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Role of Nitric Oxide in the Pathogenesis of Chronic Pulmonary Hypertension

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Hampl, Václav, and Jan Herget. Role of Nitric Oxide in the Pathogenesis of Chronic Pulmonary Hypertension. *Physiol Rev* 80: 1337–1372, 2000.—Chronic pulmonary hypertension is a serious complication of a number of chronic lung and heart diseases. In addition to vasoconstriction, its pathogenesis includes injury to the peripheral pulmonary arteries leading to their structural remodeling. Increased pulmonary vascular synthesis of an endogenous vasodilator, nitric oxide (NO), opposes excessive increases of intravascular pressure during acute pulmonary vasoconstriction and chronic pulmonary hypertension, although evidence for reduced NO activity in pulmonary hypertension has also been presented. NO can modulate the degree of vascular injury and subsequent fibroproduction, which both underlie the development of chronic pulmonary hypertension. On one hand, NO can interrupt vascular wall injury by oxygen radicals produced in increased amounts in pulmonary hypertension. NO can also inhibit pulmonary vascular smooth muscle and fibroblast proliferative response to the injury. On the other hand, NO may combine with oxygen radicals to yield peroxynitrite and other related, highly reactive compounds. The oxidants formed in this manner may exert cytotoxic and collagenolytic effects and, therefore, promote the process of reparative vascular remodeling. The balance between the protective and adverse effects of NO is determined by the relative amounts of NO and reactive oxygen species. We speculate that this balance may be shifted toward more severe injury especially during exacerbations of chronic diseases associated with pulmonary hypertension. Targeting these adverse effects of NO-derived radicals on vascular structure represents a potential novel therapeutic approach to pulmonary hypertension in chronic lung diseases.

I. INTRODUCTION

Chronic injury to the pulmonary vasculature results in sustained pulmonary hypertension. Although various forms of pulmonary hypertension are a significant medical problem, mechanisms of development of this syndrome are unclear. Consequently, current options for effective prevention and therapy are limited.

One of the fastest growing areas of biomedical research during the last decade has been the biological role of endogenously produced nitric oxide (NO). Enormous evidence has accumulated showing an important role of

this simple molecule in a wide variety of physiological functions, including regulation of vascular tone and mesenchymal cell growth.[1] Lots of work has also been devoted to finding out the role played by NO in the normal and hypertensive pulmonary circulation. Reviewing the findings of that work is the objective of this article. The available data support the possibility that in pulmonary hypertension, endogenous NO can act not only to suppress the increase in vascular tone, but depending on conditions not yet well characterized, also to promote the vascular wall injury. The large, promising, and rapidly expanding area of the therapeutic manipulations of NO activity in pulmonary hypertension (inhaled NO gas, gene therapy) is not covered in this review; the interested reader is referred to recent reviews published elsewhere (54, 75, 121, 145, 164, 180, 194, 214).

A. Pulmonary Hypertension

As reviewed extensively elsewhere (295, 389), pulmonary hypertension is defined clinically as a condition of elevated pulmonary arterial pressure and/or pulmonary vascular resistance. It is a syndrome common to a variety of lung and heart diseases, such as chronic obstructive lung disease, lung fibrosis, adult respiratory distress syndrome, mitral stenosis, or congenital heart defects. Pulmonary hypertension also exists in a primary form, which is a relatively rare but serious disease (1, 66). Pulmonary hypertension secondary to pulmonary or cardiac diseases significantly worsens the prognosis of the primary disease (389).

Pulmonary hypertension presents an increased load to the right ventricle, which consequently hypertrophies and tends to fail. In fact, right heart failure is the most common cause of death in pulmonary hypertension (389).

The elevated vascular resistance in pulmonary hypertension is a result of an increase in vascular tone and of structural remodeling of the peripheral pulmonary arteries. The remodeling affects both vascular smooth muscle, which hypertrophies and proliferates, and vascular wall connective tissue, which increases in amount and undergoes qualitative changes. The endothelium is often affected as well. All of that reduces vascular lumen and thus increases vascular resistance to blood flow. It also makes the vascular wall less compliant. A relative pathogenetic significance of the functional and structural components varies with the type and stage of the disease. In the developed pulmonary hypertension, the importance of structural remodeling prevails (298), as suggested by the relative resistance of various types of pulmonary hypertension to vasodilator therapy (see Refs. 275, 276 for review).

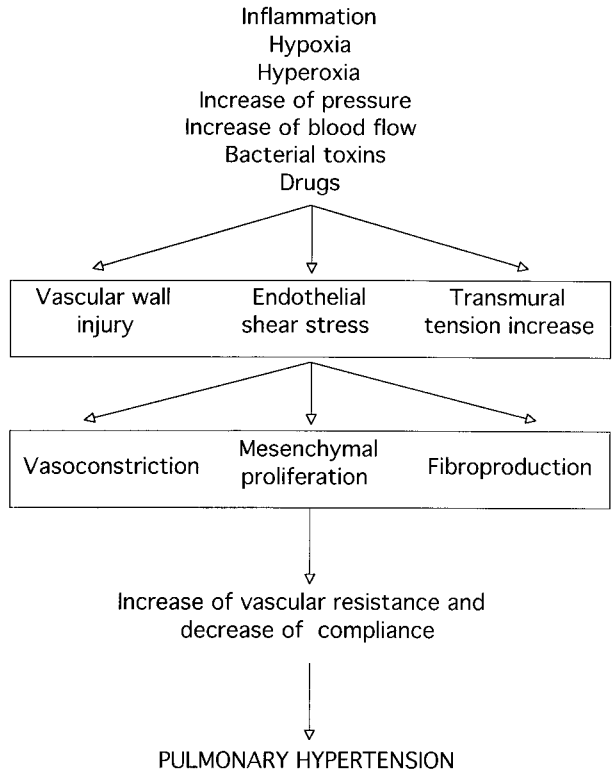

FIG. 1. Major mechanisms causing pulmonary hypertension.

Although chronic pulmonary hypertension is caused by a variety of pathogenetic factors, they all lead to vasoconstriction and structural remodeling of surprising uniformity. It suggests that at least part of the pathogenetic chain is similar despite the diverse origins of the disease. Injury to the pulmonary vascular wall and resulting reparatory processes are likely to be such a common phenomenon.

Various causes of chronic lung vascular injury were studied in different models of experimental pulmonary hypertension (for review see Refs. 139, 143, 296). Three major mechanisms (Fig. 1), namely vascular wall injury, abnormal shear stress of endothelial cells due to locally increased blood flow, and increase of transmural pressure across the vascular wall, interact in most cases of pulmonary hypertension, although their relative importance may differ.

As discussed in detail in recent reviews (17, 146, 290, 315, 324, 330, 353, 382, 389), a variety of intercellular and intracellular messenger molecules appear to be involved in the mechanism of pulmonary hypertension. However, their exact interplay is unclear, as is the primary stimulus to switch it on. Recent data point to alterations in the

[1] Robert F. Furchgott, Louis J. Ignarro, and Ferid Murad were jointly awarded the 1998 Nobel Prize in Physiology and Medicine for their discoveries concerning NO as a signaling molecule in the cardiovascular system.

metabolism of vascular wall matrix proteins as a significant part of the mechanism of pulmonary hypertension (290). The extracellular matrix is an important denominator of mesenchymal cell migration, growth, and differentiation (331).

An important factor in the development of pulmonary hypertension is chronic or intermittent alveolar hypoxia. Lung tissue hypoxia accompanies most of the lung and heart diseases associated with pulmonary hypertension. It is not surprising, therefore, that exposure of animals to hypoxic environment has been the most often used experimental model of chronic pulmonary hypertension.

A distinction should be made between the effects of acute and chronic hypoxia. Acute hypoxia causes pulmonary vasoconstriction, which is one of the hallmarks of pulmonary vascular regulation. Systemic vessels generally respond to hypoxia with vasodilation or no change in tone. Hypoxic pulmonary vasoconstriction reduces blood flow to poorly ventilated areas of the lung in favor of the flow into the better ventilated regions. That helps to optimize lung ventilation/perfusion matching and consequently also the oxygenation of the blood. Hypoxic pulmonary vasoconstriction is a local lung regulatory mechanism. It is fast in onset (165) and is readily reversible upon reoxygenation.

Chronic hypoxia causes pulmonary hypertension, which often has a vasoconstriction component too. However, morphological remodeling of the pulmonary vascular wall appears to be more important than vasoconstriction (298).

B. NO and NO Synthases

NO is a simple molecule with one unpaired electron, i.e., it is a free radical. In the presence of oxygen, NO undergoes oxidation, which follows a second-order kinetics. Hence, when NO levels are high, it is oxidized within seconds. On the other hand, when NO levels are relatively low, as is often the case in vascular tissues (30, 167, 217), its half-life can be hours or more. Oxidation end-product of NO is nitrite (NO_2^-) in aqueous solutions and nitrate (NO_3^-) in the presence of oxyhemoglobins (e.g., in blood) (157). NO and its oxidation products are often collectively referred to as NO_x. The biologically relevant aspects of NO chemistry were recently reviewed in detail elsewhere (15, 119, 156, 178).

NO is an important endogenous vasodilator produced by endothelial cells. It also serves as a neurotransmitter. High levels of NO and products of its interaction with oxygen free radicals are toxic, a fact which is utilized by cells of the immune system to kill invading bacteria or tumor cells.

NO is produced in mammalian cells by an oxygen-dependent, five-electron oxidation of a terminal guanidino nitrogen of L-arginine. Aside from NO, the reaction yields L-citrulline. The multistep reaction is catalyzed by a single heme-containing enzyme, NO synthase (NOS; EC 1.14.13.39), which exists in three isoforms. All isoforms are active as homodimers, are stereospecific (D-arginine is not a substrate), and require reduced nicotinamide adenine dinucleotide phosphate, 6(*R*)-5,6,7,8-tetrahydrobiopterin, flavin adenine dinucleotide, and flavin mononucleotide as cofactors. Isozyme I (subunit molecular mass ~160 kDa), encoded by a gene located on the human chromosome 12, is constitutively expressed in many central and peripheral neurons and is therefore often called neuronal NOS (nNOS). It can also be present in certain epithelial and vascular smooth muscle cells (including pulmonary) (340). Its activity is regulated by calcium-dependent binding of calmodulin. Type II NOS (~130 kDa; encoded by a gene on human chromosome 17) is inducible by a variety of factors related to inflammation and therefore is often referred to as inducible NOS (iNOS). It is regulated at the level of gene expression; once expressed, it produces NO at a high rate independently of the intracellular concentration of the free calcium ion ($[Ca^{2+}]_i$). The third isoform, NOS III or endothelial NOS (eNOS; ~133 kDa), is encoded by a gene on human chromosome 7. In most endothelial cells and several other cell types, it is expressed constitutively, but the rate of the eNOS gene transcription and translation can be modulated by numerous factors, such as the shear stress of the endothelial surface (reviewed in Ref. 94). The eNOS enzyme activity is regulated by calcium-dependent binding of calmodulin and by tyrosine phosphorylation (111). Although the initial research of the physiology of NO in the vasculature focused on the eNOS, recent data suggest that at certain situations the remaining two NOS isoforms may also contribute to the vascular regulation (36, 291). More detailed discussions of NOS enzymology are available elsewhere (92, 94, 95, 150, 243, 250, 356).

The target tissue effects of NO depend on its quantity. At high concentrations, NO readily reacts with oxygen and especially with superoxide, forming highly reactive, cytotoxic substances, such as peroxynitrite. At lower concentrations, NO serves regulatory roles via activation of soluble guanylate cyclase, resulting in increased cGMP levels in target cells. In vascular smooth muscle, cGMP causes relaxation by reducing $[Ca^{2+}]_i$ and by downregulating the contractile apparatus (Fig. 2). These actions are mostly (although not exclusively; Ref. 97) mediated by type I (soluble) cGMP-dependent protein kinase (150, 398).

The reduction of $[Ca^{2+}]_i$ by cGMP is accomplished in several ways. One is inhibition of calcium influx. Voltage- and receptor-operated calcium channels of the sarcolemma are directly phosphorylated and inactivated by the

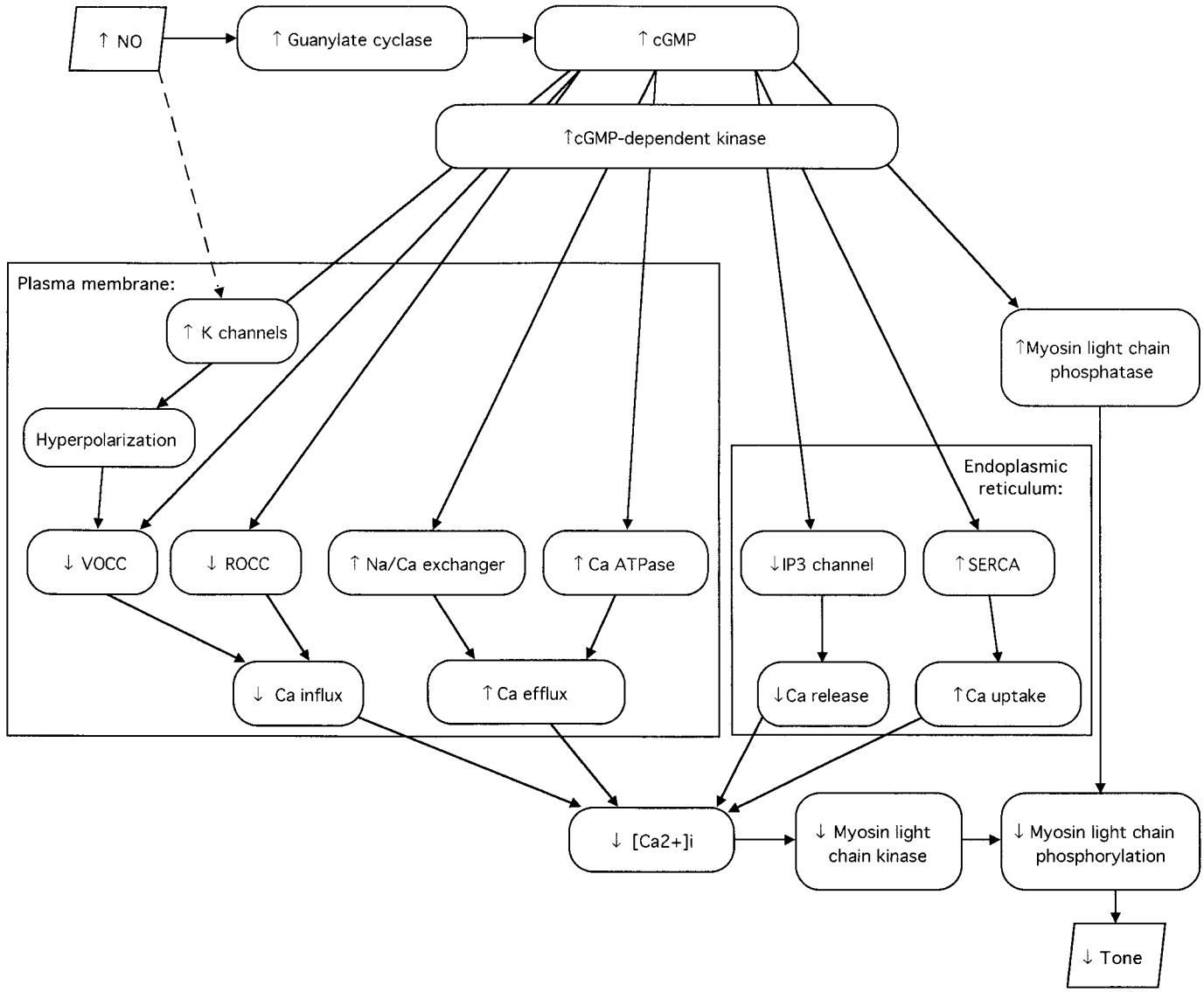

FIG. 2. Mechanisms of nitric oxide (NO)/cGMP-induced vasodilation. VOCC, voltage-operated calcium channels; ROCC, receptor-operated calcium channels; SERCA, sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase; IP_3 channel, inositol 1,4,5-trisphosphate-gated calcium channel; $[Ca^{2+}]_i$, intracellular free calcium ion concentration.

cGMP-dependent protein kinase (9, 28). In addition, cGMP-dependent protein kinase activates potassium channels of the sarcolemma (12, 13, 39), causing membrane hyperpolarization (13) and consequently reducing calcium influx through the voltage-operated calcium channels. In addition to the reduction of the extracellular calcium influx, cGMP also diminishes calcium release from the sarcoplasmic reticulum by blocking the inositol 1,4,5-trisphosphate-sensitive calcium release channel (186).

Enhanced calcium extrusion from the cytosol into the extracellular space also contributes to the vasorelaxant effect of cGMP. Ca^{2+}-ATPase and the sodium/calcium exchanger of the sarcolemma are both stimulated by cGMP (108, 109). Calcium sequestration into the sarcoplasmic reticulum is also potentiated by cGMP via phospholamban-mediated activation of the Ca^{2+}-ATPase of the sarcoplasmic reticulum (9, 374).

In vascular smooth muscle, tension depends on the phosphorylation status of the regulatory myosin light chain. Activation of the myosin light chain phosphatase is another means by which cGMP reduces vascular tone (202, 397, 398). In various tissues cGMP acts also by altering the activity of phosphodiesterases deactivating cAMP; in smooth muscle, however, this mechanism has not been documented. More details on the NO-cGMP signal transduction system can be found in extensive recent reviews (150, 246, 329).

II. NITRIC OXIDE IN THE REGULATION OF THE BASAL TONE OF THE NORMAL PULMONARY VESSELS

Because NO is a vasodilator continuously produced in many vascular beds, it has been suggested that a reduction of resting pulmonary vascular NO synthesis could be responsible for pulmonary hypertension (7, 71). Thus, to evaluate the role of NO in pulmonary hypertension, it is useful to first examine the basal NO production in the normal pulmonary circulation.

With respect to basal vascular tone, there is a qualitative contrast between the pulmonary circulation of a fetus and newborn on one side and that of an adult on the other. In the fetus, pulmonary vascular tone is similarly high as in the systemic circulation. In the neonatal period, pulmonary vascular tone decreases rapidly so that in older infants, children, and adults it is usually minimal. This review focuses primarily on the pulmonary circulation that has completed the perinatal transition from the fetal state. The role of NO in the fetal and neonatal pulmonary circulation, reviewed in detail elsewhere (2, 4, 89, 181, 307, 350), is summarized only briefly.

A. Adults

After a neonatal period, a normal, healthy pulmonary circulation is typically fully dilated. This statement is based not only on the well-known fact of a high flow and low pressure and resistance in the pulmonary circulation, but especially on a common finding that administration of vasodilators (in doses sufficient to cause profound systemic vasodilation) have very little or no effect on the pulmonary circulation of most healthy individuals (5, 69, 76, 104, 154, 317). The mechanisms responsible for this low basal tone of the pulmonary vessels are not clear. When NO was discovered as an endogenous vasodilator, an intriguing idea appeared that it could be a high continuous release of NO that keeps (or helps to keep) pulmonary vessels dilated. As discussed below, most of the data available today are not consistent with this possibility.

Approaches used to assess the role played by NO in the regulation of the normal, basal tone of the healthy pulmonary circulation include measuring pulmonary vasomotor effects of NOS inhibitors, studies of NOS mRNA and protein expression, and the use of transgenic mice.

1. Effect of NOS inhibitors

The rationale behind using NOS inhibitors is as follows. If a continuous, basal production of NO (a vasodilator) helps to keep pulmonary vascular tone and resistance low, then inhibition of this basal production should increase pulmonary vascular resistance. Analogous reasoning led to the discovery of the continuous, "tonic" release of NO in the systemic vasculature when it was noticed that administration of NOS inhibitors caused systemic vasoconstriction (114–117, 294, 377). In the pulmonary vasculature, the reported effects of NOS inhibitors are variable. It is likely that differences in species and in NOS expression in different vascular segments contribute to the discrepancies in the literature. Therefore, in an attempt to bring some order into the plethora of findings, the following discussion of the effects of NOS inhibitors on the resting pulmonary vascular tone is sorted by species and experimental preparation.

a) rat. *I) Isolated perfused lungs.* The initial attempts to elucidate the role of endothelium-derived relaxing factor (EDRF)/NO in the pulmonary circulation were started before the relatively selective NOS inhibitors, such as N^{ω}-monomethyl-L-arginine (L-NMMA) or N^{ω}-nitro-L-arginine methyl esther (L-NAME), became available. Brashers et al. (38) utilized the finding that EDRF activity was inhibited by the lipoxygenase antagonists eicosatetraynoic and nordihydroguaiaretic acids and by the antioxidant hydroquinone. Using isolated rat lungs, they found that the resting vascular tone was not significantly altered by these substances at doses effective in blocking the responses to endothelium-dependent vasodilators.

The first study utilizing an L-arginine-derived specific blocker to investigate the effect of NOS inhibition on the basal pulmonary vascular tone was published by Archer et al. (16). They found that L-NMMA (4.7×10^{-4} M) did not alter baseline perfusion pressure in isolated rat lungs perfused with Krebs-albumin solution at a constant flow rate. The same dose of L-NMMA completely prevented the vasodilator response of preconstricted pulmonary vasculature to bradykinin, known to be EDRF dependent (52, 107), confirming that the dose was effective in inhibiting NOS. These data indicated that, unlike in the systemic vasculature, there is no physiologically significant basal synthesis of NO in the normal rat pulmonary circulation.

The finding that L-NMMA causes minimal or no vasoconstriction is isolated rat lungs perfused with artificial solution was subsequently independently confirmed (18, 26, 44, 74, 137) and expanded by using other NOS inhibitors and variations of the technique. For example, when blood was used instead of an artificial solution as a perfusate for the isolated rat lungs, L-NMMA again caused no physiologically significant increase in vascular resistance (20, 23, 100, 210, 309, 362, 412). Using both salt solution- and blood-perfused rat lungs, numerous authors found minimal or no vasoconstrictor response to more potent NOS inhibitors, namely, L-NAME (20, 73, 83, 134, 135, 158, 319, 359, 376, 406) and N^{ω}-nitro-L-arginine (L-NA) (84, 134, 265, 302, 304, 318). Using a sensitive videomicroscopy system in isolated perfused rat lungs, Suzuki, Yamaguchi, and colleagues (359, 406) found that L-NAME altered neither the resting pulmonary vascular resistance nor the

resting diameters of the pulmonary precapillary arterioles (20–30 μm).

A few authors found an increased vascular resistance in isolated rat lungs after administration of L-NA (130) or L-NAME (21, 311, 394), usually using relatively high doses ($>3 \times 10^{-4}$ M). We believe that this vasoconstriction may have been caused predominantly by the nonspecific, NOS-unrelated effects of higher doses (11, 40, 209, 282, 371) because lower doses, which do not elicit pulmonary vasoconstriction, are sufficient to inhibit endothelium-dependent vasodilation (16, 74, 83, 84, 135, 158, 210, 265, 304, 318). In one study, the effects of L-NAME and L-NA were directly compared between the lung and kidney isolated from the same rat (134). Doses of both NOS inhibitors, which elicited massive vasoconstriction in the kidney, had no effect in the lung (Fig. 3). This finding confirms the presence of a continuous NO production in the renal circulation and its absence in the lung.

Dr. Taylor's group (21, 394) found a vasoconstrictor response to a relatively high dose of L-NAME in lungs perfused at constant pressure only when using higher viscosity perfusates (blood or salt solution containing >10% albumin), but not with perfusates of low viscosity. They argued that basal NO production existed in the pulmonary circulation as a result of shear stress, which was greatly reduced in lungs perfused with low viscosity solutions (because shear stress is a function of perfusate viscosity). This idea is intriguing because shear stress is a known and potent stimulus for endothelial NO production in vitro (60, 138, 167, 189, 192, 200, 236). However, Uncles et al. (376) found that even high doses of L-NAME had no effect on resting vascular tone in isolated rat lungs perfused with artificial perfusates of viscosity equal to that of the whole blood. On the other hand, in their experiments L-NAME caused pulmonary vasoconstriction when blood (even diluted, of relatively low viscosity) was used as a perfusate (376). Thus the issue of viscosity and shear stress remains controversial. In any case, these studies do not explain the findings of many authors of a minimal or no response to lower, yet effective, doses of NOS inhibitors in lungs perfused with blood (20, 23, 83, 210, 309, 319, 362, 412).

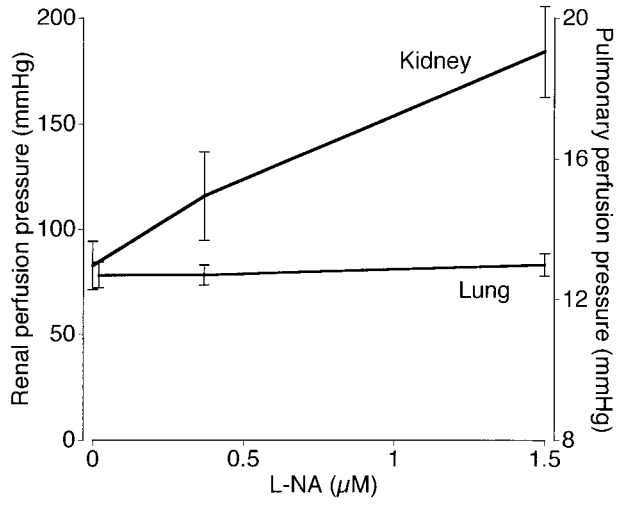

FIG. 3. Doses of N^{ω}-nitro-L-arginine (L-NA), capable of causing marked renal vasoconstriction, do not produce pulmonary vasoconstriction. Lung and kidney isolated from the same rat were perfused at constant flow rate (so that changes in perfusion pressure directly reflect changes in vascular resistance). Data are means ± SE. [Data from Hampl et al. (134). Reprinted by permission of Blackwell Science, Inc.]

To summarize, most studies show that low doses of NOS inhibitors, effective in inhibiting endothelium-dependent vasodilation, do not cause a significant vasoconstriction in isolated perfused rat lungs.

II) Intact rats. The studies on intact rats show no significant pulmonary vasoconstrictor response to acute administration of L-NA (265) and L-NAME (98, 155) in doses (5–15 mg/kg iv) effective in increasing systemic vascular resistance. Higher doses of L-NAME (30–300 mg/kg iv) increased pulmonary arterial pressure in catheterized rats when lung flow was held constant (153). When pulmonary blood flow was not controlled, there was no significant change in pulmonary vascular resistance in response to L-NAME (153). Chronic oral treatment with L-NAME significantly increased systemic, but not pulmonary, arterial pressure in normal rats (132). L-NMMA, on the other hand, increased pulmonary vascular resistance in conscious rats when administered acutely (50 mg/kg iv) (229). The reason for this discrepancy is obscure, but it is quite likely that differences in technique do not play a role because the same laboratory using the same technique found no response to L-NAME (98) and a significant response to L-NMMA (229). One possible explanation is that L-NMMA has more nonspecific, NOS-unrelated effects than L-NA or L-NAME, and these, rather than reduction of NO synthesis, produce pulmonary vasoconstriction.

III) Isolated pulmonary arteries. Both unchanged (61, 198, 215, 265, 322, 363, 388) and increased (16, 127, 169, 322, 349, 405, 409) tension in response to NOS inhibitors have been reported in the isolated rat pulmonary arteries. Salameh et al. (322) found a constrictor response to L-NA in pulmonary arteries of one rat strain and its absence in another. Possible reasons for the discrepancies between vasoconstrictor response to NOS inhibition in many studies with isolated pulmonary arteries and its absence in most of the studies on isolated lungs or intact rats (see above) have not been directly addressed. It is likely that three factors may play a role: vessel size/type (conduit vs. resistance), resting passive tension, and precontraction by agonists.

Most of the studies in the isolated vessels utilize large, conduit arteries, which contribute relatively little to the total pulmonary vascular resistance in the whole lung. The total resistance is controlled mostly by the small,

peripheral vessels (68). The large arteries express more eNOS (see sect. II*A2*) and are more reactive to exogenous NO (13) than the smaller, more peripheral arteries. Several (61, 198, 215, 363), even though not all (349), studies performed on small, distal pulmonary arteries found no significant contractile response to NOS inhibitors. On the other hand, unchanged tension after administration of NOS inhibitors has also been described on several occasions in large pulmonary arteries in vitro (265, 388).

The degree of passive stretch of the vascular ring preparations is another potentially confounding factor. In most studies, the resting, passive stretch is set to a level that results in the largest constrictor response to depolarization induced by an elevation of the extracellular potassium concentration. For pulmonary arteries, the passive stretch force found in this manner is 500 mg or more, most often around 800 mg. With the use of the Laplace equation, the wall tension can be used to calculate the corresponding transmural pressure (245, 270). A stretch force of 500 mg corresponds to a transmural pressure of ~30 mmHg in the large pulmonary arteries and of ~50 mmHg in the smaller pulmonary arteries (270). In vivo, pulmonary arterial pressure above 20 mmHg is diagnosed as pulmonary hypertension (295). In other words, vasoconstrictor reactivity of the isolated pulmonary vessels is maximal at wall stretch levels corresponding to markedly elevated pulmonary arterial pressure. When this high level of wall stretch is used as a baseline, the administration of NOS inhibitors does not accurately address the problem of NO synthesis in the normal, resting pulmonary vasculature, even though vessels isolated from normal rats are used. Instead, the finding of a vasoconstrictor response to NOS inhibitors under these conditions (16, 127, 169, 349, 405, 409) indirectly supports the idea discussed below that basal NO synthesis is elevated in situations associated with increased pulmonary arterial wall tension. To yield more direct information about the role of NO in the normal, resting pulmonary vasculature, measurements would be needed of the responses to NOS inhibitors at a range of stretch values including those corresponding to the normal physiological transmural pressures. MacLean and McCulloch (215) found a negligible response of the rat pulmonary resistance arterial rings to L-NAME when transmural tension was set to 235 mg (equivalent to intravascular pressure of ~16 mmHg).

Similarly, although it is customary to use the pulmonary arterial rings precontracted with various agonists (typically, phenylephrine or norepinephrine), the nature of the interaction between the NOS inhibitors and the preexisting active tension is poorly defined. Consequently, it is not clear which degree of precontraction in vitro models the resting, usually fully dilated pulmonary circulation (5, 69, 76, 104, 154, 317) and which precontraction is more similar to a vasoconstricted state in vivo. Thus the interpretation of the results is uncertain.

B) DOG. Most of the available evidence indicates that L-NA and L-NAME do not cause pulmonary vasoconstriction in the dog. This is true in the isolated perfused left lower lobe (129), isolated whole lung (21, 64), and intact anesthetized (205) and conscious (256) dogs. On the other hand, Perrella and co-workers (278, 279) found significant pulmonary vasoconstrictor response to L-NMMA in anesthetized dogs. Thus, as in the intact rat, pulmonary vasoconstriction in dogs appears to be produced by L-NMMA, but not by L-NA or L-NAME, supporting the possibility that L-NMMA's vasoconstrictor action could be due to its more pronounced NOS-unrelated effects.

C) CAT. Originally, L-NAME (100 mg/kg iv) was shown to cause vasoconstriction in the pulmonary circulation of the cat (70, 231). The same laboratory recently published an elegant study in cats demonstrating that a novel NOS inhibitor, L-N^5-(1-iminoethyl)-ornithine, increased pulmonary vascular resistance only at high doses (>10 mg/kg iv). These higher doses had no more inhibitory effect on the endothelium-dependent vasodilation to acetylcholine, bradykinin, and substance P than lower doses (1–10 mg/kg iv) that were without effect on the resting pulmonary vascular resistance (70) (Fig. 4). Thus NOS-unrelated effects of L-N^5-(1-iminoethyl)-ornithine seem to be responsible for the pulmonary vasoconstriction. The authors themselves interpret their results as showing that the "basal release of NO does not play an important role in the maintenance of baseline tone in the pulmonary vascular bed of the cat" (70).

D) RABBIT. Data in rabbits are conflicting. On one hand, Persson and co-workers (280, 281, 392) found a significant

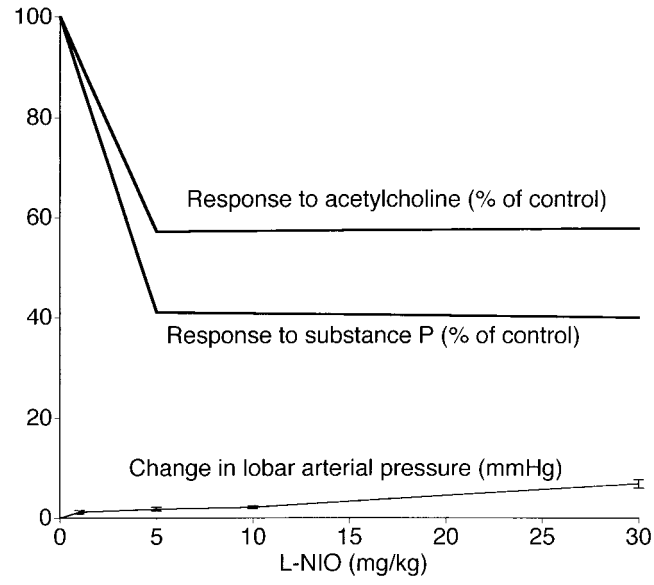

FIG. 4. Higher doses of a nitric oxide synthase (NOS) inhibitor, L-N^5-(1-iminoethyl)-ornithine (L-NIO), required to produce pulmonary vasoconstriction, are not more effective in reducing endothelium-dependent vasodilation than lower doses, which do not change pulmonary vascular resistance in intact cats. [Data from DeWitt et al. (70).]

vasoconstrictor response to L-NAME (30 mg/kg iv) in open-chest rabbits. On the other hand, other groups reported an unchanged vascular resistance in isolated rabbit lungs perfused either with a buffer solution or with blood after administration of L-NMMA (128) or L-NAME (172, 208, 400). Sprague et al. (346) suggested that erythrocytes may respond to mechanical deformation during their passage through microvessels by releasing cAMP that, in turn, evokes vascular NO synthesis. They found that rabbit and human (but not dog) erythrocytes release cAMP in response to mechanical deformation. L-NAME produced vasoconstriction in isolated rabbit lungs in the presence of human, but not dog, erythrocytes (346).

E) PIG. An increased pulmonary vascular resistance in response to L-NA and L-NAME was reported in anesthetized pigs (8, 380) and in isolated pig lungs (63, 64). However, a lack of a vasoconstrictor effect of L-NAME was also found in anesthetized pigs (77).

F) SHEEP. Today, sheep are the only species where NOS inhibitors appear to consistently cause pulmonary vasoconstriction; we are unaware of studies in which NOS inhibitors would have not increased pulmonary vascular resistance in sheep. L-NA and L-NAME (20–25 mg/kg iv) were reported to cause pulmonary vasoconstriction in an intact adult sheep (185, 211, 237) and in isolated sheep lung (64).

G) HORSE. In resting horses, L-NAME (20 mg/kg iv) caused a minimal rise in pulmonary arterial pressure (218). In this study, the pulmonary arterial pressure value before L-NAME administration is not given, but it is said to be similar to that in horses in a control study (no L-NAME given), where it was 31.3 ± 1.2 mmHg. After L-NAME administration, the pulmonary arterial pressure was 31.4 ± 1.0 mmHg. At the same time, L-NAME increased systemic blood pressure by 36 mmHg. Thus the pulmonary response to L-NAME was relatively negligible in comparison with the systemic effect. This conclusion is further reinforced by the results in exercise, showing that the pulmonary vascular pressure-flow relationship was unaffected by L-NAME (218).

H) MOUSE. In mice, acute administration of L-NAME (100 mg/kg iv) caused systemic vasoconstriction, but both pulmonary arterial pressure and pulmonary vascular resistance were unchanged (354). Similarly, when L-NAME was infused in mice by minipumps for 5 days (100 mg · kg^{-1} · day^{-1}), systemic arterial pressure and vascular resistance were significantly elevated, whereas pulmonary arterial pressure and vascular resistance were not (354). Endothelium-dependent vasodilation to acetylcholine was inhibited. In isolated mouse lungs perfused at constant flow rate, acute L-NA administration (10^{-4} M) did not alter baseline perfusion pressure (80).

The mouse is a species with an advantage of a technical feasibility of producing individuals with targeted gene disruption. Steudel et al. (354) were the first to study the pulmonary circulation in mice with targeted disruption of the gene encoding eNOS. As expected, the eNOS −/− mice had impaired endothelium-dependent vasodilator response to acetylcholine and marked systemic hypertension. They also had a somewhat higher mean pulmonary arterial pressure (19.0 ± 0.8 mmHg) than the wild-type mice (16.4 ± 0.6 mmHg) and significantly elevated total pulmonary resistance due to reduced cardiac output (354). Fagan and co-workers (79, 80) confirmed that the mice with the null mutation of the eNOS gene had increased right ventricular systolic pressure compared with the wild-type mice. Right ventricular systolic pressure was normal in nNOS −/− mice and elevated in mice with iNOS gene disruption, although to a lesser degree than in the eNOS −/− mice (80). However, it is useful to keep in mind that the studies by Fagan and co-workers (79, 80) were performed at an altitude of ~1,600 m. It has been shown previously that even the very mild hypoxia experienced at that altitude is sufficient to elicit pulmonary hypertension in a susceptible rat strain (326). It is thus possible that the elevated right ventricular systolic pressure found in eNOS −/− mice in mild hypoxia (79, 80) may reflect the role of eNOS in limiting the pulmonary hypertensive response to chronic hypoxia (discussed in sect. IV).

It is striking in the study of Steudel et al. (354) that the transgenic eNOS-deficient mice have moderate pulmonary hypertension whereas the wild-type mice treated with NOS inhibitor have none. An explanation of this paradox can be related to the role of NO in the neonatal pulmonary circulation (see sect. II*B*). Endogenous NO production is known to be essential for a successful transition of the fetal (high pressure, low flow) pulmonary circulation into the postnatal (low pressure, high flow) one (3, 59). Thus it is likely that the postnatal transition of the pulmonary circulation of the eNOS-deficient transgenic mice was impaired in a manner similar to that shown in newborn lambs treated with NOS inhibitors (3, 59). In this respect, the moderate pulmonary hypertension seen in the transgenic eNOS-deficient mice (354) appears to be a consequence of an incomplete postnatal transition of the pulmonary circulation rather than a consequence of the absent NO production at the time of measurement. In contrast, the acute and the 5-day-long treatment of the adult wild-type mice with L-NAME did not affect the neonatal development of the pulmonary circulation and only could influence the NO synthesis at around the time of measurements. Thus, with respect to studying the role of NO in the pulmonary vascular tone regulation in adults, the results with NOS inhibitors in wild-type mice could be more relevant than the results with transgenic eNOS-deficient mice, which are invaluable in confirming the importance of NO for normal development of the pulmonary circulation. This possibility is supported by the observation that the increased pulmonary vascular resis-

tance in the transgenic eNOS-deficient mice was not reduced by exogenous NO (354).

I) HUMAN. As in other species, data on the effects of NOS inhibition on the human pulmonary circulation are somewhat conflicting.

Stamler et al. (348) reported that L-NMMA caused pulmonary vasoconstriction in healthy human volunteers. However, their study showed a substantially lower reactivity of the pulmonary, compared with systemic, circulation to L-NMMA (Fig. 5). Pulmonary vascular resistance was increased only by the highest dose used (1 mg · kg^{-1} · min^{-1} iv), which represented the limit tolerable by the systemic circulation (31, 348). The pulmonary vascular resistance increased as a result of a drop in cardiac output; pulmonary arterial pressure was unchanged. Systemic arterial pressure and vascular resistance were significantly increased not only by this higher dose, but also by doses as much as 10 times lower, which had no effect on the pulmonary vascular resistance (Fig. 5). Thus, in humans, as in other species (see above), L-NMMA in doses sufficient to inhibit NO production in the systemic vessels had no effect on the pulmonary circulation. Perhaps the nonspecific, NOS-unrelated effects of L-NMMA could be important in the pulmonary vasoconstriction seen at high doses.

Using intravascular Doppler sonography, Celermajer et al. (47) observed dose-dependent decreases in segmental pulmonary blood flow in response to locally infused L-NMMA in six children with congenital heart disease and normal pulmonary vascular resistance.

Data obtained on in vitro human preparations are also inconclusive. On one hand, a relatively high dose of L-NMMA (10^{-4} M) did not increase tension of resting isolated small human intrapulmonary arteries (61). On the other hand, a vasoconstrictor response to a lower dose of L-NAME (10^{-5} M) was found in isolated perfused human lungs (64). Nonetheless, this response was only partially reverted by excess L-arginine, suggesting the possibility of participation of NOS-unrelated effects of L-NAME. Samples of tissue obtained from human patients tend to be inhomogeneous, possibly contributing to discrepancies among studies.

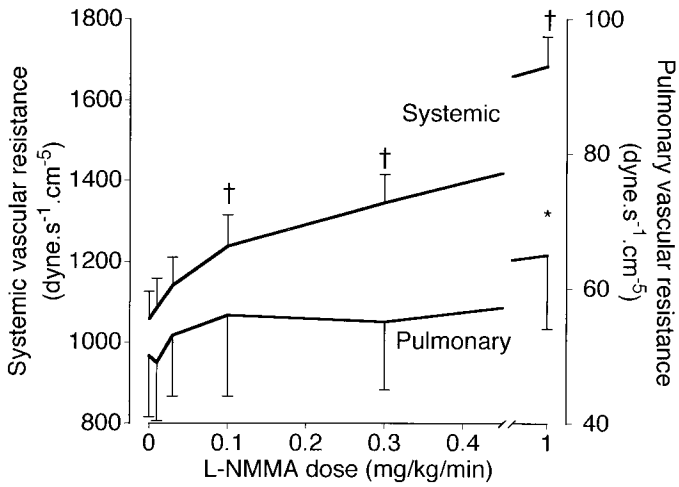

FIG. 5. N^{ω}-monomethyl-L-arginine (L-NMMA) causes pulmonary vasoconstriction in human volunteers only at a high dose, whereas lower doses are sufficient for systemic vasoconstriction. Data are means ± SE. *$P < 0.05$. †$P < 0.01$. [Data from Stamler et al. (348).]

Plasma concentration of an endogenous NOS inhibitor, N^{G},N^{G}-dimethylarginine, is elevated in patients with chronic renal failure to a level which inhibits NOS in vitro (378). Pulmonary hypertension does not belong among the complications of chronic renal failure (22). Although very indirect, this fact is consistent with the possibility that NO is less important in the regulation of the pulmonary than systemic vascular tone in humans.

J) INTERIM SUMMARY: EFFECTS OF NOS INHIBITORS ON BASAL PULMONARY VASCULAR TONE IN ADULTS. The studies of the effects of NOS inhibitors on the resting pulmonary vascular tone in adults of different species are summarized in Table 1. In the rat, numerous studies using the isolated buffer-perfused lungs consistently show no response to NOS inhibitors. The same finding is most often reported in blood-perfused lungs, even though a significant vasoconstriction has also been repeatedly found in this preparation. Similarly, reports of no significant response appear to prevail in the intact rat, although a vasoconstriction has also been seen. The studies on the isolated rat pulmonary arterial rings in vitro, showing both an increase and no change in tone after NOS inhibition, are difficult to evaluate conclusively because of the lack of characterization and normalization of the passive and active tension.

The canine pulmonary circulation is not reactive to NOS inhibitors in most studies, although again, there are exceptions. In the cat, vasoconstriction has been shown in response to L-NAME, but not to L-N^{5}-(1-iminoethyl)-ornithine at a dose sufficient to inhibit NO-dependent vasodilation. In the rabbit, a vasoconstrictor response to NOS inhibitors has been shown in open-chest studies but not in isolated lung experiments. In pig and sheep, virtually all published studies except one (77) found a pulmonary vasoconstrictor response to NOS inhibitors. In the horse and mouse, on the other hand, there is minimal or no response to L-NAME. In humans, the limited evidence available so far appears to support the existence of a vasoconstrictor response to L-NMMA and L-NAME. However, only the highest L-NMMA dose tolerated by the systemic circulation is effective in the pulmonary circulation in conscious volunteers (348), and L-NMMA does not constrict isolated human peripheral pulmonary arterial rings in vitro (61).

Hence, in most species NOS inhibitors do not consistently cause significant pulmonary vasoconstriction in doses that minimize the risk of nonspecific effects yet are effective in NOS inhibition. However, the presence of a

TABLE 1. *Effects of NOS inhibitors on basal pulmonary vascular tone in various preparations in adults*

Species	Preparation	NOS Inhibitor	Effect	Reference Nos.
Rat	Conscious	L-NMMA	+	229
		L-NAME	=	98
	Anesthetized	L-NAME	=†	132, 153, 155
		L-NA	=	265
	Isolated lungs			
	Blood perfused	Nonselective*	=	38
		L-NMMA	=	20, 23, 100, 210, 309, 362, 412
		L-NAME	=	20, 83, 319, 359, 406
			+	21, 311, 376, 394
		L-NA	=	84
			+	130
	Buffer perfused	L-NMMA	=	16, 18, 26, 44, 74, 137
		L-NAME	=	21, 73, 134, 135, 158, 376, 394
		L-NA	=	134, 265, 302, 304, 318
	Pulmonary arterial rings	L-NMMA	=	61
			+	16, 405
		L-NAME	=	215, 363, 388
			+	349, 405, 409
		L-NA	=	200, 265, 322
			+	127, 169, 322
Dog	Conscious	L-NA	=	256
	Anesthetized	L-NMMA	+	278, 279
		L-NA	=	204
	Isolated lungs			
	Blood perfused	L-NAME	=	21, 64
	Buffer perfused	L-NAME	=	64
	Isolated lobe			
	Blood perfused	L-NA	=	129
Cat	Anesthetized, controlled lung flow	L-NAME	+	70, 231
		L-NIO	= +	70
Rabbit	Open chest	L-NAME	+	280, 281, 392
	Isolated lungs			
	Blood perfused	L-NAME	=	208
			+‡	346
	Buffer perfused	L-NMMA	=	128
		L-NAME	=	172, 208, 400
Pig	Anesthetized	L-NAME	=	77
			+	379
		L-NA	+	8
	Isolated lungs			
	Blood perfused	L-NAME	+	64
		L-NA	+	63
	Buffer perfused	L-NMMA	+	64
		L-NAME	+	64
Sheep	Conscious	L-NAME	+	237
		L-NA	+	185
	Anesthetized	L-NAME	+	237
		L-NA	+	211
	Isolated lungs			
	Blood perfused	L-NAME	+	64
	Buffer perfused	L-NAME	+	64
Horse	Conscious	L-NAME	=	218
Mouse	Anesthetized	L-NAME	=	354
	Isolated lungs			
	Buffer perfused	L-NA	=	80
Human	Conscious	L-NMMA	= +	348
	Anesthetized	L-NMMA	+	47
	Isolated lungs			
	Buffer perfused	L-NAME	+	64
	Pulmonary arterial rings	L-NMMA	=	61

=, Absence of a significant change of resting vascular tone; +, significant vasoconstriction; = +, only the highest dose caused pulmonary vasoconstriction; L-NIO, L-N^5-(1-iminoethyl)-ornithine; L-NMMA, N^{ω}-monomethyl-L-arginine; L-NAME, N^{ω}-nitro-L-arginine methyl ester; L-NA, N^{ω}-nitro-L-arginine; NOS, nitric oxide synthase. * Eicosatetraynoic acid, nordihydroguaiaretic acid, and hydroquinone were used as nonselective inhibitors in the study of Brashers et al. (38). † In the study of Hyman et al. (153), L-NAME increased pulmonary arterial pressure but not pulmonary vascular resistance when lung blood flow was not controlled. ‡ In the study of Sprague et al. (346), L-NAME caused vasoconstriction in the presence of human, but not dog, erythrocytes.

significant pulmonary vasoconstrictor response to NOS inhibitors has been reported more often in certain species, namely, the pig, sheep, and human. Thus the crucial question whether the response in humans is substantially similar to that in experimental animals remains without a definitive answer.

2. NOS expression

In rats, NADPH diaphorase staining (a classical method for constitutive NOS localization), immunohistochemical studies using eNOS antibodies, and in situ hybridization with eNOS mRNA probe found essentially no NOS in the endothelium of the small, peripheral pulmonary arteries (those most responsible for pulmonary vascular resistance) (173, 201, 375, 402, 403). In contrast, eNOS expression was considerable in large pulmonary vessels, while <25% of medium-sized vessels showed eNOS staining (Fig. 6). No NOS immunostaining was detected in the smooth muscle of the pulmonary vessels of all sizes (403). Inducible NOS mRNA was not detectable by reverse-transcriptase polymerase chain reaction in the pulmonary arterial wall of normal rats (44).

Data on NOS expression in humans are contradictory. Kobzik et al. (184) found variable NADPH diaphorase staining in the endothelium of large pulmonary arteries and no staining in the pulmonary microvasculature. That resembles the results in the rat (173, 201, 402, 403). On the other hand, Giaid and Saleh (125) reported dense eNOS immunostaining in pulmonary arteries of all sizes. Several details of their technique were challenged by Xue and Johns (401), who themselves observed only a weak eNOS immunostaining in normal human pulmonary vessels.

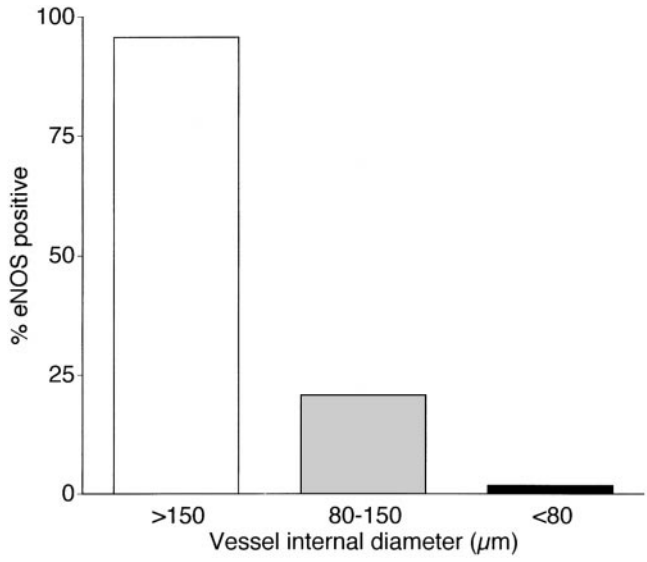

FIG. 6. Endothelial NOS (eNOS) expression is marked in the large pulmonary vessels of the rat and negligible in the peripheral ones. [Data from Xue et al. (403).]

An interesting possible source of NO in the pulmonary circulation might be NO produced in the paranasal sinuses (213) and inhaled with each breath. When autoinhalation of nasal NO was prevented in patients recovering from open heart surgery by having them breathe through their mouth, their pulmonary vascular resistance was slightly but significantly higher than when they breathed through their nose (336). However, pulmonary vascular resistance was slightly reduced in only 4 of 12 intubated, ventilated patients (who cannot inhale NO from their nose) when the air derived from the patient's own nose was aspirated and led into the inhalation limb of the ventilator (212). Hence, this interesting idea and its relevance to the normal, healthy pulmonary circulation needs more experimental clarification.

B. Fetal and Neonatal Pulmonary Circulation

Pulmonary circulation in the fetus differs substantially from that in the adult. In the adult, where blood is oxygenated in the lung, the whole cardiac output flows through the lung at a low pressure. Vascular resistance is very low. In the fetus, the oxygenation of the blood does not take place in the lung, and the lung receives only a fraction of cardiac output at high pressure. Vascular resistance is high. In these aspects the normal fetal pulmonary circulation bears more similarities to the systemic vascular beds or to the hypertensive pulmonary circulation of the adult than to the normal adult pulmonary vascular bed. Correspondingly, the role of NO may differ between normal fetal and adult pulmonary circulation. A detailed discussion of the role of NO in the fetal and neonatal pulmonary circulation, available in relevant reviews (2, 4, 89, 181, 307, 350), is beyond the scope of this review; we briefly summarize only the aspects important for a comparison with the situation in the adult.

eNOS immunostaining is dense and eNOS mRNA level is high in the fetal pulmonary circulation; they both decrease postnatally (131, 148, 173, 258, 404). Lung eNOS mRNA levels and immunoreactivity are high in the fetal rat, highest around the first postnatal day, and minimal in adult rats (148, 173, 404) (Fig. 7). There is even evidence for iNOS expression and hemodynamically relevant activity of iNOS in the fetal pulmonary circulation (291, 404), reminiscent of the iNOS expression in pulmonary hypertension in adults (see sect. IV*C*). NOS inhibitors cause pulmonary vasoconstriction in fetal (3, 182, 241) and newborn (67, 88, 251, 252, 277) lambs, piglets, and guinea pigs. The magnitude of this response decreases with postnatal age (277).

Endogenous NO synthesis plays an important role in the transition of the pulmonary circulation from the high-resistance fetal state to the low-resistance postnatal one at birth. The postnatal decline of pulmonary vascular

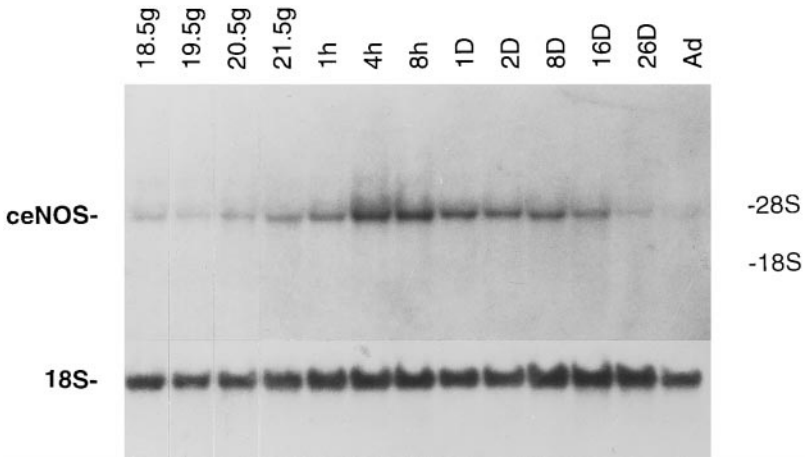

FIG. 7. Pulmonary eNOS gene expression culminates at the perinatal period and decreases with postnatal age. RNA extracted from lungs of individual fetal (days 18.5, 19.5, 20.5, and 21.5 of 22-day gestation), postnatal (1, 4, and 8 h and 1, 2, 8, 16, and 26 days after birth), and adult (Ad) rats were hybridized with radiolabeled NOS cDNA and 18S oligonucleotide probes (the later to confirm integrity of the extracted RNA). [From Kawai et al. (173).]

resistance in lambs is attenuated by acute (3, 59, 182, 241) and chronic (90) L-NA treatment. The pulmonary vasodilation at birth is caused to a great extent by the increase in lung P_{O_2}. The oxygen-induced decline in the pulmonary vascular resistance in late fetal lambs is attenuated by L-NA (49, 59, 233, 241, 364).

Thus the available evidence is consistent with the view that basal NO synthesis in the pulmonary circulation is high in the fetus, culminates at the time of birth, declines postnatally, and is relatively small during adulthood.

III. ROLE OF NITRIC OXIDE IN PULMONARY VASOCONSTRICTION

Vasoconstriction is among the factors contributing to the development of most forms of chronic pulmonary hypertension. It is therefore useful to briefly explore the NO activity during pulmonary vasoconstriction.

One of the most physiologically important pulmonary vasoconstrictor stimuli is alveolar hypoxia, and much of our knowledge of the pulmonary vasoreactivity comes from experiments studying hypoxic pulmonary vasoconstriction. Because oxygen is needed for NO synthesis, hypoxia has been hypothesized to cause pulmonary vasoconstriction by inhibiting basal NO synthesis in the pulmonary vasculature. Indeed, the apparent Michaelis constant (K_m) of the eNOS for oxygen, 7.7 μM (299), predicts that NO output can be significantly reduced when P_{O_2} drops below ~30 mmHg. However, as discussed below, there is a solid evidence available today that physiologically relevant degrees of acute hypoxia actually potentiate NO synthesis in the pulmonary circulation, as do other vasoconstrictor stimuli. This increased NO production limits the vasoconstrictor-induced increase in intravascular pressure. Apparently, the effect of the reduced substrate (O_2) availability on the eNOS activity can be overridden during pulmonary vasoconstriction by other regulatory mechanisms, such as the elevated $[Ca^{2+}]_i$ (133), at least if hypoxia is not too severe. In this context it is useful to keep in mind that in vivo, P_{O_2} in the adult pulmonary circulation does not drop below ~30 mmHg even in hypoxia as extreme as that experienced during exercise on the summit of Mt. Everest (358).

It should also be noted that in contrast to the pulmonary vessels, the production of NO in the distal airways is reduced during ventilatory hypoxia (128). It is possible that at least a portion of this NO is produced by the neuronal isoform of NOS. Its K_m for oxygen (23.2 μM) is higher than that of the endothelial isoform (299). Even less severe degrees of hypoxia therefore may suppress its activity. However, pharmacological inhibition of the airway NO production does not mimic hypoxic pulmonary vasoconstriction (128), suggesting that the hypoxic decrease in airway NO synthesis is not responsible for the increase in the pulmonary vascular tone.

The studies of the effects of NOS inhibitors on pulmonary vasoconstriction are summarized in Table 2. As already mentioned, the initial attempts to determine the role of EDRF in the pulmonary circulation started before the selective NOS inhibitors became available. It was shown that several putative blockers of the EDRF-cGMP pathway, including eicosatetraynoic and nordihydroguaiaretic acids (lipoxygenase antagonists), hydroquinone (antioxidant), and methylene blue (gyanlylate cyclase inhibitor), potentiated the hypoxic vasoconstriction in the isolated rat lungs (38, 228). The baseline tone was unaffected.

After the more specific, L-arginine-based NOS inibitors became available, Archer et al. (16) were the first to show that L-NMMA, while not changing the baseline vascular tone, significantly potentiates the vasoconstrictor responses of isolated rat lungs to hypoxia and angiotensin II (Fig. 8). A logical interpretation is that vasoconstriction increases the normally low NO synthesis in the pulmonary circulation. This observation was subsequently confirmed

TABLE 2. *Effects of NOS inhibitors on acute pulmonary vasoconstriction in various preparations*

Species	Preparation	Stimulus	Effect	Reference Nos.
Rat	Conscious	Hypoxia	+	98, 229
	Anesthetized	Hypoxia	+	155
		U46619	+	153
	Isolated lungs	Hypoxia	+	16, 18, 20, 23, 38, 73, 83, 100, 132, 137, 158, 210, 265, 309, 359, 362, 406, 412
		Angiotensin II	+	16, 18, 83, 132, 158, 210, 362
		Endothelin-1	+	20
		U46619	+	302, 318
		U46619	= +	395
		Serotonin	=	83
		Almitrine	+	20
		Dexfenfluramine	+	390
	Pulmonary arterial rings	Hypoxia	+	16
			=	198
			−	169, 322
		Phenylephrine	+	11, 169, 265
		Norepinephrine	=	11
Mouse	Isolated lungs	Hypoxia	+	80
Dog	Conscious	U46619	+	256
	Anesthetized	Hypoxia	+	41, 204, 205, 278
	Isolated lobe	Hypoxia	+	129
Rabbit	Anesthetized	Hypoxia	+	347
	Open chest	Hypoxia	+	280, 281, 392
	Isolated lungs	Hypoxia	+	128
		U46619	+	128, 172, 208
		Angiotensin II	+	128
Pig	Anesthetized	Hypoxia	+	77, 102
	Pulmonary arterial rings	Hypoxia	+	263
Human	Conscious	Hypoxia	+	31
	Pulmonary arterial rings	Phenylephrine	+	61

L-NMMA, L-NAME, or L-NA was used in all studies except in that of Brashers et al. (38), where eicosatetraynoic acid, nordihydroguaiaretic acid, and hydroquinone were used. +, Vasoconstriction potentiated by NOS inhibitors; =, unchanged vasoconstriction; = +, response was potentiated only in lungs perfused at constant flow, but not at constant pressure; −, reduced vasoconstriction. Other definitions are as in Table 1.

many times in isolated rat lungs using L-NMMA, L-NAME, and L-NA for NOS inhibition and hypoxia, angiotensin II, endothelin-1, dexfenfluramine, or a thromboxane analog U46619 as vasoconstrictor stimuli (18, 20, 23, 73, 83, 100, 137, 158, 210, 265, 302, 309, 359, 362, 390, 395, 406, 412). Vasoconstrictor responses to acute hypoxia and angiotensin II were also potentiated in lungs isolated from rats after treating them with L-NAME for 3 wk (132). Acute administration of L-NAME or L-NMMA potentiated hypoxic pulmonary vasoconstriction in awake (98, 229) and anesthetized (155) rats. Pulmonary vasoconstrictor response to U46619 was potentiated by L-NAME in anesthetized rats (153). NOS inhibitors added to the bath of isolated rat pulmonary arterial rings in vitro contracted by various stimuli cause further increase in tension (11, 16, 127, 169), although no change (198) or even a decrease (169, 322) in hypoxic contraction has also been observed in this preparation. Using intravital videomicroscopy, Suzuki et al. (359) observed no potentiation of the hypoxic contraction of very small pulmonary arterioles (~25 μm) by L-NAME, although the total pressor response of the whole lung was markedly increased. This suggests that NO production is stimulated only in a portion of vessels affected by vasoconstriction.

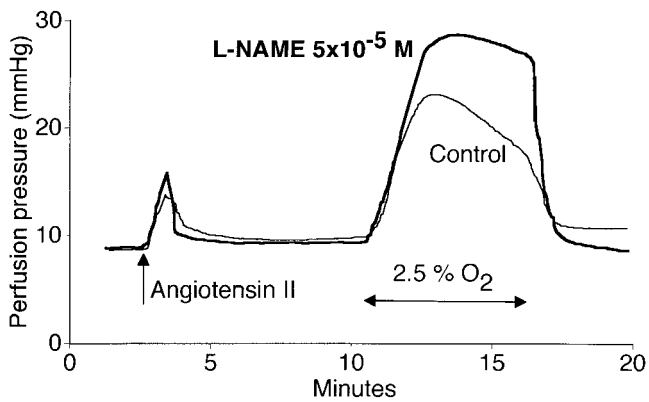

FIG. 8. Inhibition of NOS by N^{ω}-nitro-L-arginine methyl ester (L-NAME) potentiates pulmonary vasoconstrictor reactivity to angiotensin II and hypoxia. A representative tracing is shown of an experiment in isolated rat lungs perfused with Krebs-albumin solution at constant flow rate (so that increases in pressure directly reflect vasoconstriction). Thin line, control run without L-NAME; thick line, a subsequent run in the presence of 5×10^{-5} M L-NAME. Two subsequent control runs did not differ from one another in a separate group of lungs.

A potentiation of the pulmonary vasoconstrictor responses to hypoxia and U46619 by L-NA, L-NAME, or L-NMMA was observed in conscious (256) or anesthetized

(41, 204, 205, 278) dogs and in isolated left lower lobe of the dog lung (129). Increased reactivity to hypoxia, U46619, or angiotensin II was also found after NOS inhibitors administration in intact (347) and open-chest rabbits (280, 281, 392) and in isolated rabbit lungs (128). Hypoxic pulmonary vasoconstriction was increased by L-NAME in intact pigs (77, 102) and by L-NMMA in isolated pig intrapulmonary arteries (263). Hypoxic pulmonary vasoconstriction potentiated by L-NMMA was also reported in human patients (31). Phenylephrine-increased tension was further elevated by L-NMMA in human small pulmonary arteries in vitro (61).

Hypoxic vasoconstriction is potentiated by L-NA in isolated perfused mouse lungs (80). Lungs isolated from transgenic mice with targeted disruption of the eNOS gene show hypoxic vasoconstriction that is about twice as large as that seen in lungs of wild-type mice, but cannot be increased further by L-NA (80). This suggests that eNOS is the main source of NO produced in the pulmonary circulation during acute hypoxia. Hypoxic vasoconstriction is normal in lungs isolated from both nNOS −/− mice and iNOS −/− mice (80).

The potentiation of pulmonary vasoconstriction by NOS inhibitors suggests that vasoconstriction increases NO synthesis in the pulmonary circulation. A more indirect proof for this conclusion was added by Cohen et al. (58). They found that hypoxic vasoconstriction was reduced in isolated rat lungs by an inhibition of cGMP phosphodiesterase, which did not change the baseline tone. Because cGMP phosphodiesterase inactivates NO's second messenger, cGMP, this finding is consistent with increased NO levels during hypoxic pulmonary vasoconstriction, although other factors than NO can elevate cGMP.

Direct measurements of NO and its oxidation products (NO_x) in the lung effluent during acute hypoxia are infrequent. Grimminger et al. (128) found unchanged NO_x in perfusate of isolated rabbit lungs during ventilation with a hypoxic gas, although hypoxic pulmonary vasoconstriction in that study was markedly potentiated by L-NMMA. Naoki et al. (249), on the other hand, reported a significant increase in perfusate NO_x during acute hypoxia in isolated rat lung. The reason for the discrepancy is unknown, although it might be related to gradual improvements in the sensitivity of NO detection.

The mechanism whereby vasoconstriction increases NO synthesis in the pulmonary circulation has not been much studied. Wilson et al. (395) found that L-NMMA potentiated the U46619-induced vasoconstriction only in lungs perfused at constant flow, but not at constant pressure. One of the key differences between constant flow and constant pressure perfusion is that vasoconstriction increases shear stress only in the former. Therefore, these data suggest that the stimulus for increased NO synthesis during pulmonary vasoconstriction is the increase in shear stress. Shear stress is known to be a potent stimulus for endothelial NO synthesis (60, 138, 167, 189, 192, 200, 236). However, additional factors must be at play because NOS inhibitors potentiate vasoconstriction in isolated pulmonary arterial rings in vitro (11, 16, 127, 169, 265), where there are no changes in shear stress. Hypoxia itself, in the absence of changes of shear stress or vascular smooth muscle tone, increases NO synthesis in cultured pulmonary artery endothelial cells (133). Further supporting a direct, shear stress-independent influence of hypoxia on the eNOS in the pulmonary vasculature is a recent report by Le Cras et al. (200). Using rats, they found that eNOS is upregulated by chronic hypoxia equally in the normal right lung and in the left lung, in which blood flow (and therefore shear stress) had been severely reduced by creating a left pulmonary artery stenosis (see also sect. IV*C*).

IV. NITRIC OXIDE SYNTHESIS IN CHRONIC PULMONARY HYPERTENSION

The methods used to study the role of NO in chronic pulmonary hypertension include measurements of the effects of NOS inhibitors (or eNOS gene deficiency) on pulmonary vascular tone, measurements of NOS activity, studies of NOS expression, and evaluation of reactivity to endothelium-dependent vasodilators.

A. Effects of NOS Inhibitors

Several laboratories have shown that acute administration of L-NMMA, L-NAME, or L-NA to isolated adult rat lungs, which is usually without much effect in normal, control rats (see sect. II*A1*), causes a marked vasoconstriction in lungs of rats with chronic hypoxic pulmonary hypertension (20, 158, 265, 319, 375, 376) (Fig. 9). Similar presence of a marked vasoconstrictor response to NOS inhibitors (minimal in controls) was reported in intact rats with chronic hypoxic pulmonary hypertension (155, 265) and in pulmonary arterial rings isolated from such rats (215, 265, 388). In contrast, one study found that constriction in response to L-NA was reduced by chronic hypoxia in rat conduit pulmonary artery rings in vitro (169).

An increase in vascular resistance in response to L-NMMA or L-NA was also found in isolated lungs of rats with pulmonary hypertension induced by an injection of an alkaloid, monocrotaline (100, 265, 375), and of a rat strain with spontaneous pulmonary hypertension (375). These findings suggest that the increased vasoconstrictor reactivity to NOS inhibitors is related to the presence of pulmonary hypertension rather than to chronic hypoxia itself. This conclusion is further supported by the observation that the hyperreactivity to NOS inhibitors persists

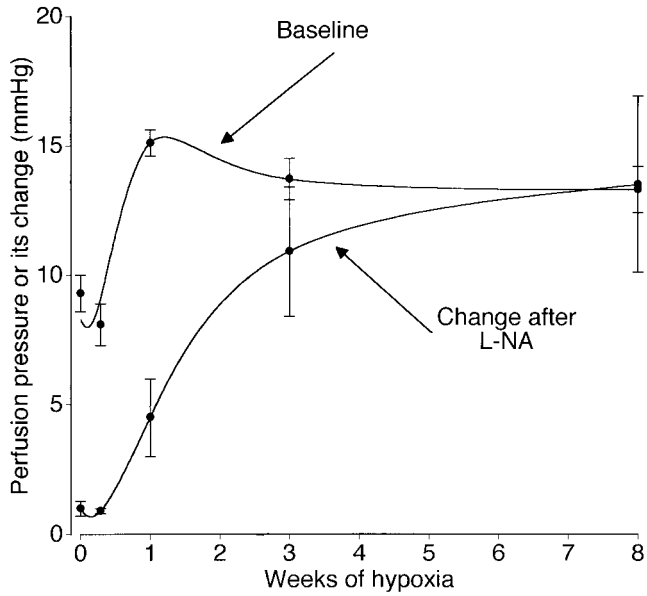

FIG. 9. Vasoconstrictor response to L-NA in isolated rat lungs increases with progression of chronic hypoxic pulmonary hypertension. Baseline perfusion pressure in the isolated rat lungs and its increase upon acute addition of L-NA are shown (means ± SE). [Data from Oka et al. (265).]

after 2 days of recovery from chronic hypoxia (when some degree of pulmonary hypertension is still present) (265). Pulmonary resistance arterial rings isolated from chronically hypoxic rats constrict significantly in response to L-NAME only when they are passively stretched to a tension corresponding to pulmonary hypertension (215). On the other hand, vasoconstrictor response to L-NA was not found in control lungs even after increasing flow rate to such a degree that the perfusion pressure reached the level found in chronically hypoxic lungs (20, 265). That suggests that the high intravascular pressure per se is not responsible. The vasoconstrictor response to NOS blockers in isolated lungs can be prevented by endothelin receptor blockers, as has been shown in rats with pulmonary hypertension induced by chronic hypoxia (247) or monocrotaline injection (100). The authors of these studies interpreted the data as showing that endogenous NO synthesis is elevated in the hypertensive lung and opposes vasoconstriction produced by the potent endogenous pulmonary vasoconstrictor endothelin-1 (50, 199, 287), the levels of which are increased in numerous forms of pulmonary hypertension (126, 206, 352). Endothelin is a known stimulus for NO synthesis (86, 287, 410).

Aside from studies showing an increased pulmonary vasoconstrictor response to NOS inhibitors in rats with chronic hypoxic pulmonary hypertension, there are also publications reporting a lack of such a potentiation (302, 304, 318, 412). The doses of L-NMMA and L-NA in these studies were sufficient to inhibit endothelium-dependent vasodilation (304) or to potentiate reactivity to vasoconstrictor stimuli (302, 318, 412). Cremona et al. (64) found no significant difference in vasoconstrictor response to L-NAME between isolated lungs from healthy human donors and lungs of patients with pulmonary hypertension. The reason for this discrepancy from other studies is unclear. Nevertheless, it is safe to conclude that experiments studying the effects of NOS inhibitors on the resting pulmonary vascular tone in adults do not support NO deficiency in pulmonary hypertension (Table 3). In the neonates, where the role of NO in the basal pulmonary vascular tone regulation seems different than in adults (see sect. II*B*), there is support both for (85) and against (29) reduced lung NO production in pulmonary hypertension.

Recently, mice with a targeted disruption of the eNOS gene began to be utilized to study the role of eNOS in pulmonary hypertension (79, 355). Steudel et al. (355) found that after 3–6 wk of hypoxia, several indices of pulmonary hypertension were greater in the eNOS-deficient than in the wild-type mice, including right ventricular systolic pressure, pulmonary arterial pressure, incremental total pulmonary vascular resistance, pulmonary vascular remodeling, and right ventricle free wall weight and thickness. The difference between the eNOS-deficient and wild-type mice was especially marked in the propor-

TABLE 3. *Effects of NOS inhibitors on resting pulmonary vascular tone in various types of pulmonary hypertension in adults*

Species	Preparation	Type of PHT	Effect	Reference Nos.
Rat	Intact	Chronic hypoxia	+	155, 265
	Isolated lungs	Chronic hypoxia	+	20, 158, 265, 319, 375, 376
			=	302, 304, 318, 412
		Monocrotaline	+	100, 265, 375
		Spontaneous	+	375
	Pulmonary arterial rings	Chronic hypoxia	+	215, 265, 388
			−	169
Human	Isolated lungs	Primary and secondary	=	64

PHT, pulmonary hypertension; +, =, and −, a greater, equal, and smaller, respectively, vasoconstriction in response to NOS inhibitors as compared with the control group (no PHT).

tion of muscularized small pulmonary vessels. This proportion is often considered an essential factor determining the severity of pulmonary hypertension (297). Fagan et al. (79) reported that eNOS-deficient mice were especially sensitive to a relatively mild degree of chronic hypoxia, whereas with severe hypoxia these authors did not find right ventricular systolic pressure to be significantly different between the wild-type and genetically manipulated mice. As in the study of Steudel et al. (355), the proportion of muscularized small pulmonary vessels was increased by chronic hypoxia considerably more in the eNOS-deficient than in the wild-type mice (79).

B. Measurements of NOS Activity

Only a few studies measured NOS activity in adult pulmonary hypertension. Isaacson et al. (158) found negligible accumulation of NO_x in the recirculating artificial perfusate of lungs isolated from control rats. In lungs isolated from rats with chronic hypoxic pulmonary hypertension, however, NO_x accumulation in the perfusate was significantly elevated (158, 325, 375) (Fig. 10). The faster NO_x accumulation in the perfusate of lungs of chronically hypoxic rats can be prevented by endothelin type B receptor antagonism (325). This suggests that endothelin-1, known to be upregulated in pulmonary hypertension (126, 206, 352), activates NOS via its action on type B receptors. Sato et al. (325) also found that perfusate NO_x accumulation is not accelerated in lungs of chronically hypoxic rats if they are ventilated with anoxic gas. The relevance of this finding to less severe hypoxia, compatible with long-term survival, remains to be elucidated. Although acute hypoxia decreases otherwise elevated plasma NO_x in intact rats with chronic hypoxic pulmonary hypertension (325), the contribution to this finding of NO produced in the systemic circulation is likely but unknown. The tendency for an increased release of NO into the perfusate of the isolated rat lungs did not reach significance in monocrotaline-induced pulmonary hypertension (375).

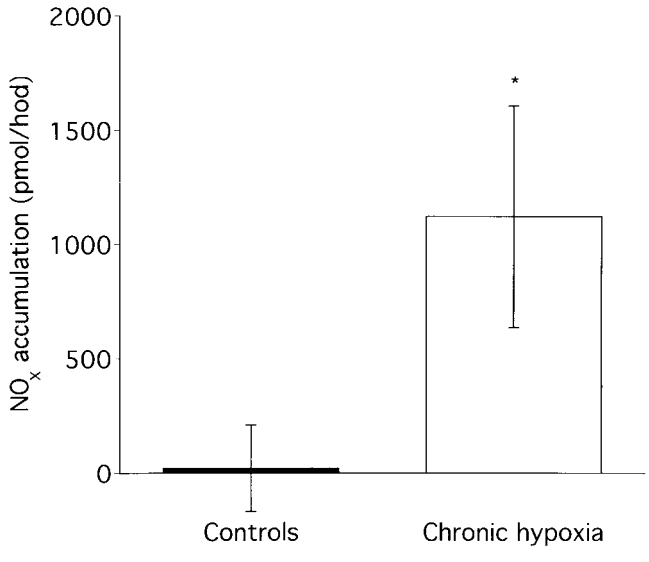

FIG. 10. Accumulation of NO and its oxidation product, nitrite, in the perfusate of isolated rat lungs is increased in chronic hypoxic pulmonary hypertension. Nitrite plus NO (NO_x) was measured by chemiluminescence. Nitrite was first reduced to NO by potassium iodide at low pH. Data are means ± SE. *$P < 0.05$. [Data from Isaacson et al. (158).]

NOS activity in a whole lung homogenate, measured as [^{3}H]arginine to [^{3}H]citrulline conversion, was doubled in rats with chronic hypoxic pulmonary hypertension compared with normoxic controls in two studies (338, 403) and unchanged in the third (99). It is interesting that the increase was in the cytosolic fraction, suggesting that the isoenzyme responsible for it might have been iNOS (403). The [^{3}H]arginine to [^{3}H]citrulline conversion was similar in lung homogenates of normoxic and chronically hypoxic piglets (327).

Plasma concentration of the NO end-oxidation product nitrate (157) is elevated in congenital heart disease patients with high flow pulmonary hypertension (361, 383). In fact, plasma nitrate levels correlate positively with pulmonary arterial pressure (and independently also with pulmonary blood flow) in children with ventricular septal defect (361). The plasma nitrate concentration in these patients is reduced dramatically after corrective surgery (361).

NO production in the rat main pulmonary artery, measured as cGMP accumulation in the presence of inhibitors of other sources of cGMP than NO, was reduced by chronic hypoxia (339). The hemodynamic relevance of this finding, however, is probably small, as is the contribution of the main pulmonary artery to the overall resistive properties of the whole pulmonary circulation. A potentially confounding factor in this study is the extreme hyperoxia (P_{O_2} = 680 mmHg) in which the vessels were incubated.

Although NOS activity appears elevated in most of the pulmonary vasculature in chronic pulmonary hypertension, this increase in NO production is lower than that found after activating iNOS in severe inflammation. From the published data, NO production in chronic hypoxic pulmonary hypertension can be estimated at less than ~100 nmol/h in a whole rat lung (158, 338, 375, 403) (Fig. 10). In ischemia-reperfusion injury, tissue NO concentration can reach micromolar values within hours (216).

C. NOS Expression

NADPH diaphorase staining and eNOS immunohistochemistry revealed that whereas the endothelium of the small, peripheral pulmonary arteries of normal, control rats contains little eNOS (see sect. II*A2*), the same vessels are rich in eNOS in adult rats with pulmonary hyperten-

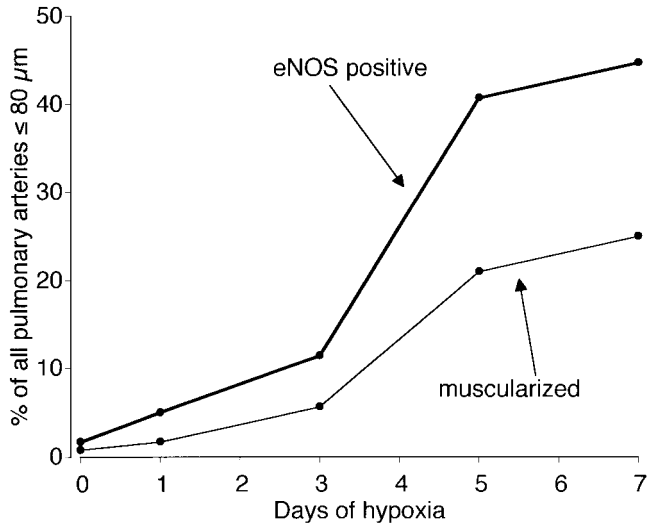

FIG. 11. eNOS expression in small pulmonary arteries of the rat increases early in the course of chronic hypoxic exposure. Chronic hypoxic pulmonary hypertension increases the proportion of both muscularized and eNOS immunostaining small (≤80 μm) pulmonary arteries. [Data from Xue et al. (403).]

sion induced by monocrotaline injection (301, 375) or by chronic hypoxia (201, 300, 301, 375, 402, 403) (Fig. 11). The rat strain with a spontaneous form of pulmonary hypertension also shows a prominent eNOS staining in the peripheral pulmonary arteries (375). In the chronic hypoxic model, the percentage of small pulmonary vessels positive for eNOS antibody is increased, as compared with normoxic controls, as early as after 1 day of hypoxia, before any significant changes in vascular morphology can be detected (402) (Fig. 11). The percentage of eNOS-positive peripheral vessels further increases during several more days of hypoxia (402), but then it stabilizes and does not change much between 2 and 4 wk of hypoxia (403). The increase in eNOS expression in the microvasculature during pulmonary hypertension is limited to small arteries; small-venule eNOS expression does not change (300, 301). In the large pulmonary arteries, eNOS is present in both control and chronically hypoxic rats; its quantity appears higher in the latter (201, 403). It is well established that the resistive properties of the pulmonary vascular bed are mostly determined by the peripheral vessels and not much influenced by the conduit portion of the vasculature.

Whole lung eNOS mRNA and protein content is also increased in rats (44, 99, 201, 300, 338, 375, 402) and piglets (327) with chronic hypoxic pulmonary hypertension and in lambs with aortopulmonary shunt-induced pulmonary hypertension (29). Exposure to hypoxia as brief as 6 h is sufficient to upregulate rat lung eNOS mRNA (122). Surprisingly, only the whole lung eNOS mRNA, but not protein, is elevated in the monocrotaline model of pulmonary hypertension in rats, despite a positive eNOS immunostaining in the peripheral pulmonary arteries (375).

In addition to the increased eNOS expression in the endothelium of the peripheral pulmonary vessels, de novo iNOS mRNA and protein expression was noted in whole lung extracts and in the vascular smooth muscle of both large and small pulmonary vessels of rats (44, 155, 201, 272, 302, 402, 403) and mice (79) with chronic hypoxic pulmonary hypertension. iNOS mRNA is also increased in pulmonary endothelium of chronically hypoxic rats (272). This iNOS induction appears slower than the eNOS upregulation, because a 6-h hypoxic exposure, which increases whole lung eNOS mRNA, does not alter whole lung iNOS mRNA content (122). The functional significance of the iNOS induction in chronic hypoxic pulmonary hypertension has been questioned by recent studies showing that relatively selective inhibitors of the inducible NOS isoform, L-N^6-(1-iminoethyl)lysine and aminoguanidine, had no effect on pulmonary hemodynamics of chronically hypoxic rats (302, 375).

Published data on the pulmonary vascular NOS expression in human pulmonary hypertension are rather scarce. Giaid and Saleh (125) found reduced eNOS expression in pulmonary vessels of all sizes in 46 patients with severe chronic pulmonary hypertension. Methodological factors could contribute to the discrepancy of their data with the above discussed experimental findings in rats, as suggested by Xue and Johns (401), who found increased eNOS immunostaining in two patients with pulmonary hypertension. Tuder et al. (372) studied 18 patients with various forms of pulmonary hypertension and found no decrease in eNOS immunostaining.

One factor possibly contributing to the discrepancy between the data of Giaid and Saleh (125) and animal studies is due to a focus on different stages of the progression of pulmonary hypertension. The studies using experimental animals investigate the early phases that are difficult to study in humans because pulmonary hypertension is typically diagnosed in later stages of the disease. More importantly, to study NOS expression, lung tissue must be obtained. In humans that means either using autopsy material from patients whose pulmonary hypertension had already led to death or a material from patients whose pulmonary hypertension was so severe as to require lung transplantation, or at least patients whose symptoms are serious enough to justify lung biopsy.

These three sources of human lung tissue were all used in the study of Giaid and Saleh (125), but the contribution of each source to the results was not specified. In any case, their data are from patients with very advanced or terminal stages of severe pulmonary hypertension. In such an advanced pulmonary hypertension, the pulmonary endothelium is severely damaged (386). Thus it is possible that the endothelial damage at the terminal stages of the pulmonary hypertension can reach such a

level as to disable the capability of the endothelial cells to keep the NOS expression up. The elevated NOS expression of the early phases of the pulmonary hypertension (suggested by the animal experiments) may thus be overridden. This speculative interpretation is supported by the observation that the intensity of eNOS immunoreactivity correlated inversely with the severity of histological changes in the pulmonary vessels of the human patients with pulmonary hypertension (125). Recently, an increased regional heterogeneity of eNOS expression in human pulmonary hypertension has been described. Mason et al. (225) found particularly high eNOS levels in pulmonary vessels affected by plexiform lesions, whereas small arterioles free of this type of lesions had lower eNOS expression compared with healthy lungs. The studies of NOS expression in pulmonary hypertension are summarized in Table 4.

The cause for the increased NOS expression (where it was found) is unclear. It could be that the decreased oxygen concentration directly stimulates NOS gene expression in a manner similar to that shown for several other genes, such as the genes encoding endothelin-1, platelet-derived growth factor β-chain, vascular endothelial growth factor, erythropoietin, or heme oxygenase-1 (190, 323). The hypoxic induction of several of these genes is mediated by a transcription factor known as hypoxia-inducible factor 1 (HIF-1) (96, 203, 333). HIF-1 is a heterodimer, both subunits of which are rapidly induced by hypoxia and quickly decay upon reoxygenation (387). The HIF-1 binding sequence is present in the 5′-flanking region of the murine and rat iNOS gene (235, 257, 333). Prolonged hypoxia induces HIF-1 expression in many pulmonary cell types, including endothelium and vascular smooth muscle (272, 408). HIF-1 binding to the iNOS gene promoter is induced in lungs of chronically hypoxic rats and in bovine pulmonary artery endothelial cells incubated in hypoxia (272). Hypoxia increases iNOS promoter activity in the pulmonary artery endothelial cells, but only if the HIF-1 binding site is intact (272). Taken together, these findings implicate HIF-1 in the hypoxic induction of iNOS in the pulmonary circulation.

The role of HIF-1 in hypoxic regulation of expression of other NOS isoforms has not yet been studied. The human eNOS gene promoter contains no homology to the published binding sequence of HIF-1 (94); however, the presence of the HIF-1 binding site upstream from the published sequence cannot be excluded. The persistence of the elevated eNOS protein and mRNA abundance in pulmonary vessels for several days after the end of the chronic hypoxic exposure (300) argue against a direct role of hypoxia in eNOS upregulation. The eNOS gene promoter contains *cis*-regulatory elements for activation protein 1 and nuclear factor κB (37, 195), transcription factors regulated (among other influences) by changes in cellular redox status, which is proportional to oxygen availability. The nNOS gene promoter contains the putative HIF-1 binding sites (94), but the limited evidence available today does not support the role of nNOS in pulmonary hypertension. The nNOS expression appears unaltered in pulmonary vessels of patients with pulmonary hypertension (124) and in mice with chronic hypoxic pulmonary hypertension (79).

The de novo iNOS expression in the pulmonary vascular smooth muscle could also be mediated by the acti-

TABLE 4. *NOS expression in pulmonary hypertension in adults*

Species	Type of PHT	Isoform	Technique	NOS	Reference Nos.
Rat	Chronic hypoxia	Not specified	NADPH diaphorase	+	403
			Immunostaining	+	403
			mRNA	+	44
		eNOS	Immunostaining	+	200, 300, 301, 375, 402
			mRNA	+	198, 201, 300, 338, 375, 402
			Protein	+	99, 201, 338, 375, 402
		iNOS	Immunostaining	+	402
			mRNA	+	155, 201, 272, 302, 402
			Protein	+	155, 201, 402
	Monocrotaline	eNOS	Immunostaining	+	301, 375
			mRNA	+	375
			Protein	−	375
	Spontaneous	eNOS	Immunostaining	+	375
			mRNA	=	375
			Protein	−	375
Mouse	Chronic hypoxia	iNOS	mRNA	+	79
Human	Primary and secondary	Not specified, eNOS	NADPH diaphorase, immunostaining, mRNA	−	125
		eNOS	Immunostaining	+	401
				− +	225
				= +	372

+ and −, Greater and smaller NOS expression, respectively, in the group with PHT than in a control group; − +, in the study of Mason et al. (225), endothelial NOS (eNOS) expression was increased in plexiform lesions, but reduced in other portions of the vasculature; iNOS, inducible NOS.

vation of cytokines that are known to induce iNOS expression in vascular smooth muscle (123, 168). Both vascular wall injury and hypoxia are capable of activating various cytokines. Recent data point to the possibility that NO synthesis can be stimulated by fragments of proteins forming vascular wall matrix, the turnover of which is increased in pulmonary hypertension (see sect. V*C1*C). Increased intravascular pressure has been described as a stimulus for iNOS induction in systemic vessels (48); whether this mechanism plays a role in the pulmonary hypertension is unknown.

One factor to consider as possibly contributing to the eNOS upregulation is an increased shear stress of the pulmonary endothelium. In vitro, shear stress enhances both the eNOS activity (60, 138, 167, 189, 192, 200, 236) and expression (37, 94, 255, 293, 335, 367, 396, 399).

Shear stress is directly proportional to flow rate and indirectly to vessel caliber. The total blood flow through the lung usually does not change substantially in the initial phases of the pulmonary hypertension, whereas the vessel caliber decreases because of vasoconstriction and medial (and sometimes intimal) thickening. Therefore, pulmonary endothelium is probably exposed to increased shear forces during pulmonary hypertension. However, eNOS mRNA and protein expression were found identically increased in the hypoperfused left and hyperperfused right lungs of chronically hypoxic rats with surgical stenosis of the left pulmonary artery (200), implying that chronic hypoxia increases eNOS expression independently of changes in shear stress. In addition, eNOS was not found increased in normoxic rats with surgically created chronic pulmonary blood flow elevation with normal pulmonary arterial pressure (78).

D. Endothelium-Dependent Vasodilation

The role of NO in chronic pulmonary hypertension was first studied using the reactivity to endothelium-dependent vasodilators as an indicator of NO activity. However, it is important to consider that the level of a continuous, basal production of NO may be more important in terms of the mechanism of pulmonary hypertension than the response to endothelium-dependent agonists. For example, the classic endothelium-dependent vasodilator acetylcholine (106) is probably present in the pulmonary circulation in vivo only in minimal amounts because the cholinergic innervation of the resistance pulmonary vessels is scarce (305). Alterations in reactivity to a substance that the pulmonary circulation does not encounter in significant amounts in vivo may not be physiologically important. In addition, endothelium-dependent vasodilation is not always completely NO dependent; other factors, such as yet unidentified endothelium-derived hyperpolarizing factor, may participate significantly in different species and vascular beds (381), including rat lung (10). The changes in reactivity to endothelium-dependent vasodilators in chronic pulmonary hypertension may reflect altered receptor density or activity rather than or in addition to changes in NOS activity. Moreover, recent reports of increased iNOS expression in pulmonary hypertension (see sect. IV*C*) raise the possibility that endothelium-dependent vasodilation can be blunted by the increased iNOS activity, because it has been shown in systemic vessels (48). In fact, a paradoxical decrease in endothelium-dependent systemic vasodilation is present even in mice overexpressing the endothelial NOS isoform (264). For all these reasons, the data on endothelium-dependent vasodilation should be used for conclusions about the role of NO in pulmonary hypertension only with caution.

Many studies found a reduced reactivity to endothelium-dependent vasodilators in pulmonary hypertension. Most of these were performed using acetylcholine (or carbachol) in rings isolated from conduit pulmonary arteries of chronically hypoxic rat (45, 62, 169, 221, 223, 310). However, endothelium-independent vasodilation (to sodium nitroprusside) was also reduced in some of these studies (62, 221, 310). This argues for a reduced ability of the vascular smooth muscle to relax rather than for a decreased ability of the endothelium to produce NO in response to the agonist. The impaired reactivity to acetylcholine in pulmonary arterial rings from chronically hypoxic rats can be partially restored by cyclooxygenase inhibition (221) or by a prostaglandin H_2/thromboxane A_2 receptor antagonist (223), suggesting that an increased production of a vasoconstrictor prostanoid may contribute to the reduced acetylcholine reactivity. Dinh-Xuan and co-workers (71, 72) found reduced responsiveness to acetylcholine in subsegmental pulmonary arteries (1.2–3.4 mm external diameter) isolated from hypoxemic human patients with end-stage chronic obstructive lung disease. The degree of reduction correlated with the degree of vascular wall damage. Endothelium-independent vasodilator response to sodium nitroprusside was unaltered. Adnot and co-workers (7, 74) found a reduction of reactivity to acetylcholine and to a calcium ionophore A23187 (which activates eNOS in a receptor-independent manner), but not to sodium nitroprusside, in isolated perfused lungs of chronically hypoxic rats. Abolished responsiveness to acetylcholine and normal reactivity to sodium nitroprusside was reported in conduit pulmonary arteries isolated from piglets with pulmonary hypertension elicited by chronic pulmonary venous obstruction (334).

On the other hand, several studies reported unchanged pulmonary reactivity to endothelium-dependent vasodilators in pulmonary hypertension (303, 318). Still others found a significant increase (76, 99, 132, 158, 265, 268, 300, 301, 304, 384). These studies, unlike most of the

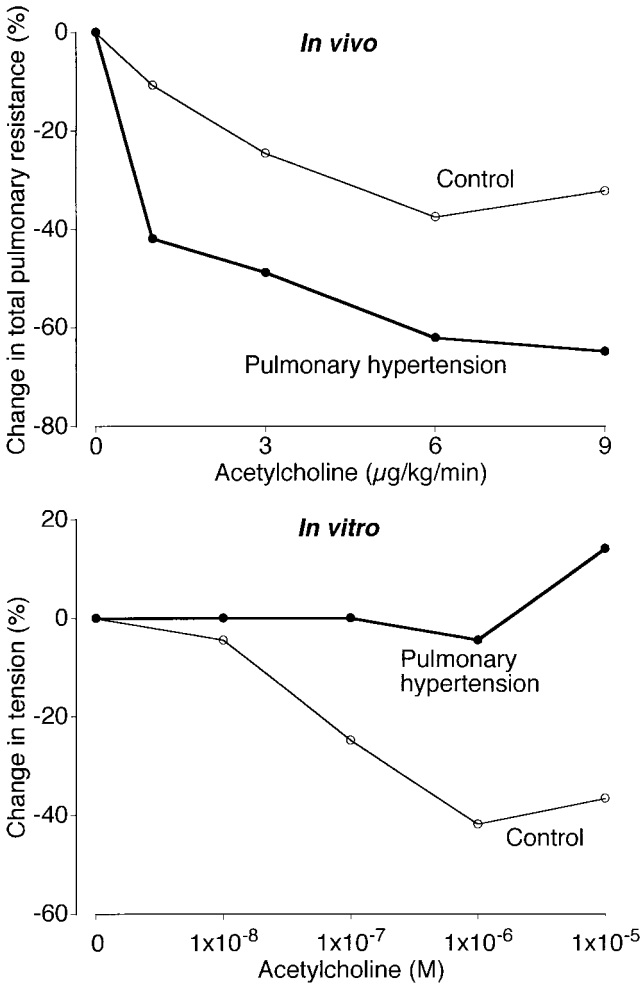

FIG. 12. Endothelium-dependent pulmonary vasodilation in response to acetylcholine is potentiated by chronic hypoxic pulmonary hypertension in intact calf lungs in vivo and reduced in their isolated pulmonary arterial rings in vitro. [Data from Orton et al. (268).]

studies showing inhibition of endothelium-dependent vasodilation, were done on intact pulmonary vascular bed (isolated lungs or whole animals). In fact, Orton et al. (268) demonstrated that pulmonary vasodilation in response to acetylcholine was potentiated in intact calves with chronic hypoxic pulmonary hypertension but reduced in their isolated conduit pulmonary arteries (Fig. 12). A similar finding was reported in rats by Oka et al. (265). However, MacLean and McCulloch (215) found a markedly potentiated vasodilatation in response to acetylcholine in pulmonary resistance arterial rings isolated from chronically hypoxic rats compared with normoxic controls.

In humans, Adnot et al. (6) found similar pulmonary vasodilator responses to acetylcholine and NO in pulmonary hypertension due to chronic obstructive lung disease, indirectly suggesting that NO-dependent vasodilation was preserved.

Taken together, it appears that in pulmonary hypertension, endothelium-dependent vasodilation is often impaired in larger pulmonary arteries, whereas in the whole adult pulmonary circulation it is, in most cases, preserved or potentiated (Table 5).

E. Interim Summary: Changes of NO Synthesis in Pulmonary Hypertension

The data reviewed so far indicate that pulmonary vascular NO synthesis is low when pulmonary vascular resistance is low (normal adult, see sect. II*A*) and increased when pulmonary vascular resistance is increased (fetus, sect. II*B*; acute pulmonary vasoconstriction, sect. III; and chronic pulmonary hypertension, sect. IV). It is likely that this elevated NO synthesis protects the thin-walled pulmonary vessels against excessive increases in intravascular pressure and a consequent danger of mechanical damage. However, it is plausible that the price for this protection is an exposure of the vascular wall to the toxic effects of the NO radical, which, when prolonged, may contribute to the vascular injury. This possibility is discussed below.

TABLE 5. *Effect of pulmonary hypertension on endothelium-dependent vasodilation*

Species	Type of PHT	Preparation	Effect	Reference Nos.
Rat	Chronic hypoxia	Isolated lungs	−	7, 74
			=	303, 318
			+	76, 99, 132, 158, 265, 300, 304
		Pulmonary arterial rings	−	45, 62, 169, 221, 265, 310, 223
			+	215
	Monocrotaline	Isolated lungs	+	301
Cattle	Chronic hypoxia	Intact	+	268, 384
		Pulmonary arterial rings	−	268
Pig	PVO	Pulmonary arterial rings	−	334
Human	COPD	Pulmonary arterial rings	−	71, 72

PVO, pulmonary venous obstruction; COPD, chronic obstructive lung disease; +, =, and −, a larger, equal, or smaller response, respectively, to endothelium-dependent vasodilators as compared with a control (no pulmonary hypertension) group.

V. REMODELING OF THE PULMONARY VASCULAR BED IN PULMONARY HYPERTENSION

The mechanisms of hypertrophy and proliferation of vascular smooth muscle and deposition of matrix proteins in pulmonary hypertension have been debated for several years. The emphasis changed from the importance of physical stretch of the vascular wall and "work hypertrophy" of vascular smooth muscle due to sustained vasoconstriction to the consequences of the injury to the pulmonary vascular wall (142). In this section we discuss the experimental evidence relevant to the hypothesis that radical injury to the walls of peripheral pulmonary arteries initiates the process of vascular mesenchymal proliferation and structural remodeling. This radical injury may result from the interaction of NO and reactive oxygen species released in the injured tissue.

A. Role of Injury to the Pulmonary Vascular Wall

Injury to the alveolocapillary barrier resulting in transvascular fluid leak was described in models of pulmonary hypertension induced by hypoxia (220, 343, 351, 391), monocrotaline (152, 175, 357), increased pulmonary blood flow (289), or chronic lung embolism (366). Experimental pulmonary inflammation causes sustained pulmonary hypertension (144, 147). In patients with chronic lung diseases, pulmonary hypertension often worsens during acute respiratory attacks (87).

Lung tissue injury and inflammation are accompanied by an increased production of oxygen radical species (for review, see Refs. 179, 321). The participation of reactive radicals in the pathogenesis of chronic pulmonary hypertension ranges from an easily acceptable principle, as in the case of hyperoxia (166) and monocrotaline-induced pulmonary hypertension (174, 296), to the less intuitive possibility in the case of chronic hypoxia.

Data on oxygen radical production in acute hypoxia (minutes to hours) are contradictory; both an increase (32, 56, 91, 341, 368, 379) and a decrease were reported (14, 271). Less data are available for more chronic exposures to hypoxia (days to weeks), but they are all consistent with increased levels of oxygen radical production in the lung and their participation in the vascular remodeling of pulmonary hypertension. Nakanishi et al. (248) reported a biochemical evidence of oxidant stress in lung tissue of rats exposed to a hypoxic environment. Using a sophisticated approach of an on-line chemiluminiscence HPLC assay of phosphatidylcholine hydroperoxide, Hoshikawa et al. (151) brought a direct evidence of oxidant stress of lung tissue in chronically hypoxic rats. In addition, the authors were able to reduce hypoxic pulmonary hypertension in rats by an antioxidant *N*-acetyl-L-cysteine. Pulmonary artery blood pressure, right ventricle weight, and muscularization of peripheral pulmonary arteries were all lower than in untreated hypoxic rats. We have the same experience: rats given *N*-acetyl-L-cysteine in drinking water (20 g/100 ml) during their 2-wk exposure to hypoxia had lower pulmonary arterial blood pressure and right ventricular weight than chronically hypoxic rats not given *N*-acetyl-L-cysteine (140). A similar finding was reported earlier by Reid and co-workers (197). They, however, used dimethylthiourea as the antioxidant; therefore, their results have to be interpreted as evidence for participation of oxidative damage in pulmonary hypertension only with caution because of dimethylthiourea's toxicity (197). Experiments in vivo (393) and in vitro (373) showed that rat alveolar macrophages are primed to produce more reactive oxygen species after they are exposed to hypoxia for several days. Therefore, in contrast to acute hypoxia, where the reports are contradictory, it is likely that chronic hypoxia results in oxidant injury to lung vasculature.

The increased production of oxygen radicals is consistent with the vascular remodeling of pulmonary hypertension despite the experiments showing inhibition of cellular proliferation by high levels of reactive oxidant species in vitro (81). The main argument for this statement is the results of experiments with chronic hyperoxia, where the production of radical oxygen species in lung tissue is definitely increased (103, 149, 162, 342). Animals kept in hyperoxic atmosphere (>80% O_2 in inspired air) develop pulmonary vascular remodeling similar to that described after exposure to hypoxic environment (166, 297).

B. Effects of NO on Radical Injury of Lung Vessels

Interactions of nitric oxide and other radicals released in tissue injury are multifaceted, and NO may oppose or enhance the oxidant tissue damage depending on relative quantities of NO and oxygen radicals produced and on the activity of antioxidant defense mechanisms. NO may reduce lung vascular damage by oxygen radicals. However, in conditions of high concentration of oxygen radical species it may act synergistically and worsen the tissue damage by products of NO and oxygen radicals interaction.

1. NO opposes oxidant lung injury

NO appears to be one of the factors that modulates the increased transvascular fluid and protein transport in lung injury. On a cellular level, the endothelial permeability was studied in vitro on a monolayer of endothelial cells. The increase in permeability was induced by H_2O_2. Lower amounts of NO released by 10 mM sodium nitroprusside had a protective effect. Large amounts released

by a 10 times higher dose, however, worsened the endothelial barrier dysfunction (232). On a whole organ level, NO inhalation prevented oxidant injury induced by superoxide in isolated rabbit lungs; inhibition of NO production had an opposite effect (171).

An often used experimental model of lung oxidant damage in vivo is inhalation of gas mixtures containing a high concentration of oxygen. The effects of combined inhalation of NO and O_2 are controversial. Some studies show that ventilation with NO and O_2 reduced hyperoxic injury (230, 253); others found injury to be worsened (306). The controversy may be explained by the concentrations of NO used. In the study of Garat et al. (110), lower concentrations of NO (10 ppm) mitigated, whereas high concentrations (100 ppm) worsened hyperoxic lung injury. Combined inhalation of NO (at a very low dose of 2 ppm) and O_2 was used with some benefits in patients with chronic obstructive lung disease (407). Arterial oxygenation was higher in patients treated with O_2 plus NO than in patients with oxygen therapy without NO.

Studies that use NO synthesis inhibitors support the possibility of NO having a cytoprotective effect in hyperoxia. L-NAME decreased tolerance to hyperoxia in newborn (283) and adult (42) rats.

Hypoxic injury to endothelial cells induces lipid peroxidation of plasma membrane (32). NO interrupts chain reactions involved in lipid peroxidation (for review, see Ref. 313). If a local concentration of NO is higher than that of superoxide, lipid peroxidation is depressed. On the other hand, if the NO levels are exceeded by those of superoxide, NO stimulates superoxide-induced lipid peroxidation and tissue injury by converting superoxide to a highly reactive peroxynitrite (314).

NO decreases polymorphonuclear activation and adherence (238). It has been reported that NO reduces superoxide (312) and H_2O_2 production in activated neutrophils (93). A direct inhibition of membrane-bound NADPH-oxidase by NO (55, 93) or reduced cell viability (65) may be the explanation. The inhibitory effect depends again on the ratio of NO and oxygen radical levels. In conditions with high production of oxygen radicals, NO increases peroxynitrite formation by polymorphonuclear cells, and it enhances their ability to produce neutrophil-mediated oxidant injury (46).

2. *By interacting with superoxide, NO produces more potent radicals*

NO has a relatively low reactivity for a free radical species (183). Its toxicity is often a consequence of a reaction with superoxide anion to yield secondary products that are more reactive and cytotoxic.

Superoxide reacts rapidly with nitric oxide yielding peroxynitrite. The reaction is more than three times faster than dismutation by superoxide dismutase, and it is near the diffusion limit. Peroxynitrite is a short-lived substance (<1 s). Under physiological conditions it is in equilibrium with peroxynitrous acid (reviewed in Ref. 244). Peroxynitrous acid readily reacts with biological substrates similarly as hydroxyl radical.

Peroxynitrite formation from superoxide and nitric oxide occurs in vivo (24). It has been described in acute lung injury in humans (188). Production of peroxynitrite was found in several experimental diseases in animals (for review, see Ref. 360). Production of peroxynitrite was demonstrated in macrophages (159), neutrophils (43), endothelial cells (187), and vascular smooth muscle cells (35). The chemistry and biological actions of peroxynitrite have been recently reviewed (25, 313, 360).

Endothelial and vascular smooth muscle cells actively generate superoxide into the extracellular space (219, 227, 320), whereas NO freely diffuses across the cell membrane. Peroxynitrite can therefore be formed in extracellular space near the endothelial surface (24, 25). The effects of peroxynitrite may be more important on the abluminal side of the endothelium because in the vascular lumen NO is very effectively scavenged by hemoglobin.

Production of superoxide determines NO levels by controlling peroxynitrite formation. Dismutation of superoxide increases NO concentration in the tissue (24). Antioxidants (Cu/Zn superoxide dismutase, ascorbate, reduced glutathione, and α-tocopherol) protect NO against superoxide anions (207).

3. *Interim conclusions: NO and lung vascular injury*

The increased production of NO in pulmonary hypertension may have a protective role against radical injury to the pulmonary vascular wall. On the other hand, products of NO and oxygen radical interaction may worsen and perpetuate the vascular damage. This is especially likely to occur in the presence of an active inflammation (e.g., during acute exacerbations of chronic obstructive lung disease) in which NO production is further markedly elevated. The balance between cytotoxic and cytoprotective effects depends on relative amounts of individual radicals. The amount of radicals produced in pulmonary hypertension, however, appears lower compared with acute oxidant lung injury seen in adult respiratory distress syndrome or sepsis. In pulmonary hypertension, chronic "reparatory" mesenchymal proliferation and fibroproduction prevail.

C. Effects of NO on Remodeling of the Pulmonary Vascular Wall

1. *Production of vascular matrix proteins*

The fibroproduction that occurs in peripheral blood vessels during pulmonary hypertension alters rheological

properties of the pulmonary vascular bed. In animal studies, hypoxic pulmonary hypertension was reduced after impairment of collagen metabolism by β-aminopropionitrile or *cis*-hydroxyproline (141, 176, 177). The amount of collagen and elastin in the walls of blood vessels is a result of a balance between synthesis and breakdown of these proteins. Both processes are accelerated in pulmonary hypertension (27, 224, 234, 284).

A) COLLAGEN SYNTHESIS. In vitro experiments on isolated mesenchymal cells show that NO inhibits collagen formation. It has been demonstrated in chondrocytes (260), mesangial cells (369, 370), and pleural mesothelial cells (269). It is questionable, however, whether the effect is specific to collagen synthesis or whether it results from the known nonselective inhibitory effect of high doses of NO on proteosynthesis.

The suppressive effects found in vitro contrast with the results of experiments in intact animals. Schaffer et al. (328) showed that wound fibroblasts synthesize NO. Inhibition of NOS in vivo impaired the accumulation of collagen in the wound. It was not due to inhibition of fibroblast proliferation. The wound NO synthesis can be further increased by stimulation with lipopolysaccharide or interferon-γ. The stimulation of NO synthesis is followed by enhancement of collagen production (328). Similarly, there is an increase in NO production in rat lungs with radiation-induced pneumonitis. Treatment with L-NAME decreased the progression of radiation pneumonitis and inhibited the expression of procollagen-α_1 type III mRNA in lungs (260).

B) COLLAGEN DEGRADATION. The turnover of matrix collagen in the walls of pulmonary arteries is increased in pulmonary hypertension (27). We have recently observed increased collagenolytic activity in the peripheral pulmonary arteries isolated from rats with chronic hypoxic pulmonary hypertension (259). Native collagen molecules are degraded by matrix metalloproteinases (MMP) (262). Our recent data indicate that a synthetic MMP inhibitor, batimastat, reduces chronic hypoxic pulmonary hypertension in rats (J. Herget, J. Novotná, and V. Hampl; unpublished data). Metalloproteinases are secreted in a latent form in which a prodomain is folded over and covers the catalytic site containing a zinc atom. The conformation is maintained by thiol bounds between the cysteine residues of the prodomain and zinc in the catalytic site. Radicals including NO and peroxynitrite react with thiol groups. Therefore, nitric oxide, oxygen radicals, and peroxynitrite may be involved in activation of collagenolytic metalloproteinases (292).

Recent experimental evidence from in vitro experiments supports this view. Peroxynitrite is a strong activator of human neutrophil procollagenase (MMP-8) at 1–20 $\times$ 10^{-6} M concentrations (266), which can exist within vascular wall in vivo (159). Exogenous NO, however, activated MMP-8 (266) and pro-MMP-1 and -9 (267) only at high (mM) concentrations. The activation was strongly potentiated by a low concentration of reduced glutathione (267).

Peroxynitrite at low doses also stimulated the collagenolytic activity of MMP-2 and MMP-9 secreted by vascular smooth muscle cells (292). Immunoblotting of activated metalloproteinases with antinitrotyrosine antibodies showed tyrosine nitration, which is considered a "footprint" of peroxynitrite action (25).

In the tissues, metalloproteinase activity is inhibited by specific tissue inhibitors of metalloproteinases. Peroxynitrite fragments the tissue inhibitor of metalloproteinase-1 (101). That may result in an increase of collagenolytic activity.

Recently Kato et al. (170) described a peroxynitrite-promoted nitration of collagen type I in vitro. It is not clear whether similar modification of collagen molecules appears in vivo in vascular matrix fibers and whether it has any functional consequences. It may be speculated that nonenzymatic modifications of collagen molecule may decrease its resistance to proteolytic enzymes (239).

After the native collagen molecules are cleaved by specific metalloproteinases, they can be further degraded by other nonspecific proteases (57). Collagen degradation products stimulate lung collagen metabolism in in vitro conditions (113) and also in intact rabbit lungs (112). Application of a synthetic inhibitor of metalloproteinases reduced remodeling of an injured rat carotid artery (411). Therefore, data from various tissues suggest that increased collagen cleavage, which can be induced by NO-derived radicals, may participate in the increased collagen deposition in pulmonary hypertension. This possibility needs to be verified by future experiments.

C) ELASTIN. Increased lung elastolytic activity was thoroughly studied by the group of Dr. Rabinovitch in pulmonary hypertension induced by hypoxia (224) or monocrotaline (365). The possible pathogenetic consequences for development of pulmonary hypertension were reviewed recently (290). There is little information on the effect of nitric oxide on elastolytic activity. Kumar et al. (193) found no effect of nitric oxide, superoxide, or H_2O_2 on cytokine-stimulated elastolytic activity in macrophages. Faury et al. (82) showed that elastin degradation products elicit vasodilatation by stimulating NO production (Fig. 13). Elastin degradation products, therefore, may participate in the increased NO release in chronic pulmonary hypertension.

2. Proliferation of vascular smooth muscle in peripheral pulmonary arteries

Application of authentic NO or NO donors during the development of pulmonary hypertension inhibits the proliferation of vascular smooth muscle cells in pulmonary blood vessels. Chronic hypoxic pulmonary hypertension

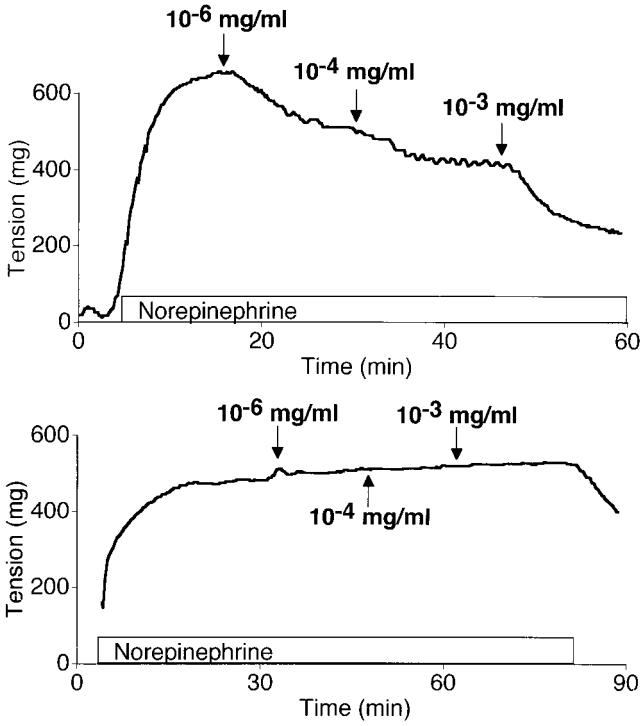

FIG. 13. Soluble fragments of elastin (κ-elastin) elicit endothelium-dependent vasodilatation in isolated aortic rings. Endothelium-intact (*top panel*) and denuded (*bottom panel*) aortic rings were precontracted with 10^{-6} M norepinephrine, and 10^{-6}, 10^{-4}, and 10^{-3} mg/ml κ-elastin was added to the bath. L-NAME (10^{-5} M) had a similar inhibitory effect on the relaxations (not shown) as the endothelial denudation. [Data from Faury et al. (82).]

in rats was mitigated by addition of 10 ppm NO to the hypoxic atmosphere for the whole duration of the hypoxic exposure (191). There was significantly less smooth muscle in the peripheral pulmonary arteries than in hypoxic rats not treated with NO (191). Also Roos et al. (311) reported that rats exposed to chronic hypoxia and inhalation of 20 ppm NO had a smaller remodeling of peripheral pulmonary arteries and increase in right ventricular weight than hypoxic rats not treated with NO. Unlike these morphological markers, however, the hemodynamic indices of pulmonary hypertension were unaffected by NO inhalation. As an explanation of this controversy, the authors offer an assumption that endogenous NO production is downregulated in rats who lived in an hypoxic environment enriched with NO. Because of inhibition of endogenous NO production, their vascular tone increases after the weaning from the NO-enriched environment. Attenuated proliferation of vascular smooth muscle in peripheral pulmonary arteries was shown also in neonatal rat pups exposed simultaneously to chronic hypoxia and NO (20 ppm) (308). However, Maruyama et al. (222) did not find any effect of inhalation of 10 or 40 ppm NO for more than 2 wk after the induction of pulmonary hypertension by a monocrotaline injection (222). In contrast, a continuous subcutaneous infusion of molsidomide, a NO donor drug, partially prevented the growth of pulmonary vascular smooth muscle in rats with monocrotaline-induced pulmonary hypertension (226).

There are no reports to demonstrate a prolonged therapeutic application of NO on pulmonary vascular wall structure in already developed pulmonary hypertension or during recovery of vascular changes after the relief from exposure to chronic hypoxia. Information on the effect on recovery would be of interest because hypoxia in chronic lung diseases is usually not continuous; rather, it is temporarily worsened during acute exacerbations. Mechanisms similar to those active in tissue reperfusion may play a role during the reoxygenation after exposure to hypoxia. NO can attenuate reperfusion lung injury (19, 53).

It is commonly accepted that NO inhibits proliferation of vascular smooth muscle cells in culture (118, 240, 254, 261, 337). A later work of one of these groups showed, however, that antimitotic effect of NO is restricted to cells that had been repeatedly passaged in culture (136). In a primary culture of vascular smooth muscle cells, NO stimulated mitogenesis through the amplification of fibroblast growth factor-2 production (136). Experiments in systemic vessels in vivo support the inhibitory role of NO. Increasing NO levels at the site of vascular injury by gene transfer of NOS (385) or by a local infusion of NO donor (51, 332) decreased vascular smooth muscle proliferation. Mice with targeted disruption of the eNOS gene respond to carotid artery injury with vascular smooth muscle hyperplasia, unlike the wild-type mice (316).

We reviewed above the data indicating that the lung NO production is increased in chronic hypoxic pulmonary hypertension and we argue in this section that increased NO and its interactions with other radicals may initiate the mesenchymal remodeling of the pulmonary vasculature. This is in controversy with the reports that inhalation of NO during the exposure to hypoxia attenuates vascular remodeling (191, 308, 311). We suggest two possible explanations.

First, during chronic hypoxia alone, the vascular tissue NO concentration can rise to a level which (in integration with other factors) promotes proliferation, whereas higher levels, achieved by additional exogenous NO supplementation, can be antiproliferative (perhaps due to cytotoxicity). Such a dual effect has been shown in vitro (232). The second possible explanation is related to the different factors that are at play during the early development and during the subsequent stage of a relatively stable pulmonary hypertension. During the first, progressive stage, endogenous NO can contribute to the oxidative vascular wall injury, as discussed above. Exogenous NO can be expected to aggravate this process. After approximately the first week (in the chronic hypoxic

rat model), the pulmonary hypertension becomes more stable. The progressive increase in pulmonary arterial blood pressure and vascular muscularization is replaced by a near-steady state. At this point, endogenous NO may partially oppose the increased tone and, by reducing transmural tension, it can even partially reverse the previously established vascular remodeling. This effect can be potentiated by exogenous NO. This way the net effect of exogenous NO can be a reduced remodeling at the end of the exposure to chronic hypoxia despite the possibility of a potentiation of the initial insult to the vascular wall.

Apart from L-arginine being a substrate for NO synthase, it is also metabolized by arginase to urea and L-ornithine and then to polyamines (spermine, spermidine, and putrescine). These two pathways of L-arginine metabolism are linked (242). Polyamines are involved in cell growth and differentiation (for review, see Ref. 163). Although it is intriguing to speculate on possible interactions of the increased NO synthesis in pulmonary hypertension and proliferative effects of polyamines, no experimental data are available.

NO may affect vascular wall structure in other ways in addition to its influence on the radical-induced pulmonary vascular wall injury and subsequent reconstruction. It can alter or mediate the response of cultured vascular smooth muscle or endothelium to various growth factors (273, 274, 345), although a lack of such an effect has also been reported (34). There is evidence that NO stimulates the mitogen-activated protein kinase cascade (196) that is known to be involved in smooth muscle hypertrophy (reviewed in Ref. 288). The relevance of these results, obtained mostly on the systemic vessels, for pulmonary hypertension has not been systematically studied.

Prolonged exposure to elevated NO levels downregulated cGMP-dependent protein kinase (344). It has been shown that upregulation of this enzyme in vascular smooth muscle cells converts them from a dedifferentiated, "synthetic" phenotype to a more contractile-like morphology (34). It can be hypothesized that NO-induced downregulation of the cGMP-dependent protein kinase has an opposite effect on vascular smooth muscle morphology, i.e., would promote the synthetic phenotype. The vascular remodeling of pulmonary hypertension is associated with increased proportion of vascular smooth muscle cells exhibiting the synthetic phenotype (reviewed in Ref. 353). But again, the relevance of this proposed mechanism for pulmonary hypertension has not been directly addressed.

NO can cause apoptosis in vascular smooth muscle (105, 120, 161, 285, 286). Although the programmed cell death might intuitively be expected to reduce vascular wall thickness (and thus possibly act against the development of pulmonary hypertension), there is evidence of association of apoptosis with vascular wall remodeling (33, 160, 285). The cause and effect relationship is not clear at present, but one possibility could be that NO-induced apoptosis elicits reparative proliferation. Most likely, the balance between apoptosis and proliferation determines cell count in injured tissues (160). Alteration of one of these processes might be expected to result in excessive vascular wall hypertrophy. Currently, there are no studies directly linking apoptosis to the mechanism of pulmonary hypertension, but research in this direction may prove useful in near future.

3. Interim conclusions: NO and pulmonary vascular remodeling

The data concerning the involvement of NO in the vascular remodeling of pulmonary hypertension are insufficient and contradictory. Judging from experiments in various tissues, NO can have both antiproliferative and proproliferative effects. In general, experiments using repeatedly passaged cells usually show that NO inhibits growth, whereas isolated cells in primary cultures tend to proliferate in the presence of NO. Primary cultures may be closer to the in vivo situation in that the cells have not been deprived of contact with other cell types and matrix proteins for too long. The composition of matrix proteins is an important modulator of mesenchymal proliferation. An increased collagenolytic and elastolytic activity was reported in pulmonary vessels from animals with pulmonary hypertension. There are data to support the possibility that the collagenolytic and elastolytic activity can be stimulated by NO-derived radicals. Products of this activity, in turn, may potentiate NO synthesis. More experiments are needed to clarify this issue.

The finding that chronic inhalation of low concentrations of NO partly prevented experimental pulmonary hypertension suggests that the endogenous NO production, elevated in chronic hypoxic pulmonary hypertension, may have predominantly a protective effect. It is likely, however, that additional insults, such as exacerbations of hypoxia or inflammation, further increase NO production to a level at which adverse effects of NO and its related species may prevail.

VI. GENERAL SUMMARY AND CONCLUSIONS

Pulmonary hypertension is a consequence of an elevation of pulmonary vascular tone (which is normally minimal) and of thickening of the pulmonary vascular wall (which is normally thin). After a potent endogenous vasodilator, NO, was discovered, suggestions appeared that a continuous, basal production of NO keeps normal pulmonary vessels dilated. It was also speculated that reduction of this basal NO synthesis is the mechanism of pulmonary hypertension. Because chronic hypoxia occurs in many forms of pulmonary hypertension and it elicits pulmonary hypertension in experimental models, it

was hypothesized that diminished availability of oxygen as a substrate for NOS reduces NO production in chronic hypoxic pulmonary hypertension.

A high basal NO production in the pulmonary vessels, however, was not confirmed by most of the subsequent experiments. A large number of studies have shown that inhibitors of NOS, which elicit a substantial vasoconstriction in the systemic vascular beds, have no significant effect in the pulmonary circulation. These findings indicate that a continuous NO release contributes to the basal tone regulation in the systemic circulation, but not in the lung vessels. This conclusion has been further strengthened by studies of NOS localization. They showed minimal NOS mRNA and protein expression in the peripheral pulmonary vessels, which are the ones mostly responsible for the overall rheological properties of the pulmonary circulation. In humans, both NOS expression in lung vessels and pulmonary vasoconstrictor reactivity to NOS inhibitors appear higher than in most experimental animals. However, humans are no exception from the observation that the role of NO in vascular tone regulation is significantly less in pulmonary than in systemic circulation.

In comparison with adults, a higher basal NO release is present in the late fetal pulmonary circulation, where pressure and resistance are high and thus resemble the situation found in adult pulmonary hypertension. NO is important in the perinatal transition of the pulmonary circulation to the low pressure state. Further studies of the mechanisms involved in this transition may help to understand the role of NO in pulmonary hypertension in adults.

Pulmonary vascular NOS expression and activity are elevated in experimental models of pulmonary hypertension in adults. In contrast, human studies with near-terminal stages of chronic pulmonary hypertension found reduced NOS expression and activity. It is likely that NO synthesis is elevated in the earlier phases of pulmonary hypertension (modeled in experimental animals) and reduced due to gross endothelial damage at advanced and terminal states (studied in humans). Unfortunately, direct measurements of NO synthesis in the pulmonary circulation are technically difficult, particularly in humans, and are done less often than needed for reliable conclusions.

All in all, the available data are consistent with the view that pulmonary vascular NO synthesis is relatively low when tone and resistance are low (in normal adults). When tone and resistance are elevated, NO synthesis in the pulmonary circulation rises. That is the case in the normal fetus and in the adult during acute vasoconstriction and (at least earlier stages of) chronic pulmonary hypertension. Traditionally, most of the research has focused on the activity of eNOS. In chronic pulmonary hypertension, vascular smooth muscle iNOS represents an additional source of NO. Because of its central location in the vascular wall, it may be more important in affecting the components of vascular media and adventitia than NO from the endothelium, which can be more easily scavenged by hemoglobin in the blood. This issue can be expected to attract more attention in future experiments.

There is evidence indicating that the primary trigger of the remodeling of peripheral lung arteries in pulmonary hypertension is an injury to the vascular wall of an oxidative nature. This injury can be worsened by NO. The combination of increased NO production with oxygen-derived radicals (also increased in pulmonary hypertension) may yield highly reactive compounds, peroxynitrite and its metabolites. Metabolism of these oxidants, once they are produced, is poorly controlled by cellular defense mechanisms. They are primarily cytotoxic and can trigger matrix protein reconstruction. However, NO can also protect against oxidant-induced injury and morphological remodeling. The ratio between local tissue levels of NO and oxygen radicals determines which effect of NO, protective or adverse, prevails. In chronic lung diseases associated with pulmonary hypertension, this balance is unstable, and cellular compensatory mechanisms may be more easily exhausted. This may be particularly true during acute attacks of respiratory infections that often interrupt the periods of tissue repair.

NO may affect the mechanism of pulmonary hypertension in more ways that just by altering the radical injury to the pulmonary vascular wall. It can influence or mediate the response of vascular smooth muscle or endothelium to various growth factors. Stimulation of the mitogen-activated protein kinase cascade by NO might also be expected to play a role. By downregulating the cGMP-dependent protein kinase, NO may contribute to the switch of the pulmonary vascular smooth muscle cells from a contractile to a synthetic phenotype, which is a known feature of vascular wall hypertrophy. The importance of NO-induced apoptosis in pulmonary hypertension is currently unknown but appears a promising direction for future research.

Selective reduction of pulmonary vascular tone by inhaled NO has been used therapeutically to overcome pulmonary hypertensive crises with remarkable success. This approach, extensively reviewed elsewhere (75, 121, 145, 180, 214), is less suitable for treatment of more chronic forms of pulmonary hypertension due to technical difficulties. In addition, given the two-faceted role of NO in the pathogenesis of pulmonary hypertension in chronic lung diseases, it may not always be beneficial. More research is needed to find out whether attempts to limit lung vascular tissue injury due to interaction of elevated endogenous NO and oxygen radicals are more appropriate in some forms of chronic pulmonary hypertension.

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