Hypoxic pulmonary endothelial cells release a diffusible contractile factor distinct from endothelin

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Hypoxic pulmonary endothelial cells release a diffusible contractile factor distinct from endothelin. Am. J. Physiol. 274 (Lung Cell. Mol. Physiol. 18): L657–L664, 1998.—Hypoxia (0% O2) evokes a late-phase, endothelium-dependent contractile response in porcine isolated pulmonary arteries that may be caused by a cyclooxygenase-independent, endothelium-derived contractile factor. The aim of this study was to further analyze the mechanism underlying this hypoxic response. Proximal porcine pulmonary arterial rings were suspended for isometric tension recording in organ chambers. Hypoxia (0% O2) caused a late-phase, endothelium-independent contractile response that was not inhibited by the endothelin (ET)A-receptor antagonist BQ-123 (10^-6 M), by the ETB-receptor antagonist BQ-788 (10^-7 M), or by their combination. In contrast, ET-1 caused a concentration-dependent contraction of arterial rings that was inhibited by BQ-123 (10^-6 M) and a relaxation that was abolished by BQ-788 (10^-7 M) or by endothelial cell removal. Therefore, the endothelium-dependent contraction to hypoxia is not mediated by ET. Hypoxia caused only relaxation in endothelium-denuded rings. However, when a pulmonary valve leaflet, a rich source of pulmonary endothelial cells, was placed into the lumen of endothelium-denuded rings, hypoxia caused a late-phase contractile response that was similar to that observed in arterial rings with native endothelium. This hypoxic contraction persisted in the presence of indomethacin (10^-5 M) and N-nitro-L-arginine methyl ester (3 x 10^-5 M) to block cyclooxygenase and nitric oxide synthase, respectively. These results suggest that hypoxic contraction of pulmonary arteries is mediated by a diffusible, contractile factor released from hypoxic endothelial cells. This contractile mediator is distinct from ET.

endothelium; hypoxia; endothelinA receptor; endothelinB receptor; pulmonary valve leaflet; pulmonary artery; endothelium-derived contractile factor

THE ROLE OF ENDOTHELIUM in mediating or modulating hypoxic pulmonary vasoconstriction has not been clearly defined. Hypoxia causes an immediate, transient contraction of isolated pulmonary and systemic arteries that is endothelium dependent and mediated by inhibition of the basal activity of the endothelium-derived dilator nitric oxide (NO) (11, 13, 17, 21, 30). Hypoxic constriction in the intact pulmonary circulation does not involve this mechanism (2, 5), which might suggest that the constriction is endothelium independent. Indeed, hypoxia can cause a direct contraction of isolated vascular smooth muscle cells (23, 25), presumably by inhibiting K+ currents (27, 40). Although modulation of endothelium-derived dilators may not mediate hypoxic pulmonary constriction, altered activity of endothelium-derived contractile mediators may play an important role in initiating or amplifying smooth muscle constriction in response to hypoxia. Kovitz et al. (21) observed a late-phase, endothelium-dependent contraction to hypoxia in porcine isolated arteries that is not mediated by inhibition of dilator mediators. On the basis of, in part, a theoretical analysis, Kovitz et al. concluded that this hypoxic contraction was mediated by an endothelium-derived contractile factor (EDCF). A similar mechanism was proposed for a late-phase hypoxic contraction in rat isolated pulmonary arteries (22). Previous studies have suggested that the EDCF endothelin (ET) may play a role in hypoxic pulmonary vasoconstriction: 1) production of ET can increase during hypoxia (20) and 2) inhibition of ET receptors can attenuate hypoxic vasoconstriction in the intact pulmonary circulation (4, 6, 8, 26). However, this proposal remains controversial (9, 16, 38). The aim of the present study was to further analyze the mechanism underlying the late-phase hypoxic contraction and to determine whether the mediator was ET.

MATERIALS AND METHODS

Blood Vessel Preparation

Male pigs (~25 kg) were anesthetized with ketamine (700 mg im) followed by pentobarbital sodium (12.5 mg/kg iv). The pigs were then killed by exsanguination through the femoral arteries. Proximal pulmonary arteries (8- to 12-mm ID) were isolated and cleared of adherent connective tissue (21). Rings were obtained by dividing the vessel into 5-mm-long segments. In some rings, the endothelium was removed by gently rubbing the intimal surface with a cotton swab and was confirmed during the course of each experiment by the loss of a relaxant response to acetylcholine (10^-6 M) or bradykinin (10^-7 M). The arterial rings were suspended between two stainless steel stirrups in organ chambers filled with 25 ml of modified Krebs-Ringer bicarbonate solution composed of (in mM) 118.3 NaCl, 4.7 KCl, 1.2 MgSO4, 1.2 KH2PO4, 2.5 CaCl2, 25.0 NaHCO3, 11.1 glucose, and 0.016 Ca-EDTA (control solution). Chambers were maintained at 37°C and pH 7.4 and were gassed with 16% O2-5% CO2-balance N2. One of the stirrups was anchored in the chamber, and the other was connected to a strain gauge (model FT03, Grass, Quincy, MA) for the measurement of isometric force (Grass polygraph model 7E). Arterial rings were stretched at 10-min intervals to reach the optimal resting tone, previously determined to be...
5 g (21). Once the rings were stretched to the optimal tone, the contractile response to 60 mM KCl was determined. KCl was then removed from the organ chambers, and the tone was allowed to return to prestimulation levels.

Experimental Protocols

Role of ET. To analyze the influence of ET-receptor antagonists on the responses to ET or hypoxia, the rings were incubated for 30 min in the presence and absence of BQ-123 (10^{-6} M; ETA-receptor antagonist) and/or BQ-788 (3 \times 10^{-7} M; ETB-receptor antagonist) before the arteries were exposed to ET or phenylephrine. At these concentrations, the antagonists are potent and selective inhibitors of the ET receptor subtypes (14, 15). The ET-receptor antagonists had no effect on baseline tone and did not influence the contractile response to phenylephrine. Control and antagonist-treated rings were studied in parallel, and only one exposure to ET or hypoxia was studied in each ring. Concentration-response curves to ET were determined under quiescent conditions by increasing the concentration of ET in half-log increments (10^{-10} to 3 \times 10^{-7} M) once the response to the previous concentration had stabilized. To facilitate analysis of ET-induced relaxation, the response to ET (3 \times 10^{-9} M) was determined during contraction of the rings with phenylephrine. Arterial rings were contracted with phenylephrine to 50% of their maximal response to KCl, which approximates 50% of the maximal response to phenylephrine (21).

Responses to hypoxia were evaluated as previously described (21). During contraction of the arteries with phenylephrine (to 50% of KCl maximum), O2 tension was decreased in a stepwise fashion from 16 to 10, 4, and 0%. The level of O2 was decreased once the response to the previous level had stabilized. Responses to hypoxia were also evaluated as an abrupt change from 16 to 0% O2. At 16% O2, PO2 was 120–127 mmHg; at 10% O2, PO2 was 77–81 mmHg; at 4% O2, PO2 was 40–43 mmHg; and at 0% O2, PO2 was 8–12 mmHg.

Transfer experiments. To determine whether the hypoxic mediator was a diffusible mediator, transfer experiments were performed with pulmonary valve leaflets as a source of endothelial cells. Valve leaflets were dissected from the pulmonary outflow tract and stored at 4°C in a control solution until required in the experimental protocol. A concentration response to phenylephrine was determined in endothelium-denuded and endothelium-containing arterial rings. The phenylephrine was removed from the chambers, and the tension of the rings was allowed to return to baseline. The passive tension (5 g) was then relaxed by 2 g, the organ baths were lowered, and a pulmonary valve leaflet was carefully placed inside the endothelium-denuded arterial rings (Fig. 1). Control rings were treated in the same manner except no leaflet was inserted. The organ chambers were raised, and the passive tension was returned to 5 g. Arterial rings were then contracted to the 50% effective dose level of tension with phenylephrine and exposed to hypoxia. Pulmonary valves and arteries were obtained from the same animals.

The influence of valve placement on the reactivity of pulmonary arterial rings to phenylephrine was also determined in separate experiments. Concentration-response curves to phenylephrine were generated before and after placement of valve leaflets. In some experiments, valve leaflets were first incubated in distilled water (4°C for 4 h) to damage the endothelium and to determine the stearic effects of the valve on vascular reactivity.

Drugs and Solutions

Acetylcholine, N-nitro-L-arginine methyl ester (L-NAME), and phenylephrine hydrochloride were obtained from Sigma. Bradykinin was obtained from Calbiochem. BQ-123 and BQ-788 were obtained from American Peptide, and ET-1 was obtained from Peninsula Laboratories. The drugs were dissolved in distilled water and kept on ice during the course of the experiment except for ET and bradykinin, which were dissolved in acetic acid followed by dilution in distilled water (final chamber concentration of acetic acid was 0.001% vol/vol); BQ-123, which was dissolved in dimethyl sulfoxide followed by dilution in distilled water (final chamber concentration of dimethyl sulfoxide was 0.01% vol/vol); and BQ-788, which was dissolved in methanol followed by dilution in distilled water (final chamber concentration of methanol was 0.04% vol/vol). These concentrations of solvents had no effect on the hypoxic reactivity or the responses to constrictor or dilator agonists. All chemicals were the highest purity available. Stock solutions were prepared each day. All concentrations are expressed as the final molar concentration in the organ chamber.

Data Analysis

Results are expressed as means ± SE. Concentration-response curves to ET determined under quiescent conditions are expressed as a percentage of the maximum response to KCl. Responses to ET or hypoxia determined during contraction to phenylephrine are expressed as a percentage of the precontraction. To compare concentration-response curves to ET, the effective concentration of agonist causing X% of the maximal contraction to KCl (ECX,KCl) was determined by regression analysis of the concentration-effect curves. Antagonist dissociation constants (Kd) were determined with the formula Kd = [B]/(CR - 1), where [B] is the concentration of antagonist and CR is the ratio of agonist concentrations producing equal responses in the presence and absence of the antagonist (19). Statistical evaluation of the data was performed with Student’s t-test for paired or unpaired analysis. When more than two means were compared, analysis of variance was performed. If a significant F value was found, Scheffé’s test for multiple comparisons was employed to identify differences among groups. Values were considered to be statistically significant when P is <0.05. When mentioned in the text, n refers to the number of animals from which blood vessels were taken.
RESULTS

Responses to ET-1

Under quiescent conditions, ET-1 caused a concentration-dependent contraction of pulmonary arterial rings with endothelium. Endothelium removal increased the response to ET-1, causing an upward shift in the concentration-effect curve (Fig. 2).

The response to ET-1 was inhibited by the ET<sub>A</sub>-receptor antagonist BQ-123 (10<sup>-6</sup> M), which caused a rightward shift of the concentration-effect curve with no change in the maximum response in rings with and without endothelium (Fig. 2). The –log K<sub>B</sub> for BQ-123 calculated at the EC<sub>50,KCl</sub> level was 6.99 ± 0.18 in endothelium-denuded rings (n = 5 animals). In endothelium-denuded rings, contractions with ET-1 were not significantly influenced by BQ-788 (3 × 10<sup>-7</sup> M) either in the presence or absence of BQ-123 (10<sup>-6</sup> M; Fig. 2). However, in endothelium-intact arterial rings, the addition of BQ-788 (3 × 10<sup>-7</sup> M) significantly enhanced the response to ET in both the presence and absence of BQ-123 (10<sup>-6</sup> M).

In arterial rings with endothelium that were contracted with phenylephrine, ET-1 (3 × 10<sup>-9</sup> M) caused a biphasic response consisting of an initial relaxation followed by a sustained contraction (Fig. 3). The relaxation was abolished by the ET<sub>B</sub>-receptor antagonist BQ-788 (3 × 10<sup>-7</sup> M) or by removal of the endothelium. The contraction with ET was increased by BQ-788 (3 × 10<sup>-7</sup> M) or by endothelium removal and was abolished by the ET<sub>A</sub>-receptor antagonist BQ-123 (10<sup>-6</sup> M). BQ-788 (3 × 10<sup>-7</sup> M) had no effect on the response to ET in endothelium-denuded rings (Fig. 3).

Effect of ET-Receptor Antagonists on Responses to Hypoxia

During a contraction with phenylephrine (50% maximal response), moderate hypoxia (10–4% O<sub>2</sub>) caused a graded relaxation that was similar in rings with and without endothelium. Severe hypoxia (0% O<sub>2</sub>) caused a transient increase followed by a slowly developing and sustained increase in tension in endothelium-contain-
ing rings but no significant change in tension in denuded rings (Fig. 4). The ET-receptor antagonists BQ-123 (10^{-6} M) and/or BQ-788 (3 \times 10^{-7} M) did not affect the vascular responses to moderate or severe hypoxia (Fig. 4).

Transfer of Endothelial Mediators From Pulmonary Valve Leaflets

During contraction with phenylephrine (to 50% of maximal response), bradykinin (10^{-9} to 10^{-6} M) caused relaxation of endothelium-containing rings but no change in tension of endothelium-denuded arterial rings (Fig. 5). The placement of a pulmonary valve leaflet, a rich source of endothelial cells, into the lumen of the endothelium-denuded arterial ring restored a vasodilator response to bradykinin (Fig. 5). This suggests that the pulmonary valve leaflet is a suitable bioassay system for analyzing production of endothelium-derived mediators.

As previously demonstrated (21), abrupt exposure to severe hypoxia (16–0% O_2) caused relaxation of endothelium-denuded rings contracted with phenylephrine (to 50% of maximal response; Fig. 5). However, the presence of a pulmonary valve leaflet in endothelium-denuded rings converted this response into a slowly developing sustained contractile response (Fig. 5). This response was similar to the endothelium-dependent response observed in pulmonary arteries with native endothelial cells (21).

In arterial rings without endothelium contracted with phenylephrine (to 50% of maximal response), moderate hypoxia (10–4% O_2) caused a graded relaxation, and severe hypoxia (0% O_2) caused no further significant change in tension (Fig. 6). Placement of a pulmonary valve leaflet in endothelium-denuded rings did not alter the response of the arterial ring to moderate hypoxia (10–4% O_2) but uncovered a late-phase contraction to severe hypoxia (0% O_2; Fig. 6). The magnitude of this valve-dependent, late-phase hypoxic contraction was the same as that observed in rings with native endothelium. This valve-dependent contraction was still observed in the presence of indomethacin (10^{-5} M) and L-NAME (3 \times 10^{-5} M) to inhibit cyclooxygenase and NO synthase, respectively (Fig. 6).

The placement of a pulmonary valve leaflet in the lumen of an endothelium-denuded ring depressed contractile responses to phenylephrine, causing a parallel rightward shift in the concentration-response curve.

Fig. 4. Effects of moderate (10 and 4% O_2) and severe (0% O_2) hypoxia on tension of proximal porcine pulmonary arteries with and without endothelium (endo) in absence (control; A) and presence of ET_A-receptor antagonist BQ-123 (10^{-6} M; B), ET_B-receptor antagonist BQ-788 (3 \times 10^{-7} M; C) or their combination (D). During normoxia (16% O_2), arterial rings were contracted to EC_{50,KCl} level of tension with phenylephrine. O_2 tension was then decreased in a stepwise manner (from 16 to 10, 4, and 0% O_2), allowing time for tone to stabilize at each level. Protocol was performed without interruption, and contractile tension was determined at 1-min intervals for entire time course of each experiment. However, time course for response to each level of O_2 is presented only for lowest common denominator of time followed by a break in the curve. Last 6 min of each O_2 level are then presented before continuation to next level. Results are means or means \pm SE expressed as a percentage of normoxic response to phenylephrine at selected time points; n = 7 animals.
without affecting the maximum response (Fig. 7). The inhibitory effect of the valve was not influenced by L-NAME (3 x 10⁻⁵ M) or indomethacin (10⁻⁵ M) by prior incubation of the valve leaflet in distilled water (4°C for 4 h; Fig. 7).

**DISCUSSION**

In porcine isolated pulmonary arteries, severe hypoxia (0% O₂) evokes a late-phase, endothelium-dependent contractile response that is not mediated by inhibition of dilator mediators (21). On the basis of, in part, a theoretical analysis, Kovitz et al. (21) suggested that this hypoxic contraction was mediated by a cyclooxygenase-independent EDCF. In the present study, the EDCF ET-1 caused contraction of the proximal porcine pulmonary artery by stimulating smooth muscle ETA receptors and relaxation by stimulating endothelial ETB receptors. However, inhibition of the ETA receptor (and/or the ETB receptor) did not inhibit the late-phase, endothelium-dependent contraction to hypoxia (0% O₂). A pulmonary valve leaflet, used as a source of endothelial cells, restored the hypoxic contraction to endothelium-denuded rings. Our results suggest, therefore, that the late-phase, endothelium-dependent hypoxic constriction is mediated by a diffusible EDCF distinct from ET.

ET-1, -2, and -3 are synthesized by many different cell types, although ET-1 appears to be produced exclusively by endothelial cells (1, 39). There are also three known ET receptors, two of which (ETA and ETB) have been cloned with 60% homology. ETA and ETB receptors are located on vascular smooth muscle and mediate contraction (1, 33, 36), whereas ETB receptors are also located on endothelial cells mediating relaxation via increased NO and/or prostacyclin production (12, 31). A putative ETc receptor, selective for ET-3, has been cloned (18) and may contribute to vascular smooth muscle contraction. Hypoxia has been demonstrated to increase ET production in isolated resistance arteries (28) and intact lungs (34) and may (20) or may not (24) increase ET-1 production from cultured endothelial cells. There has generally been agreement that ET-receptor antagonists attenuate the pulmonary hypertension and vascular remodeling associated with chronic hypoxia (4, 6, 8). However, the role of ET in acute hypoxic vasoconstriction is controversial. The ETA-receptor antagonist BQ-123 attenuated acute hypoxic vasoconstriction in an in vivo rat study (26), although this has not been a consistent finding. In isolated canine or rat arteries, blockade of ETA receptors with BQ-123 did not alter the contractile response to hypoxia (9, 16, 38). However, these and other studies of isolated arteries have focused on immediate and transient endothelium-dependent contractions to hypoxia that are mediated by inhibition of dilator mediators rather than by the release of an EDCF. Our in vitro model of hypoxic constriction, which appears to be mediated by an EDCF, is therefore a novel model to evaluate a role for ET in hypoxic pulmonary constriction.

Our results demonstrate, in the proximal porcine pulmonary artery, that ET-1-induced contraction was inhibited by BQ-123, with a KB (100 nM) consistent with antagonism of ETA receptors (3, 14). ET-1 also caused a relaxation that was abolished by endothelial removal and by BQ-788, a selective ETB-receptor antagonist (15). Therefore, in the proximal porcine pulmonary artery, ET-1 mediates contraction via the ETA receptor on the smooth muscle and relaxation via endothelial ETB receptors (35). Despite their potency at inhibiting contractile responses to ET-1, the ET-receptor antagonists did not inhibit the endothelium-dependent, late-phase hypoxic contraction in the proximal pulmonary artery, implying that ET does not play a role in mediating this response. However, it is possible that hypoxia augmented the activity of a distinct ET recep-
tor on vascular smooth muscle that was not inhibited by these antagonists (e.g., ET-C). Indeed, BQ-123 caused a nonparallel shift in the ET-1 concentration-response curve, being more potent at low compared with high levels of tension. For example, in endothelium-denuded rings, BQ-123 caused a significantly greater shift in the curve at the EC30,KCl level compared with the EC 100,KCl level of tension [log shifts in the concentration-response curve of 1.3 ± 0.2 (20-fold shift) and 0.55 ± 0.14 (3.5-fold shift), respectively; P < 0.05; n = 5 rings].

This pattern was not influenced by blockade of ETB receptors (BQ-788) and may therefore reflect ET-1-induced activation of a non-ETA, non-ETB receptor of low activity (e.g., ET-C) (10). However, ET-1 (3 × 10⁻⁹ M), when given to endothelium-denuded rings during anoxia (0% O₂), caused a contraction that was still abolished by the ETₐ-receptor antagonist BQ-123 (10⁻⁶ M; data not shown). These results indicate that hypoxia does not uncover a novel ET receptor and that ET-1 does not mediate the late-phase, endothelium-dependent contraction to hypoxia.

To further characterize the nature of the hypoxic mediator, transfer experiments were performed with pulmonary valve leaflets as a source of endothelial cells. Although endothelium-denuded pulmonary arterial rings normally relax in response to hypoxia, the presence of a valve leaflet in endothelium-denuded rings restored the endothelium-dependent contraction to severe hypoxia (0% O₂). The contraction persisted after L-NAME and indomethacin, confirming that the contraction was not mediated by inhibition of endothelial dilator mediators but rather indicated the release of a diffusible contractile factor by the endothelium. Placing the leaflet into an endothelium-denuded ring caused a rightward shift in the phenylephrine concentration-effect curve that was unaffected by L-NAME and indomethacin or by pretreatment of the valve with distilled water. This suggests that the leaflet does not release significant amounts of dilator mediators under basal conditions but that it does exert a slight mechanical effect on arterial contractility. Indeed, although the presence of the valve in endothelium-denuded arteries restored the late endothelium-dependent contractile

Fig. 6. Transfer of a hypoxic endothelium-derived contractile factor from pulmonary valve leaflets to endothelium-denuded (w/o endo) arterial rings. Effects of moderate (10 and 4% O₂) and severe (0% O₂) hypoxia on tension of endothelium-containing rings, endothelium-denuded rings, and endothelium-denuded rings containing a pulmonary valve leaflet were studied. Arterial rings were contracted to EC30,KCl level of tension with phenylephrine before hypoxic exposure (A). In some experiments, N-nitro-L-arginine methyl ester (3 × 10⁻⁵ M) and indomethacin (10⁻⁵ M) were present before and during exposure to phenylephrine and hypoxia (B). O₂ tension was then decreased in a stepwise manner (from 16 to 10, 4, and 0% O₂), allowing time for tone to stabilize at each level. Protocol was performed without interruption, and contractile tension was determined at 1-min intervals for entire time course of each experiment. However, time course for response to each level of O₂ is presented only for lowest common denominator of time followed by a break in the curve. Last 6 min of each O₂ level are then presented before continuation to next level. Results are means or means ± SE expressed as a percentage of normoxic response to phenylephrine at selected time points; n = 5 animals.

Fig. 7. Effect of valve leaflet on contractile responses of pulmonary arterial rings without endothelium to phenylephrine. Before placement of pulmonary valve leaflet, endothelial denudation was confirmed by lack of response to acetylcholine or bradykinin, and phenylephrine concentration-effect curves were determined. Some valve leaflets were treated with distilled water (4°C, 4 h) to damage endothelial cells. N-nitro-L-arginine methyl ester (L-NAME) and indomethacin (INDO) were administered after placement of valve leaflet and 30 min before administration of phenylephrine. Results are means ± SE expressed as a percentage of maximum contraction to KCl (60 mM); n = 4 animals.
response, it did not restore the initial, transient endothelium-dependent contraction to moderate hypoxia (21) (Fig. 6). This transient response is mediated by hypoxic inhibition of the basal activity of endothelium-derived NO (21).

The role of endothelium in mediating or modulating hypoxic pulmonary vasoconstriction in vivo has not been clearly defined. Hypoxia has been demonstrated to directly produce contraction in cultured pulmonary arterial smooth cells in the absence of endothelium (25). Hypoxia may initially increase intracellular Ca\(^{2+}\) through the release of stores in the sarcoplasmic reticulum or by directly inhibiting the delayed rectifier K\(^+\) channels in smooth muscle cells, leading to depolarization, opening of voltage-operated Ca\(^{2+}\) channels, and the influx of extracellular Ca\(^{2+}\) (27, 40). The Ca\(^{2+}\) influx may stimulate further sustained increases in intracellular Ca\(^{2+}\) via Ca\(^{2+}\)-induced Ca\(^{2+}\) release from internal stores, resulting in smooth muscle contraction (7, 27, 40). The endothelium, by releasing a contractile factor, may amplify this response (29). The mechanisms controlling the activity of this hypoxic mediator were not investigated in the present study. The mediator could be released in response to hypoxia (20, 21) or it could be released continuously, with hypoxia modulating its activity on the vascular smooth muscle. The late-phase, endothelium-dependent contraction to hypoxia was observed in pulmonary but not in systemic arteries. Isolated porcine iliac arteries (n = 6) exposed to anoxia for 45 min relaxed by 97 ± 3% of the phenylephrine contraction. This is in keeping with previous findings in isolated rat systemic arteries (22). Differences in the activity of this factor may therefore contribute to the heterogeneous effects of hypoxia in the pulmonary and systemic circulations and to pathophysiological increases in pulmonary vascular resistance.

In summary, hypoxic pulmonary endothelial cells release a diffusible, cyclooxygenase-independent contractile factor that mediates hypoxic constriction in isolated pulmonary arteries. This factor is distinct from ET. The nature of the factor and the mechanisms controlling its activity remain to be determined.

This study was supported by National Heart, Lung, and Blood Institute Grant HL-09385-01 to S. P. Gaine.

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Received 23 June 1997; accepted in final form 30 December 1997.

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