Phosphodiesterase Isoforms in the Pulmonary Arterial Circulation of the Rat: Changes in Pulmonary Hypertension

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ABSTRACT

Phosphodiesterase (PDE) activity was determined in pulmonary arteries removed from control and chronic hypoxia-induced pulmonary hypertensive rats. The main, first-branch, intrapulmonary and resistance pulmonary arteries were studied. We measured total cAMP PDE activity and cGMP PDE activity, as well as that of individual isoforms (PDE1–5). cAMP PDE activity in chronic hypoxic rats was increased in first-branch and intrapulmonary arteries from hypoxic rats. No changes were observed in the main or resistance pulmonary arteries. Similarly, cGMP PDE activity was increased in the main, first-branch and intra-pulmonary arteries of the hypoxic rats. No changes in cGMP PDE activity were observed in resistance arteries. There was evidence for PDE1–5 activity in all pulmonary arteries. The increased cAMP PDE activity in first-branch and intrapulmonary vessels was associated with an increase in cilostamide-inhibited PDE (PDE3) activity. Increased total cGMP PDE in main pulmonary artery was associated with increases in Ca

PDE enzymes hydrolyze cyclic nucleotides to the corresponding 5'-nucleotide and represent the only known means whereby cells can inactivate cyclic nucleotides (Fulle and Garbers, 1996). PDE enzymes have a variety of functions, the ultimate effect of this action being functional changes within the cell (Scott, 1991). There is currently much interest in establishing the molecular details of abnormalities in cyclic nucleotide-mediated signaling in disease states, the ultimate aim being therapeutic intervention and treatment of clinical problems (Levitzki, 1996).

Cyclic nucleotides are synthesized by families of enzymes called guanylyl and adenylyl cyclases (Fulle and Garbers, 1994; Sunahara et al., 1996). PDE enzymes hydrolyze cyclic nucleotides to the corresponding 5'-nucleotide and represent the only known means whereby cells can inactivate cyclic nucleotides. Current classification of PDEs is as follows: PDE1 enzymes can hydrolyze both cAMP and cGMP, and their activity is stimulated by Ca

PDE2 enzymes also can hydrolyze both cAMP and cGMP. However, the hydrolysis of cAMP by these enzymes is stimulated by micromolar concentrations of cGMP, and these enzymes can

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ABBREVIATIONS: PDE, phosphodiesterase; EHNA, erythro-9-(2-hydroxy-3-nonyl) adenine; PHT, pulmonary hypertension; LV, left ventricle; RV, right ventricle; TV, total ventricle; PMSF, phenylmethylsulfonyl fluoride; EDRF, endothelium-derived relaxing factor; IBMX, isobutylmethylxanthine
be specifically inhibited by EHNA (Podzuweit et al., 1995; Michie et al., 1996). PDE3, PDE4 and PDE7 enzymes hydrolyze cAMP specifically. However, whereas micromolar concentrations of cGMP inhibit PDE3 activity, the activities of PDE4 and PDE7 are unaffected by such levels of cGMP. PDE3 and PDE4 isoenzymes can be specifically inhibited by cilostamide and rolipram, respectively (Bolger, 1994; Manganelli et al., 1995b). There are no known selective inhibitors for PDE7 enzymes. Indeed, PDE7 activity is even insensitive to inhibition by IBMX, a compound that is able to inhibit all other PDE classes in a nonselective fashion. PDE5 and PDE6 enzymes hydrolyze GMP specifically, and PDE5 isoenzymes are selectively inhibited by zaprinast (Gillespie and Beavo, 1989).

PDE inhibitors have become a focal point in the search to find novel vasodilators for clinical use (Cortijo et al., 1993; Torphy, 1994; Banner and Page, 1995; Giembycz, 1996; Christensen and Torphy, 1994). Inhibitors of the PDE5 isoforms, such as zaprinast and dipyridamole, are receiving increasing attention as facilitators of pulmonary arterial vasodilation (Clarke et al., 1994; Thusu et al., 1995). However, it is known that PDE isoforms 1, 3, 4 and 5 are present in human pulmonary arteries and that inhibitors of PDEs 3, 4 and 5 relax preconstricted human pulmonary arteries (Rabe et al., 1994). It is not known, however, how pulmonary vascular remodeling affects PDE isoform activity. Here we have determined the PDE isoforms present in the rat pulmonary arterial circulation in order to examine how the activity of these isoforms is altered after the development of PHT.

Materials and Methods

Chronic hypoxic rats. Male Wistar rats 28 to 30 days old (at the start of the experiment) were placed in a specially designed perspex hypobaric chamber (Royal Hallamshire Hospital Sheffield). This was depressurized, over 2 days, to 550 mbar (oxygen concentration equivalent to 10%). The temperature of the chamber was maintained at 21–22°C, and the chamber was ventilated with air at approximately 45 l/min. Rats were maintained in these hypoxic/hypobaric conditions for 2 weeks. Age-matched control animals were held in room air.

PHT was assessed by measuring the ratio of RV weight to TV weight. The RV was carefully dissected free, cleaned of the surrounding parenchyma and then incubated separately in Krebs solution for 30 min at 37°C before PDE analysis was performed.

Assay of PDE activity. Tissues were rapidly frozen in liquid N2 before being homogenized in ice-cold Krebs-bicarbonate solution (119 mM NaCl, 4.7 mM KCl, 0.6 mM MgSO4, 1.2 mM KH2PO4, 2.5 mM CaCl2, 25 mM NaHCO3 and 11.1 mM glucose). Figure 1 indicates the different regions of the pulmonary arterial bed that we studied. These were the main pulmonary artery (4–5 mm I.D.), first-branch pulmonary artery (2–3 mm I.D.), intrapulmonary arteries (0.2–2 mm I.D.) and resistance arteries (100–300 μm I.D.). These were dissected free, cleaned of the surrounding parenchyma and then incubated separately in Krebs solution for 30 min at 37°C before PDE analysis was performed.

First-branch pulmonary artery

Intrapulmonary arteries

Resistance arteries

Fig. 1. Diagrams indicating the locations of the vessels used in this study.

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been shown to act as a much more potent inhibitor of PDE2 when this enzyme is maximally stimulated by cGMP (Michie et al., 1996). This strategy has been employed before to analyze changes in PDE class activity and has been discussed in some detail previously (Spence et al., 1995; Erdogan and Houslay, 1997).

We also describe changes in PDE activity using cGMP as substrate, with Ca\(^{2+}\)/calmodulin to activate PDE1, EHNA to inhibit PDE2 and zaprinast (10 \(\mu\)M) to inhibit PDE5 selectively. We note that PDE1 activity results from expression of three genes, with isoforms that have different selectivity for cAMP and cGMP. This makes it important to analyze activities with both substrates (Beavo, 1995).

**Statistics.** Statistical comparisons of the means of groups of data were made by one-way analysis of variance (ANOVA). \(P < .05\) was considered significant. All values are shown as means \(\pm\) S.E.M.

**Drugs.** \(^3\)H-cAMP and \(^3\)H-cGMP were supplied by Amersham International, Amersham, Bucks. Activated charcoal, *Hannah ophiophagus* snake venom, dowex-1-chloride, IBMX, bovine brain calmodulin, zaprinast and BSA were supplied by Sigma Chemical Co. (Poole, UK). Rolipram was a gift from Roche (Basel, Switzerland), and EHNA was a gift from Dr. T. Podzuweit, W. G. Kerckhoff Institute, Bad Neuheim, Germany. Cilostimide was a gift from Pfizer, UK.

**Results**

**PHT.** The RV/TV ratio of the control rats was 0.247 \(\pm\) 0.006. That of the CH rats was 0.393 \(\pm\) 0.006 (\(P < .001\)), which indicated the development of severe PHT.

**Total PDE activity.** Figure 2A shows total cAMP hydrolysis by PDE enzymes. When main pulmonary arteries are analyzed, total activity is found to be comparable in control and hypoxic rats. Similarly, no significant difference between activity in control rats and that in hypoxic rats was observed in pulmonary resistance arteries. However, total cAMP PDE activity was increased by \(\sim 60\%\) in first-branch and intrapulmonary arteries from hypoxic rats.

Total cGMP hydrolysis is shown in figure 2B. PDE activity was increased by \(\sim 50\%, \sim 60\%\) and \(\sim 120\%\) in the main pulmonary artery, first-branch and intrapulmonary arteries, respectively, isolated from hypoxic rats. There was no difference in cGMP PDE activity in resistance arteries from control and hypoxic rats.

**PDE isoform activity.** Table 1 shows activity of individual cAMP PDE isoforms.

**Main pulmonary artery: Ca\(^{2+}\)/calmodulin-stimulated PDE (PDE1) activity was extremely low in control animals but increased markedly in the chronic hypoxic rats despite an absence of change in total activity. PDE3 activity was increased by 18%.

**First-branch pulmonary artery: The increase in total PDE activity in the hypoxic rats was associated with a \(\sim 92\%\) increase in cilostamide-inhibited PDE (PDE3) activity.

**Intrapulmonary artery:** The increase in total PDE activity in the hypoxic rats was associated with a 68% increase in cilostamide-inhibited PDE3 activity.

**Resistance artery:** There was no significant change in total PDE activity, but a small (33%) decrease in rolipram-inhibited PDE4 activity occurred.

**Activity in the hypoxic rats was associated with a 70% increase in zaprinast-inhibited PDE5 activity.

**Intrapulmonary artery:** The large increase in total PDE activity in the hypoxic rats was associated with a 115% increase in zaprinast-inhibited PDE5 activity.

**Resistance artery:** There was no significant change in PDE activity.

In all the vessels tested, PDE activity remaining in the presence of IBMX never exceeded 10% of the total PDE activity measured. Hence there was no evidence for PDE7 activity.

**Discussion**

There was a 60% increase in the RV/TV ratio of the chronic hypoxic rats, which indicated the development of severe PHT in the rats used in this study (see MacLean et al., 1995; Sheedy et al., 1996 for a complete characterization of this model).

A wide variety of different mechanisms for controlling cyclic nucleotide synthesis in an individual cell are now known (Bentley and Beavo, 1992). Cyclic nucleotide-mediated cellu-
observed in these vessels (MacLean et al., 1996). Here we see an increase in the smooth muscle preparation is preconstricted (Rabe et al., 1990). There was a small but significant rise in cilostamide-sensitive PDE3 activity. The increase in cilostamide-sensitive PDE3 activity underlies the changes observed here may occur through either induction or post-transcriptional modification, e.g., phosphorylation of PDE3 (Manganiello, 1995a). We have previously shown that chronic hypoxia causes a decrease in cGMP, in these vessels (MacLean et al., 1996). This may well reflect changes in the nitric oxide EDRF system occurring in the chronic hypoxic rat model of PHT. For example, a loss of Ca
calmodulin-stimulated PDE1 activity emerged when cAMP was used as substrate. In the hypoxic rats, however, there was evidence of Ca++/calmodulin-stimulated PDE1 activity when cAMP was used as substrate. Because this PDE activity is activated by Ca++, one might expect the increase in expression noted here to serve to regulate cAMP levels only when appropriately stimulated by Ca+++. Ca++ levels are tightly regulated in cells and are usually only transiently elevated, so it is unlikely that this will lead to any chronic increase in cAMP levels. Little is known about the regulation of PDE1 activity. The increased activity we observed in the hypoxic rat vessels may be due to some post-translational change (Beavo, 1995). It may also be due to induction; it has been shown that PDE1 activity can be rapidly but transiently induced through the activation of specific protein kinase C isoforms (Spence et al., 1995).

**First-branch pulmonary artery.** There was an increase in cAMP PDE activity in first-branch vessels from chronic hypoxic rats. This appears to be accounted for by a profound increase in cilostamide-sensitive (PDE3) activity. The changes observed here may occur through either induction or post-transcriptional modification, e.g., phosphorylation of PDE3 (Manganiello, 1995a). We have previously shown that chronic exposure to hypoxia caused a decrease in [cGMP], in these vessels (MacLean et al., 1996). This may well reflect changes in the nitric oxide EDRF system occurring in the chronic hypoxic rat model of PHT. For example, a loss of EDRF activity in pulmonary arteries from hypoxic rats has been described (Rodman et al., 1990). Shaul et al. (1993) also demonstrated that hypoxia attenuates endothelial nitric oxide production in rat pulmonary arteries. In human pulmo-
nary arteries of all sizes removed post mortem from patients with severe primary PHT, there is reduced expression of endothelial nitric oxide synthase (Giand and Saleh, 1995). Other studies, however, suggest that there may be increased nitric oxide production in the chronic hypoxic rat lung (Isaacson et al., 1994; Xue et al., 1994). Our study suggests that the decreased [cGMP], we observed was due, at least in part, to an increase in cGMP PDE activity.

The increase in zaprinast-inhibited PDE5 activity in this model of PHT is consistent with the suggestion that PDE5 inhibitors may be of use as a treatment in cases of PHT. Evidence from animal models certainly implies that regulation of PDE5 activity may be relevant to the clinical therapy of PHT. Selective inhibitors of the PDE5 family have been shown to decrease the pulmonary arterial pressure in newborn lambs (Braner et al., 1993; Clarke et al., 1994). Zaprinast has also been used to enhance the vasodilator effect of inhaled nitric oxide in experimental PHT in lambs (Ichinose et al., 1995; Thusu et al., 1995). Hence cGMP-specific PDE plays a role in the regulation of pulmonary vascular tone, and PDE5 inhibitors can vasodilate the pulmonary vasculature even in the absence of increased endogenous PDE5 activity or PHT. An increase in PDE5 activity provides a further explanation of the effectiveness of PDE5 inhibitors as pulmonary vasodilators in animal models of PHT.

Intrapulmonary arteries. An increased cAMP PDE activity was observed, and it was related to an increase in the activity of cilostamide-sensitive PDE3. An increase in cGMP PDE activity was also observed, and this was related to an increase in zaprinast-inhibited PDE5 activity.

Pulmonary resistance arteries. Our previous studies showed that exposure to chronic hypoxia had no net effect on [cAMP], or [cGMP], (MacLean et al., 1996). Correspondingly, in this study, there was no change in total PDE activities despite a small decrease in rolipram-inhibited PDE4 that was probably offset by a small, though not significant, increase in PDE1 activity. Our previous failure to observe changes in [cGMP], in the resistance arteries was surprising in light of evidence that suggests increases in nitric oxide release in chronic hypoxic rat lungs and increased nitric oxide synthase activity in the small pulmonary arterioles (Isaacson et al., 1994; Xue et al., 1994). However, there is evidence to suggest that there may be differential control of basal nitric oxide release and agonist-stimulated release from the pulmonary resistance vessels (Cremona et al., 1994), so changes in [cGMP], may reflect net changes in both of these systems.

The functional consequence of an increase in PDE activity, and of the corresponding decrease in cAMP and cGMP, is likely to be an increase in endogenous pulmonary arterial tone and an increase in vasoconstriction in response to some critical vasoconstrictors such as 5-hydroxytryptamine and endothelin-1. Indeed, both of these functional changes are observed in these pulmonary hypertensive rats, where vascular tone of the large pulmonary arteries is profoundly increased (MacLean et al., 1995; MacLean et al., 1996). In elastic pulmonary arteries, we have shown that inhibition of endogenous nitric oxide synthase causes an increase in endogenous tone that is most marked in rats with PHT. This is also consistent with an elevation of endogenous tone in these rats (MacLean et al., 1995). We have been studying the effect of changes in cyclic nucleotide levels on pulmonary vascular tone in bovine pulmonary arteries and have shown that, indeed, increased cAMP and cGMP causes a decrease in vascular tone, whereas a decrease in cGMP and cAMP causes increased pulmonary vascular tone (Sweeney et al., 1995; MacLean et al., 1994a). These previous results, together with those of the present study, suggest that an increase PDE activity causes a decrease in cAMP and cGMP in the pulmonary vascular smooth muscle, which causes an increase in endogenous tone. An increased tone, in turn, increases pulmonary vascular reactivity to critical vasoconstrictors.

The results emphasize major differences in physiology between the resistance arteries and the larger pulmonary arteries. Indeed, we have shown this to be the case in other studies where responses to vasoactive agents, such as endothelin-1, differ depending on the size of the vessel studied (MacLean et al., 1994b). Main pulmonary arteries and first branches are more elastic in nature, and the smooth muscle is of a different phenotype from that in the resistance vessels. The intrapulmonary vessels used in our study can be equated with the elastic vessels within the lung, as described by Sasaki et al. (1995). The smaller resistance pulmonary arteries are more muscular and are distinguished from the larger, more elastic arteries by a paucity of extracellular matrix and by smooth muscle cells that contain better-developed microfilament bundles (Sasaki et al., 1995). Structural differences in smooth muscle cells and in extracellular matrix in the media between the elastic and muscular arteries may reflect the functional heterogeneity of pulmonary arteries in response to hypoxic pulmonary vasoconstriction. There is remodeling of pulmonary arteries in PHT. There is pronounced medial thickening in the large pulmonary arteries from pulmonary hypertensive rats because of hyperplasia of smooth muscle fibers situated between the elastic laminae (Heath et al., 1973). This may account for some of the changes observed, although we measured PDE activity as pmol min\(^{-1}\) mg\(^{-1}\) to control for changes in the amount of tissue.

Although rolipram-inhibited PDE4 activity was present in all the pulmonary arteries, there was little evidence for changes induced by exposure to chronic hypoxia, except for a small decrease in activity in the resistance arteries. Intriguingly, it is PDE4 isozyme activity that is thought to be prevalent in many inflammatory cells involved in asthma. PDE3 and PDE4 isozymes have been characterized in a number of inflammatory cells. The nonselective PDE inhibitor theophylline and selective PDE4 inhibitors can modify allergic inflammation in animal models of asthma and in clinical asthma; this may be due to their combined bronchodilatory and anti-inflammatory effects (Torphy, 1994; Banner and Page, 1995; Giembycz, 1996; Tenor et al., 1996). Hence, selective PDE inhibitors can be expected to affect pulmonary arteries and airways differentially, and this property may be of therapeutic value. PDE inhibitors can be envisaged combating pathophysiological defects central to the pathogenesis of PHT, where abnormal cyclic nucleotide-mediated cell signaling is involved.

In conclusion, we previously observed decreases in intracellular cyclic nucleotide levels in pulmonary arteries from pulmonary hypertensive rats. This study indicates that these changes are associated with increased PDE1, 3, and 5 activity and that there were regional differences in these changes that were confined to the elastic pulmonary arteries.
References