High Levels of Exhaled Nitric Oxide (NO) and NO Synthase III Expression in Lesional Smooth Muscle in Lymphangioleiomyomatosis

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Smooth-muscle proliferation is the hallmark of lymphangioleiomyomatosis (LAM). Although little is known about the pathogenesis of LAM, nitric oxide (NO) is a key regulator of smooth-muscle proliferation. NO is linked to the pathogenesis of other lung diseases such as asthma, in part by the finding of higher-than-normal levels of exhaled NO. If NO were involved in the abnormal smooth-muscle proliferation in LAM, we reasoned that exhaled NO from individuals with LAM would also differ from that of healthy control subjects. To evaluate this hypothesis, we studied exhaled NO in individuals with LAM in comparison with healthy and asthmatic women using a chemiluminescent NO analyzer. Women with LAM had higher exhaled NO than did healthy women but lower than asthmatic women (NO [parts per billion] median [25 to 75%]: LAM 8 [7 to 15] \([n = 28]\), control 6 [5 to 8] \([n = 21]\), asthma 14 [8 to 25] \([n = 22]\); Kruskal–Wallis \(P < 0.001\)). Immunohistochemical studies on formalin-fixed, paraffin-embedded sections of surgical and autopsy material from lungs of individuals with LAM showed diffuse NO synthase III (NOSIII) expression in the lesional smooth muscle of LAM similar to that in the vascular endothelium. NOSIII expression was limited to the vascular endothelium and bronchial smooth muscle in healthy control lungs. The increased NO and the presence of NOSIII expression in lesional smooth muscle warrants further study into the potential role for NO in the pathogenesis of LAM.

Lymphangioleiomyomatosis (LAM) is a rare disease that affects women, primarily in their reproductive years. It is a devastating illness that carries a very poor prognosis due to progressive loss of lung function and ultimately leads to death. The hallmark of the disease is the non-neoplastic proliferation of atypical smooth-muscle cells within the lung parenchyma leading to the progressive loss of lung function (1–12). Because of the rarity of LAM, little is known about its pathogenesis or effective therapies (1, 5, 10). Corticosteroids and cytotoxic agents offer no benefit (1). Given the occurrence of the disease in women of child-bearing years (1–4), reports of clinical worsening with exogenous estrogen (6), and the presence of estrogen and progesterone receptors in the proliferating LAM cells (1, 7–9), hormones—particularly estrogen—have been implicated in the pathogenesis of LAM. Hormonal manipulation, such as antiestrogen therapy, has been used with some success in treatment of LAM but the response to such therapy has been variable (1, 3, 10, 11).

Smooth-muscle proliferation is regulated by numerous cytokines and mediators, but is profoundly affected by nitric oxide (NO) (12–17). NO is a diffusible gas that is produced endogenously in the lung by a group of enzymes known collectively as NO synthases (NOSs) (EC 1.14.13.39) (18–21). These enzymes convert the amino acid L-arginine to NO and L-citrulline in the presence of oxygen and other cofactors (20). Three isoforms of NOS have been identified: neuronal (NOSI), inducible (NOSII), and endothelial (NOSIII) (18–21). Interestingly, NOSIII expression is regulated by estrogen (12, 22). In this context, we questioned whether NOSIII and NO are involved in the abnormal smooth-muscle proliferation in LAM. NO is implicated in the pathophysiology of lung diseases such as asthma (23, 24) and pulmonary hypertension (25), in part due to the findings of higher or lower levels of NO in exhaled gases. Thus, we reasoned that if NO/NOSIII plays a role in LAM, exhaled NO of individuals with LAM would differ from that of healthy control subjects. To evaluate this hypothesis, exhaled NO of individuals with LAM was compared with exhaled NO of healthy and asthmatic women, and NOS expression in the lung was evaluated.

Materials and Methods

Patient Selection

Individuals with LAM were selected based on a pathologic diagnosis by open lung biopsy or transbronchial biopsy. They were identified from membership in the LAM Foundation and evaluated during a LAM Foundation meeting. Five individuals had previously undergone a lung transplant at the time of NO measurement. The median duration since transplant in these individuals was 2 yr (range, 1.5 to 8 yr). Because LAM is a disease that affects women in childbearing age, healthy control subjects and individuals with asthma were selected on the basis of female gender and being of childbearing age. Asthma diagnosis was based on American Thoracic Society guidelines (26). Asthmatic individuals had mild to moderate asthma controlled with \(\beta_2\) agonists and/or inhaled but not oral corticosteroids. Control individuals were identified by the absence of pulmonary symptoms or history of pulmonary disease. The study was approved by the Cleveland Clinic Foundation Institutional Review Board, and informed consent was obtained from all individuals.

Exhaled NO Measurement

Levels of exhaled NO were determined by two methods. In the offline method, exhaled gas was measured by having the individuals inhale to total lung capacity (TLC) followed by exhaling against 10 cm of water pressure into a Mylar balloon while wearing nose clips. This maneuver was repeated twice with no breath hold, and again two times with a 15-s inspiratory breath hold at TLC. In the online method, individuals inhaled via the mouth to...
TLC and exhaled without delay against resistance into a mouthpiece connected directly to the analyzer while targeting a constant pressure of 12 to 13 torr. This combination of pressure and resistance resulted in a flow rate of 50 ml/s. The exhalation was maintained until a steady NO plateau of 3 s duration was detected on the computer screen (after ~ 10 to 15 s). Repeated exhalations were performed until three NO plateaus were obtained which agreed within 10% of the average value but no more than five times (27). NO was quantitated by a chemiluminescence NO analyzer (NOA 280; Sievers, Boulder, CO). The analyzer was calibrated daily using NO-free gas (zero air; Praxair, Cleveland, OH) and 8.7 parts per million NO gas (Praxair) (18). Data from the analyzer were transferred in real time to a laptop computer via a modem connection and analyzed utilizing Microsoft Excel. NO levels were performed on individuals breathing room air, or while breathing air with no NO (zero air; Praxair) when ambient NO levels were ≫ 20 parts per billion (ppb).

**Immunohistochemistry**

Formalin-fixed, paraffin-embedded sections (5 μM) were used for immunohistochemistry staining with rabbit polyclonal epitope-purified immunoglobulin (Ig) G antibody directed against human NOSIII (ABR-PA 1-037; Affinity Bioreagents, Golden, CO) after 16 min of proteinase digestion and with a mouse monoclonal IgG antibody against melanoma-specific antigen HMB-45, which has previously been reported to be present in the characteristic LAM spindle cells (4), (ENZO, Farmingdale, NY) after 8 min of proteinase digestion. Immunohistochemical staining was performed by an automated biotin-avidin peroxidase system (Ventana-ES-320) with amino-ethyl-carbonyl (Ventana, Tucson, AZ) as a chromogen. Positive controls for the NOSIII consisted of the adjacent pulmonary vasculature of LAM or control lungs. Positive controls for HMB-45 consisted of a tissue section of a breast carcinoma. Negative controls of secondary antibody only were performed on each section of tissue studied. Lungs from five patients with LAM were obtained from explanted lung or autopsy.

**Statistical Analysis**

The data are reported as means ± standard error of the mean (SEM) for normally distributed data and as median and interquartile ranges for data that were not normally distributed. Two-tailed t test statistics, analysis of variance, Kruskal–Wallis, and Mann–Whitney rank sum test were used as appropriate at a significance level of 0.05.

**Results**

**Clinical Characteristics**

The LAM, control, and asthmatic individuals were similar in age (yr, LAM 46 ± 2 [n = 28], control 41 ± 2 [n = 21], asthma 47 ± 3 [n = 22]; P = 0.23) The time since diagnosis with LAM in individuals at entry into the study varied from 1 to 17 yr (Table 1). Control and asthmatic subjects were all women in their reproductive years, to control for gender and hormonal differences. Five of the 28 LAM individuals had received lung transplantation, with the median duration since transplant 2 yr (range, 1.5 to 8 yr) (Table 1). None of the participants in the study were current users of tobacco products. Eleven individuals with LAM were ex-smokers (16 ± 4 pack-years). Three control individuals were ex-smokers (12 ± 6 pack-years). Seven asthmatic individuals were ex-smokers (20 ± 10 pack-years). Individuals with LAM had airflow limitation and decreased diffusion capacity (DLCO) (Table 1). Pulmonary function testing of asthmatics revealed mild airway obstruction (forced vital capacity [FVC] % predicted), 90 ± 4; forced expiratory volume in 1 s [FEV1] % predicted, 76 ± 4; FEV1/FVC %, 70 ± 4). One control was on levothyroxine and a β-blocker, but the rest of controls were on no medications. All asthmatic subjects were on intermittent β2 agonists and 13 out of 22 were receiving inhaled corticosteroids.

In addition to the 28 women with LAM evaluated for exhaled NO, explanted lungs from a separate group of five LAM individuals, three from transplant and two from autopsy, were obtained for NOSIII immunostaining.

<table>
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<th>Clinical characteristics of individuals with LAM</th>
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<td><strong>Age (years)</strong></td>
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<td><strong>Years since diagnosis</strong></td>
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<td><strong>FVC (% of predicted)</strong></td>
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<td><strong>Lung transplantation</strong></td>
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<td><strong>Oxygen supplement</strong></td>
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Medications:
- progesterone (17)
- leuprolide (4)
- norethindrone (2)
- megestrol acetate (2)
- inhaled corticosteroids (6)
- salmeterol (3)
- nasal inhaled corticosteroids (2)
- inhaled albuterol (3)
- inhaled ipratropium (3)
- bisphosphonate (10)
- levothyroxine (3)
- calcium (3)
- calcium (2)
- clonidine, amlodipine, nifedipine, verapamil, hydrochlorothiazide, glyburide, ferrous gluconate, famotidine (1 each)

All values are means ± SEM.
*Numbers in parentheses represent individuals receiving medications; transplanted patients were also receiving acyclovir (2); itraconazole (1); and immunosuppressive therapy, including one or more of the following: oral corticosteroids (3), cyclosporine (3), and azathioprine (2).
Exhaled NO

Offline and online exhaled NO measurements had a positive correlation ($R = 0.74$, $P < 0.001$) (Figure 1). Offline measurements with and without an inspiratory breath-hold also had a positive correlation (data not shown: $R = 0.87$, $P < 0.001$). Online NO was higher than offline. Offline measurements with an inspiratory breath-hold were used for analysis and comparison among groups. This method is reproducible over time. In four control subjects measured on two to seven separate occasions over a span of 1 mo to 3 yr, the coefficient of variation was 6.5%.

Exhaled NO from women with LAM was higher than from female control subjects but lower than levels in female asthmatics (NO [ppb] median [25 to 75%]: LAM 8 [7 to 15; $n = 28$], control 6 [5 to 8; $n = 21$], asthma 14 [8 to 25; $n = 22$]; Kruskal–Wallis $P < 0.001$) (Figure 2). Individuals with asthma who were receiving inhaled corticosteroids had significantly lower exhaled NO levels than did asthmatics not receiving inhaled corticosteroids (NO [ppb] means ± SEM: asthma off corticosteroids 25 ± 5 [$n = 9$] versus asthma on corticosteroids 14 ± 2 [$n = 13$]; $P = 0.05$) (Figure 2). In contrast, NO levels were similar among individuals with LAM on or off corticosteroids (NO [ppb] means ± SEM: LAM off corticosteroids 11 ± 1 [$n = 22$] versus LAM on corticosteroids 12 ± 3 [$n = 6$]; $P = 0.8$) (Figure 2). LAM individuals with lung transplantation had NO levels similar to other LAM individuals (NO [ppb] means ± SEM: LAM with transplantation 15 ± 3 [$n = 5$] versus LAM 11 ± 1 [$n = 23$]; $P = 0.2$).

Twenty of the 28 LAM patients were on hormonal therapy which included one or more of the following: progesterone, leuprolide, norethidone, or megesterol acetate. There was no significant difference in exhaled NO between the two groups (NO [ppb] median [25 to 75%]: LAM receiving hormonal therapy 8 [6 to 16; $n = 20$] versus LAM not receiving hormonal therapy 10 [7 to 14; $n = 8$]; $P = 0.78$). Six of the healthy controls were postmenopausal. There was no difference in NO between the pre- and postmenopausal women (NO [ppb] median [25 to 75%]: premenopausal 6 [5 to 8; $n = 15$] versus postmenopausal 6...

**Figure 2.** Exhaled NO from individuals with LAM in comparison with healthy and asthmatic individuals. Women with LAM and asthma have higher exhaled NO than do healthy women ($P < 0.001$). Asthmatics receiving inhaled corticosteroids (+cs) have lower exhaled NO than asthmatics not receiving corticosteroids (−cs) ($P = 0.05$). NO levels were similar among individuals with LAM on (+cs) or off (−cs) corticosteroids ($P = 0.8$). Each circle represents exhaled NO from a single individual.

**Figure 3.** Immunohistochemical immunoreactivity for NOSIII antibody. (A) LAM smooth-muscle cells have diffuse moderate staining for NOSIII (L) similar to the adjacent bronchial smooth muscle (B) and endothelium (arrow) (Hematoxylin counterstain; original magnification, ×20). (B) LAM smooth-muscle cells (L), bronchiolar smooth muscle (B), and endothelium of multiple vessels (arrows) have immunoreactivity to NOSIII (hematoxylin; original magnification, ×40). (C) LAM smooth-muscle cells (L) and adjacent endothelium (arrow) have immunoreactivity to NOSIII (hematoxylin; original magnification, ×200).
Exhaled NO did not correlate with time since diagnosis (NO versus years since diagnosis: $R = -0.155$ [$P = 0.596$]) or with disease severity (NO versus FVC: $R = -0.308$ [$P = 0.214$], NO versus FEV$_1$: $R = -0.0882$ [$P = 0.728$], NO versus DLCO: $R = 0.223$ [$P = 0.390$]).

**NOSIII Immunostaining**

Semiquantitative evaluation revealed that the lesional smooth muscle of LAM uniformly showed diffuse positive cytoplasmic immunoreactivity for NOSIII. However, LAM spindle cells were less immunoreactive than the adjacent vascular endothelium and airway smooth muscle (Figure 3), which was diffusely strongly positive. The density of NOSIII staining was heterogeneous within the lesions, with a suggestion of perinuclear clearing that was not seen in the bronchial smooth muscle. Figure 3C illustrates a transsection through a fascicle of LAM spindle cells that accentuates the presence of sarcoplasm around the myofiber, which may explain the clearing. Also, LAM spindle cells stained with less intensity than did the normal smooth-muscle cells, which may account for focal loss of staining as well. Control lungs were also evaluated for NOSIII immunostaining for comparison ($n = 3$). NOSIII staining was identified in the vascular and bronchial smooth muscles, and in the vascular endothelium. Unfortunately, commercially available NOSII and NOSI antibodies were not specific or sensitive enough to allow a confident interpretation of immunoreactivity of LAM cells (data not shown).

The lesional smooth muscle of LAM showed moderate to strong focal cytoplasmic immunoreactivity for HMB-45 in all five patients (data not shown) as previously reported (4). HMB-45 immunohistochemistry was performed on subsequent tissue sections of lung which were stained for NOSIII. As previously reported, HMB-45 immunoreactivity was present only in the characteristic LAM spindle cells and was not seen in the bronchial smooth-muscle cells. The cells staining positive for HMB-45 were also positive for NOSIII. Lesional smooth-muscle cells also showed focal nuclear immunoreactivity for estrogen receptors as previously described (7). In contrast, LAM smooth-muscle cells did not show immunoreactivity for progesterone receptors (data not shown).

**Discussion**

Currently, 200 to 400 individuals are diagnosed with LAM in the United States (28). The poor understanding of pathogenesis is compounded by the rarity of LAM and the unique pathology (28). However, previous studies have shown that LAM cells have estrogen and progesterone receptors, whereas normal bronchiolar or vascular smooth muscle do not stain for either receptor (1, 7–9). In this context, it has been postulated that estrogens play a role in LAM smooth-muscle proliferation.

LAM is characterized by the non-neoplastic proliferation of atypical smooth-muscle cells (LAM cells). The stimulus for proliferation is not known. However, NO is well known to mediate smooth-muscle proliferation in a variety of experimental systems (12–17). In general, NO inhibits smooth-muscle tone and cell proliferation through the activation of cyclic guanidine monophosphate (12, 21), through negative feedback control on calcium responses to growth factors (29), or by effects on programmed cell death (30). Although most studies support an inhibitory role for NO, others suggest that NO can stimulate the proliferation of smooth muscles (12). For example, physiologic levels of NO ($\mu$M) stimulate rat fetal pulmonary artery smooth-muscle cell and aortic smooth-muscle cell proliferation (12, 17).

In this study, higher-than-normal NO is demonstrated in a relatively large cohort of individuals with LAM, i.e., 5 to 10% of the total population. This raises the possibility that NO may play some role in the pathogenesis of LAM. However, the significance of high NO to pathogenesis of LAM is unclear. NO is present in the normal human lung, evidenced by NO in the exhaled air of humans and NO as S-nitrosothiol and NO$_2^-$ in the airway aspirate and bronchoalveolar lavage fluid from human lungs (18, 24, 25, 31). NO is produced by NOs, which convert L-arginine to NO and L-citrulline in a reaction that requires oxygen and nicotinamide adenine dinucleotide phosphate, and the cofactors flavin adenine dinucleotide, flavin mononucleotide, tetrahydriopterin, and calmodulin (20). Studies have identified the presence of the three NOs isoforms in the human lung (12, 31). NOSI is located in inhibitory nonadrenergic noncholinergic neurons in the lung, NOSIII in endothelial cells (12, 31), and NOSII is expressed in normal human airway epithelium (32). In general, NOSI and NOSIII activity is dependent on increases in calcium to bind calmodulin, which results in enzyme activation and picomolar levels of NO production (21). NOSII is regulated at the level of transcription and messenger RNA (mRNA) stability (32), is calcium-independent, and produces nanomolar levels of NO (20).

Interpretation of the increased levels of NO in lung diseases, such as LAM or asthma, requires an understanding of the distribution of NO in tissues. The spatial distribution of NO in an organ such as the lung will be dependent on the solubility of NO (gas–liquid interfaces), NO diffusibility, the rate at which NO is synthesized, and the rate at which NO is consumed (18). In biologic systems, up to one-third of the NO synthesized may be consumed by chemical reactions. Because NO is freely diffusible, consumption of NO can occur at different sites within the cell, extracellular fluids, and intravascular compartments. Primary reactions that may consume NO intra- and extracellularly include its reaction with oxygen, superoxide, hemoglobin, another molecule of NO, enzymes containing iron-sulfur centers, heme-containing proteins, and thiol proteins (33). In general, however, exhaled NO reflects the concentration of total NO in liquids/tissues in the lung, because at atmospheric pressures over 97% of NO is rapidly distributed from the liquid to the gaseous phase (18).

Exhaled NO from individuals with LAM was significantly lower than NO of asthmatic individuals. High NO levels in asthma are closely associated with airway inflammation (23, 24) and are attributed to increased NOSII expression in airway epithelial cells. NOSII produces 1,000-fold higher levels of NO than does NOSIII (18–20), and is inhibited at the transcriptional and translational levels by corticosteroids (18–29, 32). In this context, lower NO in LAM than in asthma suggests that the mechanism of in-
creased NO may be different. Importantly, inhaled corticosteroids significantly reduce NO in asthma whereas NO levels in LAM are similar regardless of corticosteroid use. Interestingly, NO levels in transplanted LAM individuals were also elevated; however, transplantation itself increases exhaled NO which may be related to rejection and NOSII expression (34). These observations and the fact that LAM is not an inflammatory lung process suggest that increased NO may not be related to NOSII. An alternative source of NO may be NOSIII, an isoform known to be expressed in smooth muscle and to be regulated hormonally. Both pregnancy and estradiol increase NOSIII mRNAs in several tissues (23). Strikingly, the expression of NOSIII in the fetal rodent lung increases during late gestation, which is mediated in part by estrogen (12, 35). In the present study, NOSII was diffusely present in lesional smooth muscle of LAM. Although changes in NOS expression do not automatically mean there will be elevations in exhaled NO, on the basis of the uniformity of NOSIII expression in all smooth muscles in the lung, high levels of exhaled NO may be due to the fact that LAM lungs contain an abnormal amount of muscle with NOSIII expression. However, the lack of association between hormone therapy and NO levels does not support a role for estrogen in NOSIII expression in LAM. Further studies of regulation of NO in individuals with LAM may reveal insight into the role of NO in the progressive smooth-muscle proliferation in LAM.

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References