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Investigation Of The Mechanism Of Contraction Of The Isolated Guinea Pig Pulmonary Venule Induced By Hypoxia Or Anoxia

Wayne Ross Tracey

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INVESTIGATION OF THE MECHANISM OF CONTRACTION OF THE ISOLATED GUINEA-PIG PULMONARY VENULE INDUCED BY HYPOXIA OR ANOXIA

by

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ABSTRACT

Since small pulmonary arteries are thought to be the major site of hypoxic pulmonary vasoconstriction (HPV), the response of pulmonary veins to hypoxia has not been thoroughly investigated. Therefore, the response of isolated guinea-pig pulmonary venules to hypoxia (bath Po_2: 25 torr) and anoxia (bath Po_2: 0 torr) was characterized. Pulmonary venules [effective lumen radius (ELR): 119 ± 1 μm] responded to hypoxia and anoxia with a graded, sustained, and repeatable contraction (hypoxia: 3 ± 1 mg/mm; anoxia: 27 ± 3 mg/mm), while paired femoral venules (ELR: 184 ± 7 μm) contracted to the initial anoxic challenge only (5 ± 2 mg/mm). The pulmonary venular contractions were calcium-dependent, but independent of the parenchyma.

Endothelial injury was induced by perfusion of vessel segments with either a mixture (HX/XO) of hypoxanthine (5 mM) and xanthine oxidase (0.05 U/ml), or with collagenase (2 mg/ml). HX/XO significantly ($p < 0.05$) augmented pulmonary venular contractions to hypoxia (HX/XO: 3.2 ± 1.0 mg/mm; control: 1.0 ± 0.5 mg/mm) and anoxia (HX/XO: 35.1 ± 6.6 mg/mm; control: 20.3 ± 4.0 mg/mm), while superoxide dismutase (40 μg/ml) and catalase (323 μg/ml) prevented this augmentation. Collagenase also significantly ($p < 0.05$) enhanced the anoxic contractions (collagenase: 36.0 ± 3.7 mg/mm; control: 20.9 ± 6.8 mg/mm). Neither gossypol (5 μM) or methylene blue (10 μM), nor indomethacin (5 μM) or ibuprofen (10 μM) affected pulmonary venular contractions to reduced Po_2.

Hypoxia and anoxia modestly, but significantly ($p < 0.01$), enhanced leukotriene (LT) C_4- and LTD_4-induced pulmonary venular contractions. FPL 57231 (3 μM), LY 163443 (1 μM), nordihydroguaiaretic acid (5 μM), and U-60257B (10 μM) had no effect on pulmonary venular contractions induced by decreased Po_2. Anoxia depressed spontaneous LT release from pulmonary venules. SKF-525A (500 μM) depressed contractions elicited by both decreased Po_2 and pharmacological agents; metyrapone (1 mM) was without effect. Induction of the cytochrome P-450 monooxygenase system with β-naphthoflavone did not alter pulmonary venular contractions induced by decreased Po_2.

In summary, the isolated pulmonary venule exhibits several characteristics of HPV in vivo. This model was used to show that the endothelium opposes, rather than mediates, the pulmonary venular contractions induced by decreased Po_2. Neither the leukotrienes nor cyclooxygenase metabolites of arachidonic acid mediated these contractions, and there was no evidence of a mediating role for cytochrome P-450 metabolites of endogenous substrates.

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NOMENCLATURE

AA: arachidonic acid

A II: angiotensin II

ADP: adenosine diphosphate

ANTU: α-naphthylthiourea

ATP: adenosine triphosphate

CAT: catalase

COPD: chronic obstructive pulmonary disease

DECC: diethylcarbamazine citrate

DSCG: disodium cromoglycate, cromolyn sodium

EDRF: endothelium-derived relaxing factor

ELR: effective lumen radius

EM: electron microscope

7-ERF: 7-ethoxyresorufin O-deethylation

ETYA: eicosatetraynoic acid

HPV: hypoxic pulmonary vasoconstriction

5-HT: 5-hydroxytryptamine

HX: hypoxanthine

LT: leukotriene

NADPH: reduced nicotinamide adenine dinucleotide phosphate

NDGA: nordihydroguaiaretic acid

β-NF: β-naphthoflavone

$PAco_2$: alveolar carbon dioxide tension

PAH: polycyclic aromatic hydrocarbon

PAo_2: alveolar oxygen tension

PAP: pulmonary artery pressure

Pco_2: partial pressure of carbon dioxide

PG: prostaglandin

Pi: free phosphate

Po_2: partial pressure of oxygen

PSS: physiological salt solution

Pvo_2: mixed venous oxygen tension

PVR: pulmonary vascular resistance

RIA: radioimmunoassay

SEM: standard error of the mean

SOD: superoxide dismutase

Tris: tris(hydroxymethyl)aminomethane

WT: wall tension

XO: xanthine oxidase

INTRODUCTION

Although hypoxic pulmonary vasoconstriction (HPV) was first described more than four decades ago by Von Euler and Liljestrand (1946), the mechanism has yet to be elucidated. A major difficulty in examining the mechanism of HPV has been the lack of a suitable *in vitro* model. Attempts to study HPV using isolated pulmonary vascular tissue or perfused lungs have encountered several difficulties, such as: a) non-sustained contractions (Lloyd, 1964, 1970; Madden *et al.*, 1985), b) loss of the response over time (Gorsky and Lloyd, 1967; Hauge, 1968a), c) the need to precontract isolated vessels in order to elicit contractions (Detar and Gellai, 1971; Miller *et al.*, 1988), d) a requirement for pre-treatment with high oxygen concentrations (Lloyd, 1968, 1970; Madden *et al.*, 1985; Miller *et al.*, 1988), or e) loss of the contractile response when physiological salt solutions are used (Lloyd, 1964).

In addition to the above problems, the vast majority of studies of HPV have failed to take into account the influence of the vascular endothelium on vascular tone. The endothelium is capable of releasing vasodilator substances such as prostacyclin and endothelium-derived relaxing factor (EDRF) (Furchgott, 1983), and the recently reported vasoconstrictor peptide known as endothelin (Yanagisawa *et al.*, 1988). Thus release of endothelium-derived factors during hypoxia may modulate HPV. Previous attempts to describe the role of the endothelium in HPV have produced contradictory findings. Some investigators have reported that endothelial damage serves to blunt the hypoxic contractions of isolated porcine (Holden and McCall, 1984) and rat (Rodman *et al.* 1987) pulmonary arteries, suggesting that the

endothelium contributed to the hypoxic contractions. However, Brashers et al. (1988) recently reported that inhibitors of EDRF (ETYA, NDGA, hydroquinone) potentiated the hypoxic pressor response of isolated rat lungs. A preliminary report by Yamaguchi et al. (1987) also supports the observations of Brashers et al. (1988). In addition, Madden et al. (1986a) observed that reduced Po_2 inhibited prostacyclin release from cultured bovine pulmonary artery endothelial cells. These latter observations suggest that hypoxia might elicit vasoconstriction by inhibiting a vasodilator influence of the endothelium on pulmonary vascular tone. Clearly, the involvement of the endothelium in HPV has yet to be established.

Various endogenous compounds have been suggested to mediate HPV, but most of the recent attention has focused on arachidonic acid (AA) metabolites produced via the 5-lipoxygenase (peptidoleukotrienes) and cyclooxygenase (prostaglandins and thromboxanes) pathways, and as of yet unidentified cytochrome P-450 metabolites. Although cyclooxygenase metabolites (eg. prostaglandins) are clearly able to modulate HPV, they do not appear to mediate this response. Several investigators have reported that cyclooxygenase inhibitors either have no effect on HPV (Naeije et al., 1988; Rubin et al., 1985; Walker et al., 1982a), augment the response (Vaage et al., 1975; Weir et al., 1976a), or change "nonresponder" animals to "responders" (Hales et al., 1978; Ahmed et al., 1983). Although it is unlikely that prostaglandins mediate HPV, the respective roles of the leukotrienes (LTs) or cytochrome P-450 metabolites of AA are not as clear. Leukotriene concentrations are elevated in lung lavage samples

obtained from hypoxic rats (Morganroth et al., 1984a) or infants with persistent pulmonary hypertension (Stenmark et al., 1983). In addition, several investigators have concluded that LTs play a role in HPV following observations that LT receptor or synthesis antagonists inhibit the response (Ahmed and Oliver, 1983; Morganroth et al., 1984b, 1985; Raj and Chen, 1987). On the other hand, several studies (Leffler et al., 1984; Schuster and Dennis, 1987; Garrett et al., 1987; Gottlieb et al., 1988; Lonigro et al., 1988; McCormack and Paterson, 1987) suggest that LTs do not mediate HPV. In contrast, the contribution of cytochrome P-450 metabolites in HPV has not been thoroughly investigated. Studies to date have suggested that cytochrome P-450 inhibitors reduce HPV (Sylvester and McGowan, 1978; Miller and Hales, 1979; Chang et al., 1986). However, the lack of selectivity of the antagonists used in these studies makes unambiguous interpretation of the data difficult.

The available experimental evidence suggests that the small pulmonary arteries are the major locus of HPV (Marshall and Marshall, 1983a; Voelkel, 1986) and, perhaps as a consequence, the response of pulmonary veins to hypoxia has not been studied extensively. However, pulmonary veins also contract in response to hypoxia (Furnival et al., 1970; Morgan et al., 1968; Miller et al., 1988), and may contribute up to half of the total pulmonary resistance during hypoxia (Raj and Chen, 1986). Since the role of the venous side of the pulmonary circulation in HPV is poorly understood, elucidation of the response of isolated pulmonary venules to decreased Po_2 and the mechanisms involved were the primary goals of the present study. Venules

(< 300 μm internal diameter) were examined instead of veins because the major site of HPV is thought to be in the small resistance vessels of the lung.

The particular objectives of the present study were to: 1) determine if this model may be used to predict mechanisms of HPV by characterizing the response of the pulmonary venule to decreased Po_2 and selected pharmacological stimuli; 2) investigate the effect of endothelial damage on pulmonary venular contractions to decreased Po_2; and 3) determine if cyclooxygenase, lipoxygenase or cytochrome P-450 metabolites mediate the venular contractions to reduced Po_2. Preliminary reports of our findings have been presented (Paterson et al., 1988a; Tracey et al., 1988).

HISTORICAL REVIEW

Although Plumier (1904) may have been the first to observe hypoxic pulmonary vasoconstriction (HPV), von Euler and Liljestrand (1946) were the first to recognize its significance when they proposed that HPV was a local (ie. confined to the lung) mechanism responsible for matching lung perfusion and ventilation. Shortly thereafter, Motley et al. (1947) demonstrated that breathing a gas mixture low in oxygen caused an increase in the pulmonary artery pressure (PAP) of normal subjects. Since these early observations, the mechanism of HPV has been extensively investigated, with little progress having been made.

Hypoxic pulmonary vasoconstriction is clinically important because over 80% of all cases of pulmonary hypertension relate to chronic obstructive pulmonary disease (COPD), a syndrome in which hypoxia is a major contributor to the development of pulmonary hypertension. Regional HPV should improve gas exchange by matching perfusion to ventilation and thus maintaining oxygenation of the blood. In other words, the parts of the lung that are not ventilated are not perfused. Furthermore, the pressor effect of widespread HPV may lead to recruitment of additional vessels and therefore increase the surface area involved in diffusion.

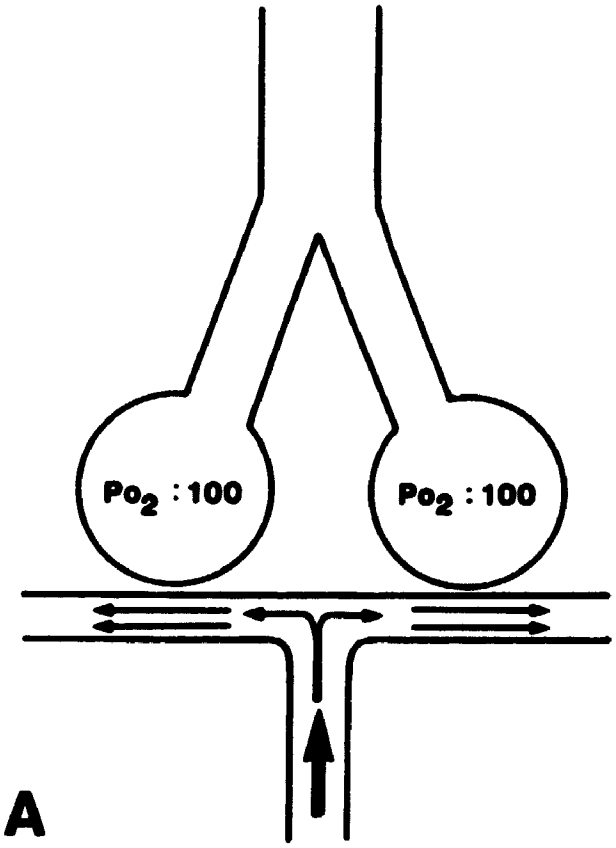
Hypoxic pulmonary vasoconstriction is unique to the pulmonary vasculature, in that the response to tissue hypoxia in the systemic circulation is vasodilation, not vasoconstriction. This fact was nicely demonstrated by Davis et al. (1981) when they transplanted neonatal lung tissue of the hamster into the cheek pouch. The transplanted pulmonary arteries continued to contract in response to

hypoxia, while the systemic arteries of the cheek pouch dilated (Davis et al., 1981).

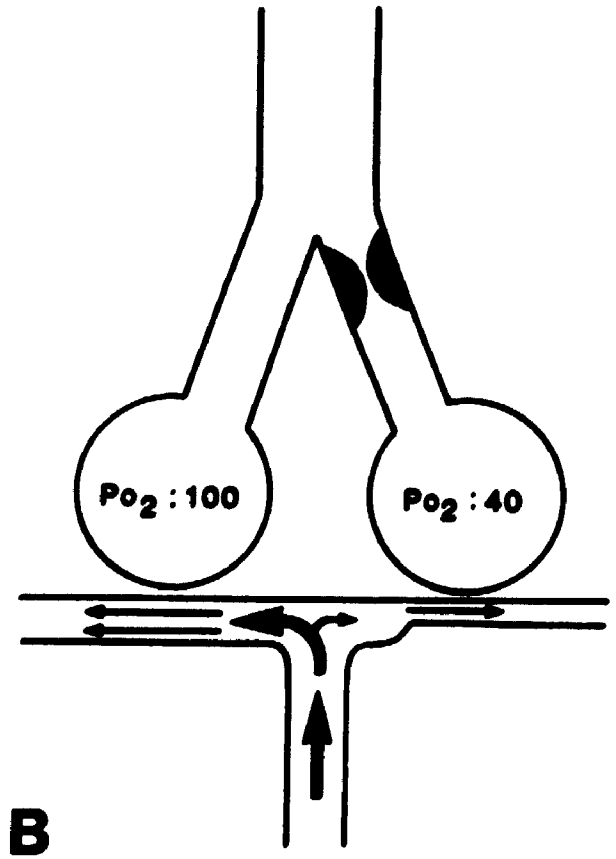
The schematic representation of two alveoli presented in Figure 1 demonstrates the physiological significance of HPV. When the Po_2 in two individual alveoli is "normal" (ie. approximately 100 torr), the bloodflow is equally divided past each alveolus (Fig. 1A). However, if the Po_2 in one of the alveoli should drop, in this example due to an airway obstruction, the adjacent pulmonary arteriole constricts, thereby shunting the blood flow away from the poorly ventilated alveolus to the one which is better ventilated (Fig. 1B). Although this is a highly simplified description, it serves to illustrate the beneficial effects of HPV on gas exchange.

In general terms, the degree of pulmonary vasoconstriction is inversely proportional to the oxygen tension, ie. the lower the oxygen tension, the greater the increase in pulmonary vascular resistance (PVR). In humans, PAP begins to increase when the alveolar Po_2 (PAo_2) drops below 60 torr (Voelkel, 1986). Peake et al. (1981) studied the response of isolated lungs from 5 different species (pigs, dogs, rabbits, cats and ferrets), and found that on average, PAP in rabbit, cat and ferret lungs began increasing at an inspired Po_2 of approximately 75 torr and peaked at an inspired Po_2 of approximately 25 torr. [In this study, the canine lungs did not respond to decreases in inspired Po_2, although other investigators have observed HPV in the dog (see below)]. The "oxygen tension-response curve" of the porcine lungs was shifted slightly to the right of the rabbit, cat, and ferret, beginning at about 100 torr and peaking at 50 torr.

FIGURE 1. Schematic representation of the basic mechanism of hypoxic pulmonary vasoconstriction. The upper diagram (A) represents two alveoli with normal Po_2's of 100 torr with a pulmonary artery running adjacent to each alveolus. Blood flow is equally divided past each alveolus. In the bottom diagram (B), one of the airways has been partially obstructed, dropping the Po_2 in the corresponding alveolus to 40 torr. The adjacent pulmonary artery constricts, shunting the majority of the pulmonary blood flow past the better ventilated alveolus.



A



B

If the inspired Po_2 was lowered below that which produced the maximum increase in PAP, the PAP then began to decline, indicating vasodilation was occurring (Peake et al., 1981). Similar observations have been made in man (Groves et al., 1987; Lockhart and Saiag, 1981). Notwithstanding the observations of Peake et al. (1981), it is often difficult to elicit HPV consistently using isolated pulmonary vessels or perfused lungs. Several difficulties may be encountered in attempting to reproduce HPV in an isolated preparation. For instance, the contractions are often not sustained (Lloyd, 1964, 1970; Madden et al., 1985) or are lost during the course of an experiment (Gorsky and Lloyd, 1967; Hauge, 1968a), isolated vessels may require either a preinduced level of tone (Detar and Gellai, 1971; Miller et al., 1988) or pretreatment with high oxygen concentrations (Lloyd, 1968, 1970; Madden et al., 1985; Miller et al., 1988) in order to elicit contractions, and finally, the contractile response may be lost when physiological salt solutions are used (Lloyd, 1964).

Since the pulmonary vasculature may be exposed to changes in either or both the PAo_2 and mixed venous Po_2 (Pvo_2), studies were performed to determine the relative importance of these two variables in HPV. The data from these experiments, using cats (Hyman et al., 1981), dogs (Hughes and Rubin, 1984) and isolated rat lungs (Marshall and Marshall, 1983a, 1983b) indicate that pulmonary vasoconstriction can be elicited by decreasing either the Pvo_2 or PAo_2 and is actually a function of both oxygen tensions. However, PAo_2 appears to be the predominant stimulus for HPV (Hales, 1985; Marshall and Marshall, 1983b), as maintaining the PAo_2 at normal to high levels can either

blunt or prevent the increase in PVR elicited by a decrease in the Pvo_2 (Duke, 1954; Hughes and Rubin, 1984; Marshall and Marshall, 1983a, 1983b; Hyman et al., 1981).

Species differences in HPV generally relate to the strength of the response. The coati mundi (Boggs et al., 1984), ferret (Peake et al., 1981), pig (Peake et al., 1981; Sylvester et al., 1980; Tucker et al., 1975) and calf (Tucker et al., 1975) are relatively strong responders, the rabbit (Peake et al., 1981; Tucker et al., 1975) and rat (Tucker et al., 1975) are intermediate responders, and the cat, dog (Peake et al., 1981), hamster (Walker et al., 1982b), guinea-pig and sheep (Tucker et al., 1975) are relatively weak responders. Hakim and Macek (1988) have recently suggested that differences in erythrocyte deformability are related to species differences in the strength of HPV, ie. species with erythrocytes that become relatively more "rigid" during hypoxia (eg. rats) have a stronger pressor response than those species whose erythrocytes are more deformable under hypoxic conditions (eg. hamsters).

Sex differences are also known to be important in determining the strength of HPV. McMurtry et al. (1973) reported that male pigs developed a slightly higher, albeit insignificant, increase in PAP than female pigs at high altitude (5,490 m) and had larger right ventricular weight/total ventricular weight ratios than females, indicating that the males did not adapt as well to hypoxic conditions. Similarly, Wetzel and Sylvester (1983) observed that the isolated lungs of postpubertal (6-7 months) male sheep (both normal and castrated) achieved a greater maximal hypoxic pressor response than

those of postpubertal female sheep. The attenuation of the response in the lungs from the female sheep was proposed to be due to the effect of female hormones (Wetzel and Sylvester, 1983). In subsequent study, Wetzel et al. (1984) found no difference in HPV in prepubertal male and female sheep lungs. In addition, they observed that administration of 17β-estradiol to either 2 month-old female or 6 month-old male sheep significantly attenuated the development of pulmonary hypertension (Wetzel et al., 1984), thus confirming their earlier hypothesis. Similar results have been reported by Peake et al. (1981), who found that isolated lungs from female cats had a blunted acute hypoxic pressor response. While investigating the structural and hemodynamic effects of chronic hypoxia, Rabinovitch et al. (1981) observed that the mean PAP following chronic hypoxia was less in adult female rats compared to adult male rats.

The literature on the influence of age on HPV is contradictory. Fike and Hansen (1987) observed that the percent increase in PAP in isolated lungs from rabbit pups (matched for either baseline PAP or flow), was greater in older pups (10-14 days) than in younger pups (3-8 days old). Since flow remains constant in such a preparation, increases in PAP should reflect increases in PVR, suggesting that in this model, HPV increases with postnatal age. Rendas et al. (1982) reported similar results using anesthetized pigs (expressed as percent change in total PVR). However, the baseline PVR of the pigs decreased with age, as did the absolute increase in total PVR (Rendas et al., 1982). On the other hand, Custer and Hales (1985) compared hypoxic changes in pulmonary perfusion distribution in neonatal and adult

sheep and found that the neonate had both a stronger regional alveolar HPV and was more sensitive to changes in PAo_2 than the adult. Similarly, both on the basis of absolute and percent increases in PVR, Lowen et al. (1987) reported that the hypoxic pressor response of the isolated rat lung tends to decline with age (Lowen et al., 1987). Finally, Rabinovitch et al. (1981) found no significant difference in the mean PAP after chronic hypoxic exposure in infant (8 days-4 weeks) and adult (9-13 weeks) rats.

The anatomical site of HPV has been a source of long-standing discussion. While it is relatively clear that the "middle" portion of the pulmonary circulation (ie. the resistance vessels and/or capillaries) contributes the majority of the increase in vascular resistance during HPV (Shirai et al., 1986; Hakim et al., 1983; Duke, 1954; Kato and Staub, 1966; Rock et al., 1985; Kapanci et al., 1974), different investigators have ascribed varying levels of importance to the subdivisions of this part of the pulmonary circulation. Duke (1954) performed one of the earliest studies to address this question, and concluded that the major site of hypoxic constriction was in the capillaries. This view has not received much support as evidence accumulated suggesting a major role for the small (< 500 μm in diameter) pulmonary vessels (Allison and Stanbrook, 1980). Kato and Staub (1966), after examining frozen sections from normal and anoxic cat lungs, found that the lumen size of the small (< 300 μm in diameter), muscular pulmonary arteries was significantly reduced. Similarly, Malik and Kidd (1976) observed that the primary site of HPV in the anesthetized dog was in the small pulmonary arteries. Marshall

and Marshall (1983a) performed a unique study when they exposed isolated rat lungs to forward and retrograde flow and measured changes in PAP in response to changes in perfusate oxygen tension. They found that only during forward flow did the PAP respond to changes in perfusate oxygen tension, suggesting that the site of HPV was on the arterial side of the circulation (Marshall and Marshall, 1983a). The data of Dawson and colleagues (1973), again obtained using forward and retrograde perfusion, but in the isolated cat lung, also suggested that hypoxia acted predominantly on the arterial side of the pulmonary circulation. In addition, Marshall and Marshall (1983b) have concluded that there is not a localized sensor for HPV, rather the response is accounted for by each individual smooth muscle cell responding to the oxygen tension in its vicinity. Several other investigators have also concluded that hypoxia acts mainly on the arterial side of the pulmonary circulation (Bergofsky et al., 1968; Glazier and Murray, 1971; Fike et al., 1988).

On the other hand, data also exists which implicates the venous side of the circulation in HPV. Using X-ray imaging, Shirai et al. (1986) observed that in anesthetized cats, both the pulmonary arteries and veins contracted in response to hypoxia, although the major increase in vascular resistance arose in the pulmonary arteries. The contractions elicited by hypoxia were maximal in arteries of 200-300 μm diameter and veins of 300-400 μm diameter (Shirai et al., 1986). Micropuncture techniques have also allowed the relative contribution of the arteries, capillaries and veins to HPV to be examined. Nagasaka and colleagues (1984) reported that in the isolated cat lung,

the relative contribution to the total increase in PVR during hypoxia was greatest in the arterial side of the circulation, followed by the venous side, and finally the blood vessels < 50 μm in diameter. Raj and Chen (1986) reported similar results using isolated lamb lungs, but they found that the venous and arterial sides of the circulation contributed almost equally to the total increase in PVR during hypoxia (41.1% and 37.7%, respectively), with the blood vessels < 80 μm in diameter contributing the least (21.1%). Raj and colleagues (1988) have also provided indirect evidence for a role of the venous side of the pulmonary circulation in HPV when they reported an increase in the lung lymph flow of hypoxic lambs. As the lymph/plasma protein ratio was reduced during hypoxia, this suggested that hypoxia increased lung lymph flow by increasing microvascular filtration pressure, possibly via constriction of the pulmonary veins (Raj et al., 1988). Other investigators have concluded that both the pulmonary arteries and veins (Morgan et al., 1968; Furnival et al., 1970), or only the veins, contract in response to decreases in Po_2 (Nisell, 1951; Rivera-Estrada et al., 1958).

One other hypothesis concerning the site of HPV has received relatively little attention. Kapanci and colleagues (1974) demonstrated the presence of contractile elements in pulmonary interstitial cells of the rat lung. These investigators (Kapanci et al., 1974) proposed that these interstitial cells contracted during hypoxia and modified pulmonary vascular conductance, presumably by compression or deformation of alveolar vessels. As support for this hypothesis, they cited the hypoxic contractions of pulmonary

parenchymal strips, but they failed to adequately exclude any contribution of airway or vascular smooth muscle to these contractions.

In summary, it is generally accepted that the site of HPV is in the small resistance vessels of the lung, and the arterial side of the circulation is predominantly responsible for the increase in PVR under hypoxic conditions. However, there is also evidence which indicates that the pulmonary veins contract in response to hypoxia, and in some cases may contribute significantly to HPV.

The magnitude of HPV is affected by several factors including temperature, blood (perfusate) elements, calcium and pH. HPV was found to be dependent on temperature in both the canine (Lloyd, 1966a; Benumof and Wahrenbrock, 1977; Daly et al., 1962) and rat (Nilsen and Hauge, 1968) lung. Decreases in perfusate temperature were found to elevate normoxic PAP and depress increases in PAP induced by hypoxia in the isolated left lower lobe of the canine lung (Lloyd, 1966a). At the same time, temperature decreases had relatively little effect on increases in PAP induced by 5-hydroxytryptamine (5-HT) and epinephrine (Lloyd, 1966a). Similar results in vivo were reported by Benumof and Wahrenbrock (1977) and these investigators suggested that since a decrease in temperature increased baseline pulmonary arterial tone, the inhibition of HPV may have been due to a reduced capacity of the pulmonary arteries to contract or for other parts of the pulmonary vasculature to accept a redistribution of blood flow. Nilsen and Hauge (1968) observed comparable reductions in HPV when the perfusate temperature of isolated rat lungs was lowered. However, Nilsen and

Hauge (1968) found the contractile response to ATP was depressed as well, although the response to bradykinin was unaffected.

Because of the early finding that HPV was blunted in lungs perfused with physiological saline, numerous experiments have been performed to try and determine what component(s) of blood serve to enhance the contractions. One of earliest studies to address this question was performed by Lloyd (1964) using isolated dog lungs. Lloyd found that hypoxia elicited a dilator response from lungs which were perfused with synthetic perfusates, while a pressor response was observed in lungs perfused with blood (Lloyd, 1964). A subsequent study demonstrated that the hypoxic pressor response was augmented in lungs perfused with either autologous plasma or physiological salt solution (PSS) plus 20% autologous plasma compared to lungs perfused with PSS plus 5% autologous plasma or PSS alone (Gorsky and Lloyd, 1967). The pressor response to 5-HT was also augmented by perfusion with autologous plasma, indicating a non-selective enhancement of pulmonary vascular reactivity (Gorsky and Lloyd, 1967). Similar results were reported by Kivity and Souhrada (1981) using isolated rat lungs.

In 1968, Hauge obtained evidence for modulation of HPV by cellular elements of the blood. Hauge found that by removing these cellular elements (including platelets) the hypoxic pressor response of isolated rat lungs was rapidly lost (Hauge, 1968a). If the cells were then added back to the perfusate, HPV was restored for a short period of time (Hauge, 1968a). In lungs that were perfused with blood until a hypoxic pressor response could no longer be elicited, replacement of

the perfusate with fresh blood did not restore the response (Hauge, 1968a). In addition, perfusion of fresh lungs with "old" blood (ie. taken from lungs in which a hypoxic pressor response could no longer be produced) did not affect the hypoxic pressor response of the fresh lungs (Hauge, 1968a). Taken together, these observations suggested that the disappearance of HPV was due to changes in the lungs themselves, although apparently a cellular constituent of the blood was able to prolong the duration of the response while not preventing its disappearance (Hauge, 1968a). In contrast to the observations of Hauge (1968a), Lloyd (1966a) did not observe any difference in the hypoxic pressor response of isolated dog lungs when perfused with plasma or blood.

A potential role for platelets in HPV was suggested by Hauge and Melmon (1968) when they observed that perfusion of isolated rat lungs with platelet-rich plasma enhanced HPV. These investigators were unable to explain this observation, although they noted that 5-HT release from platelets was probably not involved, since 5-HT antagonists did not affect the hypoxic pressor response, and total indoles in the plasma did not increase during hypoxia (Hauge and Melmon, 1968). Kivity and Souhrada (1981) found that adding platelets to 100% plasma enhanced the hypoxic pressor response of isolated rat lungs; maximal enhancement was obtained at a platelet concentration similar to that <u>in vivo</u>.

Data from subsequent studies have indicated that platelets play a minimal role in HPV. Weir <u>et al.</u> (1976b) found that HPV was enhanced in dogs which had previously been administered anti-platelet serum,

and McMurtry et al. (1977) concluded that platelets were not required for strong HPV in isolated rat lungs. In fact, McMurtry et al. (1978) found that lungs perfused with plasma and platelets were less responsive to hypoxia and angiotensin II (A II) compared to plasma alone, and suggested that the restoration of HPV which Hauge and Melmon (1968) observed with platelet-rich plasma was actually due to the use of fresh plasma. A role for erythrocytes in HPV was suggested when McMurtry et al. (1977) reported that the addition of erythrocytes to either plasma or Tyrode's solution maintained HPV at a level higher than that obtained with either plasma or Tyrode's alone. Following further experimentation, McMurtry and colleagues (1978) concluded that erythrocytes are not an integral part of HPV, but that they may prolong/enhance the response by preventing a "deterioration" of the perfusate. Unlike the earlier observations of Hauge (1968a), McMurtry et al. (1978) found that fresh lungs perfused with "old" plasma or blood were hyporesponsive to hypoxia and A II. McMurtry and colleagues had earlier reported that the addition of glucose to the perfusate inhibited the hypoxic pressor response and postulated that erythrocytes might augment HPV by uptake of glucose and/or adenosine from the perfusate (McMurtry et al., 1977). [Adenosine is released from the lung during hypoxia and is a pulmonary vasodilator (Mentzer et al., 1975)]. Recently, a mechanical role for erythrocytes in HPV has also been suggested (Hakim and Macek, 1988; Hakim and Malik, 1988; Chick et al., 1988), ie. during hypoxia, erythrocytes become more rigid, thereby impeding microvascular flow and increasing pulmonary driving pressure. Chick et al. (1988) reported that pentoxifylline (a

methylxanthine derivative), an agent which increases erythrocyte deformability, blunted HPV in anesthetized dogs. However, these investigators acknowledged that this drug had other actions which could account for their observations (Chick et al., 1988), a conclusion which was also reached by Hakim (1988).

A further possibility is that glucocorticoids found in the blood may modulate HPV (Herget and McMurtry, 1987). Herget and McMurtry (1987) reported that pretreatment of rats with dexamethasone, or inclusion of dexamethasone in the perfusate of isolated rat lungs (PSS plus albumin) augmented the hypoxic pressor reactivity of the lungs. However, addition of dexamethasone to blood perfused lungs did not alter their reactivity to hypoxia, while the pressor response of lungs from adrenalectomized rats was enhanced (Herget and McMurtry, 1987). The dexamethasone-induced augmentation of HPV in the isolated rat lungs did not appear to be due solely to inhibition of cyclooxygenase, as meclofenamate (a cyclooxygenase inhibitor) was much less effective at producing this enhancement, while causing a decrease in perfusate concentrations of cyclooxygenase metabolites similar to that obtained with dexamethasone (Herget and McMurtry, 1987).

Several other researchers have investigated the possible role of glucose and glucose metabolism in HPV, as suggested by the previous studies of McMurtry and colleagues (1977). Early studies revealed that by removing glucose from the perfusate, the hypoxic pressor response of isolated rat lungs was enhanced (Rounds et al., 1981; Stanbrook and McMurtry, 1983). Similar results have been reported by Vanhoutte (1976) using isolated canine pulmonary veins and Souhrada

and Dickey (1976) using isolated guinea-pig pulmonary arteries. Souhrada and Dickey (1976) electrically stimulated the pulmonary arteries every 5 min and found that anoxia increased the resting tension of the stimulated arteries when the glucose in the buffer solution was replaced with sucrose. These investigators reported comparable results using aortic segments, implying that the phenomenon may not have been unique to pulmonary vessels (Souhrada and Dickey, 1976). Several studies from the Cardiovascular Pulmonary Research Center in Denver have provided evidence that the oxidative production of ATP may be involved in blunting the pressor response to hypoxia, and that by removing substrate (ie. glucose) from the solution, oxidative ATP production declines and results in an augmentation of HPV. Rounds and McMurtry (1981) observed that several unrelated inhibitors of oxidative ATP production (azide, cyanide, dinitrophenol, antimycin A, and rotenone) all depressed the pressor response to both hypoxia and A II in isolated rat lungs. However, each of these agents initially elicited a transient increase in PAP which was similar to the hypoxic pressor response, albeit shorter-lived (Rounds and McMurtry, 1981). In 1981, Rounds et al., and in 1983, Stanbrook and McMurtry used different inhibitors to block the oxidative metabolism of glucose in different stages (2-deoxyglucose, iodoacetate, malonate) and found that each of these agents augmented the hypoxic pressor response. In addition, if either lactate (Stanbrook and McMurtry, 1983) or pyruvate (Stanbrook and McMurtry, 1983; Rounds et al., 1981) were added to the perfusate, HPV was inhibited in a manner similar to that observed with glucose. Stanbrook and McMurtry (1983) concluded

that the augmentation of HPV by inhibition of glucose metabolism was not due to increased anaerobic production of ATP, but rather was apparently related to a decreased production of pyruvate, thereby limiting mitochondrial oxidative phosphorylation (and therefore production of ATP via that route). Ohe and colleagues (1986) measured ATP concentrations during anoxia, using isolated rabbit pulmonary arteries in a glucose-free solution, and found that ATP concentrations declined during anoxic challenges. However, these investigators did not report whether the decline in ATP preceded or followed hypoxic increases in pulmonary artery tone, preventing any conclusions regarding cause and effect (Ohe et al., 1986). The fact that ATP levels also declined over the course of their experiments, without a corresponding increase in baseline pulmonary arterial tone (Ohe et al., 1986), suggests that the rapid decrease in ATP levels during anoxia may have been a result of the hypoxic contractions instead of their cause.

Perhaps not surprisingly, calcium has been found to play an integral role in pulmonary vascular contractions induced by hypoxia. Calcium channel blockers have been found to inhibit HPV in vivo in piglets (Escourrou et al., 1986; Redding et al., 1984), sheep (Yoshimura et al., 1987), rats (Stanbrook et al., 1984), normal man (Melot et al., 1987; Naeije et al., 1982) and patients with COPD (Simonneau et al., 1981; Burghuber, 1987), attenuate the structural changes associated with chronic hypoxia (Michael et al., 1986; Stanbrook et al., 1984) and partially reverse established hypoxic pulmonary hypertension (Stanbrook et al., 1984). In contrast, Clozel

et al. (1987) reported that intravenous diltiazem did not affect the increase in PVR elicited by hypoxia in patients with hypoxic pulmonary hypertension, even though therapeutic plasma levels of the drug were achieved. This observation may reflect a difference in the site and/or mechanism of action of various calcium channel antagonists, as the majority of the other studies mentioned were performed using nifedipine. Nifedipine does not necessarily reduce hypoxic PVR by solely decreasing PAP (Burghuber, 1987; Yoshimura et al., 1987); this reduction in PVR may also be partially due to an increase in cardiac output (Naeije et al., 1982; Redding et al., 1984).

The cellular events through which calcium levels affect HPV have not been established. Using isolated rat lungs, McMurtry and colleagues (1976) found that both verapamil and SKF-525A blunted the hypoxic pressor response, while having relatively little effect on the increases in PAP elicited by A II or prostaglandin (PG) $F_{2\alpha}$. They postulated that since A II- and $PGF_{2\alpha}$-induced contractions were presumably primarily due to intracellular release of calcium and these agents were less sensitive than hypoxia to the calcium channel antagonists, hypoxia-induced increases in PAP might reflect increases in transmembrane calcium influx. This study did not, however, differentiate between the possibilities of a calcium-stimulated release of a vasoconstrictor mediator versus a direct action of calcium influx on vascular smooth muscle contractility. In support of the latter mechanism, Harder et al. (1985) found that hypoxia elicited action potential generation in isolated cat pulmonary arteries and that verapamil abolished these action potentials along with partial

membrane repolarization. The hypoxic contractions of these arteries were also blocked by verapamil (a calcium channel blocker) and the amplitude of the contractions were directly dependent on extracellular calcium concentration (Harder et al., 1985). These observations led Harder and colleagues (1985) to suggest that hypoxia increases calcium permeability across pulmonary arterial cell membranes and that this increase in calcium permeability is at least partially responsible for the hypoxia-induced action potentials.

The influence of pH on HPV has also been investigated, and although HPV is generally augmented by acidosis and inhibited by alkalosis (Fishman, 1976), there have been conflicting findings. Liljestrand (1958) first called attention to the influence of pH on HPV when he suggested that the production of lactic acid from cells in the lung was responsible for the increase in PAP during hypoxia. Enson et al. (1964) studied the effects of altering blood pH on HPV in patients with COPD and found that alkalosis inhibited the hypoxic pressor response. In contrast, Bergofsky et al. (1962) reported that in normal human subjects, acute alkalosis produced by infusion of sodium bicarbonate or Tris did not modify the pulmonary arterial pressor response to hypoxia, and Silove and coworkers (1968) found that increased pH did not alter the hypoxic pressor response in the left lower lung lobe of the calf. Lyrene et al. (1985) and Schreiber et al. (1986) observed that alkalosis inhibited HPV in anesthetized newborn lambs and concluded that the strength of HPV was directly related to the hydrogen ion concentration and did not depend on whether the alkalosis was metabolic or respiratory (ie. decreased CO_2

tension) in origin. Similar observations were made by Lloyd (1966b) using the isolated perfused dog lung. In addition, when Lloyd (1966b) perfused the lung with acidic solutions, the hypoxic pressor response was enhanced, and he also found that the degree of change in the pressor response was unrelated to the agent used to alter the pH. However, Lloyd (1967) was unable to repeat these observations when using isolated strips of pulmonary arteries obtained from either dogs or rabbits. Decreases in pH have been reported to augment hypoxic pulmonary pressor responses in calf lungs (Rudolph and Yuan, 1966; Silove et al. 1968), cat lungs (Viles and Shepard, 1968; Barer et al., 1971), intact dogs (Malik and Kidd, 1973a), and patients with COPD (Enson et al., 1964; Harvey et al., 1967). Malik and Kidd (1973a) concluded from their experiments on intact dogs that potentiation of HPV during acidosis is due to an increase in CO_2 tension (Pco_2) rather than an increase in hydrogen ion concentration, and the inhibition of HPV during alkalosis is due to a decrease in hydrogen ion concentration rather that a decrease in Pco_2. More recently, Orchard et al. (1983) proposed that the effects of CO_2 on HPV in ventilated lungs or animals may be explained by the changes in PAo_2 which are secondary to changes in alveolar Pco_2 ($PAco_2$), ie. as $PAco_2$ increases, PAo_2 will decrease (thereby augmenting HPV) and vice-versa. These investigators were unable to alter HPV in anesthetized dogs by infusion of agents (lactic acid, $NaHCO_3$) which changed the pH of the blood, but changes in concentration of CO_2 in the inspired gas did modify the hypoxic pressor response (Orchard et al., 1983)

In contrast to the studies suggesting that acidosis enhances and alkalosis inhibits HPV, there have also been reports that acidosis outside the normal range (Marshall et al., 1984) or hypercapnia (Emery et al., 1977) can inhibit HPV. Raffestin and McMurtry (1987) recently observed that agents believed to increase intracellular pH (PMA, NH_4Cl, imidazole, methylamine, HEPES buffer, and low Cl^-) enhanced HPV in isolated rat lungs, while agents which should lower intracellular pH (amiloride and sodium acetate) blunted the response. In addition, Nagasaka et al. (1984) reported that although alkalosis inhibited the increase in pressure difference due to hypoxia on the arterial side of the feline pulmonary circulation, the hypoxia-induced increase in pressure difference on the venous side of the circulation was increased.

Formulations of the mechanism of HPV have generally fallen into two categories. First, hypoxia may elicit pulmonary vasoconstriction via a direct action on the vascular smooth muscle. Alternatively, an oxygen sensing cell releases a contractile mediator(s), which then stimulates the smooth muscle to contract. The latter possibility is difficult to disprove. For instance, even though isolated pulmonary vascular strips may contract under hypoxic conditions, a mediator released from contaminating parenchymal cells, leukocytes, or the vascular endothelium could be responsible for eliciting the contraction. An example of the caution required may be found in a report by Detar and Gellai (1971) in which isolated vascular tissue from the rabbit was used. Apparently, based on the observation that hypoxic pulmonary arterial strips contracted, while hypoxic aortic

strips relaxed, the conclusion was reached that the hypoxic contractions of the pulmonary arterial strips were due to a direct interaction between oxygen and the pulmonary vascular smooth muscle cells (Detar and Gellai, 1971). Unfortunately, these investigators did not mention whether there was any adherent parenchyma on their strips, or perform studies to exclude a contribution from the other cell types which may have been present in the preparation, thereby limiting the validity of their conclusion (Detar and Gellai, 1971). A study performed three years earlier had suggested a role for the parenchyma in hypoxic contractions (Lloyd, 1968), and subsequent studies have suggested that the endothelium may mediate or modulate these contractions (Holden and McCall, 1984). Another way of addressing the direct hypothesis might be to use cultured pulmonary vascular smooth muscle cells, but these cultures may be of limited usefulness, again because of the possible presence of contaminating cells (fibroblasts, endothelium), the likelihood of altered phenotypes, and correlation of intracellular biochemical changes with contraction in a cell which may no longer actually contract.

Bergofsky and Holtzman (1967) measured changes in the electrolyte composition during hypoxia of segments of feline pulmonary artery and vein, and femoral and carotid arteries. These investigators found that during hypoxia, the pulmonary artery lost potassium and gained sodium in a reversible manner, whereas neither the pulmonary vein nor the femoral or carotid arteries demonstrated this change in electrolyte composition (Bergofsky and Holtzman, 1967). Both the intima and adventitia of the blood vessels were removed, precluding a

contribution from the endothelium or pulmonary parenchymal cells (Bergofsky and Holtzman, 1967). In the perfused cat lung, depolarization of the vascular smooth muscle with a solution high in K^+ increased the PAP and tended to augment the hypoxic pressor response, while hyperpolarization of the smooth muscle with a solution high in Cl^- decreased the PAP and tended to inhibit the hypoxic pressor response (Bergofsky and Holtzman, 1967). Bergofsky and Holtzman (1967) interpreted these data as indicating that hypoxia depolarizes the smooth muscle so that it is closer to its excitatory threshold and more readily able to contract, although they refrained from stating that hypoxia directly elicits pulmonary vasoconstriction via membrane depolarization. Another possible explanation for the results of Bergofsky and Holtzman (1967) is that the high K^+ or Cl^- solutions may have respectively augmented or blunted the hypoxic pressor response by changing the resting length-tension characteristics of the pulmonary vascular smooth muscle; indeed this is suggested by the change in normoxic baseline PAP elicited by these solutions. In support of the observations of Bergofsky and Holtzman (1967), Madden et al. (1985) have demonstrated that isolated small pulmonary arteries of the cat are both depolarized and contracted by hypoxia; this depolarization did not appear to be due to inhibition of Na^+/K^+ ATPase, since ouabain did not affect the depolarizations. Large pulmonary arteries (> 500 μm in diameter) from the same species did not exhibit significant hypoxic vasoconstriction or depolarization (Madcen et al., 1985). Madden et al. (1985) were unable to determine if the depolarization of pulmonary arterial smooth muscle was a direct

effect of hypoxia, or indirectly mediated by a released substance. Similar results were reported by Harder et al. (1985) who postulated that hypoxia induces contraction by a mechanism involving an increased calcium conductance; their observations have already been described in the discussion on HPV and calcium.

A second hypothesis for a direct mechanism of action of hypoxia on pulmonary vascular smooth muscle proposes that hypoxia causes the energy state of the cell (defined by the phosphate potential: ATP/[ADP][Pi]) to decline, thereby eliciting HPV (Weir, 1984; Voelkel, 1986). The available data indicate that hypoxia does lower this ratio (ie. by decreasing cellular ATP content) as has been discussed previously (the influence of substrate on HPV). A conceptual problem with this hypothesis is how a decrease in cellular ATP levels could lead to contraction of the vascular smooth muscle cell, let alone a sustained contraction. There may be different intracellular pools of ATP in pulmonary vascular smooth muscle, one of which is involved in calcium sequestration (Fitzpatrick et al., 1972; Hurwitz et al., 1973). If this pool of ATP diminished during hypoxia, intracellular levels of calcium might increase, leading to smooth muscle contraction. Even if other pools of ATP which might be involved in smooth muscle contraction were to be depleted, this would not necessarily prevent contraction, as smooth muscle cells have been shown to contract in the absence of myosin light chain phosphorylation (Wagner and Ruegg, 1986; Hoar and Kerrick, 1988). In addition, a myosin light chain phosphorylation-independent activating system (ie. the formation of slowly cycling "latch"-bridges) has been suggested to

be involved in stress maintenance in skinned (Chatterjee and Murphy 1983) and intact (Aksoy et al., 1982) vascular smooth muscle. Such a system would allow stress maintenance with a greatly reduced energy expenditure (Aksoy et al., 1982) and would essentially require just enough ATP to generate the initial level of tone. The lack of such a mechanism in pulmonary vascular smooth muscle would not necessarily preclude contraction however, since even severe hypoxia may not lower the ATP supply in the involved compartment(s) to levels which are incompatible with vasoconstriction (Weir, 1984).

The search for a mediator of HPV has essentially spanned the history of recent pharmacology, ie. as a new endogenous compound with biological activity was discovered, its role in HPV was investigated. However, none of the agents tested to date have been conclusively proven to mediate HPV, although a number of potential modulators of the response have been discovered. Lloyd (1967) was one of the first to suggest the involvement of an extravascular factor. He arrived at this conclusion because isolated canine and rabbit pulmonary arteries did not contract when made hypoxic, while precontracted strips relaxed under the same conditions (Lloyd, 1967). Lloyd (1967) reasoned that if the direct effect of hypoxia on the pulmonary artery was to cause relaxation, but vasoconstriction occurred in vivo, there must be some extravascular factor released in vivo during hypoxia which elicited the vasoconstriction. In a subsequent study, Lloyd (1968) found that strips of rabbit pulmonary artery which retained a collar of parenchyma contracted when made hypoxic, while arteries free of

parenchyma did not contract, supporting his initial hypothesis (Lloyd, 1967). Unfortunately, these findings have never been confirmed.

The contribution of the autonomic nervous system in HPV, in particular the pulmonary sympathetic innervation, has also been assessed. Lloyd (1966a) observed that neither phenoxybenzamine (α-blocker) nor reserpine (catecholamine depleting agent) affected HPV in the isolated dog lung lobe, and although cooling inhibited HPV, it had little effect on responses to electrical stimulation or epinephrine. Therefore, Lloyd concluded that nerve pathways were not involved in the hypoxic pressor response (Lloyd, 1966a). Silove et al. (1968) reached similar conclusions using an in situ isolated lung lobe from the calf, ie. they found that a drop in pH augmented HPV but had no effect on norepinephrine pressor responses, and that norepinephrine and epinephrine dilated pulmonary vessels precontracted by hypoxia. In contrast, Kazemi et al. (1972) and Porcelli et al., (1977) concluded that adrenergic nerves were responsible for most of the vasoconstriction elicited by hypoxia. Kazami et al. (1972) found that sympathectomy decreased the effect of hypoxia on perfusion distribution in anesthetized dogs by approximately two-thirds, while in the cat, Porcelli and colleagues (1977) reported that phenoxybenzamine blunted HPV. However, very little data was presented by Porcelli et al. (1977) (only percent change in PVR) to support their observations, with no information on changes in cardiac output or PAP. Custer and Hales (1986) obtained some evidence indicating that sympathetic innervation may be more important in the lamb compared to the sheep, when they observed that chemical sympathectomy

with 6-hydroxydopamine reduced the effect of hypoxia on perfusion distribution by approximately 50% in the lamb, but did not affect the response in the adult. The majority of other studies in this area have supported the conclusion that adrenergic nerves do not mediate HPV. This evidence includes the persistence of the hypoxic pressor response in isolated lungs (Cutaia and Friedrich, 1987; Marshall and Marshall, 1983a, 1983b; Gorsky and LLoyd, 1967; Rounds and McMurtry, 1981) and pulmonary vessels (Lloyd, 1970; Madden et al., 1985; Vanhoutte, 1976; Holden and McCall, 1984), the persistence of HPV in transplanted lungs (Robin et al., 1987) and in animals chemically sympathectomized with 6-hydroxydopamine (Custer and Hales, 1986; Hales and Westphal, 1979), the lack of effect of α-adrenergic blockade (Malik and Kidd, 1973b) or catecholamine depletion (Hauge and Melmon, 1968) on HPV, and the fact that when the pulmonary vasculature is contracted by hypoxia, the effect of norepinephrine is either attenuated (Rorie and Tyce, 1983) or changes to vasodilation (Cutaia and Friedrich, 1987). This does not mean that HPV is totally independent of sympathetic involvement. Adrenergic stimulation may provide a basal level of tone in some instances which could amplify the hypoxic pressor response. On the other hand, considering that norepinephrine may relax the hypoxic pulmonary vasculature (Cutaia and Friedrich, 1987; Porcelli and Cutaia, 1988) and that hypoxia has been demonstrated to increase the release and overflow of endogenous norepinephrine (Rorie and Tyce, 1983), the sympathetic nervous system might instead tend to restrict the magnitude of HPV. Insofar as the pulmonary vagal innervation is concerned, Chapleau et al. (1988)

reported that chemoreceptor stimulation with hypoxic blood in anesthetized dogs interfered with the changes in pulmonary perfusion induced by hypoxia, and that vagotomy abolished this effect. Kazemi et al. (1972), on the other hand, found that vagotomy had no effect on hypoxic perfusion distribution in anesthetized dogs which were unilaterally ventilated with nitrogen and had slightly elevated systemic arterial oxygen tensions.

The postulated involvement of A II in HPV has been investigated and the hypothesis generally discarded. Berkov (1974) reported that inclusion of sub-threshold quantities of A II in the perfusate of PSS-perfused rat lungs maintained and selectively augmented the pressor response to hypoxia. Although Berkov (1974) did not suggest that A II directly mediated HPV, he stated that it could permit the release or action of another agent and was specifically required for the response to occur. Ten years later, McMurtry (1984) essentially repeated this study and concluded that A II, like several other agents (plasma, KCl, vanadate, 4-aminopyridine, norepinephrine + propranolol) non-selectively augmented pulmonary vascular reactivity of the isolated rat lung when added to the perfusion fluid. Furthermore, saralasin acetate (A II receptor antagonist) did not affect the augmentation of the hypoxic pressor response following addition of plasma to the perfusate (McMurtry, 1984). Unlike Berkov (1974), McMurtry (1984) concluded that A II did not play an integral role in HPV. Other investigators have stated that captopril (angiotensin converting enzyme inhibitor) reduces HPV in patients with COPD (Bertoli et al., 1986) and sheep (Yoshimura et al., 1987), but the reductions in PVR

could at best be considered modest (ie. no more than a 20% reduction). Another study in patients with COPD (Boschetti et al., 1985) demonstrated no significant reduction in PVR or PAP by captopril, and Prewitt and Leffler (1981) reported that captopril did not affect HPV in the cat. Several other investigators, when considering the lack of effect of saralasin acetate on hypoxic pressor responses, have concluded that A II is not the mediator of HPV (Allison and Stanbrook, 1980; McMurtry et al., 1976; Hales et al., 1977). Moreover, Rabinovitch et al. (1988) recently reported that A II infusion in rats both abolished the morphological and physiological changes induced by chronic hypoxia, and prevented acute pulmonary hypertension.

Both 5-hydroxytryptamine and histamine were at one time thought to be possible mediators of HPV. It quickly became apparent that 5-HT was a poor candidate in light of reports demonstrating that reserpine depletion of pulmonary 5-HT stores did not affect the hypoxic pressor response in the rat (Hauge and Melmon, 1968) or dog (Nayar et al., 1972), 5-HT plasma levels do not rise during hypoxia (Nayar et al., 1972), hypoxia and 5-HT have different sites of action in the pulmonary circulation (Glazier and Murray, 1971), and the inability of 5-HT antagonists to prevent HPV (Barer, 1966; Hauge, 1968b; Helgesen and Bjertnaes, 1986). Histamine received much more attention as a potential mediator of HPV, perhaps due to early reports of inhibition of the hypoxic pressor response following depletion of lung histamine content by compound 48/80 (Hauge and Melmon, 1968), attenuation of HPV by antihistamines (Hauge, 1968b), and of mast cell degranulation and histamine release under hypoxic conditions (Haas and Bergofsky, 1972).

However, Dawson et al. (1974) found that compound 48/80 eliminated HPV in the isolated cat lung, while not affecting lung histamine content or pressor responses to hypercapnia, 5-HT or norepinephrine. These data suggest that the inhibition of HPV reported by Hauge and Melmon (1968), which was attributed to histamine depletion, may have been due to some other action of compound 48/80. Hypoxia may stimulate histamine release in the dog (Tucker et al., 1976; Rengo et al., 1979), guinea-pig (Haas and Bergofsky, 1972), and man (Sudhakaran et al., 1979). In contrast, Paterson (1986) found that neither hypoxia nor anoxia affected either basal or A-23187-stimulated histamine release from dispersed porcine lung parenchymal cells. Further evidence against a role for histamine in HPV includes the observations that neither H_1 nor combined H_1 + H_2 blockade affects the hypoxic pressor response (Barer, 1966; Tucker et al., 1976; Ahmed et al., 1982), histamine and hypoxia act at different sites in the pulmonary circulation (Glazier and Murray, 1971), and in the pulmonary circulation precontracted by hypoxia, histamine infusion causes vasodilation (Tucker et al., 1976; Porcelli and Cutaia, 1988).

Although the experiments of Haas and Bergofsky (1972) suggested that mast cells might mediate HPV via the release of histamine, Tucker et al. (1977a) was not able to correlate mast cell density with the strength of the hypoxic pressor response in the calf, pig, rat, guinea-pig, dog or sheep, and the species least reactive to hypoxia (dog and guinea-pig) had the highest perivascular mast cell density. Furthermore, under hypoxic conditions, total mast cell hyperplasia was only seen in the calf, and perivascular mast cell proliferation was

observed only in the calf and pig (Tucker et al., 1977a). Bronchial, alveolar septal and systemic mast cell hyperplasia was not seen in any species, and in the guinea-pig, mast cell density actually decreased during hypoxia (Tucker et al., 1977a). Since perivascular mast cell density correlated with other indices of pulmonary hypertension (right ventricular hypertrophy, pulmonary arterial medial thickness and pressure), Tucker et al. (1977a) speculated that mast cell density might increase in response to hypertension, rather than mediating hypertension. A subsequent study found an inverse correlation between mast cell density and strength of HPV in cats, suggesting that mast cells might oppose HPV (Martin et al., 1978). In 1983, Zhu and colleagues examined the hypoxia-induced increase in right ventricular systolic pressure, right ventricular hypertrophy, and pulmonary vascular remodelling in normal mice (BALB/c) and a strain of mice which were mast cell deficient (W/W^V). These investigators found no difference in the effect of hypoxia on any of the measured indices between strains of mice (Zhu et al., 1983). Unfortunately, lung histamine levels were the same in both strains, preventing any conclusion about a role for histamine in the mediation of these changes (Zhu et al., 1983).

Mast cells are able to release many other biologically active substances beside histamine (Wasserman, 1980; Marom and Casale, 1983; Friedman and Kaliner, 1987), and several investigators have examined the effect of the mast cell stabilizer, disodium cromoglycate (DSCG), on the pulmonary hypoxic pressor response. Most of these studies have been performed in the awake sheep or lamb, and the common conclusion

is that DSCG effectively prevents acute HPV in these models (Ahmed et al., 1982, 1986; Taylor et al., 1986, 1988). However, the route of administration is important, as intravenous administration abolishes HPV (Ahmed et al., 1982; Taylor et al., 1986), whereas inhalation of the drug only partially prevented the response (Ahmed et al., 1986; Taylor et al., 1988). In contrast, Rhind et al. (1986) reported that in patients with hypoxic chronic bronchitis or emphysema, DSCG had no effect on PAP at rest or during exercise, suggesting that neither the chronic nor the exercise-induced increase in PAP was mast cell product related. In support of previous studies indicating histamine does not play a role in HPV, Rengo et al. (1979) reported that DSCG blocked hypoxia-induced histamine release in the anesthetized dog at a dose of 1 mg/kg, but a dose of 8 mg/kg was required to block HPV, suggesting that histamine release was not responsible for the increase in PVR.

In face of the overwhelming evidence that histamine does not mediate HPV, the observations which indicated that mast cell stabilization opposes HPV suggested that some other mast cell-derived product might be a potential mediator. Prostaglandins and LTs are both released from mast cells (Marom and Castle, 1983; Friedman and Kaliner, 1987; Wasserman, 1980; Paterson et al., 1976) and numerous other cell types present in the lung (Gryglewski et al., 1978; Salzman et al., 1980; Hanley, 1986; Paterson, 1986; Paterson et al., 1981; Cheng et al., 1987; Sautebin et al., 1985) and these compounds have been demonstrated to alter pulmonary vascular tone (Kadowitz et al., 1982; Hyman and Kadowitz, 1979; Sada et al., 1987; Ahmed et al., 1985; Smedegard et al., 1982; Hand et al., 1981; Voelkel et al., 1984).

Cyclooxygenase metabolites (eg. PGs), while clearly being able to modulate HPV, do not appear to mediate this response. Release of PGs, in particular prostacyclin (PGI_2) from rat lung (Voelkel et al., 1981) and dog lung homogeneates (Hamasaki et al., 1982) during hypoxia has been observed. Conzen et al. (1984), on the other hand, reported no change in plasma PGI_2 levels (measured as the stable metabolite, 6-keto-$PGF_{1\alpha}$) in the hypoxic pig, while the plasma levels of the major metabolite of $PGF_{2\alpha}$, 13,14-dihydro-15-keto-$PGF_{2\alpha}$, were elevated. Since $PGF_{2\alpha}$ is a vasoconstrictor, this observation might suggest an augmentation of HPV by a cyclooxygenase metabolite. However, most of the evidence obtained through the use of cyclooxygenase inhibitors indicates that cyclooxygenase metabolites either have little effect on HPV (Naeije et al., 1988; Rubin et al., 1985; Walker et al., 1982a) or attenuate the response (Vaage et al., 1975; Weir et al., 1976a; Hales et al., 1978; Ahmed et al., 1983; Sprague et al., 1984; Alexander et al., 1977). Rubin et al. (1985) reported that the administration of one of three different inhibitors of cyclooxygenase (indomethacin, meclofenamate, ibuprofen) or a combined cyclooxygenase/lipoxygenase inhibitor (BW755C) to anesthetized dogs had no effect on HPV. Walker et al. (1982a) reported comparable results in the conscious dog using meclofenamate and RO-20-5720. Similarly, Naeije and colleagues (1988) found that neither ibuprofen nor dazoxiben (a thromboxane synthetase inhibitor) affected pulmonary hemodynamics during hypoxia in normal subjects. Those studies which suggest the PGs oppose HPV have presented data which indicates that these compounds are capable of effects ranging from attenuation to complete prevention of the

response. For example, cyclooxygenase metabolites have been reported to attenuate the hypoxic pressor response in the isolated rat lung (Vaage et al., 1975; Weir et al., 1976a), anesthetized dog (Weir et al., 1976a), and conscious calves (Weir et al., 1976a). Within a particular species, however, there may be animals which are either weak or vigorous responders. Hales and colleagues (1978) found that cyclooxygenase inhibition with either aspirin or indomethacin, in anesthetized dogs, had no effect on HPV in those dogs classified as vigorous responders, while producing a fourfold enhancement of HPV in the weak responders. Ahmed et al. (1983) reported similar results in "nonresponder" and "responder" sheep while using indomethacin. This ability of cyclooxygenase metabolites to prevent HPV had been observed to occur in vitro by Alexander et al. (1977), prior to the in vivo studies. While investigating the labile nature of the hypoxic pressor response in the isolated canine lung, these researchers determined that the addition of aspirin or indomethacin to the perfusate could restore HPV and postulated that the generation of PGE_1 was responsible for the degeneration of the response (Alexander et al., 1977).

As it became clear that the PGs opposed HPV and therefore could not be mediators of the response, attention focussed on the LTs. Both the pulmonary parenchymal cells (Paterson et al., 1976, 1981; Sautebin et al., 1985; Cheng et al., 1987) and the pulmonary vasculature (Piper and Galton, 1984; Piper and Levene, 1986) are quite capable of releasing LTs. Initially, several studies from different laboratories indicated that the LTs might be the long sought after mediators of HPV. Ahmed and Oliver (1983) were the first to provide supporting

evidence for this hypothesis when they reported that DSCG prevented, while the LT receptor antagonist FPL 57231 both prevented and reversed, HPV in the conscious sheep. In the same year, Stenmark et al. (1983) found LT concentrations to be elevated in lung lavage samples obtained from infants with persistent pulmonary hypertension (in which hypoxia plays a major role). Morganroth et al. (1984a) and Matthay et al. (1984) subsequently reported similar observations using isolated hypoxic rat lungs, and in patients with the adult respiratory distress syndrome (in which hypoxia may be a contributing factor), respectively. Numerous studies then followed, claiming a role for the LTs in HPV in the rat (Morganroth et al., 1984b, 1985), lamb (Kulik et al., 1985; Schreiber et al., 1985; Raj and Chen, 1987), pig (Goldberg et al., 1985), and dog (Leeman et al., 1987). The most convincing evidence for a role of the LTs in HPV arises from studies in the rat or isolated rat lung (Morganroth et al., 1984a, 1984b, 1985), but the evidence from other species is rather weak, since in many of these studies, non-selective actions of the drugs in question were not ruled out (Kulik et al., 1985; Goldberg et al., 1985; Leeman et al., 1987), and/or the desired activity of LT synthesis inhibitors or antagonists was not demonstrated in the experimental preparation (Ahmed and Oliver, 1983; Schreiber et al., 1985; Goldberg et al., 1985; Raj and Chen, 1987; Leeman et al., 1987). For example, the LT receptor antagonist FPL 57231 often decreased pulmonary and/or systemic vascular resistance under normoxic conditions (Kulik et al., 1985; Goldberg et al., 1985; Raj and Chen, 1987; Leeman et al., 1987).

Aside from one study by Leffler et al. (1984) in which they reported that neither the LT synthesis inhibitors nordihydroguaiaretic acid (NDGA) or DECC, nor the LT receptor antagonist FPL 55712, inhibited the hypoxic pressor response in neonatal piglets, the early part of the eighties was dominated by the belief that LTs mediated HPV. However, opinions began to change in the late eighties as more studies appeared which were unable to demonstrate a role for LTs in this response. Several of these studies were performed using dogs or isolated canine lungs (Schuster and Dennis, 1987; Garrett et al., 1987; Lonigro et al., 1988), while others were performed using isolated ferret lungs (Gottlieb et al., 1988), pigs (McCormack and Paterson, 1988), or isolated porcine pulmonary vascular smooth muscle (Miller et al., 1988). The report by Lonigro et al. (1988) pointed out the importance of determining cause and effect before ascribing a role for a substance(s) in any phenomenon, not just HPV. These investigators found that LT concentrations increased in lung lavage fluid from anesthetized, hypoxic dogs (Lonigro et al., 1988). In animals treated with DECC, the increase in LT content of the lavage fluid was prevented, whereas the hypoxic pressor response was unaffected (Lonigro et al., 1988). These data suggest that rather than hypoxia stimulating LT release, which then elicits the pulmonary vasoconstriction, LT release is a result of some aspect of the phenomenon. However, Paterson et al. (1988b) found that the sensitivity of isolated porcine pulmonary artery and vein to added LTs was increased by lowering the Po_2, suggesting that LT release per se may not be required for these compounds to elicit HPV.

Data obtained from investigations of LT release under hypoxic conditions have also suggested that it is unlikely the LTs play a role in HPV. This is due to the observation that decreased oxygen tension depresses LT release from pulmonary parenchymal cells (Paterson, 1986; Peters et al., 1986). Paterson (1986) was unable to demonstrate spontaneous LT release from dispersed porcine parenchymal lung cells under conditions of normoxia, hypoxia or anoxia. When the cells were stimulated with A-23187, anoxia inhibited the ionophore-stimulated LT release (Paterson, 1986). Similarly, Peters and colleagues (1986) observed that reducing the buffer Po_2 from 161 to 54 torr did not elicit LT release from human lung fragments. In addition, if the lung fragments were stimulated to release LTs with goat antihuman IgE, hypoxia inhibited the LT release by 81% (Peters et al., 1986). These results are perhaps not surprising when one considers that the 5-lipoxygenation of AA requires molecular oxygen. Furthermore, even if LTs were somehow released by hypoxia in vivo, the primary site of action of these compounds appears to be in the pulmonary veins rather than the arteries (Noonan et al., 1986; Garcia et al., 1987; Schellenberg and Foster, 1984; Kadowitz and Hyman, 1984; Hanna et al., 1981; Burka and Eyre, 1977), the latter of which are generally believed to be the major site of HPV.

Arachidonic acid metabolism via the cytochrome P-450 monooxygenase system (McGiff and Carroll, 1987) may also produce compounds capable of mediating the hypoxic pressor response. A polycyclic aromatic hydrocarbon (PAH)-inducible form of cytochrome P-450 metabolizes AA to a product(s) with biological activity including the relaxation of

vascular smooth muscle (Schwartzman et al., 1985; Proctor et al., 1987) and there is evidence suggesting such a pathway exists in the lung. Domin et al. (1984) reported that a cytochrome P-450 isozyme which is orthologous to the well characterized, PAH-inducible isozyme 6 of rabbit lung is present in guinea-pig pulmonary microsomes and is induced by PAH-type compounds. Other investigators have localized a cytochrome P-450 in the cell membrane and pinocytotic vesicles of endothelial cells of rabbit lung (Serabjit-Singh et al., 1988) and a PAH-inducible form of cytochrome P-450 was found to be present in the endothelium of pulmonary arteries and veins of this species (Dees et al., 1982).

Although the cytochrome P-450 metabolites of AA have been suggested to play a role in controlling pulmonary vascular tone (Pinto et al., 1986), their possible involvement in HPV has not been thoroughly investigated. Duke and Killick (1952) were perhaps the first to obtain indirect evidence of a role for cytochrome P-450 metabolites in HPV (although this was not their intention), when they observed that ventilating isolated cat lungs with 100% CO caused pulmonary vasodilation, whereas ventilation with 100% N_2 caused vasoconstriction. The opposite effects of the two gases, both of which would produce severe hypoxia or anoxia, could be explained if a cytochrome P-450 metabolite were responsible for eliciting HPV and the production of this metabolite were prevented by CO. Sylvester and McGowan (1978) reported that HPV in perfused porcine lungs was blunted by metyrapone, SKF-525A (proadifen) and CO (all inhibitors of cytochrome P-450). However, each of these agents either altered

normoxic pulmonary vascular tone or non-selectively depressed $PGF_{2\alpha}$-induced contractions, thus precluding an unambiguous interpretation of the data. Miller and Hales (1979) on the other hand, using anesthetized dogs, found that metyrapone and CO selectively inhibited HPV, suggesting that a cytochrome P-450 metabolite(s) was responsible for mediating the response. Chang et al. (1986) also concluded that a cytochrome P-450 dependent reaction was involved in HPV when they observed that a suicide inhibitor of cytochrome P-450 (1-aminobenzotriazole) inhibited the pressor response to hypoxia in the isolated rat lung.

There is, however, at least one major problem with the hypothesis that a cytochrome P-450 metabolite(s) mediates HPV. At present, there does not appear to be any evidence suggesting the existence of a vasoconstrictor cytochrome P-450 metabolite; rather, all the metabolites tested to date demonstrate vasodilator activity (Schwartzman et al., 1985; Proctor et al., 1987; Pinto et al., 1986; Carroll et al., 1987).

Finally, the vascular endothelium is capable of regulating vascular tone and may either modulate or mediate the hypoxic pressor response. The endothelium is capable of releasing vasodilator substances such as PGI_2 and endothelium-derived relaxing factor (EDRF) (Furchgott, 1983) [which was recently proposed to be nitric oxide by Palmer et al. (1987)], and a vasoconstrictor peptide known as endothelin (Yanagisawa et al., 1988). In addition, hypoxia stimulates the release from the endothelium of a pulmonary smooth muscle mitogen(s) (Vender et al., 1987), which might contribute to the

remodelling of the pulmonary circulation during chronic hypoxia. The endothelium is also involved in the systemic vasodilator response to hypoxia (Busse et al., 1983, 1984), and the anoxic facilitation of responses to vasoconstrictor agonists in some systemic vessels (Katusic and Vanhoutte, 1986; De Mey and Vanhoutte, 1983; Rubanyi and Vanhoutte, 1985). Thus release of endothelium-derived factors during hypoxia may modulate or mediate the hypoxic pressor response.

Previous attempts to describe the role of the endothelium in HPV have produced contradictory findings. Hill and Rounds (1983) observed that α-naphthylthiourea (ANTU), a compound which (among other actions) induces endothelial injury, enhances vascular reactivity to hypoxia in isolated rat lungs. In addition, Madden et al. (1986a) reported that decreases in oxygen tension inhibited PGI_2 production from cultured bovine pulmonary artery endothelial cells, suggesting that hypoxia might elicit pulmonary vasoconstriction via inhibition of the production of an endothelium-derived vasodilator. In contrast, several other studies indicate that endothelial damage inhibits, not augments, hypoxic and anoxic contractions (Rodman et al., 1987; Madden et al., 1986b; Holden and McCall, 1984; Rubanyi and Vanhoutte, 1985), thereby implying that the endothelium may release a contractile mediator under conditions of reduced oxygenation. Although such a contractile compound (endothelin) was recently identified by Yanagisawa et al. (1988), the persistence of the contraction elicited by this substance is incompatible with the rapid fall in PVR in vivo following resoration of normoxic conditions, which suggests that endothelin is unlikely to be directly involved in HPV. In addition,

endothelin has not been demonstrated to be released from hypoxic endothelial cells, and O'Brien et al. (1987) recently reported that hypoxia or anoxia had no effect on the release of unidentified constrictor activity from cultured bovine pulmonary artery endothelial cells.

Preliminary studies of the effect of EDRF inhibitors on HPV have yielded contradictory results. Rodman et al. (1987) reported that methylene blue and hemoglobin blunted the contractions of isolated rat pulmonary arteries to decreased oxygenation. In order to obtain these contractions, however, precontraction of the arteries was required, and when the same group repeated the studies in isolated rat lungs, they observed that the EDRF inhibitors augmented the hypoxic increase in perfusion pressure (Yamaguchi et al., 1987). More recently, Brashers et al. (1988) presented evidence that NDGA, ETYA, and hydroquinone (all of which may inhibit or inactivate EDRF) augmented the hypoxic pressor response in isolated rat lungs.

Since the pulmonary arteries are usually thought to be the major site of HPV, it is not surprising that the role of the endothelium in pulmonary venous responses to lowered oxygen tension has received little attention. Although De Mey and Vanhoutte (1982) did not examine the effects of anoxia alone on isolated canine pulmonary veins, they found that anoxia enhanced the contractile response of this vessel to norepinephrine. If the endothelium was removed, anoxia caused a relaxation of the norepinephrine-precontracted pulmonary veins (De Mey and Vanhoutte, 1982), suggesting the release of a contractile factor from the endothelium. However, until now no

studies have specifically examined the role of the endothelium in pulmonary venular responses to decreased Po_2.

MATERIALS AND METHODS

Male Hartley strain guinea-pigs (260 - 420 g) (Charles River Canada Inc., St. Constant, Que.) were anesthetized with sodium pentobarbital, exsanguinated and the lungs removed. The lungs were perfused via the pulmonary artery with modified Krebs buffer [composition (mM): NaCl (118.3); KCl (4.7); $MgSO_4$ (1.2); KH_2PO_4 (1.2); $NaHCO_3$ (22.1); dextrose (11.1); $CaCl_2$ (2.5); calcium disodium EDTA (0.026)] until a blood-free perfusate was obtained. Pulmonary venules were obtained from the middle lobe of the right lung or the caudal segment of the cranial lobe of the left lung, while pulmonary arteries were obtained from the caudal lobe of the left or right lung. Pulmonary arteries or femoral venules, if used, were obtained from the same animal. Vessel segments (1.5 - 2.8 mm in length) were mounted on individual myographs adapted from those described by Hogestatt et al. (1983). Two stainless steel wires (126 μm diameter), one connected to a micromanipulator (Marzhauser MM33, Fine Science Tools, North Vancouver, B.C.), the other connected to an FTO3 isometric force transducer (Grass Instrument Co., Quincy, MA.), were inserted through the lumen of the vessel. During mounting, the vessels were handled very carefully in order to minimize the loss of endothelium. The mounted vessels were suspended in 5 ml organ baths containing modified Krebs buffer, which were equilibrated with 15% O_2 / 5% CO_2 / 80% N_2 (bath Po_2: 110 torr) and maintained at 37°C. When bath Po_2 was altered by changing the gas mixture, full equilibration was achieved within 30 sec. All gas mixtures contained 5% CO_2 in order to maintain the buffer pH between 7.36 and 7.42. Organ bath Po_2 was measured with

a Clark-style oxygen electrode (model 731) and Chemical Microsensor (model 1201) (Diamond Electro-Tech, Ann Arbor, MI.).

Vessels were allowed to equilibrate under minimal tension (50 mg) for 1 hr. The tension of the vessels was then gradually increased over 20 min to the optimal tension (as assessed by length-tension studies -- see below) for isometric recording. Vessels were allowed to equilibrate at their optimal tension for a further 40 min before experiments were begun. The bath fluid was changed every 15 - 20 min during the equilibration period and between experiments.

Initially, length-tension studies were conducted to determine the optimal tension for isometric recording. Vessel radius was incrementally increased from the resting effective lumen radius (ELR), and the contractile response to a standard dose of KCl (pulmonary venule: 35 mM; pulmonary artery: 35 mM; femoral venule: 45 mM) recorded at each radius. (Length-tension data for the pulmonary and femoral venule are presented in Chapter 1; the corresponding data for the pulmonary artery are in Appendix I, Figs. N and O).

The response of the venules to lowered oxygen tension was assessed by exposing the venules to hypoxia (bath Po_2: 25 torr) or anoxia (bath Po_2: 0 torr) for 10 min periods. This time was sufficient for the contraction to reach a stable plateau. In order to document the duration of the response, the hypoxic or anoxic exposure was continued for up to 4.5 hrs in some experiments. Following hypoxic/anoxic challenge, thirty min were allowed for recovery before further hypoxic/anoxic challenges.

pH and calcium experiments:

During experiments in which pH was altered, the bath fluid was replaced with fluid of the desired pH 30 min prior to lowering the oxygen tension. The pH of the buffer solution was lowered or raised by the addition of HCl or $NaHCO_3$, respectively. Acidotic bath fluid did not change baseline tension of the pulmonary venules. However, alkaline bath fluid caused an increase in tension which was mechanically readjusted to the original baseline before exposure of the pulmonary venules to reduced oxygenation.

To determine if the response of pulmonary venules to lowered oxygen tension was calcium-dependent, a cross-over experiment was performed using 2 venules for each experiment. One of the venules was bathed in "calcium-free" buffer (ie. $CaCl_2$ was not added to the buffer), while the other venule (the control) remained in normal buffer. Following the hypoxic and anoxic challenges, the "calcium-free" venule was changed to normal buffer, the control venule was changed to "calcium-free" buffer, and the experiment was repeated. Data obtained from venules in identical buffer solutions were pooled.

Co-incubation, transfer, and "add-back" experiments:

In co-incubation experiments, two pulmonary venules were sutured end-to-end and placed around a femoral venule (obtained from the same animal) which had been mounted on one of the myographs. The pulmonary venules did not directly contribute to, or affect the tension generated by the femoral venule when the vessels were mounted in this manner. A paired femoral venule mounted on a separate myograph served

as a control. Following the equilibration period, control femoral venules and pulmonary venule/femoral venule preparations were subjected to repeated hypoxic and anoxic challenges as previously described.

Additional experiments were performed in which bath fluid from an anoxic pulmonary venule was transferred to either an anoxic femoral venule, or a pulmonary venule under normoxic or hypoxic conditions. If bath fluid was to be transferred to a paired femoral venule, the transfer was performed during the third anoxic challenge, so that the contraction of the femoral venule to the anoxic stimulus was minimal. Gas-tight syringes were used to transfer the bath fluid.

In "add-back" experiments, bath fluid was withdrawn from an anoxic pulmonary venule and subsequently returned to the same venule after the vessel tone had stabilized. Anoxic conditions were maintained in the organ bath during this time.

Techniques used to produce endothelial damage:

Two methods were used to induce endothelial damage: 1) perfusion with collagenase (2 mg/ml) for 15 min at 25°C (modified from Jaffe et al., 1973), or 2) perfusion with a mixture (HX/XO) of hypoxanthine (5 mM) and xanthine oxidase (0.05 U/ml) for 30 min at 25°C (modified from Toth et al., 1984). Following removal of the lungs from the thoracic cavity, the vessels were cannulated with a polypropylene catheter, through which the enzyme solutions were infused at a flow rate of 0.07 - 0.14 ml/min. Pulmonary venules were perfused in a retrograde fashion, while pulmonary arteries were perfused anterogradely. If the

effects of superoxide dismutase (SOD) or catalase (CAT) on HX/XO perfusion were to be determined, SOD or CAT were initially infused into the vessel for 1.5 min at a concentration of 40 μg/ml or 323 μg/ml, respectively. HX/XO solution, which contained SOD or CAT in the same concentration, was then infused for a further 30 min. For both collagenase and HX/XO treatment, after enzyme perfusion for the specified time, the reaction was terminated by flushing with buffer. The vessel was then removed from the lung and mounted on a myograph as previously described.

When infused individually, HX, XO, SOD or CAT had no effect on the reactivity of pulmonary arteries or venules to any agent tested. In addition, none of the enzymes, either alone or in combination, altered the length-tension characteristics or the maximal response to KCl of the pulmonary artery or venule.

HX, XO, SOD and CAT were applied individually or in combination to resting or precontracted [10 μM $PGF_{2\alpha}$] pulmonary venules in the organ bath to determine whether these agents affected venular tone in the concentrations used. HX, SOD and CAT had no effect. In resting venules, XO elicited a small contraction, whereas in precontracted venules, XO caused a rapid, transient (< 5 sec) relaxation, followed by an equally rapid return of tone to slightly above the precontracted baseline. This effect was due to the ammonium sulfate contained in the XO stock solution, since the same final concentration (4.3 mM) of ammonium sulfate alone reproduced the transient responses observed with XO.

Spectrophotometric determination of rate of superoxide generation from the hypoxanthine/xanthine oxidase mixture:

The *in vitro* rate of superoxide production by the HX/XO mixture was determined spectrophotometrically (Perkin-Elmer 559A UV/VIS Spectrophotometer, Perkin-Elmer (Canada) Ltd., Downsview, Ont.). The reduction of cytochrome c^{3+} was monitored at 550 nm and 25°C, and an extinction coefficient of 21,000 cm^2/mM (Massey, 1959) was used to calculate the rate of superoxide generation. Under the conditions of this study (HX/XO in modified Krebs buffer as above, 25°C), the HX/XO mixture generated superoxide at a rate of 4.0 ± 0.2 nmol/ml/min.

Functional assessment of endothelial damage:

Several putative endothelium-dependent vasodilators (acetylcholine, bradykinin, A-23187) were used to determine whether perfusion with collagenase or HX/XO altered the dilator effects of these agents on the pulmonary arteries or venules. Vessels were precontracted with either 35 mM KCl (pulmonary artery) or 10 μM $PGF_{2\alpha}$ (pulmonary venule), following which a cumulative concentration-response curve was obtained for each vasodilator agent.

Endothelium-derived relaxing factor inhibitor experiments:

The effect of two putative EDRF inhibitors, methylene blue and gossypol, on hypoxic and anoxic pulmonary venular contractions was assessed. In each case, the drug was added to the organ bath (methylene blue: 10 μM, 15 min; gossypol: 5 μM, 30 min) before reducing the oxygen tension. Both gossypol and methylene blue

prevented acetylcholine-induced relaxations of the pulmonary artery segments, although the acetylcholine-, bradykinin- and A-23187-induced relaxations of pulmonary venular segments were not affected.

Studies on the response of pulmonary venules to leukotrienes C_4 and D_4 under normoxic, hypoxic and anoxic conditions:

Concentration-response curves to LTD_4 and LTC_4 were obtained by cumulative addition of increasing amounts of LT to the organ bath. When these experiments were performed under conditions of decreased Po_2, the level of venular tone which was due solely to the hypoxic or anoxic stimulus was used as the baseline from which the response to the LT was measured. Concentration-response curves to the LTs were expressed as a percent of the venular contraction to a maximal KCl stimulus (120 mM).

Lipoxygenase, cyclooxygenase and cytochrome P-450 inhibitor experiments:

In order to determine the contribution of cyclooxygenase, lipoxygenase, or cytochrome P-450 metabolites to pulmonary venular hypoxic and anoxic contractions, the cyclooxygenase inhibitors indomethacin or ibuprofen, the 5-lipoxygenase inhibitors NDGA or U-60257B, the LT receptor antagonists FPL 55712 or LY 163443, or the cytochrome P-450 inhibitors metyrapone and SKF-525A were added to the organ bath before decreasing the bath Po_2. Each drug was incubated with the pulmonary venules for 15 min, except for indomethacin,

metyrapone and SKF-525A, which were incubated for 20, 30 and 30 min, respectively.

In order to confirm the activity of the LT receptor antagonists FPL 57231 and LY 163443, these compounds were incubated with pulmonary venules for 15 min prior to performing a LT concentration-response study. In separate experiments, either FPL 57231 or LY 163443 was cumulatively added to pulmonary venules which were precontracted with 1 μM of LTC_4 or LTD_4.

Assessment of inhibition of pulmonary venule leukotriene synthesis by nordihydroguaiaretic acid and U-60257B:

The activity of the 5-lipoxygenase inhibitors NDGA and U-60257B was confirmed by radioimmunoassay (RIA) (TRK.910 Leukotriene $C_4/D_4/E_4$ [^{3}H] assay system, Amersham Canada Ltd., Oakville, Ont.). Six to eight pulmonary venules were obtained as previously described. The parenchyma was removed from each venule, then each venule was cut in segments approximately 1 mm in length. The venule segments were randomly assigned to one of two polypropylene tubes, each containing 1 ml of Krebs buffer: a "control" tube [preincubation with the drug vehicle for 15 min, followed by stimulation with A-23187 (6 μM) for 20 min]; or a "drug" tube (preincubation with NDGA or U-60257B for 15 min, followed by stimulation with A-23187). The solutions were incubated at 37°C and under normoxic (Po_2: 110 torr) conditions. One hundred μl of the supernatant from each treatment tube was added to duplicate RIA tubes containing 100 μl of tracer, antiserum and assay buffer. For each experiment, a separate standard curve was also

constructed using duplicates of 12.5, 25, 50, 100, 200, 400 and 800 pg of cold LTC_4 per tube, along with 100 μl of tracer, antiserum and assay buffer. Duplicate total counts and non-specific binding tubes contained 100 μl of tracer and 300 μl of assay buffer per tube, while duplicate zero standard tubes contained 100 μl of tracer and antiserum, and 200 μl of assay buffer. All tubes were incubated overnight at 4°C. After the overnight incubation, 250 μl of charcoal was added to each standard, sample, non-specific binding and zero standard tube, 250 μl of assay buffer was added to the total counts tubes, and the tubes were centrifuged. The supernatants from each tube were then decanted into scintillation vials along with 10 ml of ACS II scintillant fluid (Amersham Canada Ltd., Oakville, Ont.). Each vial was then counted for 4 min in a Beckman LS 7800 β-scintillation counter (Beckman Instruments Canada Inc., Mississauga, Ont.). The total quantity of sulfidopeptide LTs in each sample was determined using a standard curve of % Bound/Free versus log [pg of LTC_4/tube]. The quantity of LTs was then normalized for the wet weight of the tissue in each experimental tube.

Studies of leukotriene release from pulmonary venules under normoxic and anoxic conditions:

Leukotriene release from pulmonary venules under normoxic and anoxic conditions was also assessed by RIA. Pulmonary venules were prepared for RIA as previously described and randomly assigned to one of three polypropylene tubes, each containing 1 ml of Krebs buffer: a "control" tube (Po_2: 110 torr, incubation for 30 min); an "A-23187"

tube (Po_2: 110 torr, stimulated with 6 μM A-23187 for 20 min); or an "anoxia" tube (Po_2: 0 torr, incubation for 30 min). All solutions were incubated at 37°C. One hundred μl of the supernatant was then withdrawn and subjected to RIA as previously described.

Effect of cytochrome P-450 induction with β-naphthoflavone on pulmonary venular contractions induced by hypoxia and anoxia:

The following procedures and experiments, except for the isolated pulmonary venule studies, were kindly performed by Miss Kim Woodcroft of the Department of Pharmacology and Toxicology, U.W.O. To study the effect of cytochrome P-450 induction on pulmonary venular contractions induced by decreased Po_2, guinea-pigs were injected daily for 6 days with β-naphthoflavone (β-NF) (100 mg/kg, ip) dissolved in corn oil. Control animals received injections of equal volumes of corn oil. The last injection was administered 24 hrs before sacrifice, and food and water were given ad libitum. Animals were sacrificed by CO_2 inhalation and exsanguinated. Segments of pulmonary venule for isometric studies were obtained as previously described. The remaining lung tissue was placed in phosphate-buffered isotonic (1.15%) KCl solution, pH 7.4. Microsomes were prepared as previously described (Mathews et al., 1985) and cytochrome P-450 concentrations were measured by the dithionite difference technique of Estabrook et al. (1972) using a Beckman DU-65 Spectrophotometer (Beckman Instruments Canada Inc., Mississauga, Ont.). The method of Lowry et al. (1951) was used to measure protein. 7-Ethoxyresorufin O-deethylation (7-ERF) activities were measured fluorimetrically,

essentially as described by Burke and Mayer (1974). The reaction mixture, prepared in a fluorimeter cuvette, contained 2 ml of potassium phosphate buffer (0.1 M, pH 7.45), 25 μl of microsomal suspension (1 mg protein/ml) and 4.0 μl of 7-ERF in DMSO to give a final 7-ERF concentration of 1.0 μM. Fluorescence was recorded at an excitation wavelength of 550 nm and an emission wavelength of 585 nm with a Perkin-Elmer LS-5B Luminescence Spectrometer (Perkin-Elmer Canada Ltd., Downsview, Ont.). The reaction was started by the addition of 100 μl of NADPH (2 mM) and was run at 37°C. Resorufin (5 μl of 3 μM solution in DMSO) was added as an internal standard after the reaction was continuously monitored for 1-5 min.

Theoretical Considerations:

The ELR of each venule was determined from the formula:

$$ELR = L / 2\pi$$

where r (the internal circumference of the vessel) is defined as:

$$L = 2f + d(\pi - 2)$$

f is the distance between the outer limits of the wires and d is the diameter of the wires (Hogestatt et al., 1983). The mean ELR for the vessels used in the present study were 119 ± 1 μm (pulmonary venules; n=252), 174 ± 5 μm (pulmonary arteries; n=44) and 184 ± 7 μm (femoral venules; n=42).

The active force (F) recorded by the isometric transducer (in mg) was related to the circumferential wall tension (WT) of the vessel by using the formula:

$$WT = F / 2e$$

where e is the vessel length in mm (Hogestatt et al., 1983). The compliance of this system was 20 μm per gram of applied tension and the wires remained parallel at all tensions.

Histology:

To confirm the presence of endothelial cells, randomly selected venules were stained with toluidine blue, sectioned transversely, and the sections examined using light microscopy. Endothelial cell loss from each vessel segment was kindly assessed by Dr. Ian Craig, Department of Pathology, U.W.O. A Zeiss Videoplan Computer (Carl Zeiss Canada Ltd., Don Mills, Ont.) was used to measure the percent of endothelium remaining in a minimum of eight transverse sections from each venule.

The structural integrity of the endothelium was also assessed using scanning electron microscopy (EM). For scanning EM, vessel segments were fixed in phosphate-buffered 2% glutaraldehyde, followed by 1% osmium tetroxide, and dehydration in ethanol. Segments were opened longitudinally and pinned on paraffin between the glutaraldehyde and osmium tetroxide steps. These preparations were then desiccated in a critical point dryer and sputter-coated with gold-palladium before being examined with a Hitachi S-650 scanning electron microscope. In order to assess the degree of endothelial pitting, 10 fields were randomly chosen for each treated or control venule and the number of pits counted at a magnification of 2,500X. The total number of pits in the ten fields was then averaged and expressed as number of pits/μm^2.

Drugs and solutions:

β-Naphthoflavone (Aldrich Chemical Co., Milwaukee, WI.); SKF-525A (Biomol Research Laboratories, Plymouth Meeting, PA.); superoxide dismutase (7874 U/mg protein) (Boehringer Mannheim Canada, Dorval, Que.); A-23137 (Calbiochem, La Jolla, CA.); methylene blue (Fisher Scientifi ., Toronto, Ont.); collagenase (274 U/mg protein) (Gibco Laboratories, Burlington, Ont.); 7-ethoxyresorufin (Molecular Probes, Eugene, ILL.); resorufin (Pierce Chemical Co., Rockford, ILL.); acetylcholine iodide, histamine dihydrochloride, 5-hydroxytryptamine creatinine sulfate complex, bradykinin triacetate salt, prostaglandin $F_{2\alpha}$ tris salt, gossypol, indomethacin, dimethyl sulfoxide (DMSO)(Grade I), reduced nicotinamide adenine dinucleotide phosphate (NADPH), nordihydroguaiaretic acid (NDGA), 2-methyl-1,2-di-3-pyridyl-1-propanone (metyrapone), tris(hydroxymethyl)aminomethane (Trizma base), hypoxanthine, xanthine oxidase (grade I, 27 U/ml), catalase (C-10 from bovine liver, 3100 U/mg protein), cytochrome c^{3+} (type III from horse heart) (Sigma Chemical Company, St. Louis, MO.); and ibuprofen (Upjohn Co., Kalamazoo, MI.) were purchased from the manufacturers. FPL 57231, U-60257B, and LY 163443 were kindly donated by Mr. P. Sheard (Fisons Pharmaceuticals, Leicestershire, UK), Dr. Michael Bach (Upjohn Co., Kalamazoo, MI.), and Dr. Jerome Fleisch (Eli Lilly and Co., Indianapolis, IN.), respectively. Leukotriene C_4 and D4 were gifts from Dr. Tom Jones (Merck-Frosst, Dorval, Que.). Indomethacin was dissolved in an equimolar concentration of Na_2CO_3 in distilled water and U 60257B was dissolved in distilled water with equal amounts of tris(hydroxymethyl)aminomethane. Gossypol, NDGA, A-23187, and β-NF

were dissolved in DMSO, 50% ethanol, 100% ethanol, and corn oil, respectively. Final organ bath concentrations for DMSO and ethanol did not exceed 0.05%, at which concentration DMSO and ethanol did not affect vascular reactivity. Xanthine oxidase was supplied as a suspension in 2.3 M $(NH_4)_2SO_4$. All other drugs were dissolved in buffer or distilled water and diluted as necessary in buffer. Drug concentrations were expressed as the final concentration to which the vessel was exposed.

Statistical analysis:

During any given experiment, the response of pulmonary venules to decreased Po_2 was highly consistent; however, responses varied 2-3 fold between experiments. Therefore, paired comparisons were always performed. Paired data were analyzed using the Mann-Whitney nonparametric test (Tallarida and Murray, 1981), with a Bonferroni correction if multiple comparisons were performed (Wallenstein et al., 1980). Dose-response data were analyzed by ANOVA; pairwise comparisons using Tukey's test were performed if the ANOVA revealed significant differences (Wallenstein et al., 1980). A p value of 0.05 or less was considered significant. All data are reported as the mean ± SEM.

Chapter 1: THE RESPONSES OF ISOLATED GUINEA-PIG PULMONARY VENULES TO HYPOXIA AND ANOXIA

RESULTS

Length-tension characteristics:

The length-tension characteristics of the vessels are shown in Figure 2 and Appendix I (Figs. A and B). The increase in ELR which yielded the maximum change in wall tension of the pulmonary venules was 280 μm, which corresponded to a resting tension of 390 mg (123 mg/mm). The femoral venules, unlike the pulmonary venules, did not show a distinct peak ELR for a maximal change in wall tension. Effective lumen radii 280 - 405 μm above the resting ELR of the femoral venules provided the maximum response to the EC_{50} of KCl. A resting tension of 290 mg (98 mg/mm) was chosen to be used for all further experiments on the femoral venules (corresponding to an increase in ELR of 375 μm).

Response of pulmonary and femoral venules to hypoxia and anoxia:

The pulmonary and femoral venules differed both quantitatively and qualitatively in their response to hypoxia and anoxia (Fig. 3, Appendix I: Fig. C). Under conditions of hypoxia, pulmonary venules consistently developed a sustained increase in tone. In contrast, the femoral venules did not respond to hypoxia at any time. When exposed to repeated anoxic challenges, the pulmonary venule contractions were repeatable and significantly ($p < 0.001$) larger than the contractions of paired femoral venules over all four exposures. The sustained anoxic contraction of the femoral venules in response to the first exposure to anoxia was less than 20% of the pulmonary venule

FIGURE 2. Effect of increasing effective lumen radius (ELR) on the developed (change in) wall tension in pulmonary (○) and femoral (□) venules under normoxic (15% O_2) conditions. Vessels were stimulated with an EC_{50} concentration of KCl (35 mM: pulmonary venule; 45 mM: femoral venule). The results are means ± SEM (n=6).

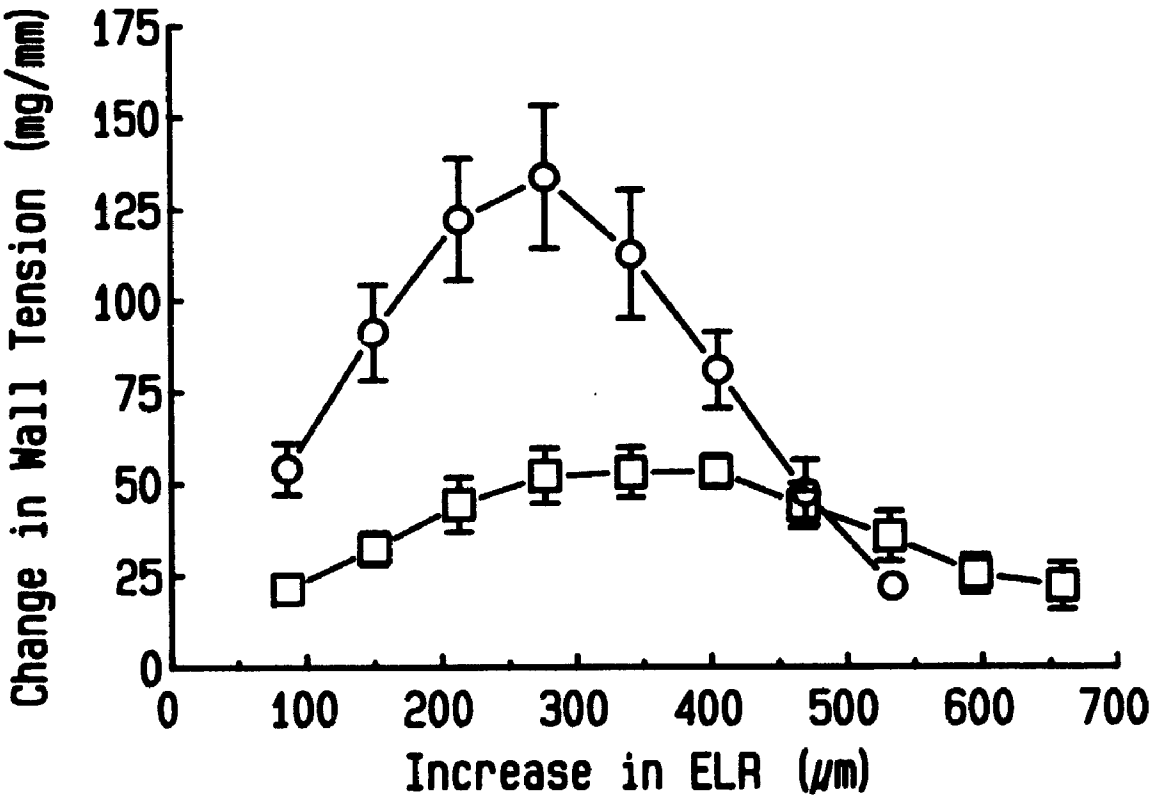
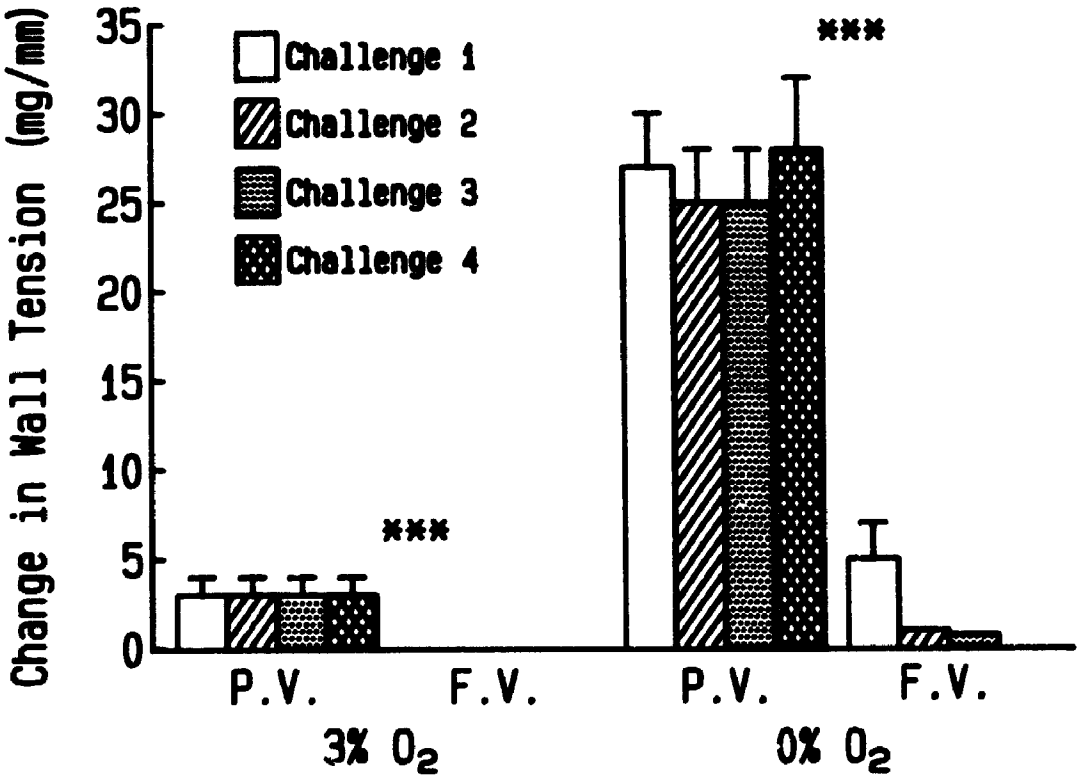

Change in Wall Tension (mg/mm)
175
150
125
100
75
50
25
0
0
100
200
300
400
500
600
700
Increase in ELR (μm)

FIGURE 3. Effects of hypoxia (3% O_2) or anoxia (0% O_2) on pulmonary (p.v.) and femoral (f.v.) venules from the same animal. Four sequential challenges of hypoxia followed by anoxia were performed on each vessel. The results are means ± SEM (n=6) and significant differences between p.v. and f.v. responses, as well as between p.v responses and zero (baseline), for each challenge are as indicated (*** $p < 0.001$).


Change in Wall Tension (mg/mm)
35
30
25
20
15
10
5
0
Challenge 1
Challenge 2
Challenge 3
Challenge 4
***
***
P.V.
F.V.
P.V.
F.V.
3% O_2
0% O_2

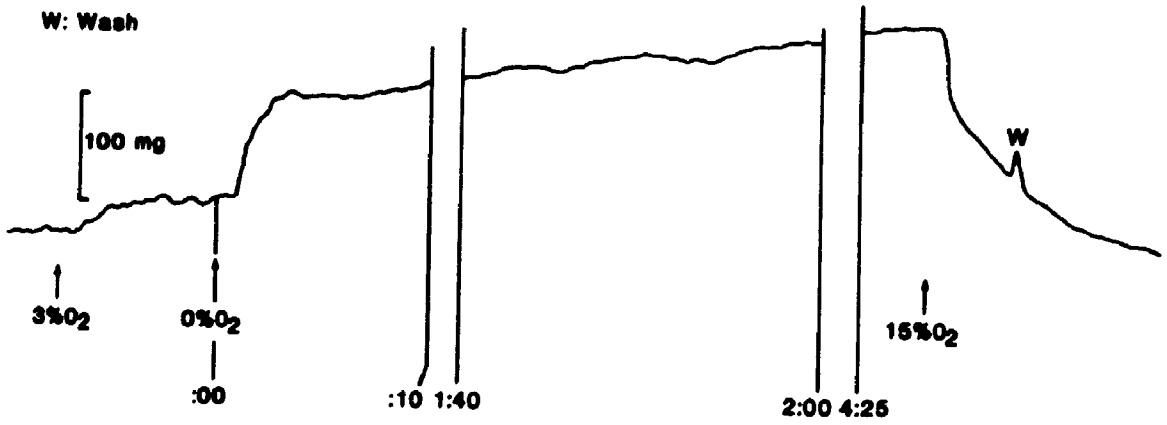
contraction, and unlike the pulmonary venules, the response of femoral venules could not be reproduced with further anoxic challenges. Qualitatively, pulmonary and femoral venules rapidly contracted when exposed to anoxia (within one minute after the gas mixture was changed). However, the pulmonary venules reached a stable plateau (27 ± 3 mg/mm), whereas the tone of the femoral venules peaked (17 ± 6 mg/mm) and then quickly declined to a lower, somewhat less stable plateau (5 ± 2 mg/mm)(n=10). This biphasic response of the femoral venules was seen only during the first anoxic challenge. The response (if any) to subsequent challenges was qualitatively similar to that of the pulmonary venules.

Characteristics of pulmonary venule hypoxic and anoxic contractions:

The pulmonary venule response to lowered oxygen tension was rapid in onset, persistent, and rapidly reversed by restoration of normoxia (Fig. 4). Pulmonary venules maintained their increased tone under hypoxic or anoxic conditions. Under anoxic conditions, tone was maintained for up to 4.5 hrs and rapidly returned to baseline when the bath was re-oxygenated. Following exposure of the venules to prolonged anoxia, the response to histamine was unchanged.

The response of venules to replacement of bath fluid during anoxia varied greatly, from no change in the contractile response in some preparations, to complete ablation of the response in others. If anoxic venular tone was decreased by washing, and the anoxic conditions were maintained, the contraction gradually returned.

FIGURE 4. Representative tracing of the response of a pulmonary venule to hypoxia (3% O_2) or anoxia (0% O_2) showing venular tension as a function of time. Venules were pre-equilibrated with 15% O_2 before producing conditions of hypoxia or anoxia.


W: Wash
100 mg
W
3%O2
0%O2
:00
:10
1:40
2:00
4:25
15%O2

However, the restoration of the response was often incomplete, varying from 60 - 100% of the original contraction.

The requirement of hypoxic and anoxic contractions for extracellular calcium ions is shown in Figure 5. Both responses were significantly ($p < 0.03$) depressed in buffer from which the $CaCl_2$ was omitted. The hypoxic and anoxic contractions also appeared to be dependent on pH (Table 1). Increasing the pH of the bath fluid enhanced hypoxic and anoxic contractions, while lowering the pH had the opposite effect.

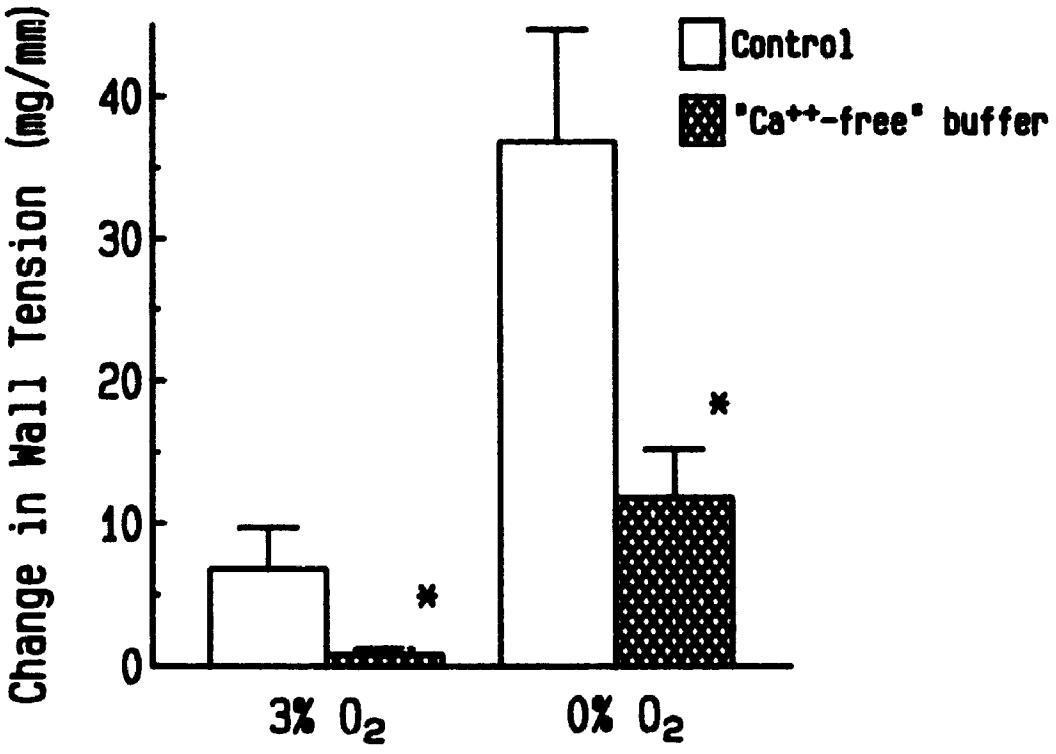
Role of the parenchyma in the response of pulmonary venules to lowered oxygen tension and reference agonists:

As illustrated in Figure 6, removal of the parenchymal cuff around the pulmonary venule had no significant effect on the hypoxia- or anoxia-induced contractions. Removal of the parenchyma also had no effect on the contractile response of pulmonary venules to 5-HT and KCl, or the dilator response to acetylcholine (Table 2, Appendix I: Figs. H-J). The maximum histamine- and $PGF_{2\alpha}$-induced contractions were modestly, but significantly ($p < 0.05$) altered following parenchymal removal (Table 2, Appendix I: Figs. K and L).

Co-incubation, transfer, and "add-back" experiments:

Although in some experiments the anoxic contractions of femoral venules which were co-incubated with pulmonary venules were variably enhanced compared with those of the paired femoral venules (Fig. 7), the mean responses were not significantly different. In addition, the

FIGURE 5. Influence of extracellular calcium ions on the contractions of paired pulmonary venules induced by hypoxia (3% O_2) or anoxia (0% O_2). Venules were incubated in either normal Krebs buffer (Control) or Krebs buffer from which $CaCl_2$ was omitted ("Ca^{++}-free buffer). The results are means ± SEM (n=4), and significant differences are as indicated (* $p < 0.03$).


Change in Wall Tension (mg/mm)
40
30
20
10
0
Control
"Ca++-free" buffer
*
*
3% O2
0% O2

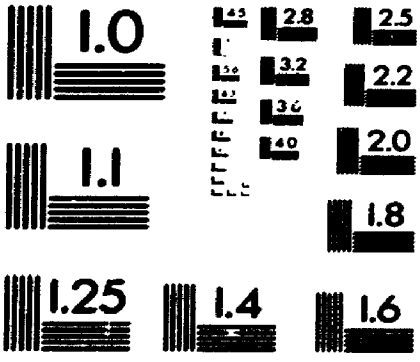

1.0
1.1
1.25
1.4
1.6
1.8
2.0
2.2
2.5
2.8
3.2
3.6
4.0

TABLE 1. Effect of pH on pulmonary venular contractions induced by hypoxia (3% O_2) or anoxia (0% O_2).

		Change in Wall Tension (mg/mm)	
	Treatment	3% O_2	0% O_2
	Control[a]	2.2	12.4
	pH 7.31	1.3	5.3
Low pH	Control	5.8	28.3
	pH 7.22	2.0	16.1
	Control	1.9	21.6
	pH 7.14	.0	8.3
	Control	2.2	12.4
	pH 7.51	4.0	20.0
High pH	Control	5.8	28.3
	pH 7.65	18.2	39.5
	Control	1.9	21.6
	pH 7.75	9.3	33.4

n=2; [a]control pH: 7.39

FIGURE 6. Effect of parenchymal removal on the contractions of paired pulmonary venules induced by hypoxia (3% O_2) or anoxia (0% O_2). The results are means ± SEM (n=6).

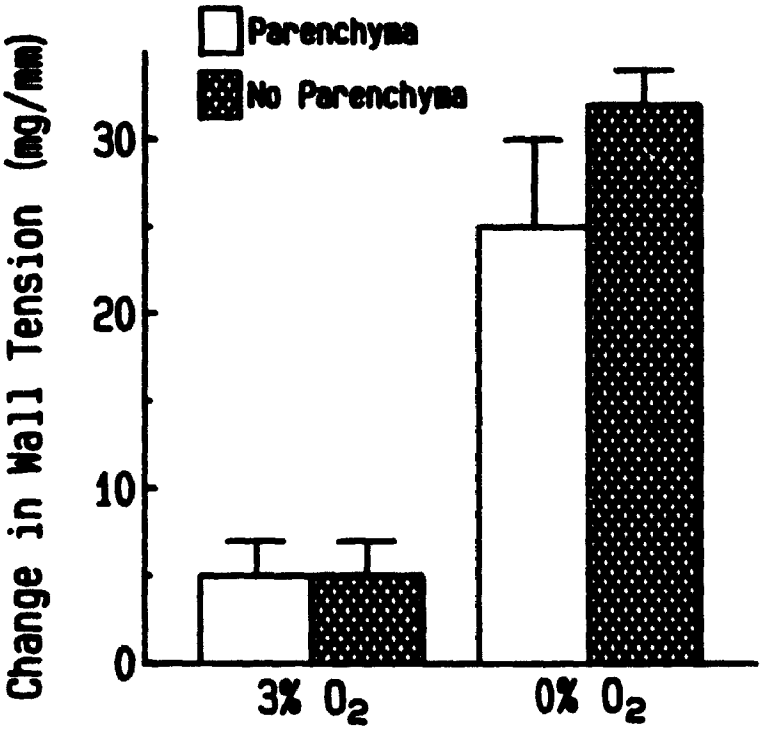
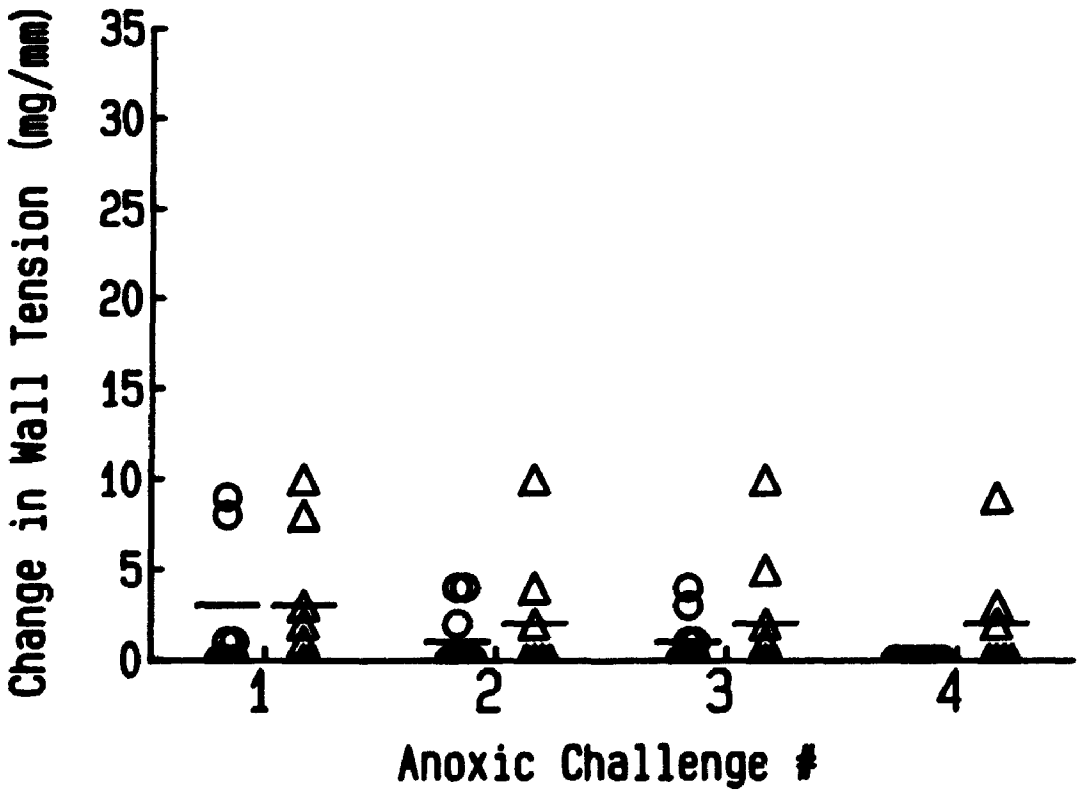

Parenchyma
No Parenchyma
Change in Wall Tension (mg/mm)
30
20
10
0
3% O_2
0% O_2

TABLE 2. Effect of parenchymal removal on the response of pulmonary venules to pharmacological agents.

	EC_{50}		Maximum Response (mg/mm)	
Drug	Control	Minus Parenchyma	Control	Minus Parenchyma
$PGF_{2\alpha}$	0.85 ± .30 μM[a]	1.82 ± .81 μM	62 ± 8	38 ± 4**
HIS	15 ± 5 μM	17 ± 4 μM	31 ± 10	47 ± 10*
5-HT	0.07 ± .02 μM	0.09 ± .02 μM	67 ± 15	69 ± 9
KCl	29 ± 1 mM	30 ± 2 mM	203 ± 20	193 ± 9
ACh[b]	0.16 ± .38 μM	0.19 ± .43 μM	-74 ± 4%	-68 ± 9%

[a]mean ± SEM, n=6, paired vessels from the same animal; [b]Relaxation following precontraction with 10 μM $PGF_{2\alpha}$; experiments were performed under normoxic (15% O_2) conditions, and significant differences from control are as indicated (* $p < 0.05$; ** $p < 0.01$).

FIGURE 7. Effect of pulmonary venule co-incubation (Δ) on the response of femoral venules (O) to anoxia. Pulmonary and femoral venules were obtained from the same animal and only the tension changes in the femoral venules were recorded. Individual responses from seven experiments are shown, with mean responses indicated by the bars.


Change in Wall Tension (mg/mm)
35
30
25
20
15
10
5
0
1
2
3
4
Anoxic Challenge #

magnitude of the anoxic contractions of co-incubated femoral venules (Fig. 7) was never comparable to that of pulmonary venular anoxic contractions (Fig. 3). Hypoxia failed to elicit contractions of femoral venules co-incubated with pulmonary venules.

Transfer of bath fluid from anoxic pulmonary venules to anoxic femoral venules, normoxic pulmonary venules, or hypoxic pulmonary venules did not elicit contractions from the recepient vessel (data not shown). In addition, the removal of anoxic bath fluid from an anoxic pulmonary venule followed by the subsequent return of the anoxic fluid to the same venule failed to alter pulmonary venular tone.

DISCUSSION

The purpose of the present study was to determine whether isolated small pulmonary veins (venules) contracted in response to lowered oxygenation, and if so, ascertain if this model can be used to predict mechanisms of HPV. We chose to use venules of < 400 μm internal diameter because small resistance vessels are believed to be the site of HPV *in vivo* (Allison and Stanbrook, 1980; Hakim *et al.*, 1983; Shirai *et al.*, 1986).

Earlier *in vitro* models of HPV have encountered many difficulties, for example, non-sustained hypoxic contractions (Lloyd, 1964, 1970; Madden *et al.*, 1985), a decline in the hypoxic response over time (Gorsky and Lloyd, 1967; Hauge, 1968a), the need to precontract isolated vessels before being able to elicit hypoxic contractions

(Detar and Gellai, 1971; Miller et al., 1988), and the equilibration of tissues at a non-physiological Po_2 (Lloyd, 1968, 1970; Madden et al., 1985; Miller et al., 1988). In contrast to these studies, isolated guinea-pig pulmonary venules exhibited a sustained (Fig. 4), repeatable (Fig. 3) response to decreased oxygenation, and did not require pre-developed tone or a high initial Po_2 in order to elicit a contraction. The lack of a comparable response in a systemic venule (Fig. 3), as well as calcium-dependence (Fig. 5), are also characteristics of both the present model and HPV in vivo (Weir, 1984).

However, there are dissimilarities between the contractions to decreased oxygenation observed in the guinea-pig pulmonary venule and HPV. In the venule, anoxia or a rather severe hypoxia (bath Po_2: 25 torr) was required to elicit contractions of the pulmonary venule; higher oxygen tensions did not alter pulmonary venular tone. Peake et al. (1981) studied HPV in vitro in isolated lungs from several species, and found pulmonary artery pressure rises as the inspired Po_2 is lowered to approximately 50-75 torr, peaks at 25-50 torr, and may decrease if the inspired Po_2 is lowered further. The reason for this difference between the oxygen tension "response curve" for HPV in isolated lungs and the contractions in our model is not clear. However, it may be that isolated blood vessels require, in general, a more severe stimulus compared to that required to elicit a comparable response in vivo. Previous studies have shown that hypoxia is no exception to this observation (Lloyd, 1970; Detar and Gellai, 1971; Miller et al., 1988). Madden et al. (1985) have described

contractions of isolated small pulmonary arteries from cats in response to oxygen tensions similar to those reported to elicit HPV in vivo, but in this study, the arteries were equilibrated at a Po_2 of 400-450 torr.

Furthermore, systemic vessels may also contract in response to profound hypoxia and anoxia (Katusic and Vanhoutte, 1986; Rubanyi and Vanhoutte, 1985), and anoxic facilitation of norepinephrine- and KCl-induced contractions has also been reported (De Mey and Vanhoutte, 1983). Although there are obvious differences between the present and previous studies, such as species (canine versus guinea-pig) and initial oxygen tension (95% O_2 versus 15% O_2 in the present study), the phenomenon which we describe may not be unique to the pulmonary venule. However, in the present study, experiments in which paired femoral and pulmonary venules were simultaneously exposed to lowered oxygenation demonstrated that the contractions were essentially confined to the pulmonary venule (Fig. 3). Due to reports suggesting a role for the endothelium in hypoxia-induced vascular contractions (Holden and McCall, 1984; Rubanyi and Vanhoutte, 1985), we ensured that the endothelium was intact in our preparations. The influence of the endothelium on the effects of lowered oxygenation in this preparation will be reported separately (see Chapter 2).

The effect of changes in pH on hypoxia- and anoxia-induced contractions of the pulmonary venule differs somewhat from previous reports on the effect of pH on HPV. In the present study, preliminary observations suggested that acidosis blunted, while alkalosis enhanced, the contractions to lowered oxygen tension (Table 1).

Although HPV is generally considered to be augmented by acidosis and inhibited by alkalosis (Fishman, 1976), there are conflicting findings. For example, there have been reports that acidosis outside the normal range (Marshall et al., 1984) or hypercapnia (Emery et al., 1977) can inhibit HPV. Raffestin and McMurtry (1987) recently observed that agents believed to increase intracellular pH enhanced HPV in isolated rat lungs, while agents which should lower intracellular pH blunted the response. In addition, Nagasaka et al. (1984) reported that although alkalosis inhibited the increase in pressure difference due to hypoxia on the arterial side of the feline pulmonary circulation, the hypoxia-induced increase in pressure difference on the venous side of the circulation was increased.

Formulations of the mechanism of HPV have generally fallen into two categories. First, hypoxia may elicit pulmonary vasoconstriction via a direct action on the vascular smooth muscle. Alternatively, an oxygen sensing cell releases a contractile mediator(s), which then stimulates the smooth muscle to contract. In view of the earlier observation of Lloyd (1968) that parenchyma was necessary to elicit hypoxic contractions of rabbit isolated pulmonary arteries, we examined the effect of parenchymal removal in our model. However, as shown in Fig. 6, removal of the parenchyma did not significantly influence the hypoxia- or anoxia-induced contractions of the pulmonary venule. The pulmonary venular responses elicited by 5-HT, acetylcholine, and KCl were similarly unaltered by removal of the parenchyma, although there were minor changes in the maximal contractions to $PGF_{2\alpha}$ and histamine (Table 2). These observations

indicate that the surrounding parenchyma is unlikely to be the source of a contractile mediator(s) released by hypoxia. However, these studies do not rule out release of such a mediator from the vascular tissue. Indeed, we observed that changing the buffer surrounding anoxic pulmonary venules sometimes reduced the venular tone, suggesting that a contractile mediator might exist. Therefore, we attempted to demonstrate the release of a such a substance. The experimental approaches consisted of co-incubation of pulmonary and femoral venules, transfer of bath fluid from an anoxic pulmonary venule to either an anoxic femoral venule or a normoxic or hypoxic pulmonary venule, and returning anoxic bath fluid to an anoxic pulmonary venule from which the fluid had earlier been withdrawn. None of these experiments demonstrated the presence of a soluble contractile mediator, although some femoral venules which were co-incubated with pulmonary venules did tend to contract to a greater degree than the paired femoral venules (Fig. 7). The failure of these experiments to conclusively demonstrate the release of a contractile mediator(s) from the pulmonary venule does not disprove the existence of such a substance; in fact, there are several possible explanations for our negative results. Any hypothetical mediator may be very unstable and therefore might not have been present in sufficient quantities to elicit contraction of recipient tissues after the 20-30 sec required to transfer the bath fluid from an anoxic pulmonary venule. If this were the case, we would have expected that the femoral venules which were coincubated with pulmonary venules would have contracted in response to hypoxic and anoxic stimulation; we did

not observe such a response. However, femoral venules may not respond to a contractile mediator originating from the pulmonary venule, or baseline wall tension (absent in the pulmonary venules which were coincubated with femoral venules) may be required for the release of such an agent.

In summary, the pulmonary venular response to decreased Po_2 exhibited many of the characteristics of HPV *in vivo*, such as rapid onset and offset of the response, persistence, repeatability, calcium dependence, and lack of a comparable response in a systemic venule. These characteristics suggest that this model may be useful in predicting mechanisms of HPV. The hypoxia- and anoxia-induced contractions of the pulmonary venule are not dependent on the parenchyma, while experiments designed to show the involvement of a contractile mediator(s) were inconclusive.

As alluded to earlier, there is evidence which suggests that the vascular endothelium may be capable of either modulating or mediating the contractile response of the pulmonary venule to reduced Po_2. The experiments described in the next chapter were designed to investigate the possible involvement of the endothelium in this phenomenon and attempt to clarify the responsible mechanism(s).

Chapter 2: THE EFFECT OF ENDOTHELIAL INJURY ON THE RESPONSES OF ISOLATED GUINEA-PIG PULMONARY VENULES TO REDUCED OXYGENATION AND REFERENCE PHARMACOLOGICAL AGONISTS

RESULTS

Effect of collagenase or hypoxanthine/xanthine oxidase on pulmonary artery and venule endothelium:

As assessed by light microscopy, venules treated with collagenase lost 54 ± 8% (n=4) of their endothelium, compared to 5 ± 1% loss ($p < 0.01$) from paired control venules.

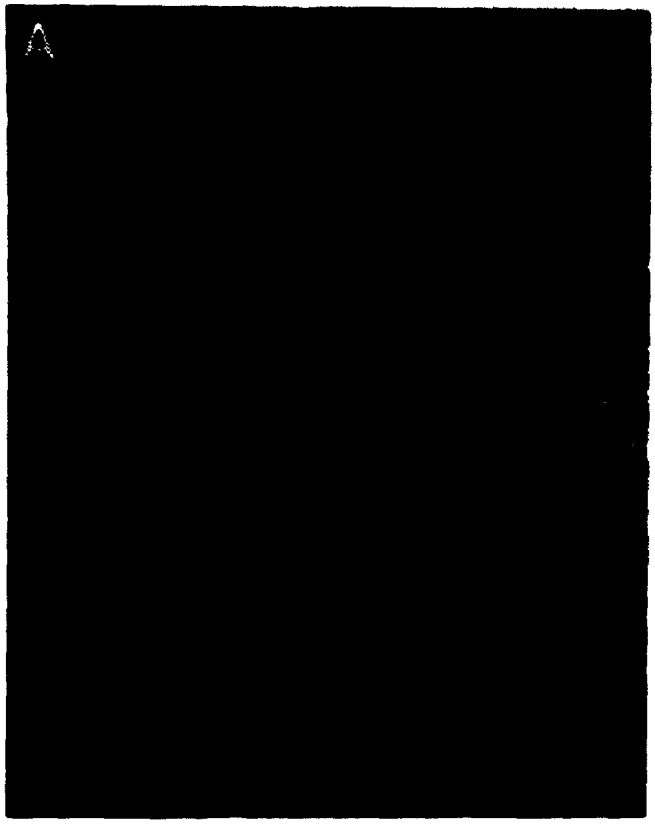
Endothelial cells of pulmonary venules exposed to HX/XO had numerous pits or craters in their cell membranes which were visualized by scanning EM (Plate 1B). Although the endothelium of control venules also demonstrated some pitting, the majority of the cells did not exhibit this lesion (Plate 1A). Pulmonary arteries treated with HX/XO also demonstrated endothelial pitting, but to a lesser degree than observed in the venules. However, the endothelium of HX/XO-treated pulmonary arteries exhibited numerous blebs which were not seen in the pulmonary venules.

Effect of hypoxanthine/xanthine oxidase or collagenase on vasodilator responses of pulmonary arteries and venules:

Relaxations of pulmonary arteries by acetylcholine and A-23187 were significantly ($p < 0.01$) reduced by exposure to HX/XO and these reductions by HX/XO could be prevented by SOD (Figs. 8 and 9), whereas the responses of HX/XO pre-treated pulmonary venules to acetylcholine (Fig. 10) and A-23187 (Fig. 11) were unchanged. Treatment of pulmonary arteries with HX/XO tended to attenuate the bradykinin-induced relaxations (Fig. 12) and changed the relaxation induced by

PLATE 1A. Scanning electron micrograph of normal pulmonary venular endothelium from a control vessel (2500 X). The endothelial cells form a continuous layer and have numerous microvilli on their surface. The series of dots on the bottom of the micrograph depict a 12.0 μm scale bar.

PLATE 1B. Scanning electron micrograph of pulmonary venular endothelium from a hypoxanthine/xanthine oxidase-treated vessel (2500 X). Numerous pits or craters are seen on the surface of the endothelial cells. The series of dots on the bottom of the micrograph depict a 12.0 μm scale bar.


A


B

FIGURE 8. Effect of perfusion with either hypoxanthine + xanthine oxidase (○; n=6) or hypoxanthine + xanthine oxidase + superoxide dismutase (□; n=5) on acetylcholine-induced relaxations (●; n=6) of KCl (35 mM) precontracted pulmonary arteries obtained from the same animal. Arteries were perfused with the enzyme solution before mounting the segments in the organ baths and experiments were performed under normoxic (15% O_2) conditions. The results are means ± SEM, and significant differences compared to control (●) are as indicated (** $p < 0.01$).

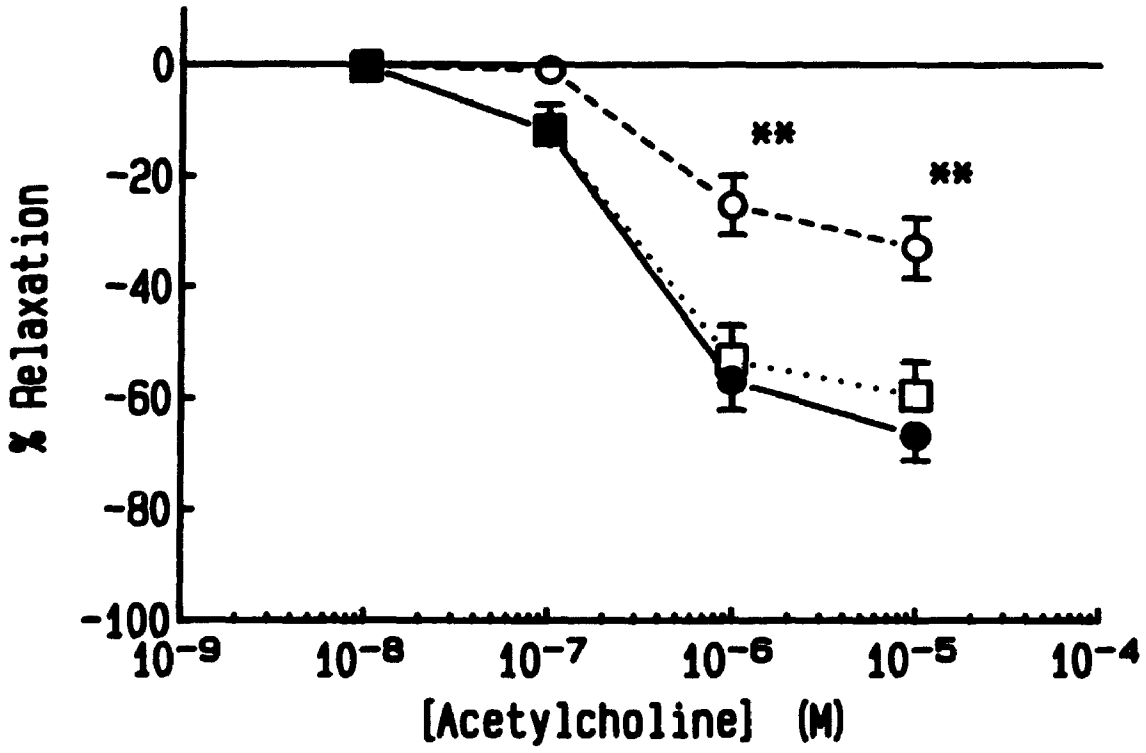

0
-20
-40
-60
-80
-100
% Relaxation
**
**
10⁻⁹
10⁻⁸
10⁻⁷
10⁻⁶
10⁻⁵
10⁻⁴
[Acetylcholine] (M)

FIGURE 9. Effect of perfusion with either hypoxanthine + xanthine oxidase (○; n=6) or hypoxanthine + xanthine oxidase + superoxide dismutase (□; n=5) on A-23187-induced relaxations (●; n=6) of KCl (35 mM) precontracted pulmonary arteries obtained from the same animal. Arteries were perfused with the enzyme solution before mounting the segments in the organ baths and experiments were performed under normoxic (15% O_2) conditions. The results are means ± SEM, and significant differences compared to control (●) are as indicated (** $p < 0.01$).

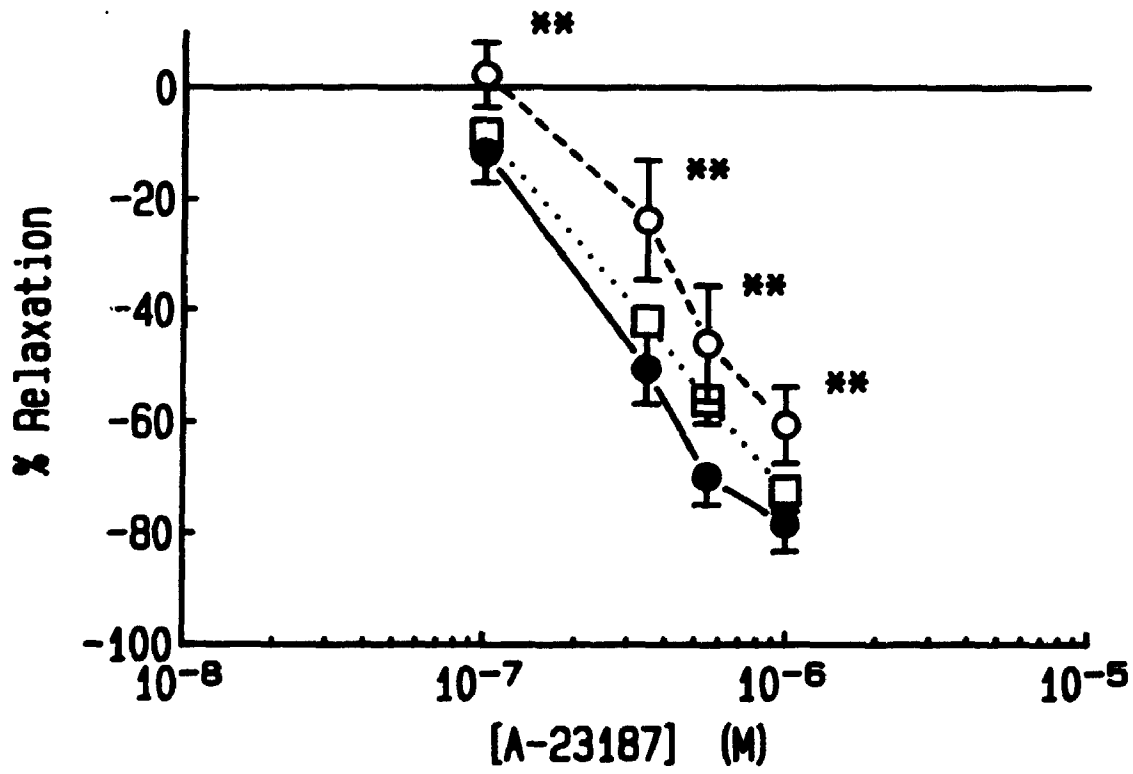

**
**
**
**
0
-20
-40
-60
-80
-100
% Relaxation
10-8
10-7
10-6
10-5
[A-23187] (M)

FIGURE 10. Effect of perfusion with either hypoxanthine + xanthine oxidase (O; n=6) or collagenase (Δ; n=6) on acetylcholine-induced relaxations (●; n=12) of $PGF_{2\alpha}$ (10 μM) precontracted pulmonary venules obtained from the same animal. Venules were perfused with the enzyme solution before mounting the segments in the organ baths and experiments were performed under normoxic (15% O_2) conditions. The results are means ± SEM.

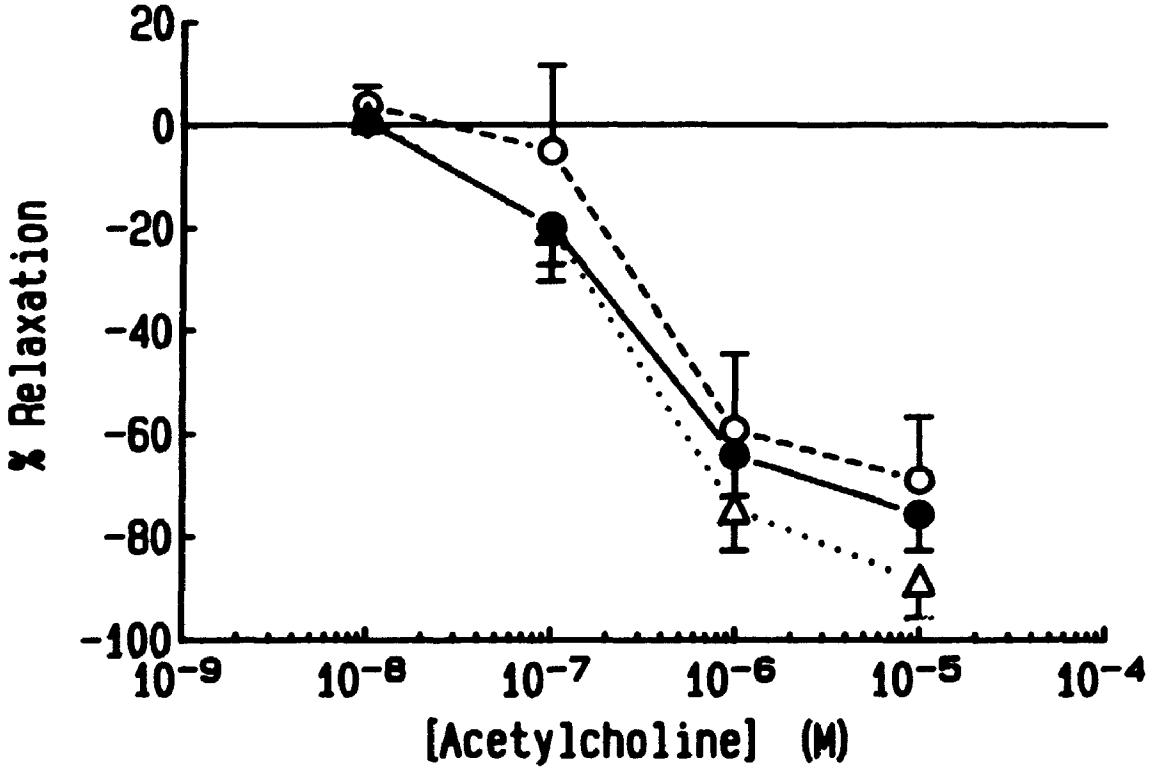

20
0
-20
-40
-60
-80
-100
% Relaxation
10-9
10-8
10-7
10-6
10-5
10-4
[Acetylcholine] (M)

FIGURE 11. Effect of perfusion with either hypoxanthine + xanthine oxidase (O; n=5) or collagenase (Δ; n=5) on A-23187-induced relaxations (●; n=9) of $PGF_{2\alpha}$ (10 μM) precontracted pulmonary venules obtained from the same animal. Venules were perfused with the enzyme solution before mounting the segments in the organ baths and experiments were performed under normoxic (15% O_2) conditions. The results are means ± SEM.

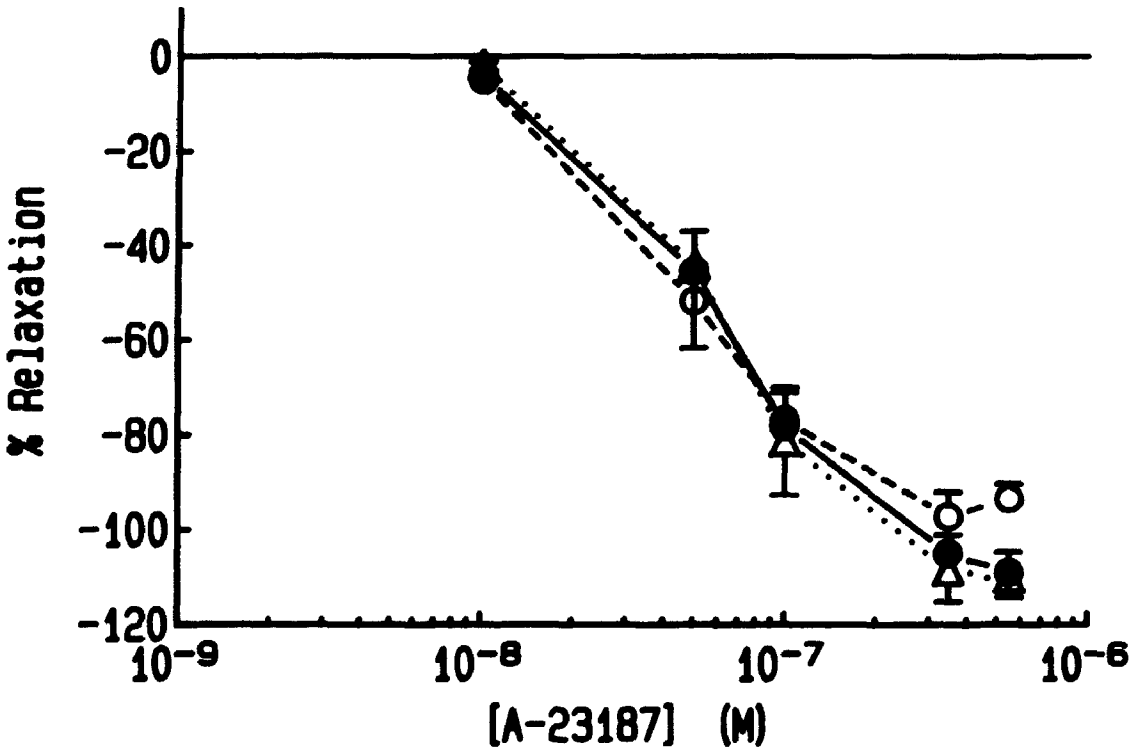

0
-20
-40
-60
-80
-100
-120
% Relaxation
10-9
10-8
10-7
10-6
[A-23187] (M)

FIGURE 12. Effect of perfusion with either hypoxanthine + xanthine oxidase (○; n=6) or hypoxanthine + xanthine oxidase + superoxide dismutase (□; n=5) on bradykinin-induced relaxations (●; n=6) of KCl (35 mM) precontracted pulmonary arteries obtained from the same animal. Arteries were perfused with the enzyme solution before mounting the segments in the organ baths and experiments were performed under normoxic (15% O_2) conditions. The results are means ± SEM, and significant differences from control (●) are as indicated (** $p < 0.01$).

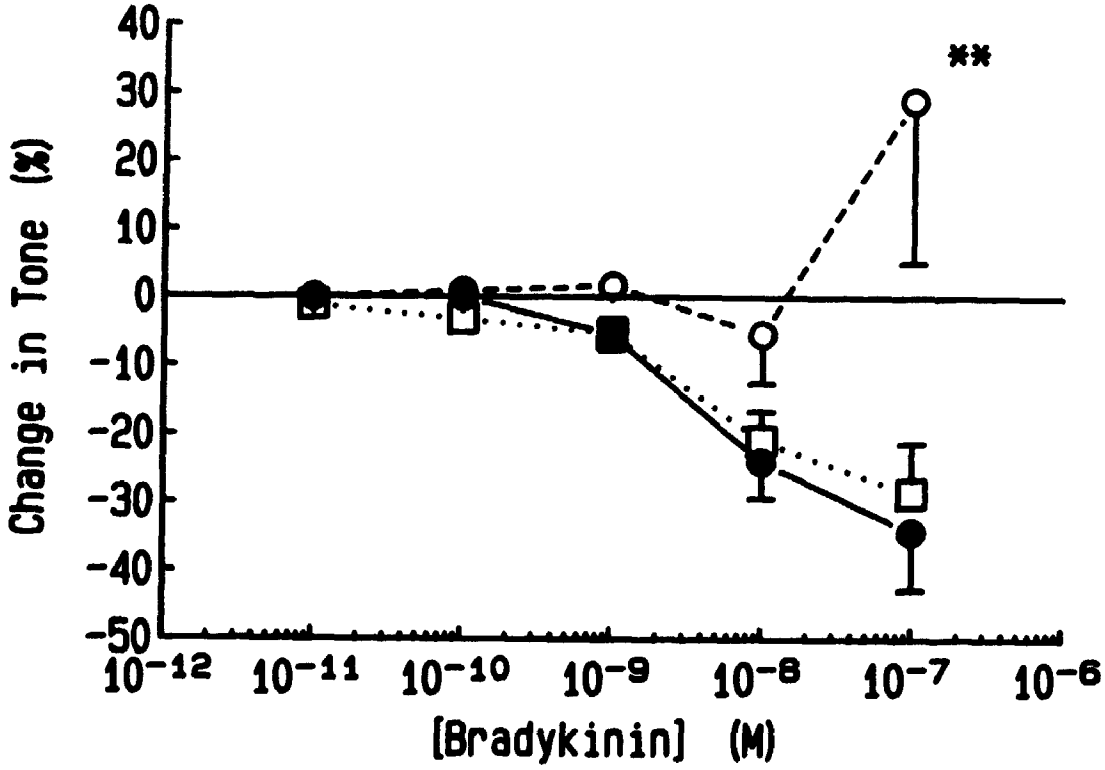
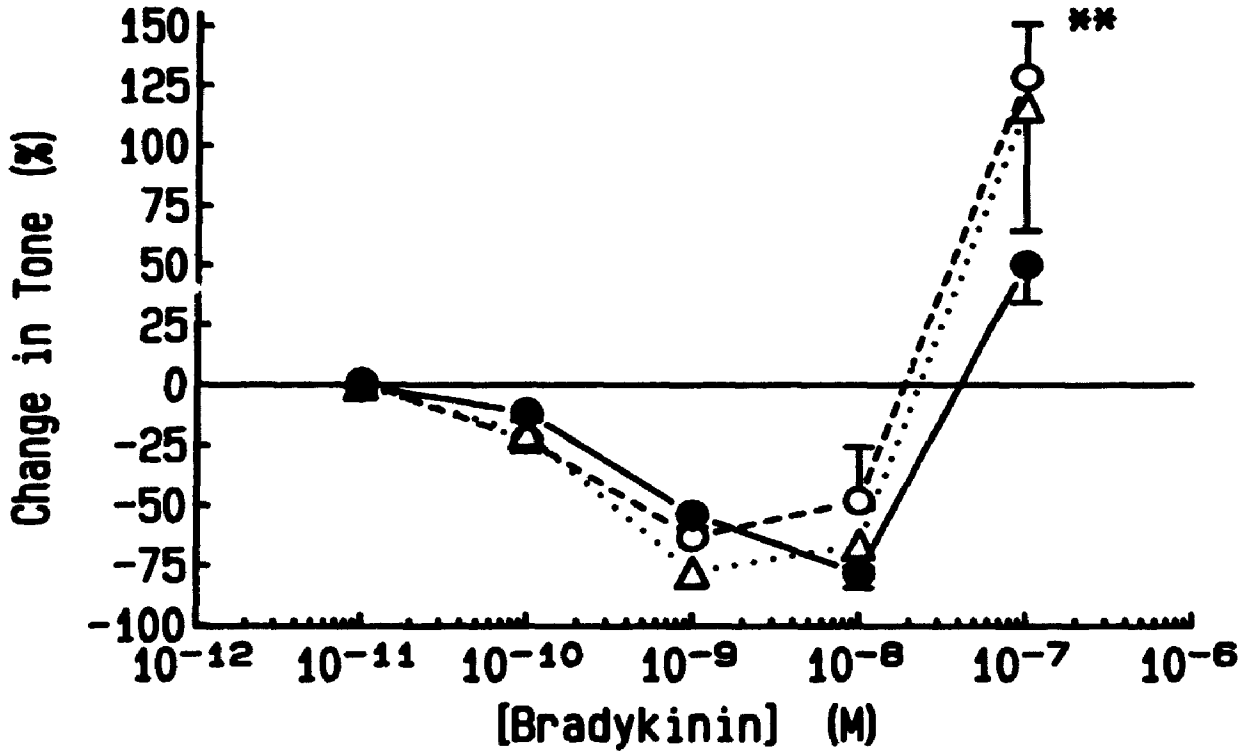

40
30
20
10
0
-10
-20
-30
-40
-50
Change in Tone (%)
**
10^-12
10^-11
10^-10
10^-9
10^-8
10^-7
10^-6
[Bradykinin] (M)

FIGURE 13. Effect of perfusion with either hypoxanthine + xanthine oxidase (○; n=8) or collagenase (△; n=4) on bradykinin-induced relaxations (●; n=12) of $PGF_{2\alpha}$ (10 μM) precontracted pulmonary venules obtained from the same animal. Venules were perfused with the enzyme solution before mounting the segments in the organ baths and experiments were performed under normoxic (15% O_2) conditions. The results are means ± SEM, and significant differences from control (●) are as indicated (** $p < 0.01$).


150
125
100
75
50
25
0
-25
-50
-75
-100
Change in Tone (%)
**
10^-12
10^-11
10^-10
10^-9
10^-8
10^-7
10^-6
[Bradykinin] (M)

0.1 μM bradykinin to a contraction. Bradykinin-induced relaxation of pulmonary venules was not affected by HX/XO treatment (Fig. 13). However, HX/XO significantly ($p < 0.01$) enhanced the pulmonary venular contractile response to 0.1 μM bradykinin.

The effects of collagenase perfusion on the responses of pulmonary venules to acetylcholine (Fig. 10), A-23187 (Fig. 11) and bradykinin (Fig. 13) were also assessed. As illustrated in the figures, collagenase (like HX/XO) did not affect the responses to these agents. However, collagenase caused a moderate, but significant ($p < 0.001$) increase in the ELR necessary to produce equal resting tensions [497 ± 22 μm vs 378 ± 16 μm (n=9) for collagenase-treated and control venules, respectively]. Nevertheless, collagenase pretreatment did not significantly reduce the maximum contraction to KCl (120 mM) [control: 203 ± 16 mg/mm versus collagenase: 169 ± 13 mg/mm; (n=9)].

Effect of hypoxanthine/xanthine oxidase or collagenase on the response of pulmonary venules to decreased oxygen tension:

Hypoxanthine/xanthine oxidase significantly ($p < 0.05$) increased the sustained hypoxia and anoxia-induced contractions of pulmonary venules (Fig. 14). Both SOD and CAT prevented the effects of HX/XO perfusion on the response of venules to lowered oxygen tension (Table 3). Collagenase treatment also significantly ($p < 0.05$) augmented the contractile response of pulmonary venules to anoxia (Fig. 14).

FIGURE 14. Effect of perfusion with either hypoxanthine + xanthine oxidase (n=10) or collagenase (n=7) on pulmonary venular contractions induced by hypoxia (3% O_2) or anoxia (0% O_2). Control and treated pulmonary venules were obtained from the same animal. Venules were perfused with the enzyme solution before mounting the segments in the organ baths. The results are means ± SEM, and significant differences from control are as indicated (* $p < 0.05$).

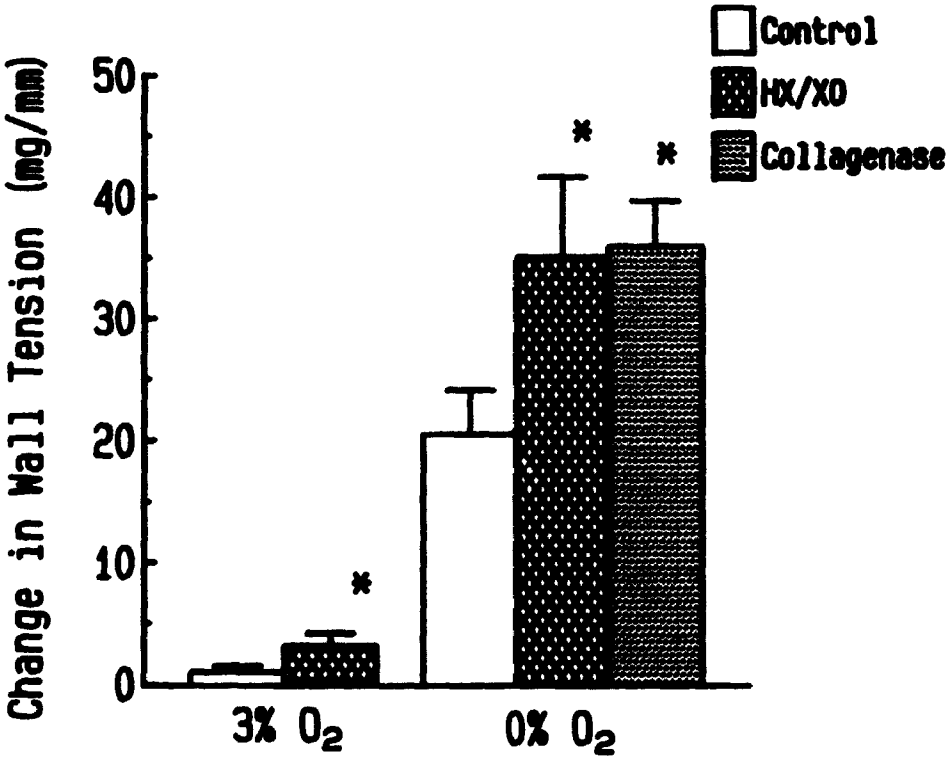

Control
HX/XO
Collagenase
Change in Wall Tension (mg/mm)
50
40
30
20
10
0
*
*
*
3% O_2
0% O_2

TABLE 3. Prevention by superoxide dismutase (SOD) and catalase (CAT) of hypoxanthine/xanthine oxidase (HX/XO)-induced augmentation of pulmonary venular contractions elicited by hypoxia (3% O_2) or anoxia (0% O_2).

		Change in Wall Tension (mg/mm)	
Treatment	n	3% O_2	0% O_2
Control	6	2.2 ± 0.9[a]	27.3 ± 7.1
HX/XO/SOD	6	2.0 ± 0.7	31.3 ± 8.5
Control	5	2.0 ± 0.5	32.8 ± 8.3
HX/XO/CAT	5	2.1 ± 0.3	31.6 ± 7.9

[a]mean ± SEM, paired vessels from the same animal.

Effect of gossypol and methylene blue on hypoxia- and anoxia-induced pulmonary venular contractions:

Methylene blue (10 μM) increased baseline tone by 31.7 ± 6.9 mg/mm (n=11) necessitating readjustment of tone to the original baseline before the Po_2 was decreased. Gossypol (5 μM) did not affect baseline tone of the venules. Neither methylene blue nor gossypol significantly affected hypoxic or anoxic contractions of the pulmonary venules (Table 4).

DISCUSSION

In the present study, two dissimilar methods of inducing endothelial damage were used: collagenase, and HX/XO perfusion. Collagenase has been used to remove vascular endothelium in previous studies from other laboratories (Jaffe et al., 1973; Furchgott et al., 1987). In addition, HX/XO perfusion was used since it has been shown that reactive oxygen species can initiate endothelial injury, whether they are derived from neutrophils (Varani et al., 1985; Weiss et al., 1981; Martin, 1984), the endothelium itself (Rodell et al., 1987; Brigham et al., 1987; Ratych et al., 1987), or an extracellular HX/XO system (Ody and Junod, 1985; Jornot and Junod, 1988). In addition, xanthine oxidase has been localized in the vascular endothelium (Jarasch et al., 1981, 1986), and Pacella and coworkers (1988) recently reported that extracellular oxidant stress converts pulmonary endothelial xanthine dehydrogenase activity to xanthine oxidase

TABLE 4. Effect of gossypol and methylene blue on pulmonary venular contractions induced by hypoxia (3% O_2) or anoxia (0% O_2).

		Change in Wall Tension (mg/mm)		
Treatment	n	3% O_2	n	0% O_2
Control	4	4.0 ± 1.1[a]	4	19.1 ± 8.6
Gossypol (5 μM)	4	4.9 ± 0.9	4	19.8 ± 4.7
Control	3	4.1 ± 1.7	11	19.4 ± 3.5
Methylene blue (10 μM)	3	6.0 ± 1.3	11	25.6 ± 5.2

[a]mean ± SEM, each vessel served as its own control.

activity. Such a reaction might augment oxidant-induced injury of the endothelium.

Collagenase perfusion removed approximately half of the venular endothelium, but also altered the length - tension characteristics of the vessels. On the other hand, HX/XO perfusion did not remove endothelial cells, but did induce modest morphological changes in individual cells as assessed by EM (Plate 1). The changes observed in the HX/XO-treated endothelium consisted of numerous craters and blebs. These alterations in pulmonary endothelial morphology also occur following exposure to several other stimuli, such as hypoxia (Hung et al., 1986), AA (Kontos, 1987), ANTU (Martin et al., 1986), or cobra venom factor (Warren and Ward, 1986), all of which may elicit endothelial injury via the production of toxic oxygen radicals. However, it has been suggested that these changes are artifacts created by the techniques used in preparing blood vessels for electron microscopy (Hollweg and Buss, 1980; Smith and Heath, 1978). Although we cannot be certain that the observed changes in the pulmonary endothelium represent oxygen radical-induced damage, the amount of endothelial damage was clearly greater (approximately three-fold difference) in HX/XO-treated vessels than in control vessels.

Since morphological changes in the vascular endothelium do not necessarily correlate with functional changes, we also assessed the effects of HX/XO and collagenase treatment on the endothelium-dependent vasodilator actions of acetylcholine, bradykinin and A-23187. Neither collagenase nor HX/XO treatment altered the relaxant effects of acetylcholine, bradykinin or A-23187 in precontracted

guinea-pig pulmonary venules (Figs. 10, 11 and 13). In contrast, HX/XO significantly blunted the actions of these vasodilators in the guinea-pig pulmonary artery (Figs. 8, 9 and 12). This inhibition was prevented by SOD, indicating involvement of superoxide radicals (Figs. 8, 9 and 12). Collagenase similarly blunted acetylcholine-, bradykinin-, and A-23187-induced relaxations of the pulmonary artery. In addition, we observed that neither gossypol nor methylene blue affected acetylcholine-, bradykinin- or A-23187-induced relaxations of pulmonary venules, but both agents effectively inhibited the relaxations induced by acetylcholine in the pulmonary artery. Therefore, although the data from the pulmonary artery experiments indicate that both HX/XO and collagenase produce functional changes in the vascular endothelium, such alterations in endothelial function are not immediately evident in the pulmonary venule, since it appears that acetylcholine, bradykinin and A-23187 dilate the pulmonary venule via an endothelium-independent mechanism(s).

Endothelium-independent relaxation of veins has been previously reported. De Mey and Vanhoutte (1982) observed that acetylcholine elicited endothelium-independent relaxations of isolated canine splenic veins (10-300 nM), but had no effect on precontracted pulmonary veins (10-300 nM) and caused only transient endothelium-dependent relaxations of precontracted saphenous and femoral veins (30-300 nM). The effects of bradykinin or A-23187 on the splenic vein were not investigated (De Mey and Vanhoutte, 1982). Other studies have shown that acetylcholine does not relax bovine intrapulmonary veins (Gruetter and Lemke, 1986a; Ignarro et al., 1986). Bradykinin

has been reported to dilate bovine intrapulmonary (Gruetter and Lemke, 1986b; Ignarro et al., 1987) and canine femoral (Furchgott, 1983) veins by an endothelium-dependent mechanism. A-23187-induced relaxation of bovine intrapulmonary (Gruetter and Lemke, 1986a) and canine femoral (Furchgott, 1983) veins also appears to be endothelium-dependent.

The role of the endothelium in pulmonary venous responses to lowered oxygen tension has received little attention. Although De Mey and Vanhoutte (1982) did not examine the effects of anoxia alone on isolated canine pulmonary veins, they found that anoxia enhanced the contractile response of this vessel to norepinephrine. If the endothelium was removed, anoxia caused a relaxation of the norepinephrine-precontracted pulmonary veins (De Mey and Vanhoutte, 1982).

In the present study, the hypoxic and anoxic contractions of the pulmonary venule were modulated by the endothelium, in contrast to the endothelium-independent effects of acetylcholine, bradykinin and A-23187. Endothelial damage, whether produced by collagenase or HX/XO, augmented the contractile response of the pulmonary venules to lowered oxygen tension (Fig. 14). In addition, the HX/XO-induced augmentation of the hypoxic and anoxic contractions of pulmonary venules was prevented by SOD or CAT, indicating the involvement of superoxide radicals and hydrogen peroxide, or possibly hydroxyl radicals generated secondarily via a Haber-Weiss reaction. Spectrophotometric analysis confirmed that the HX/XO mixture produced superoxide anion at a rate of 4.0 ± 0.2 nmol/ml/min.

Others have previously reported that the endothelium influences vascular responses to lowered oxygen tension. Hill and Rounds (1983) observed that ANTU, a compound which induces endothelial injury, enhances vascular reactivity to hypoxia in isolated rat lungs. In contrast to our results and those of Hill and Rounds (1983), other reports have indicated endothelial damage inhibits, not augments, hypoxic and anoxic contractions (Rodman et al., 1987; Madden et al., 1986b; Holden and McCall, 1984; Rubanyi and Vanhoutte, 1985). Unfortunately, comparisons between the present and earlier studies must be indirect because of important dissimilarities in the experimental approaches. For example, either a whole lung preparation was used instead of an isolated vessel (Hill and Rounds, 1983), arteries were examined instead of veins (Rodman et al., 1987; Madden et al., 1986b; Holden and McCall, 1984; Rubanyi and Vanhoutte, 1985), or a main pulmonary vessel segment was studied instead of a smaller resistance segment (Rodman et al., 1987; Holden and McCall, 1984). Nevertheless, the available data indicate that the endothelium modulates hypoxic contractions of pulmonary vessels.

Madden et al. (1986b) reported that small (approximately 300 μm in diameter) feline pulmonary arteries, after being treated with collagenase, demonstrated a reduced contractile response to hypoxia. However, the contractile response to other agents was also depressed, suggesting non-specific damage of the smooth muscle. Holden and McCall (1984) and Rodman et al. (1987) observed an inhibition of hypoxic and anoxic contractions in isolated, endothelium-denuded porcine and rat pulmonary arteries, respectively. These observations

imply that the endothelium releases a contractile mediator under conditions of reduced oxygenation. Indeed, Aichholz and coworkers (1986) reported hypoxia stimulates the release of a coronary artery vasoconstrictor from cultured bovine aortic endothelial cells, and Yanagisawa et al. (1988) recently identified an endothelium-derived contractile factor, endothelin. However, it appears unlikely that endothelin is directly involved in HPV due to the persistence of the contraction it elicits, which is incompatible with the rapid fall in pulmonary vascular resistance in vivo after normoxic conditions have been restored. In addition, O'Brien et al. (1987) found that hypoxia or anoxia had no effect on the release of constrictor activity from cultured bovine pulmonary artery or aortic endothelial cells, in contrast to the observations of Aichholz et al. (1986).

Since our results indicated that an intact, functional endothelium reduces the contractile response to lowered oxygenation, we postulated this inhibition is achieved via basal release of EDRF [recently proposed to be nitric oxide by Palmer et al. (1987)] or a vasodilator cyclooxygenase product(s). However, neither methylene blue or gossypol (Table 4) at concentrations that inhibited acetylcholine-induced relaxations of pulmonary arteries, nor indomethacin or ibuprofen (refer to Chapter 3) enhanced the hypoxic or anoxic contractions. It remains possible that an unidentified vasodilator released from the pulmonary venule endothelium reduces contractions to decreased Po_2 even in the presence of these inhibitors. Alternatively, the endothelium could modulate the hypoxic and anoxic

contractions not by releasing a vasodilator, but rather by metabolizing a vasoconstrictor mediator of the contractions.

Earlier preliminary studies of the effect of EDRF inhibitors on HPV have yielded contradictory results. Rodman et al. (1987) reported that methylene blue and hemoglobin blunted the contractions of isolated rat pulmonary arteries to decreased oxygenation. In order to obtain these contractions, however, Rodman and colleagues (1987) had to precontract the arteries, and when the same group repeated the studies in isolated rat lungs, they observed the EDRF inhibitors augmented the hypoxic increase in perfusion pressure (Yamaguchi et al., 1987). More recently, Brashers et al. (1988) presented evidence that NDGA, ETYA, and hydroquinone (all of which may inhibit or inactivate EDRF) augmented the hypoxic pressor response in isolated rat lungs. Unfortunately, important methodological differences between these and the present study do not allow any meaningful discussion as to why our respective observations with EDRF inhibitors should differ.

In conclusion, our data suggest that the endothelium modulates pulmonary venular responses to hypoxia, with endothelial damage augmenting the contractions induced by lowered oxygen tensions. If the endothelium plays a comparable role in vivo, acute or chronic damage would be associated with augmentation of the pressor response to hypoxia, with its resultant effects on gas exchange, pulmonary venular pressures, etc. Thus the role of the endothelium in hypoxic responses in vivo requires further examination.

The data presented thus far have indicated modulatory influences of several different factors, including the endothelium, on the contractile response of the isolated pulmonary venule to reduced Po_2, but have not provided any evidence of a discrete mediator of this response. The primary candidates for such a mediator are metabolites of the lipoxygenase or cytochrome P-450 pathway of AA metabolism. Therefore, the experiments described in the next chapter were designed to determine whether one or a combination of these products may indeed be the mediator of this phenomenon.

Chapter 3: THE ROLE OF LIPOXYGENASE, CYCLOOXYGENASE AND CYTOCHROME P-450 METABOLITES IN CONTRACTIONS OF ISOLATED GUINEA-PIG PULMONARY VENULES INDUCED BY HYPOXIA AND ANOXIA

RESULTS

Effect of cyclooxygenase inhibitors on pulmonary venular contractions induced by decreased oxygenation:

Neither indomethacin (5 μM) nor ibuprofen (10 μM) altered the pulmonary venular contractions to anoxia; ibuprofen also had no effect on hypoxia-induced contractions (Table 5).

Response of pulmonary venules to LTC_4 and LTD_4 under normoxic, hypoxic, or anoxic conditions:

Pulmonary venules responded in a concentration-dependent manner in the presence of LTC_4 (Fig. 15) or LTD_4 (Fig. 16). LTC_4- and LTD_4-induced venular contractions were modestly, but significantly ($p < 0.05$) enhanced by hypoxia or anoxia (Figs. 15 and 16).

Effect of FPL 57231 and LY 163443 on LTC_4- and LTD_4-induced pulmonary venular contractions:

When preincubated with the pulmonary venules, FPL 57231 (1 and 3 μM) inhibited LTD_4-induced contractions (Fig. 17), but was much less effective at antagonizing LTC_4-induced contractions (Fig. 18). FPL 57231 significantly ($p < 0.01$) attenuated both LTC_4 and LTD_4 (1 μM) preinduced pulmonary venular tone (Appendix I: Fig P), but was more potent against LTD_4 compared to LTC_4 [IC_{50}'s: 0.7 ± 0.3 μM vs 4.3 ± 1.6 μM for LTD_4 and LTC_4, respectively (n=3)].

TABLE 5. Effect of cyclooxygenase inhibitors on pulmonary venular contractions induced by hypoxia (3% O_2) or anoxia (0% O_2).

		Change in Wall Tension (mg/mm)	
Treatment	n	3% O_2	0% O_2
Control	5		12.5 ± 0.9[a]
Indomethacin (5 μM)	5		13.3 ± 2.3
Control	4	4.0 ± 1.2	20.5 ± 5.7
Ibuprofen (10 μM)	4	3.6 ± 1.3	25.4 ± 7.0

[a]mean ± SEM, each vessel served as its own control.

FIGURE 15. The effect of cumulative concentrations of LTC_4 on pulmonary venules under conditions of normoxia (□), hypoxia (○) or anoxia (△). Each pulmonary venule was administered cumulative concentrations of LTC_4 under randomly ordered conditions of hypoxia (3% O_2), anoxia (0% O_2), or normoxia (15% O_2). The results are means ± SEM (n=6) and significant differences from control (normoxia) are as indicated (* $p < 0.05$; ** $p < 0.01$).

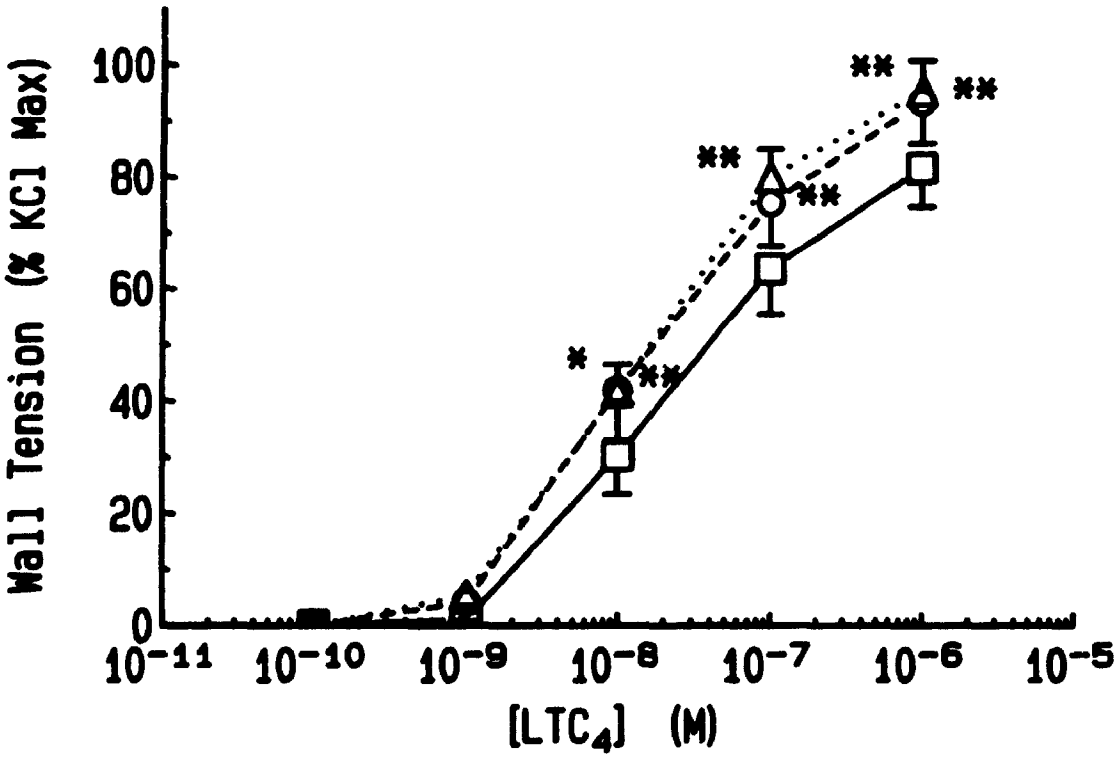

Wall Tension (% KCl Max)
100
80
60
40
20
0
10^-11
10^-10
10^-9
10^-8
10^-7
10^-6
10^-5
[LTC4] (M)
*
**
**
**
**
**

FIGURE 16. The effect of cumulative concentrations of LTD_4 on pulmonary venules under conditions of normoxia (□), hypoxia (○) or anoxia (△). Each pulmonary venule was administered cumulative concentrations of LTD_4 under randomly ordered conditions of hypoxia (3% O_2), anoxia (0% O_2), or normoxia (15% O_2). The results are means ± SEM (n=6) and significant differences from control (normoxia) are as indicated (* $p < 0.05$; ** $p < 0.01$).

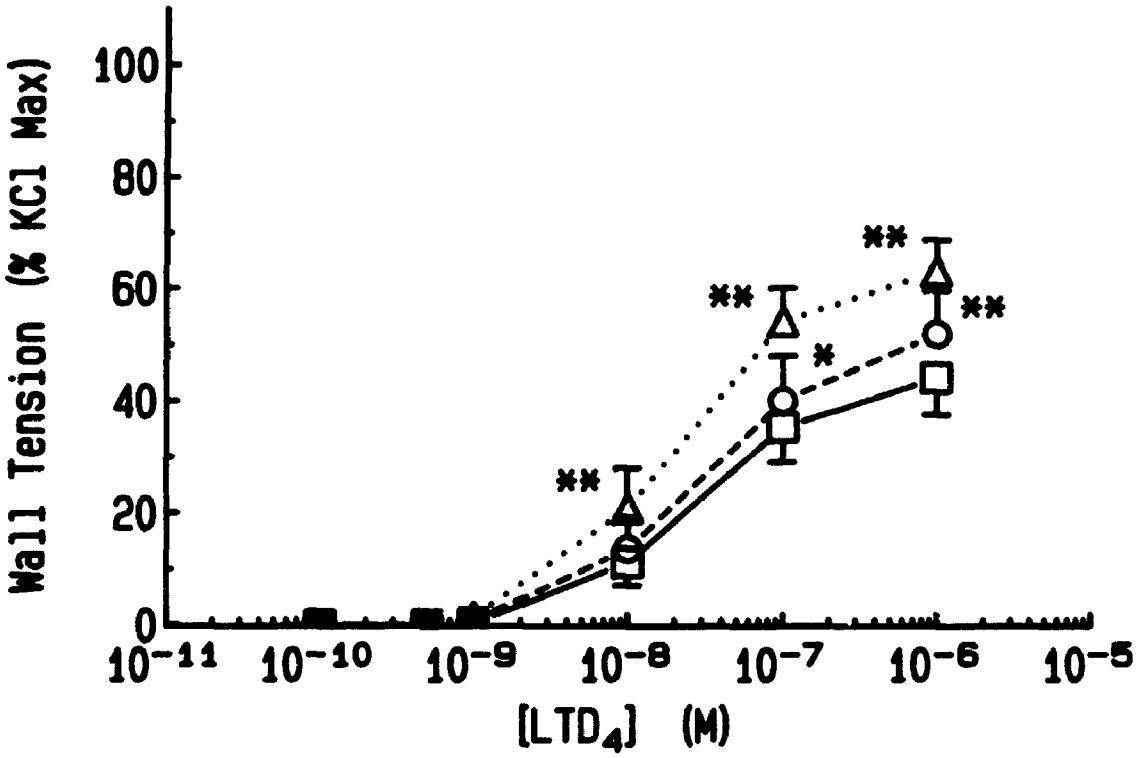

Wall Tension (% KCl Max)
100
80
60
40
20
0
10⁻¹¹
10⁻¹⁰
10⁻⁹
10⁻⁸
10⁻⁷
10⁻⁶
10⁻⁵
[LTD4] (M)
**
**
**
**
*

FIGURE 17. Effect of FPL 57231 (○, 1 μM;△, 3 μM) on LTD_4-induced contractions of pulmonary venules (□) under normoxic (15% O_2) conditions. FPL 57231 was preincubated with the venules for 15 min before adding cumulative concentrations of LTD_4 to the organ bath. Each venule served as its own control. The results are means ± SEM (control: n=12;1 μM FPL 57231: n=4; 3 μM FPL 57231: n=2) and significant differences from control are as indicated (* $p < 0.05$; ** $p < 0.01$). Statistical comparisons between 3 μM FPL 57231 and control were not performed.

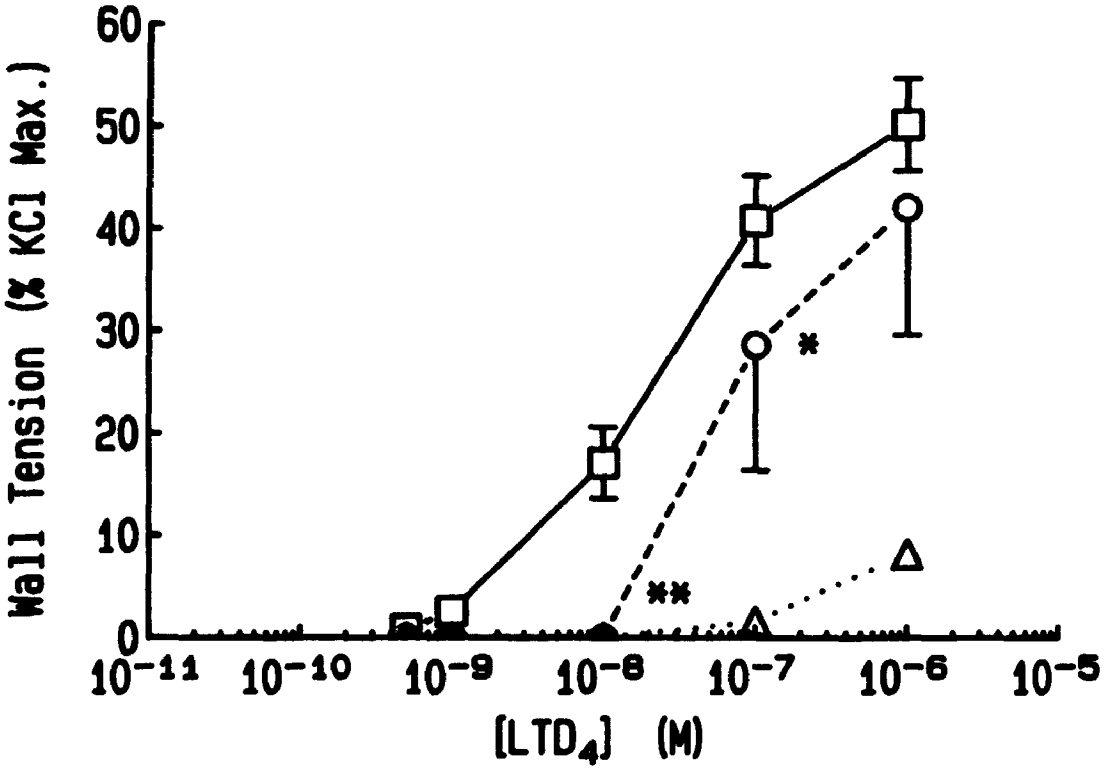
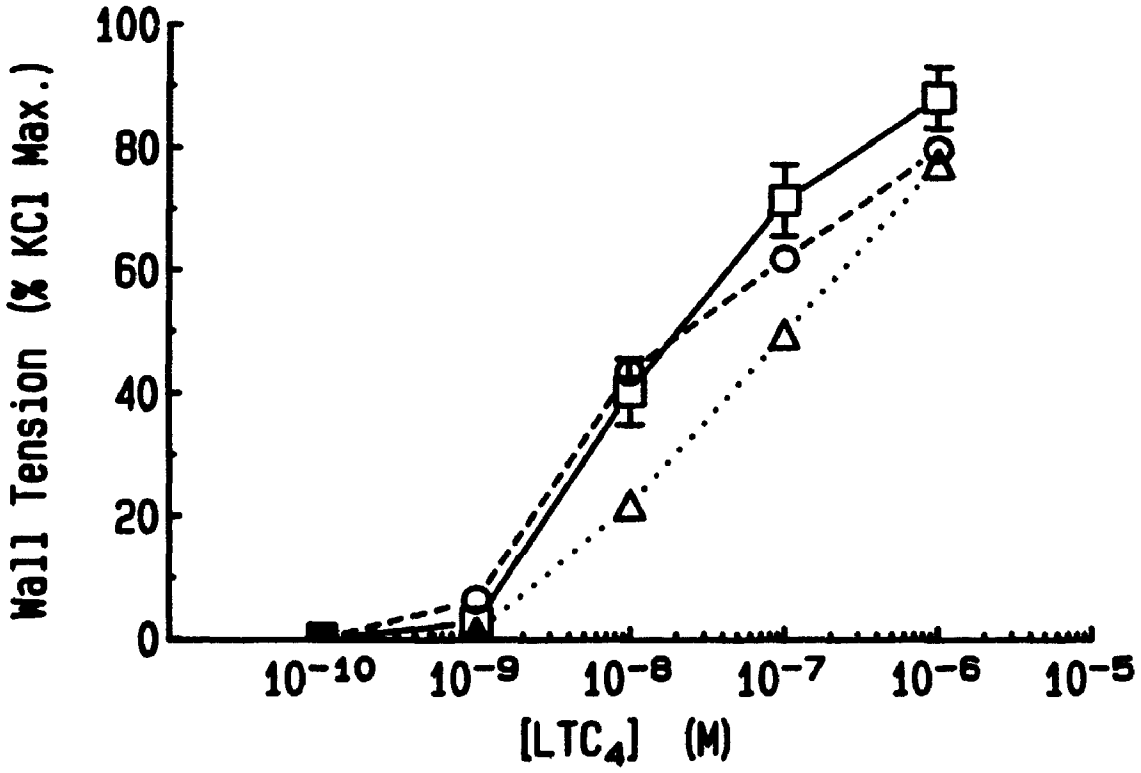

Wall Tension (% KCl Max.)
60
50
40
30
20
10
0
10⁻¹¹
10⁻¹⁰
10⁻⁹
10⁻⁸
10⁻⁷
10⁻⁶
10⁻⁵
[LTD4] (M)
*
**

FIGURE 18. Effect of FPL 57231 (Δ, 3 μM) or LY 163443 (O, 1 μM) on LTC_4-induced contractions of pulmonary venules (□) under normoxic conditions (15% O_2). Each antagonist was preincubated with the venules for 15 min before adding cumulative concentrations of LTC_4 to the organ bath. Each venule served as its own control. The results are means ± SEM (control: n=12; 1 μM LY 163443: n=2; 3 μM FPL 57231: n=2).


Wall Tension (% KCl Max.)
100
80
60
40
20
0
10⁻¹⁰
10⁻⁹
10⁻⁸
10⁻⁷
10⁻⁶
10⁻⁵
[LTC4] (M)

LY 163443 (0.5 and 1 μM), following preincubation with the pulmonary venules, inhibited LTD_4-induced venular contractions (Fig. 19), while having no effect on the contractions elicited by LTC_4 (Fig. 18). Moreover, LY 163443 significantly ($p < 0.01$) relaxed LTD_4 precontracted pulmonary venules [IC_{50}: 0.25 ± 0.04 μM (n=3)], but was ineffective against LTC_4-induced venular tone (Appendix I: Fig. Q).

Leukotriene release from pulmonary venules under conditions of normoxia and anoxia:

Control pulmonary venules, under normoxic conditions, released small, occasionally non-detectable (< 2.5 pg), quantities of immunoreactive-LTs (Table 6). However, under anoxic conditions, LT release was depressed to non-detectable levels (Table 6). When stimulated with A-23187, LT release from the pulmonary venules was increased at least 10-fold (Table 6).

Effect of NDGA and U-60257B on leukotriene synthesis by pulmonary venule segments:

Radioimmunoassay demonstrated that of the two lipoxygenase inhibitors tested (NDGA, U-60257B), only NDGA (5 μM) significantly ($p < 0.05$) inhibited A-23187-stimulated LT synthesis by the pulmonary venules (Table 7). The inhibition due to U-60257B (approximately 22%) was not statistically significant.

FIGURE 19. Effect of LY 163443 (○, 0.5 μM;△, 1 μM) on LTD_4-induced contractions of pulmonary venules (□) under normoxic conditions (15% O_2). LY 163443 was preincubated with the venules for 15 min before adding cumulative concentrations of LTD_4 to the organ bath. Each venule served as its own control. The results are means ± SEM (control: n=12; 0.5 μM LY 163443: n=2; 1 μM LY 163443: n=4) and significant differences from control are as indicated (** $p < 0.01$). Statistical comparisons between 0.5 μM LY 163443 and control were not performed.

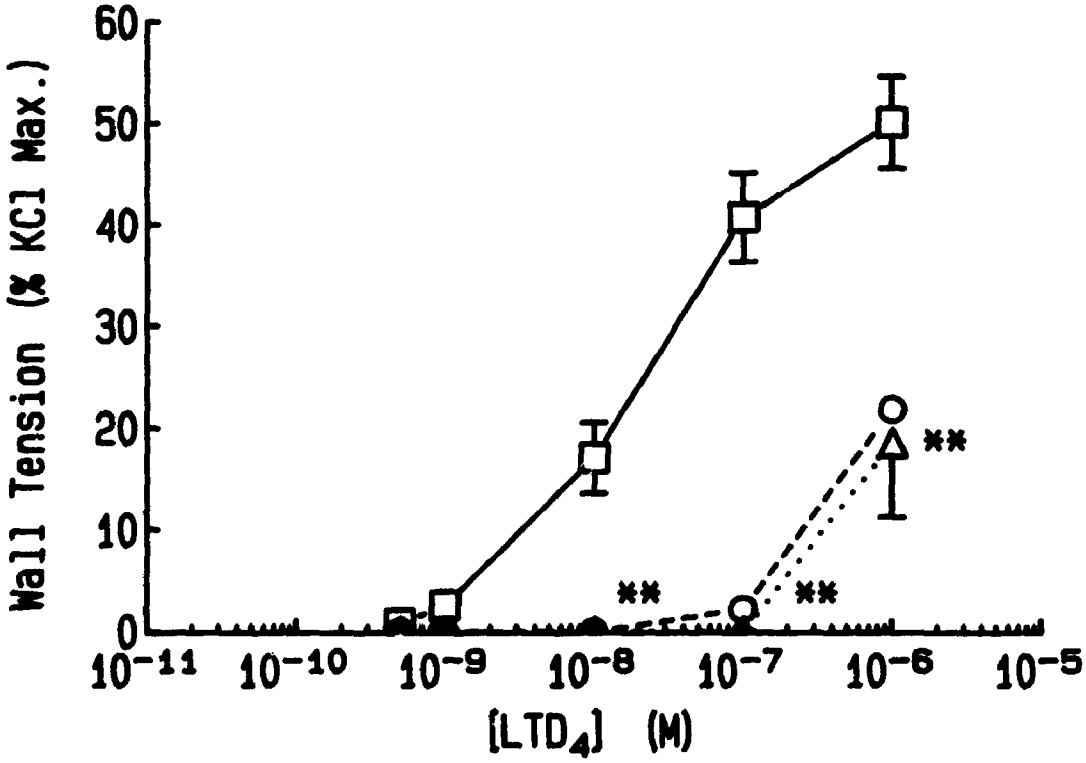

Wall Tension (% KCl Max.)
60
50
40
30
20
10
0
**
**
**
10^{-11}
10^{-10}
10^{-9}
10^{-8}
10^{-7}
10^{-6}
10^{-5}
[LTD_4] (M)

TABLE 6. Effect of anoxia (0% O_2) on leukotriene release from pulmonary venules.

Leukotriene release (C_4, D_4 and E_4)[a] (pg/mg wet tissue)		
Control[b]	A-23187[b]	Anoxia
9.0	191.1	< 2.5
< 2.5	99.3	< 2.5
12.6	145.7	< 2.5

[a]Results from three separate experiments.

[b]Control and A-23187-stimulated venules were maintained at a Po_2 of 110 torr.

TABLE 7. Effect of lipoxygenase inhibitors on A-23187-stimulated leukotriene release from pulmonary venules.

Treatment	n	Leukotriene release (C_4, D_4 and E_4) (pg/mg wet tissue)
Control	3	220.7 ± 47.2[a]
NDGA (5 μM)	3	26.9 ± 2.8*
Control	4	282.2 ± 63.7
U-60257B (10 μM)	4	220.1 ± 20.0

[a]mean ± SEM, significant differences from control are as indicated (* $p < 0.05$).

Effect of leukotriene receptor antagonists or lipoxygenase inhibitors on pulmonary venular contractions due to decreased oxygenation:

None of the agents tested (FPL 57231: 3 μM; LY 163443: 1 μM; NDGA: 5 μM; U-60257B: 10 μM), when preincubated with the pulmonary venules, hadany significant effect on contractions in response to hypoxia or anoxia (Table 8).

Effect of inhibitors of the cytochrome P-450 monooxygenase system on pulmonary venular contractions due to decreased oxygenation:

SKF-525A, at a concentration of 500 μM, significantly ($p < 0.05$) decreased venular contractions induced by hypoxia or anoxia; at 100 μM, the observed decreases were not significant (Fig. 20). However, contractions elicited by 5-HT and $PGF_{2\alpha}$ were also depressed by 500 μM SKF-525A, implying a lack of selectivity in this response (Fig. 20). Metyrapone, at 200 μM, had minimal effect on contractions in response to reduced Po_2, 5-HT or $PGF_{2\alpha}$ (Fig. 21). At a concentration of 1 mM, metyrapone tended to reduce pulmonary venular contractions induced by hypoxia, anoxia and $PGF_{2\alpha}$, but this effect was not significant (Fig. 21).

Effect of β-NF treatment on pulmonary venular contractions induced by decreased Po_2:

Although there was a doubling of 7-ERF activity in pulmonary microsomes following treatment of guinea-pigs with β-NF, the response of the pulmonary venules from these same animals to reductions in Po_2, as well as to several pharmacological agents, was unchanged (Table 9).

TABLE 8. Effect of lipoxygenase inhibitors and leukotriene receptor antagonists on pulmonary venular contractions induced by hypoxia (3% O_2) or anoxia (0% O_2).

	Change in Wall Tension (mg/mm)	
Treatment	3% O_2	0% O_2
Control	3.1 ± 0.5[a]	54.7 ± 11.4
FPL 57231 (3 μM)	2.9 ± 0.5	40.3 ± 9.9
Control	2.0 ± 0.4	27.4 ± 6.0
LY 163443 (1 μM)	3.4 ± 0.9	24.4 ± 5.2
Control	2.7 ± 0.9	20.5 ± 4.0
NDGA (5 μM)	3.5 ± 1.2	17.6 ± 3.9
Control	4.0 ± 0.6	58.1 ± 12.6
U-60257B (10 μM)	5.4 ± 0.9	56.0 ± 12.4

[a]mean ± SEM, n=6, each vessel served as its own control.

FIGURE 20. Effect of SKF-525A on the response of pulmonary venules to decreased Po_2, 5-HT and $PGF_{2\alpha}$. In each case, SKF-525A was preincubated with the venules for 30 min before the Po_2 was altered or an agent added to the organ bath. Responses to 5-HT and $PGF_{2\alpha}$ were assessed under conditions of normoxia (15% O_2). The results are means ± SEM and significant differences from control are as indicated (* $p < 0.05$) (n=3-4).

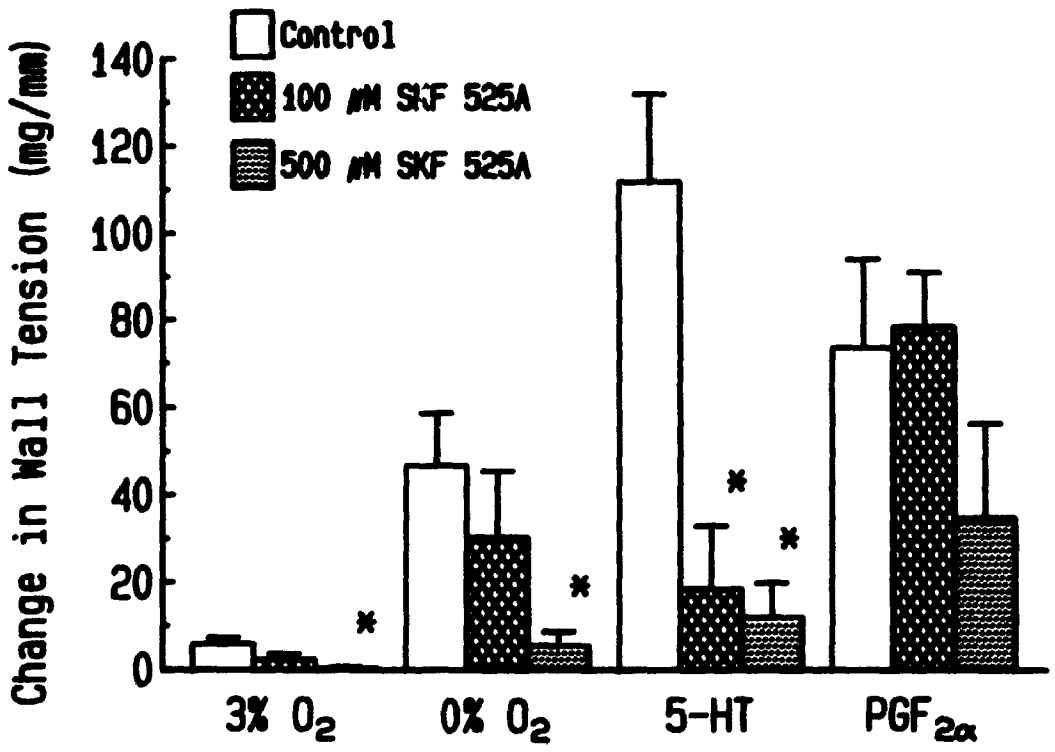

Control
100 μM SKF 525A
500 μM SKF 525A
Change in Wall Tension (mg/mm)
140
120
100
80
60
40
20
0
*
*
*
*
3% O_2
0% O_2
5-HT
$PGF_{2\alpha}$

FIGURE 21. Effect of metyrapone on the response of pulmonary venules to decreased Po_2, 5-HT and $PGF_{2\alpha}$. In each case, metyrapone was preincubated with the venules for 30 min before the Po_2 was altered or an agent added to the organ bath. Responses to 5-HT and $PGF_{2\alpha}$ were assessed under conditions of normoxia (15% O_2). The results are means ± SEM (n=3-4).

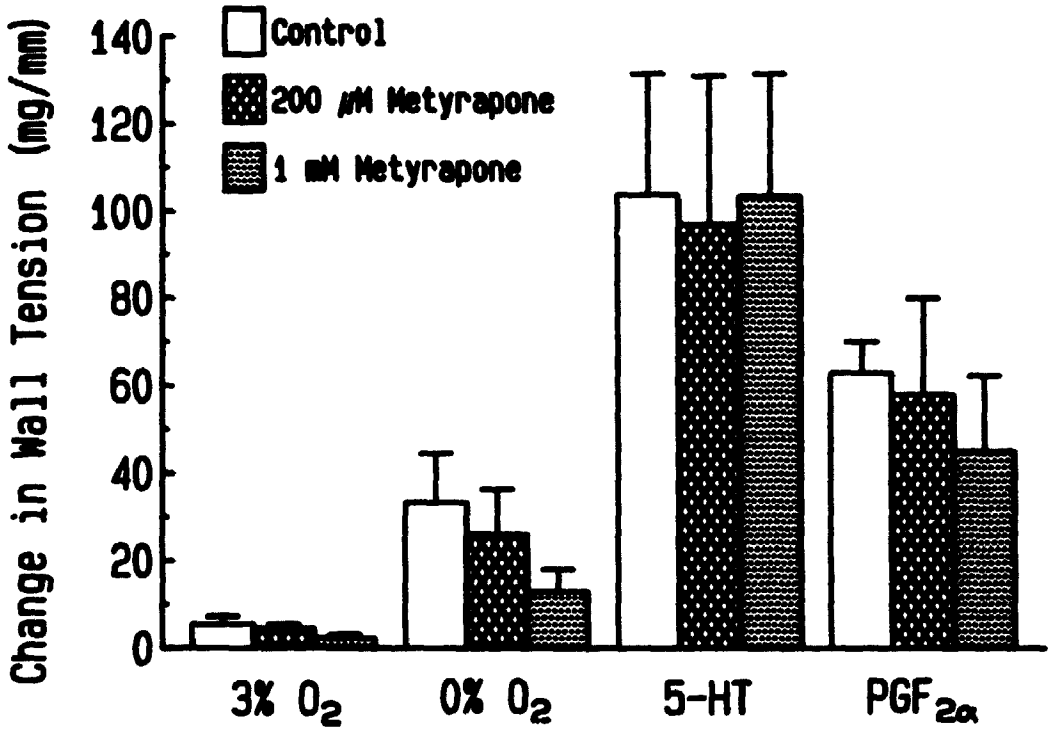

Change in Wall Tension (mg/mm)
140
120
100
80
60
40
20
0
Control
200 μM Metyrapone
1 mM Metyrapone
3% O_2
0% O_2
5-HT
$PGF_{2\alpha}$

TABLE 9. Effect of β-naphthoflavone treatment on pulmonary cytochrome P-450 activity and pulmonary venular contractions induced by hypoxia (3% O_2), anoxia (0% O_2) or pharmacological agents.

Treatment	7-ERF activity (pmol/min/mg protein)	Total P-450 (nmol/mg protein)	3% O_2	0% O_2	5-HT (0.5 μM)	$PGF_{2\alpha}$ (10 μM)	KCl (15 mM)	KCl (120 mM)
						(mg/mm)		
Control[a]	14.4	0.79	3	24	116	87	49	229
β-NF[a]	28.8	0.98	2	18	129	54	54	197

[a] n=2

DISCUSSION

Since the LTs and PGs may regulate each other's synthesis and/or action, it was first necessary to determine the involvement of PGs in pulmonary venular hypoxic and anoxic contractions before studying the possible role of the LTs. Neither of the cyclooxygenase inhibitors tested (indomethacin and ibuprofen) altered the venular contractions in response to reduced Po_2 (Table 5). These observations are consistent with recent studies indicating that PGs do not play a major physiological role in HPV (Naeije et al., 1988; Rubin et al., 1985). Although we did not confirm directly that ibuprofen and indomethacin inhibited cyclooxygenase activity in our preparation, both antagonists were used at concentrations approximately ten-fold greater than the previously reported IC_{50}'s for inhibition of PG synthesis (Shen, 1979).

Prior to addressing whether LTs mediate pulmonary venular contractions to reduced oxygenation, it was necessary to establish if the venules respond to LTs. Both LTD_4 and LTC_4 induced concentration-dependent contractions of the pulmonary venules (Figs. 15 and 16) and these contractions were modestly augmented under hypoxic or anoxic conditions. A similar augmentation of LT-induced contractions of isolated porcine pulmonary arteries and veins under conditions of reduced Po_2 was previously reported by this laboratory (Paterson et al., 1988b). Hypoxic augmentation is not unique to the LTs, as pulmonary vascular responses to histamine (Paterson et al., 1988b), $PGF_{2\alpha}$ and PGE_1 (Tucker et al., 1977b) are also enhanced by decreased

Po_2. However, the data from the present study and those of Paterson et al. (1988b) suggest that any contribution of the LTs to hypoxic vasoconstriction may reflect an increased vascular responsiveness to the LTs in addition to (or instead of) enhanced LT release.

When LT release from pulmonary venule fragments was measured by RIA, spontaneous release of these compounds was detected (Table 6). In addition, the pulmonary venules were capable of increased LT synthesis following A-23187 stimulation (Table 6), as recently demonstrated for other vessels (Piper and Levene, 1986; Piomelli et al., 1987). However, rather than stimulating LT release from the venules, anoxia actually decreased LT levels below the lower detection limit of the assay (2.5 pg) (Table 6).

Data from previous studies of LT release under conditions of decreased oxygenation, both from our laboratory (Paterson, 1986) and others (Peters et al., 1986), have indicated that LT release is depressed under such conditions. Thus, Paterson (1986) was unable to demonstrate spontaneous LT release from dispersed porcine parenchymal lung cells under conditions of normoxia, hypoxia or anoxia. When the cells were stimulated with A-23187, anoxia inhibited the ionophore-stimulated LT release (Paterson, 1986). Similarly, Peters and colleagues (1986) observed that reducing the buffer Po_2 from 161 to 54 torr did not elicit LT release from human lung fragments. In addition, if the lung fragments were stimulated to release LTs with goat antihuman IgE, hypoxia inhibited the LT release by 81% (Peters et al., 1986).

If the LTs do indeed mediate pulmonary venular contractions to reduced Po_2, preventing the synthesis of the LTs or blocking the receptors at which they act, should inhibit the contractions. We chose to test this hypothesis by using the LT receptor antagonists FPL 57231 and LY 163443, as well as the LT synthesis inhibitors NDGA and U-60257B (Piriprost). FPL 57231 is a propionic acid analog of FPL 55712 that has been demonstrated to block pharmacological responses to LTC_4, LTD_4 and LTE_4 (Sheard et al., 1982), while LY 163443 is an aryloxymethylacetophenone that selectively blocks pharmacological responses to LTD_4 and LTE_4 (Fleisch et al., 1986). Nordihydroguaiaretic acid is a 5-lipoxygenase inhibitor (Egan and Gale, 1985; Chang et al., 1984) that also inhibits cyclooxygenase activity (Chang et al., 1984) and has anti-oxidant properties (Egan and Gale, 1985). U-60257B is also a 5-lipoxygenase inhibitor, although it may antagonize LTC_4 synthetase as well (Bach et al., 1982).

Before assessing the effects of these agents on pulmonary venular contractions induced by hypoxia and anoxia, it was necessary to demonstrate that they were active in the experimental preparation. Both FPL 57231 and LY 163443, when preincubated with the venules, significantly inhibited LTD_4-induced contractions (Figs. 17 and 19). In contrast, FPL 57231 was relatively less effective at preventing LTC_4-induced pulmonary venular contractions, while LY 163443 was inactive against LTC_4 (Fig. 18). Leukotriene release from A-23187-stimulated pulmonary venule fragments was significantly inhibited by the lipoxygenase inhibitor NDGA, as assessed by RIA (Table 7).

Although U-60257B appeared to reduce LT synthesis, the reduction was not significant (Table 7).

When pulmonary venules were preincubated with FPL 57231 (3 μM), LY 163443 (1 μM), NDGA (5 μM), or U-60257B (10 μM), the hypoxic and anoxic contractions of the venules were unaffected (Table 8). Higher concentrations of FPL 57231, LY 163443, NDGA and U-60257B were not studied because they were found to non-selectively depress the contractions elicited by other pharmacological agents. As mentioned, these inhibitors, at the concentrations used, have been shown in our model to prevent either LT-induced contractions or LT synthesis. Therefore, these data, along with the observation that anoxia blunts LT release from pulmonary venules, indicate that the LTs do not mediate pulmonary venular contractions to decreased Po_2.

If our data can be extrapolated to HPV in vivo, they would support the observations of other investigators (Schuster and Dennis, 1987; Leffler et al., 1984; Gottlieb et al., 1988; McCormack and Paterson, 1988) which indicate that LTs do not appear to play an important role in HPV. Although there are several earlier reports which have suggested that LTs do mediate HPV (Ahmed et al., 1983; Morganroth et al., 1984a, 1984b; Raj and Chen, 1987), in only one of these studies (Morganroth et al., 1984a) were LT concentrations measured. Before assigning a role for the LTs in HPV, it is necessary to demonstrate that LTs are indeed released during hypoxia and that chemically dissimilar LT synthesis inhibitors prevent the synthesis of these compounds while selectively preventing HPV. Although Morganroth et al. (1984a) reported that DECC both prevented HPV in the isolated rat

lung and attenuated LTC_4 release during hypoxia, a recent study performed in anesthetized dogs (Lonigro et al., 1988) found that while DECC inhibited LT release during hypoxia, it had no effect on HPV. Lonigro and colleagues (1988) were unable to provide a reason for this discrepancy, although species and experimental design differences were considered.

These earlier studies were performed using whole animals or isolated lung preparations, and with the exception of the report by Raj and Chen (1987), no information was provided on the response of the venous versus the arterial side of the circulation. The physiological and/or pathophysiological importance of pulmonary venular constriction during HPV is still unclear, even though recent studies suggest venular constriction may contribute significantly to the total increase in pulmonary vascular resistance during hypoxia (Nagasaka et al., 1984; Raj and Chen, 1986). Although Raj and Chen (1987) observed that the pulmonary veins in isolated lamb lungs constricted during hypoxia, they concluded that thromboxane A_2 is required for venous constriction. This finding conflicts with the present study, since in our hands, cyclooxygenase inhibitors failed to prevent pulmonary venular contractions to decreased Po_2. We do not have a satisfactory explanation for this discrepancy, although a species difference is an obvious possibility.

There is yet another pathway of AA metabolism, ie. via the cytochrome P-450 monooxygenase system (McGiff and Carroll, 1987), which has recently received a great deal of attention. The metabolites of this pathway have been suggested to play a role in

controlling pulmonary vascular tone (Pinto et al., 1986); however, their possible involvement in HPV is not clear. Sylvester and McGowan (1976) reported that HPV in perfused porcine lungs was blunted by metyrapone, SKF-525A (proadifen) and carbon monoxide (CO), known inhibitors of the P-450 system. However, each of these agents either altered normoxic pulmonary vascular tone or non-selectively depressed $PGF_{2\alpha}$-induced contractions, thus precluding an unambiguous interpretation of the data. Miller and Hales (1979) on the other hand, using anesthetized dogs, found that metyrapone and CO selectively inhibited HPV.

In the present study, we investigated the effects of metyrapone and SKF-525A on pulmonary venular contractions induced by lowered Po_2. Metyrapone is a reversible inhibitor of cytochrome P-450 with the capacity to bind both the oxidized and reduced forms of the enzyme, and which may compete either with the binding of substrate and/or oxygen to the enzyme (Testa and Jenner, 1981, Rossi, 1983). SKF-525A is a reversible non-competitive inhibitor of cytochrome P-450 which usually requires conversion by the enzyme to an active metabolic intermediate before producing its inhibitory effect (Testa and Jenner, 1981). In the present study, SKF-525A non-selectively depressed pulmonary venular contractions elicited by hypoxia, anoxia, and reference agonists (Fig. 20). Metyrapone (1 mM), on the other hand, appeared to mildly depress venular contractions induced by hypoxia, anoxia and $PGF_{2\alpha}$, although these effects were not significant (Fig. 21).

The lack of selectivity of these agents in our model is not surprising, as these compounds are known to have several different actions. The non-selective effect of SKF-525A in our preparation could be explained by this compound's ability to depress vascular smooth muscle contraction (Massingham, 1973), stimulate prostacyclin (Boeynaems et al., 1987; Rees et al., 1988) and nitric oxide release (Rees et al., 1988), or interfere with calcium movement (Kalsner et al., 1970). Indeed, this latter action was the basis for the use of SKF-525A in an earlier study of the role of transmembrane calcium flux in HPV (McMurtry et al., 1976). Similarly, metyrapone is known to alter PG production and inhibit smooth muscle activity (Parnham, 1976), and to inhibit lipoxygenase activity (Pretus et al., 1985). Clearly, the pharmacological effects of these compounds must be interpreted with caution, especially at the relatively high concentrations required to elicit an effect.

Since the results of the cytochrome P-450 inhibitor experiments were equivocal, we decided to test the effect of selectively inducing an isozyme of P-450 in guinea-pig lung by the administration of β-NF, a polycyclic hydrocarbon (PAH)-type inducing agent (Domin et al., 1984), on the response of the pulmonary venules to decreased Po_2. Our rationale was based on the observation that a PAH-inducible form of cytochrome P-450 metabolizes AA to a product(s) with biological activity including the relaxation of vascular smooth muscle (Schwartzman et al., 1985; Proctor et al., 1987). In addition, Mansour and colleagues observed that pretreatment of rats (1988a) or mice (1988b) with β-NF or 3-methylcholanthrene provided protection

against the toxic effects of hyperoxia. Other investigators have localized a cytochrome P-450 in the cell membrane and pinocytotic vesicles of endothelial cells of rabbit lung (Serabjit-Singh et al., 1988). Moreover, a PAH-inducible form of cytochrome P-450 was found to be present in the endothelium of pulmonary arteries and veins of this species (Dees et al., 1982) and cytochrome P-450-dependent monooxygenase activity or metabolism of PAH-type compounds has been reported in endothelial cells from the hog (Abraham et al., 1985) and bovine aorta (Baird et al., 1980), respectively. Metabolism of PAH-type compounds has also been observed in bovine lung fibroblast-like cell cultures (Baird et al., 1980). Finally, a cytochrome P-450 isozyme which is orthologous to the well characterized, PAH-inducible isozyme 6 of rabbit lung is present in guinea-pig pulmonary microsomes and is induced by PAH-type compounds (Domin et al., 1984).

To verify that treatment with β-NF had induced the P-450 system of guinea-pig lung, 7-ERF activity was measured (Serabjit-Singh et al., 1983). As shown in Table 8, preliminary experiments showed that there was a 2-fold increase in 7-ERF activity. However, the pulmonary venular contractions induced by lowered Po_2 were not augmented (Table 8). The increase in isozyme 6-dependent activity which we observed was relatively moderate, confirming earlier observations that this form of cytochrome P-450 is relatively resistant to induction in the guinea-pig (Abe and Watanabe, 1982). Nevertheless, if this form of cytochrome-P-450-like activity did mediate pulmonary venular contractions induced by decreased Po_2, an augmentation of the contractions elicited by hypoxia and anoxia might be expected; such an

effect was not observed. Since induction of the isozyme 6 orthologue did not augment, and SKF-525A and metyrapone had little effect (if any), on the pulmonary venular contractions induced by lowered Po_2, it seems unlikely that a cytochrome P-450 metabolite(s) of AA mediates these contractions. However, our data do not totally preclude this possibility. It is possible that one of the phenobarbital-inducible forms of pulmonary cytochrome P-450, similar to forms 2 and 5 of the rabbit lung, may be involved in the contractions elicited by decreases in Po_2. However, these pulmonary isozymes, unlike their counterparts in the liver, are not induced by phenobarbital (Serabjit-Singh et al., 1983), preventing a similar study to that performed on the isozyme 6 orthologue.

In summary, we have investigated the role of cyclooxygenase, lipoxygenase and cytochrome P-450 metabolites in the pulmonary venular contractions induced by hypoxia and anoxia, and have found no evidence to support the hypothesis that any one or a combination of AA metabolites formed by these pathways mediates these contractions.

GENERAL DISCUSSION AND CONCLUSIONS

The goal of the studies described in this thesis was to describe and elucidate some of the mechanisms involved in the response of pulmonary venules to reduced Po_2. Impetus for this study was provided by recent evidence suggesting a potentially important role for the venous side of the circulation in HPV or in pulmonary diseases in which hypoxia is a contributing factor, and the relative lack of information in the literature regarding the pulmonary venous response to hypoxia. Although it was hoped that the model described in this thesis could be useful for studying mechanisms of HPV, the model has some potential limitations. Perhaps the most important limitation is the use of venular smooth muscle to study a phenomenon (HPV) which is believed to be primarily an arteriolar response. However, to the extent that venular and arteriolar smooth muscle are similar, the results from the model described in this thesis have applicability to the arterial side of the circulation. In addition, these studies have shed important light on the pulmonary venular response to decreases in oxygen tension and should provide the impetus for further investigations in this area, as well as stimulate research which compares venular and arteriolar responses to hypoxia. Future experiments should also include investigating the effect of oxygen tension gradients across the venular wall (ie. different Po_2's inside and outside the vessel) and the effect of different baseline Po_2's (ie. instead of 15% O_2, use 7.5%, 40%, 60%, 95%, etc.) on the hypoxic and anoxic contractions.

Other potential limitations of the present model are the severity of the stimulus required to elicit the contractile response, and the

fact that the apparent effects of acidosis and alkalosis in this model are opposite of those observed in man. As mentioned in the discussion in Chapter 1, the need for a relatively severe stimulus is perhaps not surprising since tissues studied *in vitro* may, in general, require a stimulus stronger than that which will produce an equal response *in vivo*. Similarly, although the effects of pH in this model are not readily explained, the effect of pH on HPV in different species (and with different experimental conditions) has by no means been consistent. The pH studies should be repeated and verified using different ranges of pH, different methods of altering the pH, and other buffering systems.

The experiments which investigated the role of the endothelium in the pulmonary venular responses to hypoxia and anoxia suggested a modulatory role for the endothelium in a physiological response, rather than a role of mediation. The other interesting observation was the apparent lack of involvement of a known endothelium-derived vasodilator which might oppose the venular contractions induced by decreased Po_2. These results must be looked at with some degree of caution, considering the lack of selectivity of the antagonists which are presently available to address such a question. However, information about various endothelium-derived vasodilators (and vasoconstrictors) is enlarging rapidly, and it would be very interesting to repeat some of these studies in the future with either more selective antagonists, or the actual compounds themselves, once their identity has been conclusively demonstrated. Nontheless, there are other studies which could be performed, such as superfusing

endothelium-denuded venular rings with the effluent from cultured endothelial cells, thereby allowing one to separate the effects of decreased Po_2 on the smooth muscle versus the endothelium. This type of approach might also shed some light on the question of whether the endothelium metabolizes a mediator of the hypoxic contractile response. Other studies should be performed using a specifically designed bioassay cascade apparatus to further investigate whether a soluble mediator of the hypoxic and anoxic contractions is indeed released, be it from the endothelium, smooth muscle, or other cell type. Notwithstanding the importance of further investigation into Po_2/endothelial/smooth muscle interactions, one should also consider pursuing the appparent endothelium-independent relaxations of the pulmonary venule. This observation has only rarely been made in the past, and it would be interesting to elucidate the mechanisms involved in this response.

The data presented in this thesis indicate that the LTs do not mediate the pulmonary venular contractions induced by decreased Po_2. Assuming that the response of pulmonary venules to hypoxia is similar to that of the pulmonary artery, these results suggest (along with several other recent studies) that LTs do not mediate HPV, at least in the *in vitro* situation. Since cytochrome P-450 metabolites have not been thoroughly investigated as potential mediators of HPV, an attempt was made to determine if a role for these metabolites might exist in the pulmonary venular contractions induced by decreased Po_2. A contribution of these metabolites was not demonstrated; however, these experiments were inconclusive and should not be taken to indicate that

continued investigation in this area is not warranted. Further experiments might be performed to examine the possible involvement of pulmonary cytochrome P-450 isozymes other than the isozyme 6 orthologue examined in the present studies. One could also attempt to superfuse pulmonary venular rings with the effluent from pulmonary or renal microsomes. This would allow one to get a better indication of whether a cytochrome P-450 metabolite actually mediates the hypoxic and anoxic contractions. In addition, there is still much room for progress in developing specific probes and antagonists for the cytochrome P-450 monooxygenase system, and once these probes become available, one should be able to address the hypothesis further.

APPENDIX I

FIGURE A. Effect of increasing effective lumen radius (ELR) on resting (■) and stimulated (35 mM KCl) (●) wall tensions of pulmonary venules under normoxic conditions (15% O_2). The results are means ± SEM (n=6).

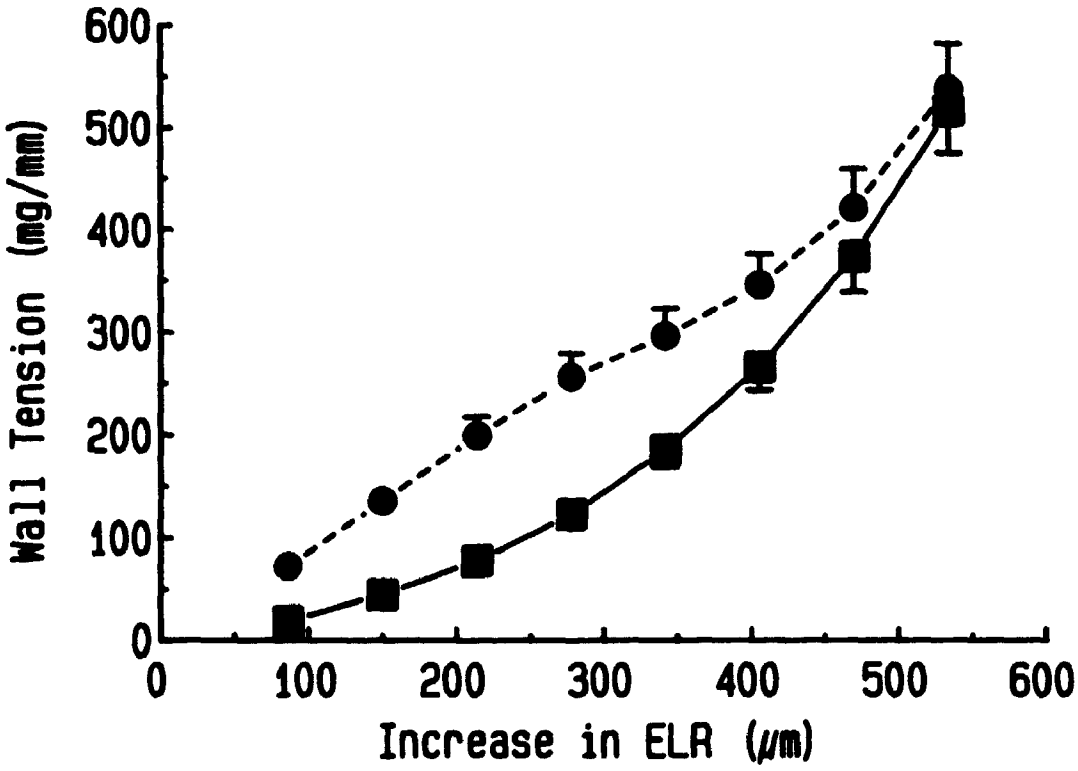

Wall Tension (mg/mm)
600
500
400
300
200
100
0
0
100
200
300
400
500
600
Increase in ELR (μm)

FIGURE B. Effect of increasing effective lumen radius (ELR) on resting (■) and stimulated (45 mM KCl) (●) wall tensions of femoral venules under normoxic conditions (15% O_2). The results are means ± SEM (n=6).

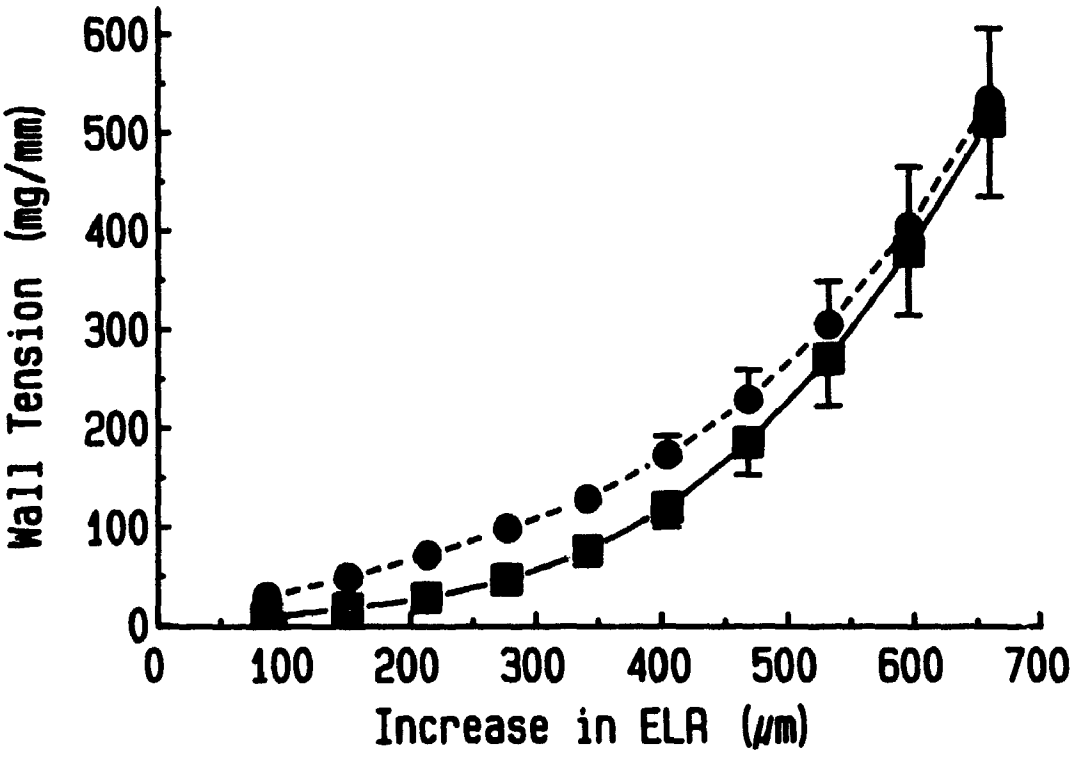

Wall Tension (mg/mm)
600
500
400
300
200
100
0
0
100
200
300
400
500
600
700
Increase in ELR (μm)

FIGURE C(i). Representative tracing of the response of pulmonary and femoral venules from the same animal to an initial challenge with hypoxia (3% O_2) or anoxia (0% O_2). Venules were equilibrated with 15% O_2 prior to producing conditions of hypoxia or anoxia.

FIGURE C(ii). Representative tracing of the response of pulmonary and femoral venules from the same animal to a second challenge with hypoxia (3% O_2) or anoxia (0% O_2). Venules were equilibrated with 15% O_2 prior to producing conditions of hypoxia or anoxia.

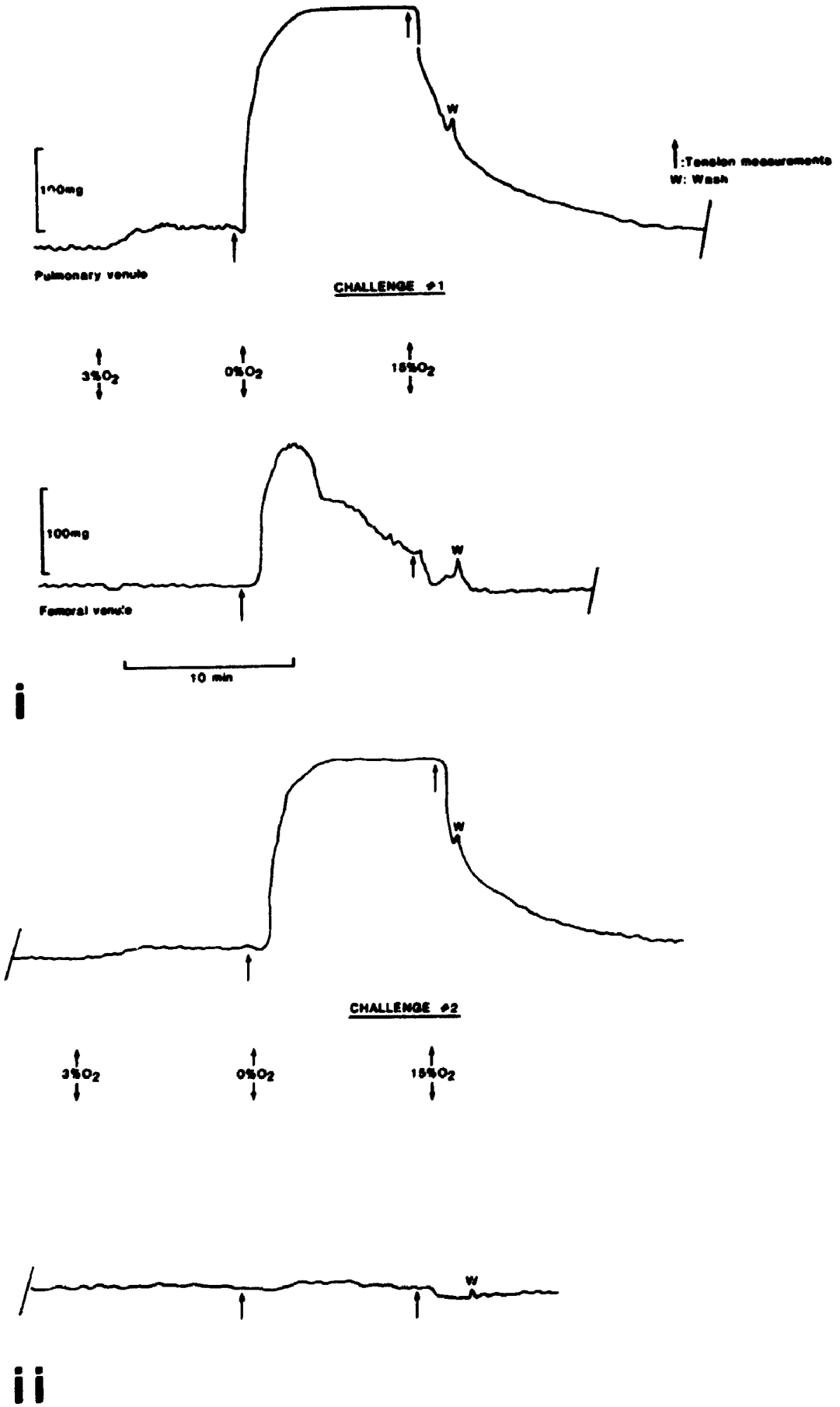

W
:Tension measurements
W: Wash
100mg
Pulmonary venule
CHALLENGE #1
3%O2
0%O2
15%O2
100mg
W
Femoral venule
10 min
i
W
CHALLENGE #2
3%O2
0%O2
15%O2
W
ii

FIGURE D. Effect of cumulative concentrations of KCl on pulmonary (□) and femoral (○) venules from the same animal under normoxic conditions (15% O_2). The results are means ± SEM. (n=12: pulmonary venule, n=9: femoral venule).

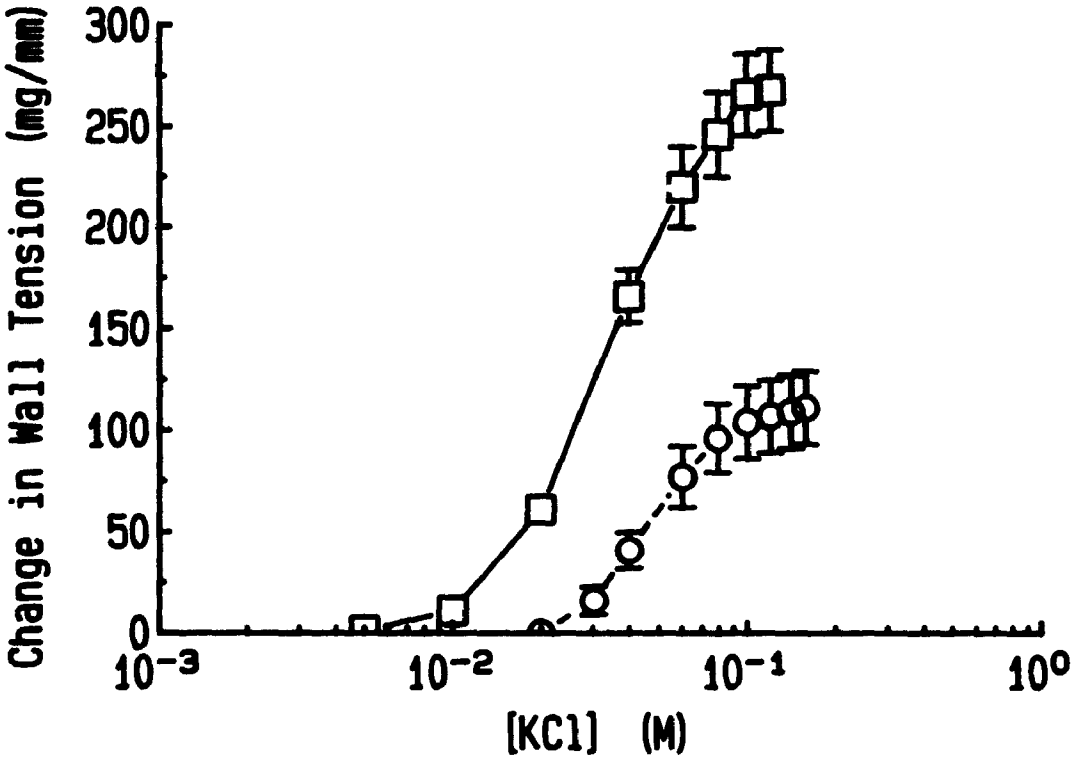

Change in Wall Tension (mg/mm)
300
250
200
150
100
50
0
10^{-3}
10^{-2}
10^{-1}
10^{0}
[KCl] (M)

FIGURE E. Effect of cumulative concentrations of histamine on pulmonary (□) and femoral (○) venules from the same animal under normoxic conditions (15% O_2). The results are means ± SEM (n=10).

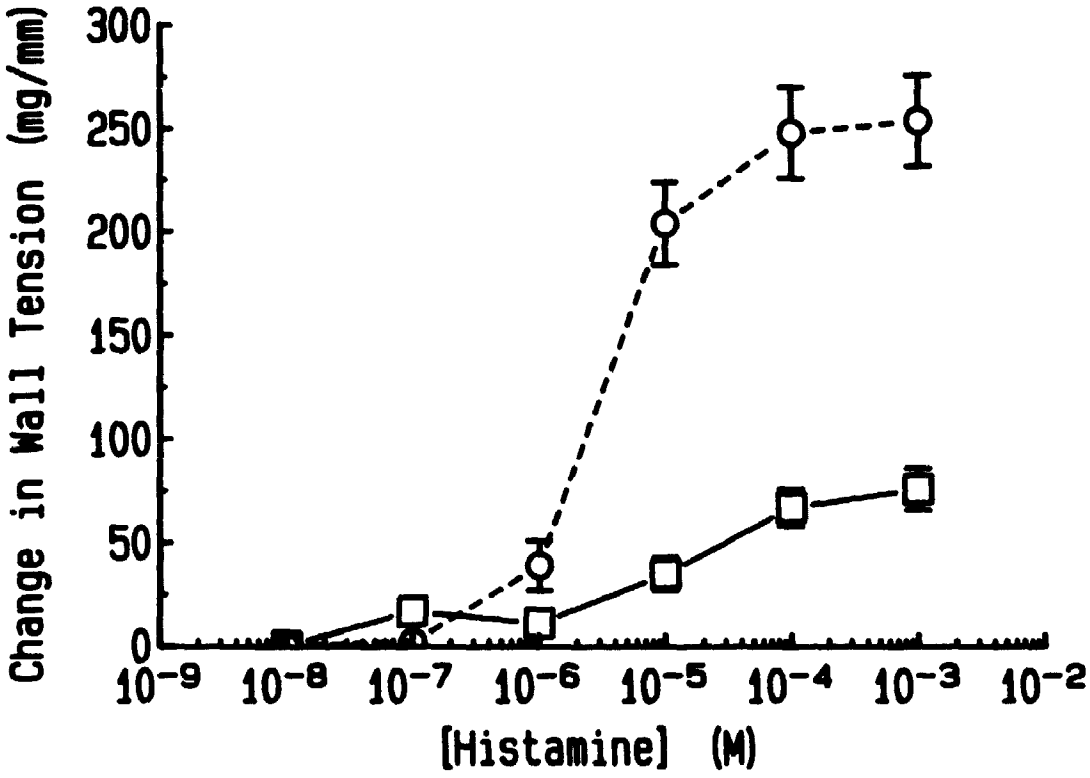

Change in Wall Tension (mg/mm)
300
250
200
150
100
50
0
10-9
10-8
10-7
10-6
10-5
10-4
10-3
10-2
[Histamine] (M)

FIGURE F. Effect of cumulative concentrations of $PGF_{2\alpha}$ on pulmonary (□) and femoral (○) venules from the same animal under normoxic conditions (15% O_2). The results are means ± SEM (n= 10: pulmonary venule, n=8: femoral venule).

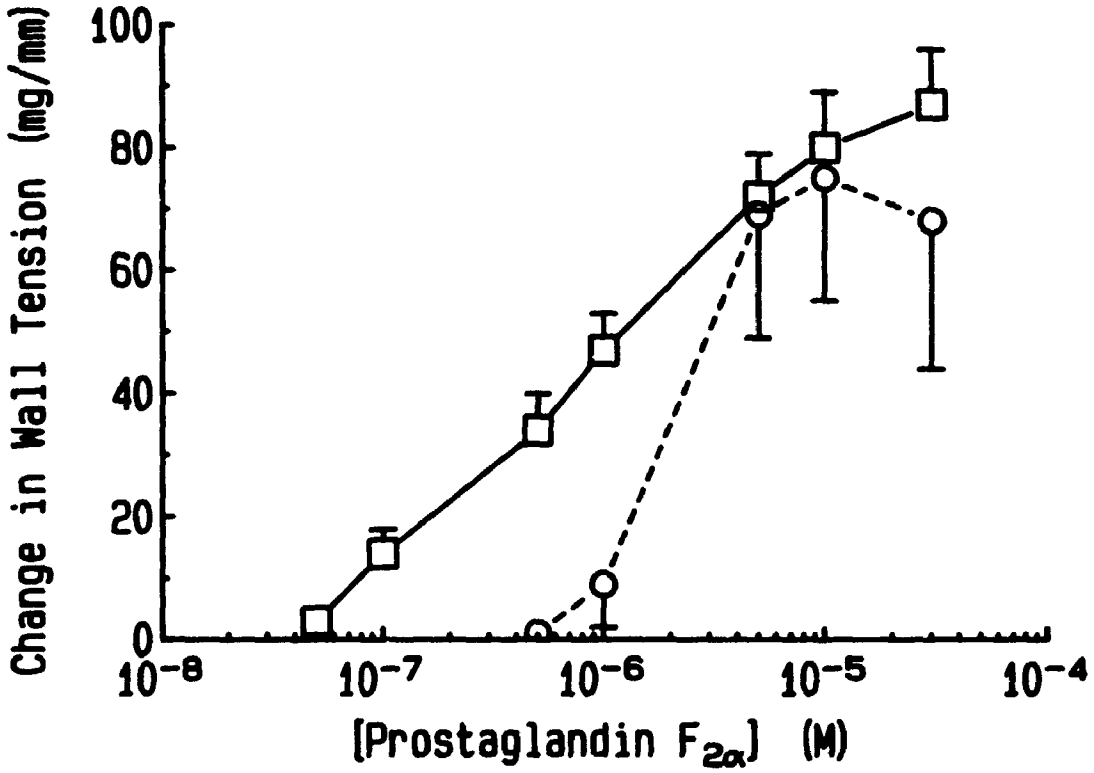

Change in Wall Tension (mg/mm)
100
80
60
40
20
0
10⁻⁸
10⁻⁷
10⁻⁶
10⁻⁵
10⁻⁴
[Prostaglandin F2α] (M)

FIGURE G. Effect of cumulative concentrations of acetylcholine on $PGF_{2\alpha}$ (10 μM) precontracted pulmonary (□) and femoral (○) venules from the same animal under normoxic conditions (15% O_2). The results are means ± SEM (n=9: pulmonary venule, n=6: femoral venule).

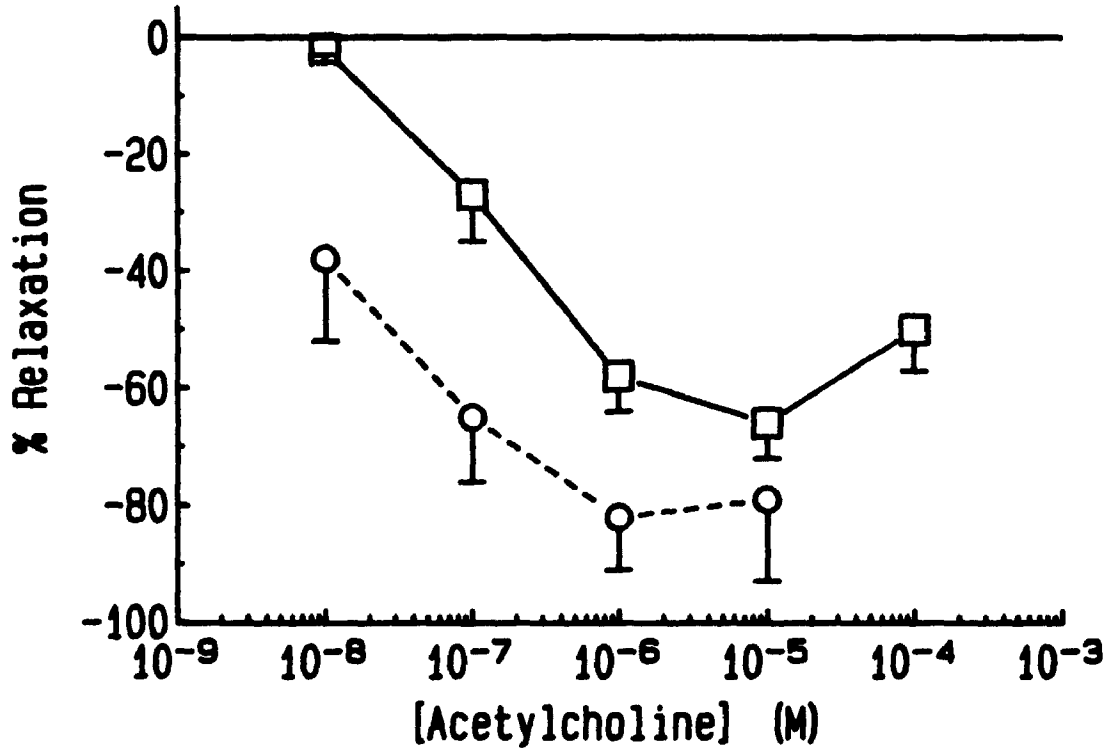

0
-20
-40
-60
-80
-100
% Relaxation
10-9
10-8
10-7
10-6
10-5
10-4
10-3
[Acetylcholine] (M)

FIGURE H. Effect of cumulative concentrations of KCl on pulmonary venules obtained from the same animal with (●) and without (○) parenchyma. Experiments were performed under normoxic (15% O_2) conditions. The results are means ± SEM (n=6).

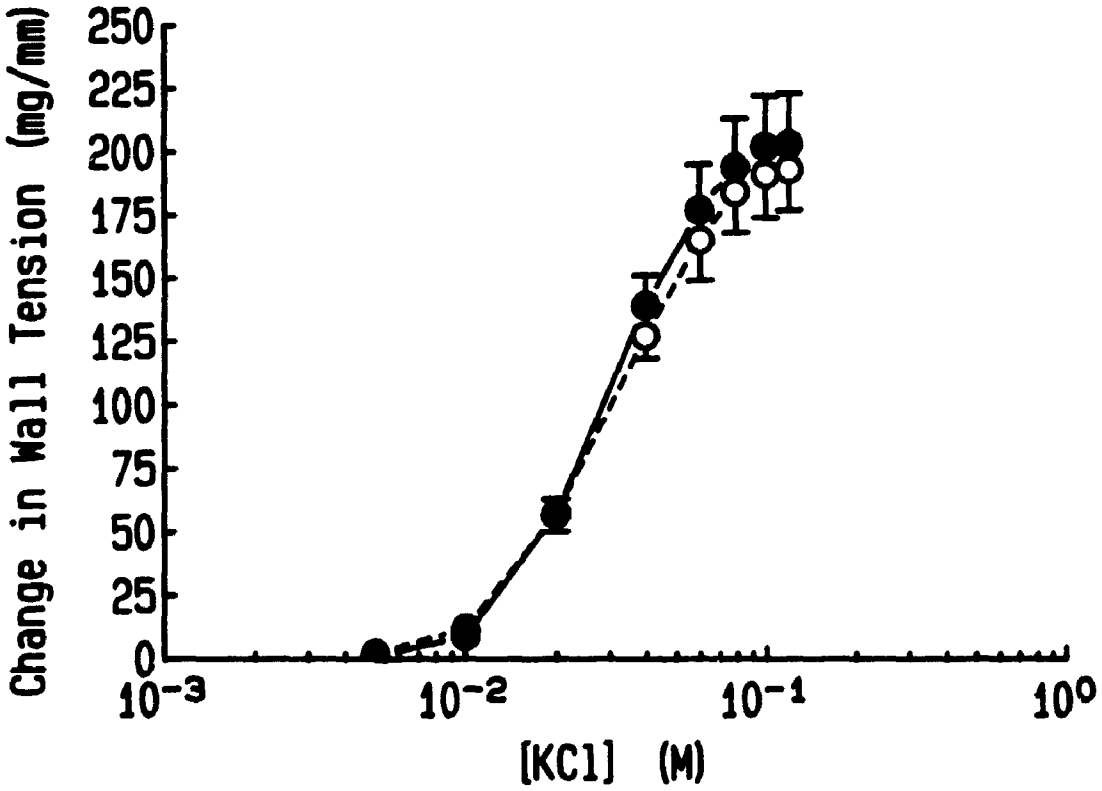

Change in Wall Tension (mg/mm)
250
225
200
175
150
125
100
75
50
25
0
10^{-3}
10^{-2}
10^{-1}
10^{0}
[KCl] (M)

FIGURE I. Effect of cumulative concentrations of 5-HT on pulmonary venules obtained from the same animal with (●) and without (○) parenchyma. Experiments were performed under normoxic (15% O_2) conditions. The results are means ± SEM (n=6).

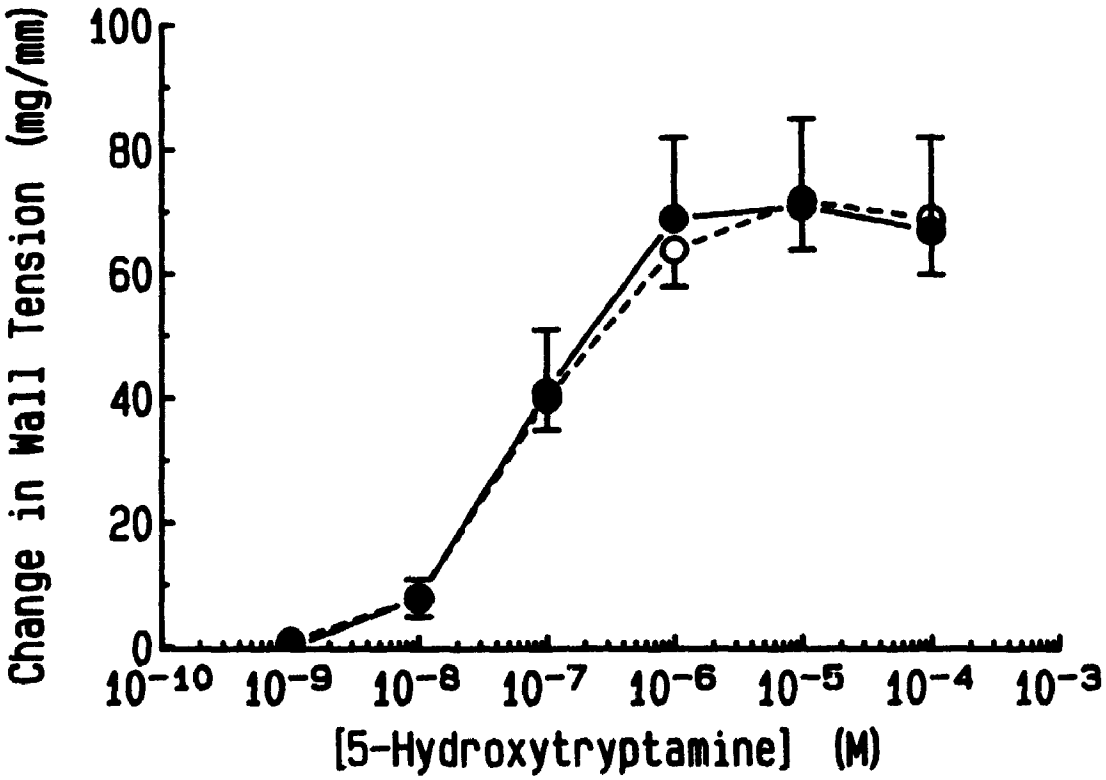

Change in Wall Tension (mg/mm)
100
80
60
40
20
0
10⁻¹⁰ 10⁻⁹ 10⁻⁸ 10⁻⁷ 10⁻⁶ 10⁻⁵ 10⁻⁴ 10⁻³
[5-Hydroxytryptamine] (M)

FIGURE J. Effect of cumulative concentrations of acetylcholine on $PGF_{2\alpha}$ (10 μM) precontracted pulmonary venules obtained from the same animal with (●) and without (O) parenchyma. Experiments were performed under normoxic (15% O_2) conditions. The results are means ± SEM (n=6).

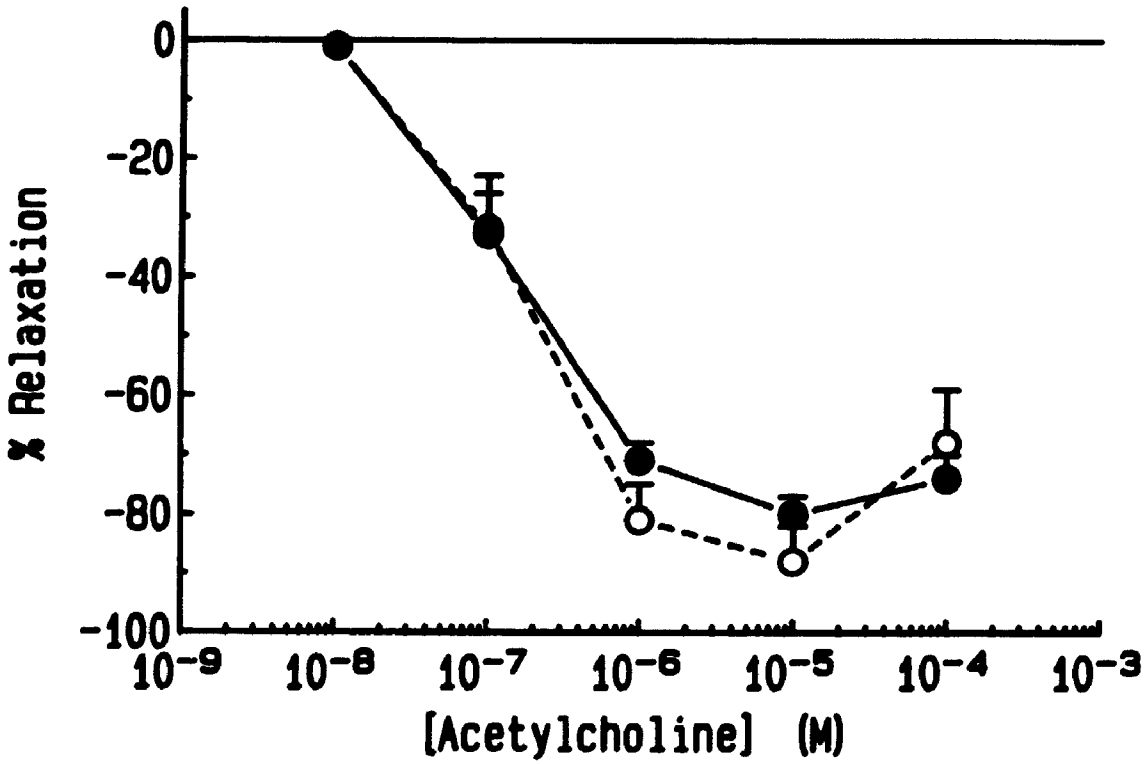

0
-20
-40
-60
-80
-100
% Relaxation
10-9
10-8
10-7
10-6
10-5
10-4
10-3
[Acetylcholine] (M)

FIGURE K. Effect of cumulative concentrations of histamine on pulmonary venules obtained from the same animal with (●) and without (○) parenchyma. Experiments were performed under normoxic (15% O_2) conditions. The results are means ± SEM and significant differences from control (●) are as indicated (* $p < 0.05$) (n=6).

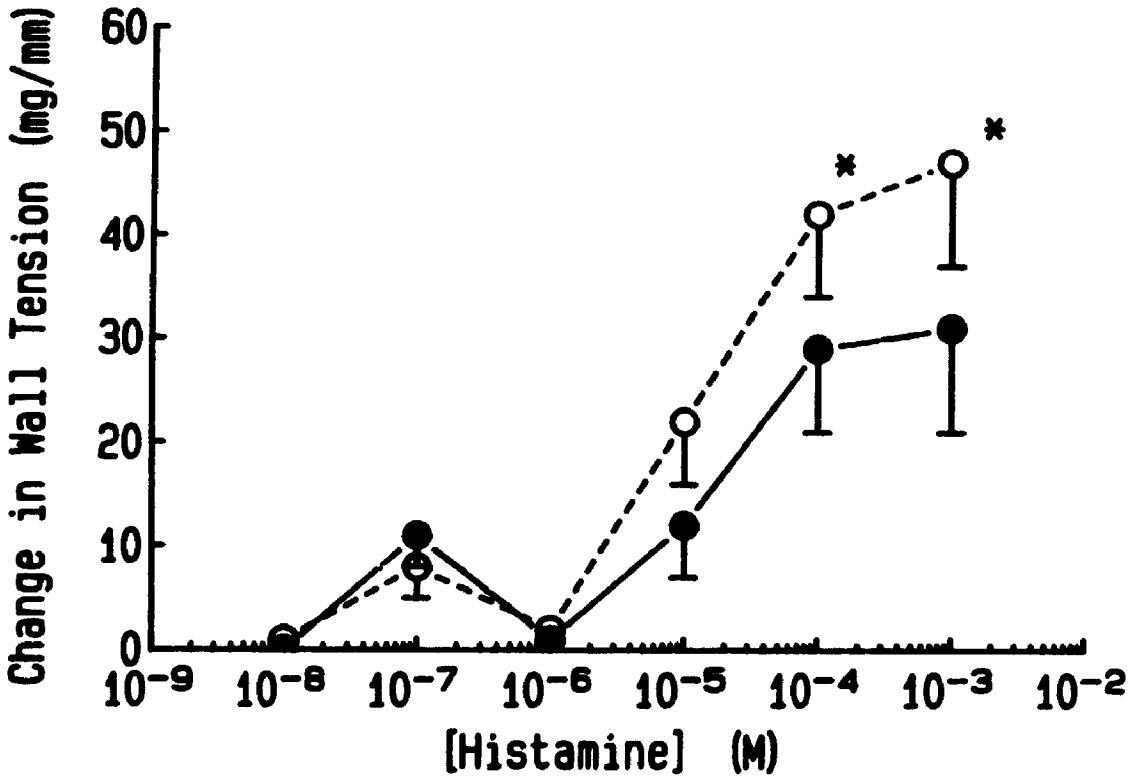

Change in Wall Tension (mg/mm)
60
50
40
30
20
10
0
*
*
10-9
10-8
10-7
10-6
10-5
10-4
10-3
10-2
[Histamine] (M)

FIGURE L. Effect of cumulative concentrations of $PGF_{2\alpha}$ on pulmonary venules obtained from the same animal with (●) and without (O) parenchyma. Experiments were performed under normoxic (15% O_2) conditions. The results are means ± SEM and significant differences from control (●) are as indicated (* $p < 0.05$; ** $p < 0.01$) (n=6).

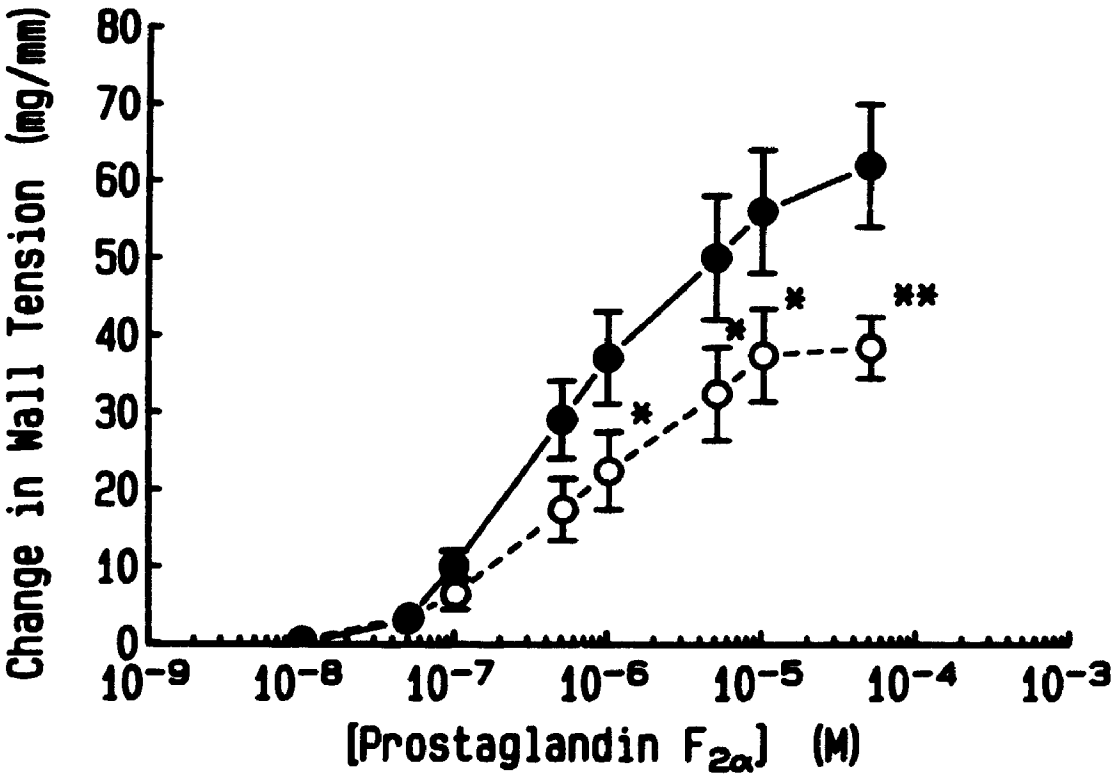

Change in Wall Tension (mg/mm)
80
70
60
50
40
30
20
10
0
10-9
10-8
10-7
10-6
10-5
10-4
10-3
[Prostaglandin F2α] (M)
*
*
*
**

FIGURE M. Response of hypoxanthine/xanthine oxidase-treated femoral venules to anoxia when coincubated with pulmonary venules (Δ) compared to the response of untreated femoral venules to anoxia (O). Femoral venules were perfused with the enzyme solution <u>in situ</u> before being mounted in the organ baths. Pulmonary and femoral venules were obtained from the same animal. Only the tension changes of the femoral venules were recorded. Individual responses from three experiments are shown, with mean responses indicated by the bars.

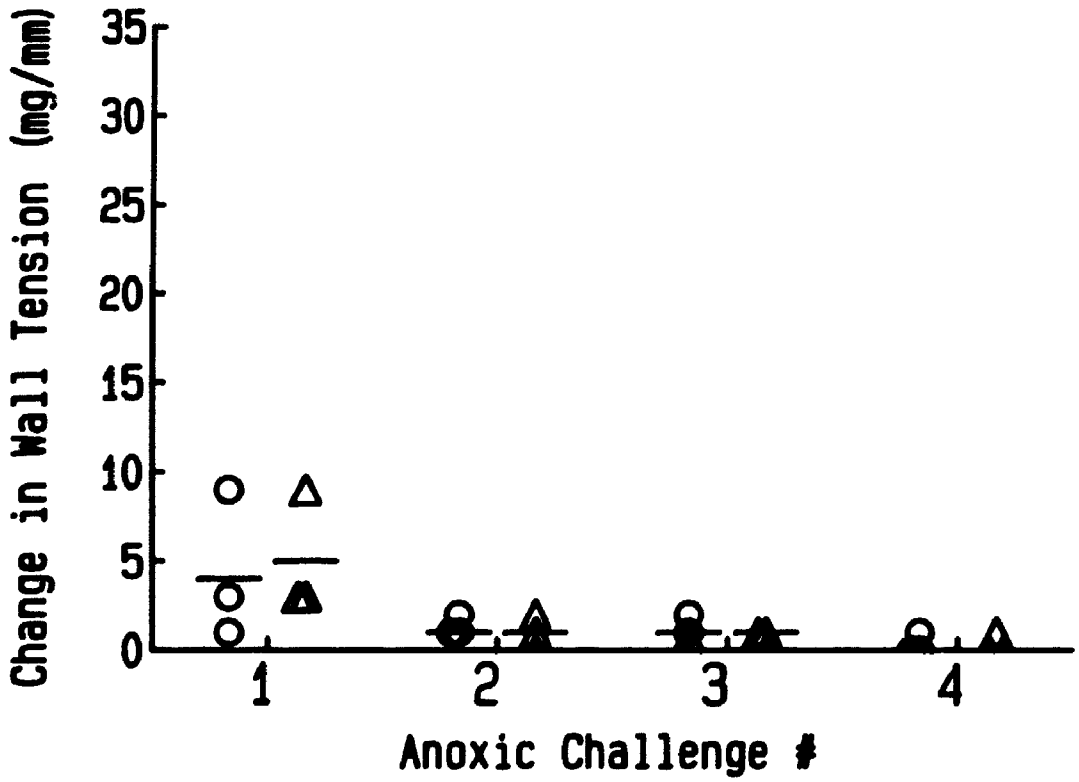

Change in Wall Tension (mg/mm)
35
30
25
20
15
10
5
0
1
2
3
4
Anoxic Challenge #

FIGURE N. Effect of increasing effective lumen radius (ELR) on resting (■) and stimulated (35 mM KCl) (●) wall tensions of pulmonary arteries under normoxic conditions (15% O_2). The results are means ± SEM (n=9).

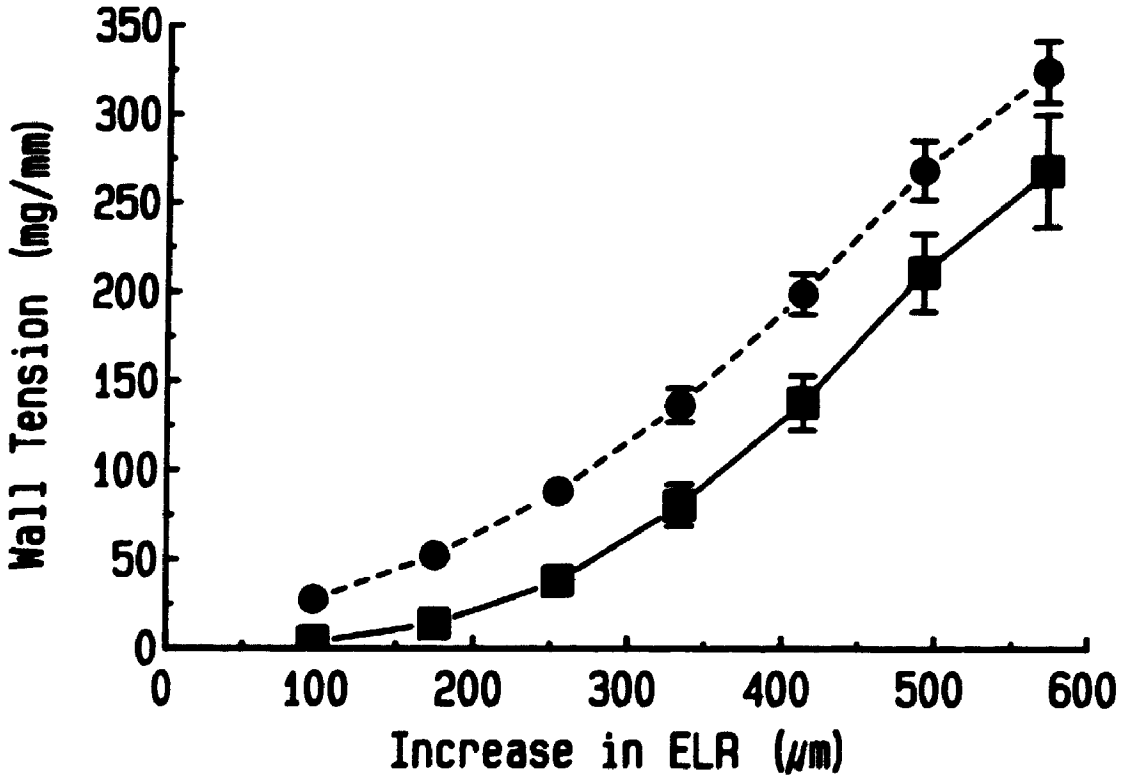

Wall Tension (mg/mm)
350
300
250
200
150
100
50
0
0
100
200
300
400
500
600
Increase in ELR (μm)

FIGURE O. Effect of increasing effective lumen radius (ELR) on the developed (change in) wall tension in pulmonary arteries (O) under normoxic conditions (15% O_2). Vessels were stimulated with 35 mM KCl. The results are means ± SEM (n=9).

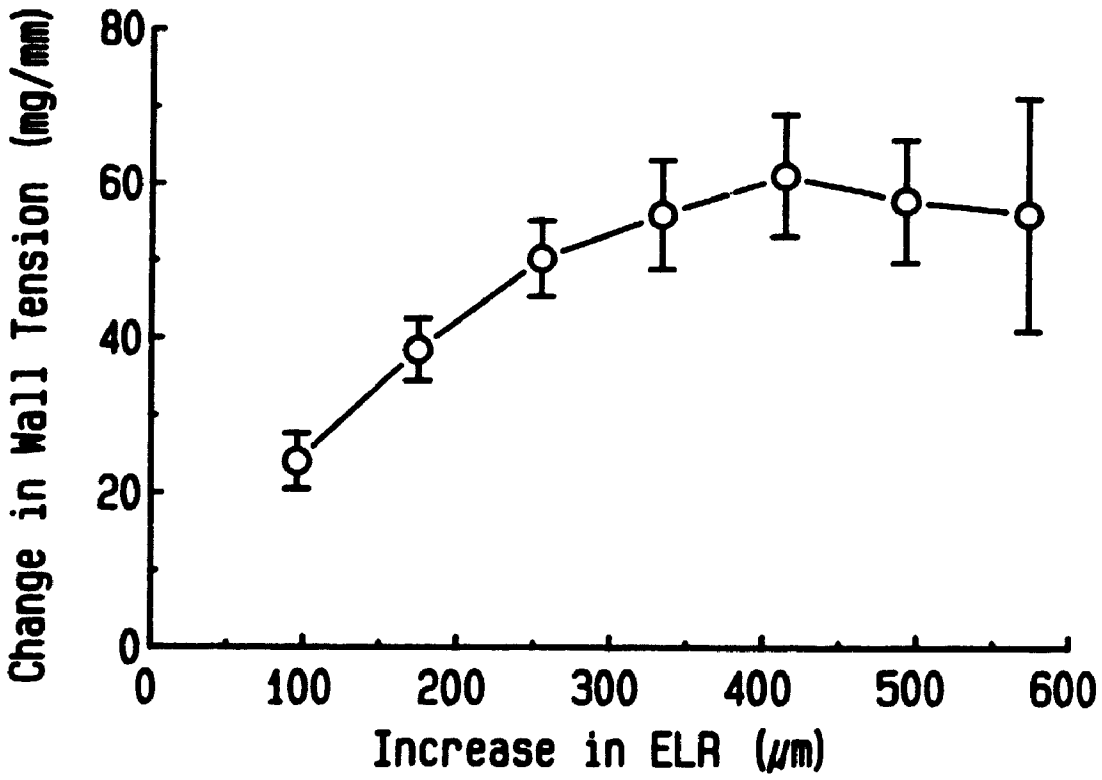

Change in Wall Tension (mg/mm)
80
60
40
20
0
0
100
200
300
400
500
600
Increase in ELR (μm)

FIGURE P. Effect of cumulative concentrations of FPL 57231 on pulmonary venules precontracted with 1 μM LTC_4 (□) or 1 μM LTD_4 (○) under normoxic (15% O_2) conditions. Each venule served as its own control. The results are means ± SEM and significant differences from control (the precontracted baseline) are as indicated (** $p < 0.01$) (n=3).

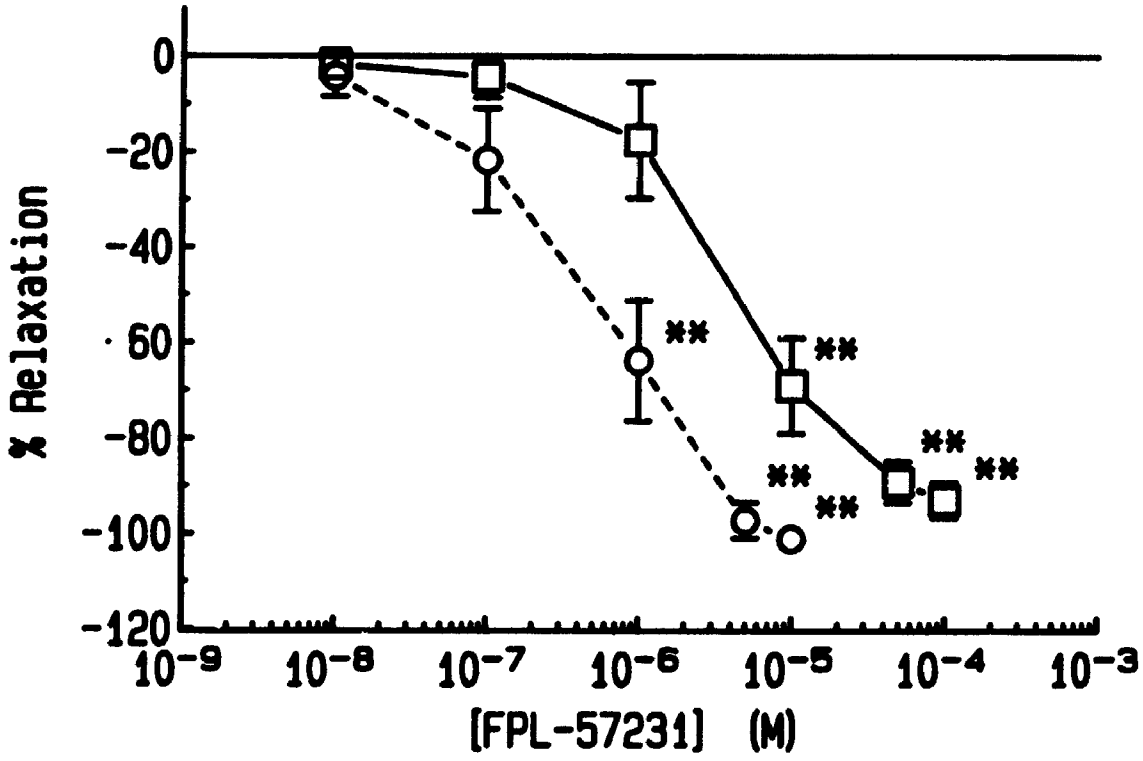
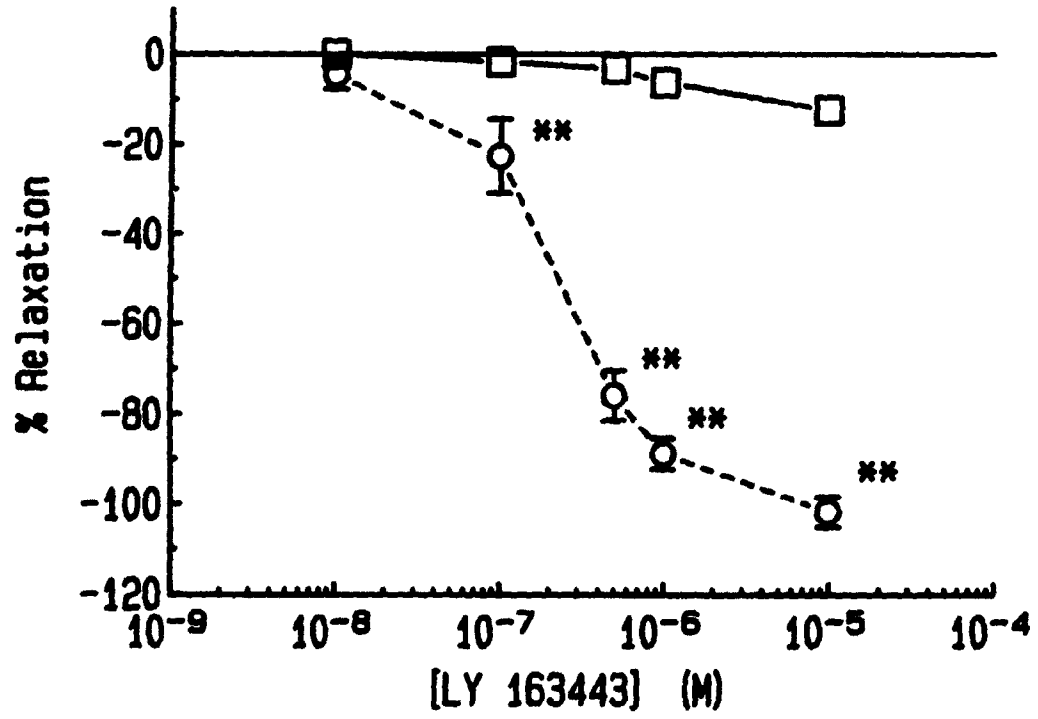

0
-20
-40
-60
-80
-100
-120
% Relaxation
**
**
**
**
**
**
10⁻⁹
10⁻⁸
10⁻⁷
10⁻⁶
10⁻⁵
10⁻⁴
10⁻³
[FPL-57231] (M)

FIGURE Q. Effect of cumulative concentrations of LY 163443 on pulmonary venules precontracted with 1 μM LTC_4 (□) or 1 μM LTD_4 (○) under normoxic (15% O_2) conditions. Each venule served as its own control. The results are means ± SEM and significa t differences from control (the precontracted baseline) are as indicated (** $p < 0.01$) (n=5: LTD_4, n=2: LTC_4).


0
-20
-40
-60
-80
-100
-120
% Relaxation
**
**
**
**
10⁻⁹
10⁻⁸
10⁻⁷
10⁻⁶
10⁻⁵
10⁻⁴
[LY 163443] (M)

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