Prostacyclin Production and Mediation of Adenylate Cyclase Activity in the Pulmonary Artery

Alterations after Prolonged Hypoxia in the Rat

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Abstract

Prostacyclin is a critical mediator of structure and function in the pulmonary circulation, causing both the inhibition of vascular smooth muscle growth and vasodilatation via the stimulation of adenylate cyclase. To examine the potential role of alterations in prostacyclin production or mechanism of action in chronic hypoxic pulmonary hypertension, we determined the effects of prolonged (7 d) in vivo hypoxia on in vitro prostacyclin synthesis and mediation of adenylate cyclase activity in rat main pulmonary arteries. In control arteries prostacyclin production exceeded that of prostaglandin (PG) E_2 by 25-fold, with 42% originating from the endothelium. Studies utilizing indomethacin revealed that endogenous prostaglandins mediate at least 69% of basal adenylate cyclase activity. Prostacyclin-stimulated enzyme activity was enhanced by exogenous GTP, indicating that this is a receptor-mediated process involving G protein amplification. Comparable dose-related responses to prostacyclin and PGE_2 suggest that these agents may activate a common receptor. After 7 d of in vivo hypoxia there was a 2.7-fold increase in in vitro prostacyclin production, with equivalent increases in synthesis in the endothelium and vascular smooth muscle. However, despite this increase there was no change in basal adenylate cyclase activity, and this was associated with attenuated sensitivity of the enzyme to prostacyclin stimulation. Concomitant diminution of the response to beta-adrenergic stimulation, with previously-demonstrated beta receptor downregulation and unaltered postreceptor-mediated activity, suggests that the blunted response to prostacyclin is due to receptor downregulation. Parallel studies of the thoracic aorta indicated that these changes are specific to the pulmonary artery. It is postulated that attenuation of the response of adenylate cyclase to prostacyclin may contribute to the structural changes and hypertension observed in the pulmonary vasculature of the rat with chronic hypoxia. (*J. Clin. Invest.* 1991. 88:447–455.) Key words: aorta • cyclic AMP • G proteins • hypertension • prostaglandin E_2

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Introduction

Hypoxic pulmonary hypertension is one of the critical mechanisms underlying persistent pulmonary hypertension of the neonate and the development of pulmonary hypertension in older children and adults with a variety of cardiac and respiratory illnesses (1, 2). This phenomenon has been well studied in the adult rat, yielding both physiologic and anatomic findings similar to those noted in the human (3–5). In the rat model, pulmonary hypertension is evident almost immediately after the onset of acute severe hypoxia, and it is also present with accompanying anatomic changes after more prolonged periods of hypoxia of milder degree. These events in the pulmonary circulation of the rat are contrasted by the maintenance of normal systemic blood pressure, mimicking the clinical experience with this problem (1, 4, 6, 7).

Studies in the rat and other species indicate that locally-produced prostacyclin is important in the regulation of vasomotor tone and vascular cell differentiation and growth in the pulmonary circulation (8, 9). These actions are mediated through the plasma membrane–bound enzyme adenylate cyclase, with prostacyclin stimulation of cyclic AMP (cAMP) production resulting in vasodilatation and the inhibition of smooth muscle cell growth (10, 11). In vivo experiments and in vitro studies with perfused isolated lung preparations have demonstrated that pulmonary production of this potent vasodilator increases with acute hypoxia, resulting in a degree of attenuation of the vasoconstrictor response (12, 13). However, despite the initial increase in prostacyclin production, further hypoxia leads to the development of pulmonary hypertension with medial hypertrophy of muscular arteries and extension of vascular smooth muscle (VSM)[1] into peripheral, normally nonmuscular, arteries (3, 4). These alterations are prevented in the rat when angiotensin II is administered during the hypoxia period, most likely related to its ability to release prostaglandins (PG) (8). This suggests that changes in PG synthesis or PG-mediated mechanisms may be involved in the pathogenesis of chronic hypoxic pulmonary hypertension.

To examine the potential role of alterations in prostacyclin production or mechanism of action in chronic hypoxic pulmonary hypertension, we determined the effects of prolonged (7 d) in vivo hypoxia on in vitro prostacyclin production and mediation of adenylate cyclase activity in rat main pulmonary arteries. PGE_2 production and mediation of adenylate cyclase activity were also investigated to determine if the results for prostacyclin are specific to that vasodilatory prostanoid. Based on the findings with acute hypoxia (12–14), we tested the hypothesis that pulmonary artery prostacyclin production is also increased after prolonged hypoxia, but there is attenuation of the response of adenylate cyclase to prostacyclin stimulation.

1. *Abbreviation used in this paper:* VSM, vascular smooth muscle.

Methods

Animal model. Adult male Sprague-Dawley rats (300–350 g) were used as described in our previous investigations of beta-adrenergic regulation of adenylate cyclase activity in the pulmonary artery (15, 16). The rats were randomly assigned to either the control group to remain in room air at normal atmospheric pressure (760 mmHg), or to the experimental hypoxia group to be kept in the same room in a steel and plexiglass hypobaria chamber for 7 d. During the first day of hypobaria the rats were subjected to room air at 570 mmHg (3/4 atm) to allow them to acclimate to the hypoxic conditions. The remainder of the hypobaria challenge was with room air at 380 mmHg (1/2 atm), equivalent to an environment with $F_iO_2 = 0.105$ at normal atmospheric pressure. A stock diet and tap water were supplied ad libitum. In this model, hypertension is present in the pulmonary circulation by 3 d of hypobaria and it persists with further hypobaric challenge, whereas systemic blood pressure remains similar to that of controls (4, 17). The experiments were performed at the end of 7 d of hypobaria because that duration of hypoxia has been employed previously by other investigators examining PG-mediated processes in the pulmonary circulation of the rat (8). In addition, we have demonstrated alterations in beta-adrenergic receptor-mediated mechanisms over that time period (16).

The rats were sacrificed by rapid decapitation at the end of the control or hypobaria study period and the main pulmonary arteries were obtained. These vessels were selected for study because they have been used previously to assess hypoxia-induced changes in mechanisms of pulmonary vasomotor tone regulation in the rat (16, 18, 19). Furthermore, PG production and adenylate cyclase activity can be determined directly in these arteries, and the endothelium can be mechanically removed before in vitro study to distinguish the roles of the endothelium and VSM in these processes (19). Adenylate cyclase activity was determined directly in lieu of examining relaxation responses to PGs because alterations in PG-mediated mechanisms may be masked by the dramatic changes in vascular reactivity that result from the structural alterations that occur with prolonged hypoxia (18). Systemic arteries (thoracic aorta) were also examined to assess the specificity of the findings for the pulmonary arteries. Vessels from separate groups of rats were used in each of the experiments described. The procedures followed in the care and euthanasia of the study animals were approved by the Institutional Review Board for Animal Research.

Determination of vascular prostaglandin production. Immediately after harvesting, the main pulmonary arteries and thoracic aortas were placed in freshly prepared Krebs-Henseleit buffer gassed with 95% O_2/5% CO_2 at 37°C. The Krebs-Henseleit buffer contained 4.8 mM KCl, 2.0 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 11.0 mM dextrose, 118 mM NaCl, and 25 mM $NaHCO_3$ at a pH of 7.4. Fat and connective tissue were removed taking care not to disrupt the endothelium, and the arteries were cut into 5–10-mg segments. The segments were rinsed and equilibrated collectively for 1 h in fresh oxygenated buffer.

The presence of functional endothelium was confirmed by examining endothelium-dependent acetylcholine stimulation of VSM guanylate cyclase activity in similarly obtained arterial segments (20). 1-min incubations in oxygenated Krebs-Henseleit buffer in the presence of 10 μM acetylcholine and the phosphodiesterase inhibitor methylisobutylxanthine (0.5 mM) resulted in a 100-fold increase in cGMP content in pulmonary and a 57-fold increase in cGMP content in systemic arterial segments. The presence of intact endothelium was also confirmed by light microscopy of 5-μm sections of randomly chosen arterial segments (18). For experiments examining the distinct roles of the endothelium and VSM, the endothelium was removed from selected segments by rubbing with a cotton-tipped applicator. Endothelium removal was verified by documenting the absence of cGMP accumulation with acetylcholine stimulation and also by light microscopy.

After collective equilibration, the segments were placed individually into polypropylene chambers containing 2.0 ml of oxygenated Krebs-Henseleit buffer at 37°C for a 1-h preincubation. At the end of the preincubation period the media was replaced with fresh oxygenated Krebs-Henseleit buffer and a 2-h incubation was performed, exchanging the buffer each hour to determine if substrate availability was rate limiting (21). As we have done previously in studies of other arteries (21), hyperoxic conditions were employed in vitro to maintain the degree of oxygenation at a level at which oxygen availability would not limit PG production (22, 23). Selected segments were incubated in the presence of 100 μM indomethacin to confirm that the PGs measured were newly synthesized. Upon collection the incubation media was placed into ice cold tubes containing 100 μg of acetylsalicylic acid and it was stored at −20°C until the time of assay for PG. The arterial segments were blotted and wet weights were determined.

Samples of the incubation media were assayed for the stable metabolite of prostacyclin, 6-keto-prostaglandin $F_{1\alpha}$, and for PGE_2 by radioimmunoassay as previously described (21, 24). Briefly, the 6-keto-$PGF_{1\alpha}$ assay procedure utilized duplicate aliquots of standard (0–200 pg) and unknown quantities of 6-keto-$PGF_{1\alpha}$ put into a mixture of Krebs-Henseleit solution and 0.1 M phosphate-buffered saline (1:1) plus 0.1% polyvinyl-pyrrolidone. Antiserum (0.1 ml, 1:4,000 titer) and 0.1 ml [^{3}H]-6-keto-$PGF_{1\alpha}$ were added and the tubes were incubated at 4°C for 12–18 h. Bound and free ligand were separated with dextran-coated charcoal and bound ligand was counted by liquid scintillation spectrometry. For the PGE_2 assay duplicate aliquots of standard (0–500 pg) and unknown quantities of PGE_2 were placed in the same mixture of Krebs-Henseleit solution and phosphate-buffered saline. Antiserum (0.1 ml, 1:14,000 titer) and 0.1 ml [^{3}H]-PGE_2 were added and the tubes were incubated, bound and free ligand were separated, and bound ligand was quantified as in the 6-keto-$PGF_{1\alpha}$ assay. The unknown quantities of 6-keto-$PGF_{1\alpha}$ and PGE_2 were determined from the standard curves generated. The intra- and interassay coefficients of variation in the 6-keto-$PGF_{1\alpha}$ assay were 5.8 and 8.9% at 250 pg/ml, and 2.8 and 8.7% at 1,000 pg/ml. For PGE_2 they were 10.8 and 13.8% at 250 pg/ml, and 7.0 and 17.7% at 1,000 pg/ml. The results for PG production are expressed as picograms per milligram wet weight · hour.

Adenylate cyclase activity assay. The main pulmonary arteries and thoracic aortas for the determinations of adenylate cyclase activity were placed in ice cold 0.25 M sucrose buffer with 10 mM Tris Base normalized to pH 7.4 with HCl (10× volume), and fat and connective tissue were removed. Arteries were pooled from two rats for each experiment. The arteries were transferred into ice cold 50 mM Hepes buffer containing 10 mM Tris HCl, 10 mM $MgCl_2$, 0.5 mM methylisobutylxanthine, 0.5 mM EDTA, 0.1 mM dithiothreitol, and 0.5 mM ascorbic acid at pH 7.4 (5× volume). The tissue was minced with scissors at 4°C and was homogenized with a Polytron at setting No. 6 for 10 s. The homogenate was filtered through two layers of gauze, and adenylate cyclase activity was measured on the resulting crude plasma membrane preparation after a 1-h equilibration period by a modification of the method of Brooker, as previously described (15, 16, 25). Further purification of the membranes was not performed because agonist stimulation of adenylate cyclase activity decreases with subsequent preparatory steps (26). In selected experiments the crude plasma membrane preparation was performed after the removal of the endothelium by the methods described above.

Briefly, adenylate cyclase activity was determined in a total volume of incubation of 50 μl, with 20 μl of crude membrane preparation, 1.0 mM ATP, 12 mM phosphocreatine, and 185 U/ml creatine phosphokinase. Incubations were performed at 30°C for 15 min and were terminated by the addition of 450 μl of ice cold 50 mM sodium acetate buffer, pH 4.7, with 1.0 mM EDTA. The amount of cAMP present at the end of the incubation was determined by radioimmunoassay after acetylation (27), and the protein content of the crude plasma membrane preparation was measured by the Lowry method using BSA as the standard (28). The quantity of cAMP produced during the incubation was calculated by subtracting the cAMP content of blanks in which the adenylate cyclase reaction was inhibited before the onset of the incubation, yielding adenylate cyclase activity expressed as picomoles cAMP/mg protein · minute. Linearity of enzyme activity versus the time of incubation and the protein content of the crude membrane preparation has been previously confirmed (15). The intraassay and

interassay coefficients of variation for the cAMP radioimmunoassay were 3.6 and 7.8%, respectively.

Basal (nonstimulated) adenylate cyclase activity was determined, as was activity with the addition of 10^{-4} M GTP, and 10^{-10} to 10^{-4} M prostacyclin or PGE_2 in the presence or absence of 10^{-4} M GTP. The GTP was added to optimize the coupling of activated receptors to the catalytic subunit of adenylate cyclase via guanine nucleotide-dependent regulatory proteins (G proteins) (29). Isoproterenol (10^{-10} to 10^{-4} M) stimulation of pulmonary artery adenylate cyclase activity was also examined to determine if the findings for prostaglandin activation of the enzyme were specific to that class of compounds, or if the findings were common to all stimulatory agonists. The incubations with isoproterenol were performed in the absence or presence of the antagonist l-alprenolol (10^{-5} M) to confirm that enzyme stimulation was via activation of beta-adrenergic receptors. Selected membrane incubations for adenylate cyclase activity were conducted in the presence of 100 μM indomethacin to inhibit endogenous prostaglandin production. All determinations were performed in duplicate.

Statistical analysis. Analysis of variance (ANOVA) with Neuman-Keuls post-hoc testing was employed to compare mean values between more than two groups (30). Nonparametric ANOVA was used when indicated. Single comparisons between two groups were performed with paired and nonpaired Student's *t* tests. Significance was accepted at the 0.05 level of probability. All results are expressed as mean±SEM.

Materials. L-Isoproterenol as the *d*-bitartrate salt, adenosine 5'-triphosphate (disodium salt, from equine muscle), phosphocreatine (disodium salt), creatine phosphokinase (from rabbit muscle, Type I) and l-alprenolol were obtained from Sigma Chemical Co. (St. Louis, MO). Guanosine 5'-triphosphate (disodium salt) was from the United States Biochemical Corp. (Cleveland, OH), and prostacyclin (sodium salt) and PGE_2 were obtained from Upjohn Co. (Kalamazoo, MI). Prostacyclin was solublized in 50 mM Tris buffer, pH 9.7, and PGE_2 was solubilized in 0.1 M PO_4 buffer, pH 7.4. Indomethacin was obtained from Merck, Sharp and Dohme (West Point, PA). All the above reagents were prepared fresh daily. Cyclic AMP [^{125}I] radioimmunoassay kits were from New England Nuclear/Dupont (Boston, MA).

Results

Vascular prostaglandin production. The rates of production of prostacyclin (6-keto-$PGF_{1\alpha}$) and PGE_2 for pulmonary and systemic arterial segments obtained from control animals, in the absence or presence of indomethacin, are shown in Fig. 1. Prostacyclin production (Fig. 1 *A*) was 2.7-fold greater in the pulmonary compared with the systemic segments. Indomethacin inhibited prostacyclin production by 96% in the pulmonary segments and by 95% in the systemic segments. PGE_2 production (Fig. 1 *B*), which was comparable in the pulmonary and systemic segments, occurred at rates that were only 3.7% and 10.2% of the rates of prostacyclin production in the two artery types, respectively. Indomethacin inhibited PGE_2 production by 82% in the pulmonary and by 96% in the systemic arterial segments. The production rates did not change from hour 1 to hour 2 of incubation, except for a slight increase in PGE_2 production by systemic arterial segments during the second hour (Table I).

The effects of 7 d of in vivo hypoxia on arterial segment prostaglandin production in vitro are depicted in Fig. 2. Prostacyclin production was increased 2.7-fold in the pulmonary segments after hypoxia (Fig. 2 *A*). This increase was specific to the pulmonary arteries in that the production of prostacyclin by the systemic arterial segments was not changed. However, the elevation in pulmonary arterial prostacyclin production after hypoxia was less dramatic during the second hour of in vitro incubation, with the production rate decreased by 33% compared to hour 1 (Table II). In contrast to the findings for prostacyclin, PGE_2 production by the pulmonary segments was not significantly changed after hypoxia, whereas it was increased by 41% in the systemic arteries (Fig. 2 *B*). There were no detectable changes in PGE_2 production by arterial segments from the hypoxic rats between hour 1 and hour 2 of in vitro incubation (Table II). Indomethacin inhibition of prostacyclin and PGE_2 production by the arterial segments from the hypoxic animals was similar to that for the segments from control animals (data not shown).

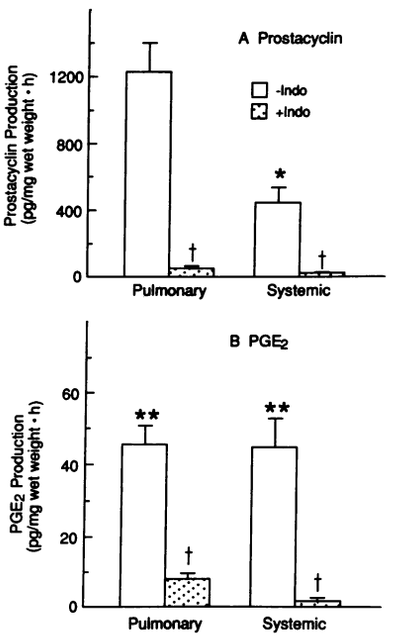

Figure 1. Prostacyclin (*A*) and PGE_2 (*B*) production in pulmonary and systemic arterial segments from control rats, in the absence (*open bar*) or presence (*stippled bar*) of 100 μM indomethacin (*INDO*). The segments were incubated in oxygenated (95% O_2/5% CO_2) Krebs-Henseleit buffer at 37°C and the prostaglandins in the incubation media were determined by radioimmunoassay. Values are means±SEM for segments from eight rats. Note the differences in the scales in *A* and *B*. *$P < 0.05$ vs. pulmonary; †$P < 0.05$ vs. no INDO; **$P < 0.05$ vs. prostacyclin.

The contribution of the endothelium and VSM to pulmonary arterial prostacyclin production under control conditions and after prolonged hypoxia is shown in Fig. 3. After 1 wk of in vivo hypoxia, prostacyclin production in intact arterial segments increased 2.4-fold in these experiments, similar to the increase observed in Fig. 2. Removal of the endothelium re-

Table I. Comparison of Prostacyclin and PGE_2 Production in Pulmonary and Systemic Arterial Segments from Control Rats during the First Versus Second Hour of Incubation

	Hour 1	Hour 2
	pg/mg wet wt · h	
Prostacyclin		
Pulmonary	1187±286	1286±136
Systemic	534±206	528±122
PGE_2		
Pulmonary	41±7	45±4
Systemic	40±6	52±8*

Values are means±SEM for segments from eight rats. The segments were incubated for 2 h in oxygenated (95% O_2/5% CO_2) Krebs-Henseleit buffer at 37°C, replacing the incubation media hourly. The prostaglandins in the incubation media were determined by radioimmunoassay. * $P < 0.05$ vs. hour 1.

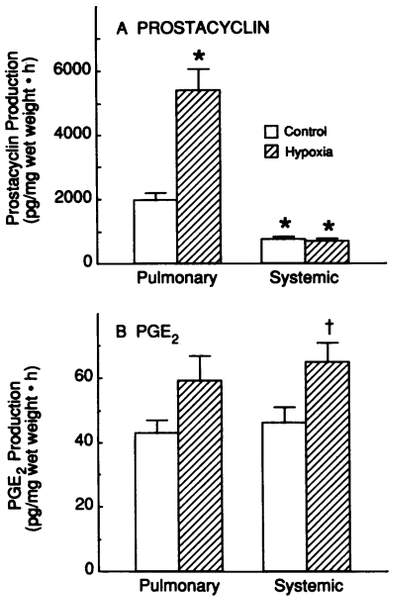

Figure 2. Effect of 7 d of in vivo hypoxia on prostacyclin (*A*) and PGE_2 (*B*) production in pulmonary and systemic arterial segments. The segments from control (*open bar*) or hypoxic rats (*hatched bar*) were incubated as described for Fig. 1. Values are means±SEM for segments from eight rats. Note the differences in the scales in *A* and *B*. $*P < 0.05$ vs. control pulmonary; $^{\dagger}P < 0.05$ vs. control systemic.

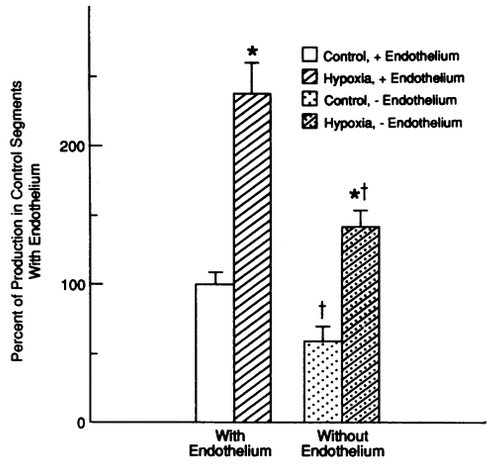

Figure 3. Effect of endothelium removal (*stippled bar*) on prostacyclin production by pulmonary arterial segments from control (*open bar*) and hypoxic rats (*hatched bar*). The segments were incubated as described for Fig. 1. Prostacyclin production is expressed as the percent of production in control segments with intact endothelium. Values are means±SEM for segments from eight rats. $*P < 0.05$ vs. control; $^{\dagger}P < 0.05$ vs. with endothelium.

sulted in a 42% decline in prostacyclin production in segments from control rats. Endothelium removal from the segments from hypoxic rats caused an equivalent 40% fall in prostacyclin production. As a result, prostacyclin production by endothelium-denuded segments was also 2.4-fold greater after in vivo hypoxia compared with controls.

Basal and GTP-stimulated adenylate cyclase activity. Basal and GTP-stimulated adenylate cyclase activity in crude plasma membrane preparations made from intact and endothelium-denuded pulmonary and systemic arteries from control animals is shown in Fig. 4. In preparations made with endothelium present, basal activity was 2.3-fold greater in pulmonary (Fig. 4 *A*) compared to systemic membranes (Fig. 4 *B*). The addition of exogenous GTP did not augment enzyme activity in either membrane type. Endothelium removal from pulmonary arteries caused a 38% decline in basal activity and a comparable 41% decline in activity in the presence of GTP. In contrast, removal of the endothelium from systemic arteries did not affect adenylate cyclase activity. As a result, basal and GTP-stimulated enzyme activity were similar in pulmonary compared to systemic membranes after endothelium removal.

Table II. Comparison of Prostacyclin and PGE_2 Production in Pulmonary and Systemic Arterial Segments from Hypoxic Rats during the First Versus Second Hour of Incubation

	Hour 1	Hour 2
	pg/mg wet wt · h	
Prostacyclin		
Pulmonary	5391±668	3598±570*
Systemic	693±82	606±68
PGE_2		
Pulmonary	59±8	48±9
Systemic	65±6	55±7

Values are means±SEM for segments from eight rats. The segments were incubated for 2 h in oxygenated (95% O_2/5% CO_2) Krebs-Henseleit buffer at 37°C, replacing the incubation media hourly. The prostaglandins in the incubation media were determined by radioimmunoassay. * $P < 0.05$ vs. hour 1.

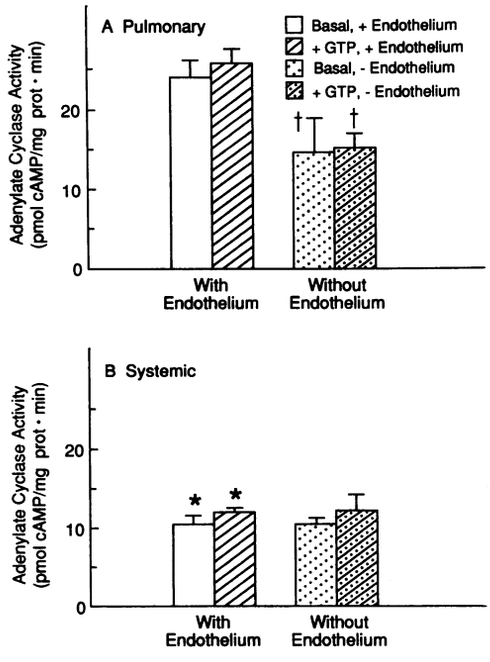

Figure 4. Effect of endothelium removal (*stippled bar*) on basal (*open bar*) and GTP-stimulated (*hatched bar*) adenylate cyclase activity in pulmonary (*A*) and systemic arteries (*B*) from control rats. Crude plasma membrane preparations were incubated at 30°C for 15 min with 1 mM ATP, 12 mM phosphocreatine, and 185 U/ml creatine phosphokinase. Cyclic AMP produced was measured by radioimmunoassay after acetylation. Adenylate cyclase activity is expressed as picomoles cAMP per milligram protein · minute. All determinations were performed in duplicate. Values are means±SEM for eight experiments. $*P < 0.05$ vs. pulmonary; $^{\dagger}P < 0.05$ vs. with endothelium.

The effects of indomethacin on basal and GTP-stimulated adenylate cyclase activity in crude plasma membrane preparations from intact pulmonary and systemic arteries of control animals are given in Fig. 5 *A*. Similar to the findings depicted in Fig. 4, basal activity was 2.6-fold greater in pulmonary compared to systemic membranes and the addition of GTP did not enhance enzyme activity in either membrane type. Indomethacin decreased basal enzyme activity in the pulmonary membranes by 69% to levels similar to those in nontreated systemic membranes. In contrast, indomethacin had no effect on basal activity in the systemic membranes. In a parallel fashion, indomethacin decreased enzyme activity in the pulmonary membranes in the presence of GTP by 53%, but it did not attenuate activity with GTP in the systemic membranes.

The effects of 7 d of in vivo hypoxia on basal and GTP-stimulated adenylate cyclase activity are evident upon comparison of the results shown in Fig. 5, *A* and *B*. There was no significant change in basal activity nor in activity with GTP in the pulmonary membranes after in vivo hypoxia. In contrast, there was a 2.3-fold increase in basal activity and a 5.2-fold increase in activity with GTP in the systemic membranes, resulting in levels of activity that were comparable with those in the pulmonary membranes from the same animals. In the pulmonary membranes, indomethacin attenuation of basal adenylate cyclase activity was less after in vivo hypoxia than in controls (43 vs. 69% attenuation, respectively, $P < 0.05$), and the decline in GTP-stimulated activity that was observed with indomethacin in controls was absent after hypoxia. In the systemic membranes, indomethacin did not attenuate basal or GTP-stimulated activity after hypoxia, similar to the findings for controls.

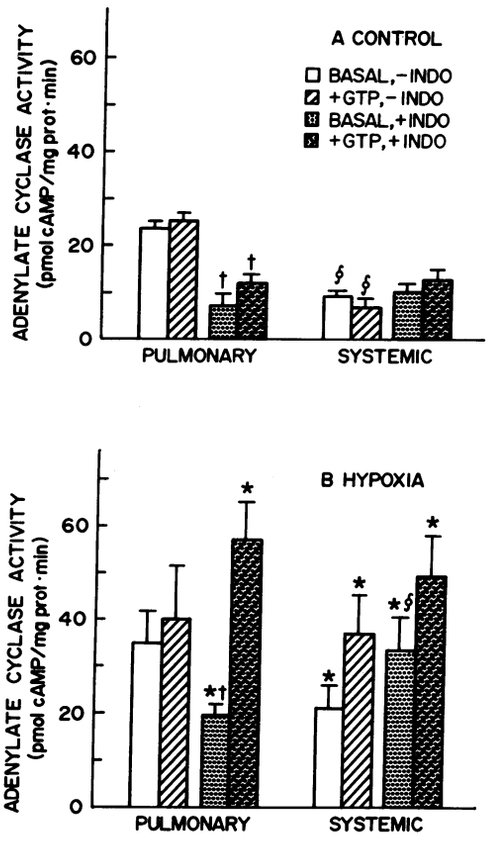

Figure 5. Basal (*open bar*) and GTP-stimulated (*hatched bar*) adenylate cyclase activity in pulmonary and systemic arteries from control (*A*) and hypoxic rats (*B*), in the absence or presence of 100 μM indomethacin (INDO, *stippled bar*). Crude plasma membrane preparations were incubated and adenylate cyclase activity was assessed as described in Fig. 4. Values are means±SEM for eight experiments. $^{*}P < 0.05$ vs. control, $^{\S}P < 0.05$ vs. pulmonary, $^{\dagger}P < 0.05$ vs. no indomethacin.

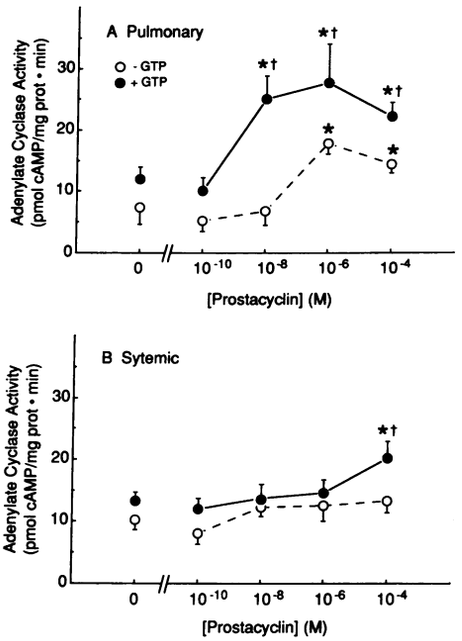

Figure 6. Prostacyclin stimulation of adenylate cyclase activity in pulmonary (*A*) and systemic (*B*) membranes from control rats, in the absence (*open circle*) or presence (*solid circle*) of 10^{-4} M GTP. The membranes, which were all treated with 100 μM indomethacin, were incubated as described for Fig. 4. Values are means±SEM for eight experiments. $^{*}P < 0.05$ vs. no prostacyclin; $^{\dagger}P < 0.05$ vs. no GTP.

Prostaglandin stimulation of adenylate cyclase activity. PG stimulation of adenylate cyclase activity was examined in membranes treated with indomethacin. The response to exogenous prostacyclin in pulmonary membranes obtained from control animals, in the absence or presence of exogenous GTP, is shown in Fig. 6 *A*. In the absence of exogenous GTP, there was no increase in adenylate cyclase activity with 10^{-10} and 10^{-8} M prostacyclin, but 10^{-6} and 10^{-4} M prostacyclin caused 2.5-fold and 2.0-fold increases in enzyme activity, respectively. The response to prostacyclin was markedly augmented by GTP in that maximal stimulation was attainable with 10^{-8} M prostacyclin, and the resulting level of enzyme activity was greater than that observed in the absence of GTP. Exogenous prostacyclin had no effect on adenylate cyclase activity in systemic membranes, except at 10^{-4} M in the presence of GTP (Fig. 6 *B*).

The response to prostacyclin was compared with that to PGE_2 in both pulmonary and systemic membranes obtained from control animals (Fig. 7). All experiments were performed in the presence of indomethacin and exogenous GTP. In the pulmonary membranes, prostacyclin and PGE_2 had similar threshold doses (10^{-8} M) and comparable maximal effects on adenylate cyclase activity, both causing 2.6-fold stimulation of

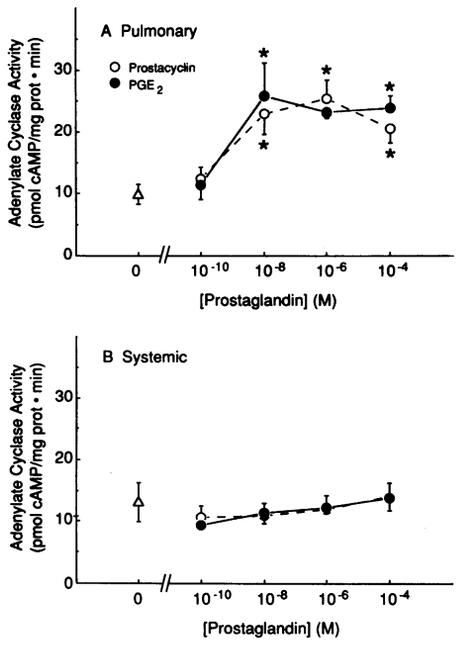

Figure 7. Comparison of the response of adenylate cyclase to prostacyclin (*open circle*) vs. PGE_2 (*solid circle*) in pulmonary (*A*) and systemic (*B*) membranes from control rats. The open triangle depicts activity in the absence of exogenous prostaglandin. The membranes were treated with 100 μM indomethacin and were incubated as described for Fig. 4 in the presence of 10^{-4} M GTP. Values are means±SEM for eight experiments. $^*P < 0.05$ vs. no prostaglandin.

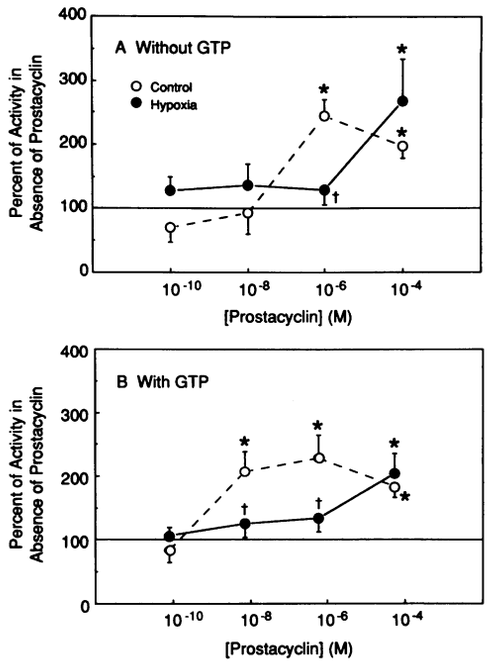

Figure 8. Effect of 7 d of in vivo hypoxia on prostacyclin stimulation of pulmonary artery adenylate cyclase activity, without (*A*) or with (*B*) 10^{-4} M GTP present. The membranes were treated with indomethacin and incubated as described for Fig. 4. Results for the control group (*open circle*) and the hypoxia group (*closed circle*) are expressed as the percent of activity in the absence of prostacyclin. Values are means±SEM for eight experiments. $^*P < 0.05$ vs. no prostacyclin; $^\dagger P < 0.05$ vs. control.

enzyme activity (Fig. 7 *A*). In these experiments neither prostaglandin caused stimulation of adenylate cyclase activity in systemic membranes (Fig. 7 *B*).

The effects of 7 d of in vivo hypoxia on prostacyclin stimulation of pulmonary artery adenylate cyclase activity are shown in Fig. 8. As described above, enzyme activity in control pulmonary membranes was stimulated by prostacyclin at and above 10^{-6} M in the absence of GTP (Fig. 8 *A*), and at and above 10^{-8} M in the presence of GTP (Fig. 8 *B*). In contrast, after 1 wk of in vivo hypoxia there was attenuated stimulation of adenylate cyclase activity with 10^{-6} M prostacyclin in the absence of GTP (Fig. 8 *A*). Furthermore, there was decreased stimulation of enzyme activity with 10^{-8} and 10^{-6} M prostacyclin in the presence of GTP (Fig. 8 *B*). Thus, the sensitivity of the response of pulmonary artery adenylate cyclase to prostacyclin was attenuated after 7 d of in vivo hypoxia. Exogenous prostacyclin did not stimulate adenylate cyclase activity in the systemic membranes from the hypoxic rats, similar to the findings for the control rats (data not shown).

Beta-adrenergic stimulation of adenylate cyclase activity. Isoproterenol stimulation of adenylate cyclase activity in pulmonary membranes from control rats and rats exposed to 1 wk of in vivo hypoxia is depicted in Fig. 9. All experiments were carried out in the presence of 10^{-4} M GTP. In membranes from control animals isoproterenol at concentrations of 10^{-8} M and above stimulated adenylate cyclase activity 2.2- to 2.4-fold. The degree of stimulation obtained was similar to that observed with prostacyclin and PGE_2, and it occurred at similar threshold doses. The addition of 10^{-5} M *l*-alprenolol inhibited the increase in adenylate cyclase activity with 10^{-8} to 10^{-4} M isoproterenol, yielding 88 to 120% of the enzyme activity in the absence of isoproterenol ($P < 0.05$), thus confirming that this process occurs via stimulation of beta-adrenergic receptors. In contrast to the results for controls, isoproterenol did not stimulate enzyme activity in the pulmonary membranes from the hypoxic animals.

Discussion

We determined the effects of 7 d of in vivo hypoxia on in vitro prostacyclin production and mediation of adenylate cyclase

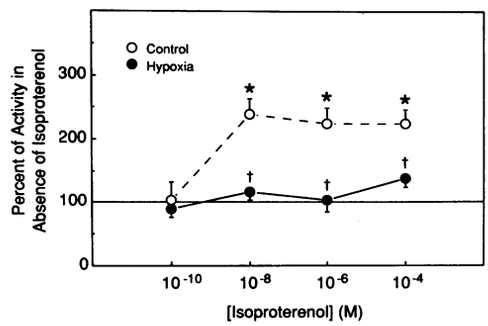

Figure 9. Effect of 7 d of in vivo hypoxia on isoproterenol stimulation of pulmonary artery adenylate cyclase activity. The membranes were incubated as described for Fig. 4 in the presence of 10^{-4} M GTP. Results for the control group (*open circle*) and the hypoxia group (*closed circle*) are expressed as the percent of activity in the absence of isoproterenol. Values are means±SEM for eight experiments. $^*P < 0.05$ vs. no isoproterenol; $^\dagger P < 0.05$ vs. control.

activity in rat main pulmonary arteries in an effort to reveal the potential role of this vasodilator in chronic hypoxic pulmonary hypertension. The observations made in control arteries increase our understanding of pulmonary artery prostacyclin synthesis and mechanism of action under normal conditions. Prostacyclin production greatly exceeded that of PGE_2, consistent with the findings of Voelkel et al. that prostacyclin is the major arachidonic acid metabolite in rat pulmonary venous effluent (12). This provides evidence that PG production by the main pulmonary artery may mimic that by the microcirculation in this model. A large component of the prostacyclin produced (42%) was derived from the endothelium, similar to the findings of Moncada et al. that 37% of prostacyclin synthesized by rabbit aorta originates in the intimal layer (31). In addition, prostacyclin production in rat pulmonary artery was greater than in thoracic aorta, whereas PGE_2 production was similar. Because prostacyclin and PGE_2 are synthesized from the same precursor (prostaglandin H_2), this indicates that prostacyclin synthetase activity may be greater in the former compared with the latter artery type (32).

Comparison of adenylate cyclase activity in control pulmonary artery and aorta suggests that the response to prostacyclin may also differ in the two arteries. In agreement with our previous results (15, 16), basal adenylate cyclase activity was greater in pulmonary artery than in aorta. However, it is now evident that this is due to differences in enzyme activation by endogenous PG because basal activity was similar when PG production was inhibited with indomethacin. In addition, the 69% decline in pulmonary basal activity with indomethacin indicates that PGs mediate the majority of basal activity in the pulmonary artery, whereas the lack of an effect of indomethacin in aortic membranes suggests that they have a minimal role in that artery. Furthermore, because basal adenylate cyclase activity in pulmonary membranes was decreased only 38% after endothelium removal (vs. 69% with indomethacin), it suggests that approximately half of the PGs that mediate basal activity are derived from the endothelium and half are from the VSM. Removal of the endothelium is more likely to have decreased the amount of agonist present than the amount of enzyme because studies of cultured cells indicate that arterial cAMP production is primarily in VSM, particularly when the proportion of cell types present is considered (33).

The contrasting roles of prostacyclin in the two arteries are further demonstrated by the reestablishment of basal levels of adenylate cyclase activity in indomethacin-treated pulmonary membranes with the addition of exogenous prostacyclin and the complete lack of response in the systemic membranes. In addition, GTP augmentation of the response to prostacyclin in pulmonary membranes indicates that the process is receptor-mediated, involving G protein amplification (29). Comparable dose-related responses to prostacyclin and PGE_2 suggest that these agents may activate the same receptor, as has been proposed in studies of cultured VSM cells (34, 35). Receptor binding characteristics have not been quantified in this model because presently-available radioligands have low affinity for vascular PG receptors (35).

Pulmonary artery prostacyclin production was markedly enhanced after in vivo hypoxia, consistent with the thesis proposed by others that the vasculature is the source of the increase in pulmonary prostacyclin that occurs with hypoxia (12–14). In the rat, the similarities between our results and findings in perfused isolated lung preparations (12) suggest that the large and small pulmonary vessels may respond comparably to hypoxia. However, such comparisons should be made with emphasis that the previous studies (12–14) examined the effects of acute hypoxia, whereas the present investigation focuses on changes that occur with prolonged hypoxia.

In addition to demonstrating the increase in pulmonary vascular prostacyclin production, the present studies indicate that this response to hypoxia was specific to the pulmonary arteries, because an increase in prostacyclin production was not observed in aorta. Furthermore, the distinct roles of the pulmonary endothelium and VSM were determined. Because the fold increase in pulmonary artery prostacyclin production after hypoxia was comparable in segments with and without intact endothelium, prostacyclin production was similarly enhanced in the endothelium and VSM.

Contrasting with the findings in arteries from control rats, prostacyclin production declined during the second hour of in vitro incubation in the pulmonary segments from the hypoxic rats, indicating that substrate availability was rate limiting. It is doubtful that these latter observations were due to an effect of the in vitro environment on prostacyclin production during the incubation period because the segments were equilibrated under the same conditions for 2 h before study. Limitations in substrate availability may have been similarly involved and perhaps even more evident in investigations by Badesch et al. (36). They reported attenuated prostacyclin production by both pulmonary arterial rings and early passage pulmonary endothelial cells and adventitial fibroblasts from neonatal calves with severe pulmonary hypertension after 14 d of hypobaric hypoxia (36). A role for substrate limitation is supported by their observation that attenuated synthesis was evident primarily when prostacyclin production was stimulated with A23187 (36), and by the finding of that substrate utilization for prostacyclin synthesis is markedly greater in the pulmonary artery of the developing compared to mature animal (37).

In addition to studies in perfused isolated lung preparations and in in vivo models (12, 13, 36), the direct effects of changes in oxygenation on cultured vascular cells have been investigated. Madden et al. demonstrated a dose-dependent inhibition of prostacyclin synthesis by bovine pulmonary endothelial cells with declining in vitro oxygenation of relatively short duration (4 h) (22). Rabinovitch et al. noted no change in prostacyclin or PGE_2 production by ovine pulmonary endothelial cells with in vitro hypoxia (1 h), but cultured pulmonary VSM cells exhibited a decline in the production of both compounds (23). In both studies these effects were more evident in pulmonary compared to systemic vascular cells (22, 23). As suggested by Madden et al. (22), the likely explanation for disparities between observations made with cultured vascular cells and those obtained with intact pulmonary vasculature in vivo or ex vivo is that the results in cultured cells reflect direct effects of decreased oxygenation, whereas findings for hypoxia in intact pulmonary vasculature may reflect the effects of increased hemodynamic stress due to changes in flow and pressure (38). In the isolated rat lung, Voelkel et al. have demonstrated that attenuation of the acute pressor response to hypoxia abolishes the increase in pulmonary prostacyclin production (12). In addition, Van Grondelle et al. have shown that rat lung prostacyclin production parallels changes in vascular flow and pressure, and that monolayers of bovine pulmonary endothelial cells produce more prostacyclin when subjected to increased shear stress (39). As such, it is postulated that the enhancement in in

vitro pulmonary artery prostacyclin production observed in the experimental group was due to increased hemodynamic stress during the period of in vivo hypoxia.

Despite the marked increase in pulmonary arterial prostacyclin production, basal adenylate cyclase activity was not changed following in vivo hypoxia. This was associated with attenuated prostacyclin mediation of adenylate cyclase activity, reflected by the finding that indomethacin had less of an effect on basal and GTP-stimulated activity after hypoxia, and by the observation that exogenous prostacyclin (except at 10^{-4} M) did not increase enzyme activity in pulmonary membranes from hypoxic animals. In contrast, there was a dramatic increase in systemic artery adenylate cyclase activity despite the lack of an increase in systemic prostacyclin production. We have previously demonstrated that this may be related to changes at the level of the G proteins, and we have speculated that this may play a role in the maintenance of normal systemic vasomotor tone during hypoxia (16).

To discern the basis for the attenuated response of pulmonary artery adenylate cyclase to prostacyclin, we examined enzyme stimulation by isoproterenol and demonstrated that the response to the beta-adrenergic agonist was also diminished. This suggests that the underlying mechanism(s) entails a step that is common to the regulatory pathways for the two agonists, each involving the interaction of activated receptors with stimulatory G proteins (29). However, because we have demonstrated previously that there is no change in the response to stimulation with NaF or forskolin (16), which activate the enzyme system at the level of the G proteins and the catalytic subunit of the enzyme, respectively (29), the attenuated response to both agonists is evidently due to alterations proximal to the G proteins. We have reported a 37% decrease in beta-adrenergic receptor density after 7 d of hypoxia in the rat (16), and a similar downregulation of prostacyclin receptors may occur in association with the increased endogenous production of this agonist. Thus, decreased receptor number may partly explain the impaired in vitro response of pulmonary artery adenylate cyclase to prostacyclin after prolonged in vivo hypoxia.

Possible changes in the vasomotor responsiveness of the rat pulmonary circulation to cAMP-dependent vasodilators have been interpreted from the results of examinations of reactivity to NE. Porcelli and Bergman reported that in perfused isolated lungs, more preparations exhibited vasodilatation after 14 d of in vivo hypoxia than in controls, and they concluded that beta-adrenergic receptor activity was enhanced (40). However, alpha- and beta-adrenergic responses were not differentiated, and in all other experimental groups (7 and 28 d of hypoxia) vasodilatation was less evident after hypoxia than in controls (40). In a more recent study of main pulmonary arteries, Tozzi et al. noted decreased contractility with NE after 10 d of hypoxia, but this finding was not specific to NE and it was prevented when collagen synthesis was inhibited (18). They suggested that changes in reactivity in this model may be primarily related to the structural modifications that occur in the arteries (18). Beta-adrenergic responsiveness has been more directly examined in studies of awake catheterized rats by Fried and Reid, who demonstrated that isoproterenol attenuates acute hypoxic pulmonary vasoconstriction but has no effect on pulmonary hypertension after 14 d of hypoxia (6). Our results with isoproterenol are consistent with their findings, but it is apparent that caution is required in interpreting alterations in vasomotor responses in arteries that have undergone structural changes (18).

The potential role of vasodilatory prostaglandins in the development of chronic hypoxic pulmonary hypertension has been examined previously in this model. Rabinovitch et al. showed that continuously administered angiotensin II prevents chronic (7 d) hypoxic pulmonary hypertension and the associated pulmonary vascular changes, most likely due to its ability to stimulate PG production (8). Our findings indicate that there is increased pulmonary artery prostacyclin production and an attenuation in prostacyclin mediation of adenylate cyclase activity with prolonged hypoxia. It is postulated that this may result in diminished prostacyclin regulation of vasodilatation and VSM proliferation, contributing to the pathogenesis of pulmonary hypertension and vascular remodeling. It is also postulated that when PG synthesis is augmented even further during hypoxia with angiotensin II, local PG concentrations are enhanced sufficiently to surpass the attenuated sensitivity of adenylate cyclase, maintaining cAMP-mediated modulation of vasomotor tone and vascular cell growth and thereby preserving normal pulmonary vascular function and structure. Thus, the determinations made in the present investigation complement the findings of Rabinovitch et al. (8) and provide additional evidence that alterations in PG-mediated processes may contribute to pulmonary hypertension with prolonged hypoxia in the rat. Interpretation of the present observations should be made, however, with the understanding that in order to assess these processes, main pulmonary arteries, and not resistance arteries, were studied in vitro. Despite evidence that the response of the rat main pulmonary artery to acute hypoxia mimics hypoxic pulmonary vasoconstriction in the perfused whole lung (19), a degree of caution may be warranted in the direct extrapolation of the present findings to the in vivo condition of chronic hypoxic pulmonary hypertension.

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