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ALTERED REACTIVITY OF PULMONARY VESSELS IN POSTOBSTRUCTIVE PULMONARY VASCULOPATHY

BY

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the degree of Doctor of Philosophy

Preface

This thesis is presented in the style that meets the requirements of the Guidelines for Thesis Preparations of the Faculty of Graduate Studies and Research, McGill University, as cited below:

"Candidates have the option of including, as part of the thesis, the text of one or more papers submitted or to be submitted for publication. These texts must be bound as an integral part of the thesis.

If this option is chosen, connecting texts that provide logical bridges between the different papers are mandatory. The thesis must be written in such a way that it is more than a mere collection of manuscripts; in other words, results of a series of papers must be integrated.

The thesis must still conform to all other requirements of the "Guidelines for Thesis Preparation". The thesis must include: A Table of Contents, an abstract in English and French, an introduction which clearly states the rationale and objectives of the study, a comprehensive review of the literature, a final conclusion and summary, and a thorough bibliography or reference list.

Additional material must be provided where appropriate (e.g. in appendices) and in sufficient detail to allow a clear and precise judgement to be made of the importance and originality of the research reported in the thesis.

In the case of manuscripts co-authored by the candidate and others, the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent. Supervisors must attest to the accuracy of such statements at the doctoral oral defense. Since the task of the

examiners is made more difficult in these cases, it is in the candidate's interest to make perfectly clear the responsibilities of all the authors of the co-authored papers. Under no circumstances can a co-author of any component of such a thesis as serve an examiner for that thesis."

This thesis is composed of six chapters. Chapter 1 is the introduction of this thesis which not only states the rationale, hypotheses and objectives of the study but also comprehensively reviews the literature concerning this research. Chapters 2 to 5 are the original manuscripts that have been either submitted for publication or accepted. Each manuscript contains an Abstract, Introduction, Materials and Methods, Results, Discussion, Acknowledgments and References, as well as Tables and Figures. These manuscripts are: Chapter 2 (Differential responses of pulmonary arteries and veins to histamine and 5-HT in lung explants of guinea pigs), Chapter 3 (Differential relaxant responses of pulmonary arteries and veins in lung explants of guinea pigs), Chapter 4 (Altered reactivity of pulmonary vessels in postobstructive pulmonary vasculopathy), and Chapter 5 (Endothelin reactivity and receptor profile of pulmonary vessels in postobstructive pulmonary vasculopathy).

Chapter 6 is the final section that discusses some general points brought by my research, and this section also contains the final conclusions of my studies.

All of the manuscripts in thesis are co-authored by the candidate and others. The candidate, as first author, did essentially all of the experimental work, literature review, and the writing of this thesis. Dr. René P. Michel supervised all the aspects of the research and corrected the writing of this thesis. Dr. David H. Eidelman guided my work using the lung explant technique. Dr. Peter Cernacek supervised the endothelin

and receptor assay experiments. Dr. Fu Hu was involved in the production of the animal model. Dr. Chong-Gang Wang taught me the lung explant technique. Mr. Wassim Kassouf was a summer student, who assisted with the relaxation experiments in the ligated guinea pigs.

Table of contents

Abstract

Chronic ligation of one pulmonary artery results in pulmonary vascular remodelling and bronchial angiogenesis, known as postobstructive pulmonary vasculopathy (POPV). In previous studies of POPV, we found that responses of pulmonary arteries to 5-HT and of veins to histamine were markedly increased, but the role of putative factors, such as structure, endothelial modulation and alterations in receptors remains unknown. First, we examined the role of these mechanisms in the differential responses of pulmonary arteries and veins of normal guinea pigs to histamine and 5-HT, using a novel lung explant technique. We found that veins contracted more to both agonists than arteries, and that H_2 receptors were responsible for the differential contractile responses of pulmonary arteries and veins to histamine, whereas endothelium-derived vasoactive substances (nitric oxide and prostacyclin) were responsible for their differential contractile responses to 5-HT. We also investigated relaxation responses, and found that endothelial-dependent NO-mediated relaxation was greater in pulmonary arteries than veins, and that acetylcholine-induced NO-mediated relaxation was reduced by the simultaneous production of cyclooxygenase-derived vasoconstrictors.

Second, we did experiments in guinea pigs with POPV, and found that the maximal contractions of pulmonary arteries to 5-HT and of veins to histamine were increased compared with controls, and that the augmented responses were not due to endothelial dysfunction nor to structural alterations, but probably to changes in the smooth muscle proper. To ascertain responses to endothelin (ET), and the role of altered receptors, specifically ET receptors, in POPV, we produced the model in rats: we found that contractions to ET-1 and ET-3 were increased and that relaxation to ET-1 was

reduced significantly only in the pulmonary arteries with POPV compared with controls; these findings were attributed, using receptor binding studies, to an augmented proportion of ET_A over ET_B receptors.

We conclude that the differential contractile responses of normal pulmonary arteries and veins to histamine and 5-HT, and the altered vascular responses to these amines and to ETs in POPV, are due primarily to differences in receptors or in endothelial modulation, rather than to disparities in vascular structure.

Abrégé

La ligature chronique d'une artère pulmonaire produit un remodelage des vaisseaux pulmonaires et une angiogénèse des vaisseaux bronchiques que l'on nomme vasculopathie postobstructive pulmonaire (VPOP). Dans des études précédentes sur la VPOP, nous avons démontré que la contraction des artères pulmonaires par la sérotonine et celle des veines par l'histamine était augmentée de façon importante; toutefois, le rôle de facteurs tels que la structure, la modulation par l'endothélium et les altérations au niveau des récepteurs demeurait inconnu. Dans un premier temps, nous avons donc examiné le rôle de ces mécanismes dans les réponses différentielles des artères et des veines pulmonaires de cobayes normaux à l'histamine et à la sérotonine, en utilisant une nouvelle technique, le poumon explanté. Nous avons trouvé que les veines se contractaient plus que les artères avec ces deux agonistes, et que les récepteurs H2 étaient impliqués dans les réponses différentielles des artères et des veines pulmonaires à l'histamine, tandis que des substances dérivées de l'endothélium telles que le monoxyde d'azote (NO) et la prostacycline étaient responsables de leurs réponses différentielles à la sérotonine.

Nous avons aussi étudié le phénomène de la relaxation et avons trouvé que les artères manifestaient une plus forte relaxation que les veines, en rapport avec le NO endothélial; de plus, la relaxation induite par l'acétylcholine via le NO endothélial, était réduite par la production simultanée de vasoconstricteurs de la cascade de la cyclooxygenase.

Dans un deuxième temps, nous avons fait des expériences chez des cobayes avec la VPOP, et avons trouvé que les contractions maximales des artères pulmonaires par la

sérotonine et des veines par l'histamine étaient augmentées comparées à celles des vaisseaux contrôles; cette augmentation de contractions n'était pas due à une dysfonction de l'endothélium ni à des altérations structurales, mais plutôt à des altérations du muscle lisse lui-même. Pour examiner le rôle dans la VPOP de l'endothéline (ET) et celui des récepteurs, particulièrement des récepteurs ET, nous avons produit le modèle chez le rat: nous avons trouvé que les contractions par l'ET-1 et l'ET-3 étaient augmentées, et que la relaxation par l'ET-1 était réduite de façon significative seulement dans les artères de la VPOP comparativement aux contrôles; grâce à des études d'affinité de récepteurs, ces résultats sont attribués à une proportion augmentée de récepteurs ET_A par rapport aux récepteurs ET_B. Nous concluons que les réponses différentes des artères et des veines pulmonaires normales à l'histamine et à la sérotonine ainsi que l'augmentation des contractions par ces amines et par l'ET dans la VPOP, sont dues principalement à des altérations des récepteurs et à la modulation par l'endothélium, plutôt qu'à des changements de la structure vasculaire.

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I am greatly indebted to many individuals who have given me excellent assistance and support to finish this thesis.

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My final thanks are to my parents, my wife, and my daughter, from whom I always receive encouragement, support and understanding. This is for them!

Chapter 1

Introduction

1.1 Postobstructive pulmonary vasculopathy

1.1.1 Definition

Postobstructive pulmonary vasculopathy (POPV) describes the complex set of vascular alterations that result from chronic unilateral ligation of one pulmonary artery. These alterations occur at three levels (Michel *et al.*, 1990; Vidone and Liebow, 1957; Weibel, 1960): one, in the bronchial vessels, that show prominent proliferation and increased blood flow; two, in the pulmonary vessels distal to the occlusion, that are remodeled and altered physiologically and pharmacologically; and three, in the pulmonary parenchyma.

The following summarizes the principal known pathological, physiological and pharmacological modifications that occur following chronic ligation of one pulmonary artery: 1) bronchial blood flow rises markedly, associated with proliferation of new bronchial collaterals around pulmonary vessels and airways, and in the pleura (Liebow *et al.*, 1950; Michel and Hakim, 1991; Weibel, 1960), 2) these new bronchial collateral anastomose with the pulmonary vessels at the precapillary level (Liebow *et al.*, 1950; Michel *et al.*, 1990; Michel and Hakim, 1991), 3) total pulmonary vascular resistance doubles, correlating with a reduction in diameter, peripheral muscularization, and increased medial thickness of the pulmonary arteries (Michel *et al.*, 1990; Michel and Hakim, 1991), 4) the expression of endothelin (ET), demonstrated by immunohistochemistry, is augmented in the endothelium of pulmonary and bronchial vessels (Giaid *et al.*, 1993), 5) the number of myoendothelial junctions in pulmonary and bronchial vessels is elevated (Michel *et al.*, 1995), 6) ventilation is reduced and lung resistance and elastance are increased (Kelly *et al.*, 1994), and 7) the reactivity of

pulmonary arteries to 5-HT and of pulmonary veins to histamine is markedly increased (Michel *et al.*, 1990). It is the latter change in POPV that constitutes the object of study of the present thesis. Let us first review the various alterations that occur in POPV.

1.1.2 The bronchial circulation in POPV.

Early studies of POPV, prior to the 1980s, had predominantly focused on the bronchial circulation. It was Virchow (1847) who first reported that the bronchial arteries supplying a lung whose pulmonary arterial blood supply has been occluded increase in size; he also postulated that the bronchial arteries must be responsible for providing nutrition to the lung because pulmonary arterial occlusion did not result in infarction. Since then it has been shown that occlusion of a pulmonary artery profoundly affects the bronchial circulation. In late 1870s, Kuttner (quoted by Schlaepfer, 1924) first experimentally ligated one pulmonary artery as a potential surgical treatment for pulmonary tuberculosis. Later on, in an experimental study in dogs, Schlaepfer (1924) observed that after ligation of the pulmonary artery, the bronchial arteries were greatly enlarged, and thereby explained the markedly increased bronchial blood flow. Thereafter, a number of investigators examined the responses of adult canine lungs to left pulmonary artery ligation (Alley *et al.*, 1961; Bloomer *et al.*, 1949; Goetz *et al.*, 1965; Liebow *et al.*, 1950, 1953; Michel *et al.*, 1990; Michel and Hakim, 1991; William and Towbin, 1955). In an early study, Bloomer (1949) demonstrated indirectly, with a bronchospirometric technique, that ligation of the pulmonary artery for between 2 weeks and 4 months caused a marked and time-dependent rise in blood flow through the bronchial arteries. Williams and Tobin (1955) measured bronchial collateral blood flow in dogs directly by cannulating the pulmonary arteries, and found that it increased from

baseline values of 1% of cardiac output to 25-30% of cardiac output by 1 year after ligation of the left pulmonary artery. Using bronchovascular casting and histological techniques, Liebow *et al.* (1950) found that, after chronic ligation of the left pulmonary artery, the bronchial arteries increased enormously not only in size but also in number, and that these were prominent around the pulmonary vessels and bronchi, and in the interlobular septa and pleura. More recently, also in the canine model of POPV, Michel and Hakim (1991) confirmed that periarterial and perivenous collaterals were very prominent. The increase in the number of bronchial vessels appears to depend on the duration of ligation. Liebow *et al.* (1950) found a rough correlation between the extent of bronchial collateralization and the duration of ligation between 2.3 and 23 months. Weibel (1960) described the early events in the rodent model of POPV: at 2 to 5 days after the ligation, there was dilatation of the bronchial arteries, followed, for up to 40 days, by new collateral vessel formation, ie. angiogenesis. Indeed, he found proliferation of the bronchial vessels at 10 to 40 days, as evidenced by the presence of mitoses in endothelial and smooth muscle cells of medium-sized and small bronchial arteries; the larger vessels were relatively unaffected.

The walls of the large central bronchial vessels in POPV were composed chiefly of a thick layer of hypertrophied and hyperplastic smooth muscle with an irregularly thickened internal elastic lamina (Liebow *et al.* 1950), while the newly formed smaller bronchial collaterals had a wall composed of loosely arranged smooth muscle cells and essentially no elastic lamina, resembling new blood vessels supplying malignant neoplasms (Michel and Hakim, 1991); these contrast with normal bronchial arteries that have, as any systemic artery, a muscular media lying on a single internal elastic lamina.

How are the new bronchial vessels connected to the pulmonary vessels? Under normal circumstances, bronchopulmonary anastomoses are primarily postcapillary, with precapillary anastomoses rare or absent at least in human, dogs, and rabbits (Charan *et al.*, 1986; Charan and Carvalho, 1992; Verloop, 1949). After occlusion of one pulmonary artery, however, precapillary anastomoses form or increase very prominently. Liebow *et al.* (1950, 1953) observed, using vascular casting and histological techniques, numerous anastomoses between the bronchial and pulmonary circulations predominantly around bronchioles after chronic ligation of the left pulmonary artery in dogs. In the rodent POPV model, 40 days after occlusion of the pulmonary artery, Weibel (1960) found prominent anastomoses between the enlarged bronchial arteries and the capillaries of the parenchyma via multiple small branches, whereas direct anastomoses between the bronchial arteries and the larger branches of the ligated arteries were relatively sparse. More recently, Michel *et al.* (1990) and Michel and Hakim (1991), using the arterial and venous occlusion technique, that enables the partitioning of total pulmonary vascular resistance into arterial, venous and middle segments, confirmed with a physiological technique that the anastomoses between the new bronchial vessels and the pulmonary vessels were located principally at the level of precapillary vessels; morphological studies done on the same experiments revealed that these anastomoses were situated in vessels of about 100 μm diameter.

After considering the chronic effects of pulmonary artery interruption, let us now examine the acute effects of this manoeuvre. There has been significant interest in this aspect of the bronchial circulation after 1980 (Agostoni *et al.*, 1987; Jindal *et al.*, 1984, 1985; Malik and Tracy, 1980). Under normal physiological conditions, bronchial blood

flow represents only about 1% of total cardiac output. After acute balloon occlusion of the left pulmonary artery in dogs, Jindal *et al.* (1984) found that bronchial blood flow increased significantly within 5 min. and that it was double the baseline value by 60 min; in their study, they used an open-chest preparation, with the left lower lobe isolated in a sling and the bronchial circulation intact. Their subsequent experiments suggested that vasodilator prostaglandins were responsible for the acutely increased bronchial blood flow (Jindal *et al.*, 1985). Using a similar isolated canine lobar model but controlling the temperature of the lobe, Agostoni *et al.* (1987) further noticed that 30 min. after pulmonary artery obstruction, the bronchial blood flow of the lobe doubled when it was kept at 27°C but did not change when it was at 39°C. In contrast, Malik and Tracy (1980), in their study of pulmonary microvascular occlusion in dogs by injection of 100 μm-diam. glass beads into the right atrium, found that bronchial blood flow did not change at 5 min, decreased to 1/3 of its baseline value at 60 min., and increased to 300% of baseline at 2 weeks. This study suggested that the response of the bronchial circulation to small-vessel occlusion differs from its response to large-vessel occlusion.

1.1.3 The pulmonary circulation in POPV.

Early studies of POPV, prior the 1980s, had paid little attention to the pulmonary vasculature, or generally assumed that it was normal (Liebow *et al.*, 1950; Schlaepfer, 1924; Weibel, 1960). Only more recently did Shure *et al.* (1984, 1985) report pulmonary vascular alterations in chronic POPV produced in adult dogs similar to those that have been described in human diseases associated with severe pulmonary arterial hypertension. The changes consisted of medial hypertrophy, intimal proliferation, and even "plexiform lesions". The latter are composed of dilated thin-walled vessels with

areas of proliferation of vessels forming a "plexus", distal to an obliterated small pulmonary arterial branch; they are usually found in severe pulmonary hypertension complicating left-to-right congenital cardiac shunts, and are the hallmark of "primary plexogenic pulmonary arteriopathy" (Jamison and Michel, 1995; Michel, 1989). The studies of Shure *et al.* (1984, 1985), however, were only published in abstract form, and the presence of plexiform lesions in POPV was never confirmed in the studies of Michel and Hakim (1991) using the identical model, although they found other changes such as medial muscle thickening, peripheral muscularization, and focal intimal thickening in the pulmonary arteries. The latter authors believe that the appearance of plexiform lesions was produced by the prominently proliferating bronchial collaterals in the vicinity of the pulmonary vessels (personal communication, R.P. Michel).

Detailed morphometric studies were undertaken of the pulmonary vessels in POPV by Michel and Hakim (1991): they divided pulmonary arteries and veins into size categories of < 50, 50-100, 101-200, 201-400, 401-600, 601-800, 801-1000, and > 1000 μm, and in each measured internal and external diameters, and medial muscle thickness, and from these values calculated percent medial muscle thickness. They found an increase in percent medial muscle thickness and a decrease in internal diameters of pulmonary arteries in POPV. These authors also assessed the extent of muscularization of pulmonary arteries and veins in each size category, by classifying them as elastic, transitional, muscular, partially muscular or nonmuscular, as well as grouping them, for the arteries at least, with respect to adjacent airways; they found that in POPV there was significant peripheral muscularization of the pulmonary arteries but not of the veins. More recently, Michel *et al.* (1995) observed that myoendothelial junctional complexes

in pulmonary arteries, veins, and in bronchial vessels, were increased in POPV, and speculated that these might play a role in the proliferation of the bronchial vessels, in the remodelling of the pulmonary vasculature, and in the pulmonary vascular hyperreactivity observed in this model.

What effect do these morphological changes have on the physiology and pharmacology of the pulmonary vasculature in POPV? The pulmonary arterial blood pressure distal to the site of ligation drops immediately after ligation, but returns to normal by three weeks, despite the lack of pulmonary arterial blood flow (Shure *et al.*, 1984). This is also of particular interest in view of the important morphological alterations in the pulmonary vessels noted in the section above: indeed, one would perhaps have expected the pulmonary arterial pressure distal to the ligation to be elevated. Pulmonary vascular resistance distal to the site of ligation, however, increases significantly: Michel *et al.* (1990) measured total, arterial, venous, and middle segment resistances with the arterial and venous occlusion technique in *in situ* perfused canine lungs four months after left pulmonary arterial ligation, and found a doubling of total pulmonary vascular resistance, attributable to a three-fold rise in pulmonary arterial resistance and a 60% rise in pulmonary venous resistance. The rise in vascular resistance could be accounted for by the aforementioned light microscopic findings such as medial thickening, peripheral muscularization, and a reduction in the internal diameter of pulmonary arteries (Michel and Hakim, 1991).

In view of the altered pulmonary vascular morphology and physiology in POPV, Michel *et al.* (1990) decided to ascertain if vascular reactivity to pharmacologic agents was also altered. They chose to examine the effects of 5-HT and of histamine, as in

previous studies, it had been shown, using the arterial and venous occlusion technique, that the pulmonary arterial segment responds specifically to 5-HT, and that the venous segment responds specifically to histamine (Hakim *et al.*, 1982). Thus they examined the responsiveness of pulmonary arteries to 5-HT and of veins to histamine in *in situ* perfused lungs of dogs four months after ligation of the left main pulmonary artery, and found that in POPV, pulmonary vascular reactivity rose markedly: indeed, arterial resistance rose by about 10 $mmHg.L^{-1}.min$ with 5-HT infusion rates of 115 $\mu g/min$ in control lobes, whereas in lobes with POPV it rose by 45 $mmHg.L^{-1}.min$ with 5-HT infusion rates of only 15 $\mu g/min$. With histamine, venous resistance rose by 12 $mmHg.L^{-1}.min$ in control lobes and by 25 $mmHg.L^{-1}.min$ in lobes with POPV with infusion rates of 616 and 159 $\mu g/min$ respectively.

The aforementioned study, however, did not provide insights into the mechanisms responsible for the pronounced hyperreactivity of the pulmonary arteries to 5-HT and of the pulmonary veins to histamine in POPV. As indicated above, the experiments carried out as part of the present thesis were designed to answer this very question. A first step to determine the mechanisms of the physiopathological changes in POPV was carried out by Giaid *et al.* (1993) in dogs following ligation of the left pulmonary artery for 3 to 15 months: the authors observed an increased expression of endothelin-1 (ET-1) in the endothelium of pulmonary arteries and veins and bronchial vessels in the lungs with POPV compared with the contralateral control side. The involvement of ET in POPV is also further explored in one set of experiments in the present thesis (Chapter 5).

1.1.4 The lung parenchyma in POPV.

Ligation of one pulmonary artery has been reported to reduce lung volume. In

in vitro static air pressure-volume studies, Chernick *et al.* (1966) have showed that total lung volume is reduced by 40-50% of control lungs for the first 35 days following ligation, largely due to atelectasis for the first two weeks as well as mechanical obstruction of the airways for the first month. By 50 days, the atelectasis resolves and lung volume returns to normal. Other investigators, however, have found that between 40 days and 3 years, lung volume is still significantly reduced, by as much as one quarter to one third (Lilker *et al.*, 1975; Schlaefer, 1924; Weibel, 1960). Schlaefer (1924) reported that the elastic tissue in the walls of the alveoli and that collagen around the bronchovascular bundles increased; according to this author, it explained the shrinkage of the lung to two-thirds its original size. Liebow *et al.* (1950) found that the pleura, the tissues surrounding the bronchi, and the alveoli were also slightly thickened, contributing to the reduction in lung volume. Although the mechanisms for the focal fibrosis and the reduced lung volume in POPV are not entirely clear, it appears that they can best be related to an element of ischemia due to the temporarily reduced total blood flow to the lung (ie. pulmonary plus bronchial), prior to the time when bronchial blood flow increases sufficiently to take over supplying all of the parenchyma (Chernick *et al.*, 1966, Shure, 1992).

The morphological parenchymal modifications in POPV may also alter the mechanical properties of the lung. Indeed, Kelly *et al.* (1994) found that pulmonary elastance and tissue resistance were significantly increased 9.5 months after ligation of the left main pulmonary artery of dogs. In addition, in the lung with POPV, the pulmonary capillaries are perfused with oxygenated blood from the bronchial arteries rather than deoxygenated blood from the pulmonary arteries. Thus ventilation of that

lung contributes little to O_2 exchange. Immediately after interrupting pulmonary arterial blood flow, there is a fall in alveolar CO_2, which leads to bronchoconstriction and an increase in lung impedance (Tisi *et al.*, 1970). As a result, inspired gas is diverted to the contralateral lung and arterial oxygen tension and carbon dioxide tension are maintained. Three months or more following ligation, Lilker and Nagy (1975) reported that ventilation was still reduced compared with control values. This chronic reduction in ventilation can probably be attributed largely to the increase in pulmonary elastance and tissue resistance, as shown in the studies of Kelly *et al.* (1994, 1995). As mentioned above, since pulmonary capillaries are perfused with arterial blood in the lung with POPV, O_2 exchange is reduced. However, this lung contributes significantly to CO_2 exchange, for example, in dogs 6 months after ligation of the left main pulmonary artery, Kelly *et al.*, (1995) found that the lung with POPV was responsible for 35% of the CO_2 elimination.

1.1.5 Clinical Relevance of POPV.

The model of POPV is relevant to several situations in clinical medicine. One is congenital unilateral absence of one pulmonary artery; these patients are often asymptomatic and diagnosed only on routine examination. In two reports (Arriero *et al.*, 1991; Morales *et al.*, 1991), the patients were found to have normal arterial blood gases and a mild restrictive defect in pulmonary function. Chest radiographs showed that the affected lung was small and that bronchial collaterals were prominent.

A second clinical situation relevant to POPV is that of chronic major pulmonary vessel thromboembolism (Kapitan *et al.*, 1990; Moser *et al.*, 1990; Presti *et al.*, 1990). Pulmonary function and gas exchange parameters measured prior to thrombarterectomy

indicated a significant reduction of PaO_2 and $PaCO_2$ and increased alveolar to arterial O_2 gradient suggesting greater compromise of gas exchange in these patients than in the animals with POPV, perhaps due to the presence of additional underlying diseases in them (Kapitan *et al.*, 1990; Presti *et al.*, 1990). Patients with this condition demonstrate an extensive increase in the bronchial collaterals to the obstructed regions of lung (Carroll, 1950; Moser *et al.*, 1983). In addition, in chronic thromboembolic pulmonary hypertension, there are at least some of the pulmonary vascular changes observed in POPV, including medial hypertrophy and intimal proliferation (Kay, 1994; Spragg *et al.*, 1982), although it is important to point out that thrombosis is not a feature of POPV, at least in the dog.

Third, the pulmonary vascular remodeling that occurs distal to the obstructed pulmonary arteries in POPV resembles the remodeling found in certain forms of pulmonary hypertension (with eg. the reduction in vascular diameters and the increase in wall/radius ratio), even though the pulmonary artery pressure in the lung with POPV remains normal. The marked increase in pulmonary vascular resistance in POPV is also a characteristic of several forms of pulmonary hypertension (Lund-Johansson, 1980). Furthermore, vascular hyperreactivity is a feature of the pulmonary vessels in both POPV and various forms of pulmonary hypertension (Halloway and Bohr, 1973; Hoshino *et al.*, 1994), so that POPV may provide additional insights into the mechanisms of hyperreactivity in several human conditions.

Four, it is of interest that pulmonary carcinomas are largely supplied by bronchial vessels (Cudkowicz, 1967). In them, bronchial blood flow can be as high as 7% of cardiac output, compared with 1% in normals. It is not known why bronchial vessels

but not pulmonary vessels undergo angiogenesis in pulmonary carcinomas, since it is well known that endothelial and smooth muscle cells of both bronchial and pulmonary vessels have the ability to readily proliferate *in vitro*. Thus POPV, with its prominent bronchial vessel proliferation, is a valuable model of angiogenesis that is relevant to the angiogenesis accompanying malignant neoplasia.

1.2 Normal control of the pulmonary circulation and of its vascular reactivity

1.2.1 General characteristics of the normal pulmonary circulation

The pulmonary circulation has a number of characteristics that distinguish it from the systemic circulation (Barnes and Liu, 1995). First, it is a low-pressure, low-resistance and highly compliant system that is well adapted for gas exchange. For example, the mean pulmonary arterial pressure in humans is about 14 mmHg, whereas the mean systemic arterial pressure is 93 mmHg (Anderson, 1993). Since the cardiac output to the pulmonary and systemic circulations is equal, the pulmonary vascular resistance is thus about 1/10 of the systemic vascular resistance. Second, anatomically, the walls of pulmonary vessels, particularly the arteries, are thinner and have less smooth muscle than their counterparts in the systemic circulation; the arrangement of the elastic laminae also differs between them, since muscular pulmonary arteries, for example, have a muscular media sandwiched between two distinct inner and outer elastic laminae, whereas in the systemic circulation, the muscular arteries have a thick media with only an inner elastic lamina. The pulmonary capillary segments are short and form a dense meshwork in the alveolar walls, whereas their systemic counterparts are long and rarely form a meshwork (Daly and Hebb, 1966). Third, pulmonary vessels have very little or no resting tone, evidenced by the fact that vasodilators have minimal effect on pulmonary vascular pressure, whereas they cause a large drop in systemic pressure (Groves *et al.*, 1987; Knapp and Gureiner, 1977; Tucker, 1979). Fourth, the pulmonary circulation is under the control of both active and passive forces; the former alter tone and vascular resistance and therefore blood flow by causing contraction or relaxation of smooth muscle, whereas the latter act independently of vascular tone. Active forces in the

pulmonary circulation include autonomic nervous influences, numerous vasoactive mediators, and gases, especially O_2, and CO_2; passive factors include changes in lung volume, pulmonary arterial and left atrial pressures, airway and interstitial pressures, and gravitational forces. In contrast, the systemic circulation is predominantly controlled by active factors such as autonomic nerves and gases, much less by passive forces (Anderson, 1993). Fifth, responses of pulmonary and systemic vessels to various physiologic and pharmacologic stimuli may be diametrically opposed: for example, hypoxia and histamine usually constrict pulmonary vessels but dilate systemic vessels (Ahmed *et al.*, 1982; Reeves *et al.*, 1995).

One important characteristic of the pulmonary circulation is its low baseline tone. Although the exact mechanisms for this are incompletely understood, several factors may contribute to it including the recruitable and distensible nature of the pulmonary vasculature, the stretch provided by the surrounding elastic parenchyma, the degradation of circulating vasoconstrictors by the pulmonary endothelium, and the production of vasodilators (Rodman and Voelkel, 1991).

1.2.2 Normal mechanisms of vascular smooth muscle contraction and relaxation

It is accepted that contraction of vascular smooth muscle depends mainly on the increase of the intracellular free calcium level ($[Ca^{2+}]_i$) (Himpens *et al.*, 1995). The increase in $[Ca^{2+}]_i$ activates Ca^{2+}/calmodulin-dependent myosin light chain kinase which phosphorylates the 20 kD myosin light chains, resulting in an increase in myosin ATPase activity and cross-bridge cycling (Allen and Walsh, 1994). As long as $[Ca^{2+}]_i$ remains high, phosphorylated myosin elicits repeated cycles of actin-mediated ATP hydrolysis (Hartshorne, 1987). A reduction of $[Ca^{2+}]_i$ below the threshold level leads to a reduction

of the Ca^{2+}/calmodulin complex, and a reduction in myosin light chain kinase activity. The phosphorylated light chains are then dephosphorylated by myosin light chain phosphatase, myosin returns to its inactive state and the muscle relaxes.

The increase in smooth muscle $[Ca^{2+}]_i$ during contraction is due to the influx of Ca^{2+} from the extracellular space or to its release from the sarcoplasmic reticulum. Two major types of calcium channels in the smooth muscle cell membrane account for the entry of Ca^{2+}: voltage-dependent Ca^{2+} channels, which are activated by membrane depolarization, and receptor-regulated Ca^{2+} channels, which are activated when agonists bind to their receptors (Hurwitz, 1986; McDonald *et al.*, 1994). The binding of agonists to receptors also activates phospholipase C via G-protein, which catalyzes phosphatidylinositol 4,5-biphosphate (PIP2) into inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG) (Berridge, 1993; Lee and Severson, 1994; Wilkinson and Hallam, 1994). IP3 binds to specific IP3 receptors on the sarcoplasmic reticulum to release Ca^{2+}.

Contraction or relaxation of vascular smooth muscle can occur without a change in $[Ca^{2+}]_i$. This happens through Ca^{2+} sensitization or Ca^{2+} desensitization of myosin phosphorylation. The binding of a ligand to its appropriate receptor activates phospholipase A_2 via a GTP-binding protein, leading to generation of arachidonic acid by hydrolysis of membrane phospholipid (Gong *et al.*, 1992). Arachidonic acid inhibits myosin light chain thereby decreasing the degradation of phosphorylated myosin, so that the muscle contracts without a change in $[Ca^{2+}]_i$. If $[Ca^{2+}]_i$ rises to 1 μM (Kitazawa and Somlyo, 1990), Ca^{2+}-calmodulin-dependent protein kinase II can be activated; the latter phosphorylates myosin light chain kinase, and its Ca^{2+} sensitivity is decreased (Tansey *et al.*, 1994).

The binding of ligands to receptors that are coupled via GTP-binding proteins to phosphatidylcholine-specific phospholipase C or D generates DAG which activates protein kinase C, leading to phosphorylation of caldesmon and calponin. The phosphorylated caldesmon and calponin have a reduced affinity for actin, thus reducing their inhibition of actomyosin ATPase activity, and leading to slow, sustained contractions (Adam and Hathaway, 1993; Winder *et al.*, 1993).

As mentioned above, when $[Ca^{2+}]_i$ is reduced below threshold level, contraction stops and the muscle relaxes. Both cGMP and cAMP signal systems reduce $[Ca^{2+}]_i$ in vascular smooth muscle and thus induce relaxation (Felbel *et al.*, 1988; Ousterhout and Sperlakis, 1987). For the cGMP pathway, NO and natriuretic peptides activate guanylate cyclase which catalyzes GTP to synthesize cGMP. NO activates soluble guanylate cyclase, whereas atrial natriuretic factor, brain natriuretic peptide and C-type natriuretic peptide activate particulate guanylate cyclase (Rosenzweig and Seidman, 1991). The increased cGMP in smooth muscle cells activates cGMP-dependent protein kinase, leading to a decrease in $[Ca^{2+}]_i$ (Vaandrager and de Jonge, 1996). For example, phosphorylation of Ca^{2+}-activated K^+ channels leads to membrane hyperpolarization, and phosphorylation of voltage-sensitive Ca^{2+} channels decreases Ca^{2+} influx (Robertson *et al.*, 1993). Other mechanisms could also be involved in cGMP-mediated smooth muscle relaxation, and these include the inhibition of phospholipase C activation, thus decreasing IP3 levels, or a reduction in the Ca^{2+} sensitivity of the contractile proteins (Felbel *et al.*, 1988; Nishikawa *et al.*, 1984; Vaandrager and de Jonge, 1996). For the cAMP pathway, beta-receptor agonists and PGI_2 activate adenylate cyclase via specific receptors and increase the intracellular cAMP level (Murray, 1990). The increased cAMP activates

cAMP-dependent protein kinase, resulting in phosphorylation of myosin light chain kinase, Ca^{2+} channels or pumps, and other proteins (Beall *et al.*, 1997). The phosphorylation of myosin light chain kinase decreases its sensitivity to activation by Ca^{2+}-calmodulin, leading to a decrease in the phosphorylation of the myosin light chain (De Lanerolle *et al.*, 1984). The phosphorylation of Ca^{2+}-dependent K^+ channels leads to membrane hyperpolarization and reduction in Ca^{2+} influx (Murray, 1990). In addition, cGMP inhibits Ca^{2+} mobilization (Abdel-Latif, 1996). This inhibition probably occurs through reduction of phospholipase C activation and increase of IP3 degradation.

1.2.3 Control by the autonomic nervous system

Pulmonary vessels are innervated by the autonomic nervous system, which includes sympathetic innervation, parasympathetic innervation, and innervation by the non-adrenergic non-cholinergic system.

1.2.3.1 Sympathetic innervation.

The sympathetic innervation of the pulmonary circulation has been well described by histological and immunofluorescent techniques. Nerve fibers are located in the adventitia and outer third of the media of the pulmonary arteries, and in the adventitia of the pulmonary veins (Hyman, 1986). In general, pulmonary vessels are less densely innervated than systemic vessels. Nerve density decreases with vessel size, and pulmonary arteries are more densely innervated than pulmonary veins in most species (Fillenz, 1970).

Extrapulmonary arteries and veins have an abundant sympathetic innervation in all mammalian species, but the extent and density of nerve fibers in intrapulmonary arteries varies considerably (McLean, 1986). For example, sympathetic nerve fibers are

absent in the intrapulmonary arteries of rats (McLean, 1986), mice, hedgehogs, and badgers (Cech, 1969), are sparse in pigs and calves (Hebb, 1969), and form an extensive and dense network in humans (McLean, 1986), guinea pigs (Kai, 1969), rabbits (Cech and Dolezel, 1967), sheep, cats (Hebb, 1969), and dogs (Kadowitz *et al*, 1981).

Norepinephrine is the principal transmitter released from postganglionic sympathetic nerve endings, whereas epinephrine is released from the adrenal glands after stimulation by preganglionic sympathetic nerves, and ACh is the neurotransmitter at the synapses between preganglionic sympathetic fibers and postganglionic sympathetic neurons. Numerous studies have shown that stimulation of sympathetic nerves or exogenous administration of norepinephrine results in pulmonary vasoconstriction. Daly (1961) first conclusively demonstrated that sympathetic nerve stimulation causes active pulmonary vasoconstriction. Kadowitz and Hyman (1973) extended his work by showing that pulmonary vasoconstriction in response to sympathetic nerve stimulation is abolished by the α-adrenoreceptor antagonist phentolamine. Another study by these authors, using selective α-adrenergic agonists and antagonists, demonstrated the existence of postjunctional α_1 and α_2 adrenoreceptors, and that vasoconstriction to norepinephrine was mediated primarily by α_1 adrenoreceptors. The same group of investigators also found that when the α-adrenoreceptors are inhibited, both sympathetic nerve stimulation and norepinephrine cause pulmonary vasodilation (Hyman *et al.*, 1981). This vasodilator response is mediated by β_2 but not β_1 adrenoreceptors. Since α-adrenoreceptors mediate pulmonary vasoconstriction and β-adrenoreceptors mediate pulmonary vasodilation, the net effect of sympathetic activation may well depend on the relative density of each type of receptor. In addition, the responses of pulmonary vessels to sympathetic stimulation

depend on their basal tone. When pulmonary vascular tone is elevated by pharmacologic agents, vasoconstrictor responses to α-agonists are reduced, whereas vasodilator responses to β-agonists are enhanced (Hyman and Kadowitz, 1986).

Under baseline conditions, sympathetic nerves play a role in the maintenance of pulmonary vascular tone, since 1) removal of sympathetic ganglia significantly decreases pulmonary vascular resistance (Duke and Stedeford, 1960), 2) α-adrenergic antagonists reduce pulmonary vascular resistance, and 3) β-adrenergic antagonists increase pulmonary vascular resistance (Murray *et al.*, 1986). Vasoconstrictor responses to sympathetic nerve stimulation differ in pulmonary arteries and veins. When Daly *et al.* (1970) perfused isolated lungs either in the forward or in the retrograde direction, they found that the arteries were the major site of increased resistance in response to sympathetic nerve stimulation. Similarly, in a subsequent study, Hakim *et al.* (1979) found that sympathetic nerve stimulation had little effect on venous segmental resistance.

1.2.3.2 Parasympathetic innervation.

Unlike the sympathetic innervation, the parasympathetic innervation of pulmonary vessels is highly species-specific. Studies using acetylcholinesterase or choline acetyltransferase staining have shown that cholinergic nerve fibers are abundant in the pulmonary vessels of rabbits, dogs, monkeys, sheep, and cats, whereas they are sparse in calves (Cech, 1969, 1973; Fillenz, 1970), and are altogether absent in human pulmonary arteries and veins (Partanen *et al.*, 1982); they are also absent in the intrapulmonary arteries of guinea pigs, mice, and rats, although they are present in the extrapulmonary arteries and large veins of these species (Cech, 1973).

Cholinergic nerves do not seem to be important in the maintenance of a low

baseline pulmonary vascular tone, since cholinergic blockade does not alter basal pulmonary arterial pressure or vascular resistance (Murray *et al.*, 1986). However, active parasympathetic nerve stimulation, or ACh administration can result in both pulmonary vasodilation or vasoconstriction, apparently depending on various factors. One of these is the species: for example, studies have shown that ACh results in pulmonary vasodilation in humans (Fritts *et al.*, 1958), cats (Nandiwada *et al.*, 1983), and calves (McMurtry *et al.*, 1976), whereas it produces pulmonary vasoconstriction in dogs (Hyman, 1986) and rabbits (Catravas *et al.*, 1984). Another factor influencing the pulmonary vascular response to ACh may be the presence of preexisting vascular tone: in cats and rabbits, ACh increases pulmonary vascular pressure under resting conditions but decreases it when tone is elevated (Hyman and Kadowitz, 1988, 1989); in contrast, in human, ACh induces a clear vasodilator response both under resting conditions and during acute hypoxic pulmonary vasoconstriction (Fritts *et al.*, 1958).

ACh induces pulmonary vasodilation by releasing NO from the endothelium, because if the endothelium is removed, it induces a small contraction (Greenberg *et al.*, 1987; McMahon and Kadowitz, 1992). In rabbits, ACh-induced pulmonary vasoconstriction is mediated by TXA_2, a product of the cyclooxygenase pathway, because it is entirely abolished by a selective TXA_2 receptor antagonist (el-Kashef and Catravas, 1986). In dogs, however, ACh-induced pulmonary vasoconstriction is potentiated by inhibition of the cyclooxygenase pathway (Catravas *et al.*, 1986).

Multiple receptor subtypes are responsible for the different effects of ACh on pulmonary vessels. Five subtypes of muscarinic (M_1 to M_5) have been identified using molecular biological techniques, and the first four, M_1 to M_4 receptors can be also be

differentiated pharmacologically, ie. by using specific agonists and antagonists (Hulme *et al.*, 1990). The muscarinic receptors mediating endothelium-dependent relaxation in human pulmonary arteries are of the M_3 type (McCormack *et al.*, 1989). The M_1, M_2 and M_4 receptors seem to be the ones mediating contraction in pulmonary vessels (el-Kashef *et al.*, 1991). In addition, M_1 and M_2 receptors have been found on sympathetic and parasympathetic nerve endings respectively, modulating norepinephrine and ACh release (Maclagan *et al.*, 1989).

1.2.3.3 Non-adrenergic, non-cholinergic innervation.

Primary sensory neurons, originating from the dorsal root ganglia, provide a perivascular network of fibers surrounding vessels throughout the body. When stimulated, usually by electrical stimulation in *in vitro* studies, these fibers cause vasodilation or vasoconstriction that is not inhibited by classical adrenergic and cholinergic inhibition, but can be abolished by tetrodotoxin (Barnes, *et al.*, 1991; Holzer *et al.*, 1995). This category of autonomic innervation has been termed non-adrenergic, non-cholinergic innervation (NANC). Vasodilation and vasoconstriction induced by NANC have been demonstrated electrophysiologically in pulmonary arteries of rats, guinea pigs, cats, and humans (Inoue and Kannan, 1988; Kubota *et al.*, 1988; Liu *et al.*, 1992a; Maggie *et al.*, 1990; Scott *et al.*, 1996). NO is one NANC transmitter mediating vasodilation (Grozdanovic *et al.*, 1994; Rand, 1992). Indeed, in the presence of adrenergic and cholinergic blockade, it has been shown that electrical stimulation induces a transient, frequency-dependent relaxation of precontracted, guinea pig pulmonary artery rings denuded of their endothelium, which is abolished by tetrodotoxin and NO synthase inhibitors (Liu *et al.*, 1992b). In human pulmonary arterial rings, however, NANC-

induced relaxation is only partially inhibited by NO synthase inhibition (Scott *et al.*, 1996). It has been shown that NO can be released directly from nerve endings, and may be released also from the endothelium after NANC activation (Klimaschewski *et al.*, 1992).

Calcitonin gene-related peptide (CGRP), a 37-amino-acid polypeptide, is considered an essential mediator of NANC vasodilation in the gastric mucosa and skin (Holzer *et al.*, 1995). In human pulmonary vessels, CGRP also seems to mediate NANC-induced relaxation (McCormack *et al.*, 1989). However, in guinea pig pulmonary arteries, CGRP does not mimic NANC-induced relaxation (Liu *et al.*, 1992a).

ATP is probably a NANC transmitter for vasoconstriction in pulmonary arteries. Indeed, in rat intrapulmonary arteries, electrical stimulation evokes a vasoconstriction that is not sensitive to adrenergic and cholinergic blockade, but is abolished by tetrodotoxin and by the ATP receptor antagonist α, ß-methylene ATP (Inoue and Kannan, 1988).

Although NANC nerve-mediated pulmonary vasomotor responses have been demonstrated *in vitro*, they have not been reported *in vivo*. Thus the role of this neural mechanism in the control of pulmonary vascular tone under physiological and pathological conditions remains unclear.

1.2.4 Endothelium-dependent regulation of pulmonary vascular tone

Since the cornerstone observation of Furchgott and Zawadzki (1980) showing that the presence of the endothelium is obligatory for ACh to evoke relaxation in isolated rings of rabbit aorta, numerous studies have confirmed that the endothelium plays a critical role in modulation of vascular tone. Endothelial cells synthesize on one hand

potent vasodilators such as NO, prostacyclin (PGI_2) and endothelium-derived hyperpolarizing factor (EDHF), and on the other, vasoconstrictors such as ET and cyclooxygenase-derived products such as PGH_2 and TXA_2 (Furchgott and Vanhoutte, 1989; Luscher *et al.*, 1992). In addition, the pulmonary endothelium converts angiotensin I to angiotensin II with angiotensin-converting enzyme, and inactivates bradykinin, norepinephrine, and 5-HT (Johnson, 1984).

1.2.4.1 Nitric oxide

NO is an important endothelium-derived vasoactive factor. As indicated above, Furchgott and Zawadzki (1980) first demonstrated that vascular relaxation induced by ACh depended on the presence of the endothelium, and that ACh-induced smooth muscle relaxation resulted from an endothelium-derived, nonprostanoid, labile relaxing factor later named endothelium-derived relaxing factor (EDRF). Endothelium-dependent relaxation was subsequently observed in arteries, veins, and microvessels of many vascular beds in response to various pharmacological stimuli including histamine, 5-HT, ET, bradykinin and substance P, and to physiological stimuli such as shear stress (Furchgott and Vanhoutte, 1989). The chemical identity of EDRF was first proposed to be NO by Ignarro *et al.* (1987a). Thereafter, Palmer and his colleagues (1987), using chemiluminescence and bioassay techniques, confirmed the presence of NO in the effluent of cultured endothelial cells, and the next year found that NO was synthesized from L-arginine (Palmer *et al.*, 1988).

NO is generated intracellularly from L-arginine in two steps: first from L-arginine to N^{ω}-hydroxy-L-arginine, then from N^{ω}-hydroxy-L-arginine to NO and L-citrulline (Stuehr *et al.*, 1991). Both steps are catalyzed by a heme-containing enzyme,

NO synthase (NOS). NOS exists in three isoforms named for the tissues in which they were first cloned and characterized: endothelial NOS (eNOS), neuronal NOS (nNOS), and macrophage inducible NOS (iNOS) (Cooke and Dzau, 1997). There are some salient structural differences between these isoforms: the NH_2-terminus of eNOS contains a consensus site for N-myristoylation that plays a significant role in the membrane localization of eNOS. This isoform also undergoes palmitoylation. These attributes explain that eNOS is membrane associated, whereas iNOS and nNOS are cytosolic. iNOS binds calmodulin with higher affinity than the other two isoforms, so that calmodulin forms a constitutive subunit to this isoform (Forstermann *et al.*, 1991; Schini and Vanhoutte, 1992). This explains the observation that eNOS and nNOS depend on exogenous calcium and calmodulin for activation, whereas iNOS does not. eNOS and nNOS are usually considered constitutive, and iNOS is considered inducible, since the latter is regulated by cytokines whereas the former two are not (Cooke and Dzau, 1997). eNOS is largely restricted to the vascular endothelium, although is also found in renal tubular epithelial cells and neurons. The activity of eNOS can be enhanced by shear stress and agonists such as ACh and histamine (Forstermann *et al.*, 1991), but inhibited by analogues of L-arginine such as N^{ω}-nitro-L-arginine (LNNA) and N^{G}-nitro-L-arginine (Moncada *et al.*, 1991).

NO induces vasodilation by stimulating soluble guanylate cyclase to produce cyclic GMP. NO has a very short life of only a few seconds, and interacts avidly with oxygen-derived free radicals, heme proteins, and sulfhydryl-containing proteins (Moncada *et al.*, 1991).

In pulmonary vessels, many vasoactive substances stimulate the endothelium to

release NO, including ACh, calcium ionophore A23187 (Gao *et al.*, 1995a,b), histamine (Abacioglu *et al.*, 1987), 5-HT (Glusa and Richter, 1993), bradykinin (Ignarro *et al.*, 1987b), substance P (Maggie *et al.*, 1990), ET (Crawley *et al.*, 1992), ATP, and ADP (Greenberg *et al.*, 1987; Liu *et al.*, 1992b). In different species, however, they may act differently. For example, ACh, a classical endothelium-derived NO stimulator, fails to relax bovine pulmonary veins (Ignarro *et al.*, 1988) and pulmonary arteries of full-term fetal and newborn lambs (Gao *et al.*, 1995a; 1995b), and even causes endothelium-dependent constriction in the pulmonary arteries of rabbits (Shirai *et al.*, 1992).

There is also great heterogeneity in agonist-induced endothelium-dependent relaxation between arteries and veins in response to the above agents. In systemic vessels, ACh, histamine, calcium ionophore A23187, 5-HT, bradykinin, substance P, and ADP generally cause greater endothelium-dependent relaxation in arteries than in veins (Furchgott and Vanhoutte, 1989; Luscher *et al.*, 1988). In pulmonary vessels of lambs, however, ACh, bradykinin, and calcium ionophore A23187 produce a greater relaxation in veins than in arteries (Gao *et al.*, 1995a,b). In other species, very few studies have been performed to compare differences of pulmonary arteries and veins in agonist-induced endothelium-dependent relaxation.

The endothelium can also release NO without known stimuli. This basal NO release has been demonstrated through inhibition of NO synthesis with analogues of L-arginine in the pulmonary circulation of most species studied, including cattle (Gold *et al.*, 1990), sheep (Bansal *et al.*, 1993; Gordon and Tod, 1993), guinea pigs (Davidson and Eldemerdash, 1990), rabbits (Gordon and Tod, 1993), and humans (Cooper *et al.*, 1996; Stamler *et al.*, 1994). In contrast, NO synthase inhibitors have no effect on the

pulmonary vascular resistance of dogs (Barnard *et al.*, 1993; Leeman *et al.*, 1994) or rats (Ferrario *et al.*, 1996). Pulmonary arteries and veins may also differ in the level of basal NO production. For example, nitro-L-arginine had no effect on the resting tension of pulmonary arteries but caused endothelium-dependent contraction of pulmonary veins in newborn (Gao *et al.*, 1995a) and adult lambs (Bansal *et al.*, 1993) and a marked endothelium-dependent contraction of isolated rings of bovine pulmonary arteries and veins (Gold *et al.*, 1990).

The vasoconstrictor effects of certain agonists such as norepinephrine, histamine, 5-HT, and ET may be counteracted by the effect of these same substances on the endothelium, releasing NO and dilating the vessels (Hoshino, *et al.*, 1994; Ito *et al.*, 1995; Namiki *et al.*, 1992; Ortiz *et al.*, 1992); inhibition of NO production increases the vasoconstrictor effects of these agonists. In contrast, contractile responses to KCl are not significantly affected by NO synthesis inhibition, since it does not stimulate the endothelium to release NO (Holecyova *et al.*, 1993; Kimura *et al.*, 1992).

1.2.4.2 Prostaglandins

Arachidonic acids are generated in the plasma membrane by phospholipase A_2, and may be metabolized via two main pathways: the 5'-lipoxygenase pathway leading to production of leukotrienes, and the cyclooxygenase pathway leading to formation of prostaglandins and TXA_2. The metabolic products generated by the cyclooxygenase pathway have pronounced effects on the pulmonary vascular bed. PGI_2 is the major vasodilator prostaglandin produced by the vascular endothelium (Moncada *et al.*, 1976). Although in some systemic vessels, the production of vasodilator PGD_2 and PGE_2 may be more than PGI_2 (Carter and Pearson, 1992), PGI_2 is the principal cyclooxygenase

product that vasodilates the pulmonary vascular bed (Jen *et al.*, 1994; Kadowitz *et al.*, 1978). Under baseline conditions, PGI_2 decreases lobar arterial pressure and vascular resistance in a dose-dependent manner; when the tone is raised, its effect is markedly enhanced (Hyman and Kadowitz, 1979). Differences between arteries and veins as well as between species may exist in the effect of PGI_2. For example, a recent study in newborn lambs showed that the effects of PGI_2 were more prominent in pulmonary veins than in arteries (Gao *et al.*, 1996); another study in rats indicated that the cyclooxygenase inhibitor indomethacin had no effect on pulmonary vascular resistance, whereas in dogs, it reduced blood flow markedly (Barnard *et al.*, 1993). Vasodilator prostaglandins may be released under basal conditions (Barnard *et al.*, 1993) or after stimulation with vasoactive agents such as histamine (Ortiz *et al.*, 1992), 5-HT (Hofman *et al.*, 1991), and ACh (Zhang *et al.*, 1995). The vasodilator effects of PGI_2, PGD_2 and PGE_2 are mediated by specific receptors on the plasma membrane of smooth muscle cells that then activate adenylate cyclase which leads to the production of the second messenger cAMP (Halushka, *et al.*, 1989).

Vasoconstrictor prostanoids such as PGH_2, PGF_2, and TXA_2 are also produced in a variety of vessels, and can account for endothelium-dependent contraction (Luscher *et al.*, 1992; Tesfamariam *et al.*, 1989). The production of these prostanoids can be elicited by physical forces, such as stretch, precursor substances of cyclooxygenase products, and certain agonists: for example, in the canine basilar artery and the rat aorta, rapid mechanical stretch induces a rapid increase in active tension in vascular rings with endothelium, but only a weak contraction in rings without it (Katusic *et al.*, 1987; Rinaldi and Bohr, 1989). Exogenous arachidonic acid induces endothelium-dependent

contraction in canine femoral and pulmonary veins, basilar arteries, and rabbit aortae that are blocked by indomethacin (Katusic *et al.*, 1988; Miller and Vanhoutte, 1985; Singer and Peach, 1983). ACh causes endothelium-dependent constriction in several types of vessels including rabbit pulmonary arteries (Altiere et al., 1986, Shirai *et al.*, 1992) and canine cerebral arteries (Shirahase *et al.*, 1987). Two studies (Altiere *et al.*, 1986; Shirahase *et al.*, 1987) have suggested that TXA_2 is the mediator since contraction could be prevented by cyclooxygenase inhibitors, by TXA_2 synthase inhibitors, or by TXA_2 antagonists. Other agonists such as histamine and 5-HT have also been reported to induce endothelium-dependent contraction in canine and rabbit basilar arteries respectively, and their effects can be blocked by indomethacin or TXA_2 antagonists (Seager *et al.*, 1992; Usui *et al.*, 1993).

1.2.4.3 Endothelium-derived hyperpolarizing factor.

Bolton *et al.* (1984) reported that an endothelium-dependent hyperpolarization accompanies the relaxation of vascular smooth muscle elicited by certain endothelial stimulants. This hyperpolarization, which is resistant to inhibitors of NOS and of cyclooxygenase, has been attributed to the release of a yet unidentified endothelial substance, termed endothelium-derived hyperpolarizing factor (EDHF). The existence of this diffusable substance has been demonstrated using a superfusion bioassay in which the source of EDHF was vascular segments or cultured endothelial cells (Chen *et al.*, 1991; Mombouli *et al.*, 1996). To date, however, the exact nature of EDHF remains unknown. In certain arteries and veins, NO may also cause hyperpolarization of the membrane of vascular smooth muscle cells by direct activation of potassium channels independent of cGMP (Bolotina *et al.*, 1994). Vasodilator prostanoids can also cause

direct hyperpolarization of vascular smooth muscle independent of cAMP (Siegel *et al.*, 1991). However, the membrane hyperpolarization induced by NO and prostanoids can be reduced by NOS or by cyclooxygenase inhibitors respectively, whereas the membrane hyperpolarization induced by EDHF is resistant to inhibitors of NOS and cyclooxygenase but not to a depolarizing solution such as 25-30 mmol/L KCl (Feletou and Vanhoutte, 1996). The physiological function of EDHF remains largely unexplored, but it seems to play a role in the local regulation of peripheral vascular resistance (Feletou and Vanhoutte, 1996).

1.2.4.4 Endothelin

Hickey *et al.* (1985) first reported that the culture medium of bovine aortic endothelial cells triggered a slowly developing and long-lasting contraction of isolated pig coronary arteries, which could not be attributed to any known vasoconstricting mediator and which was shown to be a peptide. It was isolated, sequenced, cloned and named ET by Yanagisawa *et al.* (1988). ET is a 21-amino acid peptide with a characteristic ring structure formed by two disulfide bridges (Yanagisawa *et al.*, 1988). Analysis of human genomic sequences for ET revealed the existence of three distinct genes which encode three distinct ET peptides named ET-1, ET-2 and ET-3 (Inoue *et al.*, 1989). ET-1 and ET-3 are expressed abundantly in endothelial and alveolar epithelial cells of the lung (Michael and Markewitz, 1996).

Shortly after the cloning and characterization of the three ET isoforms, two types of ET receptors were cloned, ET_A and ET_B, that mediate the effects of ET (Arai *et al.*, 1990; Sakurai *et al.*, 1990). ET_A receptors have a high affinity for ETs with an isoform selectivity of ET-1 $\geq$ ET-2 > ET-3 (Arai *et al.*, 1990). This selectivity correlates with

the pharmacological finding that putative ET_A receptors mediate vasoconstriction and ET-1 has a greater potency than other two isoforms in producing contraction (Spokes *et al.*, 1990; Takayanagi *et al.*, 1991). The cloned ET_B receptors have an equal affinity for all three isoforms (Sakurai *et al.*, 1990), and this finding is consistent with the pharmacological finding that all three ET isoforms are equipotent in relaxing vascular rings or in reducing blood pressure (Spokes *et al.*, 1989; Takayanagi *et al.*, 1991).

The existence of a third subtype of ET receptors, ET_C, with a proposed rank order of affinity of ET-3 > ET-1, ie., selective for ET-3, remains controversial. Evidence supporting the existence of this subtype is derived largely from pharmacological data concerning the relative potency of different ET isoforms in provoking a biological response (Kloog *et al.*, 1989). ET-3 was shown to be the most potent isoform in the inhibition of prolactin secretion from pituitary lactotrophs (Samson *et al.*, 1991). In cultured bovine carotid artery endothelial cells, ET-1 failed to compete with the binding of 0.25 pM ^{125}I-ET-3 to cell surfaces or to evoke increases in intracellular calcium, whereas ET-3 was effective in both cases (Emori *et al.*, 1990). To date, no receptor subtype selective for ET-3 has yet been cloned by screening cDNA libraries prepared from mammalian cells with probes derived from either ET_A or ET_B sequences. Furthermore, genomic Southern analysis of human DNA probed with gene fragments derived from ET_A or ET_B have failed to provide evidence that other closely related genes exist (Rubanyi and Polokoff, 1994).

ET receptors are abundant in pulmonary vessels (Fukuroda *et al.*, 1994; Perreault and Baribeau, 1995), but the ET_A:ET_B receptor ratio appears to vary with the species. Studies using autoradiography have shown that ET_A receptors predominate in human

pulmonary arteries (Fukuroda *et al.*, 1994) and in porcine pulmonary arteries and veins (Nakamichi *et al.*, 1992). In bovine and rabbit pulmonary arteries, ET_B receptors are the most prevalent (Fukuroda *et al.*, 1994; Hagiwara *et al.*, 1993), whereas in rat pulmonary vessels, ET_A and ET_B receptors are present in similar proportions (Eddahibi *et al.*, 1993).

ET_A receptors are usually located on the surface of vascular smooth muscle, whereas ET_B receptors are on endothelial cells (Hosoda *et al.*, 1991; Ogawa *et al.*, 1991). Exceptionally, in rabbit pulmonary arteries, ET_A and ET_B receptors coexist on smooth muscle cells (LaDouceur *et al.*, 1993).

Much data has accumulated on the effects of the ETs and their receptors by pharmacological approaches using different agonists and antagonists. As mentioned above, ET receptor subtypes can be distinguished from each other by experiments with the three ET isoforms. In addition, there are a number of antagonists that have become available. For example, BQ-123, a cyclic pentapeptide with a high affinity for ET_A receptors, is the most commonly used selective ET_A receptor antagonist (Ihara *et al.*, 1992); another is the tetrapeptide FR139317 (Sogabe *et al.*, 1993). Frequently utilized selective ET_B antagonists include IRL-1038 (Urade *et al.*, 1992) and BQ-788 (Ishikawa *et al.*, 1994).

Under baseline conditions, ETs are potent constrictors of pulmonary vessels from most species including humans (Buchan *et al.*, 1994), rats (Rodman *et al.*, 1989), pigs (Sudjarwo *et al.*, 1993), sheep (Toga *et al.*, 1992) and rabbits (Beck *et al.*, 1995). ET-1 is more potent than ET-2 and ET-3 in eliciting contractile responses (McKay *et al.*, 1991; Perreault and Baribeau, 1995), and pulmonary veins in sheep and rats constrict

more with ET-1 than arteries (Aharinejad *et al.*, 1995; Toga *et al.*, 1992). Constriction of pulmonary vessels is mediated by ET_A receptors on smooth muscle cells in rats (Uhlig *et al.*, 1995), guinea pigs (Cardell *et al.*, 1993), and humans (Buchan *et al.*, 1993). An atypical ET_B receptor-mediated contraction has been reported in swine pulmonary veins (Sudjarwo *et al.*, 1993) and in pulmonary arteries of rabbits (Beck *et al.*, 1995; Fukuroda *et al.*, 1994). It is not known whether the vasoconstriction mediated by ET_B receptors occurs through the production of endothelium-derived contracting factors.

When vascular tone is elevated, ETs induces a dose-related pulmonary vasodilation, mediated by the endothelial production of NO or by the activation of K^+ channels (Crawley *et al.*, 1992; Eddahibi *et al.*, 1993; Namiki *et al.*, 1992). This production of NO is mediated by ET_B receptors on the endothelium. ET-1 and ET-3 are equipotent relaxing agents of pulmonary vessels (Eddahibi *et al.*, 1993).

1.2.5 Histamine and 5-HT

1.2.5.1 Histamine

Histamine is a major product of mast cells and basophils released during anaphylactic reactions. About half of the mast cells in the human lung are located beneath the basement membrane of the bronchioles and bronchi, the other half in interalveolar septa (Friedman and Kaliner, 1987). This distribution of mast cells suggests that histamine has important influences on the airways, and indeed, it is a potent constrictor of airways of humans and several other species (Johnson *et al.*, 1997). Mast cells are also found in the adventitia of pulmonary vessels.

Histamine either contracts or dilates pulmonary vessels depending on existing tone and on the species. Under basal conditions, histamine is a potent constrictor of

either perfused pulmonary vascular beds and isolated pulmonary vascular rings in most species. In isolated perfused lungs of dogs (Hakim *et al.*, 1982), guinea pigs, and rabbits (Albert *et al.*, 1989; Bradley *et al.*, 1993) as well as in *in vivo* studies on sheep (Ahmed *et al.*, 1982), histamine significantly increases pulmonary vascular resistance. In vascular ring preparations, histamine contracts pulmonary vessels of humans (Mikkelsen *et al.*, 1984), guinea pigs (Okpako, 1972), rabbits (Howell and Carrier, 1986), and pigs (Levy *et al.*, 1995). The contractile effects of histamine on pulmonary arteries and veins may differ with the species: for example, in dogs and guinea pigs, histamine contracts predominantly pulmonary veins (Bradley *et al.*, 1993); in rabbits, it contracts pulmonary arteries mainly (Albert, *et al.*, 1989; Bradley *et al.*, 1993), whereas in rats, it randomly either contracts or relaxes arteries and veins (Thompson *et al.*, 1976). In addition, when vascular tone is elevated, histamine also induces relaxation (Abcioglu *et al.*, 1987; Ahmed *et al.*, 1982; Ortiz *et al.*, 1992). Constriction is mediated by H_1-receptors on vascular smooth muscle cells, relaxation by H_2-receptors on vascular smooth muscle cells or H_1-receptors on endothelial cells (Abcioglu *et al.*, 1987; Matsuki and Ohhashi, 1990; Turker, 1973). NO is responsible for the endothelium-dependent relaxation of pulmonary vessels in guinea pigs and rats (Sakuma *et al.*, 1988; Szarek *et al.*, 1992), but in humans, both NO and PGI_2 are involved in this relaxation (Ortiz *et al.*, 1992).

1.2.5.2 5-HT

5-HT is released from platelets in humans and many other mammalian species and thus can affect pulmonary vascular tone when intravascular coagulation and local thrombosis occur (Vaage, 1977). 5-HT is a potent constrictor of pulmonary vessels in most species including humans (Raffestin *et al.*, 1985), dogs (Hakim *et al.*, 1982),

rabbits, guinea pigs (Bradley *et al.*, 1993), cows (Gruetter *et al.*, 1981), and rats (Uma *et al.*, 1987). The contractile effects of 5-HT on pulmonary arteries and veins may differ between species. In *in situ* perfused lung preparations, 5-HT contracts pulmonary arteries of dogs (Hakim *et al.*, 1982) and rabbits (Albert, *et al.*, 1989; Bradley *et al.*, 1993), whereas in guinea pigs, it predominantly constricts pulmonary veins (Albert, *et al.*, 1989; Bradley *et al.*, 1993). The type of preparation may also influence the response obtained with 5-HT. For example, 5-HT contracts human pulmonary arteries and veins *in vitro* (Raffestin *et al.*, 1985) yet has no effect on pulmonary arterial pressure *in vivo* (Harris *et al.*, 1960); 5-HT contracts canine pulmonary arteries and veins *in vitro* (Gruetter *et al.*, 1981) yet does not contract pulmonary veins in *in situ* perfused lung preparations (Hakim *et al.*, 1982). This particular phenomenon is most likely the result of degradation of 5-HT by the endothelium in the perfused lungs (Hart and Block, 1989).

When vascular tone is elevated, 5-HT relaxes pulmonary vessels of cats (Neely *et al.*, 1993), and induces endothelium-dependent relaxation of isolated porcine pulmonary arteries (Glusa and Richter, 1993) and of pulmonary veins of sheep (Zhang *et al.*, 1995). The effects of 5-HT are mediated by multiple receptor types (Hoyer *et al.*, 1994). In many vascular smooth muscle preparations, constriction seems to be mediated primarily by 5-HT_{2A} and in some tissues partially by 5-HT_{2C} (previously 5-HT_{1C}) receptors. The vasorelaxant effects of 5-HT are mediated via 5-HT_1-like receptors on endothelial cells that trigger the release of NO (Cocks and Arnold, 1992; Glusa and Richter, 1993; Neely *et al.*, 1993;). PGI_2 is also released during the response to 5-HT since contraction to it increases after inhibition of cyclooxygenase activity in pulmonary arteries of dogs and rabbits (el-Kashef, 1996; Hofman *et al.*, 1991). In addition, 5-HT

can stimulate the endothelium to release contracting factors, probably TXA_2, thereby indirectly inducing contraction (Seager *et al.*, 1992; Usui *et al.*, 1993).

1.3 Mechanisms of vascular hyperreactivity

Vascular reactivity is defined as the ability of a vessel to constrict in response to a given physical or chemical stimulus (Dobrin, 1983; O'Rourke and Vanhoutte, 1996). Vascular hyperreactivity can thus be defined as a greater degree of reactivity than observed in normal vessels. When hyperreactive vessels are stimulated with, for example, pharmacological agents, there is an increase in the ease of the narrowing (supersensitivity) or/and in the magnitude of the constriction (elevation of the maximal response plateau) (Carrier and Shibata, 1977; Fleming *et al.*, 1973; Kadokami *et al.*, 1996). Possible causes for exaggerated vascular responsiveness include 1) structural alterations such as a decrease in baseline diameter or an increase in medial or intimal thickness, 2) endothelial dysfunction, and 3) an increased responsiveness of the smooth muscle itself.

1.3.1 Structural alterations and hyperreactivity

A reduction in luminal diameter and an increase in wall thickness are common morphological alterations in vascular diseases, especially hypertension (Schwartz, 1996). The structural alterations of vessels can affect the response to vasoactive substances. In most *in vivo* and in perfused lung preparations, changes in resistance have been used as a parameter of vasoreactivity. Because resistance, according to the Poiseuille equation, is inversely proportional to the fourth power of the radius, any given decrease in the radius of a narrow vessel will cause a greater increase in resistance than in the radius of a wide vessel (Mulvany, 1991). Medial and intimal thickening may encroach on the lumen and further reduce the vascular radius, thus amplifying the pressor response (Folkow, 1971). In *in vivo* studies, it has been shown that there is usually an increased

response of structurally altered vessels, and that it is typically non-specific: for example, Smeda *et al.* (1988) reported that the renal vascular bed of spontaneously hypertensive rats showed greater responses to all vasoconstrictors examined and to renal nerve stimulation.

In contrast, the *in vitro* studies that have examined vasoreactivity in structurally altered vessels and directly quantitated smooth muscle contraction in these, have shown conflicting results. A number of studies have found unaltered or even reduced reactivity in response to various agonists (Heagerty *et al.*, 1993), while several others have reported an increased reactivity (Deng and Schiffrin, 1991; Ito *et al.*, 1995; Morita *et al.*, 1996). Reasons for the conflicting results obtained in the various *in vitro* studies may be that a variety of normalization procedures were used to compare the maximal isometric forces generated by the smooth muscle, and that these procedures were not always based on sound principles (Jiang *et al.*, 1991). Potential explanations for the conflicting results between the *in vivo* and the *in vitro* studies include that most of the latter use the aorta and other large arteries, the only accessible vessels in small experimental animals, whereas the results of the *in vivo* studies appear to mainly reflect the responses of small resistance vessels (Orton *et al.*, 1988); moreover, the *in vitro* responses of vascular rings or strips may differ from the *in vivo* responses in which the normal cylindrical structure of vessels is maintained (Dobrin, 1983). In addition, there are studies that seem to exclude an important role for structural changes in altered vascular reactivity: in one, Hoshino *et al.* (1994) reported that aortic hyperreactivity to norepinephrine occurs before any structural changes in one-kidney and one-clip renal hypertensive rats. In a second study, in essential hypertensive patients, Aalkjaer *et al.*

(1987) showed that the subcutaneous resistance vessels show medial thickening but no altered response to norepinephrine. The failure of medial wall thickening of the vessels to increase the contractile response may be related to other abnormalities of the smooth muscle, for example, that smooth muscle may have a decreased Ca^{2+} sensitivity, as shown by Mulvany and Aalkjaer (1988).

1.3.2 Endothelial dysfunction and hyperreactivity

As discussed above, endothelial cells play a crucial role in regulating the tone of the underlying smooth muscle by generating relaxing factors (NO, PGI_2 and EDHF) and contracting factors (ET, TXA_2). In addition, the endothelial cells of pulmonary vessels take up and degrade vasoactive agents such as norepinephrine, 5-HT and bradykinin, convert angiotensin into angiotensin II, and participate in the metabolism of arachidonic acid (Barnes and Liu, 1995; Johson, 1984).

Several of the aforementioned endogenous vasoactive substances such as histamine, 5-HT and ET have dual effects on pulmonary vascular tone, i.e. produce both vasoconstriction and vasodilation, the latter occurring mainly through endothelium-dependent mechanisms. Thus, if the endothelium produces more contracting factors or fewer relaxing factors, vascular hyperreactivity may occur. Recent *in vitro* studies using isolated arteries have demonstrated that endothelium-dependent NO mediated relaxation is reduced in several vascular diseases such as systemic and pulmonary hypertension (Hoshino, *et al.* 1994; Morita *et al.*, 1996), and coronary atherosclerosis (Okumura *et al.*, 1996), and that this leads to exaggerated contractile responses to vasoconstricting agents. In further support of this concept, Ito *et al.* (1995) showed that the chronic administration of a NO synthase inhibitor induces coronary microvascular hyperreactivity

to 5-HT.

Many pathological conditions of pulmonary vessels may also affect the expression of the endothelium-derived contracting factor ET-1. An increased immunoreactivity in lung homogenates or endothelial cells and an increased mRNA expression of preproET-1 have been observed in fawned-hooded rats with idiopathic pulmonary hypertension (Stelzner *et al.*, 1992). Moreover, the ET_A-receptor antagonist BQ-123 prevents and reverses chronic hypoxia-induced pulmonary hypertension in rats (Dicarlo *et al.*, 1995). When rats are exposed to hypoxia for 2 days, there is a three-fold increase in ET-1 mRNA expression in the lung but not in other organs (Elton *et al.*, 1992). In POPV, there is also an increased immunoreactivity for ET-1 in pulmonary arteries and new bronchial vessels (Giaid *et al.*, 1993). ET-1 is not only a potent constrictor of pulmonary vessels but also potentiates the contractile effect of other vasoconstrictors such as norepinephrine and 5-HT (Yang *et al.*, 1990). Thus ET-1 overexpression in the vessels can lead to hyperreactivity to both agents.

1.3.3 Vascular smooth muscle and hyperreactivity

Vascular hyperreactivity may also result from changes in the intrinsic properties of the smooth muscle, including in its receptors, in the signal transduction pathways and in the contractile proteins. First, several recent studies indicate that an increased density of receptors mediating contraction accounts for an enhanced vascular contraction. For example, MacLean *et al.* (1996) reported that the pulmonary arteries of chronically hypoxic rats showed an increased contractile response to 5-HT, due to an increase in 5-HT_{2A}-receptors. Kadokami *et al.* (1996) suggested that coronary microvascular hyperreactivity to 5-HT was probably due to *de novo* expression on smooth muscle cells

of 5-HT_1 receptors which mediate contraction.

Second, alterations in the signal transduction pathways, eg. by increased production of IP3 or decreased production of cAMP and cGMP, may lead to vascular hyperreactivity. Huzoor *et al.* (1989) found that increased vascular sensitivity to the contractile effects of 5-HT in spontaneously hypertensive rats was linked to increased production of IP3 and IP2. MacLean *et al.* (1996) found a decreased CGMP level in large pulmonary arteries (main and first branches) and thought it might contribute to their increased contractile responses to 5-HT.

Contraction and relaxation of vessels, as detailed above, depend on the intracellular Ca^{2+} concentration. Thus an increase in intracellular Ca^{2+} levels or in the Ca^{2+} sensitization in response to agonist stimulation may lead to hyperrreactivity. Stepp and Tulenko (1994) found a five-fold increase in the sensitivity of atherosclerotic aorta segments to the constrictor effects of 5-HT, and a two-fold rise in the 5-HT-stimulated cytosolic Ca^{2+} levels and thought that these augmented levels were responsible for the elevated sensitivity to the vasoconstrictor. In another study, Satoh *et al.* (1994) found that the augmented responses of coronary arteries to 5-HT in stroke-prone spontaneously hypertensive rats were related to an increased agonist-induced Ca^{2+} sensitization. Touyz *et al.* (1996) reported that vasopressin, which induced a greater contraction in small mesenteric arteries of spontaneously hypertensive rats than of controls, also produced a greater change in $[Ca^{2+}]_i$, whereas ET-1, which did not cause a greater contraction, also failed to produce a greater elevation in $[Ca^{2+}]_i$.

Very few studies have examined the role of contractile proteins in altered smooth muscle reactivity. In a recent review, Malhotra (1994) indicated that very few studies

have demonstrated direct links between alterations in contractile proteins and altered physiological function. There are some studies in airways, however, for example, Murphy *et al.* (1991) studied the relationship between airway morphometry, the content of myosin heavy-chain and isoform stoichiometry, and the distribution of bronchoconstrictor responses in the airways of maturing swine, and found a downregulation of contractile responses during maturation, which were independent of smooth muscle receptor distribution, and were not related to morphological changes such as airway muscle mass, cellularity, changes in content of non-muscle tissues, or tissue content of functional myosin isoform. However, Stephens and his colleagues (Liu *et al.*, 1996) recently studied myosin light chain and myosin light chain kinase phosphorylation in homogenates of sensitized canine saphenous veins, which are characterized by increased active shortening capacity, maximum shortening velocity and prolonged relaxation, and found that phosphorylation of myosin light chain and total myosin light chain kinase activity were doubled. These authors also demonstrated increased myosin light chain kinase quantity in the canine model of ragweed pollen sensitized hyperresponsive airways (Jiang *et al.*, 1992). This area, therefore, is an interesting avenue for future exploration.

1.3.4 Potential mechanisms of pulmonary vascular hyperreactivity in POPV

As already alluded to in a previous section, Michel *et al.* (1990) using the arterial and venous occlusion technique, examined the responsiveness of pulmonary arteries to 5-HT and of veins to histamine in *in situ* perfused lungs of dogs four months after ligation of the left main pulmonary artery, and found that in POPV, pulmonary vascular responses increased markedly: arterial resistance rose by about 10 $mmHg.L^{-1}.min$ with

5-HT infusion rates of 115 mg/min in control lobes, whereas in lobes with POPV, it rose by 45 $mmHg.L^{-1}.min$ with 5-HT infusion rates of only 15 mg/min. With histamine, venous resistance rose by 12 $mmHg.L^{-1}.min$ in control lobes and by 25 $mmHg.L^{-1}.min$ in lobes with POPV, with infusion rates of 616 and 159 mg/min respectively.

The mechanisms for the exaggerated responsiveness of pulmonary vessels in POPV to histamine and 5-HT are unclear. As discussed above, structural alterations, endothelial dysfunction, and alterations in the smooth muscle proper could potentially contribute. Indeed, the pulmonary arteries in POPV have a narrower internal diameter and a thicker media: a reduced diameter would lead to a greater resistance change, since resistance is inversely proportional to fourth power of radius, as dictated by the Poiseuille equation; a thickened media, in addition to amplifying the pressor response through reduction of the luminal diameter (Mulvany *et al.*, 1978), may directly increase vasoconstriction. But whether smooth muscle contraction of pulmonary vessels in response to histamine and 5-HT is enhanced in POPV is unknown.

There is some evidence suggesting that endothelial dysfunction may be occurring in POPV: indeed, there is intimal thickening of the pulmonary arteries in POPV (Michel and Hakim, 1991) and the expression of ET was found to be increased by immunohistochemistry in the pulmonary and bronchial vessels in POPV (Giaid *et al.*, 1993). We have no definite proof, however, that endothelial dysfunction occurs in POPV, specifically, no evidence that endothelium-dependent relaxation is affected; thus this remains to be determined and part of the present thesis will answer this question. In addition, other mechanisms such as alterations in receptor density, signal transduction pathways, or in contractile proteins of vascular smooth muscle need to be considered in

the possible mechanisms leading to vascular hyperreactivity in POPV.

1.4 Methods of assessing vascular smooth muscle contractility and function

Since one of the aspects of the present thesis is the introduction of a novel technique, the lung explant technique as it applies to vessels, it is relevant to review the topic of the methods of assessing vascular smooth muscle contractility and function.

In general, the physiological and pharmacological behavior of vessels can be studied by two different means: first, by indirect assessment of vascular smooth muscle contraction or relaxation by measuring pressure gradients, flow rate and vascular resistance, or second by direct measurement of vascular smooth muscle contraction/relaxation, usually *in vitro* using vascular rings or strips.

1.4.1 Indirect measurement of vascular smooth muscle contractility

In most *in vivo* studies, in *in situ* or isolated perfused lungs, and in cannulated, pressurized vessel preparations, smooth muscle contraction or relaxation is assessed indirectly from changes in vascular resistance. In these preparations, blood pressure gradients and flow rates are measured, from which vascular resistance is calculated. According to the Poiseuille equation, the resistance is inversely proportional to the fourth power of radius, the change in the radius can be calculated from the change of resistance (Mulvany, 1991).

In the case of *in vivo* studies, pulmonary arterial pressure is usually measured by right heart catheterization using a Swan-Ganz catheter, pulmonary venous pressure is measured directly either with a catheter positioned in the pulmonary vein after aortic catheterization, or indirectly estimated from the pulmonary arterial wedge pressure also measured via a Swan-Ganz catheter in the pulmonary artery (Grimbert, 1988). Cardiac output can be measured using the thermodilution principle with the indicator dilution

method (Michel *et al.*, 1991). Pulmonary vascular resistance is then calculated from the pressure gradient across pulmonary vascular bed divided by the cardiac output. An analogous procedure can be used to measure changes of pulmonary vascular resistance in one lobe of an intact animal: for example, Hyman and Kadowitz (1979) did this by inserting a triple lumen balloon perfusion catheter into the arterial branch of the left lower lobe, isolated it by blowing up the balloon cuff on the catheter, and perfused the lobe with blood drawn from the femoral artery; the perfusion pressure in the lobar artery is controlled by a perfusing pump, and the venous pressure of the left lower lobe is measured with a catheter placed transseptally. This method has been extensively used in *in vivo* animal studies (McMahon *et al.*, 1993; Neely *et al.*, 1993).

The advantages of these and related *in vivo* methods are that they are physiological, but they have the drawback that there are a large number of variables that indirectly affect vascular smooth muscle function due to neural influences, release of various mediators, and due to respiration. Furthermore, any measured change in vascular resistance usually represents the average of all of the resistances of pulmonary vascular beds in the whole lung or lobe.

In *in situ* or in isolated perfused lung preparations, in addition to being able to measure total pulmonary vascular resistance, it is also relatively easy to partition this total vascular resistance into an upstream arterial segment, a downstream venous segment and a middle segment, using the arterial and venous occlusion technique (Hakim *et al.*, 1982; Michel *et al.*, 1990). This technique was first developed by Hakim *et al.* (1979) and further expanded by Hakim *et al.* (1982). These investigators found that the arterial and venous segments are relatively indistensible, and have a relatively high resistance,

whereas the middle segment is much more distensible and has a relatively low resistance (Hakim *et al.*, 1982). They were also able to show, using this method, that 5-HT and histamine specifically constricted the arterial and the venous segments respectively. Morphological and physiological studies suggest that the boundaries between arterial and venous segments on one hand, and the middle segment on the other, defined by the occlusion technique consisted of vessels with an internal diameter of about 100 μm, and that the middle segment includes extraalveolar as well as alveolar vessels (Hakim *et al.*, 1982; Michel *et al.*, 1991; Michel and Hakim, 1991).

Indirect measurements of vascular contractility may also include preparations using cannulated, perfused vessels (Halpern, 1991). Individual vascular segments either may be cannulated at one end and occluded at the other, or cannulated at both ends. This technique may represent an *in vitro* preparation that better mimics the *in vivo* situation (Halpern and Kelley, 1991). In this preparation, perfusion pressure and flow are set and readily controlled, and resistance easily calculated. The cannulated vessels have a nearly circular cross-section and more closely mimic vessels *in vivo*, in contrast to experiments in which vessels are mounted in a wire myograph, and form two flat sheets of tissue.

1.4.2. Direct measurement of vascular smooth muscle contractility

In *in vitro* experiments, with the exception of the isolated vessel segment techniques mentioned above, vascular smooth muscle contraction or relaxation is usually quantitated directly by use of vascular rings or strips supported on two wires. The variables that affect smooth muscle function can thus be controlled effectively (Bevan and Osher, 1972). Vascular rings or strips can be studied either isometrically or isotonically

with a preset length (preload). Isotonic experiments are performed by applying a nearly constant load to the tissues after the onset of contraction. This method has certain advantages: first, it tends to produce rather steep dose-response curves, and thus desirable for bioassays because it is more sensitive (Paton, 1975); second, this approach represents a more physiological type of response than isometric recording since in most *in vivo* situations, vessels change their length rather than only their tension. Isotonic preparations also have certain disadvantages: first, the choice of the postload is difficult, because if too small, the maximal response will be too great, whereas if too large, the sensitivity will be too low (Nilsson and Sjoblom, 1985); second, with isotonic recording, it is very difficult to adjust the postload and preload of the tissues independently (Paton, 1975).

The isometric recording method measures the development of tension while the length is virtually fixed. Compared with the isotonic recording method, this approach has certain advantages: first, the tissues remain at an constant position on their length-tension relationship; second, the suitable length and resting tension are easy to control (Mulvany, 1991). This method, however, is less physiological than the isotonic recording, for the reasons mentioned above.

The most common question that arises concerning the aforementioned *in vitro* preparations is how to set the initial length (preload) of the vessels so that experimental responses parallel their behavior *in vivo*. In their review, Halpern and Kelley (1991) summarize the principal approaches to setting preload: one is to find experimentally the preload that maximizes the active response to a given agonist; the problem with this approach is that a suitable preload for one agonist may not be suitable for another. A

second approach is to set a tension that corresponds to the known *in vivo* transmural pressure of the vessel, based on the law of Laplace ($T = P \times r$), in which T is tension, P is the transmural pressure and r is the radius of the vessel; in some vessels, however, the transmural pressure may not be known. A third method is to choose a circumference corresponding to the *in situ* dimensions; unfortunately, under most circumstances, it is not known.

Once a given value for the circumference of the vessel has been set, concentration-response curves are usually generated. The characteristics of these curves, and data generated from them, including the threshold dose, the slope, and the maximal response are useful in assessing the behavior of the vascular smooth muscle. Significant disadvantages to these *in vitro* techniques include the fact that 1) the passage of a wire through the vascular lumen tends to damage the endothelium (DeMey and Gray, 1985), 2) the isolation of small vessels is usually very technically demanding, and 3) the vessels mounted on wires are no longer in their physiological environment. One potential way to get around some of these drawbacks is to study vessels in their natural environment using a lung explant technique.

1.4.3 The cultured lung explant technique to assess vascular reactivity

The lung was initially cultured as an explant using classical organ-culture techniques (Rosin, 1947). These techniques have been useful to study cellular interactions and the effect of hormones or of pharmacological agents in embryonic and fetal lungs (Farrell and Bourbon, 1986; Gross and Wilson, 1989; McAteer *et al.*, 1983). Explants from mature mammalian lungs, however, are much more difficult to manipulate because the intrinsic elasticity of the lungs, when they are air-filled, causes the tissue to

roll up rather than lie flat on a culture dish. Furthermore, mature explants handled with standard tissue culture techniques have been of short duration and of limited value because the alveolar walls collapse and the normal anatomy and function of the tissue are lost (Davis, 1967; Weinhold *et al.*, 1979). Hackney *et al.* (1967) solved some of these problems by inflating mature rabbit lungs with warm fluid agar. The agar solidifies when cooled, providing internal support for the alveolar walls, and preserving the normal architecture of the lung. The firmness of the agar also enables the hand-cutting of very thin (200 to 300 μm) sections with minimal distortion. Because the explants were so thin, Hackney *et al.* (1967) found that viable lung tissue with a normal architecture could be studied directly under a high resolution microscope. In addition, these thin explants survived for days when cultured between a coverslip and cellophane. Guerrero *et al.* (1977) further improved the above technique by using a visking dialysis membrane underneath the explants and maintaining the tissues submerged in culture medium, in a 95% O_2:5% CO_2 gas mixture; the explants survived for 4 weeks, as verified by morphology and by the Erythrosine B test. Fisher and Placker (1987) prepared lung explants by inflating them with 0.5% agarose and culturing them on a sterile gelatin support sponge saturated with media, rather than submerged in the liquid; in this manner, explants lasted for 28 days. In serum-free culture media, Siminski *et al.* (1992) further prolonged the culture period to 9 weeks by supporting the explants on gelatin and gently turning them over every other day.

Recently, Eidelman and his colleagues (Almirall *et al.*, 1997; Cowley and Eidelman, 1997; Dandurand *et al.*, 1993; Wang *et al.*, 1997) have applied the lung explant technique to study airway contraction and dilation in rats and mice. Excised

lungs were inflated with 0.5-2.0% agarose through the airways and embedded in 4% agarose. The lung-agarose block was sectioned into 0.5-1.0 mm-thick transverse slices. These slices were supported on 30 mm culture plate inserts within a six-well plate containing 2 ml of bicarbonate-buffered culture medium and incubated overnight at 37°C in 5% CO_2-95% air. Imaging of airways was achieved using a conventional video camera attached to an inverted microscope and images stored on optical discs. Various parameters such as luminal area, internal diameter and perimeter could be measured. Using this technique, Dandurand *et al.* (1993) found that the maximal responses of airways to methacholine were dependent both on the degree of lung inflation as well as on the concentration of agarose used, suggesting that airway-parenchymal interdependence mechanisms are involved during bronchoconstriction. Indeed, this technique may represent an *in vitro* preparation that better mimics the *in vivo* situation: the airways have a nearly circular cross-section supported by the surrounding parenchyma as they do *in vivo*, unlike ring preparations in which two flat sheets of tissue free from the surrounding lung are mounted in a wire myograph. The epithelial cells of the airways remain intact, and the preload and postload are provided by the surrounding parenchyma (Dandurand *et al.*, 1993).

The explant technique also has some disadvantages. First, if explants are too thick ($>$ 1 mm), it is difficult to image the airway cross-sections, whereas if they are too thin ($<$ 0.5 mm), the airways may not have an intact wall. Second, the presence of air bubbles within the parenchyma precludes the acquisition of quality images hours after slicing. This is remedied by culturing the explants overnight (Cowley and Eidelman, 1997). Third, there is a substantial degree of heterogeneity in the within-animal EC_{50}

To our knowledge, no studies using lung explants have been performed on pulmonary vessels. Whether this novel technique could be adapted to pulmonary vessels would depend on how these vessels are different from their *in vivo* counterparts. Indeed, one important difference between pulmonary vessels in lung explants versus the *in vivo* situation is the absence of intravascular pressure; in contrast, for the airways, this problem does not exist, since these are normally filled with air, and airway pressure is minimal. In defense of the technique, however, intravascular pressure is very low in pulmonary arteries and even lower in pulmonary veins, and the basal tone of pulmonary vessels is low (Rodman and Voelkel, 1991). Thus, it is very likely that the stretch from the surrounding parenchyma, filled with agarose, would keep the pulmonary vessels open, despite the absence of pulmonary vascular pressure.

In summary, a review of the literature indicates that the lung explant technique has the potential of being at least as good as some of the other *in vitro* techniques to assess vascular smooth muscle contractility in the lung, and indeed may avoid some of their drawbacks, although it will present some of its own.

1.5 Rationale, hypotheses, and objectives

In the above sections of the introduction, we have reviewed POPV and indicated that the main purpose of the experiments of this thesis was to investigate the mechanisms of hyperreactivity of the arteries to 5-HT, and of the veins to histamine that had been found in this unusual model of bronchial vascular angiogenesis and vasculopathy in the lung. Based on the aforementioned review of normal and abnormal pulmonary vascular reactivity, and considering the potential mechanisms that may explain the hyperreactivity of the pulmonary vessels in POPV, we hypothesized that

1) In POPV, structural alterations of the pulmonary vessels contribute to their hyperreactivity to histamine and 5-HT;
2) The endothelium plays an important role in the hyperreactivity of pulmonary vessels to histamine and 5-HT in POPV;
3) The reactivity of pulmonary vessels to ETs in POPV is also altered, and this can be attributed to alterations in structure and/or ET receptor density and proportion of subtypes.

In order to test these hypotheses, based on our previous outline of the methods to study vascular hyperreactivity, we decided to adopt the lung explant technique as a novel method to study vascular reactivity in this model, rather than use vascular strips and rings, since it enabled us to leave the vessels in their natural environment and to examine relatively small intraparenchymal vessels. The introduction of a new technique, however, requires studies on normal animals. For this reason, chapters 2 and 3 are devoted to the study of vascular reactivity in normal guinea pigs, concentrating on the same pharmacologic agents that we had used for the canine model of POPV, ie. 5-HT

and histamine. It was also the occasion to examine the differential reactivity of arteries and veins to these two pharmacologic agents, and to ascertain, in normal lungs, mechanisms for any observed differential reactivities. The choice of the guinea pig as the principal animal was motivated by its relatively small size, by the fact that other studies are available in the literature on it, and by the fact that the veins of guinea pigs have minimal amounts of cardiac muscle in their walls, unlike the lungs of some of the rodents.

Based on these considerations, the principal objectives of the experiments in this thesis were to:

1) Adapt the lung explant technique to the study of pulmonary vessels;
2) Examine the roles of structure, of the endothelium, of histamine receptors, and of selected 5-HT receptors in explaining the differential contractile responses of pulmonary arteries and veins to histamine and 5-HT in normal guinea pigs;
3) Characterize the role of endothelium-dependent relaxation in pulmonary arteries and veins of guinea pigs;
4) Examine the responses of pulmonary arteries and veins to histamine and 5-HT in POPV, and delineate the mechanisms involved in the altered vasoreactivity, which potentially include structure, endothelium-dependent relaxation, and intrinsic ability of the smooth muscle to relax with NO.
5) Test the responses of pulmonary arteries and veins to ET-1 and ET-3 in POPV, and examine mechanisms of the altered vasoreactivity, particularly ET receptor density and proportion of subtypes.

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Chapter 2

Differential responses of pulmonary arteries and veins to histamine and 5-HT in lung explants of guinea pigs

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Summary

1 The mechanisms by which histamine and 5-HT differentially contract pulmonary arteries and veins are unclear. In lung explants from 26 guinea pigs, we compared responses of pulmonary arteries and veins to histamine, 5-HT and KCl, and examined potential determinants for the differential responses. Lungs were filled with agarose, sectioned into ~1 mm-thick slices, and vascular luminal areas measured by image analysis.

2 Constriction to histamine and 5-HT peaked at 20 sec in arteries and veins, followed by relaxation only in arteries. Both agents also produced concentration-dependent constriction, greater in veins than arteries. KCl constricted arteries and veins equally.

3 The histamine H_1 antagonist chlorpheniramine (10^{-4} M) abolished contractions to histamine; the H_2 antagonist cimetidine enhanced maximal responses and sensitivity of arteries and veins to histamine, and diminished the differences between their maximal responses; the NO synthase inhibitor N^{ω}-nitro-L-arginine (LNNA) increased the maximal responses of arteries and veins, and the differences between their responses; indomethacin had no effect.

4 Contractions to 5-HT were abolished in arteries and markedly reduced in veins by the 5-HT_2 antagonist ketanserin (10^{-4} M); LNNA potentiated the maximal responses of arteries but not of veins; indomethacin increased the maximal responses of arteries but reduced them in veins.

5 By morphometry, arteries had a greater medial thickness and luminal diameter than veins.

6 The data suggest that in guinea pigs, H_2 receptors are responsible for the differential

contractile responses of pulmonary arteries and veins to histamine, whereas endothelium-derived vasoactive substances are responsible for their differential contractile responses to 5-HT.

Keywords: vasoconstriction; pulmonary artery; pulmonary vein; histamine; 5-HT; nitric oxide; lung culture; morphometry.

Introduction

Differential alterations in the reactivity of pulmonary arteries and veins to pharmacological agents can influence perfusion, ventilation-perfusion relationships and vascular resistance in the lung, all of which may affect fluid exchange and raise right ventricular afterload. The biogenic amines histamine and 5-HT are known to differentially constrict pulmonary arteries and veins in several species: for example, in dogs, histamine contracts pulmonary veins, and 5-HT contracts pulmonary arteries (Bradley *et al.*, 1993; Hakim *et al.*, 1982; Michel *et al.*, 1990); in rabbits, both amines primarily contract pulmonary arteries (Bradley *et al.*, 1993; Albert *et al.*, 1989), whereas in guinea pigs, they predominantly contract pulmonary veins (Bradley *et al.*, 1993).

The determinants for the differential responses of pulmonary arteries and veins to histamine and to 5-HT are not known. Putative explanations include differences in receptor subtypes and density (van Nueten *et al.*, 1984), in the endothelium that produces several vasoactive substances affecting vascular tone (Feletou *et al.*, 1995; Furchgott and Vanhoutte, 1989; Gao *et al.*, 1995), and difference in structure (Bradley *et al.*, 1993). Indeed, not only do histamine and 5-HT contract vascular smooth muscle but they also relax it by activation of specific receptors on smooth muscle and endothelial cells (Cushing and Cohen, 1992; Neely *et al.*, 1993; Toda, 1990). For example, histamine constricts vessels by activating H_1 receptors on smooth muscle and relaxes them directly through H_2 receptors on smooth muscle, and indirectly via H_1 receptors on endothelial cells (Toda, 1990) that release relaxing factors such as nitric oxide (NO) and prostacyclin (PGI_2) (Furchgott and Vanhoutte, 1989; Sakuma *et al.*, 1988). Arteries and veins differ in their dilatory responses (Furchgott and Vanhoutte, 1989; Gao *et al.*, 1995), and this

difference could contribute to their differential contractile responses to these two amines. Structural differences of pulmonary arteries and veins could also contribute to their differential contractile responses to histamine and 5-HT (Ferencz, 1969).

Recently, the lung explant technique was applied to study airway constriction by Dandurand *et al.* (1993). In this preparation, small airways and vessels are readily and directly visualized by light microscopy and the structural relationships between vessels, airways and parenchyma are preserved; moreover, the explants can be fixed and morphometric measurements made on the same vessels stimulated with pharmacological agents.

Therefore the principal aims of the present study were, using lung explants, to 1) test the differential reactivity of intrapulmonary arteries and veins of guinea pigs to histamine and 5-HT, 2) explore potential mechanisms for the differential responses, specifically endothelial modulation, receptor subtypes, and vascular structure.

Methods

Preparation of the lung explants

The procedure was slightly modified from that previously described for airways (Dandurand *et al.*, 1993). A total of 26 male adult Hartley strain guinea pigs weighing 474 $\pm$ 11 g (mean $\pm$ SE) were used for these studies.

All the animals were anesthetized with pentobarbital (40 mg kg^{-1} ip), heparinized through the dorsal vein of the penis (3000 U kg^{-1}) and intubated through a tracheostomy with sterile polyethylene tubing 1.9 mm in diameter. Their anterior chest wall and upper abdomen were sterilized with 70% ethanol, the abdomen was opened, and they were

exsanguinated by cutting the abdominal aorta. After removal of the anterior chest wall, the right ventricle was punctured and a cannula advanced into the main pulmonary artery and the pulmonary vessels washed *in situ* with 10 ml Ringer's lactate containing 20 U ml^{-1} heparin. Thereafter, the heart and lungs were excised *en bloc* and the lungs inflated to near total lung capacity with 1% agarose in bicarbonate-buffered culture medium (BCM, 48 ml kg^{-1} body weight) (Dandurand *et al.*, 1993). The preparation was left to cool for 20 min at 4 °C. Then the lungs were separated from the heart, placed in a sterile 50 ml syringe, the needle end of which had been removed, and embedded in 4% agarose in bicarbonate buffered minimum essential medium at 37°C (Dandurand *et al.*, 1993). After 30 min at 4°C, the lung-agarose block was sectioned with a hand-held microtome blade into 0.5-1.0 mm-thick transverse slices. These were examined with an inverted microscope (IMT-2, Olympus, Tokyo, Japan) and those that contained at least one cross-section of a vessel were placed in a 30 mm culture well insert within a six-well plate containing 2 ml of BCM and incubated overnight at 37°C in 5% CO_2-95% air.

Image acquisition

The culture dish inserts containing the lung explants were transferred to six-well plates containing 2 ml of HEPES-buffered culture medium (HCM) (Dandurand *et al.*, 1993), and placed on the stage of an inverted microscope (LH50A, Olympus, Tokyo, Japan). Arteries and veins were identified and imaged with a video camera (CDS, Sony, Nagano, Japan) and images recorded with a video disk recorder (TQ2026F, Panasonic, Osaka, Japan). To distinguish arteries from veins, we used the following criteria: 1) the arteries usually accompanied airways, whereas veins were at a distance from them, and 2) arterial walls were thick and their inner lining was slightly wrinkled, whereas veins

were thinner and wrinkles were inconspicuous. In addition, we confirmed the identities of the vessels by histological examination (see below).

Experimental protocol

First, in 13 guinea pigs, we compared the responses over time and concentration-responses of arteries and veins to histamine and 5-HT. For the responses over time, baseline images of the vessels were first generated; then 10^{-3} M histamine or 10^{-4} M 5-HT (37°C) were added directly to the surface of the lung explants and images were gathered every 10 sec for the first min, then every min for another 4 min. For the concentration-response curves, drugs were added in a cumulative manner: after generating baseline images of the vessels, 10^{-11} M histamine or 5-HT were added to the explants. Twenty sec later (preliminary experiments showed that 20 seconds was the time to peak responses for most concentrations), images of the vessels were taken. Then 10^{-10} M was added and images again taken. This procedure was repeated until final concentrations of 10^{-3} M for histamine and 10^{-4} M for 5-HT were reached.

Second, in four guinea pigs, concentration-responses were generated to KCl using a similar protocol, except that after the baseline images were generated, 4 mM KCl was added to the explants, and images were taken at 10 min, corresponding to the time of peak contraction. The sequential addition of incremental concentrations was stopped at a final concentration of 60 mM.

Third, we examined the effects of the NO synthase inhibitor N^{ω}-nitro-L-arginine benzyl ester (LNNA, 10^{-4} M), of the cyclooxygenase inhibitor indomethacin (10^{-5} M), of the H_1 receptor antagonist chlorpheniramine (10^{-4} M), and of the H_2 receptor antagonist cimetidine (10^{-4} M) on the concentration-responses to histamine, in seven

guinea pigs (including the four guinea pigs that were used also for the KCl study). We also studied the effects of LNNA (10^{-4} M), of indomethacin (10^{-5} M), and of the 5-HT_2 receptor antagonist ketanserin (10^{-4} M) on the concentration-responses to 5-HT, in another six guinea pigs. The lung explants were preincubated with the drugs for 30 min.

In each animal, we usually used 24 explant slices. In each explant, for the responses over time, we studied one vessel, whereas for the concentration-responses, we usually observed one artery and/or one vein, and in a few instances two veins. Each vessel was studied only once. On average, two to three arteries and two to three veins from each animal were used for each treatment.

Image and data analysis

The stored images were digitized using a 80386 Intel-based microcomputer equipped with a frame-grabber board (PIP1024B, Matrox, Montreal, QC, Canada). The digitized images were then transferred to a scientific work station (RS6000, IBM, Armonk, NY), and measurements of luminal area were made with Galileo Image Processing Software (Inspiraplex, Montreal, QC, Canada). The responses of arteries or veins to histamine, 5-HT and KCl were calculated as a percentage change in luminal areas over baseline. Thus a 100% response indicated complete vessel luminal closure and 0% no effect.

From these responses, time course and concentration-response curves of arteries and veins were constructed by plotting the mean values against time and concentrations respectively. The maximal responses and EC_{50} values were determined from each individual vessel, and the latter were expressed as negative log molar (pD_2) values.

Drugs

All drugs were purchased from Sigma Chemical, St. Louis, MO, except ketanserin

tartrate, which was purchased from Research Biochemicals, Natick, MA. Histamine (dihydrochloride), 5-HT (hydrochloride), chlorpheniramine (maleate), cimetidine, KCl, ketanserin, and N^{ω}-nitro-L-arginine benzyl ester were prepared as stock solutions in HCM, from which dilutions were prepared fresh daily. Indomethacin was initially dissolved in ethanol and then diluted with HCM. Each of the drugs was added in 20 μl volumes to the 2 ml of medium, and their concentrations were expressed as values after dilution by those 2 ml of medium.

Histology and morphometry

At the end of the experiments, the explants from the first 13 guinea pigs were fixed by immersion in 10% buffered formalin, processed using standard histological techniques and embedded in paraffin. Five μm-thick sections were cut and stained with hematoxylin-eosin and, in selected ones, with Van Gieson's elastic stain. The arteries and veins whose responses to histamine and 5-HT we had studied were identified based on maps drawn at the time of image acquisition. Morphometric measurements were then made on those vessels that had an intact wall, using previously described methods (Michel, 1982; Michel and Hakim, 1991): with an ocular micrometer on a optical microscope (Leitz, Wetzlar, Germany), we measured the inside diameter at a magnification of x100, and the medial muscle thickness of both walls at the same position, at a magnification of x250 to x400 (for greater precision); the sum of the inside diameter and of the medial muscle thickness of both walls equalled the outside diameter. The few veins that contained cardiac muscle in their wall were excluded. Morphometric measurements were made on a total of 68 arteries and 75 veins.

In addition, in selected sections of vessels from five guinea pigs, we ascertained

that the endothelial cells were intact by staining for the von Willebrand factor with a rabbit polyclonal antibody as previously described (Doornekamp *et al.*, 1996).

Statistical analysis

Data are presented as means ± SE. These means were obtained by averaging data from each animal, and only this average was used for statistical analyses, with "n" being the number of animals from which the vessels were obtained. Because these means were reasonably normally distributed, parametric tests were used for statistical analyses. To compare the curves of the concentration-responses and of the responses over time between arteries and veins or between control and treated groups from the same type of vessels, two-way analysis of variance (ANOVA) was used. If the F value was significant, the Tukey test was applied to ascertain significance at each concentration or time point. The comparisons of maximal responses and pD_2 values were performed by two-way ANOVA. When only two means were compared, Student's paired or unpaired *t* test was used. All the analyses were performed with proprietary software (Systat, Evanston, IL). Differences were considered statistically significant at $P < 0.05$.

Results

Responses over time to histamine and 5-HT

As shown in Fig. 1, after challenge with 10^{-3} M histamine or 10^{-4} M 5-HT, all vessels reached their maximum contraction at about 20 sec. Arteries and veins, however, differed in their responses to each of the two drugs: veins showed a similar degree of contraction to both histamine and 5-HT and remained constricted for the entire 5 min study period. The contraction of the arteries, in contrast, after reaching its peak at the

same time, waned over time and reached a plateau at about 60 sec for 5-HT, and continued to fall slowly with histamine. The veins responded more than the arteries to histamine and 5-HT when the whole curves were analyzed and at all time points to 5-HT except at 10 sec; the differences in response to histamine were statistically significant at 180 sec and beyond.

Cumulative concentration-responses to histamine, 5-HT and KCl

These results are plotted in Figures 2-4. Histamine and 5-HT produced concentration-dependent contraction of arteries and veins. Although the shapes of the curves for the responses to histamine and 5-HT were similar, 5-HT was approximately 100 times more potent than histamine. The most important observation was that for both drugs, the veins constricted significantly more ($P < 0.01$) than the arteries. The second observation was that contraction of the arteries was reversed at the higher concentrations, a finding essentially absent or very inconspicuous in the veins. This occurred at concentrations above 10^{-5} M for histamine and 10^{-7} M for 5-HT. In contrast, KCl (4 to 60 mM) caused a concentration-dependent contraction of similar degree in arteries and veins (Fig. 4).

Effects of chlorpheniramine, cimetidine, LNNA and indomethacin on responses to histamine

The effects of these four drugs on the concentration-responses to histamine are in Fig. 5. At 10^{-4} M, the H_1 receptor antagonist, chlorpheniramine, abolished histamine-induced contraction of both arteries and veins. Cimetidine, the histamine H_2 receptor antagonist, not only significantly enhanced the sensitivity (pD_2 values) and the maximal responses of arteries and veins ($P < 0.05$), but also abolished the differences between their maximal responses (Table 1). LNNA, the NO inhibitor, significantly increased the

maximal responses of the arteries and veins without affecting the sensitivity; the increase in the maximal response, however, was greater in the veins than in the arteries (Table 1). In contrast, indomethacin, the inhibitor of cyclooxygenase, affected neither the sensitivity nor the maximal responses.

Effects of ketanserin, LNNA and indomethacin on responses to 5-HT

The effects of ketanserin, LNNA, and indomethacin on the concentration-responses are in Fig. 6. At 10^{-4} M, ketanserin prevented the contraction in arteries and markedly reduced it in veins. LNNA significantly potentiated the maximal responses of the arteries but not of the veins, whereas indomethacin increased the maximal responses of the arteries but reduced them in the veins significantly ($P < 0.05$, Table 1). Thus both LNNA and indomethacin abolished the differences in the maximal responses to 5-HT between arteries and veins, and in addition, indomethacin diminished the differences between their pD_2 values (Table 1).

Histology and morphometry

Representative images of an artery and of a vein from the explants and the corresponding light photomicrographs from the histology are shown in Fig. 7. Light microscopy revealed that the intrapulmonary arteries were muscular in type, and that they had a thick and complete inner elastic lamina, a media composed of compact smooth muscle cells with irregular elastic fibers in the larger arteries, and a thin attenuated external elastic lamina often seen only with Van Gieson's elastic stain. The veins were also muscular in type but differed from the arteries since their media was thinner, and their internal elastic lamina was also much thinner or frequently absent; in addition, there was no external elastic lamina. The two veins that had cardiac muscle in their walls were

excluded from the analyses. The morphometric measurements revealed that the inside and outside diameters and medial thickness of arteries were greater than those of veins (Table 2, $P < 0.05$). The immunostaining for the von Willebrand factor confirmed that the endothelium was preserved in arteries and veins.

Discussion

In the present study, we used lung explants to examine *in vitro* the responses of intrapulmonary arteries and veins to histamine and 5-HT, and to test potential determinants for the differential responses. The principal findings were: 1) for the responses over time, histamine and 5-HT both produced peak constriction of arteries and veins at 20 sec, after which it waned in the arteries only; for the concentration-responses, constriction of the veins was greater than that of the arteries; 2) both cimetidine and LNNA enhanced responses of arteries and veins to histamine, but only cimetidine abolished the differences between their responses; 3) LNNA potentiated the maximal responses of arteries but not of veins to 5-HT, and indomethacin increased the maximal responses of arteries and reduced them in veins; 4) responses of arteries and veins to KCl were similar; 5) by morphometric analysis, the diameters and medial thickness of the arteries were significantly greater than those of the veins.

Lung explants provide a convenient means to assess directly intraparenchymal vessel constriction or dilatation, either by continuous recording (responses over time) or at given time points after incremental concentrations (concentration-responses). In this preparation, pulmonary arteries and veins are within the framework of an intact and supporting parenchyma as *in vivo*. The lumina of the pulmonary vessels remain open and

are nearly circular, most likely related to their low baseline tone and to the preload provided by the stretch from the surrounding parenchyma that is filled through the airways with agarose. Although the control of vascular tension and preload are perhaps not as precise as in vascular strip or ring preparations, we believe this is offset by the provision of the aforementioned stretch of the parenchymal distension that is closer to the *in vivo* situation. The afterload to the vessels is also presumably provided by the surrounding parenchyma. Because of the likelihood that the load from the parenchyma remains constant, the contraction of the vessels in the lung explants probably resembles most closely isotonic rather than isometric contraction *in vitro* (Paton, 1975) and is supported by the fact we are measuring a reduction in vessel size.

In the explants, airways, arteries and veins were readily visualized and differentiated (Dandurand *et al.*, 1993): airways were identified by their thin walls and beating cilia, whereas arteries were distinguished from veins by their wall structure and position within the acinus. Their identities could be confirmed by histological examination.

The major difference that we observed between arteries and veins was a significantly greater constriction of the veins to histamine and to 5-HT, particularly in the concentration-responses (Figs. 2 and 3). In isolated perfused guinea pig lungs, Bradley *et al.* (1993) also found that veins responded more than arteries to histamine and 5-HT, and they attributed this partly to the greater amount of smooth muscle in the vein, based on responses to KCl. Indeed, since KCl contracts vessels by depolarizing the smooth muscle cell membrane independent of specific receptors (Karaki and Weiss, 1988) or of the endothelium (Holecyova *et al.*, 1993; Kimura *et al.*, 1992), the degree

of contraction it produces may well depend on smooth muscle mass. Our findings (Table 2) that the mean smooth muscle thickness of the arteries was greater than that of the veins, and that the arteries and veins contracted equally in the response to KCl (Fig. 4), however, appear to contradict the conclusions of Bradley *et al.* (1993); part of the difference may be due to the variable structure of the arterial media with its alternating thick and thin areas, compared with the venous media with nearly even thickness, which may contract more effectively (Ferencz, 1969; McLaughlin *et al.*, 1966). A more plausible explanation for the apparent discrepancy between the data of Bradley *et al.* (1993) and ours is that they measured changes in resistance that, in accordance with Poiseuille's equation, varies with the inverse fourth power of radius. We found that the veins that we studied had a smaller diameter than the arteries (Table 2) so that the resistance of the former would increase more with the same percent muscle contraction. Thus, our findings that the mean smooth muscle thickness of the arteries was greater than that of the veins, and the similar magnitude of contraction with KCl seem to exclude a significant role of smooth muscle mass in the greater venous responses to histamine and 5-HT.

Our findings that arteries and veins differed in their responses to histamine and 5-HT but not to KCl suggest that differences in receptor densities or in the production of endothelium-derived vasoactive substances are responsible for their differential contractile responses to these two amines (Karaki and Weiss, 1988; Kimura *et al.*, 1992). For histamine, there is considerable evidence for a dual receptor mechanism in both pulmonary and systemic vessels (Abacioglu *et al.*, 1987; Toda, 1990; Turker, 1973). Histamine contracts vessels via H_1 receptors on smooth muscle and relaxes them via H_1

receptors on endothelial cells and H_2 receptors on smooth muscle (Toda, 1990). We found this dual effect of histamine clearly demonstrated in guinea pig pulmonary vessels, since the H_1 receptor antagonist blocked the contraction and the H_2 receptor antagonist significantly augmented it. In isolated perfused lungs of guinea pigs, Turker (1973) also showed that H_2 receptor antagonists increased the histamine-induced pressor response. Our results extend these studies by suggesting that the role of H_2 receptors was more prominent in arteries than in veins since cimetidine diminished the differences in their maximal contractile responses to histamine (Fig. 5 and Table 1).

Our data also indicate that NO synthase inhibition significantly enhanced the contractile responses of pulmonary arteries and veins to histamine, but that cyclooxygenase inhibition had no effect, suggesting that NO rather than PGI_2 was released to modulate the responses to histamine in these tissues. These results agree with previous findings in guinea pig pulmonary arteries that histamine-induced relaxation is abolished by NO synthase inhibitors but not by indomethacin (Sakuma *et al.*, 1988) and extend them by showing that inhibition by NO of the contractile response to histamine was more prominent in veins than arteries, since the concentration-response curve was shifted about one log interval leftward only in the veins, and that the difference in the maximal contraction of arteries and veins was increased.

Although multiple 5-HT receptor subtypes exist (Hoyer *et al.*, 1994), our results suggest that the contraction of pulmonary vessels of guinea pigs with 5-HT is primarily mediated by 5-HT_2 receptors, since ketanserin abolished the contraction in arteries and markedly reduced it in veins. This finding is consistent with previous observations in perfused lungs of guinea pigs and cats, and in pulmonary arterial rings of rabbits (el-

Kashef, 1996; Neely *et al.*, 1993; Selig *et al.*, 1988). The partial return of the contraction of the veins at the high concentrations of 5-HT, despite the presence of ketanserin (Fig. 6), could be due to the production of contractile cyclooxygenase substances. Supporting evidence for this statement includes the fact that indomethacin suppressed 5-HT-induced maximal contractions (Table 1); furthermore, in a separate study, we found that acetylcholine also induced the release of contractile cyclooxygenase products in the pulmonary vessels of guinea pigs (Shi *et al.*, 1997).

Unlike their effects on the responses to histamine, LNNA and indomethacin increased the maximal responses of arteries to 5-HT, whereas they either had no effect on the veins or even reduced their maximal responses. This finding suggests that NO and vasodilator cyclooxygenase substances were responsible for the reduced responses to 5-HT in the arteries, and for the differences in contractility to this amine compared with the veins. Thus, in the latter, NO did not modulate the contractile response to 5-HT and vasoconstrictor cyclooxygenase products appeared to contribute partially to their greater contraction. An increase in contraction to 5-HT after inhibition of NO synthase and cyclooxygenase activity has been reported in pulmonary arteries of dogs and rabbits (el-Kashef, 1996; Hofman *et al.*, 1991). However, the involvement of the NO and the cyclooxygenase pathways in differential responses to 5-HT between pulmonary arteries and veins has not been reported, and may account for their differential contractile responses, since both LNNA and indomethacin abolished differences in the maximal response and the latter also abolished differences in the pD_2 values.

Our histological studies enabled us to confirm the identities of the vessels that we had studied pharmacologically and to measure them. There are few descriptions of

guinea pig pulmonary vessels in the literature (Ferencz, 1969; McLaughlin *et al.*, 1966) and these indicate that they are similar to those of rats. The principal characteristics are that the arteries have a variable muscle thickness (Ferencz, 1969) (Fig. 7), nevertheless thicker than the veins (Table 2). Although this property should confer upon them the ability to contract more, we observed the opposite. The veins have a thinner but more regular media and the large veins also have cardiac muscle in their walls (Ferencz, 1969), although it does not extend as far distally as in the rat (Best and Heath, 1961); the veins containing cardiac muscle were excluded from our pharmacological or morphologic data. The effect of cardiac muscle on reactivity is not clear. Cheung (1981) found in guinea pigs that pulmonary venous smooth muscle was electrically quiescent, and that electrical stimulation elicited action potentials in cardiac muscle, but not in smooth muscle. Therefore it is likely that the two types of muscle respond differently to pharmacological stimuli as well, and that a study of intrapulmonary veins should not *a priori* include those with cardiac muscle in their wall.

In conclusion, we have demonstrated in lung explants of guinea pigs that pulmonary veins contract more than arteries to histamine and 5-HT, and that receptors mediating vasodilation and endothelial modulation appear to contribute more to their differential contractile responses to these amines than structure. Furthermore, the differences are drug-specific: for histamine, the differences between arteries and veins are related to H_2 receptors on smooth muscle, whereas for 5-HT, they are due to NO and dilator prostaglandins.

Pulmonary veins had long been considered as conduit vessels with little reactivity. In fact, they contribute significantly to total pulmonary vascular resistance (Hakim *et al.*,

1982). Moreover, recent studies indicate that in several species, pulmonary veins exhibit equal or even greater reactivity than arteries in response to a variety of stimuli: for example, the pulmonary veins of rats contract more than arteries during hypoxia (Zhao *et al.*, 1993), ferret pulmonary veins react more to platelet-activating factor (Gao *et al.*,1995), and in most species, pulmonary veins contract more to endothelin (Levin, 1995). These studies, together with our data, suggest that pulmonary veins respond prominently to a number of constrictors, which could lead to an increase in microvascular pressure, contributing to the formation of pulmonary edema under pathological conditions.

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Tables and Figures

TO Dr. Michel

Table 1. Effects of LNNA and indomethacin as well as cimetidine on maximal responses (Rmax) and pD_2 values of pulmonary arteries and veins to histamine and 5-HT.

	Arteries		Veins	
	Rmax (%)	pD_2	Rmax (%)	pD_2
Histamine only	39.3 ± 4.5	6.1 ± 0.2	52.1 ± 3.0^{+}	5.7 ± 0.3
Cimetidine	65.6 ± 6.3*	6.7 ± 0.3*	60.1 ± 5.8*	6.6 ± 0.3*
LNNA	49.6 ± 3.8*	6.2 ± 0.1	67.2 ± 4.8*$^{+}$	5.9 ± 0.2
Indomethacin	39.5 ± 6.7	6.1 ± 0.2	48.7 ± 4.2+	5.8 ± 0.2
5-HT only	27.1 ± 5.4	8.0 ± 0.1	43.4 ± 4.8^{+}	7.4 ± 0.1^{+}
LNNA	37.9 ± 4.9*	7.9 ± 0.2	44.1 ± 4.4	7.1 ± 0.2^{+}
Indomethacin	34.6 ± 5.6*	7.7 ± 0.2	34.1 ± 4.4*	7.4 ± 0.2

Values were means ± SE of six or seven guinea pigs. * $P < 0.05$ vs. histamine or 5-HT alone, and $^{+}$ $P < 0.05$ arteries vs. veins with a same treatment.

Table 2. Baseline video image diameter and histological measurements in pulmonary vessels of guinea pigs.

	Baseline video image I.D.	I. D.	O. D.	M. T.
Arteries	427 ± 23*	406 ± 21*	522 ± 23*	56 ± 3*
Veins	288 ± 21	228 ± 17	323 ± 18	47 ± 3

Values, in μm, were means ± SE of 13 guinea pigs. * $P < 0.05$ versus veins. Only those vessels used to study responses to histamine and 5-HT and amenable to histological measurements were included. I.D.: inside diameter; O.D.: outside diameter; M.T.: medial thickness. O.D. = I.D. + 2 M.T.

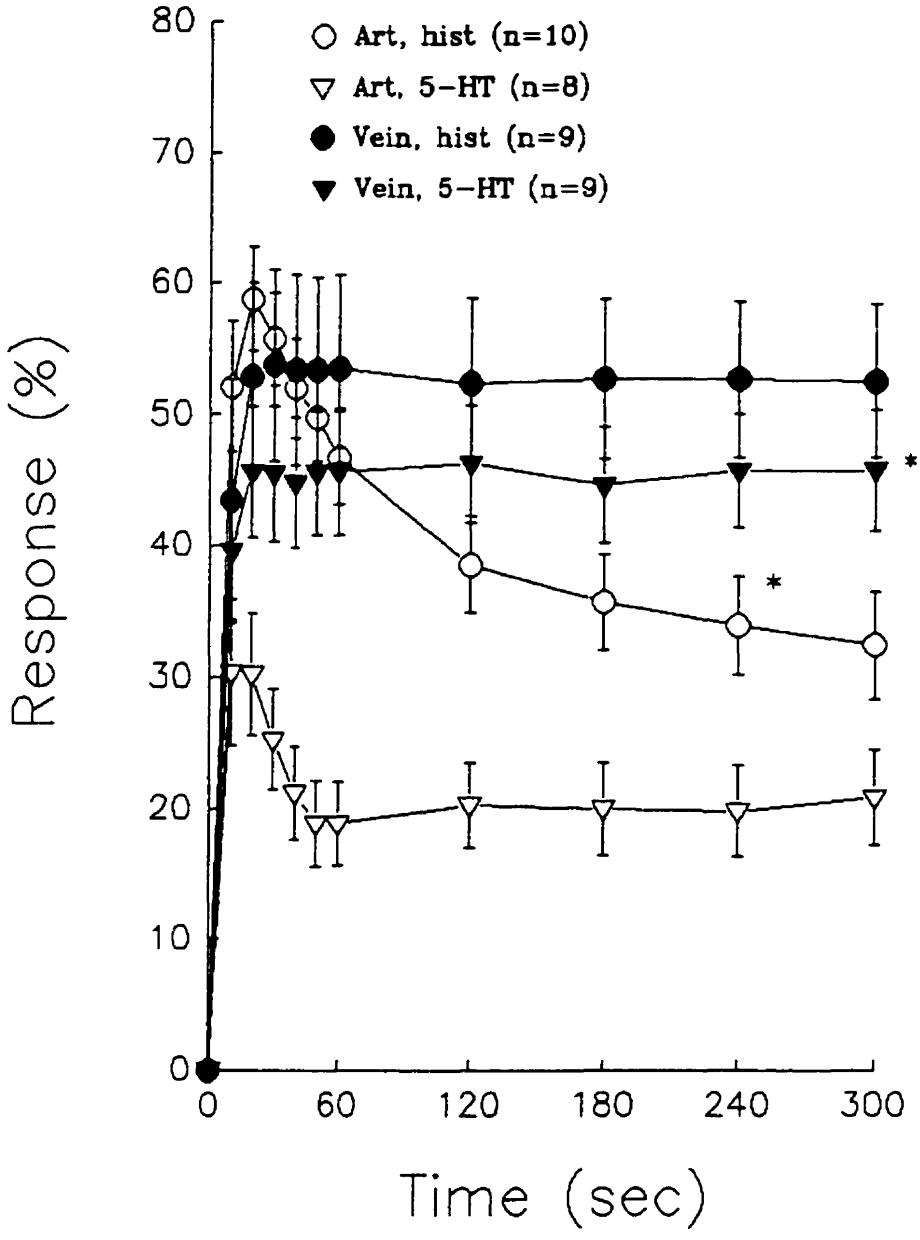

Fig. 1. Responses of pulmonary arteries and veins with time to histamine (hist) and 5-HT in lung explants. Responses were calculated as a percent change over baseline in luminal areas and expressed as means ± SE. Both agents constricted arteries and veins, with the peak at 20 sec, after which only the arteries relaxed. * $P < 0.05$ compared with arteries. n, number of animals.

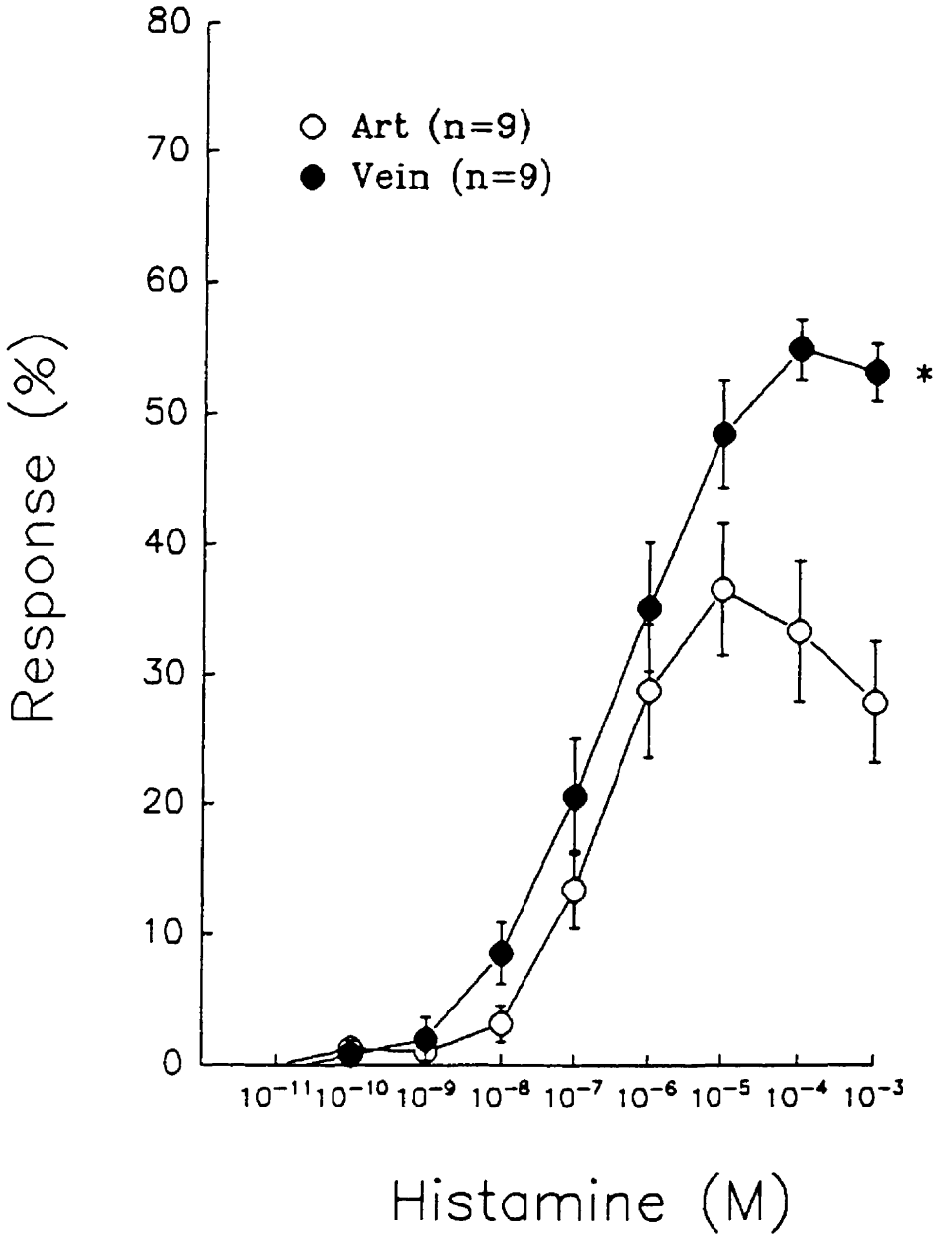

Fig. 2. Cumulative concentration-responses of pulmonary arteries and veins to histamine. In arteries, histamine caused concentration-dependent constriction at concentrations up to 10^{-6} M and dose-dependent relaxation from 10^{-5} M to 10^{-3} M. Veins constricted more than arteries for the whole curve and at 10^{-5} to 10^{-3} M. * $P < 0.05$ compared with arteries. n, number of animals.

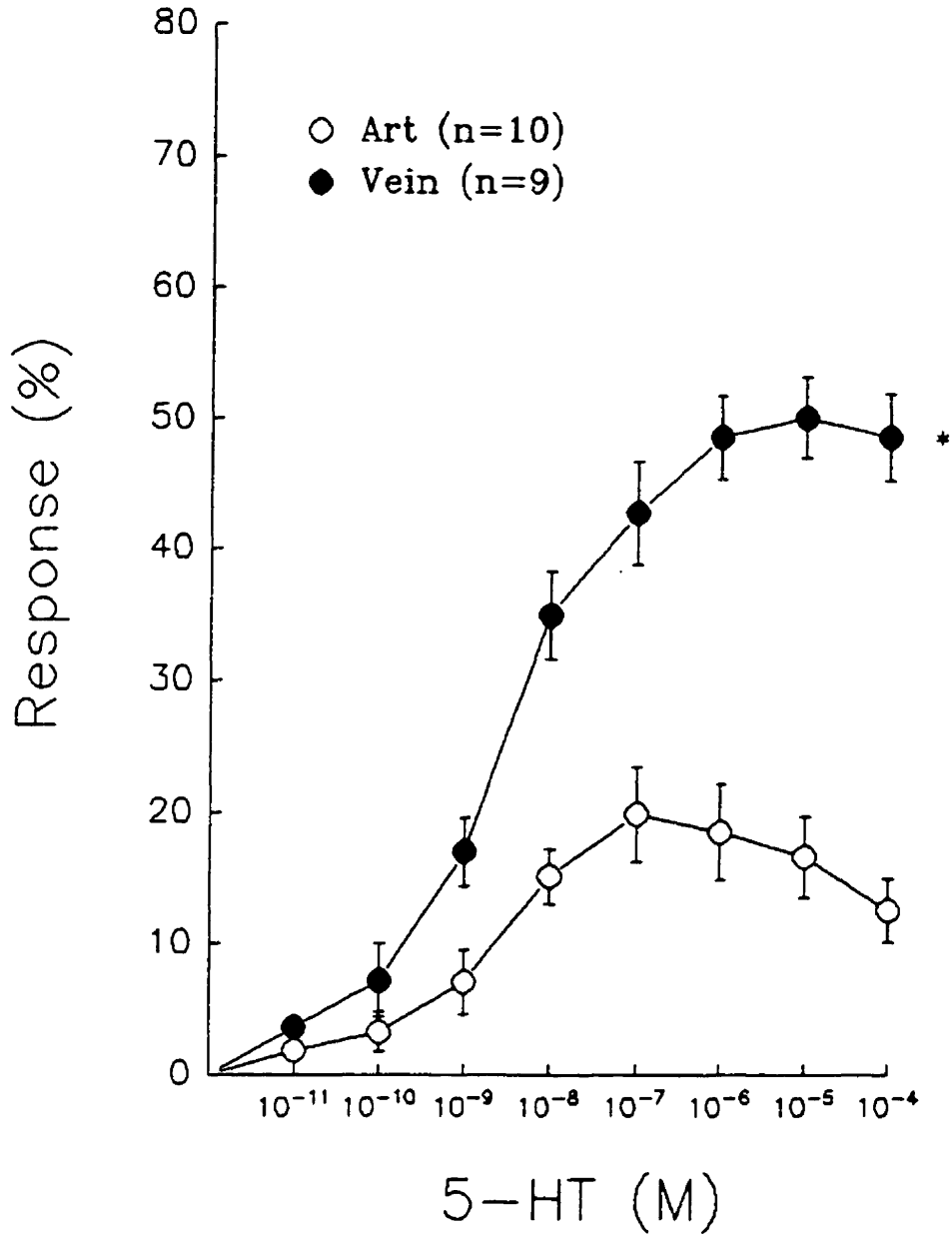

Fig. 3. Cumulative concentration-responses of pulmonary arteries and veins to 5-HT. In arteries, 5-HT induced a concentration-dependent constriction up to 10^{-7} M, and relaxation from 10^{-6} M to 10^{-4} M. Veins constricted more than arteries from 10^{-9} to 10^{-4} M and did not relax. * $P < 0.05$ compared with arteries.

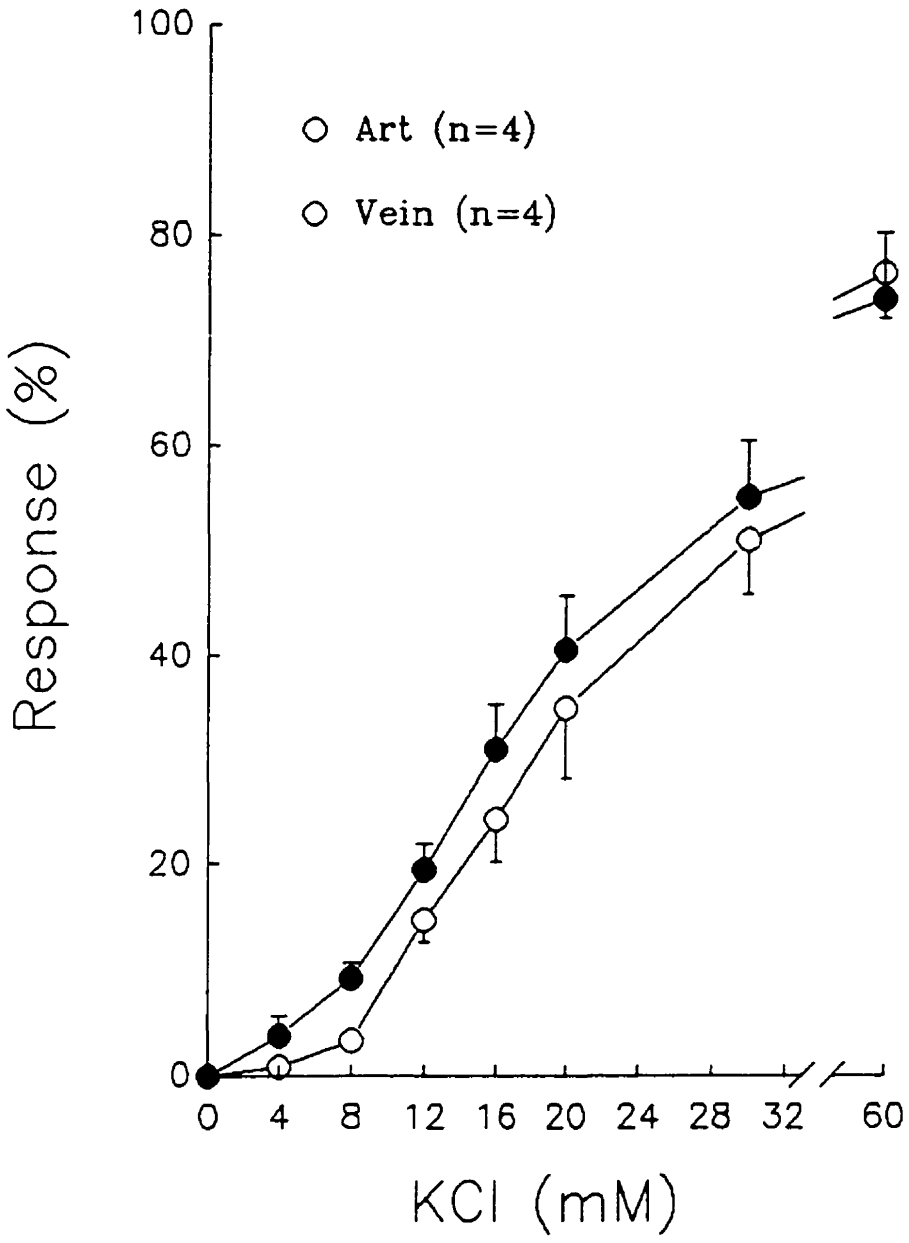

Fig. 4. Cumulative concentration-responses of pulmonary arteries and veins to KCl. In both, KCl caused a similar concentration-dependent constriction ($P > 0.05$).

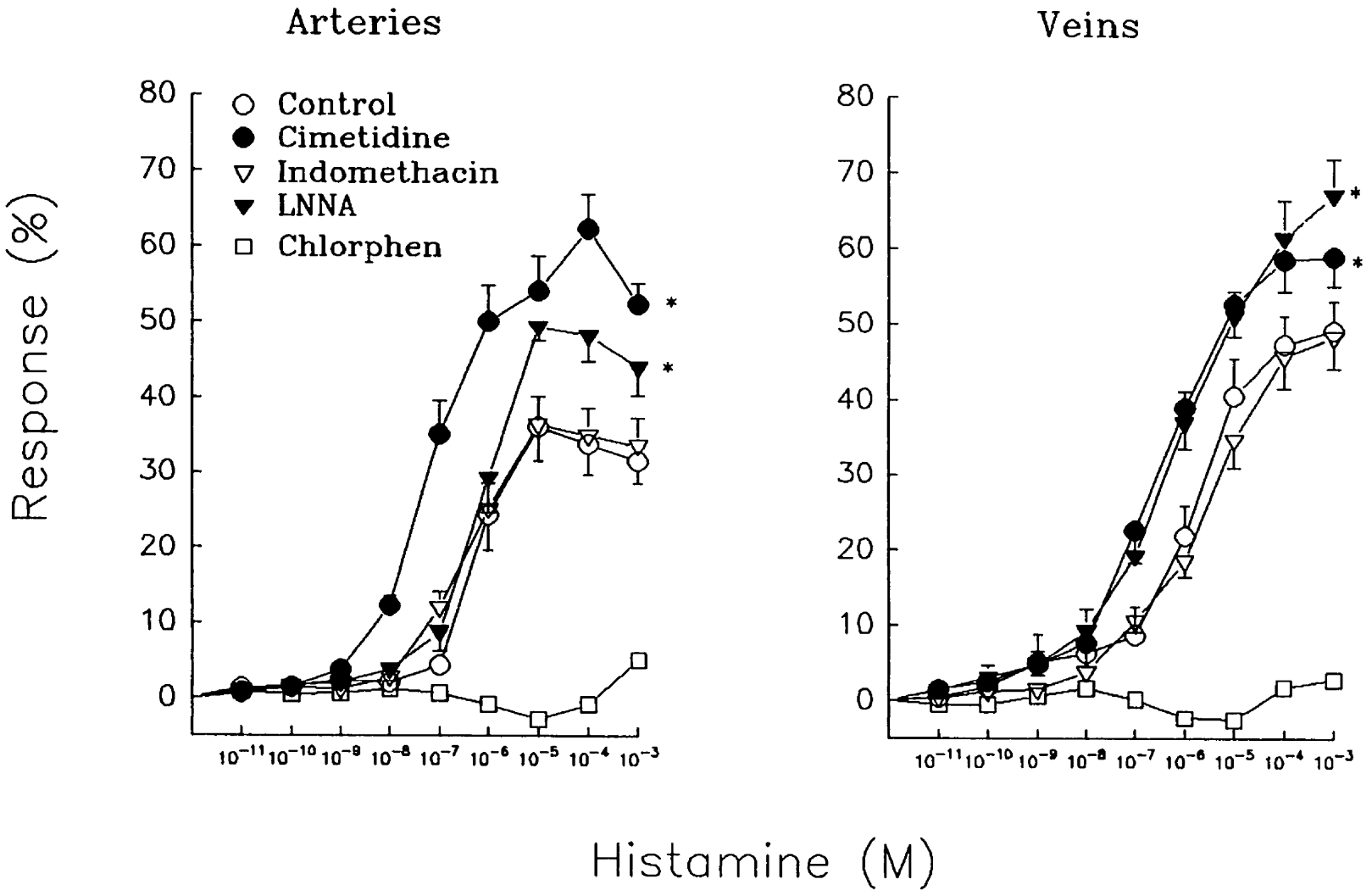
Arteries
Veins
Control
Cimetidine
Indomethacin
LNNA
Chlorphen
Response (%)
Histamine (M)
0
10
20
30
40
50
60
70
80
10^{-11} 10^{-10} 10^{-9} 10^{-8} 10^{-7} 10^{-6} 10^{-5} 10^{-4} 10^{-3}
*
*
*
*

Fig. 5. Cumulative concentration-responses of pulmonary arteries and veins to histamine after pretreatment with chlorpheniramine, cimetidine, LNNA or indomethacin. Results are means ± SE in seven guinea pigs. * $P < 0.05$ compared with histamine alone. Chlorphen, chlorpheniramine.

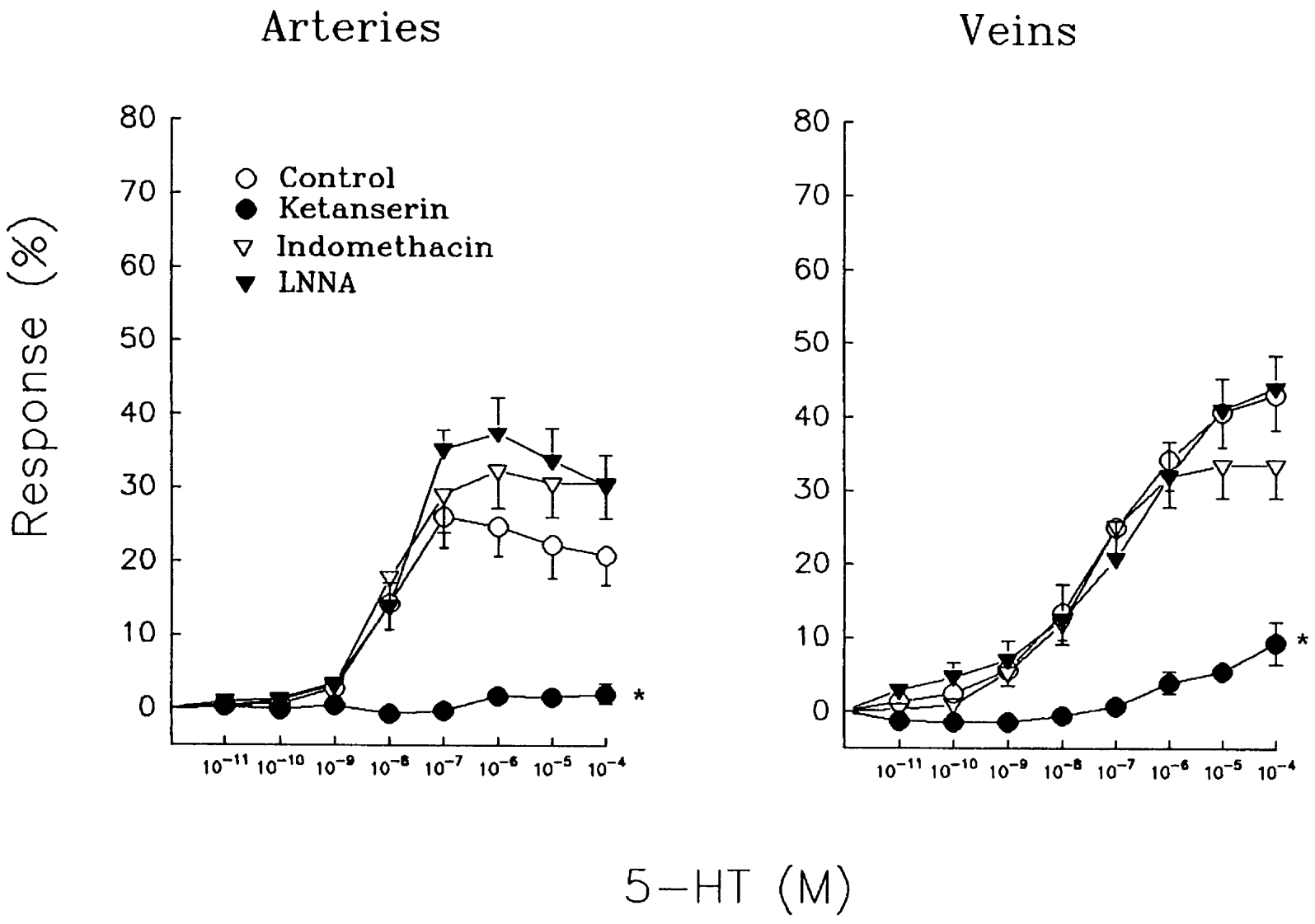
Arteries
Veins
Response (%)
80
70
60
50
40
30
20
10
0
Control
Ketanserin
Indomethacin
LNNA
*
*
10^{-11} 10^{-10} 10^{-9} 10^{-8} 10^{-7} 10^{-6} 10^{-5} 10^{-4}
5-HT (M)

Fig. 6. Cumulative concentration-responses of pulmonary arteries and veins to 5-HT after pretreatment with ketanserin, LNNA or indomethacin. Results are means ± SE of six guinea pigs. * $P < 0.05$ compared with 5-HT alone.

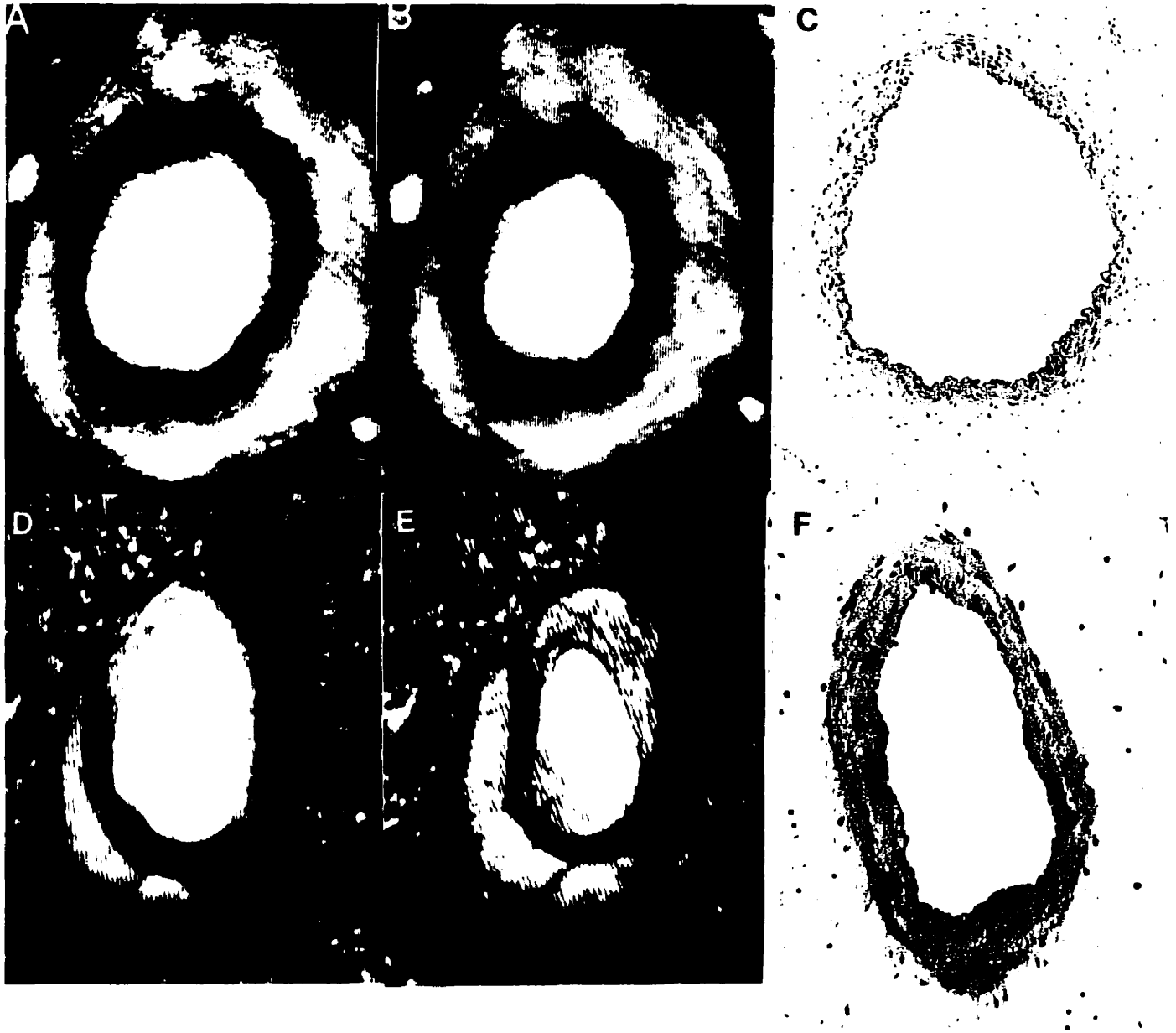
A
B
C
D
E
F

Fig. 7. Video images and light microscopy of an artery (A-C) and a vein (D-F). Video images of an artery at baseline (A) and after addition of 10^{-7} M 5-HT (B). Light photomicrograph of the same artery (C). Video images of a vein at baseline (D) and after addition of 10^{-7} M histamine (E). Light photomicrograph of the same vein (F). A, B, D, E, X 77. C, F, hematoxylin and eosin X 80.

In Chapter 2, we successfully adapted the lung explant technique to study pulmonary vasoconstriction in normal guinea pigs and found: 1) histamine and 5-HT contracted pulmonary veins more than arteries, whereas KCl equally contracted arteries and veins; 2) The H_2 antagonist cimetidine enhanced maximal responses and sensitivity of arteries and veins to histamine, and abolished differences between their maximal responses; the NO synthase inhibitor LNNA increased the maximal responses of arteries and veins, and the differences between their responses; indomethacin had no effect; 3) LNNA potentiated the maximal responses of arteries but not of veins to 5-HT; indomethacin increased the maximal responses of arteries but reduced them in veins. These results suggest that the contractile responses of pulmonary arteries and veins to histamine and 5-HT are differently modulated by endothelium-derived vasoactive substances. This difference between pulmonary arteries and veins in endothelial modulatory function is related to either different production of endothelium-derived vasoactive factors or different responsiveness to them. Endothelial NO-mediated relaxation is the major pathway in pulmonary vessels. In Chapter 3, we thus compared relaxant responses of pulmonary arteries and veins to pharmacologic agents that activate different steps of the NO pathway.

Chapter 3

Differential relaxant responses of pulmonary arteries and veins in lung explants of guinea pigs

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ABSTRACT

The endothelium regulates vascular tone through release of relaxing or contracting factors, with nitric oxide (NO) being a major endothelium-derived relaxing factor. In the present study, we determined the differential abilities and mechanisms of pulmonary arteries and veins of normal guinea pigs to relax after precontraction using a lung explant technique. Excised lungs of 15 guinea pigs were filled through the airways with 1 % agarose, cut into 1 mm-thick slices and cultured overnight; luminal areas of vascular cross-sections were measured with an image analysis system. Vessels were precontracted with U46619 and responses to histamine, acetylcholine (ACh), sodium nitroprusside (SNP) and papaverine examined; we also determined the effects of nitro-L-arginine (LNNA) and of indomethacin on ACh-induced responses. We found that histamine relaxed arteries more than veins and that ACh relaxed only arteries. LNNA pretreatment abolished ACh-induced relaxation of arteries and caused ACh-induced contraction of veins, whereas indomethacin markedly augmented ACh-induced relaxation of arteries (maximal relaxation: 48.5 $\pm$ 4.7% vs. 19.2 $\pm$ 5.1% without it) and induced a dose-dependent relaxation of veins (maximal relaxation: 17.0 $\pm$ 4.1%). SNP induced a significantly greater relaxation of arteries than veins, whereas papaverine relaxed them equally. We conclude that in guinea pigs endothelial NO-mediated relaxation is greater in pulmonary arteries than in veins, and that ACh-induced NO-mediated relaxation is reduced by the simultaneous production of cyclooxygenase-derived vasoconstrictors.

Keywords: endothelium; acetylcholine; histamine; nitric oxide; pulmonary artery; pulmonary vein.

INTRODUCTION

Differential abilities of pulmonary arteries and veins to dilate to pharmacological agents may influence perfusion, ventilation-perfusion relationships, and vascular resistance in the lung, all of which may affect fluid exchange and right ventricular afterload. The endothelium regulates the tone of the underlying vascular smooth muscle by synthesizing and releasing endothelium-derived relaxing and contracting factors (Furchgott and Vanhoutte, 1989; Yang *et al.*, 1991). Nitric oxide (NO), a chemically unstable radical, is a major endothelium-derived relaxing factor synthesized from L-arginine (Furchgott and Vanhoutte, 1989). Acetylcholine (ACh), histamine, bradykinin and other agents dilate vascular smooth muscle by stimulating the endothelium to release NO (Furchgott and Vanhoutte, 1989; Sakuma *et al.*, 1988). In contrast, nitrogen-containing vasodilators such as sodium nitroprusside (SNP) act on smooth muscle directly by release of NO (Rao and Cederbaum, 1995).

In the pulmonary vasculature, the endothelium-derived NO-mediated relaxation of arteries and veins differs among species: in lambs and pigs, it plays a larger role in veins than in arteries (Bansal *et al.*, 1993; Feletou *et al.*, 1995); in cattle, it modulates pulmonary arterial and venous tone similarly (Ignarro *et al.*, 1988), whereas in ferrets, it acts predominantly in arteries (Gao *et al.*, 1995). These differences can be related either to the ability of the endothelium to release NO or to the responsiveness of the vascular smooth muscle to it (Ignarro *et al.*, 1991; Yang *et al*, 1991). In a previous study, Sakuma *et al.* (1988) demonstrated that histamine and ACh induced endothelium-derived NO-mediated relaxation in pulmonary arteries of guinea pigs; what happens in pulmonary veins is not known. Therefore, in the present study, we aimed to compare

the relaxant responses of intrapulmonary arteries and veins of guinea pigs to pharmacologic agents that activate the different steps of the NO pathway, ie. endothelial-dependent and independent, with those of papaverine that, like NO, also increases cytosolic cGMP content but does so via inhibition of phosphodiesterase activity (Aoki *et al.*, 1994; Cumiskey and Feigenson, 1983).

To investigate further the differential responses of arteries and veins to these relaxing agents, and their mechanisms, we opted for an *in vitro* lung explant technique in guinea pigs, previously used to study airway constriction in the rat (Dandurand *et al.*, 1993). In this preparation, small airways and vessels are readily and directly visualized by light microscopy and the structural relationships between vessels, airways and parenchyma are preserved.

METHODS

Preparation of the lung explants. The procedure was slightly modified from that previously described for airways (Dandurand *et al.*, 1993). A total of 15 adult male Hartley strain guinea pigs weighing 556 $\pm$ 62 g (mean $\pm$ SE) were used for these studies. All the animals were anesthetized with pentobarbital (40 mg/kg ip), heparinized through the dorsal vein of the penis (3000 u/kg) and intubated through a tracheostomy with sterile polyethylene tubing 9 cm long and 1.9 mm in diameter. Their anterior chest wall and upper abdomen were sterilized with 70% ethanol, the abdomen was opened and they were exsanguinated by cutting the abdominal aorta. After removal of the anterior chest wall, the right ventricle was punctured and a cannula advanced into the main

pulmonary artery and the pulmonary vessels washed *in situ* with 10 ml Ringer's lactate containing 20 U/ml heparin. The heart and lungs were excised *en bloc* and the lungs inflated to near total lung capacity with 1% agarose in bicarbonate-buffered culture medium (BCM, 48 ml/kg body weight) at 37°C, prepared as described previously (Dandurand *et al.*, 1993). The preparation was left to cool for 20 min at 4°C. Then the lungs were separated from the heart, placed in a sterile 50 ml syringe the needle end of which had been removed, and embedded in 4 % agarose in bicarbonate buffered minimum essential medium at 37°C (Dandurand *et al.*, 1993). After 30 min at 4°C, the lung-agarose block was sectioned witha hand-held microtome blade into 0.5-1.0 mm-thick transverse slices. These were examined with an inverted microscope (IMT-2, Olympus, Tokyo, Japan) and those that contained at least one cross-section of a vessel were placed in a 30 mm culture well insert within a six-well plate containing 2 ml of BCM and incubated overnight at 37°C in 5% CO_2-95% air.

Image acquisition. The culture dish inserts containing the lung explants were transferred to six-well plates containing 2 ml of HEPES-buffered culture medium (HCM) (Dandurand *et al.*, 1993), and placed on the stage of an inverted microscope (LH50A, Olympus, Tokyo, Japan). Arteries and veins were identified and imaged with a video camera (CDS, Sony, Nagano, Japan) and images recorded with a video disk recorder (TQ2026F, Panasonic, Osaka, Japan). To distinguish arteries from veins, we used the following criteria: 1) the arteries usually accompanied airways, whereas veins were at a distance from them, and 2) arterial walls had a thick media and their inner lining was slightly wrinkled, whereas veins were thinner and wrinkles were inconspicuous.

Experimental protocol. First, in all explants, baseline images of the vessels were

generated. Then they were precontracted with 1 or 3 x 10^{-6} M U46619, the thromboxane A_2 analogue, added directly to the surface of the lung explants and images were gathered every 10 sec for the first min, then every min for another 4 min; thereafter, they were followed for a further 15 min to ensure stable contraction, for a total of 20 min.

To test the dilator responses of these precontracted vessels, cumulative dose-response curves were constructed by adding histamine solution in half-log unit intervals from 10^{-11} M to 10^{-7} M, and by adding ACh, SNP and papaverine solutions in one-log unit intervals from 10^{-11} M to 10^{-4} M. In addition, to determine the differential roles of the NO and of the prostaglandin pathways, some vessels were preincubated with N^{ω}-nitro-L-arginine benzyl ester (LNNA, 10^{-4} M) or with indomethacin (10^{-5} M) for 30 min before generating dose-response curves to ACh. In addition, as a control, we tested the effects of the LNNA and of indomethacin on the vessels in their baseline state, without precontraction.

In each explant, for the responses over time, we studied one vessel, whereas for the dose-responses, we usually observed one artery and/or one vein, and in a few instances two veins. We studied a total of 75 arteries and 88 veins from 123 explants. The numbers of animals used in each step of the protocol are indicated in the figures.

Image and data analysis. The stored images were digitized using a 80386 Intel-based microcomputer equipped with a frame-grabber board (PIP1024B, Matrox, Montreal, QC, Canada). The digitized images were then transferred to a scientific work station (RS6000, IBM, Armonk, NY), and measurements of luminal area were made with Galileo Image Processing Software (Inspiraplex, Montreal, QC, Canada). The contractile

responses of arteries or veins to U46619 were calculated as a percentage of complete vessel closure, using the equation:

Response = [1 - (residual area after drug/ baseline area)] x 100.

Thus a 100% response indicated complete vessel luminal closure and 0% no effect.

The responses to ACh, histamine, SNP or papaverine were expressed as a percentage of vessel precontraction induced by U46619 using the equation:

Response = [(area before dilator - area after dilator)/ (baseline area - area before dilator)] x 100.

Here -100% indicated a return to baseline state (ie. before precontraction) and 0% full persistence of the pre-contracted state.

From these responses, time course and dose-response curves of arteries and veins were constructed by plotting the mean values against time and concentrations respectively. The EC_{50} values were determined from each individual vessel and expressed as negative log molar (pD_2) values.

Drugs. All drugs were purchased from Sigma Chemical, St. Louis, MO. Histamine (dihydrochloride), ACh (chloride), SNP, papaverine (hydrochloride), and LNNA (benzyl ester) were prepared as stock solutions in HCM, from which dilutions were prepared fresh daily. Indomethacin was dissolved in ethanol and then diluted with HCM. For U46619 (9,11-dideoxy-11α,9α-epoxymethanoprostaglandin $F_{2\alpha}$), the stock solution at a concentration of 10^{-4} or 3 x 10^{-4} M was used directly. The concentrations of all drugs were then expressed as values after dilution of the 20 μl by the 2 ml of medium (ie. 100-fold).

Statistical analysis. Data are presented as means ± SE, with "n" being the number of animals from which the vessels were obtained, and with which all statistical analyses were done. To compare the curves of the dose-responses and of the responses over time between arteries and veins or between control and treated groups from the same type of vessels, two-way analysis of variance (ANOVA) was used. If the F value was significant, the Tukey test for unpaired observations or Student's paired *t* test for paired observations were applied to ascertain significance at each concentration or time point. The comparison of maximal responses or pD_2 values was performed by two-way block ANOVA, with Student's paired *t* test or the Tukey test as *post hoc* tests. All the analyses were performed using proprietary software (Systat, Evanston, IL). Differences were considered statistically significant at $P < 0.05$.

RESULTS

Responses to U46619. Fig. 1 shows the contractile responses to 10^{-6} M U46619: the veins contracted rapidly and attained a plateau at 20 sec that lasted about 120 sec, followed by a slowly increasing contraction. The arteries reached their peak of contraction at the same time as the veins, but in contrast, the responses waned substantially up to 120 sec, thereafter also slowly increasing their contraction; overall the veins constricted to a greater degree than the arteries ($P < 0.01$). By 20 min, both arteries and veins reached a plateau of contraction, greater in the latter ($P < 0.05$).

Responses to histamine. In the arteries after precontraction with U46619, histamine (10^{-11} to 10^{-7} M) produced dose-dependent relaxation in arteries and veins,

significantly greater in the arteries (Fig. 2), but at 10^{-7} M, it started to contract the arteries and veins.

Responses to ACh and effect of LNNA and indomethacin. In precontracted arteries, ACh caused a dose-dependent relaxation (Fig. 3), with maximal responses of 19.2 ± 5.1% and pD_2 values of 8.1 ± 0.7 (Table 1). In the arteries pretreated with LNNA, ACh caused further constriction instead of relaxation; indomethacin, however, markedly augmented ACh-induced relaxation (Fig. 3 and Table 1). In precontracted veins, ACh had no significant effect ($P > 0.05$), although it caused a slight contraction at 10^{-5} and 10^{-4} M (Fig. 4). In veins pretreated with LNNA, however, ACh induced constriction, whereas after indomethacin, it produced a dose-dependent relaxation with a maximal relaxation response of -17.0 ± 4.1% (Fig. 4 and Table 1).

Neither LNNA nor indomethacin alone had significant effects on the baseline luminal areas of arteries (-1.6 ± 0.6% and 3.4 ± 0.8%, respectively) or of veins (-1.3 ± 1.0% and -1.9 ± 0.5%, respectively).

Responses to SNP and papaverine. SNP produced dose-dependent relaxation in both arteries and veins, significantly greater ($P < 0.05$) in the former between 10^{-8} and 10^{-4} M (Fig. 5). The pD_2 values were also significantly greater ($P < 0.05$) for arteries than veins (Table 1). Papaverine also caused relaxation of both arteries and veins, the extent of which did not differ significantly between either vessel types for the whole curves or in the maximal relaxation responses ($P > 0.05$, Table 1, Fig. 6).

DISCUSSION

In the present study, we examined the differential responses of intrapulmonary arteries and veins to ACh, histamine, SNP and papaverine after precontraction with U46619. We found that 1) ACh relaxed arteries but had no significant effects on veins, and the effect in the former was mediated by NO, not by dilator prostaglandins, 2) histamine and SNP relaxed arteries more than veins, and 3) papaverine relaxed arteries and veins equally.

The lung explant technique has been successfully used to study constriction of the airways in rats (Dandurand *et al.*, 1993). Like the airways, the pulmonary arteries and veins in this preparation have a nearly circular cross-section within the framework of an intact and supporting parenchyma as they do *in vivo*. The reasons that the pulmonary vessels remain open and nearly circular despite the absence of intraluminal pressure are most likely their low baseline tone and the preload provided by the stretch from the surrounding parenchyma filled through the airways with agarose. In preliminary experiments in rats, we also perfused the vessels with agarose to increase their preload and, although we found an increased contractile response to 5-HT, the quantitative differences between arteries and veins were unchanged. Thus, in the present study, we opted not to inflate the vessels with agarose due to the possibility that we might reduce drug access to the endothelium or that we might damage the latter.

In the pulmonary vessels of adult guinea pigs, we found that neither LNNA nor indomethacin had much effect on baseline vascular areas. This indicates that neither NO nor prostacyclin modulate their baseline vascular tone significantly. In rats and dogs, NO synthase inhibitors have also been found to be without significant effects on pulmonary vascular tone under baseline conditions (Ferrario *et al.*, 1996; Leeman *et al.*,

1994). In mammalian systemic resistance vessels and in adult ovine pulmonary veins, however, inhibition of basal NO production does induce contraction (Bansal *et al.*, 1993; Wennmalm, 1994).

Satoh and Inui (1984) first reported that histamine induced endothelium-dependent relaxation in guinea pig pulmonary arteries; Abacioglu *et al.* (1987) found that H_2 receptors on smooth muscle contributed to histamine-induced relaxation even though their effect was much weaker than that produced by H_1 receptors on the endothelium. A subsequent study indicated that NO was the mediator responsible for histamine-induced endothelium-dependent relaxation, and that it was unaffected by indomethacin (Sakuma *et al.*, 1988). Our results extend these studies by showing that histamine also relaxed pulmonary veins, although significantly less than arteries. In a separate study in lung explants, we found that LNNA but not indomethacin potentiated the contractile responses of pulmonary arteries and veins to histamine (Shi and Michel, 1997). Thus, the histamine-induced relaxation in guinea pig pulmonary veins was also primarily mediated by NO but not by PGI_2. Moreover, in the present study, we found that at higher concentrations (above 10^{-7} M), histamine contracted pulmonary arteries and veins (Fig. 2). This observation is in accordance with the findings of Abacioglu *et al.* (1987) in main pulmonary artery strips of guinea pigs.

It has been suggested that ACh produces endothelium-dependent relaxation of pulmonary arteries in newborn and adult guinea pigs (Davidson and Eldemerdash, 1990; Sakuma *et al.*, 1988, Sata *et al.*, 1990). The mediators involved in this relaxation, however, have not been completely elucidated. Sakuma *et al.* (1988) reported that the NO synthase inhibitor N^{ω}-monomethylarginine antagonized only 64% of ACh-induced

relaxation. In the present study, however, we found that it was completely abolished by the NO inhibitor LNNA. One explanation for the discrepancy between the data of Sakuma *et al.* (1988) and ours is that the different analogues of L-arginine could affect endothelium-dependent relaxation differentially (Cheng *et al.*, 1994); the other may be that we used a higher concentration of this inhibitor and/or that responses of smaller intrapulmonary arteries differ from those of larger ones. Our findings that indomethacin potentiated the relaxation of arteries and caused relaxation of the veins with ACh was unexpected and suggests that the relative contribution of vasoconstrictor cyclooxygenase products was greater than that of vasodilator cyclooxygenase products during the response. This contrasts with the results in dogs reported by Miller and Vanhoutte (1985) who found that arachidonic acid relaxed pulmonary arteries but contracted veins, and that these effects were abolished by inhibitors of the cyclooxygenase pathway and by denudation of the endothelium. Although ACh is a classical agonist of endothelium-dependent relaxation in most blood vessels, it fails to produce relaxation in some, for example in bovine pulmonary veins (Ignarro *et al.*, 1988) and in newborn ovine pulmonary arteries (Gao *et al.*, 1995), even causing endothelium-dependent constriction. Contraction has also been reported in the pulmonary vessels of rabbits (Altiere *et al.*, 1986) and in coronary arteries of most species (Kalsner, 1989), with thromboxane A_2 being the putative mediator since constriction could be prevented by cyclooxygenase inhibitors, thromboxane A_2 synthase inhibitors and thromboxane A_2 antagonists (Altiere *et al.*, 1986). In addition, our findings seem to exclude an important role for prostacyclin, another endothelium-derived relaxing factor, in the ACh-induced relaxation of guinea pig pulmonary arteries.

Compared with the arteries in the present study, the pulmonary veins of guinea pigs showed a weaker relaxation to ACh, and the relaxation occurred only after inhibition of the cyclooxygenase pathway with indomethacin. This relaxation was probably also mediated by NO, since prostacyclin had been inhibited with indomethacin during the response and the NO synthase inhibitor LNNA enhanced ACh-induced contraction in these veins. The weaker relaxant response of the veins could also be explained by the lower reactivity of their smooth muscle to NO or to a reduced ability of the endothelium to produce NO. We investigated this using SNP, that acts like exogenous NO (Rao and Cederbaum, 1995), and indeed found that the veins responded less to SNP than the arteries. This finding is in agreement with previous findings in isolated perfused lungs of rats and pigs (Cigarini *et al.*, 1989, Roos *et al.*, 1994), as well as in systemic vessels (Ignarro *et al.*, 1991). Although the smaller relaxant response of the veins could be due in part to the greater precontraction to U46619 (Stork and Cocks, 1994), this is unlikely because papaverine, that increases cytosolic cGMP content by inhibiting the activity of phosphodiesterase, independent of the endothelium and the NO pathway (Aoki *et al.*, 1994; Kramer and Wells, 1979), relaxed arteries and veins equally. Thus after inhibition of the cyclooxygenase passway, the differences between arteries and veins in response to ACh lie in the reduced responsiveness of the venous smooth muscle to NO, with a component of the reduced ability of the venous endothelium to release NO.

In conclusion, our data demonstrate that in guinea pigs, endothelial NO-mediated relaxation is greater in pulmonary arteries than in veins and that ACh-induced relaxation was reduced in the arteries and masked in the veins by constricting factors from the cyclooxygenase pathway. Differences in NO-mediated relaxation of pulmonary arteries

and veins may also contribute to their differential contractile responses. Indeed, the data of Bradley *et al.* (1993) in isolated perfused lungs, and our own findings in lung explants (Shi and Michel, 1997), reveal that pulmonary veins of guinea pigs constrict more to histamine and serotonin than arteries. Since histamine and 5-HT stimulate the release of endothelial NO, in addition to contracting vascular smooth muscle (Furchgott and Vanhoutte, 1989), the reduced release of NO by the venous endothelium and the diminished responsiveness of the venous smooth muscle to NO may produce a smaller relaxant effect to antagonize the vasoconstriction. Furthermore, the present study, together with others (Miller and Vanhoutte, 1985; Yang *et al.*, 1991), has indicated that endothelium-derived contracting factors contribute to the differential relaxant responses of arteries and veins. Since pulmonary veins are the major site of action of several vasoconstrictors (Bradley *et al.*, 1993, Gao *et al.*, 1995; Zhao *et al.*, 1993), if NO-mediated relaxation in them is smaller and/or if they produce more contracting substances, an exaggerated increase in microvascular pressure could result, potentially contributing, for example, to the formation of pulmonary edema under pathological conditions.

Acknowledgements.

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Tables and Figures

Table 1. Maximal relaxation responses (Rmax) and pD_2 values to ACh, SNP and papaverine of pulmonary arteries and veins in guinea pigs.

	Rmax (%)		pD_2	
	Arteries	Veins	Arteries	Veins
Histamine	12.3 ± 1.4*	5.7 ± 1.6	8.9 ± 0.3	8.6 ± 0.2
ACh	19.2 ± 5.1	contraction	8.1 ± 0.7	contraction
ACh + indomethacin	48.5 ± 4.7*+	17.0 ± 4.1	7.3 ± 0.5	8.3 ± 0.4
ACh + LNNA	contraction	contraction	contraction	contraction
SNP	69.7 ± 3.1*	40.2 ± 5.2	7.2 ± 0.3*	6.6 ± 0.2
Papaverine	46.9 ± 5.3	33.2 ± 7.7	5.9 ± 0.3	5.7 ± 0.2

Values are means ± SE of four to six animals. * $P < 0.05$ arteries vs. veins; + $P < 0.05$ compared with ACh alone.

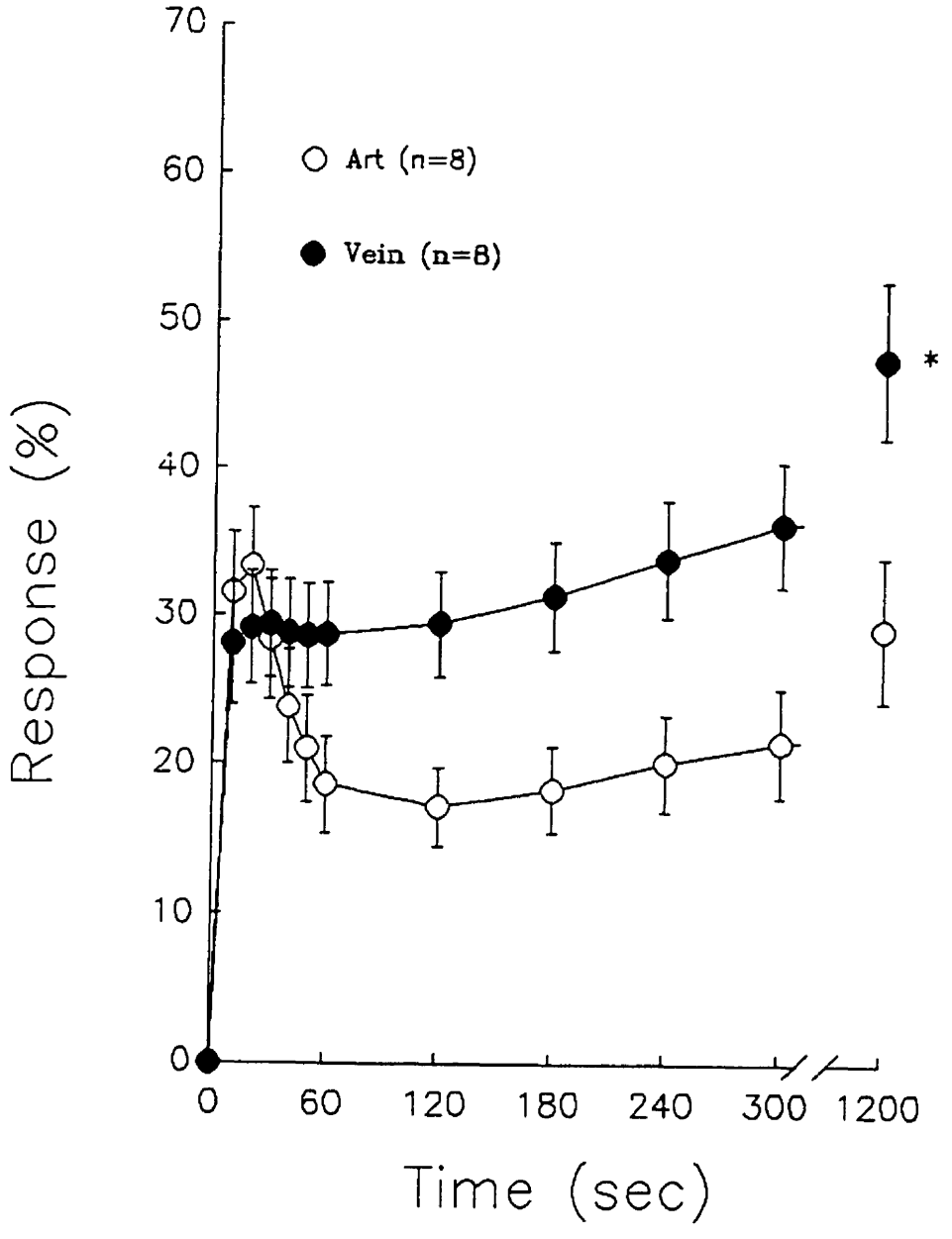

Fig. 1. Responses with time to 10^{-6} M U46619 of pulmonary arteries and veins in lung explants. U46619 constricted arteries and veins, with the peak at 20 sec, after which only the arteries relaxed. After 120 sec, both arteries and veins constricted gradually to reach steady levels at 20 min. n, number of animals. $P < 0.05$ vs. arteries.

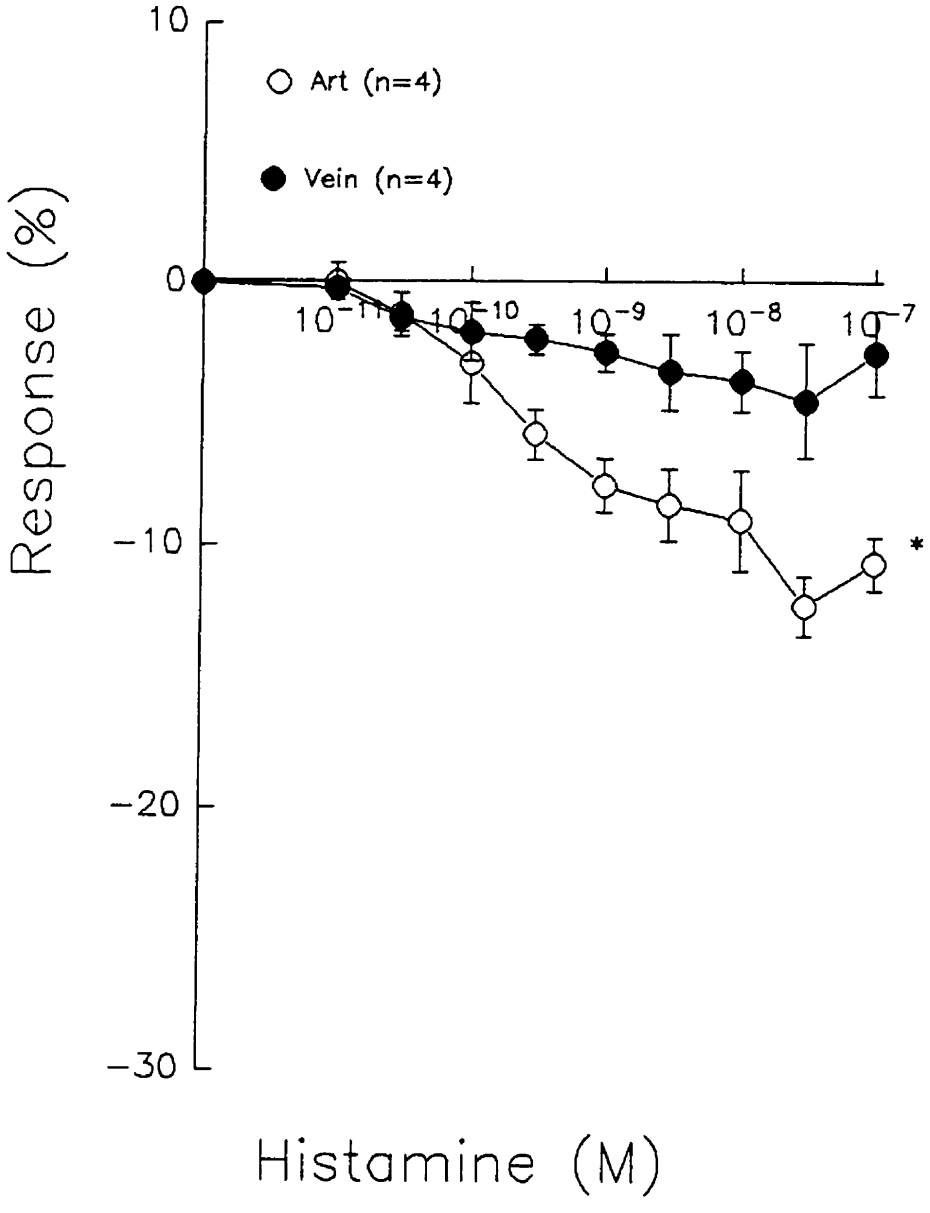

Fig. 2. Responses of pulmonary arteries and veins to histamine following precontraction with U46619. Histamine relaxed arteries more than veins. * $P < 0.05$ vs. veins. n, number of animals.

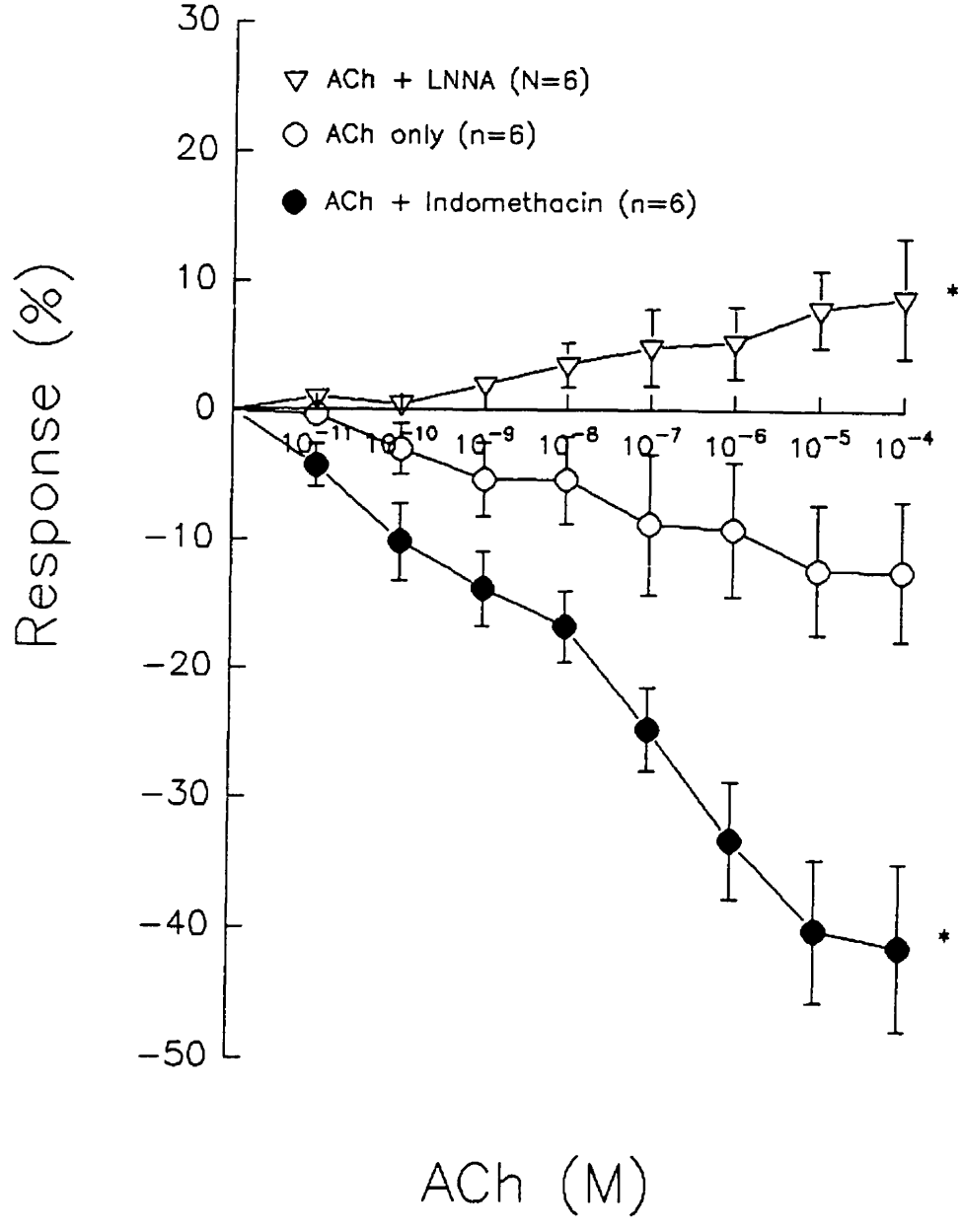

Fig. 3. Cumulative dose-responses of pulmonary arteries to ACh following precontraction with U46619. ACh induced a dose-dependent relaxation. The cyclooxygenase inhibitor indomethacin significantly potentiated ACh-induced relaxation, whereas the nitric oxide synthase inhibitor LNNA resulted in further contraction. * $P < 0.05$ versus ACh only. n, number of animals.

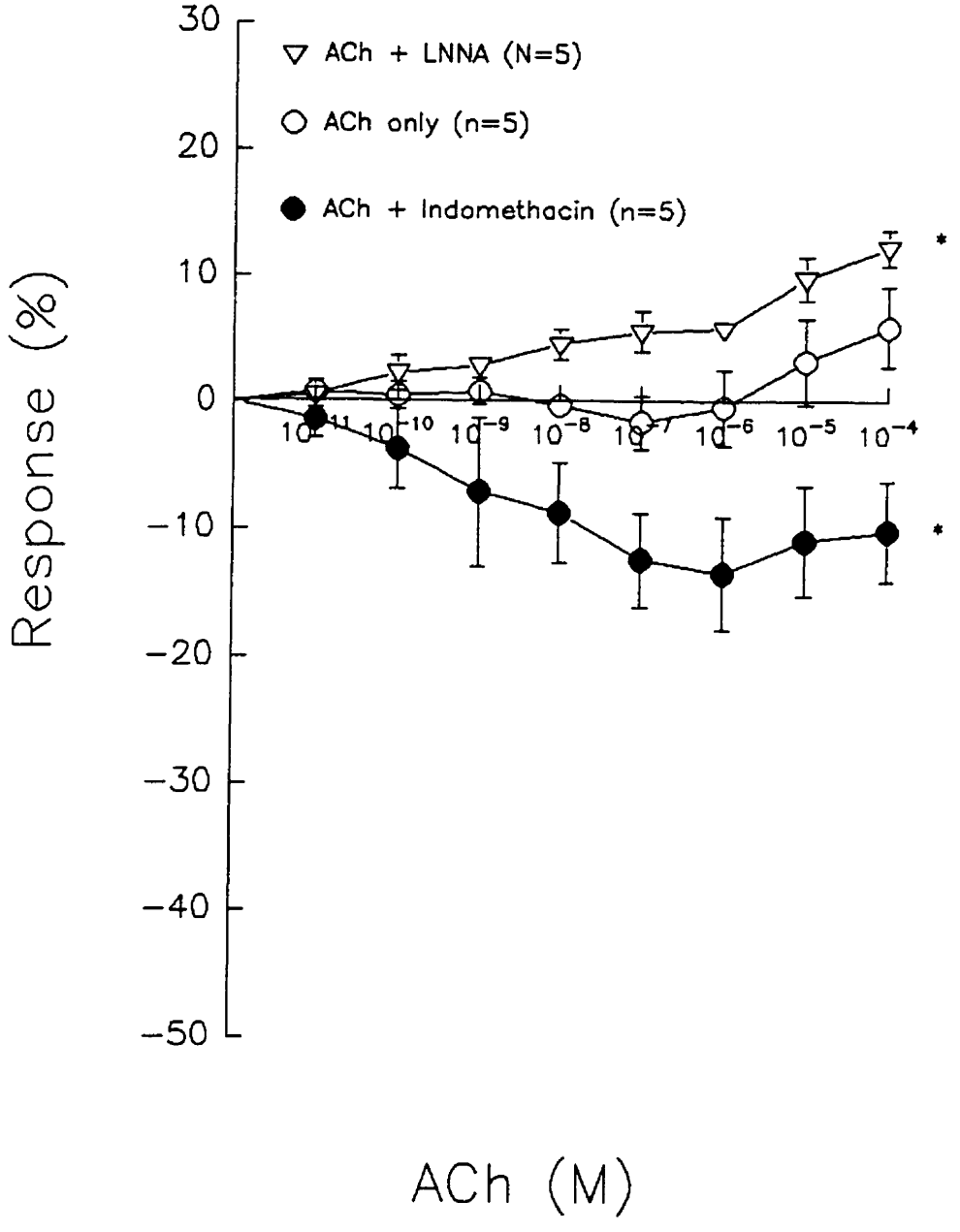

Fig. 4. Cumulative dose-responses of pulmonary veins to ACh following precontraction with U46619. ACh failed to induce relaxation. In veins pretreated with indomethacin, ACh induced a dose-dependent relaxation, whereas in those pretreated with LNNA, ACh induced a significant contraction. * $P < 0.05$ vs. ACh only. n, number of animals.

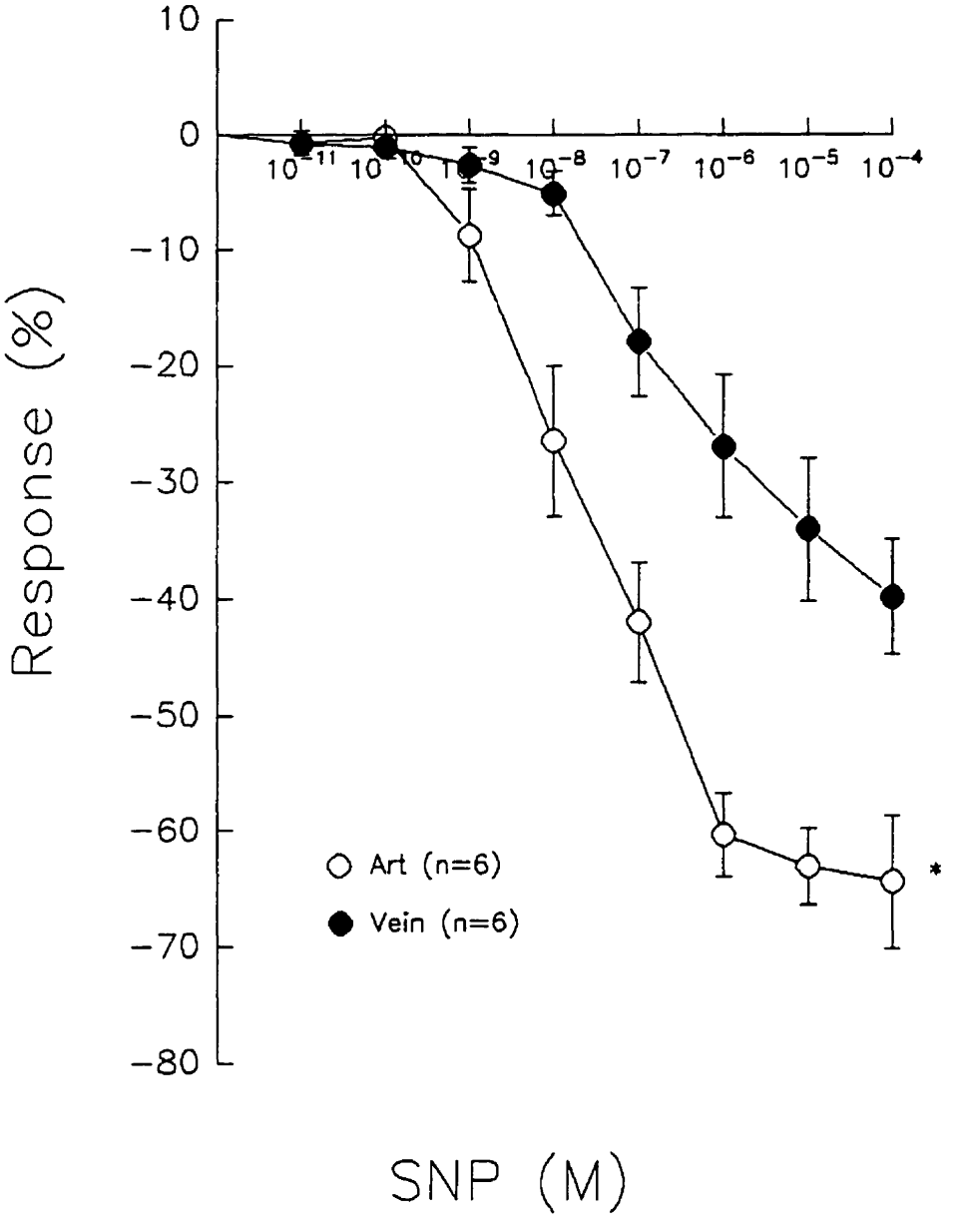

Fig. 5. Responses of pulmonary arteries and veins to nitroprusside (SNP) following precontraction with U46619. SNP relaxed arteries more than veins from 10^{-8} to 10^{-4} M. * $P < 0.05$ vs. veins. n, number of animals.

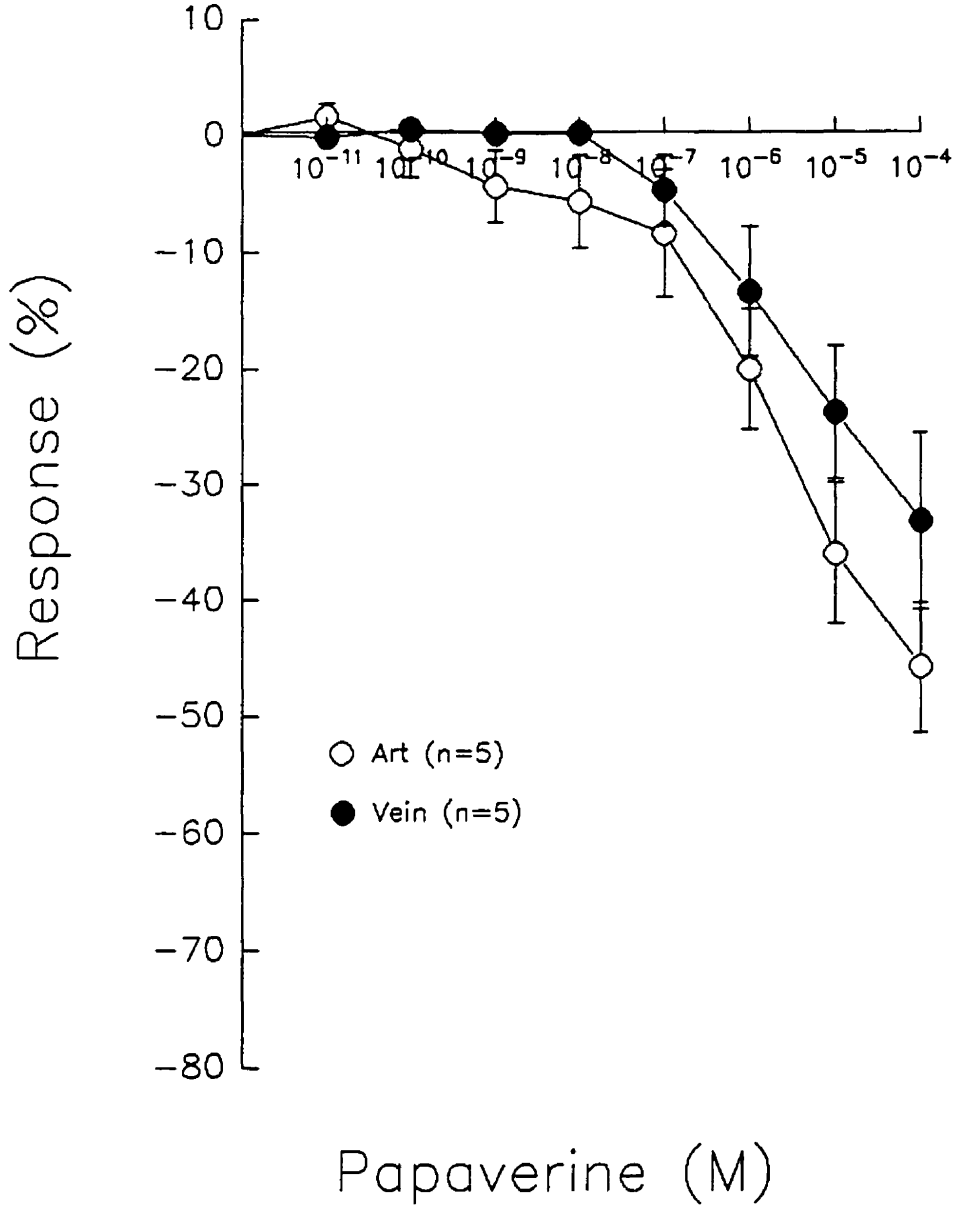

Fig. 6. Responses of pulmonary arteries and veins to papaverine following precontraction with U46619. Papaverine relaxed arteries and veins equally. n, number of animals.

After using the lung explant technique to study pulmonary vasoconstriction and vasodilation in normal guinea pigs, both as a baseline for the studies planned for POPV, and to delineate mechanisms of differential arterial and venous responses in normal animals, in chapter 4, we investigated some of the potential mechanisms responsible for the previously observed increased vasoreactivity in POPV (see Introduction, Chapter 1). Specifically, we examined the contractile responses of pulmonary arteries and veins to histamine and to 5-HT, and the relaxant responses to ACh and SNP in lungs with POPV, comparing them to the contralateral control lungs.

Chapter 4

Altered reactivity of pulmonary vessels in postobstructive pulmonary vasculopathy

Weibin Shi, Fu Hu, Wassim Kassouf, and René P. Michel

Abstract

Chronic ligation of one pulmonary artery results in pulmonary vascular remodeling and bronchial angiogenesis, collectively known as postobstructive pulmonary vasculopathy (POPV). We previously reported that the responses of pulmonary arteries to 5-HT and of veins to histamine were markedly augmented in POPV in dogs. To further investigate this phenomenon in individual vessels, POPV was produced in guinea pigs by ligating the left main pulmonary artery. Using a lung explant technique, we compared contractile responses of pulmonary arteries and veins to histamine and 5-HT, and dilator responses to acetylcholine (ACh) and sodium nitroprusside (SNP) with those of contralateral control lungs. We found that the maximal contractions of arteries to 5-HT (24.4 $\pm$ 2.6 %) and of veins to histamine (53.9 $\pm$ 4.7 %) were significantly increased in POPV of 3 months duration compared with controls (16.8 $\pm$ 1.5 %, 40.8 $\pm$ 5.0 % respectively). Relaxation of U46619-precontracted arteries with ACh was enhanced at 10 months but not at 1 month after ligation. The relaxant effect of SNP on veins was increased at 1 month after ligation but was not altered in arteries. Morphometry revealed reduced diameters of arteries and veins without significant increase in vascular medial muscle thickness. Our data suggest that the enhanced contractile responses of pulmonary vessels to histamine and 5-HT in POPV were not due to endothelial dysfunction nor to structural alterations, but were probably due foremost to changes in the smooth muscle *per se*.

Introduction

Chronic ligation of one pulmonary artery causes structural remodeling of the pulmonary vascular bed and angiogenesis of the bronchial vessels (Michel *et al.*, 1990; Vidone and Liebow, 1957; Weibel, 1960). These characteristic alterations are termed postobstructive pulmonary vasculopathy (POPV). Clinically, POPV resembles large pulmonary arterial embolism, some forms of pulmonary hypertension, and conditions with elevated bronchial blood flow (Michel *et al.*, 1990). The morphological changes of the pulmonary vascular bed in POPV consist of medial thickening, a reduction in the diameter of pulmonary arteries, muscularization of nonmuscular arteries, patchy intimal thickening, and an increased density of myoendothelial junctions (Michel and Hakim, 1991; Michel *et al.*, 1995). These alterations could profoundly influence the reactivity of pulmonary vessels to vasomotor stimuli. Indeed, in the canine model of POPV, we had previously found a markedly augmented responsiveness of pulmonary arteries to 5-HT and of veins to histamine (Michel *et al.*, 1990).

The mechanisms for the exaggerated responsiveness of pulmonary vessels in POPV to substances such as histamine and 5-HT are unclear. Putative explanations include structural alterations such as a decreased baseline diameter and increased medial thickness (Mulvany *et al.*, 1978), endothelial dysfunction (Hoshino *et al.*, 1994; Ito *et al.*, 1995), and alterations in the smooth muscle proper (Stepp and Tulenko, 1994). In the canine model of POPV, the hyperreactivity of the pulmonary vessels, assessed by a disproportionate increase in segmental pulmonary vascular resistance (Michel *et al.*, 1990), could be caused by their narrower internal diameter and thicker media without necessarily enhanced smooth muscle contractility (Mulvany *et al.*, 1978). Thus an *in*

vitro system, in which we could measure smooth muscle contraction directly rather than indirectly with a parameter such as vascular resistance, is preferable to determine whether smooth muscle constriction is truly enhanced in POPV. Recently, we successfully adapted a lung explant technique, used to study airway constriction in the rat (Dandurand *et al.*, 1993), to pulmonary vessels of normal guinea pigs (Shi *et al.*, 1997). In this preparation, vascular smooth muscle contraction or relaxation can be directly measured; moreover, the explants can be fixed and morphometric measurements made on the same vessels studied with the pharmacological agents.

Endothelial cells modulate the responses of the underlying smooth muscle to vasoactive agonists by releasing endothelium-derived relaxing and contracting factors (Furchgott and Vanhoutte, 1989). Nitric oxide (NO) is a major endothelium-derived relaxing factor (Palmer *et al.*, 1987), and acetylcholine (ACh), histamine, bradykinin and several other agents stimulate the endothelium to release NO (Furchgott and Vanhoutte, 1989). Recent *in vitro* studies on isolated arteries have demonstrated that endothelium-dependent NO mediated relaxation is reduced in several vascular diseases, leading to exaggerated contractile responses to vasoactive substances (Hoshino, *et al.* 1994; Ito *et al.*, 1995). Thus in POPV, a similar mechanism could account for the hyperreactivity to histamine and 5-HT, since in addition to directly constricting vascular smooth muscle, these agents stimulate the endothelium to release NO and thereby reduce the constriction (Furchgott and Vanhoutte, 1989). As mentioned above, the direct responses of pulmonary vessels in POPV have not been studied.

Thus the principal aims of the present study were to 1) directly compare the contractile responses of individual intrapulmonary arteries and veins to histamine and to

5-HT in POPV lungs with those in contralateral control lungs using the lung explant technique; and 2) determine whether structural alterations and/or endothelial dysfunction contributed to alterations in the contractile responses.

Methods

Surgical procedure for pulmonary artery ligation. Animals were used according to a protocol approved by the McGill University Animal Care Committee. The procedure previously described for dogs was adapted to guinea pigs (Michel *et al.*, 1990). Briefly, 24 male Hartley guinea pigs (Charles River, St. Constant, QC, Canada) weighing 556 $\pm$ 30 g were anesthetized with pentobarbital sodium (35 mg/kg, ip), and placed in the prone position for intubation. A 14 gauge polyethylene tubing with a needle stylet angled at about 15° was passed through the mouth into the trachea, and animals were ventilated with 30% O_2 at 60 breaths/min with a tidal volume of 5-7 ml by means of a Harvard Apparatus rodent ventilator model 680 (Millis, MA).

For the ligation, the animals were placed in the supine position and using sterile technique, a left thoracotomy performed via the third or fourth intercostal space. The left pulmonary artery was ligated with a 4 O silk suture approximately 2 mm beyond its bifurcation from the main pulmonary artery. The chest was closed and the lung was reexpanded with negative suction and positive pressure ventilation. Postoperatively and daily for 3 days, 5 mg/kg trimethoprim and 25 mg/kg sodium sulfadiazine were injected subcutaneously.

Preparation of the lung explants. The procedure was slightly modified from that previously described for airways (Dandurand *et al.*, 1993). The animals were anesthetized with pentobarbital (40 mg/kg, ip), heparinized through the dorsal vein of the penis (3000 U/kg) and intubated through a tracheotomy with sterile polyethylene tubing 1.9 mm in diameter. The abdomen was opened and animals were exsanguinated by cutting the abdominal aorta. After removal of the anterior chest wall, the pulmonary

vessels were washed *in situ* with 10 ml Ringer's lactate containing 20 U/ml heparin through a catheter inserted into the main pulmonary artery for the right control lungs, or the left atrium for the left ligated lungs. The heart and lungs were excised *en bloc* and the lungs inflated to near total lung capacity with 1% agarose in bicarbonate-buffered culture medium (BCM) at 37°C. The preparation was left to cool for 20 min at 4°C. Then the lungs were separated from the heart, and in some animals (those used for the study of the dilator responses - see below), the volumes of the left and right lungs were measured by fluid displacement (Ringer's lactate) in a sterile 50 ml syringe, before being embedded in 4% agarose in bicarbonate buffered minimum essential medium. After 30 min at 4°C, the lung-agarose block was sectioned with a hand-held microtome blade into 0.5-1.0 mm-thick transverse slices. These were examined with an inverted microscope (IMT-2, Olympus, Tokyo, Japan) and those that contained at least one cross-section of a vessel were placed in a 30 mm culture well insert within a six-well plate containing 2 ml of BCM and incubated overnight at 37°C in 5% CO_2-95% air.

Image acquisition. The culture dish inserts containing the lung explants were transferred to six-well plates containing 2 ml of HEPES-buffered culture medium (HCM) (Dandurand *et al.*, 1993) and placed on the stage of an inverted microscope (LH50A, Olympus). Arteries and veins were identified and imaged with a video camera (CDS, Sony, Nagano, Japan) and images recorded with a video disk recorder (TQ2026F, Panasonic, Osaka, Japan). To distinguish arteries from veins, we used the following criteria: 1) the arteries usually accompanied airways, whereas veins were at a distance from them, and 2) arterial walls had a thick media and their inner lining was slightly wrinkled, whereas veins were thinner and wrinkles were inconspicuous. The identities

of the vessels were confirmed by histological examination (see below).

Experimental protocol. Vasoconstrictor responses to histamine and 5-HT. In this experiment, we tested vasoconstrictor responses to histamine and 5-HT in 10 guinea pigs, 3 months after ligation. After generating baseline images of the vessels, 10^{-11} M histamine or 5-HT were added to the vessels. Twenty sec later (which corresponded to the time at which the peak contractile response occurred), images of the vessels were taken. This procedure was repeated with increasing concentrations up to 10^{-3} M for histamine and 10^{-4} M for 5-HT.

Vasodilator responses to ACh and SNP. Vasodilator responses to ACh and SNP were performed in two groups of guinea pigs; group 1 consisted of 6 animals ligated for 1 month, and group 2 consisted of 8 animals ligated for 10 months. All vessels were first precontracted with U46619 at 3×10^{-6} M in group 1 and at 10^{-5} M in group 2; preliminary experiments showed that at these concentrations, U46619 produced contraction about 80% of maximum and that the contraction was stable for at least 20 min. Cumulative concentration-responses to ACh or SNP were generated in one log unit intervals from 10^{-11} to 10^{-4} M using the same procedure as for histamine and 5-HT above.

In each animal, we usually used 12 explants from each lung and in each explant observed one artery and/or one vein, and in a few instances two veins; each vessel was only studied once. In the experiments of the constrictor responses to histamine and 5-HT, we studied 62 arteries and 66 veins from 82 explants of the control lungs and 36 arteries and 64 veins from 68 explants of the lungs with POPV; in group 1 of the experiments of the dilator responses, we studied 32 arteries and 41 veins from 43 explants of the control lungs and 34 arteries and 32 veins from 47 explants of the POPV

lungs; and in group 2, 38 arteries and 63 veins from 67 explants of the control lungs and 36 arteries and 64 veins from 68 explants of the POPV lungs.

Image and data analysis. The stored images were digitized using a 80386 Intel-based microcomputer equipped with a frame-grabber board (PIP1024B, Matrox, Montreal, QC, Canada). The digitized images were then transferred to a scientific work station (RS6000, IBM, Armonk, NY), and measurements of luminal area were made with Galileo Image Processing Software (Inspiraplex, Montreal, QC, Canada). The contractile responses of arteries or veins to histamine, 5-HT and U46619 were expressed as percent change in luminal area over baseline and the responses to ACh and SNP were expressed as percentage reversal of vessel precontraction induced by U46619.

From these responses, concentration-response curves of arteries and veins were constructed by plotting the mean values against concentrations. The potency and maximum responses were determined from each individual vessel, and potency was expressed as pD_2, the negative log of the molar concentrations of EC_{50}.

Histology and morphometry. At the end of each experiment, the explants were fixed by immersion in 10% buffered formalin, processed using standard histological technique and embedded in paraffin. Five μm-thick sections were cut and stained with hematoxylin-eosin and, in selected instances, with Van Gieson's elastic stain. The arteries and veins that were used for pharmacological study were identified based on maps drawn at the time of image acquisition. Morphometric measurements were then made on those vessels that had an intact wall, using previously described methods (Michel, 1982; Michel and Hakim, 1991), with an ocular micrometer on a optical microscope (Leitz, Wetzlar, Germany). We measured the internal diameter (ID) at a

magnification of 100 or 250 X and medial smooth muscle thickness (MT) at a magnification of 250 or 400 X at the same position; the sum of ID and 2 x MT was the external diameter (ED). We made measurements on 21 arteries and 23 veins from the control lungs and 19 arteries and 17 veins from the lungs with POPV in lungs ligated for 1 month, 46 arteries and 36 veins from the control lungs and 34 arteries and 46 veins from the POPV lungs ligated for 3 months, and 27 arteries and 36 veins from the control lungs and 20 arteries and 29 veins from the POPV lungs ligated for 10 months.

Drugs. All drugs were purchased from Sigma Chemical, St. Louis, MO. Histamine (dihydrochloride), 5-HT (hydrochloride), ACh (chloride) and SNP were prepared as stock solutions in HCM, from which working dilutions were prepared fresh daily. For U46619 (9,11-dideoxy-11α,9α-epoxymethanoprostaglandin $F_{2\alpha}$), the stock solution was used directly.

Statistical analysis. Data are presented as means $\pm$ S.E.M, with "n" indicating the number of animals from whom the vessels were obtained; this was the same "n" used for all the analyses, and the contralateral right lungs were the controls. Statistical analyses were performed using proprietary software (Systat, Evanston, IL). For the comparisons of concentration-response curves between POPV and control lungs, two-way analysis of variance (ANOVA) was used. When the F value was significant ($P < 0.05$), the Tukey test or Student's paired *t* test was used to examine differences at each concentration. For comparisons of maximal responses, pD_2 values, we used Student's paired or unpaired *t* test. Differences were considered statistically significant at $P < 0.05$.

Results

Effects of pulmonary artery ligation. Between the left pulmonary artery ligation and the final experiment, the guinea pigs gained weight in proportion to the duration of ligation: 156 ± 41 g after 1 month ligation, 380 ± 34 g after 3 months ligation, and 536 ± 21 g after 10 months ligation. The volumes of the left POPV lungs, 10.8 ± 0.3 ml at 1 month and 10.8 ± 0.8 ml at 10 months, were significantly lower ($P < 0.05$) than those of the right control lungs, 21.3 ± 0.7 ml and 28.9 ± 1.8 ml at 1 and 10 months respectively.

Arteries and veins in the explants were readily identifiable by the criteria described above. Representative video and corresponding light microscopic images of arteries from right and left lungs are in Fig. 1 A - F. Light microscopy revealed that the pulmonary arteries were muscular in type and had a thick and complete inner elastic lamina, a media composed of compact smooth muscle cells, and a thin attenuated external elastic lamina often seen only with Van Gieson's elastic stain. The veins were also muscular in type, but their media, relative to arteries, was thinner, and their internal elastic lamina was also much thinner or frequently absent. The architecture and morphology of the control right lungs were normal with few bronchial vessels around the larger pulmonary vessels and airways, and inconspicuous lymphatic vessels. In contrast, the left lungs with the ligated pulmonary artery showed a marked increase in bronchial blood vessels and lymphatics in the adventitia of pulmonary arteries and veins and in the walls of airways. The parenchyma was normal in the right lungs, but focally fibrotic in the left lungs.

The results of the morphometric measurements are in Table 1. The internal

diameters were smaller in both arteries and veins of lungs with POPV compared with controls ($P < 0.05$). The external diameters were also reduced in the POPV lungs compared with the controls ($P < 0.05$). There was no significant effect of either POPV, or of the duration of ligation on medial muscle thickness of arteries and veins ($P > 0.05$).

Vasoconstrictor responses to histamine and 5-HT. At 3 months following ligation of the pulmonary artery, histamine produced concentration-dependent constriction of pulmonary arteries and veins in both control and POPV lungs (Fig. 2). The constriction of the veins to histamine was significantly enhanced in POPV. In addition, the maximal responses of pulmonary veins were significantly greater in POPV than in the controls ($P < 0.05$, Table 2), although the pD_2 values did not differ significantly from the controls. In contrast to the veins, with histamine, the arteries had significantly reduced pD_2 values in POPV (Table 2), and at concentrations of 10^{-8} and 10^{-7} M (Fig. 2), histamine produced significantly less contraction of arteries in POPV than of control arteries ($P < 0.05$); their maximal responses, however, were similar to those of controls.

Like histamine, 5-HT produced a concentration-dependent contraction of arteries and veins (Fig. 3). After reaching a peak, the contraction of the arteries in the control lungs waned in a concentration-dependent fashion; in distinction, the contractile responses remained stable in the lungs with POPV. In contrast to histamine, the maximal responses of the arteries in POPV to 5-HT were significantly greater than of control arteries (Table 2), although the pD_2 values in the control and POPV lungs did not differ. The effect of 5-HT on veins was not modified by POPV.

Vasodilator responses to ACh and SNP. ACh produced concentration-dependent

relaxation of pulmonary arteries precontracted with U46619; the relaxant effect of ACh in POPV lungs did not differ from controls at 1 month (Fig. 4) but was significantly enhanced at 10 months after ligation (Fig. 5). ACh did not relax veins of either control or POPV lungs; indeed in both, it caused contraction. Unlike ACh, SNP induced a concentration-dependent relaxation of both arteries and veins: the relaxant effect on veins in POPV lungs was greater than in controls at 1 month ($P < 0.05$, Fig. 6) but not at 10 months after ligation (Fig. 7). The pD_2 values of arteries and veins for ACh and SNP, however, were not significantly altered by POPV (Table 2).

Discussion

The data of this study reveal three principal effects of POPV. One, there was an increased constriction of pulmonary arteries to 5-HT and of veins to histamine, a reduced sensitivity of arteries to histamine, and a failure of the constriction in the arteries to wane at higher concentrations of 5-HT, as they did in controls. Two, not only was there no evidence of endothelial dysfunction, but the relaxation of the arteries to ACh was enhanced at 10 months after ligation; moreover, relaxation of the veins to SNP was increased at 1 month after ligation. Three, the diameters of the arteries and veins were significantly reduced at all times after ligation, with no significant medial thickening.

Postobstructive pulmonary vasculopathy has been previously produced primarily in dogs (Michel *et al.*, 1990; Michel and Hakim, 1991; Vidone and Liebow, 1957), although Weibel (1960) produced it in the rat. Qualitatively, the morphologic changes in guinea pigs are the same as in canine lungs: the numbers of bronchial vessels and lymphatics around airways and pulmonary vessels are increased in lungs with POPV compared with controls (Michel and Hakim, 1991; Kelly *et al.*, 1994). Morphometric measurements in the guinea pigs, however, differed from those in dogs in several ways. First, in canine lungs with POPV, only arterial internal diameters were reduced (Michel and Hakim, 1991), whereas in the present study, both arterial and venous diameters were reduced. The likely reason for the prominent reduction in vascular diameters in the guinea pigs is the 50-60% reduction in lung volume after ligation. Second, medial thickness was increased in the canine pulmonary arteries in POPV, whereas in the present study, it was not significantly altered. In his study in rats, Weibel (1960) studied the development of the bronchial collateral circulation after pulmonary artery ligation and

found that 2 and 5 days after ligation, the bronchial arteries enlarged, and that between 5 and 40 days, new bronchial vessels proliferated; the structural changes of pulmonary vessels, however, were not presented in detail.

We found previously in *in situ* perfused canine lungs with POPV a markedly increased pressor response of arteries to 5-HT and of veins to histamine, with no significant effect of 5-HT on veins or of histamine on arteries (Michel *et al.*, 1990). In the present *in vitro* study of POPV in guinea pigs, maximal responses of arteries to 5-HT, and of veins to histamine were also increased. In contrast, histamine was less potent in the POPV arteries than in controls, and 5-HT constricted the veins of control and POPV lungs to the same degree. These results point to drug-specific and vessel-specific alterations of the responses to these two pharmacologic agents.

What are the mechanisms of the increased reactivity to vasoactive agents such as histamine and 5-HT in POPV? One obvious potential explanation could be the structural alterations either in the pulmonary vessels themselves or in the surrounding parenchyma. In our previous studies in the *in situ* perfused canine lobes, medial thickening, peripheral muscularization and a reduced luminal diameter could be invoked to explain part of the hyperresponsiveness of the arteries to 5-HT (Michel and Hakim, 1991). Indeed, according to the law of Laplace, the transmural pressure against which vessels contract is proportional to tension and inversely proportional to radius, and thus the pressor response will be greater with a smaller lumen even though the tension generated in the smooth muscle is unchanged. Similarly, for a given degree of contraction, a narrower vessel will increase its resistance more than a wider vessel, since resistance, according to the Poiseuille's equation, is inversely proportional to radius to the fourth power

(Mulvany, 1991). The above reasoning, however, does not apply to lung explants in which we measured smooth muscle contraction directly, not pressure or resistance.

In the present study, although the reduction in vascular luminal diameter was significant in POPV, the pharmacologic specificity of the hyperreactivity that we observed argues against the possibility that it was due to this structural alteration: indeed, despite their reduced diameters, the maximal responses of the arteries were greater only to 5-HT not to histamine, whereas the veins showed an increased maximal response only to histamine not to 5-HT. Furthermore, although medial muscle thickening is certainly capable of enhancing contractility (Mulvany *et al.*, 1978), this was not observed in our study (Table 1), and thus the specifically altered constrictor responses occurred despite an unchanged muscle thickness. With regards to parenchymal alterations, we have described in POPV an elevated lung elastance and resistance (Kelly *et al.*, 1994) that, if anything, should increase the resistance against which the vascular smooth muscle must contract. Thus the fact that we observed an increased contractility to the pharmacologic agents argues against the alterations in the surrounding parenchyma explaining our results. To summarize, we believe that structural alterations play a minor role, if any, in explaining the augmented reactivity of POPV in the present study.

A second potential explanation for the increased vasoconstriction could be defective endothelium-derived NO-mediated relaxation, since it is known that denudation of the endothelium or inhibition of NO production increases constrictive responses to histamine and 5-HT (Hofman *et al.*, 1991; Ortiz *et al.*, 1992). In the present study, we used ACh, that acts through release of NO in guinea pig pulmonary vessels (Sakuma *et al.*, 1988; Shi *et al.*, 1997), to test endothelium-derived NO production, and SNP, that directly

releases NO (Rao and Cederbaum, 1995), to test the responsiveness of vascular smooth muscle to NO, and found that ACh dilated pulmonary arteries but not veins and that SNP dilated both. The finding that the responses of pulmonary arteries to ACh and SNP were not altered one month after pulmonary artery ligation suggests that endothelium-derived NO-mediated relaxation was intact, and thus that endothelial dysfunction was not present. Ten months after ligation, however, dilator responses to ACh were augmented, due either to increased endothelial NO production or to increased responsiveness of vascular smooth muscle to NO. The fact that the responses to SNP were not altered in POPV suggests that an increased release of NO rather than an increased response of the smooth muscle to it explains the enhanced response to ACh. In the pulmonary veins, ACh produced contraction but not relaxation. This further contraction in pulmonary veins of guinea pigs was probably the result of the release of vasoconstrictor prostanoids because, in a separate study, we found that it could be abolished by indomethacin (Shi *et al.*, 1997).

SNP caused greater relaxation of the veins after one month of POPV compared with controls. Other investigators have also found increased responsiveness to vasodilators of small but not large pulmonary arteries in hypertensive animals. For example, Orton *et al.* (1988) in neonatal calves with severe pulmonary hypertension, found that maximal responses to ACh were increased in structurally altered resistance vessels *in vivo*, but reduced in isolated lobar pulmonary arteries. Wanstall *et al.* (1993) reported that in rats with monocrotaline-induced pulmonary hypertension, maximal relaxation to SNP was increased in arteries with an internal diameter of 200 - 500 μm, but reduced in main pulmonary arteries; the existence of an inherent tone in the small arteries was proposed to explain the hyperresponsiveness. The arteries in POPV share some structural

alterations with those in pulmonary hypertension, including a reduced internal diameter and an increased wall/radius ratio (Michel and Hakim, 1991). The internal diameters of the arteries that we studied were under 400 μm diameter, within the range of small vessels in the monocrotaline-treated pulmonary hypertensive rats (Wanstall *et al.*, 1993).

The increment in endothelial NO-mediated relaxation of the pulmonary arteries in POPV of 10 months duration was unexpected, and obviously cannot explain the hyperreactivity of the pulmonary vessels in POPV. This increased relaxation, however, is conducive to the perfusion of pulmonary vessels, and thus could perhaps represent an adaptive response to the reduced blood flow, as observed in other conditions (Isaacson *et al.*, 1994). Moreover, it complements and provides an explanation for the results, in the arteries of lungs with POPV, of the reduced sensitivity to the contractile effects of histamine (Table 2) and of the reduced responses at the lower concentrations of this agent (10^{-8} and 10^{-7} M, Fig. 2). Indeed, it is known that histamine simultaneously stimulates the endothelium to release NO while contracting the pulmonary vessels of guinea pigs (Abacioglu *et al.*, 1987; Sakuma *et al.*, 1988); thus, if endothelial NO-mediated relaxation is increased, sensitivity to histamine would be expected to decrease. This histamine-induced endothelium-dependent relaxation is most prominent at lower concentrations (as observed in the present study), since at higher concentrations, it is masked by its predominant contractile effect (Abacioglu *et al.*, 1987; Shi *et al.*, 1997).

Although there was variation in the duration of ligation between the constrictor and the dilator experiments, we believe that the results from the effects of the latter can be used to interpret the findings in the experiments with the former. Indeed, in our previous experiments in dogs, the physiological results were consistent over a wide range of

duration of ligation. Thus, in the present study, we opted for a ligation period of 3 months to investigate constriction, as this was similar to the canine lungs. To examine potential mechanisms, however, we decided on two time periods, 1 and 10 months, similar to the extremes of the experiments in canine lungs, to ascertain if there was a time-dependent effect. The postligation duration of 3 months falls between the time points of 1 and 10 months, at which time there were increases in the endothelial NO-mediated relaxation in pulmonary arteries.

With 5-HT, we found that in POPV, there were both an increased maximal contraction and a failure of the arteries to relax at high concentrations (Fig. 3). The increased contraction of the arteries to 5-HT in POPV may be partly explained by their failure to relax at the high concentrations, because the control arteries showed pronounced relaxation at higher concentrations of 5-HT (Fig. 3). Cushing and Cohen (1992) showed that high concentrations of 5-HT (above 10^{-7} M) produced concentration-dependent relaxation of canine coronary arteries devoid of endothelium, although the receptor responsible for this effect had not been characterized. Similar findings were reported in guinea pig airways *in vitro* by Baumgartner *et al.* (1990) who found that 5-HT contracted smooth muscle at low concentrations and relaxed it at higher concentrations directly via activation of 5-HT_{2A} receptors, independent of the epithelium. Thus in POPV, receptors on smooth muscle mediating relaxation at high concentrations of 5-HT or signal transduction pathways may be impaired, leading to failure of relaxation and persistence of contraction.

In summary, we found in the present study that chronic ligation of one pulmonary artery results in alterations in pulmonary vascular structure and in vasoreactivity.

Although alterations in vasoreactivity are a common phenomenon of vascular diseases, the mechanisms and pathological significance for these remain elusive. Structural alterations, including decreased vascular diameter and increased medial thickness, are frequently invoked to explain an increased vasoreactivity (Mulvany *et al.*, 1991). The present study suggests that the exaggerated vessel-specific vasoconstriction to 5-HT and to histamine in POPV, however, are not attributable to the structural alterations. This study also suggests that endothelial NO-mediated relaxation of pulmonary vessels in POPV is intact, and even augmented. Since neither altered structure nor endothelial dysfunction appear to explain the increased vasoreactivity in POPV, we speculate that it could be caused by altered receptors on the smooth muscle or to abnormal signal transduction pathways, fruitful avenues for further exploration.

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Footnotes:

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Tables and Figures

Table 1. Effects of postobstructive pulmonary vasculopathy (POPV) on morphometric measurements of pulmonary arteries and veins in guinea pigs.

	Arteries				Veins			
	VID	ID	MT	ED	VID	ID	MT	ED
1 month								
Control	500 ± 54	419 ± 45	53 ± 3	524 ± 49	353 ± 25	274 ± 9	44 ± 3	361 ± 12
POPV	404 ± 44*	302 ± 19*	56 ± 5	414 ± 19*	311 ± 25*	206 ± 18*	50 ± 5	306 ± 26*
3 months								
Control	585 ± 37	571 ± 40	48 ± 2	666 ± 40	535 ± 31	407 ± 35	41 ± 3	487 ± 35
POPV	366 ± 33*	343 ± 32*	51 ± 3	444 ± 32*	389 ± 44*	308 ± 36*	42 ± 4	391 ± 34
10 months								
Control	581 ± 31	453 ± 38	47 ± 3	546 ± 38	454 ± 21	337 ± 23	39 ± 4	414 ± 24
POPV	375 ± 28*	301 ± 36*	56 ± 5	414 ± 33*	280 ± 28*	217 ± 22*	51 ± 4	319 ± 23*

Data, in μm, are means ± S.E.M. of 6 to 10 animals. 1, 3 and 10 months refer to duration of pulmonary artery ligation; the contralateral lungs were the controls. VID, video image internal diameter; ID, internal diameter in histological sections; MT, medial wall thickness; ED, external diameter (ED = ID + 2 x MT). * $P < 0.05$ versus controls.

Table 2. Effects of postobstructive pulmonary vasculopathy (POPV) on responses of pulmonary arteries and veins of guinea pigs to histamine, 5-HT, ACh and SNP.

	Arteries		Veins	
	Rmax (%)	pD_2	Rmax (%)	pD_2
Histamine				
Control	35.3 ± 4.7	6.71 ± 0.12	40.8 ± 5.0	6.62 ± 0.11
POPV	38.2 ± 5.9	6.16 ± 0.12*	53.9 ± 4.7*	6.39 ± 0.08
5-HT				
Control	16.8 ± 1.5	8.61 ± 0.12	38.5 ± 6.0	8.44 ± 0.13
POPV	24.4 ± 2.6*	8.98 ± 0.31	44.5 ± 5.2	8.54 ± 0.15
1 month				
ACh				
Control	-31.8 ± 8.4	7.88 ± 0.27	23.9 ± 4.3	7.58 ± 0.30
POPV	-29.5 ± 5.1	7.62 ± 0.29	14.1 ± 2.9	7.22 ± 0.33
SNP				
Control	-75.7 ± 3.9	7.48 ± 0.21	-36.3 ± 4.0	6.72 ± 0.17
POPV	-74.3 ± 7.0	7.43 ± 0.18	-56.8 ± 8.3*	6.85 ± 0.13
10 months				
ACh				
Control	-41.8 ± 8.5	7.81 ± 0.15	27.9 ± 4.5	7.71 ± 0.17
POPV	-69.8 ± 9.2*	8.02 ± 0.26	11.3 ± 4.6	7.49 ± 0.17
SNP				
Control	-56.7 ± 8.3	7.91 ± 0.12	-34.9 ± 5.7	6.85 ± 0.16
POPV	-67.1 ± 5.4	7.97 ± 0.36	-39.0 ± 4.0	6.84 ± 0.14

Values are means ± S.E.M. of 6 to 10 animals. 1 and 10 months refer to duration of pulmonary artery ligation; the contralateral lungs were the controls. Negative values denote relaxation; Rmax, maximal responses; * $P < 0.05$ versus controls.

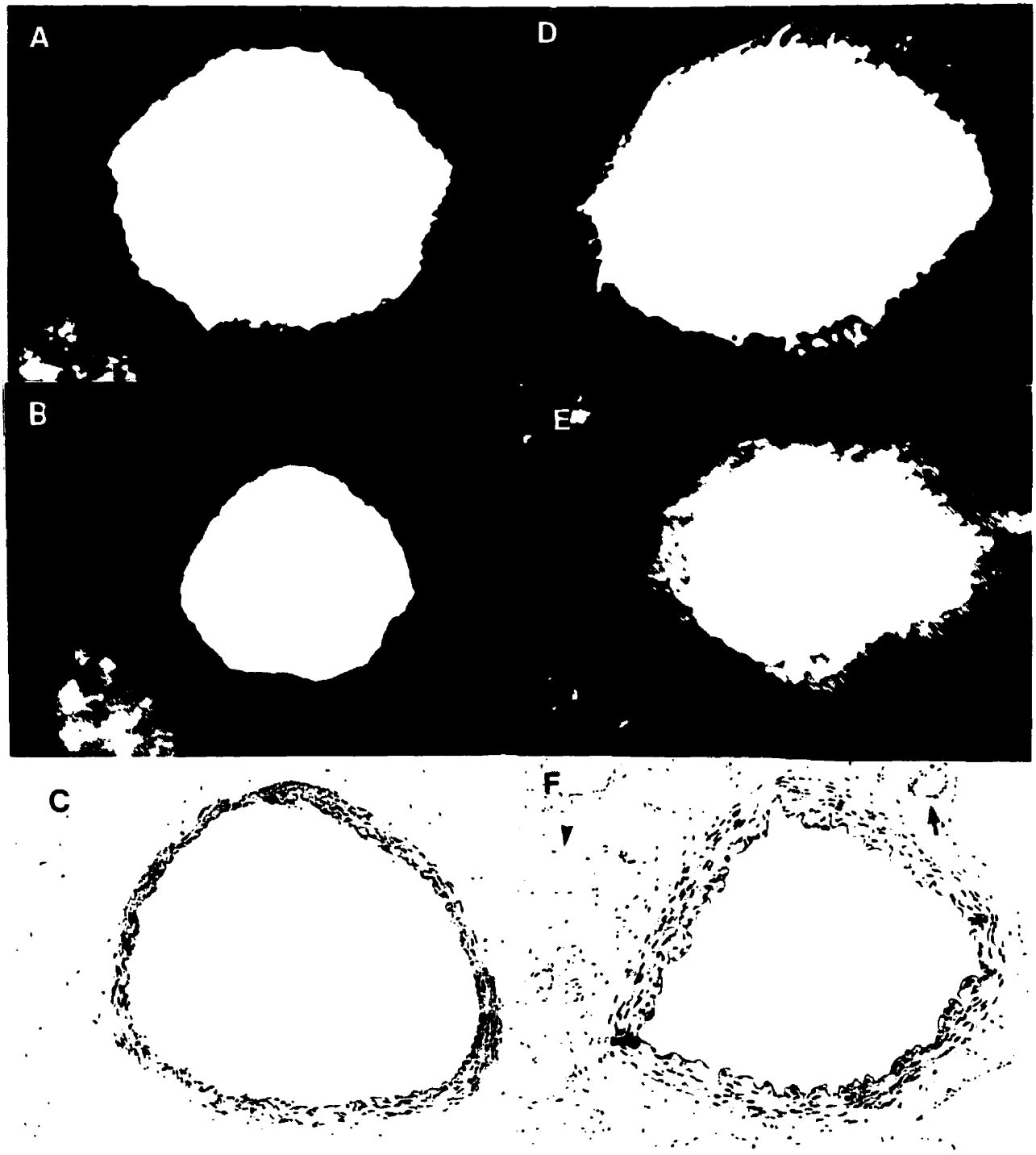
A
D
B
E
C
F

Fig. 1. Representative photomicrographs of video images and light microscopy of arteries from control (A-C) and ligated lungs (D-F). Video images of a control artery at baseline (A) and after addition of 10^{-6} M 5-HT (B). Light photomicrograph of the same artery (C). Video images of a POPV artery at baseline (D) and after addition of 10^{-7} M 5-HT (E). Light photomicrograph of the same artery (F). Note bronchial vessel (arrow) and lymphatic (arrowhead). A, B, X 80; C, X 150; D, E, X 200; F, X 250.

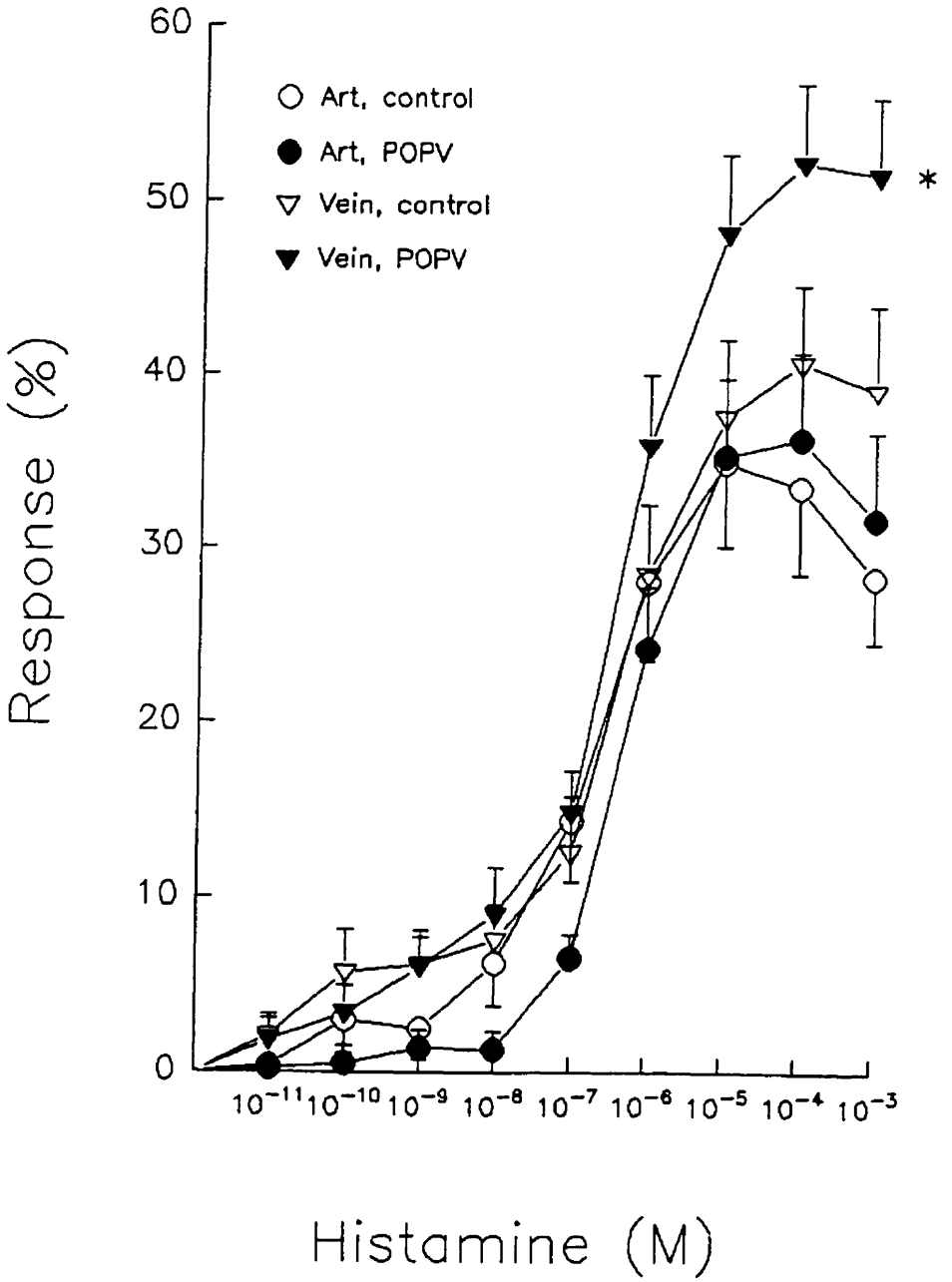

Fig. 2. Constrictive responses of pulmonary arteries (Art) and veins (Vein) to histamine in lung explants from guinea pigs after 3 months pulmonary artery ligation. Responses were calculated as percentage changes in luminal area over baseline and expressed as means ± S.E.M. of 9 or 10 animals. POPV, postobstructive pulmonary vasculopathy; Control, contralateral lungs. * $P < 0.05$ versus control.

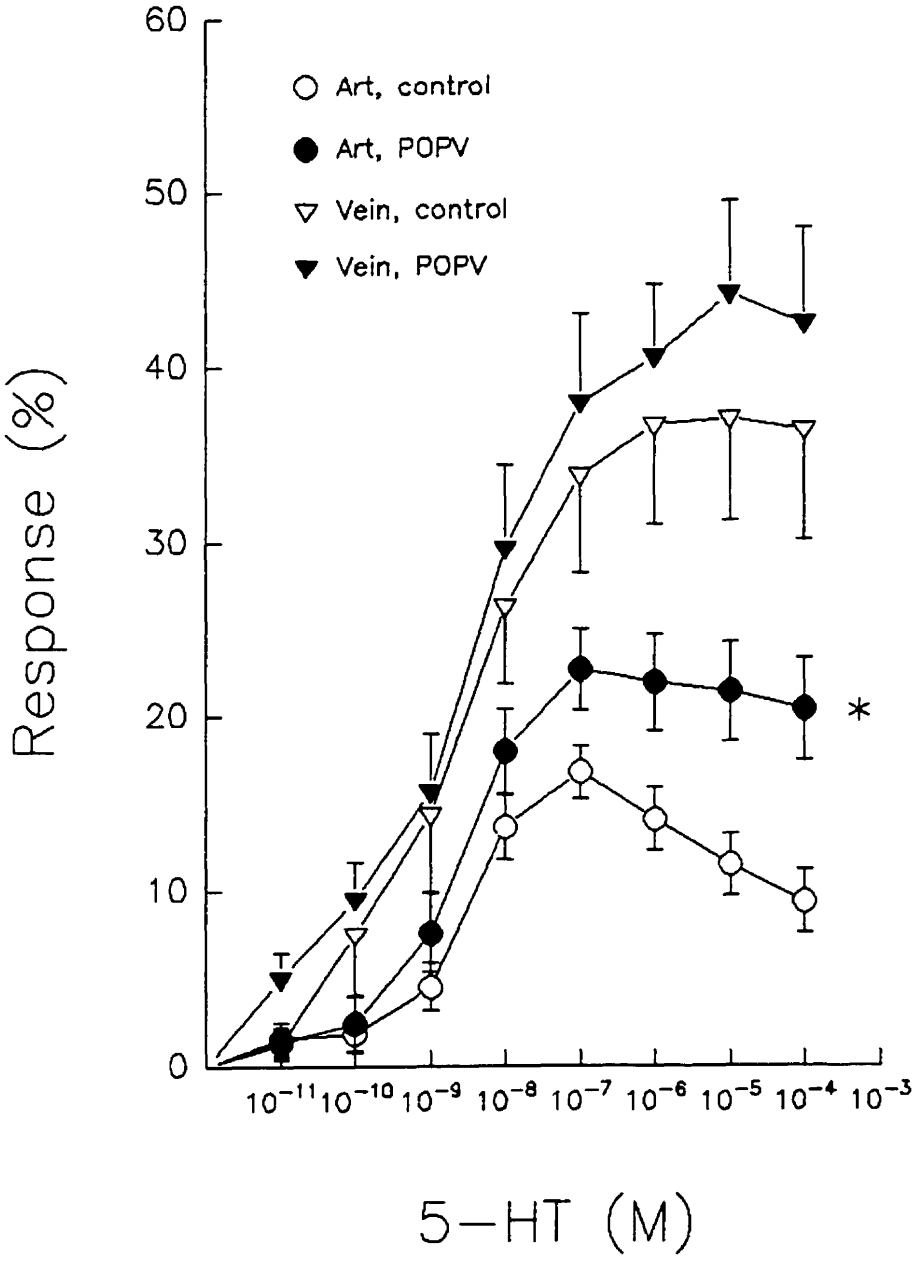

Fig. 3. Constrictive responses of pulmonary arteries and veins to 5-HT in lung explants from 9 guinea pigs after 3 months pulmonary artery ligation. * $P < 0.05$ versus control.

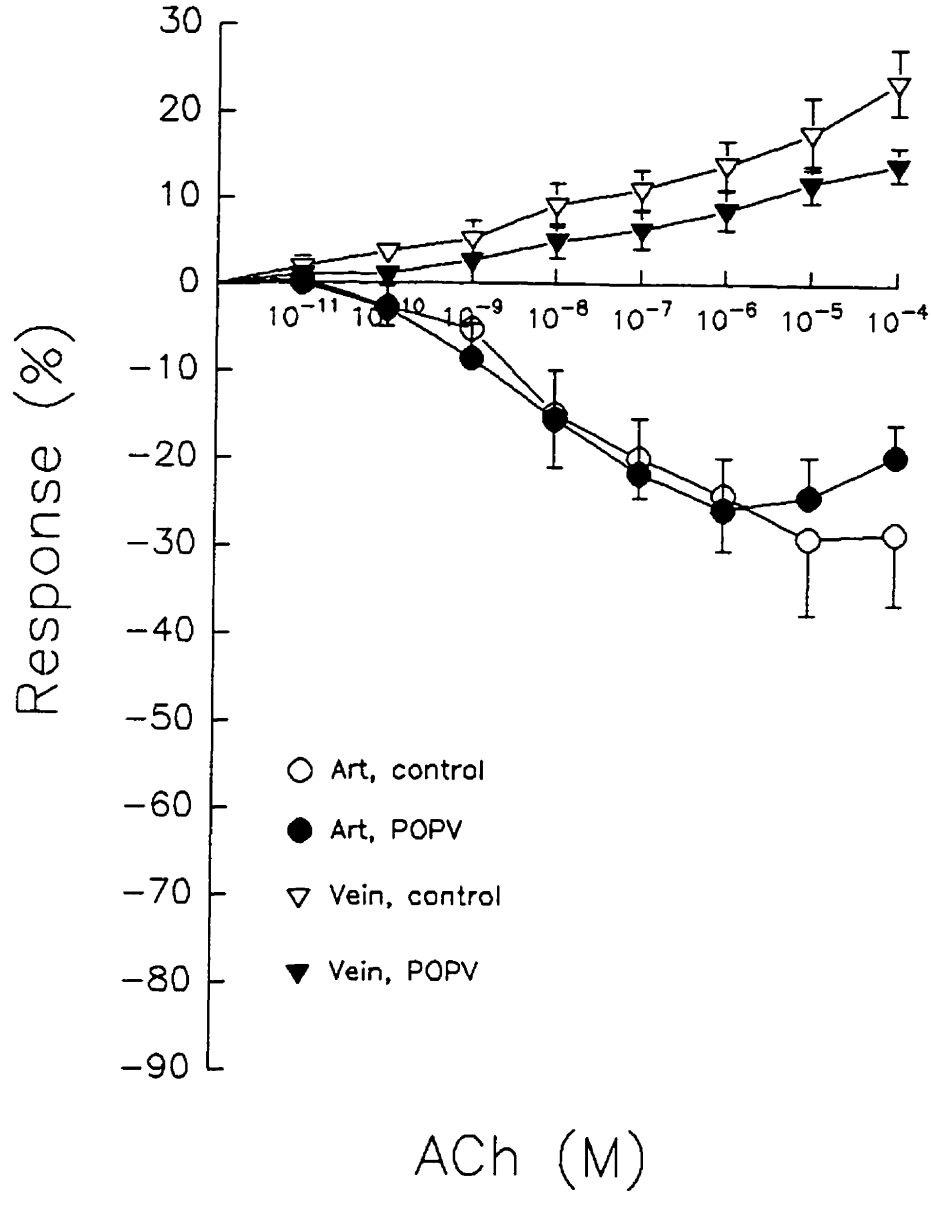

Fig. 4. Responses of pulmonary arteries and veins to acetylcholine (ACh) in lung explants from guinea pigs after 1 month pulmonary artery ligation. Relaxation was expressed as percentage reversal of precontraction to U46619. Data are means ± S.E.M. of 6 animals.

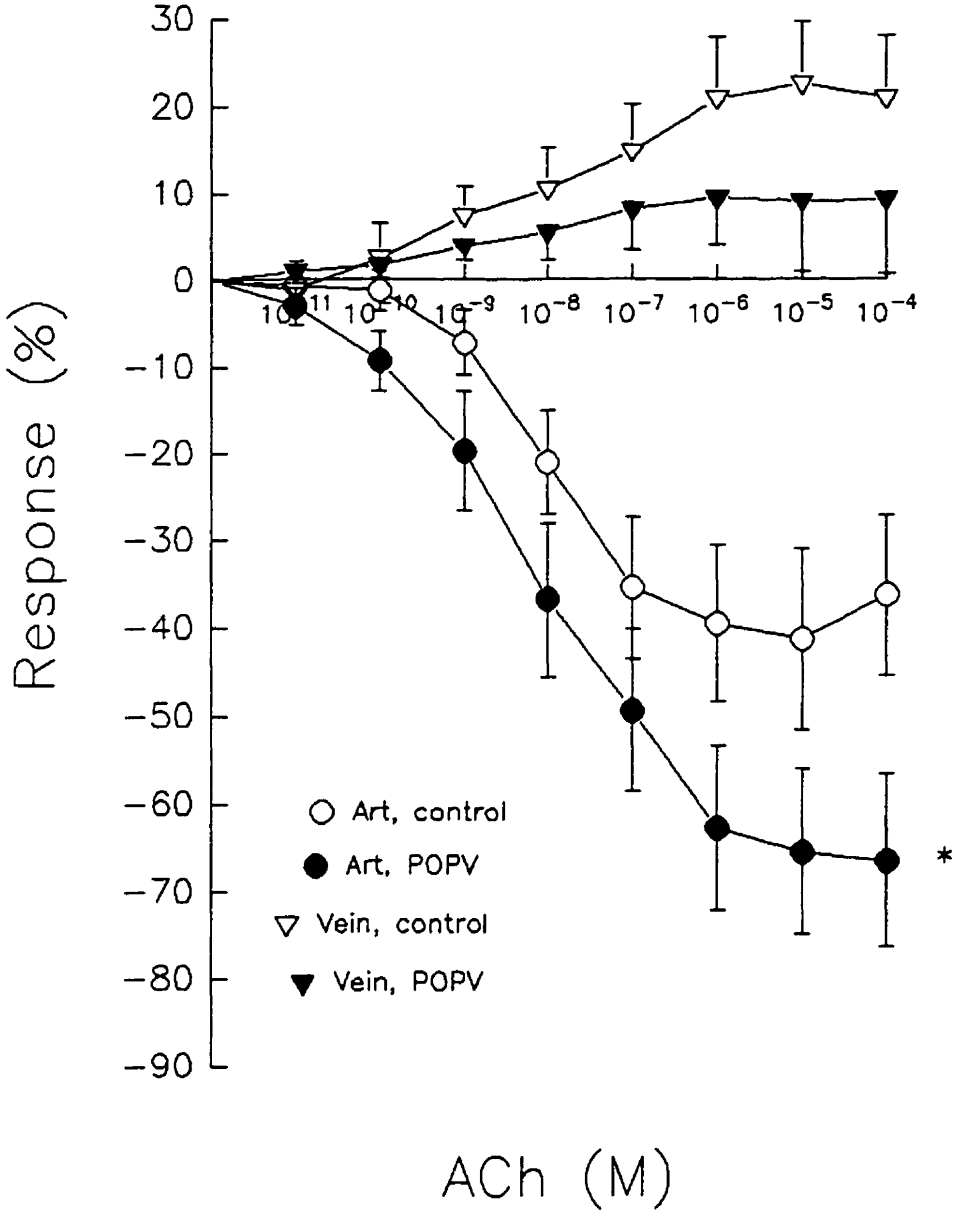

Fig. 5. Responses of pulmonary arteries and veins to ACh after precontraction with U46619 in guinea pigs after 10 months pulmonary artery ligation. Data are means ± S.E.M. of 8 animals. * $P < 0.05$ versus control.

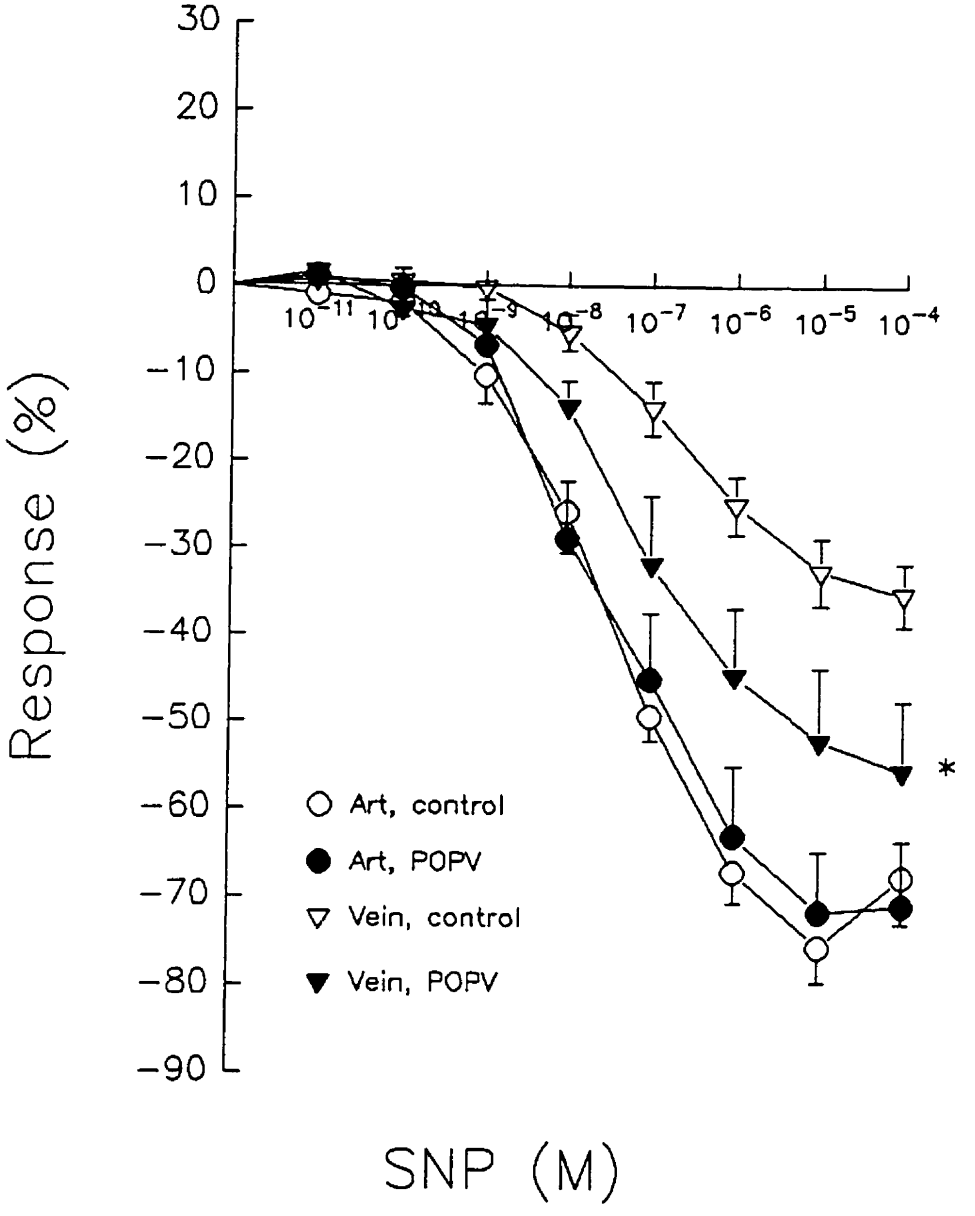

Fig. 6. Responses of pulmonary arteries and veins to sodium nitroprusside (SNP) after precontraction with U46619 in guinea pigs after 1 month pulmonary artery ligation. Relaxation was expressed as percentage reversal of precontraction to U46619. Data are means ± S.E.M. of 6 animals. * $P < 0.05$ versus control.

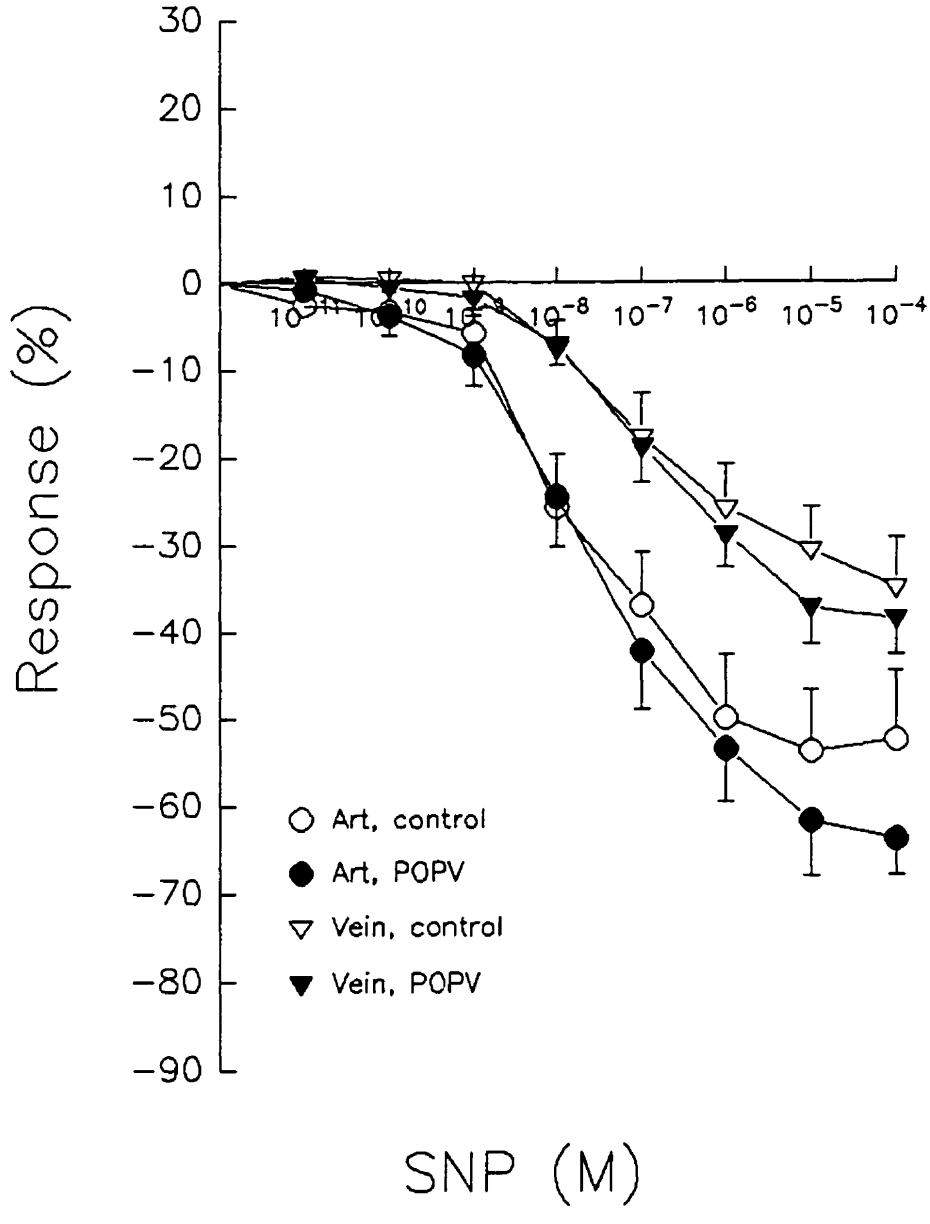

Fig. 7. Responses of pulmonary arteries and veins to SNP after precontraction with U46619 in guinea pigs after 10 months pulmonary artery ligation. Data are means ± S.E.M. of 8 animals.

In Chapter 4, we found that maximal contractions of guinea pig pulmonary arteries to 5-HT and of veins to histamine were significantly increased in POPV, and that these alterations were not due to endothelial dysfunction nor to structural changes. The increased contraction to 5-HT of the arteries in POPV, however, was probably due to alterations of receptors mediating relaxation at high concentrations. Because the receptors for this 5-HT-mediated relaxation have not been yet characterized, and because there are multiple receptor subtypes, with few specific antagonists (Hoyer *et al.*, 1994), we opted to pursue another avenue of investigation in the area of receptors, and decided on endothelins.

Like histamine and 5-HT, endothelins are synthesized and inactivated in the lung, are potent pulmonary vasoconstrictors, and have been implicated as mediators in several pulmonary vascular and allergic diseases (see Introduction, Chapter 1). Thus pulmonary vascular responses to ETs could be altered in POPV. There are two types of receptors that mediate the effects of endothelins: ET_A receptors, which mediate vasoconstriction, and ET_B receptors, which mediate relaxation (Uhlig *et al.*, 1995). Since ET_A and ET_B receptors mediate opposite effects, if the ratio of ET_A/ET_B receptors is altered by POPV, vasoreactivity may change. Therefore in Chapter 5, we tested the hypothesis that POPV increased pulmonary vascular responses to ETs and that alterations in ET receptors mediated the elevated responses.

Chapter 5

Endothelin reactivity and receptor profile of pulmonary vessels in postobstructive pulmonary vasculopathy

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ABSTRACT

Chronic ligation of one pulmonary artery results in pulmonary vascular remodelling and bronchial angiogenesis, collectively known as postobstructive pulmonary vasculopathy (POPV). To determine if the reactivity of pulmonary vessels to endothelins (ETs) was altered in POPV and to explore potential mechanisms, we ligated the left main pulmonary artery of 18 rats. Four weeks later, using a lung explant technique, we compared POPV lungs and control lungs for contractile responses of intrapulmonary vessels to ET-1 and ET-3 and for relaxant responses to ET-1 and sodium nitroprusside (SNP) after precontraction with U46619. Morphometric measurements were made on vessels studied pharmacologically. Competition receptor binding studies with ^{125}I-ET-1 and unlabeled ET-1 and BQ-123 were performed using membrane proteins of pulmonary vessels. We found, in arteries, that contractile responses to ET-1 and ET-3 were significantly increased, and that relaxant responses to ET-1 but not to SNP were reduced; in veins, only relaxation to SNP was increased. Morphometry showed that arteries and veins in POPV had reduced diameters without altered muscle thickness. Receptor binding studies showed that the proportion of ET_A receptors in arteries was significantly increased in POPV (66%) versus controls (54%). We conclude that in POPV, reactivity to ET-1 and ET-3 is increased primarily related to an augmented proportion of ET_A receptors.

Index terms: pulmonary arteries; pulmonary veins; receptor binding; lung explant; vascular remodelling.

INTRODUCTION

Chronic ligation of one pulmonary artery causes structural remodelling of the pulmonary vascular bed and angiogenesis of the bronchial vessels (Liebow *et al.*, 1950; Michel and Hakim, 1991; Michel *et al.*, 1990; Weibel, 1960). These characteristic alterations are termed postobstructive pulmonary vasculopathy (POPV). The pathological changes of the pulmonary vascular bed in POPV consist of medial thickening, a reduction in the diameter of pulmonary arteries, muscularization of nonmuscular arteries, patchy intimal thickening, and an increase in myoendothelial junctions (Michel and Hakim, 1991; Michel *et al.*, 1995). These alterations could profoundly influence the reactivity of pulmonary vessels to vasomotor stimuli. Indeed, in the canine model of POPV, we had previously found that the responsiveness of pulmonary arteries to 5-HT and of veins to histamine was augmented (Michel *et al.*, 1990). Like these two biogenic amines, endothelins (ETs) are synthesized and inactivated in the lung. ETs are potent vasoconstrictors, and are thought to play a role in pulmonary vascular diseases (Dupuis *et al.*, 1996, Michael and Markewitz, 1996, Myauchi *et al.*, 1993). Therefore we postulated that pulmonary vascular responses to ETs could also be altered in POPV.

ET-1 and its isoforms ET-2 and ET-3 are 21-amino acid peptides with a characteristic ring structure formed by two disulfide bridges (Michael and Markewitz, 1996). ET-1 and ET-3 are abundantly expressed in endothelial and alveolar epithelial cells of the lung (Michael and Markewitz, 1996). They constrict pulmonary vessels mainly via ET_A receptors in rats (Uhlig *et al.*, 1995), guinea pigs (Cardell *et al.*, 1993) and humans (Buchan *et al.*, 1994). In contrast, when vascular tone is increased with hypoxia or U46619, ET-1 and ET-3 act as endothelium-dependent dilators, mediated by

ET_B receptors on the endothelium, likely acting through production of nitric oxide or activation of K^+ channels (Crawley *et al.*, 1992; Eddahibi *et al.*, 1993;, Namiki *et al.*, 1992). Since ET_A and ET_B receptors have opposite effects on vascular tone, alterations in the proportion of these two receptors in POPV could lead to abnormal vascular responses to ETs.

Thus the principal aims of the present study were to 1) compare the contractile responses to ET-1 and ET-3 in lungs with POPV with those in the contralateral control lungs, 2) compare in POPV and control lungs dilator responses, after precontraction with U46619, to ET-1 and sodium nitroprusside (SNP), the latter being an endothelium- and receptor-independent vasodilator (Rao and Cederbaum, 1995), and 3) explore the mechanisms underlying the differences in reactivity, specifically vascular structure and receptor density.

MATERIALS AND METHODS

Surgical procedure for pulmonary artery ligation. The procedure previously described for dogs was adapted to rats (Michel *et al.*, 1990). Briefly, 18 male Sprague-Dawley rats (300-400 g) were anesthetized with pentobarbital sodium (35 mg/kg, ip), intubated and ventilated with 30% O_2 at 60 breaths/min with a tidal volume of 5-7 ml. Under sterile conditions, the left pulmonary artery was ligated with a 4 O silk suture approximately 2 mm beyond the bifurcation from the main pulmonary artery through a left thoracotomy performed in the third or fourth intercostal space. The chest was closed in layers with 4 O Dexon sutures. The lung was reexpanded with negative suction and positive pressure ventilation. Postoperative care was provided for the animals for four weeks by the McIntyre Animal Resources Center, McGill University before the final

experiments were performed.

Contractile and relaxant responses of pulmonary vessels in lung explants.

1) Preparation of the lung explants. The responses of pulmonary arteries and veins to ET-1, ET-3 and SNP were examined in lung explants from six rats (body weight: 483 ± 9 g) four weeks after ligation. The lung explants from the left lungs with the ligated pulmonary artery and from the contralateral control right lungs were prepared as previously described for airways (Dandurand *et al.*, 1993). The animals were anesthetized with pentobarbital (40 mg/kg, ip), heparinized through the dorsal vein of the penis (3000 U/kg) and intubated through a tracheotomy with sterile polyethylene tubing. Their anterior chest wall and upper abdomen were sterilized with 70% ethanol, the abdomen was opened and they were exsanguinated by cutting the abdominal aorta. After removal of the anterior chest wall, the pulmonary vessels were washed *in situ* with 10 ml Ringer's lactate containing 20 U/ml heparin. The heart and lungs were excised *en bloc* and the lungs inflated to near total lung capacity with 1% agarose in bicarbonate-buffered culture medium (BCM) (Dandurand *et al.*, 1993). The preparation was left to cool for 20 min at 4 °C. Then the lungs were separated from the heart, placed in a sterile 50 ml syringe, the needle end of which had been removed, and embedded in 4% agarose in bicarbonate buffered minimum essential medium at 37°C. After 30 min at 4°C, the lung-agarose block was sectioned with a hand-held microtome blade into 0.5-1.0 mm-thick transverse slices. These were examined with an inverted microscope (IMT-2, Olympus, Tokyo, Japan) and those that contained at least one cross-section of a vessel were placed in a 30-mm culture well insert within a six-well plate containing 2 ml of BCM and incubated overnight at 37°C in 5% CO_2 + 95% air.

The next morning, the culture dish inserts containing the lung explants were transferred to six-well plates containing 2 ml of HEPES-buffered culture medium (HCM) (Dandurand *et al.*, 1993), and placed on the stage of an inverted microscope (LH50A, Olympus, Tokyo, Japan). Arteries and veins were identified and imaged with a video camera (CDS, Sony, Nagano, Japan), and images recorded with a video disk recorder (TQ2026F, Panasonic, Osaka, Japan). To distinguish arteries from veins, we used the following criteria: 1) the arteries accompanied airways, whereas veins were at a distance from them, and 2) arterial walls had a thick media and their inner lining was slightly wrinkled, whereas veins were thinner and wrinkles were inconspicuous. In addition, we confirmed the identities of the vessels by histological examination (see below).

2) Experimental protocol. First, to examine contractile responses, cumulative dose-responses to ET-1 and ET-3 of the pulmonary vessels in the explants of control right lungs and POPV left lungs were studied: after generating baseline images of the vessels, 10^{-10} M ET-1 or ET-3 were added to the surface of the explants. Five min later (which, according to preliminary experiments, corresponded to the time at which the peak contractile responses occurred), images of the vessels were taken. Then $3x10^{-10}$ M ET-1 or ET-3 were added, and images again taken. This procedure was repeated by half-log intervals until the final concentration of $3x10^{-6}$ M was reached.

Second, to study relaxation responses to ET-1 and SNP, arteries and veins were precontracted with $3x10^{-6}$ M U46619, a thromboxane A_2 analogue. Thirty min later, cumulative dose-response curves were constructed by adding ET-1 solution in half-log unit intervals from $3x10^{-11}$ M to 10^{-8} M or by adding SNP solution in one-log unit intervals from 10^{-11} M to 10^{-4} M.

In each explant, we usually observed one artery and/or one vein, and in a few instances two veins. We examined 57 arteries and 54 veins from 79 explants in the control lungs and 55 arteries and 49 veins from 87 explants in the POPV lungs.

3) Image and data analysis from the explants. The stored images were digitized using a 80386 Intel-based microcomputer equipped with a frame-grabber board (PIP1024B, Matrox, Montreal, QC, Canada). The digitized images were then transferred to a scientific work station (RS6000, IBM, Armonk, NY), and measurements of luminal areas were made with Galileo Image Processing Software (Inspiraplex, Montreal, QC, Canada). The contractile responses of arteries or veins to ET-1, ET-3 or U46619 were calculated as a percentage of complete vessel closure using the equation:

Response = [1 - (residual area after drug/baseline area)] x 100.

Thus a 100% response indicated complete vascular luminal closure and a 0% response indicated no effect.

The relaxant responses to ET-1 and SNP were expressed as a percentage of precontraction induced by U46619 using the equation:

Response = [(area before dilator - area after dilator)/ (baseline area - area before dilator)] x 100.

Here, -100% indicated a return to baseline state (ie. before precontraction), and 0% full persistence of the pre-contracted state.

From these responses, time course and dose-response curves of arteries and veins were constructed by plotting the mean values against time and concentrations respectively. The EC_{50} values were determined from each individual vessel and expressed as negative log molar (pD_2) values.

Light microscopy and morphometry of lung explants. All explants used for experiments were fixed by immersion in 10% buffered formalin, processed using standard histological technique and embedded in paraffin. Five μm-thick sections were cut and stained with hematoxylin-eosin. The arteries and veins that were used for pharmacological study were identified based on maps drawn at the time of image acquisition. Morphometric measurements were then made on those vessels that had an intact wall, using previously described methods (Michel and Hakim, 1991): with an ocular micrometer on a optical microscope (Leitz, Wetzlar, Germany), we measured the internal luminal diameter at magnifications of 100 or 250 X and, at the same position, the medial smooth muscle thickness at a magnification of 400 X (for greater precision); the sum of the internal diameter and twice the medial thickness equalled the external diameter.

Endothelin receptor binding. Pulmonary arteries and veins from 12 rats (body weight: 499 $\pm$ 13 g) four weeks after ligation were used to prepare cell membranes as previously described (Monge *et al.*, 1995). Briefly, arteries and veins were dissected out separately from the hilum down to about 100 μm diameter under a dissecting microscope (Zeiss, Oberkochen, Germany) and snap-frozen in liquid nitrogen. The tissues from each animal were homogenized separately on ice in 2 ml cold Tris-buffer (in mM: 25 Tris HCl, 2 $MgCl_2$, 250 sucrose, 5 HEPES, pH 7.4) with a Polytron (Brinkman, Rexdale, ON, Canada) at 13,000 rpm in 6 bursts of 15 sec each. The homogenate was centrifuged at 1,000 g for 10 min at 4 °C, and the supernatant was collected and centrifuged at 35,000 g for 30 min at 4 °C. The resulting pellet was resuspended in Tris-buffer, aliquoted and stored at -80 °C. Amounts of protein were determined by the dye-binding

method with bovine serum albumin as the standard.

The type, density and affinity of ET receptors in pulmonary vessels were assessed by competitive binding experiments, performed in duplicate. Due to the small sample size, we pooled the membrane proteins from the 12 rats two by two for a total of five or six experiments. Fifty μl of ^{125}I-ET-1 (14 to 25 pM) were added to each tube containing either 50 μl of Tris-buffer or increasing concentrations of unlabeled ET-1 or BQ-123. The binding reaction was initiated by adding 100 μl membrane protein (0.7-2.5 μg/tube for arteries and 3-4 μg/tube for veins) to a final incubation volume of 200 μl. After 3 h at room temperature, the reaction was stopped by addition of 1 ml cold phosphate-buffered saline containing 0.5% bovine serum albumin and rapid centrifugation at 12,000 g for 5 min. Radioactivity of the resulting pellet was determined in a gamma counter (Packard Minaxi model 5530, Missisauga, ON, Canada) with an efficiency of 80%. Nonspecific binding was measured in the presence of $2x10^{-7}$ M unlabeled ET-1. The dissociation constant (Kd) and maximal binding (Bmax) for ET-1 were obtained using the ReceptorFit Competition program (London, Chagrin Falls, OH).

Statistical analysis. Data are presented as means $\pm$ SE, with the "n" indicating the number of animals from which the vessels were obtained; this "n" was the one used for the analyses. Contralateral right lungs were the controls (17, 18). Statistical analyses were performed using proprietary software (Systat, Evanston, IL). For the comparisons of dose-response curves, maximal responses and pD_2 between control right lungs and POPV left lungs, or between arteries and veins in the same lungs, two-way analysis of variance (ANOVA) was used. When the F value was significant ($P < 0.05$), the Tukey test or Student's paired *t* test was used to examine differences at each

concentration. For comparisons of Kd, Bmax and morphometric measurements, we used Student's paired *t* test. Differences were considered statistically significant at $P < 0.05$.

RESULTS

Contractile responses to ET-1 and ET-3. The principal finding was that in POPV the contractile responses to ET-1 and ET-3 of pulmonary arteries were significantly increased compared with controls ($P < 0.05$): the increase in the responses was significant at ET-1 concentrations from 10^{-8} to $3x10^{-6}$ M (Fig. 1) and at ET-3 concentrations from $3x10^{-9}$ to $3x10^{-6}$ M (Fig. 2). Moreover, in POPV lungs, maximal responses of pulmonary arteries to ET-1 and ET-3 were also significantly increased, although the pD_2 values were not altered (Table 1). In contrast, pulmonary veins in POPV did not show altered responses to either ET-1 or ET-3 (Figs. 1 and 2, Table 1).

When we compared responses of pulmonary arteries with those of pulmonary veins in control lungs, the latter had significantly increased maximal responses to both ET-1 and ET-3 (Table 1). In POPV lungs, however, maximal responses of pulmonary arteries and veins were not significantly different. In addition, the maximal responses and pD_2 values of both pulmonary arteries and veins were greater for ET-1 than for ET-3 in control lungs; in POPV lungs, however, only pD_2 values for ET-1 of pulmonary arteries were greater than those for ET-3 (Table 1).

Relaxant responses to ET-1 and SNP. Pulmonary arteries and veins in control and POPV lungs were submaximally contracted with U46619: the contractile responses were 19.2 ± 4.4% and 29.5 ± 3.8% for control and POPV arteries, respectively, and 18.7 ± 3.3% and 29.1 ± 3.7% for control and POPV veins, respectively; the degree of constriction to U46619 was significantly greater in POPV arteries and veins than in

controls ($P < 0.05$). In arteries following U46619, ET-1 caused dose-dependent relaxation at concentrations up to 10^{-9} M for control and up to $3x10^{-10}$ M for POPV lungs that was significantly smaller in POPV than in the control (Fig. 3, Table 1). In veins, ET-1 also caused dose-dependent relaxation up to $3x10^{-10}$ M for the control and 10^{-9} M for POPV, but maximal relaxation was not significantly different between them (Fig. 3, Table 1). Above 10^{-9} or $3x10^{-9}$ M, ET-1 started to contract the arteries and veins. In contrast, SNP produced a pronounced, dose-dependent relaxation of both arteries and veins that was not altered by POPV in arteries but significantly enhanced in veins (Fig. 4).

Histology and morphometry. The identity of arteries and veins in the explants were confirmed by histology. The architecture and morphology of the control right lungs were normal with few bronchial vessels around the larger pulmonary vessels and airways. In contrast, the left lungs with the ligated pulmonary artery showed a marked increase in the number of bronchial blood vessels in the adventitia of pulmonary arteries and veins and in the walls of airways. These findings are similar to those previously reported in dogs (Michel and Hakim, 1991). The parenchyma was normal in the right lungs, but there was mild focal fibrosis in the left lungs. The results of the morphometric measurements of the pulmonary vessels are in Table 2. The internal diameters were smaller in both arteries and veins of lungs with POPV compared with controls ($P < 0.05$). The external diameters were also reduced in POPV lungs compared with controls ($P < 0.05$). Medial muscle thickness was not altered in either arteries or veins.

ET receptor binding experiments. ^{125}I-ET-1 binding was completely displaced by

unlabeled ET-1 and partially displaced by BQ-123 in both arteries and veins (Fig. 5). The displacement curves of ^{125}I-ET-1 by ET-1 and BQ-123 were monophasic and best fitted a one-site model. In arteries, the inhibition of ^{125}I-ET-1 binding with BQ-123 was significantly greater ($P < 0.05$) at concentrations of 10^{-7} and 10^{-6} M in POPV lungs than in controls (Fig. 5). The inhibition of ^{125}I-ET-1 binding with ET-1 in arteries and veins and with BQ-123 in veins, however, was not significantly different for POPV and control lungs (Fig. 5). Binding capacity (Bmax) and affinity (Kd) for ET-1, and ET_A as a percentage of total ET receptors are shown in Table 3. The percentage of ET_A receptors were significantly increased in POPV arteries but not in veins, although the Bmax and Kd of arteries and veins for ET-1 did not differ significantly between control and POPV lungs.

DISCUSSION

In the present study, we compared the *in vitro* responses to ETs, morphometric measurements and ET receptor binding of pulmonary arteries and veins in lungs with POPV with those of the contralateral control lungs. Our principal findings were that in POPV 1) contractile responses of pulmonary arteries but not of veins to ET-1 and ET-3 were increased; 2) relaxation responses to ET-1 in precontracted pulmonary arteries were reduced whereas their responses to SNP were unchanged; 3) by morphometric analysis, the diameters of the arteries and veins were reduced, without alterations in medial thickness; 4) receptor binding experiments showed an increased proportion of ET_A receptors in the arteries, but not in the veins.

Previously, postobstructive pulmonary vasculopathy has been produced primarily in dogs (Liebow *et al.*, 1950; Michel and Hakim, 1991; Michel *et al.*, 1990), although

Weibel (1960) produced it in the rat. Qualitatively, the morphologic changes in rats resemble those in canine lungs: the numbers of bronchial vessels around airways and pulmonary vessels are increased in lungs with POPV compared with controls (Michel and Hakim, 1991). Morphometric measurements of pulmonary vessels in rats, however, differed from those in dogs. First, in the latter, only arterial internal diameters were reduced, whereas in the present study, both arterial and venous diameters were lower. The likely reason for the prominent reduction in vascular diameters in rats is the reduction in lung volume that followed ligation (Shi *et al.*, 1995). Second, the medial thickness of the pulmonary arteries in the dogs with POPV was increased, whereas in rats, it remained normal (Table 2). Weibel (1960) reported that 2 to 5 days after pulmonary artery ligation, the bronchial arteries enlarge in rats, and between 5 and 40 days, new bronchial vessels grow; in this report, however, the structural changes in pulmonary vessels were not described in detail.

In the contralateral control lungs of rats, we found that contractions to ET-1 and ET-3 were greater in pulmonary veins than in arteries, as previously observed by others (Aharinejad *et al.*, 1995). We also found that ET-1 was a more potent constrictor of control pulmonary arteries and veins than ET-3; Perreault and Baribeau (1995) reported similar findings in normal piglet lungs. Since ET_A receptors are known to mediate constriction in the pulmonary vessels of rats (Uhlig *et al.*, 1995), the greater affinity of ET-1 over ET-3 for ET_A receptors may explain their different constrictor effects in pulmonary vessels (Michael and Markewitz, 1996). In addition to inducing contraction, ET-1, in isolated perfused lung preparations of rats precontracted with U46619 or hypoxia, has been shown to relax pulmonary vessels through ET_B receptors on

endothelial cells (Eddahibi *et al.*, 1993; Hasunuma *et al.*, 1990). The present study confirms these observations in *in vitro* preparations of pulmonary arteries and veins. The right contralateral lungs, although perfused with an increased blood flow in POPV, are believed to be normal both physiologically and morphologically, as indicated by our previous studies (Kelly *et al.*, 1994; Michel and Hakim, 1991; Michel *et al.*, 1990, 1995). Indeed, in dogs, we compared lungs from normal dogs with contralateral control lungs from dogs with POPV, and found minimal differences in hemodynamics and lung mechanics (Kelly *et al.*, 1994; Michel *et al.*, 1990). The increased flow to the right lungs is probably handled primarily by the recruitment of pulmonary alveolar vessels, and the pulmonary arterial pressure remains normal (Michel *et al.*, 1990).

In lungs with POPV, our first important finding was that the pulmonary arteries but not the veins showed significantly increased contractile responses to ET-1 and ET-3. Increased vasoreactivity to ET-1 has also been reported in pulmonary arteries of pulmonary hypertensive rats (MacLean *et al.*, 1995), in portal veins of spontaneously hypertensive rats (Kamata *et al.*, 1990) and in aortas of hypercholesterolemic rabbits (Merkel and Blder, 1992). The second principal finding in POPV was that relaxation to ET-1 of the pulmonary arteries was reduced whereas relaxation to SNP, that acts directly on smooth muscle (Rao and Cederbaum, 1995), was unchanged. Decreased relaxant responses to ETs have also been found in pulmonary vessels of chronic hypoxic rats (Eddahibi *et al.*, 1993).

How do we explain the increased contractile responses to ET-1 and ET-3, and the decreased relaxation to ET-1 in POPV? One putative explanation for our findings could be an altered pulmonary vascular structure. Indeed, in pulmonary vessels of the canine

POPV model (Michel and Hakim, 1991) and in vessels from rats with systemic hypertension (Mulvany *et al.*, 1978), medial thickening and peripheral muscularization were invoked to explain part of the altered vascular response. However, our present morphometric measurements revealed that the medial thickness of arteries and veins in the rodent model of POPV was not significantly altered, suggesting that the augmented reactivity to ETs in POPV is unrelated to vascular medial thickening. The reasons for these morphologic differences between the canine and the rodent models of POPV are unclear, but could be related to species differences or to the duration of ligation, much longer in the dog (Michel and Hakim, 1991). The fact that medial thickening was not altered, however, does not preclude that vascular remodeling may still have occurred (Mulvany *et al.*, 1978). This aspect, not specifically the aim of the present study, is being pursued separately. Although the luminal diameters of both pulmonary arteries and veins were significantly reduced in POPV, we do not believe that this played an important role in the altered responsiveness of the arteries. The principal reason, as illustrated in Figures 1 to 3, is that despite the fact that both arteries and veins had a reduced luminal diameter, the altered pharmacologic responses, to both constrictor and dilator agents, were observed only in the arteries in POPV, not in the veins. The impaired relaxation in the arteries was also specific to ET-1 and not to SNP, and correlated with the increased ET_A:ET_B receptor ratio that was observed in POPV. To further address the issue of reduced diameter, we performed regression analysis of maximal responses to ET-1 or ET-3 against internal diameter of the corresponding video images in each category of vessel in control and POPV lungs, and found no correlation between them (data not shown), supporting the notion that an altered diameter *per se*

does not explain our results.

A second potential mechanism to explain our findings in POPV is the increase in the proportion of ET_A receptors. Indeed, we found that the percentage inhibition of ^{125}I-ET-1 binding sites with BQ-123 was significantly increased in POPV arteries compared with controls, indicating that the ET_A:ET_B receptor ratio of was increased (Ihara *et al.*, 1992), although there were no significant differences in total binding sites for ET-1 between control and POPV lungs. This increased ratio can be invoked, on one hand, to explain the increased pulmonary arterial constrictor responses to ET-1 and ET-3 in POPV since ET_A receptors on vascular smooth muscle mediate vasoconstriction (DiCarlo *et al.*, 1995; O'Donnell and Kay, 1995; Uhlig *et al.*, 1995). The fact that the ET_A:ET_B receptor ratio was similar in veins of control and POPV lungs explains the absence of a difference in their ability to constrict to ET-1 and ET-3. The altered receptor ratio in the arteries, on the other hand, could explain their reduced relaxation responses to ET-1, since these are mediated by ET_B receptors on endothelial cells (O'Donnell and Kay, 1995; Uhlig *et al.*, 1995). It could be argued that our finding a reduced relaxation response to ET-1 in POPV is secondary to a generalized endothelial dysfunction that may accompany POPV. In a separate study in guinea pigs (Shi *et al.*, 1996), however, we found that the endothelium-dependent relaxation of pulmonary arteries to acetylcholine (which acts through different receptors) was not impaired, indicating that there is no generalized endothelial dysfunction in POPV.

In our study, one could envisage the possibility that the reduced relaxant responses of POPV arteries to ET-1 were due to their greater degree of precontraction to U46619 (Stork and Corks, 1994). We think that this is unlikely because 1) these

arteries did not also show reduced relaxation to SNP and 2) the pulmonary veins in POPV were also more precontracted to U46619 and yet they did not show a reduced maximal relaxation. The reduced relaxation of pulmonary arteries in POPV probably occurred through mechanisms other than their increased contractility to ET-1, since concentrations required for relaxation were much lower than those for contraction. The reasons for the greater venous relaxation to SNP in POPV are unclear, although this finding was also observed in guinea pigs (Shi *et al.*, 1996).

In summary, we found in the present study increased constriction to ET-1 and ET-3 and decreased relaxation to ET-1 of pulmonary arteries in POPV, and attribute this to the increased ET_A:ET_B receptor ratio, rather than to structural changes. Although alterations in vasoreactivity are a common phenomenon in vascular diseases, their mechanisms and pathological significance remain elusive. Structural alterations of vessels, including decreased vascular diameter and increased medial thickness, are frequently invoked to explain the increased vasoreactivity (Mulvany *et al.*, 1978). Our findings point to a role for ETs in POPV and may provide insights permitting the investigation of mechanisms of abnormal vasoreactivity in other diseases.

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Tables and Figures

Table 1. Maximal responses (Rmax) and pD_2 values for ET-1, ET-3 and SNP of pulmonary arteries and veins in POPV.

	Arteries		Veins	
	Rmax (%)	PD_2	Rmax (%)	PD_2
Contraction				
ET-1				
Control	31.0 ± 3.8‡	7.67 ± 0.07‡	51.0 ± 6.6$^{+\ddagger}$	7.64 ± 0.07‡
POPV	41.0 ± 4.2*	7.80 ± 0.09‡	47.7 ± 4.0	7.50 ± 0.09
ET-3				
Control	23.5 ± 4.1	7.34 ± 0.07	34.1 ± 5.4^{+}	7.24 ± 0.05
POPV	39.8 ± 3.0*	7.45 ± 0.07	46.0 ± 7.1	7.35 ± 0.08
Relaxation				
ET-1				
Control	35.5 ± 9.4	9.62 ± 0.15	12.5 ± 3.1	9.96 ± 0.15
POPV	10.0 ± 1.7*	9.91 ± 0.12	12.9 ± 5.7	9.37 ± 0.35
SNP				
Control	49.9 ± 7.5	7.15 ± 0.41	44.8 ± 7.9	7.39 ± 0.34
POPV	57.7 ± 6.4	7.22 ± 0.44	60.8 ± 6.1*	7.85 ± 0.41

Values were means ± SE of five or six animals. * $P < 0.05$ versus controls; $^{+}$ $P < 0.05$ versus arteries in the same lungs; ‡ $P < 0.05$ versus ET-3 in the same type of vessel and condition (ie. control and POPV).

Table 2. Baseline video image diameter and histological measurements of pulmonary arteries and veins in POPV.

	Video image ID	ID	ED	MT
Arteries				
Control	439 ± 29	468 ± 17	557 ± 17	44 ± 1
POPV	353 ± 21*	349 ± 23*	432 ± 24*	42 ± 3
Veins				
Control	384 ± 17	319 ± 27	352 ± 26	17 ± 2
POPV	316 ± 17*	278 ± 20*	312 ± 20*	17 ± 1

Values in μm were means ± SE of six animals. * $P < 0.05$ POPV versus control. ID, internal diameter; ED, external diameter; MT, medial thickness. Note that ED = ID + 2*MT.

Table 3. Dissociation constants (Kd) and binding capacities (Bmax) for ET-1 and percent ET_A receptors in arteries and veins from competitive binding between ^{125}I-ET-1 and unlabelled ET-1 or BQ-123 in POPV.

	Kd (pM)	Bmax (fmol/mg protein)	ET_A (%)
Arteries			
Control	248 ± 79	1712 ± 533	54.5 ± 2.9
POPV	247 ± 63	1767 ± 571	66.1 ± 3.8*
Veins			
Control	172 ± 25	1421 ± 281	59.2 ± 8.9
POPV	218 ± 25	1138 ± 162	61.4 ± 3.0

Values were means ± SE of 5 (veins) or 6 (arteries) experiments. * $P < 0.05$ POPV versus control.

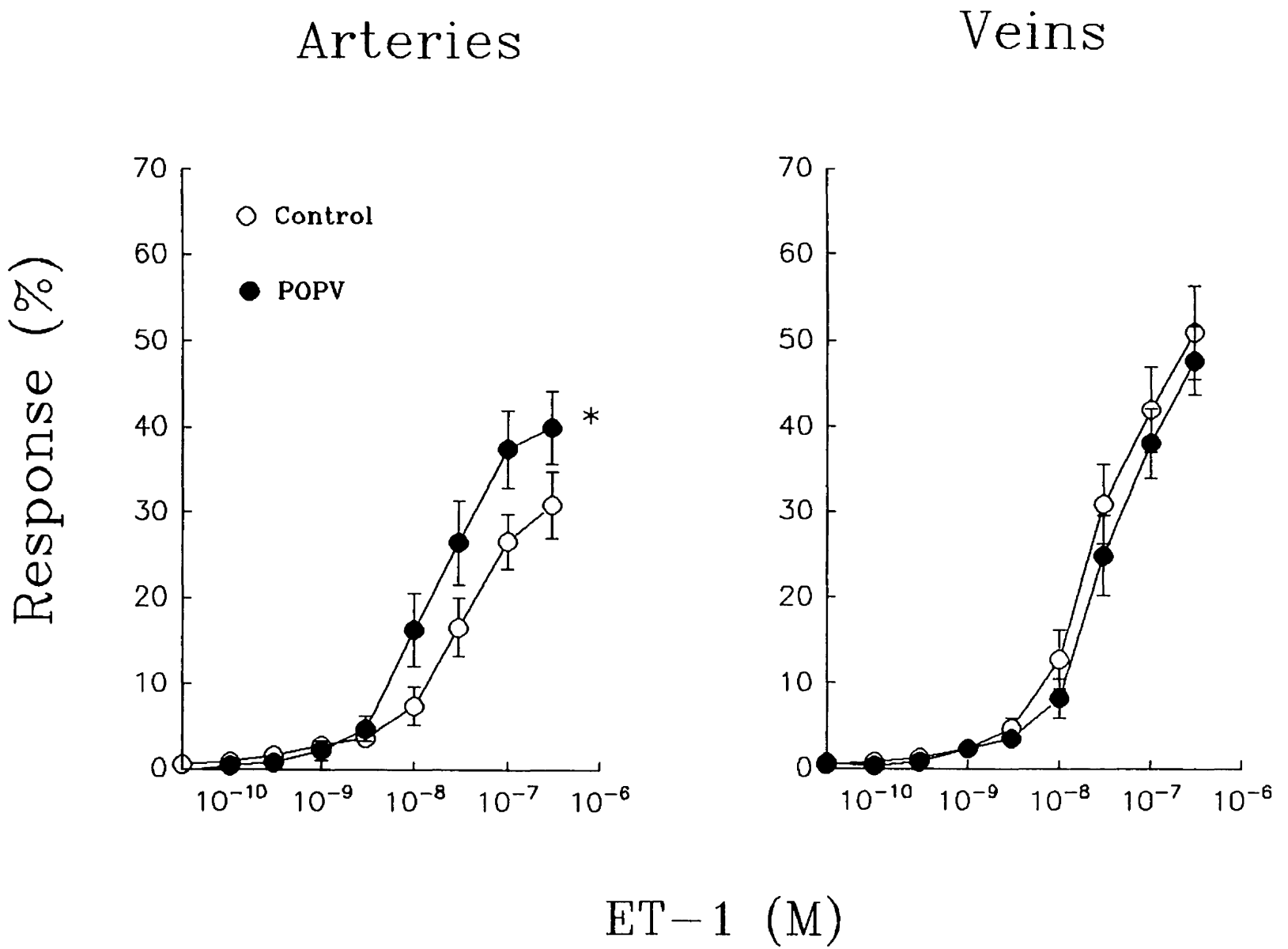
Arteries
Veins
Response (%)
Control
POPV
*
70
60
50
40
30
20
10
0
10^{-10}
10^{-9}
10^{-8}
10^{-7}
10^{-6}
ET−1 (M)

Fig. 1. Cumulative dose-responses of pulmonary arteries and veins to ET-1. Responses were calculated as percent of complete luminal closure and expressed as means $\pm$ SE of six animals. * $P < 0.05$ versus controls. The responses to ET-1 of arteries but not of veins were significantly enhanced in POPV.

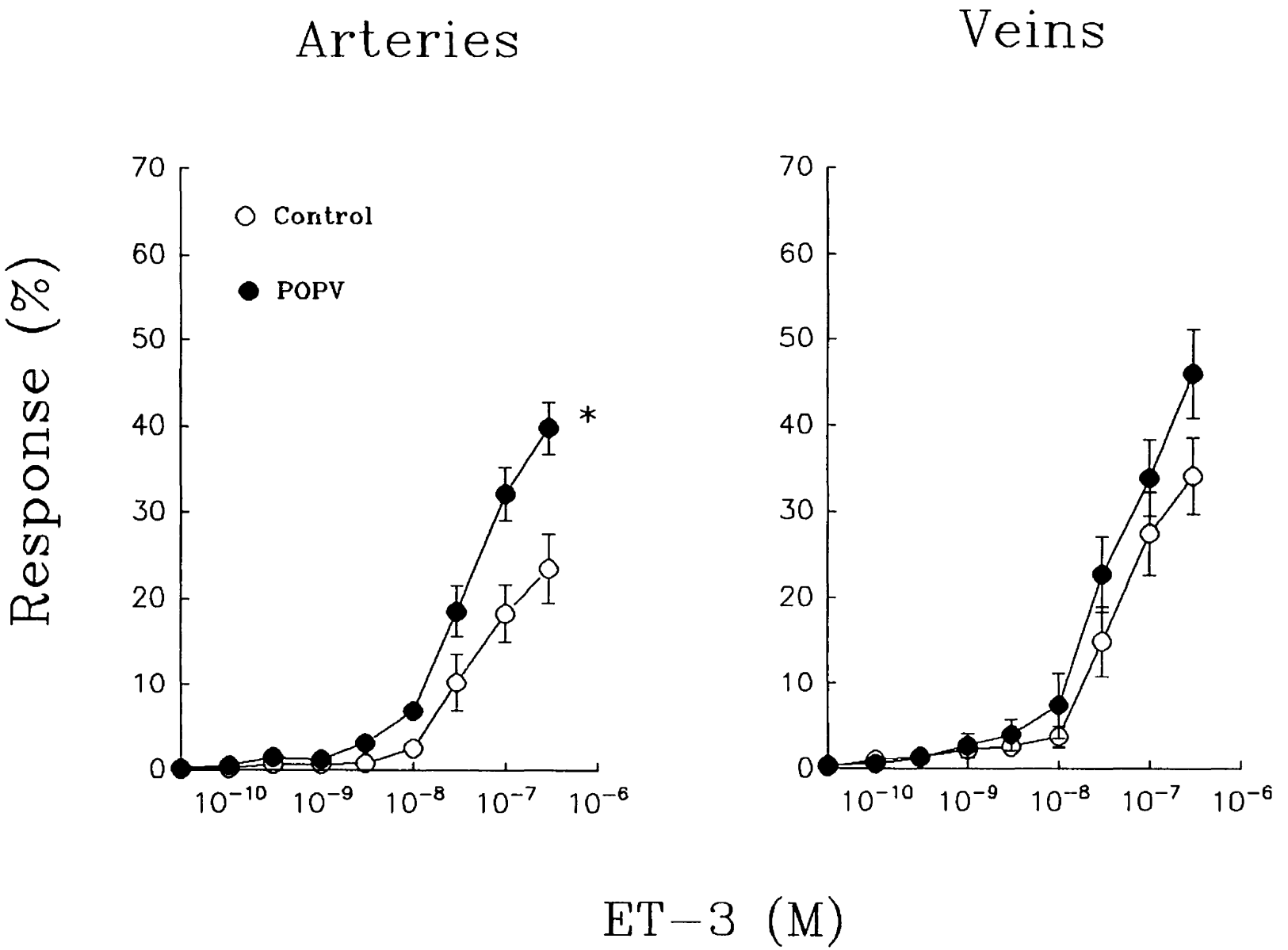
Arteries
Veins
Response (%)
Control
POPV
*
70
60
50
40
30
20
10
0
10^{-10}
10^{-9}
10^{-8}
10^{-7}
10^{-6}
ET−3 (M)

Fig. 2. Cumulative dose-responses of pulmonary arteries and veins to ET-3. Responses were expressed as means $\pm$ SE of six animals. * $P < 0.05$ versus controls. The responses to ET-3 of arteries but not of veins were significantly enhanced in POPV.

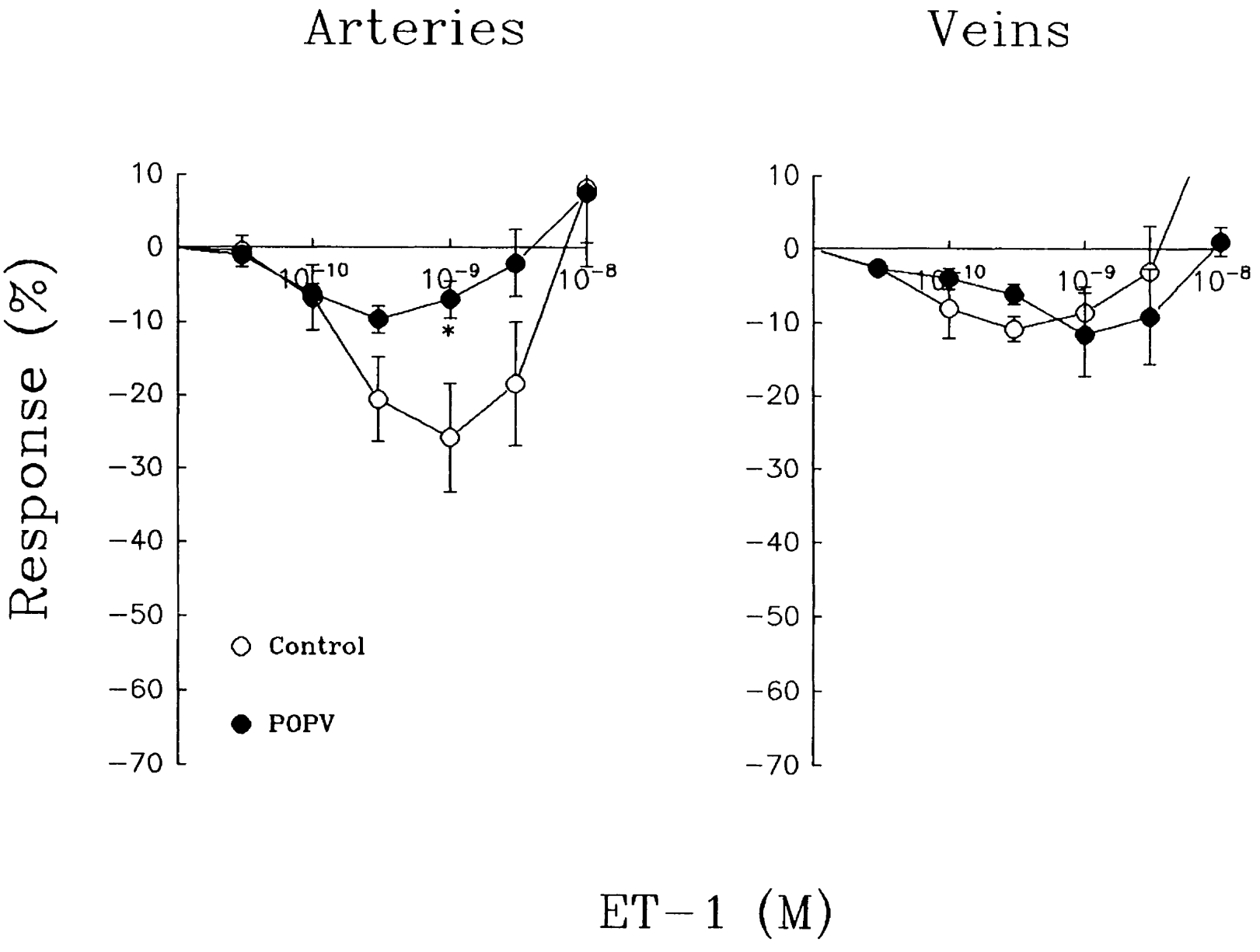
Arteries
Veins
Response (%)
10
0
−10
−20
−30
−40
−50
−60
−70
10^{-10}
10^{-9}
10^{-8}
*
Control
POPV
ET−1 (M)

Fig. 3. Responses of pulmonary arteries and veins to ET-1 after precontraction with U46619. Responses were calculated as percentage of the precontraction and were expressed as means ± SE of five or six animals. * $P < 0.05$ versus controls. The relaxation to ET-1 of arteries but not of veins was significantly reduced in POPV.

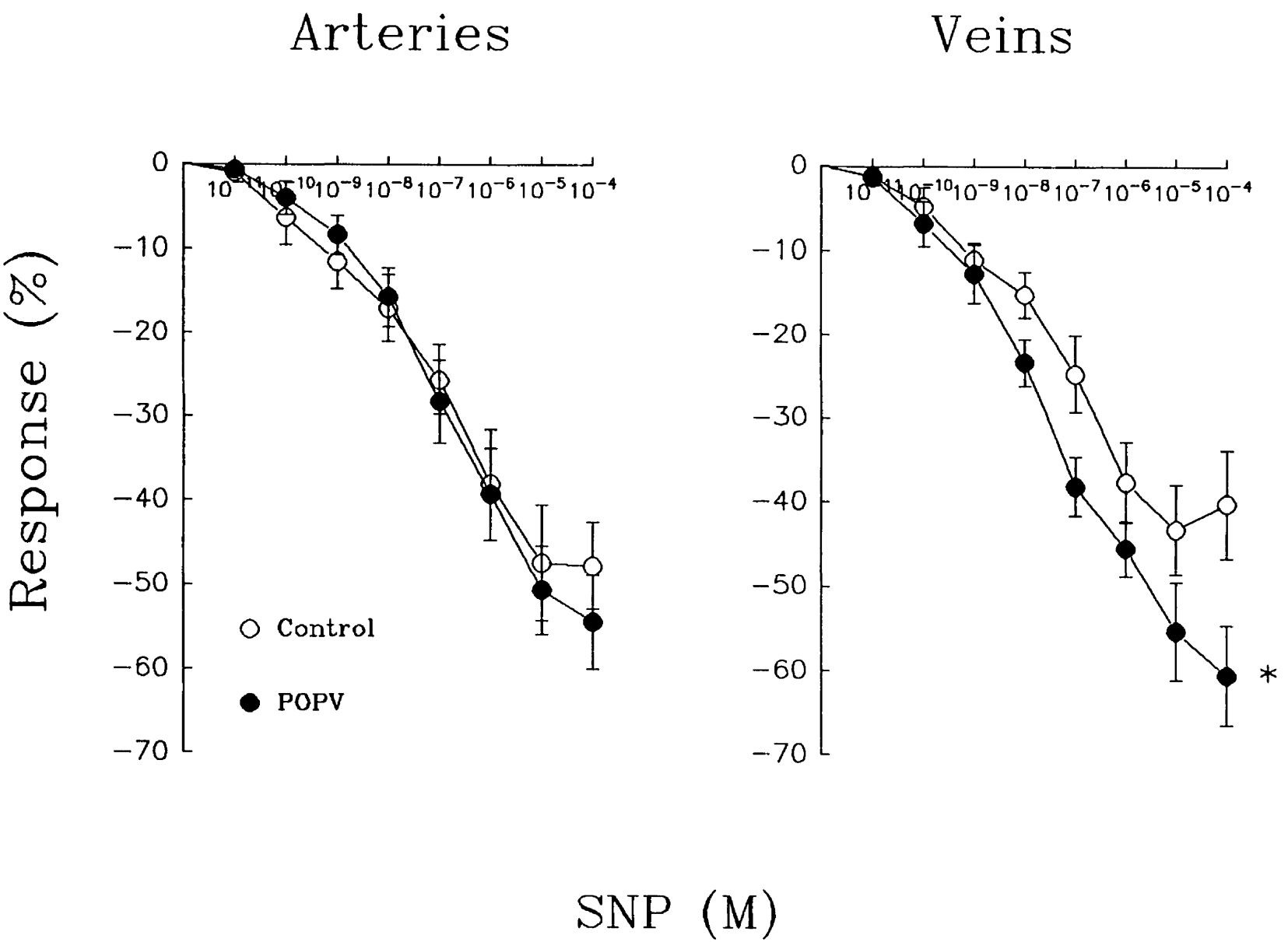
Arteries
Veins
Response (%)
0
−10
−20
−30
−40
−50
−60
−70
10^{-11} 10^{-10} 10^{-9} 10^{-8} 10^{-7} 10^{-6} 10^{-5} 10^{-4}
Control
POPV
*
SNP (M)

Fig. 4. Responses of pulmonary arteries and veins to sodium nitroprusside (SNP) after precontraction with U46619. Responses were calculated as percentage of the precontraction and were expressed as means ± SE of six animals. * $P < 0.05$ versus controls. Relaxation was not altered in arteries but enhanced in veins in the POPV lungs.

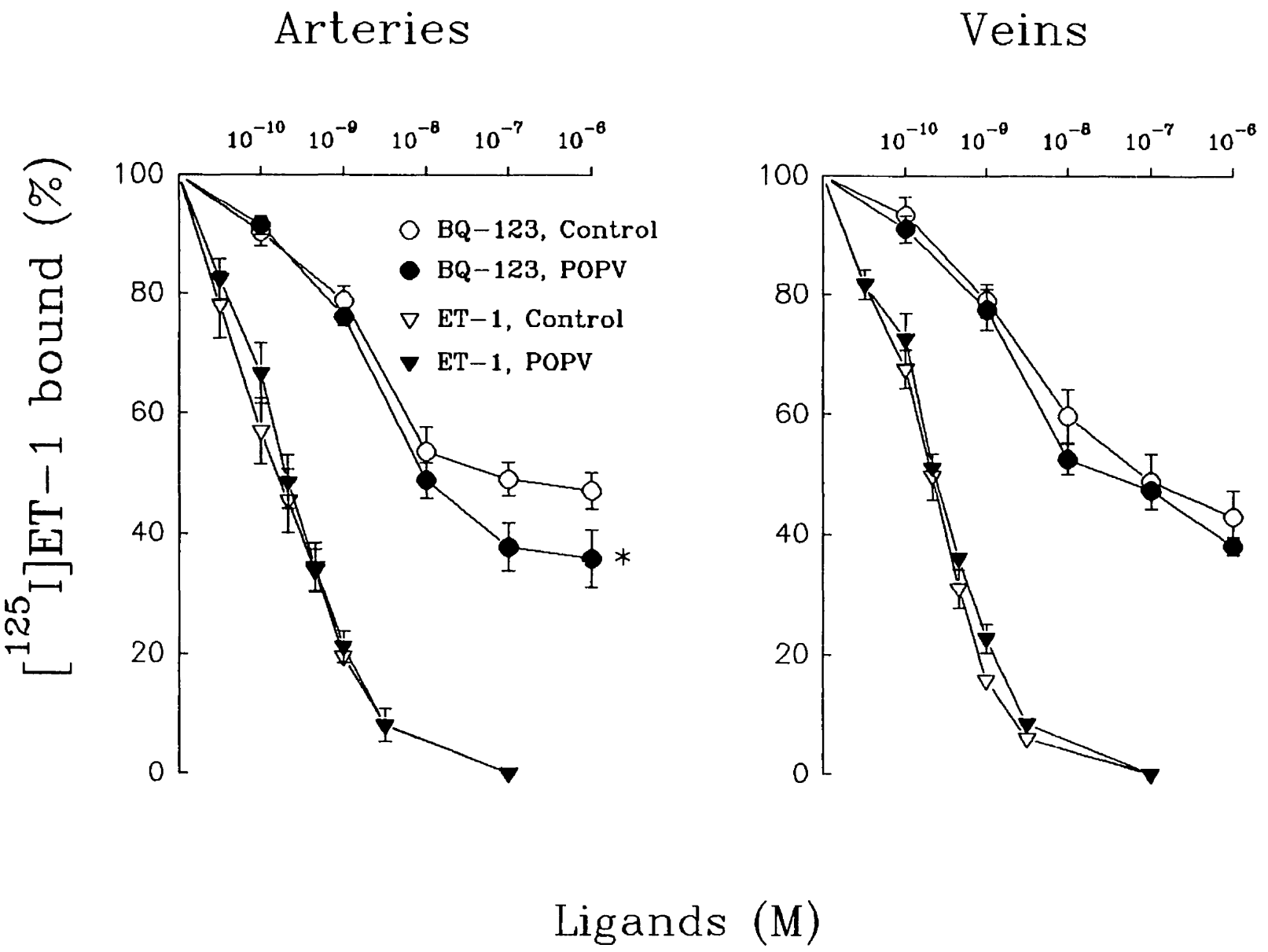
Arteries
Veins
$[^{125}I]ET-1$ bound (%)
100
80
60
40
20
0
10^{-10}
10^{-9}
10^{-8}
10^{-7}
10^{-6}
BQ-123, Control
BQ-123, POPV
ET-1, Control
ET-1, POPV
*
Ligands (M)

Fig. 5. Competitive inhibition of ^{125}I-ET-1 binding by unlabeled ET-1 and BQ123 in membrane proteins from pulmonary arteries and veins. Data were expressed as means $\pm$ SE of five or six experiments. * $P < 0.05$ versus control.

Chapter 6

General discussion and conclusions

The main purpose of the studies herein outlined was to investigate the mechanisms of hyperreactivity of pulmonary arteries to 5-HT and of veins to histamine in POPV. In the first part, which includes chapters 2 and 3, we examined the role of vascular structure, of endothelial modulation and of receptor density in the differential contractile responses of normal pulmonary arteries and veins to histamine and 5-HT using a novel lung explant technique. In the second part, contained in chapters 4 and 5, we investigated the mechanisms of pulmonary vascular hyperreactivity to histamine, 5-HT and ETs in POPV.

The principal findings of these studies were: one, the lung explant technique is a convenient and effective method to study pulmonary vasoreactivity; two, in normal guinea pigs, H_2 receptor subtypes and endothelium-derived vasoactive substances are responsible for the differential contractile responses of pulmonary arteries and veins to histamine and 5-HT respectively; three, also in normal guinea pigs, endothelium-derived NO-mediated relaxation is greater in pulmonary arteries than in veins, and ACh-induced relaxation is reduced by production of mediators from the cyclooxygenase pathway; four, in guinea pigs with POPV, we found enhanced contractile drug- and vessel-specific responses of pulmonary vessels to histamine and to 5-HT, and that these were not due to endothelial dysfunction nor to structural alterations; and five, we found augmented contractile responses of pulmonary arteries, not of veins, to ET-1 and ET-3 in POPV, that were most probably related to an elevated ET_A:ET_B receptor ratio.

The lung explant technique provides an effective means to examine the responsiveness of pulmonary arteries and veins to vasoactive substances. This technique may represent an *in vitro* method that best mimics the in *vivo* situation: the vessels have

a nearly circular cross-section supported by the surrounding parenchyma as they do *in vivo*, their endothelial cells remain intact, and both the preload and postload of the vessels are provided by the surrounding parenchyma. One difference with the *vivo* situation is that in the vessels of the lung explants, there is no intravascular pressure or blood flow, that could potentially affect the contractile or relaxant responsiveness of the vessels. This drawback is offset, however, by the fact that the basal tone of the pulmonary vessels is very low (Rodman and Voelkel, 1991), and that the stretch and tethering provided by the surrounding parenchyma appears to keep them open; indeed, in our lung explants, we found that vasodilators had little effect on the baseline area of the pulmonary vessels. Furthermore, with this technique, we found results comparable with those obtained by the more traditional methods. For example, our findings that histamine and 5-HT contracted guinea pig pulmonary veins more than arteries are consistent with those of Bradley *et al.* (1993) in the perfused lung preparation. In addition, in the contralateral control lungs of rats with POPV, we found that ET-1 and ET-3 contracted pulmonary veins more than arteries, which is in agreement with the findings of Aharinejad *et al.* (1995). Although some heterogeneity in the within-animal responses of pulmonary vessels was also present in the studies reported herein, as it had been in the studies of Dandurand *et al.* (1993) for airways (see also Introduction), it did not interfere in the plotting and analysis of the concentration-response curves.

Histamine and 5-HT are known to differentially constrict pulmonary arteries and veins in several species (Albert *et al.*, 1989; Bradley *et al.*, 1993; Michel *et al.*, 1990). The underlying mechanisms, however, have been poorly understood. Possible explanations have included differences in receptor density (van Nueten *et al.*, 1984), in

endothelial modulation (Furchgott and Vanhoutte, 1989), and in the amount of smooth muscle (Bradley *et al.*, 1993). The results of our study on this topic demonstrated that H_2 receptors appear to be responsible for the differential contractile responses of pulmonary arteries and veins to histamine, since cimetidine abolished the differences in their maximal contraction. Although inhibition of NO synthase with LNNA significantly enhanced the contractile responses of pulmonary arteries and of veins to histamine, it increased rather than decreased the differences in their maximal contraction. In contrast to their effects on the responses to histamine, LNNA and indomethacin increased the maximal responses of arteries to 5-HT, whereas they either had no effect on the veins or even reduced their maximal responses. Thus, we believe that NO and vasodilator cyclooxygenase products are responsible for the reduced responses to 5-HT in the arteries, and for the differential responses of arteries and veins to 5-HT. Indeed, in the veins, NO did not modulate the contractile responses to 5-HT and furthermore, vasoconstrictor cyclooxygenase products appeared to contribute partially to their greater contraction. Although an increase in 5-HT-induced contraction after inhibition of NO synthase and cyclooxygenase activity has been reported in pulmonary arteries of dogs and rabbits (el-Kashef, 1996; Hofman *et al.*, 1991), the present findings that the NO and the cyclooxygenase pathways were so specifically involved in the responses of pulmonary arteries and veins to 5-HT had not been reported.

Endothelium-derived NO modulated the responses of pulmonary vessels to histamine and 5-HT in guinea pigs, as evidenced by the fact that LNNA pretreatment increased their contraction to these two amines (see chapter 2). Although previous studies, using histamine and ACh, have demonstrated endothelium-derived NO-mediated

relaxation in pulmonary arteries of guinea pigs (Davidson and Eldemerdash, 1990; Sakuma *et al.*, 1988; Sata *et al.*, 1990), there is no information on the relaxation in pulmonary veins. The results of our studies indicated that histamine also relaxed pulmonary veins, although significantly less than arteries, and that ACh relaxed the veins only after the pretreatment with indomethacin. The finding that indomethacin increased the relaxation of arteries and caused relaxation of veins following ACh suggests that the relative contribution of vasoconstrictor cyclooxygenase products was greater than that of vasodilator cyclooxygenase products during the response. This finding has not been reported in the pulmonary vessels of guinea pigs. The weaker relaxation of pulmonary veins to ACh after inhibition of the cyclooxygenase pathway with indomethacin was probably due to the lower reactivity of their smooth muscle to NO, since the veins responded less to the exogenous NO donor SNP than did the arteries.

Previously, in *in situ* perfused canine lungs, Michel *et al.* (1990) found a markedly increased response of arteries to 5-HT and of veins to histamine in POPV, as measured by a disproportionate increase in pulmonary vascular resistance. These augmented responses could potentially be explained by the reduction in the luminal diameters of the vessels without necessarily an increased contractility of the smooth muscle (Folkow, 1971). Indeed, resistance, according to Poiseuille's equation, is inversely proportional to radius to the fourth power (Mulvany, 1991), and for a given degree of contraction, a narrower vessel will increase its resistance more than a wider vessel. We found, in the studies presented in chapter 4 using the lung explant technique, that the maximal contractions of arteries to 5-HT and of veins to histamine were still increased, and thus the results could be extrapolated to the perfused lung preparation, so

that factors other than the reduction in diameter that were also responsible for the increased contractility: the smooth muscle truly does contract more to the same pharmacologic stimulus. These increased contractile responses were not due to endothelial dysfunction since endothelium-dependent relaxation was intact or even enhanced. Although the reduction in vascular luminal diameter was significant in POPV, the pharmacologic specificity of the hyperreactivity argues against the possibility that it was due to this structural alteration: indeed, the veins in the lungs with POPV did not react more to 5-HT, and the arteries did not constrict more to histamine. If only a change in diameter had been responsible for the increased contraction, then they would also have reacted more to these. A failure of the constriction in the arteries to wane at higher concentrations of 5-HT, as they did in controls (Fig. 3, Chapter 4), could be responsible also in part for the increased contraction. Cushing and Cohen (1992) showed that the relaxation produced by high concentrations of 5-HT (above 10^{-7} M) in canine coronary arteries was endothelium-independent and mediated by specific receptors, but these receptors have not been characterized yet.

In the present studies, we used concentrations that went up to 10^{-3} M for histamine and 10^{-4} M for 5-HT. Although these are considered to be in the pharmacological range, and therefore somewhat high, they can be reached potentially under certain pathological conditions (Pandey *et al.*, 1995; Crystal and West, 1991). Furthermore, the use of these fairly elevated concentrations eliminates the possibility that the waning of contraction that was seen in the arteries was due to reduced effective drug concentrations. Moreover, as mentioned above, these concentrations have been used in previous studies (Cushing and Cohen, 1992; Baumgartner *et al.*, 1990).

As shown in chapter 5, we found that in pulmonary arteries but not in veins of rats with POPV, contractile responses to ET-1 and ET-3 were significantly increased and that relaxant responses to ET-1 but not to SNP were reduced. Since the medial thickness of the arteries and veins in the rodent model of POPV was not significantly altered, the augmented reactivity to ETs was unrelated to vascular medial thickening. Although the reduction of vascular luminal diameter was significant in POPV, the pharmacologic specificity of the hyperreactivity argues against the possibility that it was due to this structural alteration: although both arteries and veins had a reduced luminal diameter, the altered responses to ETs were observed only in the arteries, not in the veins. The increased ET_A:ET_B receptor ratio that we observed in the arteries with POPV can be invoked to explain the increased constrictor responses to ET-1 and ET-3 and the decreased dilator responses, since ET_A receptors on vascular smooth muscle mediate vasoconstriction and ET_B receptors on endothelial cells mediate vasodilation (DiCarlo *et al.*, 1995; O'Donnell and Kay, 1995; Uhlig *et al.*, 1995).

The following summarizes the contributions of the studies reported in the present thesis to original knowledge on the pulmonary circulation:

1. The lung explant technique was successfully adapted to study the reactivity of intrapulmonary arteries and veins in normal guinea pigs and rats, and in animals with POPV;

2. Vasodilator H_2 receptors play important roles in the differential contractile responses of pulmonary arteries and veins to histamine, and endothelium-derived NO and cyclooxygenase products account for their differential responses to 5-HT in guinea pigs;

3. In normal guinea pigs, endothelial NO-mediated relaxation is greater in pulmonary arteries than in veins, and ACh-induced NO-mediated relaxation is reduced by the simultaneous production of cyclooxygenase-derived vasoconstrictors;

4. In the guinea pig model of POPV, constriction of pulmonary arteries to 5-HT and of veins to histamine was increased, and this was not due to endothelial dysfunction nor to structural alterations, but was probably due foremost to changes in the smooth muscle *per se*.

5. In the rodent model of POPV, contractile responses of pulmonary arteries to ET-1 and ET-3 were increased, and their relaxant responses to ET-1 were reduced; this altered vasoreactivity is believed to be related to the increased ET_A:ET_B receptor ratio.

In conclusion, the differential contractile responses of normal pulmonary arteries and veins to histamine and 5-HT, and the altered vascular responses to these amines and to ETs in POPV, are due primarily to differences in receptors or to differences in endothelial modulation, rather than to disparities in vascular structure.

Further studies planned for the future include an examination of the roles of signal transduction pathways, specifically IP3, cAMP and cGMP in the hyperreactivity of pulmonary vessels to histamine, 5-HT and ETs, an investigation of the development of the altered vasoreactivity, and a study of the relationship between contractile proteins and vasoreactivity in POPV. In addition, one interesting finding in the present set of studies is that the constriction of the pulmonary arteries but not of the veins waned over time and at high concentrations after reaching their peak in response to histamine, 5-HT and U46619 in guinea pigs. Since this phenomenon appears to occur in response to several

different vasoconstrictors, differences between arteries and veins in the signal transduction pathways, beyond the level of the receptor may be responsible. Further studies may lead to a better understanding of their different properties under physiological and pathological conditions.

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Abbreviations

5-HT: 5-hydroxytryptamine

ACh: acetylcholine

ANOVA: analysis of variance

BCM: bicarbonate-buffered culture medium

cAMP: cyclic adenosine 3',5'- monophosphate

cGMP: cyclic guanosine monophosphate

CGRP: calcitonin gene-related peptide

DAG: diacylglycerol

ED: external diameter

EDHF: endothelium-derived hyperpolarizing factor

EDRF: endothelium-derived relaxing factor

ET: endothelin

ET_A: endothelin receptor type A

ET_B: endothelin receptor type B

ET_C: endothelin receptor type C

HCM: HEPES-buffered culture medium

ID: internal diameter

IP2: phosphatidylinositol 4,5-biphosphate

IP3: inositol 1,4,5-triphosphate

LNNA: N^{ω}-nitro-L-arginine

MT: muscle thickness

NANC: non-adrenergic, non-cholinergic innervation

NO: nitric oxide

NOS: NO synthase

eNOS: endothelial NOS; nNOS: neuronal NOS; iNOS: inducible NOS

°C: degree Celsius

PGI_2: prostacyclin

POPV: postobstructive pulmonary vasculopathy

SNP: sodium nitroprusside

TXA_2: thromboxane A_2

U46619: 9,11-dideoxy-11α,9α-epoxymethanoprostaglandin $F_{2\alpha}$

VID: video image internal diameter