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The NADPH Oxidase Inhibitors Iodonium Diphenyl and Cadmium Sulphate Inhibit Hypoxic Pulmonary Vasoconstriction in Isolated Rat Pulmonary Arteries

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Summary

Interest surrounds the role of an NADPH oxidase-like enzyme in hypoxic pulmonary vasoconstriction (HPV). We have studied the effects of the NADPH oxidase inhibitors iodonium diphenyl (ID) and cadmium sulphate ($CdSO_4$) upon HPV of isolated rat pulmonary arteries (n = 73, internal diameter 545±23 μm). Vessels were preconstricted with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, 0.5 or 5 μM) prior to a hypoxic challenge. ID (10 or 50 μM), $CdSO_4$ (100 μM) or vehicle (50 μl) was added for 30 min before re-exposure to $PGF_{2\alpha}$ and hypoxia. ID and $CdSO_4$ significantly inhibited HPV. In vessels preconstricted with 5 μM $PGF_{2\alpha}$, ID (10 and 50 μM) reduced HPV from 37.4±5.6 % to 9.67±4.4 % of the contractile response elicited by 80 mM KCl ($P<0.05$) and from 30.1±5.0 % to 0.63±0.6% 80 mM KCl response ($P<0.01$), respectively. $CdSO_4$ (100 μM) reduced HPV from 29.4±4.0 % to 17.1±2.2% 80 mM KCl response ($P<0.05$). In vessels preconstricted with 0.5 μM $PGF_{2\alpha}$, ID (10 and 50 μM) reduced HPV from 16.0±3.15% to 3.36±1.44 % 80 mM KCl response ($P<0.01$) and from 15.0±1.67 % to 2.82±1.40 % 80 mM KCl response ($P<0.001$), respectively. Constriction to $PGF_{2\alpha}$ was potentiated by ID. ID and $CdSO_4$, at concentrations previously shown to inhibit neutrophil NADPH oxidase, attenuate HPV in isolated rat pulmonary arteries. This suggests that an NADPH oxidase-like enzyme is involved in HPV and could act as the pulmonary oxygen sensor.

Key words

NADPH oxidase • Hypoxic pulmonary vasoconstriction • Iodonium diphenyl cadmium sulphate • Isolated rat pulmonary artery

Introduction

Hypoxic pulmonary vasoconstriction (HPV) is the mechanism by which perfusion is matched with ventilation in the lung. It is an essential regulatory function, ensuring that a low alveolar oxygen tension is not converted into a low arterial oxygen tension, thus avoiding systemic tissue hypoxemia. In addition HPV ensures constriction of the fetal pulmonary vasculature, and its reversal enables conversion to the perfused state at birth. Failure of HPV can be responsible for the ventilation-perfusion mismatch, which occurs in numerous diseases. Under pathophysiological conditions characterized by global alveolar hypoxia, sustained

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pulmonary vasoconstriction may contribute to vascular remodeling and development of pulmonary hypertension.

HPV was first reported in 1946 (von Euler and Liljestrand) and since then the underlying mechanism has remained elusive. Although numerous endogenous vasoactive agents have been considered as mediators of HPV, it now seems likely that such agents merely modulate the response, since hypoxic responses can be obtained in isolated pulmonary artery smooth muscle cells in culture (Zhang *et al.* 1997). This provides clear evidence that the whole process of HPV, from hypoxic sensing through to smooth muscle contraction, is intrinsic to these cells.

A reduction in the production of reactive oxygen species (ROS) under hypoxia has been suggested as a potential effector mechanism of HPV (Archer *et al.* 1989a). ROS have been shown to be produced in the isolated rat lung, and that their generation is markedly reduced under acute hypoxic conditions (Archer *et al.* 1993). This reduction in ROS production under hypoxia has been proposed to regulate pulmonary arterial tone *via* two mechanisms. Firstly the redox hypothesis (Archer *et al.* 1986, 1993) suggests that varying the production of ROS alters the redox potential of the pulmonary arterial smooth muscle cell, thereby controlling the function of membranous potassium channels which contain conformationally important, redox-sensitive cysteine residues (Ruppersburg *et al.* 1991). Indeed reducing agents and hypoxia decrease, whereas H_2O_2 and oxidizing agents increase, the open probability of both voltage-gated and calcium-dependent potassium channels in pulmonary arterial smooth muscle (Post *et al.* 1993, Yuan *et al.* 1994, Reeve *et al.* 1995, Park *et al.* 1995). Secondly, ROS such as hydrogen peroxide (H_2O_2) have been shown to elicit pulmonary arterial vasodilatation at physiological concentrations (Burke and Wolin 1987). Consequently, under hypoxic conditions a reduction in ROS production would result in either a reduction in potassium channel function or vasodilatory control triggering contraction. Both hypotheses are plausible transduction mechanisms for HPV, but the identity of the source of the ROS production – effectively the oxygen sensor, is unclear. Both the mitochondrial electron transport chain (Archer *et al.* 1993) and an NADH oxidoreductase (Mohazzabh and Wolin 1994) have been proposed.

In contrast an alternative mechanism for HPV has been suggested, proposing the enzyme NADPH oxidase as the pulmonary oxygen sensor. Marshall *et al.* (1996) identified a NADPH oxidase similar to that found in neutrophils in cultured pulmonary artery smooth muscle cells. In the same paper they also demonstrated that, in contrast to whole lung preparations, ROS production is increased in these cells under hypoxic conditions. Furthermore, generation of ROS could be inhibited by the NADPH oxidase inhibitor diphenyleneiodonium (DPI). Since ROS have previously been demonstrated by numerous groups to elicit pulmonary arterial smooth muscle contraction (Rhoades *et al.* 1990, Chakraborti and Chakraborti 1995, Sheehan *et al.* 1993, Archer *et al.* 1995, Jones *et al.* 1997, Jin and Rhoades 1997), an alternative mechanism for HPV was proposed: NADPH oxidase is activated under hypoxia triggering production of vasoconstrictive ROS. This second hypothesis is also supported by observations that DPI inhibits HPV in numerous preparations including isolated rat pulmonary arteries (Thompson *et al.* 1998), the isolated perfused rat lung (Thomas *et al.* 1991) and the isolated perfused rabbit lung (Grimminger *et al.* 1995).

However, DPI is a poor pharmacological tool as it is non-specific, inhibiting both Ca^{2+} and K^+ currents (Weir *et al.* 1994) and endothelium-dependent vasodilation (Wang *et al.* 1993), although distinct separation between such co-effects and NADPH oxidase inhibition with DPI has been demonstrated (Thomas *et al.* 1991, Grimminger *et al.* 1995). In addition, DPI is insoluble in water and our evidence suggests that the use of the vehicle DMSO may also alter hypoxic responses (Thompson *et al.* 1998). Consequently, this may be overcome by the use of the water soluble analogue of DPI – iodonium diphenyl (ID). Iodonium compounds inhibit NADPH oxidase by hydrolyzing to the NADPH binding site at the flavoprotein-FAD complex of the enzyme (O'Donnell *et al.* 1993). An alternative method of inhibiting NADPH oxidase is provided by cadmium and zinc ions which, at concentrations of 0.1-2 mM, reversibly inhibit proton conductance through the proton channel of the enzyme (Demaurex *et al.* 1993, DeCoursey and Cherney 1993). Channel blockade results in attenuation of superoxide generation since oxidase activity is tightly coupled to the proton efflux *via* this route (Henderson and Chappell 1996). Consequently, such agents provide a novel mechanism of NADPH oxidase inhibition. The sites of action of these various NADPH oxidase inhibitors are shown in Figure 1.

The aim of the present study is to clarify the role of NADPH oxidase in HPV utilizing the alternative

inhibitors ID and cadmium sulphate ($CdSO_4$). In our hands the response of isolated rat pulmonary arteries to hypoxia is characterized by four clear phases (Woodmansey *et al.* 1993, Zhang *et al.* 1995) which are greatly increased in magnitude once the vessels have been primed with a suitable agonist (Hoshino *et al.* 1988, Zhang *et al.* 1995). Following constriction to the agonist, hypoxic stress results in an initial small vasodilation (Phase 1), followed by a large hypoxic contraction which occurs for approximately 5 min (Phase 2) before further vasodilation (Phase 3). Eventually a second smaller sustained hypoxic contraction develops, after approximately one hour (Phase 4) (Fig. 2). Phase 2 is considered to be the physiologically relevant contraction occurring over the hypoxic oxygen tension range (Teng and Barer 1995). Consequently, the effects of ID and $CdSO_4$ were studied upon this phase of the hypoxic response.

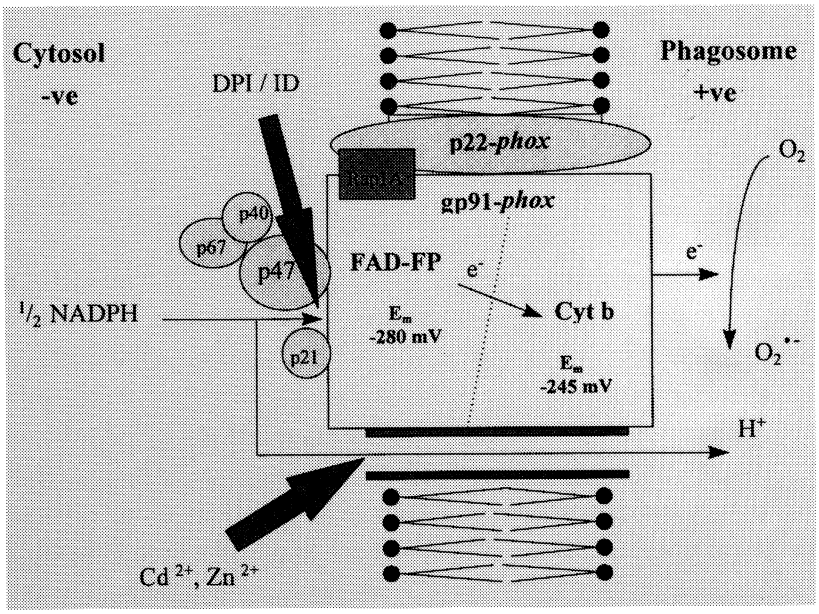

Fig. 1. *Diagrammatic representation of neutrophil NADPH oxidase showing the route of passage for the protons (H^+) and electrons (e^-) generated from the oxidation of NADPH. The enzyme consists of an α and β subunit (p22-phox and gp91-phox, respectively), the latter containing a flavoprotein and FAD moiety (FAD-FP), cytochrome b_{558} (Cyt b) and an associated proton channel. Full activity of the enzyme requires the translocation of polypeptide activating factors p67-phox (p67), p47-phox (p47) and p40-phox (p40) and the G proteins $p21^{rac1/2}$ (p21) and Rap1A. Inhibitors of the enzyme complex include the iodonium compounds diphenyleneiodonium (DPI) and iodonium diphenyl (ID)which competes with NADPH for binding at the flavoprotein-FAD complex, and cadmium and zinc ions (Cd^{2+}, Zn^{2+}) which reversibly block the proton channel.*

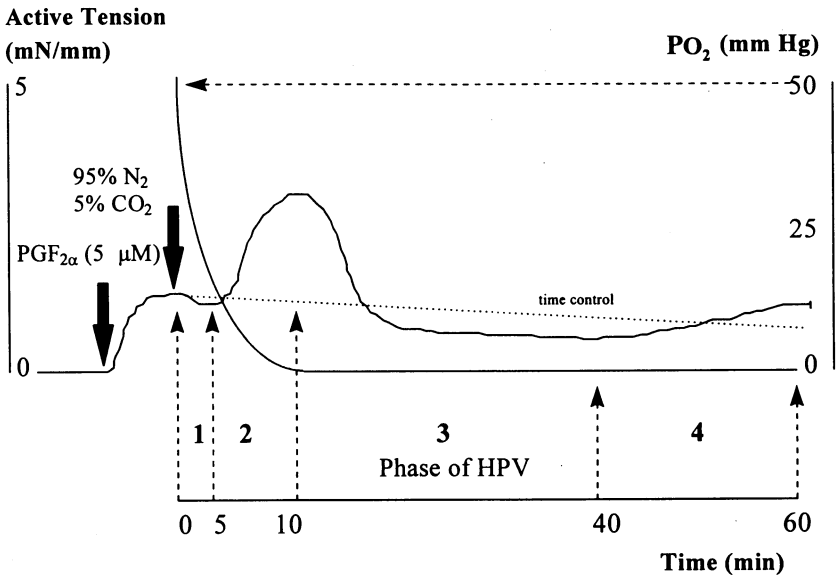

Fig. 2. *Schematic representation of the four phase response of HPV in isolated rat pulmonary arteries mounted in a wire myograph, following priming with $PGF_{2\alpha}$ (5 μM). The time-scale and corresponding PO_2 values are shown.*

Methods

Male Wistar rats (n = 38, mean body weight 242±8 g) were anesthetized by intraperitoneal injection of sodium pentobarbitone (15 mg/100 g body weight), the chest was opened and the lungs were removed and placed in physiological saline solution (PSS). Endothelial intact pulmonary arteries (n = 73, mean internal diameter 545±23 μm) from the transitional elastic segment (Sasaki *et al.* 1995) were carefully dissected from the lungs and mounted on two 40 μm stainless steel wires in the jaws of an automated wire myograph (Cambustion UK, Ltd) (Rogers *et al.* 1992). Vessel characteristics were obtained from length-tension plots which were stored within the myograph software and on the basis of these, individual arteries were loaded to a resting tension of 17.5 mm Hg, equivalent to the *in vivo* pressure. Vessels were washed by three changes of the PSS in the myograph bath, which was bubbled continuously with 95% O_2 / 5% CO_2, and left to equilibrate for 1 h.

Following equilibration, vessels were exposed to 80 mM potassium chloride (KCl) until a maximal contraction had been produced, the PSS changed three times and the vessels allowed to relax back to baseline. Vessels were then re-exposed to 80 mM KCl and the average of these two contractions recorded. Subsequent contractions were standardized *via* expression as a percentage of these KCl responses. After washing and the original baseline tension regained, vessels were exposed to acetylcholine (10 μM) following preconstriction with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, 100 μM) to confirm endothelial integrity prior to one of four protocols being undertaken.

Protocol 1 –Effect of iodonium diphenyl upon HPV

Pulmonary arteries (n = 16) were exposed to a priming concentration of $PGF_{2\alpha}$ (5 μM). After a maximal tension had been produced to this concentration, the myograph bath was sealed and the gas bubbling through it changed to 95% N_2 / 5% CO_2. Once Phase 2 of the hypoxic response had been produced, the vessels were washed by three changes of PSS and the original gas applied. Once the resting baseline tension had been regained, vessels were exposed to either ID (10 or 50 μM) (n = 6) or vehicle (distilled water 50 μl) (n = 4) for 30 min before repetition of the priming concentration of $PGF_{2\alpha}$ and application of the test gas mixture.

Protocol 2 –Effect of iodonium diphenyl upon HPV at a lower level of preconstriction

Pulmonary arteries (n = 22) were exposed to a priming concentration of $PGF_{2\alpha}$ (0.5 μM). After a maximal tension had been produced to this concentration, the myograph bath was sealed and the gas bubbling through it changed to 95% N_2 / 5% CO_2. Once Phase 2 of the hypoxic response had been produced, the vessels were washed by three changes of PSS and the original gas applied. Once the resting baseline tension had been regained, vessels were exposed to either ID (10 or 50 μM) (n = 9) or vehicle (distilled water 50 μl) (n = 4) for 30 min before repetition of the priming concentration of $PGF_{2\alpha}$ and application of the test gas mixture.

Protocol 3 –Effect of iodonium diphenyl upon HPV in vessels preconstricted to the same level

Pulmonary arteries (n = 12) were exposed to a priming concentration of $PGF_{2\alpha}$ (5 μM). After a maximal tension had been produced to this concentration, the myograph bath was sealed and the gas bubbling through it changed to 95% N_2 / 5% CO_2. Once Phase 2 of the hypoxic response had been produced, the vessels were washed by three changes of PSS and the original gas applied. Once the resting baseline tension had been regained, each vessel was exposed to ID (50 μM) for 30 min. After this period $PGF_{2\alpha}$ was added cumulatively until the same level of preconstriction as seen in the control contractions was produced. The test gas mixture; 95% N_2 / 5% CO_2 was then reapplied to produce the test hypoxic contraction.

Protocol 4 –Effect of cadmium sulphate upon HPV

Pulmonary arteries (n = 24) were exposed to a priming $PGF_{2\alpha}$ concentration (5 μM). After a maximal tension had been produced to this concentration, the myograph bath was sealed and the gas bubbling through it changed to 95% N_2 / 5% CO_2. Once Phase 2 of the hypoxic response had been produced, the vessels were washed by three changes of PSS and the original gas applied. Once the resting baseline tension had been regained, vessels were exposed to either $CdSO_4$ (10 or 100 μM) (n = 8) or vehicle (distilled water 50 μl) (n = 8) for 30 min before repetition of the priming concentration of $PGF_{2\alpha}$ and application of the test gas mixture.

Solutions and drugs

PSS consisted of (in mM); NaCl 120, KCl 4.7, $MgSO_4$ 1.17, $NaHCO_3$ 25, KH_2PO_4 1.18, glucose 5.5, $CaCl_2$ 2.5 and 26.9 μM EDTA dissolved in distilled water. All PSS reagents were obtained from Sigma, UK.

$PGF_{2\alpha}$ was obtained from Pharmacia & Upjohn Ltd. UK, KCl from BDH Laboratory Supplies, Poole and $CdSO_4$ from Sigma, UK. ID was obtained from Lancaster Synthesis Ltd, Lancashire. All were diluted to the required concentration in distilled water.

Statistical analysis

Values are expressed as mean±S.E.M. and were compared using the Mann-Whitney-Wilcoxon test or by Student's paired or unpaired t test where appropriate. Significance was assumed with values of $P<0.05$.

Table 1. The effect of ID (0, 10 or 50 μM) upon contractions to $PGF_{2\alpha}$ (5 μM) and HPV (Phase 2) in vessels used in Protocol 1.

Drug	$PGF_{2\alpha}$ (5 μM) pre ID (% contraction)	$PGF_{2\alpha}$ (5 μM) post ID (% contraction)	HPV (Phase 2) pre ID (% contraction)	HPV (Phase 2) post ID (% contraction)
Vehicle	57.8±6.0	50.0±7.7	24.3±2.6	29.6±2.2
ID (10 μM)	33.1±6.9	64.8±7.7*	37.4±5.6	9.67±4.4*
ID (50 μM)	43.9±6.8	94.0±9.5*	30.1±5.0	0.63±0.6**

*Data are mean ± S.E.M. Significantly different from vehicle: * $P<0.05$, ** $P<0.01$ (Mann-Whitney-Wilcoxon test).*

Table 2. The effect of ID (0, 10 or 50 μM) upon contractions to $PGF_{2\alpha}$ (0.5 μM) and HPV (Phase 2) in vessels used in Protocol 2.

Drug	$PGF_{2\alpha}$ (0.5 μM) pre ID (% contraction)	$PGF_{2\alpha}$ (0.5 μM) post ID (% contraction)	HPV (Phase 2) pre ID (% contraction)	HPV (Phase 2) post ID (% contraction)
Vehicle	7.55±1.12	7.55±1.01	9.10±2.14	6.75±0.81
ID (10 μM)	4.02±0.70	25.6±7.24**	16.0±3.15	3.36±1.44**
ID (50 μM)	5.99±0.47	22.7±7.14*	15.0±1.67	2.82±1.40***

Data are mean ± S.E.M. Significantly different from vehicle: * $P<0.05$, ** $P<0.01$, *** $P<0.001$ (Mann-Whitney-Wilcoxon test).

Results

No significant differences were seen between the responsiveness of any group of vessels studied. Mean response to KCl was 2.78±0.20 mN/mm vessel diameter. The 30 min incubation period with either ID or $CdSO_4$ had no effect on pulmonary artery baseline tension at any concentration.

In vessels used in Protocol 1 which were preconstricted with 5 μM $PGF_{2\alpha}$ prior to exposure to hypoxia, ID (10 and 50 μM) potentiated the response to $PGF_{2\alpha}$ and inhibited HPV (Phase 2) (Table 1). Percentage inhibition following ID (10 and 50 μM) was 74 % and 98 %, respectively. Similar results were seen in vessels used in Protocol 2; ID (10 and 50 μM) potentiated the response to 0.5 μM $PGF_{2\alpha}$ and again inhibited HPV

(Phase 2) (Table 2). Percentage inhibition following ID (10 and 50 μM) in this second series of experiments were 79 % and 81 %, respectively.

In Protocol 3 initial exposure to $PGF_{2\alpha}$ (5 μM) produced a contraction of 21.9±4.6 % 80 mM KCl response and the Phase 2 hypoxic contraction was 26.9±3.3 % 80 mM KCl response. A similar level of preconstriction (20.7±2.2 % 80 mM KCl response) was produced by addition of 1.42±0.15 μM $PGF_{2\alpha}$ following administration of 50 μM ID. The subsequent Phase 2 hypoxic contraction was reduced to 1.58±0.69 % 80 mM KCl response ($P<0.001$) (Fig. 3) constituting 92 % inhibition.

In Protocol 4 specific attenuation of HPV (Phase 2) occurred after treatment with $CdSO_4$ (100 μM). Percentage inhibition following $CdSO_4$ (100 μM) was 42 %. The response to $PGF_{2\alpha}$ (5 μM) was unaffected (Table 3).

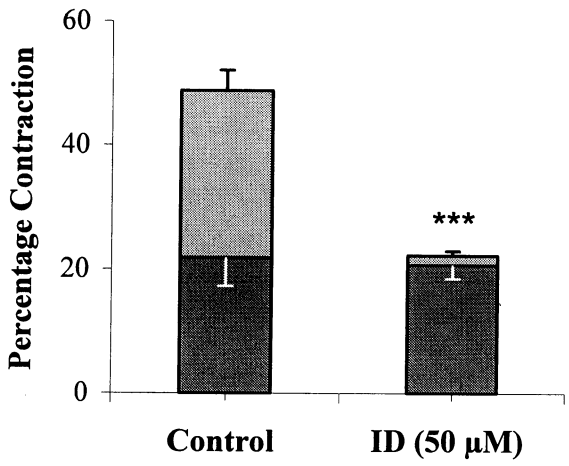

Fig. 3. *The responses to $PGF_{2\alpha}$ (dark shading) and to hypoxia (light shading) before and after 50 μM ID. Control constriction to $PGF_{2\alpha}$ was with 5 μM, test preconstriction was with 1.42 μM. Responses are as percentage of contraction to 80 mM KCl ± S.E.M. *** $P<0.001$ (Mann-Whitney Wilcoxon rank test).*

Table 3. The effect of $CdSO_4$ (0, 10 or 100 μM) upon contractions to $PGF_{2\alpha}$ (5 μM) and HPV (Phase 2) in vessels used in Protocol 4.

Drug	**$PGF_{2\alpha}$ (0.5 μM) pre $CdSO_4$ (% contraction)**	**$PGF_{2\alpha}$ (0.5 μM) post $CdSO_4$ (% contraction)**	**HPV (Phase 2) pre $CdSO_4$ (% contraction)**	**HPV (Phase 2) post $CdSO_4$ (% contraction)**
Vehicle	31.1±4.2	30.5±3.2	24.5±2.3	21.1±2.4
$CdSO_4$ (10 μM)	39.1±1.9	41.1±3.8	21.9±3.3	18.3±4.1
$CdSO_4$ (100 μM)	34.4±4.0	31.9±4.0	29.4±4.0	17.1±2.2*

*Data are mean ± S.E.M. Significantly different from vehicle: * $P<0.05$ (Mann-Whitney-Wilcoxon test).*

Discussion

This work demonstrates that the NADPH oxidase inhibitors ID and $CdSO_4$ attenuate HPV in isolated rat pulmonary arteries, providing strong evidence for the involvement of an NADPH oxidase in the generation of this response. ID has a dual action also potentiating contractions to $PGF_{2\alpha}$ but $CdSO_4$, a blocker of the NADPH oxidase-associated proton channel, selectively inhibited HPV. We believe this to be one of few studies to demonstrate specific inhibition of HPV by modulators of NADPH oxidase function and the first to demonstrate efficacy by this novel approach.

The initial results of this study (Protocol 1) showed ID (10 and 50 μM) to inhibit Phase 2 of HPV in isolated rat pulmonary arteries preconstricted with $PGF_{2\alpha}$ (5 μM). However following exposure to ID (50 μM), contraction to $PGF_{2\alpha}$ (5 μM) was also potentiated, to almost 100 % of the initial mean 80 mM KCl contraction. Consequently, this degree of preconstriction could be responsible for the subsequent reduction in Phase 2 of HPV and not ID itself, since the vessels could now theoretically be maximally contracted. Consequently, this protocol was repeated using a lower precontractile concentration of $PGF_{2\alpha}$ (0.5 μM) which would not result in the same maximal potentiation (Protocol 2).

Results from this second set of experiments were comparable to the original data, except that the responses were smaller and no longer approached the maximal contractile potential of the vessels. Thus we could now conclude that the reduction in HPV was caused by administration of ID and not due to the vessels being maximally contracted. However, since ID also potentiated the response to $PGF_{2\alpha}$, we were concerned that the increase in tension following administration of the second dose of $PGF_{2\alpha}$ was causing an inhibition of HPV. We feel this to be an unlikely event, since it can be seen by comparison of these two experiments that hypoxia-induced constriction *in vitro* is proportional to the level of preconstriction. This is best highlighted in Figure 4 where HPV following the ID-induced potentiation of the response to $PGF_{2\alpha}$ (0.5 μM) is clearly lower than the HPV which occurs following preconstriction to a similar level in the absence of ID. Consequently, the observed inhibition of HPV by ID would appear to be a characteristic of the drug. Furthermore, ID would appear to have a potent action since it inhibits HPV from a potentiated primary tension which would be expected to produce a similarly potentiated hypoxic contraction (Fig. 4). However, HPV of rat pulmonary arteries was recently reported to be a function of the stretch tension (Ozaki *et al.* 1998) and therefore the change in primary tone could account for the subsequent inhibition of HPV.

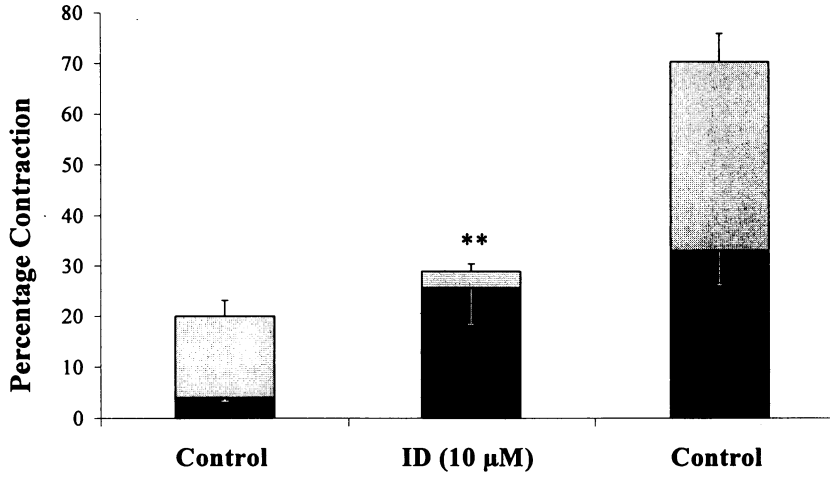

Fig. 4. *The responses to $PGF_{2\alpha}$ (dark shading) and to hypoxia (light shading). The centre bar demonstrates the dual effect of ID (10 μM), potentiating contraction to $PGF_{2\alpha}$ (0.5 μM) and attenuating HPV (Phase 2) compared to untreated control on the left (P<0.01). This elevation in primary tone following exposure to ID (10 μM) has no inhibitory effect on HPV as compared to the control bar on the right. Here preconstriction with $PGF_{2\alpha}$ (5 μM) produces a similar level of preconstriction to that produced by $PGF_{2\alpha}$ (0.5 μM) following ID (10 μM), but the hypoxic contraction is markedly higher (P<0.01). Hence attenuation of HPV is attributed to the inhibitory action of ID. Responses are expressed as percentage of contraction to 80 mM KCl ± S.E.M. ** = P<0.01 (Mann-Whitney Wilcoxon rank test).*

The third set of experiments (Protocol 3) further addressed the effect of varying the preconstriction on HPV. Following control responses to $PGF_{2\alpha}$ (5 μM) and hypoxia, administration of ID (50 μM) was followed by cumulative addition of $PGF_{2\alpha}$ to yield a similar preconstriction level, before re-exposure to hypoxia. A highly significant inhibition of Phase 2 of HPV was seen from this similar level of preconstriction, thus providing further evidence that the NADPH inhibitor ID attenuates HPV *in vitro*.

In Protocol 4 application of $CdSO_4$ resulted in specific attenuation of HPV at concentrations similar to those previously reported to elicit NADPH oxidase proton channel inhibition and attenuation of ROS production (Demaurex *et al.* 1993, DeCoursey and Cherney 1993). These findings further substantiates the theory that inhibition of ROS generation *via* an NADPH oxidase results in disruption of HPV, implying this process to be integral to the oxygen sensing process of the pulmonary circulation.

NADPH oxidase was originally described in neutrophils, as the enzyme responsible for the bactericidal production of superoxide radicals into the phagosome (Fig. 1). Neutrophil NADPH oxidase is a multi-subunit enzyme, consisting of a membrane spanning cytochrome b (β-subunit – gp91*phox*, α-subunit – p22*phox*) and adjacent proteins p40*phox*, p47*phox*, p67*phox* and $p21^{rac}$. Also present are a flavoprotein and FAD moiety and an associated H^+ channel (Henderson and Chappell 1996). Cytochrome b is an electron acceptor, which has an atypically low redox potential (–245 mV), making it suitable for the reduction of

oxygen into superoxide. The function of the cytosolic factors has yet to be fully identified, although it is clear that they do play an important role in activation of the oxidase. Involvement of a flavin moiety is well established. FAD has been shown to increase, and analogues to reduce the activity of NADPH oxidase (Parkinson and Gabig 1988). Evidence suggests that DPI inhibits NADPH oxidase by hydrolyzing to the NADPH binding site at the flavoprotein-FAD complex of the enzyme (O'Donnell *et al.* 1993).

Previous work by our group has shown DPI to inhibit HPV in isolated rat pulmonary arteries, although the solvent DMSO also produced a similar effect (Thompson *et al.* 1998). The difference between the DPI- and DMSO-mediated inhibition however indicated a DPI-sensitive portion. Thomas *et al.* (1991) showed 1 μM DPI to significantly lower and 4 μM DPI to completely inhibit HPV in the isolated perfused rat lung. This was shown to be relatively specific since contractions to the thromboxane analogue U46619 were unaffected. Contractions to angiotensin II were, however, also inhibited by 4 μM DPI although the IC_{50} of DPI for angiotensin II was more than twice that for hypoxia. Grimminger *et al.* (1995) also showed DPI to inhibit HPV in the isolated perfused rabbit lung, but at lower doses DPI actually increased HPV *via* inhibition of nitric oxide production, a phenomenon previously reported at high concentrations of DPI (Wang *et al.* 1993). Higher concentrations of DPI have also, however, been shown to inhibit Ca^{2+} and K^+ currents (Weir *et al.* 1994).

The problems associated with the lack of specificity of DPI have only been partially solved by the use of the water soluble analogue ID. Despite showing potent inhibition of HPV following administration of 10 and 50 μM ID we have also demonstrated equally potent, albeit opposite effects, on $PGF_{2\alpha}$ responses. The mechanism by which ID sensitizes the $PGF_{2\alpha}$ response is likely to be *via* nitric oxide synthase (NOS) inhibition previously reported as side effect of DPI (Wang *et al.* 1993, Grimminger *et al.* 1995). NOS inhibition would remove the endogenous vasodilatory control which prevents excessive constriction, thus subsequent contractions would be elevated. ID-mediated inhibition of HPV is unlikely to be caused by NOS inhibition since this would also elevate hypoxic contractions. Indeed NOS inhibition has been shown in a number of cases to enhance HPV in isolated rat, pig and human pulmonary arteries (Archer *et al.* 1989b, Ogata *et al.* 1992, Ohe *et al.* 1992) and in the isolated perfused rat lung (Archer *et al.* 1989b, Robertson *et al.* 1990). Further work from our laboratory using similar vessels in an identical preparation to this study, clearly demonstrated that NOS inhibition by L-NAME has no effect on any phase of HPV but does potentiate $PGF_{2\alpha}$ preconstriction (Jones and Morice 1998). The fact that HPV was not elevated also highlights the mechanistic differences between regulation of hypoxia- and $PGF_{2\alpha}$-mediated contractions.

Specific, albeit less marked, inhibition occurred following treatment with the NADPH oxidase proton channel blocker $CdSO_4$. However, the attenuation of HPV by $CdSO_4$ still constituted a 42% inhibition, being reduced from 29.4 % to 17.1 % 80 mM KCl response.

In the neutrophils, inhibition of the NADPH oxidase proton channel results in attenuation of ROS production with proton release being blocked and the oxidation of NADPH terminated (Henderson *et al.* 1987). The specificity of action for such compounds against HPV may portray an optimal mechanism of inhibiting ROS generation *via* an NADPH oxidase, or alternatively may represent inhibition of NADPH oxidase, which is not associated with the co-effects of iodonium compounds.

In conclusion, this study supports the hypothesis of Marshall *et al.* (1996) that a gp91*phox*-containing NADPH oxidase similar to that found in neutrophils acts as an oxygen sensor in the pulmonary vasculature, through the generation of vasoconstrictive ROS under hypoxia. Recent evidence would suggest that the classical gp91*phox* subunit of NADPH oxidase is not involved in HPV, since oxygen sensing is preserved in gp91*phox* knockout mice (Archer *et al.* 1999). However such animals are lacking only the neutrophil form of gp91*phox*. This enzyme may not be expected to account for the oxygen sensing process underlying HPV, since no evidence exists of such responses being adversely affected in sufferers of chronic granulomatous disease who also lack the neutrophil form of gp91*phox*. Several homologues of gp91*phox* are likely to exist in humans. Indeed several human gene sequences highly homologous to gp91*phox* have recently been deposited in GenBank. Consequently, it is likely that a non-neutrophil gp91*phox* homologue acts as the pulmonary oxygen sensor.

Acknowledgements

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