Evidence for Endothelin Involvement in the Pulmonary Vasoconstrictor Response to Systemic Hypoxia in the Isolated Rat Lung

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ABBREVIATIONS: PIP, pulmonary inflation pressure; PPP, pulmonary perfusion pressure; LW, lung weight; ET, endothelin; HPV, hypoxic pulmonary vasoconstriction; ECE, endothelin-converting enzyme.

Abstract

We investigated the effect of systemic hypoxia (Krebs-Henseleit solution gassed with 5% CO₂/95% N₂) on an isolated, perfused rat lung. Hypoxia resulted in a slowly developing sustained increase in pulmonary perfusion pressure (PPP) accompanied by an increase in lung weight (LW). The endothelin (ET) receptor antagonists BQ123 (3 and 10 μM), BQ788 (3 μM) and bosentan (1.5 and 5 μM) all attenuated the hypoxia-induced increases in LW and PPP. In addition, phosphoramidon (1 μM), an ET-converting enzyme inhibitor, also significantly attenuated the hypoxia-induced increases in PPP and LW. The use of two agents that alter peptide secretion, phalloidin (10 and 50 nM) and colchicine (100 nM), and the peptide synthesis inhibitor cycloheximide (5 μM) all significantly attenuated the hypoxia-induced increases in PPP and LW. The increase in PPP and LW after the onset of hypoxia was accompanied by an increase in perfusate levels of ET-1 compared with normoxic time-matched controls. The results show that in this model, systemic hypoxia is capable of causing a sustained vasoconstriction and increased LW. The fact that these increases can be attenuated by an ET-converting enzyme inhibitor, ET receptor antagonists and agents that block peptide synthesis and secretion, together with the increase in perfusate levels of ET-1, suggests that ET production and release contribute to the changes seen.

Hypoxia causes dilation in systemic arteries but constriction of the pulmonary vasculature (Wadsworth, 1994). This HPV is an important physiological response to maintain the ventilation perfusion ratio and facilitate optimal oxygen uptake by the pulmonary circulation (Fishman, 1976).

Recent reports have indicated that there are two main components to HPV: a rapid short-lasting constriction of ~5 min (phase 1; Jensen et al., 1992), which is commonly seen in precontracted preparations, and a slower, more-sustained phase (phase 2), which is endothelium dependent in most species (Jin et al., 1992; Ward and Robertson, 1995). The transient phase 1 HPV appears to be due to K⁺ channel blockade (Weir and Archer, 1995), but the mechanisms underlying the phase 2 response are not clear.

It is the phase 2 constriction that is probably of greatest importance from a pathological standpoint because chronic pulmonary hypoxia and the associated vasoconstriction lead to intimal thickening and hypertrophy of pulmonary vessels. This culminates in the development of pulmonary hypertension and right ventricular hypertrophy (Barnes, 1994).

There is considerable evidence to implicate ETs in the response of the lung to chronic hypoxia (3–4 weeks); long term treatment with ET receptor antagonists attenuates the development of pulmonary hypertension and associated structural changes in the pulmonary circulation and heart (Bonvallet et al., 1993; Chen et al., 1993; Eddahibi et al., 1995; Ferri et al., 1995; Goerre et al., 1995). However, it is not known how quickly the ETs become involved in this response.

The development of a salt-perfused, isolated lung model, with an intact microcirculation, would be advantageous to investigate the mechanisms involved in the sustained phase 2 HPV. In the present study, we describe such a model. We have also investigated the possible role of endogenous ETs in the responses to acute hypoxia seen in this rat lung model. This has been done by using the ET receptor antagonists BQ123 (Ihara et al., 1992), BQ788 (Ishikawa et al., 1994) and bosentan (Clozel et al., 1994), and the ECE inhibitor phosphoramidon (Fukuroda et al., 1990; Matsumura et al., 1990; McMahon et al., 1991; Sawamura et al., 1991). In addition, we used two agents that interfere with secretion (phalloidin and colchicine; Borisuy and Taylor, 1967; Kurose et al., 1993) and peptide synthesis (cycloheximide; Obrig et al., 1971), both of which have been shown to reduce ET release in other
situations (Kitazumi et al., 1991; Tasaka and Kitazumi, 1994).

Preliminary study results have been presented to the British Pharmacological Society (Smith et al., 1995a, 1995b)

Methods

Isolated, ventilated, perfused rat lung. Lungs were isolated and perfused as previously described (Lal et al., 1994). Male Wistar rats (310–340 g) were anesthetized with sodium pentobarbitone (100 mg/kg i.p.). Heparin (500 IU) was then administered intravenously via the tail vein; 5 min later, the chest was opened, and the pulmonary artery cannulated with a stainless steel cannula via the right ventricle. The left atrium and main mass of the ventricles were removed to allow free efflux of the perfusate. The trachea was cannulated, and the lungs were removed and placed in a warming jacket at 37°C. Lungs were perfused via the pulmonary artery at a constant rate of 5 ml/min with Krebs-Henseleit solution of the following composition: 4.7 mM KCl, 1.2 mM potassium dihydrogen phosphate, 1.25 mM CaCl₂, 1.2 mM magnesium sulphate, 118 mM NaCl, 25 mM sodium bicarbonate and 11.1 mM glucose, gassed with 20% O₂/5% CO₂/75% N₂ (normoxic) or 95% N₂/5% CO₂ (hypoxic). All ventilation and perfusion tubing was of the low gas permeability type (Tygon R3603 and PharMed 65). PPP was recorded via a pressure transducer (model PDCR, Druck Ltd., Groby, Leicestershire, UK) connected to the pulmonary arterial cannula. The tracheal cannula was connected to a ventilator (miniature animal ventilator; Harvard Apparatus, Edenbridge, Kent, UK), and lungs were ventilated with room air at a stroke volume of 1 ml and a rate of 28 inflations/min (with no positive end-expiratory pressure). A pressure transducer (Druck model PDCR) attached to the tracheal cannula facilitated measurement of PIP. In addition, lungs were suspended from a force displacement transducer (Dynamometer UF-1, Piden Controls Ltd., Canterbury, Kent, UK), for continual measurement of changes in lung weight. All responses were recorded on a Lectromed (Letchworth, Herts, UK) MX6 pen recorder. Random controls were carried out during the course of this study. Lungs were allowed to stabilize for 15 min before the start of the experiment.

Single-pass perfusion. After the initial stabilization period, perfusion continued for an additional 15 min before the onset of hypoxia. Hypoxia was initiated by switching to a Krebs-Henseleit solution previously equilibrated with 95% N₂/5% CO₂. In studies involving drug treatment, the drug was perfused for 15 min before the onset of hypoxia and for the remainder of the hypoxic period (70 min). During the 15-min pretreatment period, none of the agents used had any effect on basal PPP or LW. At the start and finish of two phalloidin and two colchicine experiments, a bolus dose of bradykinin (40 or 50 nmol) was administered via the pulmonary artery to assess vascular reactivity.

Recirculating perfusion. After a 15-min stabilization period, lungs were perfused in a recirculating manner (recirculating volume, 50 ml) under normoxic conditions for an additional 15 min. The lungs were then exposed to hypoxia by switching the gas mixture from 20% O₂/5% CO₂/75% N₂ to 5% CO₂/95% N₂. In studies involving drug treatment, the drug was perfused for 15 min before the onset of hypoxia and for the remainder of the hypoxic period.

ET extraction. ETs were extracted from the recirculated perfusate through the use of a modified acid extraction method (Rolinski et al., 1994). Twenty-five milliliters of perfusate was acidified with glacial acetic acid to give a final concentration of 10% v/v, and the sample was centrifuged (2500 × g at room temperature for 15 min). The supernatant was applied to a washed (3 ml of methanol, 3 ml of distilled water, 3 ml of 10% v/v acetic acid) Amprep (Amersham, Bucks, UK) ethyl C2 minicolumn under negative pressure. The column was then washed with 3 ml of 10% v/v acetic acid and 6 ml of ethyl acetate. The ETs were eluted from the column with 3 ml of methanol/0.05 M ammonium bicarbonate 80/20 (v/v) and dried overnight in a vacuum oven.

ET-1 assay. Dried samples of ET were reconstituted in 250 μl of sample buffer, and ET-1 was measured with an R and D Systems Europe (Oxford, UK) human ET-1 enzyme-linked immunosorbent assay. Reconstituted samples were assayed within 1 hr and assayed in duplicate, and results were measured with a plate reader (Multiskan MC340; Titerette, Helsinki, Finland). The enzyme-linked immunosorbent assay was sensitive to <10 pg/ml ET-1 and shows <1% cross-reactivity with big ET-1 and 14% cross-reactivity with ET-3.

Statistical analysis. Results are expressed as mean absolute values ± S.E.M. for PPP (in mm Hg). Changes in LW ± S.E.M. (in g) are referred to the initial weight at the end of the equilibration period.

Results

Single-Pass Perfusion

Hypoxic vs. hypoxic controls. In the normoxic control group, 70-min perfusion resulted in an increase in PPP from 6.6 ± 0.6 to 8.4 ± 0.5 mm Hg and an increase in LW of 0.4 ± 0.1 g (n = 7). Neither of these changes was significant. In hypoxic lungs, which were perfused for 70 min with a hypoxic solution, PPP increased from 8.2 ± 0.8 to 15.4 ± 1.4 mm Hg (n = 10, P < .001), and LW increased by 2.4 ± 0.6 g (n = 10, P < .001; see figs. 1 and 2). Both of these changes are also significantly greater than those seen over the same time period in the control normoxic group (P < .001). Over the time course of these experiments, there was no significant difference in PIP in either normoxic or hypoxic lungs; therefore, the data have been omitted.

Effects of drug treatment. Phosphoramidon (fig. 3), 1 (μM) significantly attenuated both hypoxic vasoconstriction, from 15.4 ± 1.4 to 8.9 ± 0.8 mm Hg (n = 4–10, P < .01), and the increase in LW, from 2.4 ± 0.6 to 1.1 ± 0.3 g (n = 4–10, P < .05), compared with time-matched hypoxic controls.

We next considered BQ123, BQ788 and bosentan. At concentrations of 3 and 10 μM, BQ123, an ET₄ receptor antagonist, reduced the increase in PPP from 15.4 ± 1.4 to 7.8 ± 0.4 and 9.2 ± 0.8 mm Hg, respectively (n = 6–10, P < .001). BQ123 also attenuated the hypoxia-induced increase in LW from 2.4 ± 0.6 to 2.1 ± 0.4 and 1.0 ± 0.2 g in a concentration-dependent manner (n = 6–10); however, compared with time-matched hypoxic controls, this reduction in LW was significant (P < .01) only at the higher concentration used (fig. 3).

At a concentration of 3 μM, BQ788, an ET₃ receptor antagonist, reduced PPP from 15.4 ± 1.4 to 9.6 ± 0.4 mm Hg (n = 5–10, P < .01) compared with time-matched hypoxic controls. In addition, BQ788 attenuated the hypoxia-induced increase in LW from 2.4 ± 0.6 to 0.6 ± 0.1g (n = 5–10, P < .001).

The mixed ET₄/ET₃ receptor antagonist bosentan (1.5 and 5 μM) significantly reduced the hypoxia-induced increase in...
LW from 2.4 ± 0.6 to 0.85 ± 0.1 (1.5 μM) and 0.5 ± 0.1 g (5 μM; n = 4–10, P < .01) compared with time-matched hypoxic controls. However, only the lower concentration of bosentan (1.5 μM) produced a significant inhibition of HPV (from 15.4 ± 1.4 to 9.0 ± 1.2 mm Hg, n = 4–10, P < .05) compared with time-matched controls (fig. 3).

Compared with time-matched hypoxic controls, phalloidin (10 and 50 nM), an F-actin stabilizer, and the microtubule-disrupting agent colchicine (100 nM) attenuated the hypoxia-induced increase in PPP from 15.4 ± 1.4 to 8.8 ± 0.8 and 9.9 ± 0.8 mm Hg, respectively (n = 5–10, P < .01; fig. 4). Colchicine and the higher concentration of phalloidin also significantly attenuated the increases in LW from 2.4 ± 0.6 to 0.7 ± 0.2 and 0.2 ± 0.1 g, respectively (n = 5–10, P < .01; fig. 4). In some experiments, bradykinin (40 nmol, n = 2 for both phalloidin and colchicine) was added before the addition of phalloidin and colchicine and at the end of the experiment to assess vascular reactivity. In these experiments, the increase in PPP elicited by bradykinin was the same at the start and end of the experiment (10.8 vs. 12.5 mm Hg for phalloidin and 9.5 vs. 14.0 mm Hg for colchicine).

The protein synthesis inhibitor cycloheximide (5 μM) significantly reduced the hypoxia-induced increases in PPP, from 15.4 ± 1.4 to 6.9 ± 0.4 mm Hg, and in LW, from 2.4 ± 0.6 to 0.65 ± 0.2 g (n = 4–10, P < .01), compared with time-matched hypoxic controls (fig. 4).

**Recirculating Perfusion**

**Normoxic vs. hypoxic controls.** Normoxic recirculating perfusion had no significant effect on PPP or LW over the time of the experiment. PPP fell from 7.4 ± 0.7 to 7.1 ± 0.7 mm Hg (n = 7) after 85 min of recirculating perfusion. LW increased by 0.17 ± 0.05 g over the same period (n = 7).

Hypoxic perfusion resulted in an increase in PPP from 8.6 ± 0.6 to 11.4 ± 0.7 mm Hg (n = 7, P < .05); this was accompanied by an increase in LW of 1.7 ± 0.7 g (n = 7, P < .001).

**Effects of drug treatment.** The addition of the ET receptor antagonists BQ123 (10 μM), BQ788 (3 μM) and bosentan (1.5 μM) to the recirculating perfusate attenuated the hypoxia-induced increases in PPP (8.5 ± 0.4 mm Hg, n = 3, P < .05; 7.8 ± 0.3 mm Hg, n = 6, P < .001 and 8.1 ± 0.7, n = 4, P < .05, respectively, compared with the hypoxic control, 11.4 ± 0.7 mm Hg, n = 7) and LW (0.5 ± 0.01 g, n = 4, P < .05; 0.3 ± 0.1 g, n = 6, P < .01 and 0.3 ± 0.1 g, n = 4, P < .01, respectively, compared with the hypoxic control, 1.7 ± 0.7 g, n = 7).

**ET-1 levels.** The 85-min normoxic perfusion resulted in an ET-1 level of 0.23 ± 0.03 pg/ml perfusate (n = 9). After 85 min of hypoxic perfusion, the ET-1 level had significantly increased to 0.85 ± 0.07 pg/ml of perfusate (n = 7, P < .001; fig. 5).
Discussion

The results of these experiments demonstrate that the isolated, perfused rat lung model can respond to systemic hypoxia with increases in vascular resistance and LW. The HPV seen after exposure to systemic hypoxia is slow to develop and sustained, probably corresponds to the phase 2 response reported by Jin et al. (1992) and thus is suitable for investigating the mechanisms underlying this response. The lack of a rapid (phase 1) constrictor response cannot be explained, although in most reports, this response has been investigated in preconstricted pulmonary artery ring preparations (Rodman et al., 1989; Weir and Archer, 1995).

The finding that the ECE inhibitor phosphoramidon (Fukuroda et al., 1990; Matsumura et al., 1990; McMahon et al., 1991; Sawamura et al., 1991) inhibited the increases in PPP and LW caused by hypoxia suggests that the hypoxic responses seen in this model may involve conversion of big ET or ETs to the mature peptide or peptides. Vemulapalli et al. (1992) reported that phosphoramidon inhibited ET-1 release induced by ischemia-hypoxia in an isolated guinea pig lung preparation. However, the same group also reported that pulmonary hypoxia in anesthetized rats did not prevent, but actually increased, the rise in plasma ET-1 in the presence of phosphoramidon (Vemulapalli et al., 1994). The reason for this is not clear, but in whole-animal studies, the source of the ET-1 is not readily identifiable.

Further evidence for a role of endogenous ETs in the response to hypoxia was obtained by using the selective ETα receptor antagonist BQ123 (Ihara et al., 1992), the selective ETβ receptor antagonist BQ788 (Ishikawa et al., 1994) and...
earlier, we did not see this acute response when hypoxia was induced systemically.

Phalloidin showed a concentration-related inhibition of the hypoxia-induced increases in PPP and LW. This indicates that F-actin may be involved in the secretion of ET or ETs from the endothelial cells (Kitazumi et al., 1991; Tasaka and Kitazumi, 1994). However, phalloidin has also been shown to selectively block the increase in vascular permeability caused by exogenous ET-3 in the rat mesentery (Kurose et al., 1993); thus, phalloidin is probably exerting two distinct effects, both of which may have a role in the responses seen.

The microtubule disrupting agent colchicine (Borisy and Taylor, 1967) also prevented the increases in PPP and LW associated with systemic hypoxia. These inhibitory effects suggest that the microtubular system is involved in these responses to hypoxia. Therefore, two agents, colchicine and phalloidin, both of which have been reported to interfere with ET secretion from cells (Kitazumi et al., 1991; Tasaka and Kitazumi, 1994), have inhibited the response to hypoxia in the isolated lung.

The actions of colchicine and phalloidin in inhibiting HPV cannot be attributed to nonselective depression of vascular smooth muscle contraction because when bradykinin was added at the end of some of the experiments, the responses observed were similar to preantagonist responses or the responses reported in normoxic lungs (Lal et al., 1994).

The protein synthesis inhibitor cycloheximide (Obrig et al., 1971) also prevented the increases in PPP and LW associated with hypoxia. These observations suggest that the changes seen after hypoxia involve de novo peptide synthesis and argue against secretion of preformed peptide from storage vesicles.

In studies using the recirculating system, the increases in PPP and LW were not as great as the increases seen in the single-pass system. The reason for this is not clear, but it could be due to the accumulation of vasoactive compounds in the recirculating perfusate.

The results obtained in the recirculating system with the ET receptor antagonists (BQ123, BQ788 and bosentan) are in agreement with the results obtained in the single-pass system. In the recirculating system, it has been demonstrated that systemic hypoxia increases the levels of ET-1 in the perfusate. This is in agreement with a number of other studies that demonstrate increased plasma levels of ET-1 in response to various forms of hypoxia in both humans (Goerre et al., 1995) and rats (Li et al., 1994; Shirikami et al., 1991). Our data suggest that the lung could be an important source of this plasma ET-1. Cultured pulmonary artery endothelial cells have been shown to increase ET-1 release after hypoxic exposure (Hieda and Gomez-Sanchez, 1990; Kourtembanas et al., 1991). However, Demiryurek et al. (1994) reported that hypoxia did not increase ET-1 release from large conductance pulmonary artery rings preconstricted with ET-1 (Xia and Nye, 1995).

It contrast to our data, Takeoka et al. (1995) demonstrated that BQ123 had no effect on HPV in an isolated rat lung; however, they studied the acute 5-min response to alveolar hypoxia. It is thought that the initial phase 1 HPV is due to depolarization of the cell via closure of an oxygen-sensitive potassium channel (Weir and Archer, 1995), and as stated
It would be useful to be able to remove the endothelium from the perfused lung to help identify the cell type responsible for the ET-1; however, in preliminary experiments using pulmonary arterial emboIism, we have not been able to do this and maintain a viable preparation.

In a previous study in which we used the same perfusion conditions, we have shown that bolus doses of ET-1 injected via the pulmonary artery increase PIP in the rat lung (Lal et al., 1995). The fact that increases in PIP were not seen in the present hypoxia experiments may indicate that ET production is confined to the vasculature of the lung to cause a selective increase in PIP. Because the vascular endothelium probably is the source of the released ET-1, the amount passing into the bronchial circulation may be insufficient to cause constriction.

We and others have previously shown that ET-1 injected via the pulmonary artery causes increases in PIP and LW, primarily by stimulating ETA receptors, which are predominantly located on the venous side of the circulation. Because BQ788 inhibits both the increase in PIP and LW, this suggests that ET_A receptors are involved in the observed increase in LW. The increases in LW associated with hypoxia or administered ET-1 have been attributed to a hydrostatic edema resulting from pulmonary venoconstriction (Filep et al., 1993; Lal et al., 1995). However, results from the present study with the lower doses of BQ123 (3 μM) and phallolidin (10 nM) indicate that the increase in LW can still occur in the absence of changes in PPP. The reasons for this require further investigation, but it is possible that ETs could have a direct effect to increase permeability via the stimulation of ET_A receptors.

In summary, the present results show that in the perfused rat lung, systemic hypoxia produces a sustained increase in pulmonary vascular resistance with an associated increase in edema formation, as indicated by the change in LW. De novo synthesis of ETs and subsequent secretion via the microtubular system appear to be involved in these responses, although the location of the ET-1 synthesis remains unclear. Thus, this simple preparation provides an ideal medium in which to analyze the events leading to ET-1 release after hypoxic challenge.

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References


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