29 s, and heating at 5°C/s to 86°C for fluorescence acquisition. The amplification program for ETβ consisted of heating at 20°C/s to 95°C, cooling at 20°C/s to 55°C, annealing at 55°C for 5 s, heating at 20°C/s to 72°C, elongation at 72°C for 14 s, and heating at 5°C/s to 79°C for fluorescence acquisition. The specificity of the amplimer in each reaction was confirmed by melting curve analysis, with initial gel confirmation that this large peak corresponded to the expected amplimer (representative melting curve analysis for ETβ shown in Fig. 4C) (38). The contribution to fluorescence signal of any nonspecific products and/or primer dimers was eliminated by increasing the temperature to 2°C below the melting temperature of the specific product, which eliminated any other minor cDNAs (which have lower melting temperatures) (38). Copy numbers of mRNA were calculated from serially diluted standard curves generated from cDNA or RNA templates (17, 38). Serial dilutions (1:10) over a range of three orders of magnitude were used to generate the standard curves. The serially diluted standards were simultaneously amplified with the unknown samples to generate a linear standard curve by the fit-points method of analysis with five points. Standard curves for both ETα and ETβ consistently had correlation coefficients of 0.97–1.00 (representative sample for ETβ is shown in Fig. 4B). Control samples run in triplicate had a variance of ~10%. All biological samples fell on the standard curves, and copy numbers of the unknown samples were calculated with the Lightcycler software (version 3). ETα and ETβ values were normalized to the copy numbers of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (G3PDH) and multiplied by 100 (%) to correct any variation in amount of RNA utilized per assay. All samples for an individual receptor subtype were analyzed in the same assay, eliminating any contribution of interassay variability.

**ET-1 ligand-receptor maximal binding assay.** Control and hypertensive L2 cells were pretreated with BQ-610 and BQ-788 alone and together for 2 h before isolation of crude cell membrane fractions (CMFs) as previously described (42). Maximal ET-1 receptor binding ([B]max) was examined by mixing 20 μg of CMF protein with 0.01, 0.02, 0.04, 0.06, or 0.1 μCi of [125I]-labeled ET-1 (Amersham Pharmacia Biotech) at 37°C for 1 h. The reaction was performed in a total volume of 200 μl in 50 mM Tris buffer. Controls for nonspecific binding were carried out in parallel using 20 μg of CMF protein mixed with 0.5 μM unlabeled ET-1 plus 0.002, 0.02, and 0.1 μCi [125I]-ET-1. The reaction was terminated by passing the reaction mix through a Glass Fiber C-grade filter paper (Whatman, Maidstone, England) and washing it in PBS. The bound radioactive disintegrations on the filter paper were then counted on an autoradiocounter for 125I. The counts were correlated to saturation curves using Ligand software, and the Bmax of ET-1 receptors was calculated for each experimental group.

**Assessment of ETα and ETβ by flow cytometry.** Confluent monolayers of L1 and L2 cells in 100 dishes were treated with a modified cell dissociation buffer (Life Technologies, Rockville, MD) containing 0.5% EDTA disodium salt, 6% glycerol, and 0.06 M sodium citrate for 15 min at room temperature. The dissociated cells were centrifuged at 800 rpm for 10 min at 4°C, and the pellet was resuspended in 1 ml of cold 1% BSA in PBS. A further 1 ml of cold PBS-1% BSA was added, and the cell suspensions were divided into two microcentrifuge tubes. The samples were washed twice by centrifugation for 15 s at 4°C, and each cell pellet was resuspended in 100 μl of cold PBS-1% BSA. Sheep ETα and ETβ receptor antibodies (Oxford Biomedical Research, Oxford, MI) were added to each sample to give a final concentration of 1 μg/106 cells, and the samples were incubated at 4°C for 1 h. The cells were then washed twice in cold PBS-1% BSA; rabbit V(ab)/2 anti-sheep IgG (heavy + light)-FITC (Southern Biotechnology Associates, Birmingham, AL) was used as the secondary antibody, which has lower melting temperatures (38). Copy numbers that were treated with the secondary antibody alone (background controls). Flow cytometric measurements were performed at a Becton-Dickinson FACStar+ instrument, equipped with a single 5-W argon-ion water-cooled laser tuned to 488 nm (blue green) and three fluorescence detectors. The data were analyzed using Cell Quest software (Becton-Dickinson, San Jose, CA). Each sample was measured in duplicate. All cells were included in the analysis (3). Specific fluorescence (Fsp) was calculated by subtracting the mean fluorescence of all the cells in each sample reacted with the secondary antibody alone (Fspbackground) from that obtained with the specific antibodies (Fsptotal). Percent specific signal (%Fsp) was also calculated using the formula: %Fsp = Fspbackground/Fsptotal × 100 (38).

**ET-1 extraction and measurement of intracellular ET-1.** Intracellular levels of mature ET-1 were determined in homogenates from both L1 and L2 cells as described previously (42). Briefly, at the end of the experiment, cells were washed three times in serum-free medium and trypsinized, and cell number was counted using a hemocytometer. After centrifugation at 1,000 rpm for 10 min, the cell pellets were homogenized in 0.1% Triton X-100 with a Teflon glass pestle. The homogenates were centrifuged at 3,000 rpm for 15 min, and the supernatants were collected for solid-phase extraction. The cell supernatants were acidified by 2 M HCl and loaded in Sep-Pak C18 cartridges, which were prewashed with 100% methanol, acetonitrile-5 mmol/l trifluoroacetic acid (TFA), and distilled H2O with 5 mmol/l TFA. Each cartridge was then washed in distilled H2O with 5 mmol/l TFA and eluted with 2 ml of 80% methanol containing 0.1% TFA. The samples were divided into two tubes and dried under vacuum. Levels of ET-1 were determined in 100-μl aliquots of L1 and L2 homogenates using a commercially available ET-1 ELISA system (Biotrak ELISA System; Amersham International). The ELISA was performed according to the manufacturer’s instructions. The assay is sensitive to values of >1 fmol and cross-reacts with 100% of ET-1 and ET-2 (ET-2 is not generally found in the walls of the pulmonary vasculature) but not with ET-3 (<0.001% cross reactivity). Each sample was run
RESULTS

In previous studies we have documented that 12 days of CAE causes the development of sustained pulmonary hypertension that is characterized by a doubling of PVR (32). The sheep utilized in the present study showed a similar significant increase in PVR after 12 days of CAE (controls, 2.0 ± 0.10 units; 12 days of CAE, 4.67 ± 0.47; n = 3). The control animals exhibited no increase in PVR over a 12-day period.

Morphology of L1 and L2 cells in control and hypertensive sheep. Light microscopy revealed that 12 days of CAE resulted in a striking increase in the thickness of both the L1 and L2 layers of the main pulmonary artery (Fig. 1). Marked hypertrophy and hyperplasia of the L1 and L2 cells were apparent as was an increase in extracellular ground substance.

As shown previously, L1 and L2 cells cultured from the main pulmonary artery of control sheep showed cell-specific differences in morphology. By phase-contrast microscopy, L2 cells exhibited the characteristic spindle shape and “hill-and-valley” formation of mature smooth muscle, whereas the L1 cells were initially rhomboidal and later formed a multilayer of rhomboidal and large stellate cells (Fig. 2, A and C) (41). L1 cells cultured from the hypertensive sheep maintained a shape and size similar to control cells (Fig. 2D), whereas the hypertensive L2 cells were larger and more rounded than control cells (Fig. 2B), and the typical hill-and-valley formation was less pronounced.

$ET_A$ and $ET_B$ receptor gene expression in control and hypertensive L1 and L2 cells. Both L1 and L2 cells from control sheep express the $ET_A$ and $ET_B$ genes, and quantitative real-time RT-PCR revealed that the copy number for both $ET_A$ and $ET_B$ genes (0.8 ± 0.14 × 10^{12} copies/200 ng RNA and 1.1 ± 0.17 × 10^{12}, respectively) in L2 cells was significantly higher than that obtained for L1 cells (0.05 ± 0.007 × 10^{12} copies/200 ng RNA and 0.12 ± 0.08 × 10^{12}, respectively) (P < 0.05). Similar findings were demonstrated when these data were related to G3PDH (Fig. 3).

In L2 cells from hypertensive sheep, expression of the $ET_A$ and $ET_B$ genes remained higher than in hypertensive L1 cells (Fig. 3). Although copy number of the $ET_A$ gene in the hypertensive L2 cells was similar to controls (hypertensive, 1.1 ± 0.23 × 10^{12} copies/200 ng RNA), expression of the $ET_B$ gene was twice that of controls (hypertensive, 2.2 ± 0.47 × 10^{12}, P < 0.05).

Copy number of the $ET_A$ gene in the hypertensive L1 cells was similar to control values. Copy number for the $ET_B$ gene in hypertensive L1 cells tended to be higher than in controls, but this did not reach significance. Similar findings were found when copy numbers were related to G3PDH (Fig. 3). Representative real-time RT-PCR standard curves (0.1 ng–1 μg) for $ET_B$ and curves for control and hypertensive L2 cells are shown in Fig. 4. When fluorescence is related to cycle number, the hypertensive curve is shifted to the left compared with the control indicating an increase in $ET_B$ expression (Fig. 4A).

$ET_A$ and $ET_B$ expression by FACS analysis on control and hypertensive L1 and L2 cells. FACS analysis of control and hypertensive L1 and L2 cells, when expressed as $F_{sp}$, revealed no significant difference between the $ET_A$ and $ET_B$ receptors save for a modest trend to an increase in $ET_B$ expression in the hypertensive L2 cells compared with the controls (Fig. 5A); distribution of $ET_A$ also tended to fall in the hypertensive cells. The modest nature of these changes no doubt reflects a variability in expression in primary cell lines both between sheep and between clones from a single animal. However, when the data were expressed as percent-specific fluorescence, a modest but significant increase in $ET_B$ was apparent for the hypertensive L2 cells compared with controls (Fig. 5B), confirming the real-time RT-PCR data.

$ET_1$ receptor $B_{max}$ by L2 cells from control and hypertensive sheep. $B_{max}$ of ET-1 for cellular membranes from control L2 cells was 289.3 ± 28.4 fmol/mg protein at baseline. Pretreatment with BQ-610 alone or in combination with BQ-788 caused a significant
decrease in B\textsubscript{max} below baseline (P < 0.05). Inhibition by the ET\textsubscript{B} antagonist failed to show a fall in ET-1 binding. In hypertensive cells, treatment with the ET\textsubscript{A} and ET\textsubscript{B} inhibitors alone or in combination led to a significant reduction in B\textsubscript{max} below their baseline value (Fig. 6).

**Internalization of ET-1 by L1 and L2 cells from control and hypertensive sheep.** Exposure of L2 cells from control animals to various concentrations of ET-1 resulted in a dose-dependent increase in intracellular ET-1 with a 50% effective dose (ED\textsubscript{50}) of 2 nM (Fig. 7A). L2 cells from hypertensive sheep also showed a dose-dependent increase in intracellular ET-1, but the level of uptake was 10-fold less than that seen for control cells (ANOVA P < 0.05), giving an ED\textsubscript{50} of 20 nM.

Measurement of ET-1 in L1 cells from control sheep after exposure to various concentrations of exogenous ET-1 revealed no increase in intracellular ET-1 (Fig. 7B). Similarly, L1 cells from the hypertensive sheep failed to take up ET-1.

**ET-1 receptor antagonists and ET-1 internalization by L2 cells from control and hypertensive sheep.** Because only L2 cells responded to exogenous ET-1, experiments with ET\textsubscript{A} and ET\textsubscript{B} receptors antagonists were performed only on those cells. Pretreatment of control L2 cells with various concentrations of either the ET\textsubscript{A} antagonist BQ-610 or the ET\textsubscript{B} antagonist BQ-788 revealed that the IC\textsubscript{50} for BQ-610 in our system was 0.5 μM (Fig. 8) and that for BQ-788 was 25 μM (data not shown). The concentration of each receptor antagonist giving the IC\textsubscript{50} was chosen for further experiments.

Basal levels of intracellular ET-1 in control and hypertensive L2 cells were not altered by exposure to either BQ-610 or BQ-788 (Fig. 9). Exposure of control L2 cells to exogenous ET-1 resulted in a significant increase in intracellular ET-1 (P < 0.05, Fig. 9A); pretreatment with either BQ-610 or BQ-788 significantly suppressed the increase in intracellular ET-1 by 18 and 25%, respectively. Simultaneous exposure to both antagonists caused an additive inhibitory effect.
et-1 remained significantly elevated above basal levels ($P < 0.05$).

Basal levels of intracellular ET-1 in the hypertensive L2 cells were similar to those in control cells (Fig. 9B), although uptake by hypertensive cells was $\sim50\%$ less than by control cells ($P < 0.05$). Unlike the response of the control cells, pretreatment of the hypertensive L2 cells with either BQ-610 or BQ-788 failed to inhibit the ET-1-stimulated increase in intracellular ET-1 (Fig. 9B). Simultaneous exposure to both antagonists, however, caused significant and striking inhibition of the ET-1-stimulated increase in intracellular ET-1 such that the level of intracellular ET-1 returned to basal values.

**DISCUSSION**

In a previous study, our group identified differences in the ET-1 system in L1 and L2 cells from control sheep (41). L1 cells, like endothelial cells, were found to synthesize and release ET-1, whereas L2 cells had less synthetic capacity for ET-1 but, rather, had the propensity to internalize exogenous ET-1. The present study extends these findings and examines $ET_A$ and $ET_B$ gene and surface expression, $B_{max}$ sites, and activity as assessed by ET-1 internalization by L1 and L2 cells from the main pulmonary artery of control sheep and those with sustained pulmonary hypertension induced by CAE. The present in vitro study documents that the hypertensive L2 cells, as seen in vivo, maintain their hypertrophic appearance, whereas the L1 cells from the thickened subendothelial layer revert to an appearance similar to controls. In addition, we show that L2 cells from control and hypertensive animals exhibit significantly higher basal expression of both the $ET_A$ and $ET_B$ genes than L1 cells and that $ET_B$ gene and surface expression in hypertensive L2 cells...
was significantly increased compared with controls. The present findings also confirm that L2, but not L1, cells from control sheep internalize exogenous ET-1 in a dose-dependent manner and further demonstrate that L2 cells from hypertensive sheep internalize less ET-1 than controls. In addition, we found that in control cells, the ETA receptors were mainly responsible for binding ET-1 to the L2 cells, but after 18 h of exposure to ET-1, both receptors played a role in internalization. Using ETA and ETB receptor antagonists, our study also demonstrates that in hypertensive cells, both receptors bound radiolabeled ET-1; internalization of ET-1, however, was inhibited only when the ETA and ETB antagonists were used in combination.

Function of ETA and ETB receptors. In humans, ETA and ETB are encoded by distinct genes located on chromosomes 4 and 13, respectively (32). In pulmonary vascular smooth muscle cells, the ETA receptor is linked to vasoconstriction as well as hypertrophy and smooth muscle proliferation, whereas the ETB receptor is linked to clearance of ET-1 from the circulation as well as both vasoconstriction and vasodilation (22, 36, 39). Studies in the pulmonary vasculature suggest that the actions of ET-1 are mediated by at least three receptors: one ETA receptor and two ETB receptor subtypes. Pharmacological studies in several species indicate that the endothelial ETB receptor (designated ETB1) mediates vasodilatation, whereas the ETB2 receptor expressed in smooth muscle acts as vasoconstrictor (5, 7, 9).

ETA and ETB receptors are G protein-coupled receptors that undergo agonist-stimulated desensitization or inactivation (26). Desensitization is thought to prevent both overstimulation and damage to activated cells as well as saturation by weak stimuli. Phosphorylation has been identified as the major mechanism for rapid desensitization for many G protein-coupled receptors. In humans, ETA receptors have been shown to have a prolonged period of desensitization occurring within 5 min of exposure to ET-1; sensitization is restored between 80 and 120 min (8). This prolonged desensitization period seems to be unique to the ETA receptor and may explain, at least in part, the prolonged contraction of smooth muscle cells in response to ET-1 (6). The ETB receptors, on the other hand, are rapidly deactivated (via phosphorylation and dehydration) and lose >80% of their initial activity within 5 min of agonist stimulation. This rapid deactivation of
ETB has been linked to the short-term vasodilatory effect of ET-1 (7). The present study shows that both ETA and ETB receptors bind and are responsible for internalization of exogenous ET-1; however, our data in hypertensive cells suggest that both receptors types have become desensitized.

**ETA and ETB receptors in L1 and L2 cells from control sheep.** Both ETA and ETB receptors have been identified in pulmonary and systemic vascular smooth muscle cells, e.g., rat aorta (30), human internal mammary artery and vein, porcine coronary arteries (37), and rat and human pulmonary artery (28). In humans, ETA receptors were found in both resistance and conduit arteries, and in the main pulmonary artery, ETA receptors predominated (90%) (5). Tracheal smooth muscle from rats and mice, however, exhibited approximately equal numbers of the two receptor subtypes (18), whereas ETB receptors were 50% more numerous than ETA in rat pulmonary artery smooth muscle cells (23). Thus the distribution and density of ET-1 receptors on various smooth muscle types may vary between species and location (5, 31).

The present study demonstrates that L1 and L2 cells from control sheep express both the ETA and ETB genes. The significantly lower expression of both genes in L1 cells compared with L2 cells substantiates our previous suggestion that the L1 cell contributes little to the maintenance of vascular tone in the main pulmonary artery. The paucity of ET-1 receptors in L1 cells also corroborates our finding that these cells fail to internalize exogenous ET-1.

Our binding studies confirm the notion that activity of the ETA receptors predominates in L2 cells from the main pulmonary artery of control sheep. With long-term exposure, however, it seems that both receptors participate equally in the internalization process. This difference is likely to reflect the effects of long- and short-term exposure, the ETB receptor becoming more active when exposure to ET-1 is prolonged perhaps as a result of an increase in the requirement for internalization, and degradation of ET-1. Previous studies in isolated, perfused rat lungs (29) have also shown that both receptors play a role in clearance of ET-1 (14).

**ETA and ETB receptors in hypertensive L2 cells.** Alterations in ET-1 receptors have been demonstrated in several models of CPH. For example, increased ETA gene expression was demonstrated in rat lungs and increased ETB gene expression in the rat main pulmonary artery after exposure to hypoxia for 48 h (25). After 14 days of hypoxia, increased expression of both ETA and ETB was found in the distal pulmonary arteries of rats, ETA expression occurring mainly in the media and ETB in the intima; no change in expression of either receptor was found in the large muscular pulmonary arteries (39). In contrast to the hypoxic models, in monocrotaline-induced CPH, ETB receptor mRNA was shown to be decreased in the lung (46).

The present study demonstrates a selective and significant increase in ETB gene and surface expression in and receptor binding by the hypertensive L2 cells compared with controls. Further confirmation of the increase in ETB receptors on hypertensive L2 cells is shown by our finding of an increase in early internalization of ET-1 by ETB. Similar increases in ETB gene expression have been reported in the pulmonary arteries of patients with chronic thromboembolic pulmonary hypertension (44). The ETB signaling pathway has been linked to the regulation of ppET-1 gene expression in rat mesangial cells (19). The present study confirms this notion since, in a previous study, we showed that ppET-1 gene expression in the main pulmonary artery was decreased in hypertensive sheep compared with controls.

Although our studies demonstrate that, in the short term, internalization and clearance of ET-1 by ETB in the hypertensive cells may be increased, our studies with the ETA and ETB receptor antagonists suggest that, in the longer term, this scenario does not occur.

**Fig. 9.** Effect of the ETA and ETB receptor antagonists BQ-610 and BQ-788 alone and in combination on uptake of exogenous ET-1 (10 nM) in L2 cells from control sheep (A) and hypertensive sheep (B). Cells were pretreated with antagonists for 2 h, washed, and then exposed to ET-1 for 18 h (hatched bars). Controls (open bars) did not receive ET-1 treatment. Data are expressed as means ± SE, n = 6, except for the experiments with both antagonists where n = 3. *P < 0.05 compared with ET-1 alone.
Rather, our data demonstrate that ET-1-stimulated internalization of exogenous ET-1 is decreased in hypertensive L2 cells compared with controls and that the sensitivity of the individual ET-1 receptors is lost. It is only when the ETA and ETB receptors are added simultaneously that suppression of ET-1 uptake occurs. Similar findings have been demonstrated in isolated perfused lungs from rats with hypoxic CPH (23).

One possible explanation for the failure of each antagonist alone to inhibit ET-1 uptake is that the concentration of antagonists used in the study was not sufficient to inhibit ETA and ETB receptors in hypertensive L2 cells. This would seem unlikely, because both antagonists failed to exert an inhibitory effect. Another possibility is that phosphorylation of the receptor/ligand complex and/or receptor recycling are altered in the hypertensive cells. This notion requires further study.

Another possible explanation is that concurrent treatment with the ETA and ETB antagonists involves simultaneous activation and heterodimerization of the two receptors (26). Recent studies suggest that ET-1 binds simultaneously to both an ETA and ETB receptor (2, 11). This heterodimerization between two fully functional G protein-coupled receptors is suggested to result in a new receptor/ligand binding complex with functional properties different from those of either receptor alone (21). Thus it is possible that when the individual ETA and ETB receptors of hypertensive L2 cells are desensitized to exogenous ET-1, their simultaneous stimulation results in heterodimerization of ETA and ETB receptors and internalization of ET-1 by a different pathway. This new pathway would seem to be extremely effective, since intracellular levels of ET-1 in the hypertensive L2 cells returned almost to basal values. Further studies are needed to elucidate this possible explanation.

In summary, we have shown that gene and surface expression and activity of the ETB, but not the ETA, receptors in L1 and L2 cells from control and hypertensive sheep are different. Control and hypertensive L2 cells exhibit significantly higher steady-state basal expression of both the ETA and ETB genes than L1 cells. In agreement with this finding, we found that only the L2 cells internalize exogenous ET-1. In the short term, the ETA receptors are mainly responsible for binding of ET-1, but with longer exposure, both receptors contribute to internalization. In hypertensive L2 cells, we showed that ETB gene and surface expression and Bmax by this receptor were significantly elevated above baseline. Uptake of exogenous ET-1 by these cells, however, was less pronounced than in control cells. Furthermore, we found that only concurrent treatment with both ETA and ETB antagonists suppressed ET-1 uptake in the hypertensive cells, suggesting an alteration in their structure. We conclude that changes in gene and surface expression and activity of the ETB receptors in L2 cells from hypertensive sheep may contribute to the development and maintenance of CPH through an altered sensitivity to locally produced ET-1.

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