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MINIREVIEW

Nitric Oxide Synthase in Pulmonary Hypertension: Lessons from Knockout Mice

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Summary

Nitric oxide (NO) is implicated in a wide variety of biological roles. NO is generated from three nitric oxide synthase (NOS) isoforms: neuronal (nNOS), inducible (iNOS), and endothelial (eNOS) all of which are found in the lung. While there are no isoform-specific inhibitors of NOS, the recent development and characterization of mice deficient in each of the NOS isoforms has allowed for more comprehensive study of the importance of NO in the lung circulation. Studies in the mouse have identified the role of NO from eNOS in modulating pulmonary vascular tone and in attenuating the development of chronic hypoxic pulmonary hypertension.

Key words

Nitric oxide • Knockout mice • Pulmonary hypertension • Pulmonary vasoreactivity • Vascular remodeling

Introduction

Since the identification of nitric oxide (NO) as an intrinsic endothelium derived vasoregulator (Palmer *et al.* 1987), NO generated from nitric oxide synthase (NOS) has been found in many different cell types and implicated in a wide variety of physiologic and biologic functions. Its central role in maintaining vascular tone in the systemic as well as pulmonary circulation has been intensively investigated in the last decade.

NO is a highly reactive molecule produced from L-arginine by one of three nitric oxide synthase (NOS) enzymes, neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS). NO is a readily diffusible gas with a variety of biologic activities in the lung including vasodilation, bronchodilation, anti-adherence, anti-mitogenic, and anti-inflammatory properties.

All three NOS isoforms are found in the mammalian lung and may contribute to regulation of pulmonary vascular tone as has been suggested by a variety of inhibitor studies and more recently using mice with targeted gene deletions of NOS. The role of NO in the pulmonary circulation depends on the cell type in which NOS is expressed and the localization of that cell in the lung. However, because of the close proximity of the airway and vascular structures of the lung, NO from either the vessel wall or the neighboring airway and lung parenchyma may contribute to regulation of pulmonary

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vascular tone. While controversial, eNOS protein may be decreased in patients with primary pulmonary hypertension which correlates with the severity of the pathologic lesion and the increase in pulmonary vascular resistance (Giad and Saleh 1995, Xue and Johns 1995).

In the lung, nNOS is found in non-adrenergic, non-cholinergic nerve cell bodies and termini in airway epithelium and pulmonary vessels as well as in airway and vascular smooth muscle (Guembe and Villaro 1999, Sherman *et al.* 1999, Shaul *et al.* 1995). iNOS is also found in the airway epithelium, airway and vascular smooth muscle and in great abundance in alveolar and airway macrophages (Sherman *et al.* 1999, Degnim and Nakayama 1996, Watkins *et al.* 1997). eNOS is found predominantly in vascular endothelium but is also expressed in airway epithelium (Shaul *et al.* 1995). Both nNOS and eNOS are calcium-dependent constitutively expressed enzymes, whereas iNOS may be induced by a variety of inflammatory mediators to produce high levels of NO in a non-calcium dependent manner. All three enzymes generate NO from the conversion of L-arginine to L-citrulline, a fact exploited by analogues to L-arginine that act as inhibitors of NO production from NOS. However, while there are inhibitors that may be relatively selective for one NOS isoform over the others, there are no isoform specific inhibitors available. While much work has been done using inhibitors of NOS, these compounds may have other non-NOS inhibitory effects as well as being difficult to administer to whole animals continuously.

The generation of targeted deletion of specific stem cell genes has allowed for the study of the role of NO from the individual forms of NOS without the confounding effects of non-selective NOS inhibitors. Mice lacking nNOS (nNOS-/-), iNOS (iNOS-/-), and eNOS (eNOS-/-) have been generated (Huang *et al.* 1993, 1995, MacMicking *et al.* 1995). These knock-out mice have helped to clarify the importance of NO in both the normal and abnormal murine pulmonary circulation.

Role of nitric oxide in regulation of pulmonary vascular tone

The importance of endothelium-derived factors in modulating pulmonary vascular tone was first suggested in 1981 when acetylcholine and bradykinin caused pulmonary vasodilation that was dependent on intact pulmonary vascular endothelium (Chand and Altura 1981). The importance of the vascular endothelium in modulation of pulmonary vascular tone to a variety of vasodilators was studied extensively and found to depend on release of an endothelium-derived relaxing factor leading to activation of cGMP and later identified as NO (Cherry and Gillis 1987, Feddersen *et al.* 1986, Ignarro *et al.* 1986, Palmer *et al.* 1987).

However, the role of NO in maintaining normally low vascular tone and reactivity is less certain as acute administration of NOS inhibitors raised resting pulmonary vascular tone in humans (Stamler *et al.* 1994, Celermajen *et al.* 1994) and in some but not all other species studied (Isaacson *et al.* 1994, Hasunuma *et al.* 1991, Nishiwaki *et al.* 1992, Barnard *et al.* 1993, Persson *et al.* 1990, Fineman *et al.* 1991). NOS inhibition augments the vasoconstrictor responses of lungs to hypoxia and other vasoconstrictors (Archer *et al.* 1989, Hasunuma *et al.* 1991, Brashers *et al.* 1988, Sprague *et al.* 1992, Lippton *et al.* 1992). Conversely, exogenous administration of NO does not decrease already low resting pulmonary vascular tone, but vasodilates lungs preconstricted with hypoxia and other stimuli. This suggests that NO is not uniquely responsible for maintaining low pulmonary vascular tone, but it is important in modulating responses to vasodilators and vasoconstrictors (Din-Xuan and Herve 1996). In these studies, the non-selective nature of the NOS inhibitors makes it uncertain as to which NOS isoform is producing the NO that may play the predominant role in attenuating vasoconstriction and regulating basal pulmonary vascular tone.

The use of mice with targeted deletions of the genes for the three isoforms of NOS has allowed studies of which NOS isoform is responsible for modulation of pulmonary vascular responses. In the systemic circulation, loss of eNOS results in systemic hypertension (Huang *et al.* 1995) which is not worsened by additional inhibition of other NOS isoforms (Kojda *et al.* 1999). Responses to the endothelium-dependent vasodilator acetylcholine is impaired in the aorta and carotid arteries of eNOS-deficient mice (Faraci *et al.* 1998, Waldron *et al.* 1999, Hussain *et al.* 1999, Huang *et al.* 1995), but surprisingly not in the cerebral, femoral or mesenteric arteries (Meng *et al.* 1996, Chataigneau *et al.* 1999, Hussain *et al.* 1999, Waldron *et al.* 1999). Conversely, overexpression of eNOS causes systemic hypotension (Ohashi *et al.* 1998). In the cerebral circulation of eNOS-deficient mice, vasodilation to acetylcholine can be inhibited by non-selective NOS inhibitors suggesting that upregulation of another NOS isoform, most likely nNOS,

plays a compensatory role in maintaining the response (Waldron *et al.* 1999, Meng *et al.* 1996). In contrast, in the femoral and mesenteric arteries, another endothelium-dependent mechanisms such as generation of an EDHF or cyclo-oxygenase product, are likely responsible for maintaining vasodilation (Chataigneau *et al.* 1999). Additionally, there is an increased vasodilator sensitivity to exogenously administered NO in the systemic circulation of mice lacking eNOS (Faraci *et al.* 1998, Waldron *et al.* 1999, Hussain *et al.* 1999, Lake-Bruse *et al.* 1999) which is possibly mediated through enhanced sensitivity of soluble guanylate cyclase for NO (Faraci *et al.* 1998, Hussain *et al.* 1999). Although controversial, these changes in the systemic circulation have also been seen in mice lacking only one copy of the normal eNOS gene suggesting a gene dosing effect where a relative reduction in eNOS is sufficient to confer abnormal vascular responses (Faraci *et al.* 1998, Kojda *et al.* 1999). Responses to endothelium-dependent vasodilators have been restored in eNOS-deficient aortic rings by eNOS gene transfer (Lake-Bruse *et al.* 1999).

In the pulmonary circulation, Steudel *et al.* (1997) showed that isolated pulmonary artery rings from eNOS-deficient mice did not dilate to acetylcholine but did respond to NO-donor sodium nitroprusside. The wild-type pulmonary artery rings treated with non-selective NOS inhibitor actually contracted to acetylcholine. The authors therefore suggest that because there was no similar vasoconstriction to acetylcholine in eNOS-deficient mice, other sources of NO may be important in maintaining low pulmonary vascular tone and limiting vasoreactivity.

To address the role of other NOS isoforms in modulating pulmonary vascular tone, we developed an isolated mouse lung preparation where pulmonary artery pressure is measured under constant flow and changes in pulmonary artery pressure reflect changes in pulmonary vascular resistance (Fagan *et al.* 1999b). Thus, the relative vasoconstrictor response to acute hypoxia could be measured in all three types of NOS knock-out mice to determine which isoform had the predominant role in modulating acute pulmonary vasoreactivity. We found that eNOS-deficient mice had a slightly higher pulmonary artery perfusion pressure at baseline and a near doubling of hypoxic vasoconstriction compared to wild-type mice. In contrast, there was no significant difference in hypoxic pulmonary vasoconstriction between iNOS- or nNOS-deficient mice and wild-type mice.

To determine if there was compensation by other NOS isoforms in the eNOS-deficient lungs that would attenuate the hypoxic vasoconstriction, we treated the lungs with a non-selective NOS inhibitor and did not find a further increase in vasoconstriction. Wild-type mouse lungs had a marked increase in hypoxic vasoconstriction following non-selective NOS inhibition but not after relatively selective iNOS inhibition with aminoguanidine. This confirmed the central role of eNOS-derived NO in modulating the hypoxia response. As was expected, endothelium-dependent vasodilation to bradykinin was impaired in eNOS-deficient lungs, but the vasodilatory response to exogenously administered NO was intact. In contrast to the systemic circulation, we did not observe an increased sensitivity to exogenously administered NO in the eNOS-deficient lung.

Thus, in the murine pulmonary circulation it appears that eNOS is the major contributor of NO for modulation of pulmonary vascular tone, and the loss of eNOS leads to increased basal pulmonary vascular tone and enhanced vasoconstrictor response to hypoxia.

Role of NO in development of pulmonary hypertension

An imbalance between vasodilators and vasoconstrictors in the lung has been suggested as one mechanism for the development of pulmonary hypertension. The successful use of NO as a vasodilator in the treatment of pulmonary hypertension suggests that this imbalance may exist. Although controversial (Giad and Saleh 1995, Xue and Johns 1995), evidence that eNOS levels and endothelium-dependent vasorelaxation are decreased in human primary pulmonary hypertension suggest a role for decreased NO in the pathogenesis of this disease (Uren *et al.* 1992). Increasing NOS substrate (L-arginine) in patients with pulmonary hypertension leads to vasodilation (Mehta *et al.* 1995), while non-selective NOS inhibition increases pulmonary vascular resistance (Cremona *et al.* 1994). Taken together, these observations suggest that NO production in patients with pulmonary hypertension is not absent but likely inadequate to oppose vasoconstrictors and prevent the development and progression of this disease.

In animal studies, chronic blockade of NO in normoxic or hypoxic rats by administration of non-selective NOS inhibitor causes systemic hypertension, a decrease in cardiac output, but not pulmonary hypertension (Hampl *et al.* 1993). This suggests little role for NO in preventing the development of normoxic or hypoxic pulmonary hypertension. However, in hypoxic pulmonary hypertension of rats, the expression of all

three NOS isoforms is increased with eNOS increased in pulmonary resistance vessels and temporarily related to vascular remodeling (Ignarro *et al.* 1986, Xue *et al.* 1994, Shaul *et al.* 1995, LeCras *et al.* 1996, 1998, Xue and Johns 1996, Tyler *et al.* 1999, Resta *et al.* 1999). This upregulation of eNOS may be directly due to hypoxia or to altered mechanical forces in the vessel wall (LeCras *et al.* 1998, Resta *et al.* 1999). The functional significance of the increase in eNOS expression is controversial (Adnot *et al.* 1991), but most studies support an increase in endothelium-dependent vasodilation and NO metabolites in chronically hypoxic rat lungs (Hampl *et al.* 1993, Resta and Walker 1996, Isaacson *et al.* 1994). However, inhaled NO prevents or attenuates the generation of hypoxic pulmonary hypertension in rats (Roos *et al.* 1996, Roberts *et al.* 1995, Kouyoumdjian *et al.* 1994), suggesting that, despite the increase in eNOS expression, endogenous NO activity is insufficient to prevent hypoxic pulmonary hypertension. We have recently reported that in hypertensive rat lungs with increased eNOS levels, NO metabolite levels are decreased if the lung is ventilated with hypoxia (Sato *et al.* 1999). This is in agreement with previous studies suggesting that acute as well as chronic hypoxia interfered with release of endothelium-derived nitric oxide (Nelin *et al.* 1996, Shaul *et al.* 1993). Previous studies reporting an increase in NO metabolites in hypoxic rats were done under normoxic ventilation (Isaacson *et al.* 1994, Resta and Walker 1996). Thus, in hypoxia, low O_2 levels may limit NO production and thereby contribute to the development of pulmonary hypertension *in vivo*.

The use of gene targeted knock-out mice to determine the importance of NO in the development of pulmonary hypertension in normoxia and hypoxia has recently been reported. Using an open-chest, deeply anesthetized, hyperoxic ventilated technique to measure systemic and pulmonary hemodynamics, Steudel *et al.* (1997) observed that eNOS-deficient mice raised at sea level developed a mild degree of pulmonary hypertension (16% increase in pulmonary artery pressure) that was largely accounted for by an increase in total pulmonary resistance and associated with a decrease in cardiac output. The increase in pulmonary vascular tone was not acutely reversed by either inhaled NO or intravascular NO donors. Similar to previous studies in rats, treatment of wild-type mice for 5 days with a non-selective NOS inhibitor caused systemic hypertension and a reduction in cardiac output but no increase in pulmonary artery pressure. The authors suggested that the selective loss of eNOS increased pulmonary vascular tone to a greater degree than did treatment with a non-selective NOS inhibitor and caused mild pulmonary hypertension. They concluded that NO does play an important role in maintaining normally low pulmonary vascular tone.

Using a different technique, we also reported the development of pulmonary hypertension in eNOS-deficient mice raised under conditions equivalent to sea level (Fagan *et al.* 1999a). Using a closed-chest, sedated, non-hyperoxic technique, we measured right ventricular pressure as an index of pulmonary hypertension. We also observed the presence of mild pulmonary hypertension in eNOS-deficient mice. However, when we measured right ventricular pressures in the mildly hypoxic environment in Denver, CO (~1500m) we observed a marked increase in the severity of pulmonary hypertension (33 % increase) in eNOS-deficient mice compared to similarly raised wild-type mice. This suggested that while the loss of eNOS in normoxic conditions resulted in mild pulmonary hypertension, exposure to even modest, physiologically relevant hypoxic stress led to marked increases in pulmonary pressure. Thus NO from eNOS is important in maintaining basal pulmonary vascular tone, and loss of eNOS renders that animal more susceptible to the development of hypoxic pulmonary hypertension.

In contrast to the observation of Steudel *et al.* (1997) we found that a low dose of inhaled NO completely reversed the pulmonary hypertension in the eNOS-deficient mice in Denver (Fagan *et al.* 1999a). This indicated that the pulmonary hypertension was largely due to sustained vasoconstriction. The apparent discrepancy between our observation and that of Steudel *et al.* (1997) may be explained by methodologic differences. Our animals were studied while breathing normoxia instead of hyperoxia. Since oxygen is a vasodilator, the lungs of eNOS-deficient mice in Steudel's study may have been maximally vasodilated prior to the addition of NO.

Since eNOS is upregulated in chronic hypoxia as a possible compensatory mechanism, and eNOS-deficient mice appear to be more sensitive to modest hypoxia, loss of eNOS-derived NO may increase the severity of pulmonary hypertension in severely hypoxic mice. In a second study, Steudel *et al.* (1998) observed increased right ventricular systolic pressure in eNOS-deficient mice following three weeks of exposure to hypoxic gas (11 % O_2) while being ventilated with 100 % O_2. Consistent with their previous report, acute

administration of inhaled NO did not cause pulmonary vasodilation in either normoxic or hypoxic wild-type or eNOS-deficient mice. Additionally, chronic administration of low dose NO during hypoxic exposure did not change right ventricular pressure, but did prevent the development of right ventricular hypertrophy in both wild-type and eNOS-deficient mice. Upon re-exposure to NO, a decrease in right ventricular pressure was seen in both wild-type and eNOS-deficient mice only if they had previously been exposed to NO. Steudel *et al.* (1998) hypothesized that rebound increases in pulmonary artery pressure upon removal from NO accounted for these findings. They conclude that the effects of loss of a vasodilator such as eNOS-derived NO may be more apparent when a stress, such as severe hypoxia, requires an increase in vasodilator tone to oppose the prevailing vasoconstrictor tone.

Steudel's finding of greater pulmonary hypertension in chronically hypoxic eNOS-deficient mice is in contrast to our observations of no difference between eNOS-deficient and wild-type mice after four weeks of sustained hypoxia (Fagan *et al.* 1999a). The reason for this difference is unclear, but we did observe an increase of iNOS message in eNOS-deficient mice that was further augmented by hypoxia. Thus, a compensatory increase in NO production from iNOS may have attenuated the development of augmented pulmonary hypertension in eNOS-deficient mice. This is also supported by a similar finding of increased iNOS protein in eNOS-deficient mice at baseline and following hypoxia (Quinlan *et al.* 1998). Additionally, while we did not observe an increase in right ventricular diastolic pressure, the presence of right ventricular failure and systolic dysfunction may have limited the severity of pulmonary hypertension. Steudel *et al.* (1998), however, reported that there was no difference in right ventricular cardiac output between eNOS-deficient mice and wild-type mice following three weeks of hypoxia.

Our finding of no difference in the severity of hypoxic pulmonary hypertension in eNOS-deficient vs. wild-type mice may also be due to methodological differences between our studies and those of Steudel *et al.* (1997, 1998). In our studies, mice were spontaneously breathing and not exposed to hyperoxia at the time of hemodynamic measurement. As has been suggested in studies of rats, re-exposure to oxygen may lead to a marked increase in NO due to the upregulation of NOS isoforms, which occurs during hypoxia. We have preliminary evidence to suggest that, like in the rat, there is an increase of eNOS message and protein in wild-type mice following hypoxia. This agrees with a previous report that eNOS protein is increased in hypoxic wild-type mice (Quinlan *et al.* 1998). Thus, in Steudel's study the use of hyperoxia and increased NO production by eNOS may have accounted for the lower right ventricular pressure in wild-type vs. eNOS-deficient mice.

We have also studied the effect of loss of other NOS isoforms on the development of pulmonary hypertension. We had previously demonstrated that loss of eNOS, but not iNOS or nNOS, led to increased vasoconstriction to acute hypoxia. To determine if the loss of other NOS isoforms contributed to the development of *in vivo* pulmonary hypertension we measured right ventricular pressure in mice deficient in iNOS and nNOS (Fagan *et al.* 1999b). There was no difference between wild-type and nNOS-deficient mice, but there was a small increase in right ventricular pressure in iNOS-deficient compared to wild-type mice, which was significantly less than that of eNOS-deficient mice. We speculate that this may be due to loss of inhaled NO from the upper airway and possibly the lower airway resulting in increased pulmonary vascular tone. In humans, iNOS is the major contributor to upper airway NO and bypassing the sinuses results in a significant increase in pulmonary vascular tone (Lundberg *et al.* 1995, 1998).

While it appears that complete loss of eNOS does increase basal pulmonary vascular tone and contributes to the development of pulmonary hypertension especially under physiologically relevant hypoxia, it is unlikely that complete loss of eNOS will occur in human forms of pulmonary hypertension. It is, however, possible that partial loss of eNOS may occur in humans either congenitally or as an acquired trait. Thus, we studied the pulmonary vascular consequences of loss of one allele of eNOS. We observed that loss of one allele resulted in a 50 % reduction of eNOS protein and elevation in right ventricular pressures indistinguishable from eNOS-deficient mice (Fagan *et al.* 1999a). This finding agrees with studies in the systemic circulation which show that loss of one allele leads to abnormal vascular responses (Faraci *et al.* 1998). This finding is relevant to the pathogenesis of human primary pulmonary hypertension where eNOS may be decreased. Several different eNOS gene polymorphisms have already been linked to vascular disease, including enhanced responses to vasoconstrictors, systemic hypertension, and myocardial infarction (Plantefeve *et al.* 1999, Miyamoto *et al.* 1998, Hingorani *et al.* 1999). We speculate that in humans either genetic or acquired reduction of pulmonary

vascular eNOS may exacerbate the development of pulmonary hypertension in response to physiologically relevant hypoxia.

In summary, eNOS-derived NO appears to be important in modulating vascular tone of the pulmonary circulation and may be important in the development of pulmonary hypertension possibly by increasing the sensitivity of the pulmonary circulation to vasoconstrictors such as hypoxia. While other NOS isoforms appear to be upregulated, in a compensatory manner, they are insufficient to overcome the effect of the loss of eNOS.

Role of NO in pulmonary vascular remodeling

A prominent feature of pulmonary hypertension is remodeling of the pulmonary arteries. The plexiform lesion characterizes human primary pulmonary hypertension, while medial and adventitial hypertrophy of proximal pulmonary arteries and neo-muscularization of peripheral pulmonary arteries occurs in experimental hypoxic pulmonary hypertension (Meyrick and Reid 1980). Both Steudel *et al.* and we have found a subtle but significant neo-muscularization of peripheral pulmonary arteries but no medial hypertrophy of larger pulmonary arteries in both chronically hypoxic wild-type and eNOS-deficient mice (Fagan *et al.* 1999a, Steudel *et al.* 1998). This differs from a previous report of significant medial thickening in hypoxic mice (Hales *et al.* 1983). While it remains unclear why this discrepancy exists, the severity of pulmonary vascular remodeling in mice appears to be less robust than that in other animal species. However, as demonstrated in both Steudel *et al.* and our own studies, pulmonary vascular remodeling is more prominent in the eNOS-deficient than in the wild-type mice.

The importance of eNOS in limiting vascular remodeling following injury has been suggested by several studies in the systemic circulation. In the femoral artery, intimal thickening following cuff placement was exaggerated in eNOS-deficient mice compared to wild-type and was attenuated by the presence of estrogen (Moroi *et al.* 1998). Following ligation of the carotid artery, contralateral carotid artery remodeling (increase in smooth muscle cell proliferation) was increased in eNOS-deficient mice compared to wild-type (Rudic *et al.* 1998). These findings might be explained by the recent report that NO induced expression of cyclin-dependent kinase inhibitor p-21 through a non-cGMP mediated mechanism (Ishida *et al.* 1999). Additionally, impaired remodeling may be due to decreased vascular smooth muscle cell migration by altering the expression of matrix metalloproteinases by NO, as demonstrated using eNOS gene transfer (Gurjar *et al.* 1999). Angiogenesis required for adequate wound healing may also be impaired in eNOS-deficient mice (Lee *et al.* 1999). Impaired vascular development may contribute to major limb reduction abnormalities seen in one study of eNOS-deficient mice (Gregg *et al.* 1998).

While the remodeling in the pulmonary circulation of mice was subtle, increased muscularization of distal pulmonary arteries may contribute to the development of increased pulmonary arterial vasoreactivity and pressure following mild hypoxia (Fagan *et al.* 1999a).

Conclusions

In summary, the use of mice with targeted deletions of the NOS isoforms has helped to clarify the role of NO in the pulmonary circulation. Specifically, eNOS-derived NO plays an important role in the maintenance of normally low pulmonary vascular tone, whereas the loss of eNOS causes enhanced responses to acute hypoxia, impairs endothelium-dependent vasorelaxation and leads to enhanced susceptibility to the development of hypoxic pulmonary hypertension.

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