Endothelin 1: Mitogenic Activity on Pulmonary Artery Smooth Muscle Cells and Release from Hypoxic Endothelial Cells (43342)

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Abstract. **Endothelin, a recently described vasoconstrictor, has been shown to be a mitogen for vascular smooth muscle cells from systemic arteries and might play a role in pulmonary vascular remodeling produced by chronic exposure to hypoxia. We examined the effects of endothelin on proliferation of pulmonary artery smooth muscle cells and the effects of hypoxia and normoxia on the synthesis and secretion of endothelin by endothelial cells. Our results indicate that endothelin significantly increased the incorporation of [^{3}H]thymidine by porcine pulmonary artery smooth muscle cells (122 ± 4% to 168 ± 13% of controls, with concentrations of endothelin from 1 to 1000 ng/ml). When tested on bovine pulmonary artery smooth muscle cells, endothelin increased [^{3}H]thymidine incorporation over controls by approximately 140%; cell counts were increased by 107 ± 4% and 122 ± 7% at doses of 100 ng/ml and 1000 ng/ml, respectively. The secretion of endothelin by porcine endothelial cells was not affected by hypoxia (3520 ± 138 pg/ml/10^6 cells in hypoxia vs 3770 ± 326 pg/ml/10^6 cells in normoxia). Transforming growth factor-β1 stimulated the release by normoxic, and to a lesser degree by hypoxic, porcine endothelial cells of endothelin (4716 ± 43 pg/ml/10^6 cells vs 4074 ± 106 pg/ml/10^6 cells). Taken together, our results indicate that endothelin is weakly mitogenic for pulmonary artery smooth muscle cells, but may not significantly contribute to the remodeling seen in hypoxic pulmonary hypertension.**

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Endothelin (ET) was first isolated by Yanagisawa *et al.* (1) in conditioned medium from vascular endothelial cells (EC). ET is a potent vasoconstrictor and has also been shown to stimulate DNA synthesis in systemic vascular smooth muscle cells (SMC) (2–5). Whether ET has similar proliferative effects on cells from the pulmonary circulation has not been established. If it does and if hypoxia stimulates synthesis and/or release of ET from the endothelium, this peptide could play a major role in pulmonary vascular remodeling responses to chronic hypoxia. Therefore, the purpose of our study was 2-fold: first, to assess the role of ET on the growth characteristics of pulmonary artery smooth muscle cells (PASMC); and second, to assess whether hypoxia affects the synthesis and release of ET by pulmonary artery endothelial cells (PAEC). Our data indicate that ET has mild mitogenic activity on PASMC and hypoxia does not increase the release of ET by PAEC. The possible role of ET in hypoxic pulmonary vascular remodeling is discussed.

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Materials and Methods

Reagents. RPMI 1640 medium and Fungizone (amphotericin B) were purchased from Gibco Laboratories (Grand Island, NY). Fetal calf serum (FCS) was obtained from Hyclone Laboratories, Sterile System (Logan, UT). Penicillin G potassium was obtained from Squibb Pharmaceuticals (Princeton, NJ). Streptomycin sulfate was from Pfizer Co. (New York, NY). [^{3}H]Thymidine (sp act 20 μCi/mmol, concentration 1.0 mCi/ml) was purchased from New England Nuclear Research (Boston, MA). Endothelin 1 (ET-1), a human and porcine endothelin, was obtained from Peninsula Laboratories, Inc. (Belmont, CA). Transforming growth factor (TGF)-β1 was purchased from R & D Systems, Inc. (Minneapolis, MN).

Isolation and Culture of Cells. Cells were derived

from the main pulmonary arteries obtained from pigs and calves immediately after sacrifice. EC were cultured according to previously described methods (6) and were identified by their typical "cobblestone" morphology under phase contrast microscopy, by positive immunofluorescence staining for factor VIII antigen, and by the presence of angiotensin-converting enzyme. A modification of the method of Ross (7) was used for the isolation and culture of SMC. Bovine and porcine pulmonary arteries were rinsed twice with phosphate-buffered saline (PBS) (37°C) containing antibiotics (300 units/ml of penicillin G potassium, 300 μg/ml of streptomycin sulfate, and 1.2 μg/ml of amphotericin B) and slit longitudinally on a sterile petri dish. The luminal side of the vessel was scraped with a scalpel to remove EC, and the abluminal layer was peeled away and cut into 1-cm^2 strips. Two or three such strips were placed in six-well tissue culture dishes (Costar, Cambridge, MA) containing RPMI 1640 medium with antibiotics and 10% FCS. SMC migrate out from the explant after 2–3 weeks in culture. The vessel explants were removed from the wells and cells were refed with RPMI 1640 containing antibiotics and 10% FCS every 2–3 days until they reached confluence. Cells were harvested and then stored in liquid N_2 for subculture. Third and fourth passage cells were used in these experiments. SMC were characterized by morphology and fluorescent antimuscle actin staining of first-passaged cells.

Experimental Design. SMC were seeded into 35-mm culture dishes (density of 0.5×10^5 cells/well) containing 2 ml of RPMI 1640 medium with antibiotics and supplemented with 10% FCS. Cells were allowed to attach and grow for 3 days, after which period the cells were growth-arrested by changing the culture medium to RPMI 1640 supplemented with only 0.5% FCS. After 2 days of serum deprivation, cell number did not increase by more that 5% from baseline, confirming quiescence. We then studied the proliferation of these SMC when exposed to various concentrations of ET-1 (1–1000 ng/ml) supplemented with 0.5% FCS. The addition of small amounts of FCS to culture media of cells exposed to ET-1 was prompted by preliminary experiments that revealed deleterious effects of ET-1 on SMC (change in cell shape and occasional cell detachment) in the absence of serum. This was particularly the case for porcine PASMC.

Cell Growth. SMC growth was assessed by cell number determination using a Coulter counter (cells were counted after 3 days of exposure to ET-1) or by measuring [^{3}H]thymidine incorporation as a reflection of DNA replication. For the latter, [^{3}H]thymidine was added at a concentration of 0.1 μCi/ml directly to 35-mm petri dishes in which SMC were growing. After 24–36 hr of incubation at 37°C, the radioactive supernatant was removed and the cells were washed three times with PBS. One ml of 1% perchloric acid was added to each dish. The cells were then scraped with a rubber policeman, sonicated, and centrifuged, and the supernatant was aspirated. Complete removal of cells from each petri dish was assessed by phase contrast microscopy. One milliliter of 2 *M* KOH was used to dissolve the pellet and this was then transferred to counting vials containing 0.4 ml of 6 *N* HCl. Ecolite (20 ml; ICN Biomedicals, Inc., Irvine, CA) was added to the vials, and the radioactivity was counted in a Beckman LS 7500 liquid scintillation counter (Beckman Instruments, Irvine, CA).

Endothelial Cell-Conditioned Medium. EC were grown to confluence in 35-mm petri dishes containing 2 ml of RPMI 1640 medium with 10% FCS. Two days after the cells reached confluence, the medium was replaced by 2 ml of fresh serum-free medium after three to four thorough washings of the cells. The EC were placed in humidified, airtight incubation chambers (Billups-Rothenberg, Del Mar, CA) and exposed to hypoxia (3% O_2) or normoxia (20% O_2), 5% CO_2, and balance N_2, in the presence or absence of TGF (100 p*M*), a cytokine that has been shown to stimulate the release of ET-1 in EC culture medium (8). The chambers were maintained in a New Brunswick incubator at 37°C for the duration of the exposure period (2 days). The medium was then collected and centrifuged, and the supernatant was filtered (0.22-μm Disposable Syringe Filter, Corning Glass Works, Corning, NY) to obtain endothelial cell-conditioned medium (ECCM). Approximately 1 ml of medium was conditioned by 10^6 cells. The amount of ET-1 in ECCM was measured by radioimmunoassay. There was no evidence of EC injury or cell detachment by phase contrast microscopy or by trypan blue exclusion.

Endothelial Cell Lysates. After collection of the conditioned medium, EC were scraped off the dish with a rubber policeman, collected in PBS (1 ml/10^6 cells) and subjected to ultrasonic lysis. The resulting solution was then centrifuged and the supernatant was collected for measurement of ET-1 by radioimmunoassay.

Radioimmunoassay. The radioimmunoassay kits (kit RIK6901) for ET-1 were obtained from Peninsula Laboratories and were used according to the manufacturer's recommendations. Standard curves were obtained with known concentrations of ET-1. ET-1 levels from hypoxic and normoxic ECCM and from cell lysates were then determined.

Mitogenic Assays. The mitogenic activity of ET-1 was expressed as percentage of controls using the following formula:

$$\frac{X}{R} \times 100 \quad (\%) \qquad \text{[Eq. 1]}$$

where *R* represents cell counts (Coulter counter) or cpm ([^{3}H]thymidine uptake) of control cells exposed to RPMI 1640 with 0.5% FCS, and *X* represents cell

counts or cpm of cells exposed to ET-1 supplemented with 0.5% FCS.

Statistical Analysis. All values are shown as means ± SE. Unpaired Student's *t* test was used for the results shown on Table I. Fisher's multiple comparison test (Statview 512+; Brainpower, Inc., Calabasas, CA) was used to determine significant changes occurring between control cells and cells exposed to various doses of ET-1 (Fig. 1). Significance in all cases was assumed at $P < 0.05$. Unless specified otherwise, the following results are representative experiments of at least three separate experiments each using a minimum of four culture dishes for each experimental condition tested.

Results

Effect of Endothelin on Smooth Muscle Cell Growth. We tested the effect of ET-1 on the growth characteristics of quiescent SMC from pig and bovine pulmonary arteries using both [^{3}H]thymidine incorporation by SMC and actual SMC counts. On porcine PASMC, ET-1 significantly increased [^{3}H]thymidine incorporation in a dose-dependent manner (from 122 ± 4% at 1 ng/ml up to 168 ± 13% at 1000 ng/ml) (Fig. 1). [^{3}H]Thymidine incorporation by bovine PASMC was also significantly increased to 140 ± 7% at 10 ng/ml and 138 ± 25% at 100 ng/ml. When SMC growth was assessed by actual cell counts, only bovine PASMC were stimulated to grow by ET-1. The mitogenic effect of ET-1 on bovine PASMC after 3 days of exposure was mild but significant (107 ± 4% and 122 ± 7% at doses of 100 ng/ml and 1000 ng/ml, respectively). There was, however, no increase in porcine PASMC counts at any dose of ET-1, following 1, 3, or 5 days of exposure.

To test the possibility that degradation of ET-1 in culture medium over 24 hr might prevent a more vigorous mitogenic effect on SMC, we exposed porcine PASMC to ET-1-containing medium (100 ng/ml), which was freshly constituted and changed every 6 hr over a 24-hr period. The mitogenic effect of 100 ng/ml of ET-1 assessed by [^{3}H]thymidine incorporation was 163 ± 18% ($n = 4$) in this experiment. This was not significantly different from the mitogenic effect of 100 ng/ml of ET-1 applied without change for 24 hr (157 ± 19%).

Measurement of ET-1 in ECCM and Cell Lysates. We measured the amount of ET-1 released in ECCM and contained in cell lysates after exposure of EC, in the presence or absence (basal release) of TGF-β1 (100 pM), to normoxia and hypoxia. TGF-β1 has been shown to increase the basal release of ET-1 by PAEC (8) and was, therefore, used in the present study as a positive control. The results are shown in Table I.

Basal release. There was no significant difference in the amount of ET-1 released in ECCM from normoxic or hypoxic EC, whether in porcine or bovine species. There were relatively small amounts of ET-1 detected in cell lysates from porcine or bovine PAEC. However, it should be noted that since PBS was added to EC (1 ml/10^6 cells) for collection prior to ultrasonic lysis, the concentrations of ET-1 in cell lysates are underestimated and cannot be compared with concentrations in ECCM. Hypoxic and normoxic porcine PAEC contained similar amounts of ET-1. Normoxic bovine PAEC contained significantly more ET-1 compared with hypoxic PAEC.

Stimulation by TGF-β1. TGF-β1 significantly increased the amount of ET-1 contained in porcine PAEC and the amount released in ECCM, compared with basal values in hypoxia or normoxia. Hypoxic porcine PAEC stimulated by TGF-β1 contained more ET-1 intracellularly, but released less ET-1 in ECCM, as compared with normoxic porcine PAEC also stimulated by TGF-β1.

Discussion

Exposure of animals to acute hypoxia induces pulmonary vasoconstriction, which is reflected by a rise in mean pulmonary artery pressure. This is reversible if hypoxia is of short duration. With chronic hypoxia, however, pulmonary hypertension becomes irreversible and vessels undergo "remodeling." This phenomenon is characterized by hyperplasia and hypertrophy of smooth muscle cells in the medial layer of pulmonary

Table I. Amount of ET-1 Contained in Hypoxic and Normoxic ECCM and Cell Lysates[a]

	O_2 Tension	Porcine PAEC		Bovine PAEC	
		3%	20%	3%	20%
Basal condition	ECCM	3520 ± 138	3770 ± 326	1410 ± 127	1690 ± 30
	Cell lysates	80 ± 2	75 ± 3	27 ± 4[b]	57 ± 3
Stimulation with TGF-β1 (100 pM)	ECCM	4074 ± 106[b,c]	4716 ± 43[c]	ND	ND
	Cell lysates	166 ± 10[b,c]	132 ± 2[c]	ND	ND

[a] Amount of endothelin in pg/ml/10^6 cells. Each value represents the mean ± SE of four replicate dishes for each experimental condition. ND, not done.
[b] $P < 0.05$ versus corresponding 20% O_2.
[c] $P < 0.05$ versus corresponding basal release.

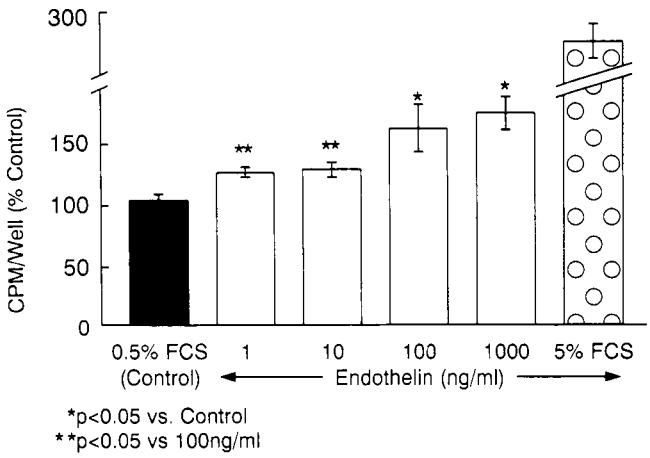

Figure 1. Effect of ET-1 on [^{3}H]thymidine incorporation by porcine PASMC. ET-1 increased the incorporation of [^{3}H]thymidine by previously growth-arrested PASMC in a dose-dependent fashion (122 ± 4% of control for 1 ng/ml of ET; 124 ± 6% for 10 ng/ml; 157 ± 19% for 100 ng/ml; 168 ± 13% for 1,000 ng/ml). The data represent the means ± SE from at least four different experiments using four dishes for each experiment. The range of cpm for control cells was 10,000 to 12,000. The mitogenic activity of 5% FCS is shown for comparison.

arteries, as well as extension of the muscle layer to distal arteries that are normally nonmuscular (9). Although several agents have been shown to mediate vasoconstriction (e.g., prostaglandins, thromboxane, leukotrienes C_4 and D_4, histamine, serotonin, and angiotensin II) (10), none has been clearly established as the predominant vasconstrictor in hypoxia. Similarly, various factors with mitogenic activity for SMC, such as PDGF and EGF, have been identified (11, 12). None of these factors has been clearly linked to the pathogenesis of vascular remodeling seen in hypoxia. Recently, a putative mitogen for smooth muscle cells, derived from hypoxic EC was reported (13). This awaits further characterization. Of particular interest in the development of pulmonary hypertension are those factors with both vasoactive (vasoconstriction) and mitogenic properties. For example, both PDGF and EGF have well-established mitogenic activity through the stimulation of the protooncogenes c-*fos* and c-*myc* (14). They are also thought to cause vasoconstriction by increasing cytosolic free calcium (15, 16). The latter effect may be mediated by activation of protein kinase C (17).

Pulmonary EC release a vasconstrictor that has not been characterized, but that may have actions similar to endothelin (18–20). Endothelin has been shown to be both a potent vasoconstrictor in the systemic circulation (2) and a mild vasoconstrictor in lobar pulmonary arteries (21). Endothelin is also a mitogen for SMC from systemic arteries (2–5) and rat mesangial cells (22, 23). Endothelin, like PDGF and EGF, induces growth by stimulating c-*fos* and c-*myc* transcription (3) and causes vasoconstriction by increasing calcium influx and calcium release from intracellular sites (24) in systemic arteries. The purpose of our study was to assess whether the synthesis or release of endothelin was accelerated by exposure of EC to short-term hypoxia and whether endothelin had a mitogenic activity on SMC obtained from the pulmonary artery.

We found no significant difference, under basal condition, in the amount of ET in porcine PAEC cell lysates and ECCM from cells exposed to hypoxia, as compared with their counterparts exposed to normoxia. Similarly, bovine ECCM showed no significant difference in ET levels from cells exposed to normoxia or hypoxia. ET levels from hypoxic bovine cell lysates, however, were lower than those in normoxia; this may be related to a decreased cell synthetic activity at 48 h of hypoxia. In both animal species we tested, ET levels in ECCM were considerably higher than levels in cell lysates. This is consistent with the hypothesis that ET is constitutively released from EC and that little ET is stored intracellularly (1). There appeared to be a significantly greater amount of ET secreted by porcine PAEC as compared with bovine PAEC. However, since the radioimmunoassay was directed toward ET-1 (human and porcine endothelin), the difference might be explained by incomplete cross-reactivity in antigenic characteristics of ET from porcine and bovine cells. TGF-β1 has been shown to be a potent stimulus for the release of ET-1 by PAEC (8). When porcine PAEC were exposed to hypoxia and normoxia in the presence of TGF-β1, there was a significant increase in levels of ET-1 in ECCM and cell lysates compared with basal conditions. The release of ET-1 by hypoxic cells, however, was less than the release by normoxic cells. On the other hand, intracellular levels of ET-1 were higher in hypoxic compared with normoxic PAEC. Our experiments indicate that hypoxic porcine PAEC are able to increase their release of ET-1 in response to an agonist (TGF-β1), albeit to a lesser degree compared with normoxic PAEC. Although the mode of secretion of endothelin remains unclear (25), our results suggest that hypoxia of a duration of 48 hr does not stimulate the release of endothelin by PAEC. The failure of hypoxia to increase ET levels has also been reported in dogs *in vivo* (26).

In parallel experiments, we found that ET has a modest mitogenic activity as determined by thymidine incorporation on porcine PASMC. However, no increase in actual PASMC counts could be demonstrated with porcine PASMC. With bovine PASMC, ET showed increased growth by both thymidine incorporation and cell counts. The lack of cell proliferation of porcine PASMC in response to ET-1 may be explained by the greater fragility of these cells in medium containing ET-1 compared with bovine PASMC. Changes in cell shape (Fig. 2) and occasional cell detachment occurred more frequently with porcine PASMC than with

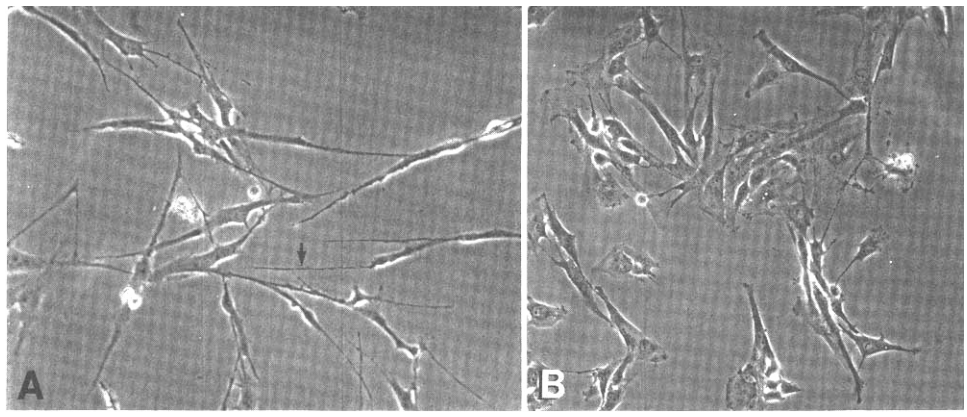

Figure 2. Effect of ET-1 on cell configuration of porcine PASMC. Sparsely seeded porcine PASMC were incubated (A) with and (B) without ET-1 (100 ng/ml) in medium supplemented with 0.5% FCS. Cells exposed to ET-1 showed significant elongation, with spindle-like cytoplasmic extension (arrow) within 4–5 hr of exposure. (Phase contrast photomicrograph, original magnification × 200.)

bovine PASMC. Whether ET-1 induces reorganization of actin fibers in vascular SMC is unclear and deserves further investigation. It should be noted that the effect of ET on the incorporation of thymidine by PASMC was seen only when a small amount of FCS was added to the cells. Similar observations have been made by other authors using rat mesangial cells and SMC from systemic arteries (23, 27). They found that ET requires the presence of low concentrations of FCS or a progression factor such as insulin-like growth factor to induce proliferation in these cells. ET, therefore, may play a role in the growth of SMC, but it requires an additional factor(s) present in the serum for its activity.

Although our experiments involved short-term exposure to hypoxia, our results do not support a potential role for ET in hypoxic vascular remodeling. Other mitogens for vascular SMC, such as PDGF, regulation of which has been shown to be affected by hypoxia (28), may play a more significant role in the pathogenesis of this disease. Taking into account the limitations of a radioimmunoassay, it is possible that qualitative changes in ET may have occurred under hypoxic conditions and may not have been detected by the assay. It should be noted that O'Brien *et al.* (18) recently described a potent vasoconstrictor released from aortic and pulmonary artery EC in culture, which may or may not be ET, whose release was not affected by hypoxia or anoxia. Although the release of ET-1 is not affected by hypoxia, it is not excluded that hypoxia might alter the expression of prepro-ET mRNA.

We have shown that both hypoxic and normoxic EC from pig and bovine pulmonary arteries release ET-like substances. Hypoxia does not accelerate the synthesis or release of ET. ET has a mild mitogenic activity on PASMC. However, taken together our results do not support a significant role of ET in the pathogenesis of hypoxic pulmonary vascular remodeling.

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