Published in final edited form as:

Am J Physiol Lung Cell Mol Physiol. 2008 January ; 294(1): L139–L148. doi:10.1152/ajplung.00384.2007.

Airway Smooth Muscle Relaxation is Impaired in Mice Lacking the p47^{phox} Subunit of NAD(P)H Oxidase

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Abstract

NAD(P)H oxidase is one of the critical enzymes mediating cellular production of reactive oxygen species and has a central role in airway smooth muscle (ASM) cell proliferation. Since reactive oxygen species also affect ASM contractile response, we hypothesized a regulatory role of NAD(P)H oxidase in ASM contractility. We therefore studied ASM function in wild type mice (C57BL/6J) and mice deficient in a component (p47^{phox}) of NAD(P)H oxidase. In histological sections of the trachea we found that the area occupied by ASM was 17% more in p47^{phox}^{-/-} than in wild type mice. After correcting for the difference in ASM content, we found that force generation did not vary between the two genotypes. Similarly, their ASM shortening velocity, maximal power, and sensitivity to acetylcholine, as well as airway responsiveness to methacholine *in vivo* were not significantly different. The main finding of this study was a significantly reduced ASM relaxation in p47^{phox}^{-/-} compared with wild type mice both during the stimulus and after the end of stimulation. The tension relaxation attained at the 20th s of electric field stimulation was respectively 17.6±2.4 and 9.2±2.3% in null and wild type mice (p<0.01 by t-test). Similar significant differences were found in the rate of tension relaxation and the time required to reduce tension by half. Our data suggest that NAD(P)H oxidase may have a role in the structural arrangement and mechanical properties of the airway tissue. Most importantly, we report the first evidence that the p47^{phox} subunit of NAD(P)H oxidase plays a role in ASM relaxation.

Keywords

airways; airway responsiveness; contractility; reactive oxygen species

Introduction

The role of airway smooth muscle (ASM) in asthma and other diseases presenting with airway hyperresponsiveness may result from an increase in its mass, the influence it has on structural remodeling of the airways, its altered functional properties, or from a combination of these features (2,23,30,44). Partial physical removal of smooth muscle from central airways by thermoplasty has been recently attempted in humans and has given promising

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This work was partially presented in preliminary forms at the 2005 annual meeting of the European Respiratory Society and at the 2007 annual meetings of the American Thoracic Society.

results in reducing airway hyperresponsiveness (13,14,38), with as yet unknown long term outcomes. The improvement of asthma symptoms by thermoplasty points out the importance of pursuing a better understanding of the factors that regulate ASM functions, which could in turn allow the development of more effective strategies to fight airway hyperresponsiveness. One emerging factor that may play a regulatory role in ASM function is the cell signaling associated with generation of reactive oxygen species (ROS), which have been implicated in asthma (20,33) as well as in ASM proliferation (7,8) and contractility (9,20).

ROS are unstable compounds containing reduced oxygen and are traditionally recognized as important mediators of inflammation (28,29) and responses to infections (36). ROS have been shown to be elevated in the exhaled air in asthmatics compared with healthy individuals and are considered indicative of the severity of airway inflammation (3,27). Their production has been suggested to increase after allergen challenge, because blood eosinophils isolated after allergen challenge produced more ROS than eosinophils isolated from the same patients before challenge (16). Moreover, generation of ROS from bronchoalveolar lavage cells correlates with the severity of asthma symptoms (24,25). However, ROS are not only the product of inflammatory cells, as increasing evidence show that they are generated by virtually any cell type, affecting many physiological processes and functioning as signaling molecules (21,39,51).

We have shown that ROS are required for ASM cell proliferation in response to mitogenic stimulation with fetal bovine serum or platelet derived growth factor (8). Our study showed that ROS are involved in the mitogen-induced activation of c-fos mRNA translation, an early event in ASM cell proliferation. We also showed that ASM cell proliferation is decreased by pharmacological inhibition of NAD(P)H oxidase or by antisense oligonucleotides to the NAD(P)H oxidase component p22^{phox}, thus suggesting that the critical endogenous source of ROS in ASM cells is NAD(P)H oxidase (7,8). In addition to p22^{phox}, we have recently reported that ASM cells also express the NAD(P)H oxidase components p47^{phox}, p67^{phox}, and Nox4 (47).

Although the role of NAD(P)H oxidase in ASM contractile function has not been investigated, several studies have addressed the effects of ROS on ASM, with sometimes contradictory results. On the one hand, it has been shown that oxidants produce bronchoconstriction and airway hyperresponsiveness *in vitro* (42) and *in vivo* (26) and that antioxidants reduce ASM contractile response to multiple contractile agonists (9). It has been suggested that increased myosin regulatory chain phosphorylation may contribute to this increase in ASM contractility by ROS, because antioxidants prevented the increase in myosin light chain phosphorylation induced by tumor necrosis factor- α (48). On the other hand, a few studies have unequivocally shown a relaxing effect of oxidants on ASM. In the presence of superoxide dismutase inhibitors, which prevented the conversion of superoxide into hydrogen peroxide, exogenous superoxide decreased the contraction induced by electric field stimulation (EFS) and relaxed bronchial smooth muscle pre-contracted with serotonin (6). Similarly, H₂O₂ was shown to relax pre-contracted tracheal smooth muscle (18). This relaxing effect of H₂O₂ was found to be associated with myosin light chain de-phosphorylation (32,41). Altogether, these studies indicate that the effect of oxidants on ASM contractility may vary with concentration, molecular species, cellular enzymatic repertoire, and possibly location of the ROS source within tissue and cell. More importantly, they suggest that ROS are likely to operate as regulatory signaling molecules in ASM contractile function.

In order to address the physiological role of endogenous ROS in ASM contraction and relaxation, we sought to first study the *in vitro* ASM mechanics in tissue with impaired ROS

generating ability. A vital knockout mouse model is available with deletion of the p47^{phox} subunit of NAD(P)H oxidase and consequently reduced generation of endogenous ROS (5,22). The present study was designed to evaluate in p47^{phox}^{-/-} mice whether the ASM functional response was altered in comparison with the relevant background wild type (C57BL/6J). We examined the ASM content by histology, the *in vitro* contraction and relaxation in response to EFS and sensitivity to ACh, and the *in vivo* airway responsiveness to methacholine.

Methods

Animals and tissue sample preparation

All animal experimentation was approved by the Duke University Institutional Animal Care and Use Committee. Wild type mice were obtained from Jackson Laboratories. The colony of p47^{phox}^{-/-} mice was established thanks to the generous gift of null mice from Dr. Steven Holland at National Institute of Allergy and Infectious Disease, NIH (22). They were generated by backcrossing over ten generations with wild type C57BL/6J and genotyped by polymerase chain reaction for selection of homozygous. For all *in vitro* experiments, animals were euthanized with Na-pentobarbital (250 mg/kg ip) and then the trachea was removed and immersed in Krebs-Henseleit (K-H) solution aerated with 95% O₂-balanced CO₂. The K-H solution contained (in mM): 115 NaCl, 1.38 NaH₂PO₄, 25 NaHCO₃, 2.5 KCl, 2.46 MgSO₄, 11.2 dextrose, and 1.9 CaCl₂. Under a dissecting microscope (SZH10 Olympus stereomicroscope), loose connective tissue was carefully removed from the outer surface of the trachea. To prepare tracheal strips, the cartilage rings were cut ventrally along the longitudinal axis of the trachea. Each tracheal strip was dissected with cartilaginous attachment on both ends. One cartilaginous end of the strip was clamped in the clip of a strip holder, which was inserted in an 80 ml water-jacked organ bath filled with oxygenated K-H at 37°C. The other end of the strip was tied, through a stainless steel hook inserted in the cartilages, to the transducer tip of a servo-controlled lever system using a 5.0 silk surgical thread. The dimensions of the strips, i.e. length, width, and thickness were measured using a video camera (Hitachi VK-C370 digital signal processor with Nikon c-mount adapter) and a Sony television monitor. The dimensions projected on the television monitor were calibrated with a ruler placed adjacent to the muscle strip in the tissue bath. All the preparative procedures were performed in K-H solution buffered to pH 7.35-7.45 by continuous aeration with 95% O₂ – 5% CO₂. For concentration response curves to acetylcholine (ACh), tracheal rings were prepared, mounted in organ baths, and connected to a force transducer (Grass FTO3). The signal was amplified (Gould transducer amplifier 13-4615-50) and the level of tension continuously recorded through a computerized data acquisition system (Astro-Med Grass PolyView).

Histology

Although we performed an accurate direct measurement of the strip cross sectional area (CSA) through projection of the strip image onto a TV monitor, the content of smooth muscle could vary between wild type and null mice. Therefore, in 5 wild type and 5 p47^{phox}^{-/-} mice we measured by histology the area occupied by smooth muscle as well as ASM thickness in transverse sections of the trachea. Sections of the trachea were obtained by fixing tracheal rings in 10% formalin. After fixation, strips were embedded in paraffin and cross sections (5 µm thick) were obtained at 300 µm step intervals along the entire length of the trachea. Slides stained with hematoxylin-eosin were viewed through an Olympus microscope (model BX40F) and pictures of the cross sections captured by a connected camera (Hitachi VK-C370). Distances and areas were calibrated using an engraved calibration slide (Olympus) and quantitative measurements of the smooth muscle area, the total area, and smooth muscle thickness were made using image analysis software

(Sigmascan, Jandel Scientific). Five thickness measurements were taken at regular intervals from each section and the average value was used for analysis. The total area of the tracheal section was measured exclusively in the posterior portion of the trachea that contained smooth muscle.

Active stress generation

Active stress generating ability was studied in tracheal strips from 16 wild type and 16 p47^{phox}^{-/-} mice. After equilibrating the strips for 60 min, EFS (15 V, 60 Hz) was produced by wire platinum electrodes positioned on both sides of the strip. A partial length-tension curve was obtained by stretching the strips at increasing length and recording the isometric response to EFS at each increment until the maximal force was generated. The maximal active tension (AT) was expressed as stress (mN/mm²) by normalizing maximal force per CSA of the strip. CSA was obtained from the measured width and thickness of each strip. The maximal rate of stress generation was obtained by performing the derivative of the force curve and measuring its maximal value.

Force-Velocity experiments

In 6 of the wild type and 6 of the p47^{phox}^{-/-} mice employed for the measure of active stress, we also studied ASM force-velocity (FV) properties. Stimulus parameters for these experiments were the same as described in the previous section, but the stimulus duration was kept to the minimum time, i.e. 5 s, that allowed application of load-clamp. Outputs from the force-displacement transducer were recorded simultaneously for force and length vs. time, using a previously described electromagnetic lever system (11). The quick-release load-clamp technique was employed to obtain FV curves. Every 6 min a stimulus was triggered and the muscle contracted isometrically. At 1.5 s after the onset of the stimulus, load-clamps to various afterloads were applied by abruptly (within 3 ms) changing conditions from isometric to isotonic. The quick-release resulted in an immediate shortening due to the smooth muscle series elastic component followed by 2 or 3 oscillations (critically damped to minimize them) that lasted for ~ 80 ms. Then the shortening due to the contractile component occurred and its maximal slope was computed and identified as the maximal velocity of shortening for each given afterload. Afterloads were applied in a random order to minimize time-dependent and history-related effects on shortening velocity and normalized to the maximal isometric stress (P_o) generated when the quick-release load-clamp was applied.

The FV relationship of each strip was obtained and fitted with a modified form of the Hill equation for ASM (49), which accounts for deviation of ASM shortening velocity at high loads from a hyperbolic FV relationship. From the best fit of the equation we calculated V_{max} , the maximal velocity of shortening at zero load. As a further index of contractility for loads between zero and P_o , we calculated the maximal power developed by each tracheal strips. This was obtained as the maximal value of the product of each applied afterload (mN·mm⁻²) and the maximal velocity of shortening ($l_{ref} \cdot s^{-1}$) reached with that given afterload. Power was normalized per maximal stress and expressed as $P_o \cdot l_{ref} \cdot s^{-1}$.

Smooth muscle relaxation

In order to study the intrinsic ability of ASM to spontaneously relax, in the same strips used to study active stress generation, we analyzed two distinct components of ASM spontaneous relaxation: the relaxation occurring in response to stimulation, i.e. the relaxation produced during a 20 s EFS, and the relaxation occurring by turning off the stimulation when force was maximal, i.e. relaxation after the end of a 10 s EFS. We used quantitative parameters that we previously developed to compare relaxation in different types of ASM (10). To analyze the relaxation phase during EFS, we measured: a) the tension relaxation at the end

of the 20 s EFS (TR_{end}) and b) the maximal rate of tension relaxation during the EFS (RTR_{st}). RTR_{st} was obtained by performing the derivative of the recorded force trace as a function of time and measuring its minimum value (maximal slope) during the stimulus. To analyze the relaxation after the end of a 10 s EFS, we measured the maximal rate of tension relaxation (RTR_{end}) and the time ($t_{1/2}$) needed for half of the active tension generated by the strip when the stimulus was turned off (AT_{end}) to be dissipated. RTR_{end} was obtained as the minimum value of the force trace derivative after the end of the stimulus.

In vitro airway smooth muscle responsiveness to acetylcholine

Cumulative concentration-response curves to ACh (10^{-9} – 10^{-3} M) were obtained in tracheal rings from 5 wild type and 5 $p47^{phox-/-}$ mice. Rings were equilibrated at a resting tension of 0.25 g for 90 minutes, after which the response to 10^{-3} M ACh was elicited. The rings were then repeatedly rinsed with fresh K-H until the tension returned to a resting value. After rinsing, cumulative concentrations of ACh were administered and response expressed as percent of the previous response to 10^{-3} M ACh. Sensitivity to ACh was calculated as EC_{50} , the concentration that evoked 50% of the maximal active tension attained during the concentration-response curve.

In vivo airway responsiveness

Airway responsiveness *in vivo* was evaluated using a flexiVent system (SCIREQ) in 6 wild type and 6 $p47^{phox-/-}$ mice. Mice were anaesthetized with an ip injection of 65 mg/kg Na-pentobarbital, the extra-thoracic trachea was exposed, and a tracheal cannula inserted through an incision of the trachea. After intubation, mice were neuromuscularly paralyzed with 0.8 mg/kg pancuronium bromide, the tracheal cannula was connected to a ventilator, and animals were ventilated with room air at a constant tidal volume of 6–8 ml/kg, at a positive end-expiratory pressure of 3 cm H_2O , and at a rate of 140 breaths/min. Measurement of airway pressure was made at a side port of the afferent limb of the ventilator wye using a differential pressure transducer. Airway resistance was monitored from pressure and volume data generated by applying a 2-s sine wave volume perturbation to the tracheal cannula with amplitude of 0.2 ml and a frequency of 2.5 Hz (low frequency forced oscillation technique). Airway resistance and static lung compliance measures were acquired at baseline and following methacholine aerosol challenge. Pressure-volume loops were generated at baseline. Three doses of methacholine aerosol (10, 25, 100 mg/ml for 70 breaths) generated by DeVilbiss ultrasonic nebulizer were delivered through the airway cannula. Between doses the lung was hyperinflated at a pressure of 30 cm H_2O to return resistance to baseline prior to the administration of the next dose of methacholine.

Drugs and chemicals

The drugs used were: Pentobarbital sodium (Abbot Laboratories), acetylcholine chloride, acetyl- β -methylcholine chloride, pancuronium bromide (Sigma), and 10% neutral buffered formalin (Trend Scientific).

Data Analysis

Data are expressed as means $\pm$ standard error of the mean except for the EC_{50} , which is expressed as geometric mean and geometric standard error of the mean. Data were compared using either Student's t-test or analysis of variance (ANOVA). The least significant difference (LSD) post hoc analysis was used to find which groups were responsible for differences revealed by ANOVA. When normality test failed for a given comparison, non parametric tests were used instead of the equivalent relevant parametric tests. The statistical software used was SigmaStat (Systat Software). Values of $p < 0.05$ were considered as statistically significant.

Results

Histology

Figure 1 shows representative microphotographs of tracheal cross sections at proximal, median, and distal portions of a mouse trachea. Both in wild type and null mice, the smooth muscle content increased gradually and substantially from the proximal to the distal trachea. The distance between the two tips of the cartilage ring also increased from the proximal to the distal portion, but this was not associated with an increase in length of the smooth muscle bundles, due to a gradual concomitant decrease of the overlapping between smooth muscle and cartilage. The average ASM thickness, ASM length, percent area occupied by ASM in the cross section of the trachea, and tracheal wall thickness are reported in table 1. On the one hand, no significant difference was found between wild type and p47^{phox}^{-/-} mice when comparing smooth muscle thickness. On the other hand, the ASM length ($P < 0.01$ by ANOVA) and percent area occupied by ASM ($P < 0.05$ by ANOVA) were significantly increased, whereas the tracheal wall total thickness was significantly reduced ($P < 0.01$ by ANOVA) in null mice compared with wild type. The area occupied by ASM in tracheas from p47^{phox}^{-/-} mice was 17% more than in tracheas from wild type mice. We used this value to correct the active stress normalization, thus accounting for the different content of ASM in the CSA of the strips. The structural differences observed in the two genotypes further support the notion that NAD(P)H oxidase may play a role in the regulation of airway structure and may be involved in airway remodeling.

Active stress generation

Representative recordings of the response to 10s EFS in tracheal strips from wild type and p47^{phox}^{-/-} mice are shown in Fig. 2. The maximal stress developed in response to EFS in p47^{phox}^{-/-} mice was 40% greater than in wild type (Fig. 3A) when normalized for strip CSA. However, when stress was corrected by the factor derived from our histological analysis to account for the different content of ASM, the difference was largely reduced, as shown in Fig. 3B. The stress at rest was respectively 2.8 ± 0.4 and 3.0 ± 0.4 mN/mm² and the maximal rate of stress generation was respectively 4.9 ± 0.7 and 4.2 ± 0.6 mN/mm²/s for wild type and null mice (not statistically different). However, when normalized by maximal active stress, the rate of stress generation was found to be significantly different (respectively 0.60 ± 0.03 and 0.49 ± 0.02 AT/s for wild type and null mice, $p < 0.01$ by t-test). This relative decrease in rate of tension development in null mice might indicate differences in the cross bridge cycling rate and a possible role of NAD(P)H oxidase in the regulation of phosphorylation/de-phosphorylation of the regulatory myosin light chain.

Force-Velocity experiments

Figure 4A shows the full FV relationship for the two groups, computed as the average of the single best fit FV curves. Maximal power was attained at afterloads of 64 and 68% P_0 for wild type and null mice, respectively, and was not different between the two genotypes (Fig. 4B). The calculated mean maximal velocity of shortening at zero load was 50% greater in p47^{phox}^{-/-} mice (0.62 ± 0.13 l_{ref}/s) than in wild type (0.38 ± 0.05), although the difference was not statistically significant ($p = 0.24$). This trend to an increase in shortening velocity for null mice occurring only at afterloads approximating zero load suggests that the resistive components that came into play after extensive shortening may be more compliant in the tissue of null mice than in wild type.

Airway smooth muscle relaxation

Airway smooth muscle relaxation was investigated in two different settings: a) by turning off the EFS at 10 s, when the maximal active stress was produced by the strip; b) by keeping the EFS on for 20 s and analyzing the relaxation during the EFS.

Figure 5 shows findings in the first setting. The maximal rate of tension relaxation (RTR_{end} , panel A) was significantly reduced ($p < 0.01$ by t-test) and the time required to reduce tension by half ($t_{1/2}$, panel B) was significantly increased ($p < 0.01$ by t-test) in $p47^{phox-/-}$ mice compared with wild type. These results show that NAD(P)H oxidase activity is required for an optimal return to resting conditions in ASM after the removal of a contractile stimulation.

Representative recordings of the response to 20 s EFS in tracheal strips from wild type and $p47^{phox-/-}$ mice are shown in Fig. 6. The maximal rate of the tension relaxation that took place during EFS (RTR_{st} , panel A) and the amount of tension relaxation at 20 s (TR, panel B) are shown in Fig. 7. Both parameters were significantly reduced ($p < 0.01$ by t-test) in $p47^{phox-/-}$ mice compared with wild type. These results show that the normally occurring spontaneous relaxation that counteracts force generation during a contractile stimulation is substantially impaired in ASM lacking the full repertoire of NAD(P)H oxidase activity.

Taken together, these data show that endogenous activity of NAD(P)H oxidase contributes to the ASM functional phenotype as an essential factor in the regulation of spontaneous relaxation.

In vitro airway smooth muscle responsiveness to acetylcholine

The concentration-dependent isometric contraction caused by ACh was similar in tracheal rings from wild type and $p47^{phox-/-}$ mice (Fig. 8). EC_{50} values for ACh were 3.09×10^{-6} (1.30 GSEM) M and 7.06×10^{-6} (1.38) M in rings from wild type and null mice, respectively. These data show that the agonist-receptor interaction and consequently the sensitivity of ASM to contractile stimulation are not affected by impairment of NAD(P)H oxidase activity.

In vivo airway responsiveness

The *in vivo* airway responsiveness to methacholine (MCh) in wild type and $p47^{phox-/-}$ mice is shown in Fig. 9A, expressed as increase in total lung resistance produced by each dose of MCh above baseline lung resistance. No difference in airway responsiveness was observed between wild type and null mice. However, baseline total lung resistance was lower ($p < 0.01$ by t-test) in null mice (0.45 ± 0.02 cmH₂O/ml/s) compared with wild type (0.65 ± 0.01), whereas the baseline static lung compliance was higher ($p < 0.01$ by t-test) in null mice (0.052 ± 0.005 ml/cmH₂O) compared with wild type (0.021 ± 0.001 ml/cmH₂O). These differences were reflected in the pressure-volume loops shown in Fig. 9B, which were obtained at baseline. On the one hand, by showing no alteration in the response to a muscarinic receptor agonist, these results confirm the data of *in vitro* ASM responsiveness to ACh. On the other hand the PV loops and the baseline R_T further suggest that impairment of NAD(P)H oxidase may affect the degree of tissue compliance through the regulation of airway structure, e.g. by affecting the extracellular matrix. Altered tissue compliance could become a crucial factor in airway responsiveness when pathologic conditions arise.

Discussion

In the present study we investigated the role of NAD(P)H oxidase activity in ASM function by evaluating structural and functional aspects of ASM in the trachea of mice lacking the $p47^{phox}$ subunit of this oxidase. Structurally, we found that the area occupied by ASM in

tracheas from p47^{phox}^{-/-} mice was 17% more than in tracheas from wild type mice. This originated from a concomitant slight increase in ASM and decrease in non muscle tissue. Functionally, we found that ASM relaxation was substantially impaired in null mice, thus providing the first evidence that the p47^{phox} subunit of NAD(P)H oxidase plays a role in ASM relaxation. Differences in contractile properties and sensitivity to acetylcholine, as well as airway responsiveness *in vivo* were not significant. However, our histological, *in vitro* shortening velocity, and *in vivo* compliance data concurrently suggest that the lack of the p47^{phox} subunit may determine an alteration of the structural-mechanical properties of non muscle tissue, which may in turn worsen airway responsiveness in pathological conditions.

The p47^{phox} cytosolic subunit is responsible for a critical step in the NAD(P)H oxidase assembly and activation and is often referred to as “the organizer” portion of this enzymatic complex. It requires phosphorylation to become active and in turn to promote enzyme activity (1). The p47^{phox} knockout mouse model reproduces the phenotype of human chronic granulomatous disease (CGD), with absence of superoxide production by phagocytes, tissue granuloma formation, and recurrent infections (22). Restoration of p47^{phox} through gene therapy recovers host defense ability (34). A role for this NAD(P)H oxidase subunit has been also shown in non-phagocytic cells, in which superoxide and its derivatives are increasingly recognized as important signaling molecules. In a mouse model of myocardial infarction induced by permanent ligation of the left coronary artery, p47^{phox}^{-/-} mice did not show the increase of NADPH oxidase activity and ROS production in left ventricular myocardium after myocardial infarction. Also, cardiomyocyte hypertrophy, apoptosis, and interstitial fibrosis were reduced in null mice, thus increasing substantially their survival rate (15). Similarly, cultured vascular smooth muscle cells isolated from p47^{phox}^{-/-} mice showed reduced superoxide generation than wild type when stimulated with bovine serum or thrombin (5). Our study shows that airway structure/mechanical properties and ASM function are altered in p47^{phox}^{-/-} mice, thus indicating a role for the p47^{phox} NAD(P)H subunit also in the pulmonary tissue. Our data further support the increasing evidence of a cell signaling role for endogenous ROS produced by NAD(P)H oxidase in a variety of physiological processes and specifically in the regulation of airway function and remodeling.

The main finding of our study was that ASM relaxation is impaired in mice lacking the p47^{phox} subunit of NAD(P)H oxidase activity. We studied two distinct aspects of ASM relaxation, the relaxation occurring when stimulation is turned off and the relaxation occurring while the stimulation is ongoing. Both these aspects of relaxation occur spontaneously in healthy adult ASM and likely share signaling mechanisms, although very little is known about their respective regulation. We have shown that the alteration of one of the two types of relaxation is not associated with alteration of the other (10). Indeed, in healthy immature guinea pig ASM, we observed a quite limited relaxation during EFS that became substantial toward adulthood, whereas the relaxation occurring after the end of the stimulation was substantial in immature ASM and decreased slightly but significantly with maturation. Because ASM relaxation during EFS had been previously reported to be impaired in allergic dogs compared with control animals (37), we suggested that this type of spontaneous relaxation may be relevant to airway hyperresponsiveness. This is further suggested by the absence of spontaneous relaxation we have recently reported in A/J mice, a strain characterized by intrinsic airway hyperresponsiveness, whereas a substantial spontaneous relaxation was observed in normo-responsive mice (12). Besides spontaneous relaxation, pharmacologically induced relaxation has been shown to be impaired in hyperresponsive airways (4,46). It remains to be investigated whether relaxation in response to bronchodilators is also impaired in p47^{phox}^{-/-} mice. What the present work demonstrates is that NAD(P)H oxidase activity is involved in the signaling pathways leading to

spontaneous relaxation. Whether this occurs through mechanisms regulating calcium sensitization, myosin regulatory chain dephosphorylation, interaction with endogenous relaxants, or other relaxation components, will be the subject of future investigations.

The structural analysis of ASM distribution along the trachea was undertaken for the purpose of correcting the calculation of active stress for possible differences in ASM content between wild type and null mice. In studies on ASM cells in culture, we have shown that ROS generated by NAD(P)H oxidase are required for ASM cell proliferation (7,8). Based on those data we expected a possible decrease in ASM content in p47^{phox}^{-/-} mice. However, instead of reduced ASM, we found a slight but significant increase in ASM content of the tracheal paries membranaceus in null mice. Indeed, we found 17% increase in ASM content in p47^{phox}^{-/-} mice and used this value to correct our normalization of force in null mice. Our study also revealed that the total thickness of the airway wall was less in null mice than in wild type, thus suggesting that NAD(P)H oxidase may be important in the formative process of airway tissues other than smooth muscle. Several explanations can be identified to account for these unexpected findings and need experimental testing. One possible explanation is that the p47^{phox} subunit is not required for ASM cell proliferation. Vascular smooth muscle null for p47^{phox} displays diminished production of superoxide anion and reduced proliferation in response to growth factors than cells from wild type (5). However, cell proliferation has not been studied in p47^{phox}^{-/-} ASM. Moreover, we have recently shown that ASM expression of p47^{phox} is low and that stimulation of ASM cell proliferation by TGF- β 1 is dependent on increased expression of the Nox4 subunit of NAD(P)H oxidase only (47). However, more direct experiments will be necessary to confirm or to exclude a participation of the p47^{phox} in ASM cell proliferation. A second possibility is that a compensatory mechanism takes place during development to assure the required production of signaling ROS, thus leading to increased Nox4 expression in p47^{phox}^{-/-} mice. Generation of ROS by Nox4 has been recently shown to occur without the contribution of NAD(P)H oxidase cytoplasmic subunits (35). Constitutive Nox4 could be functional and responsible for the proliferation of ASM in p47^{phox}^{-/-} mice. In this case, increased expression of Nox4 would account for the increased percent of ASM we have found in p47^{phox}^{-/-} mice. A further explanation could be that, like in coronary microvascular endothelial cells (31), p47^{phox} is not required for or may even reduce basal production of superoxide, but is fundamental in superoxide production induced by growth factors or agonists such as phorbol-myristate-acetate or tumor necrosis factor- α (8,43,47,48,50). If this was the case, the development of smooth muscle tissue in mice lacking p47^{phox} would be favored or not affected in absence of stimulating factors, such as in our study, whereas smooth muscle hypertrophy/hyperplasia would be inhibited following stimulation by factors involved in remodeling. Finally, the increased percent of area occupied by ASM in p47^{phox}^{-/-} tracheas can reside in the potential interaction of smooth muscle with the extracellular matrix and with other cells in the airway wall. Indeed, more than one extracellular matrix factor has been identified as strong regulator of ASM cell cycle and survival (17). Moreover, the extracellular matrix is an important component in the control of growth factor-mediated signaling, cell proliferation, and differentiation (45). The structural alterations of the airway wall that we have observed in p47^{phox}^{-/-} mice may result in a modified regulation of ASM cell proliferation by the extracellular matrix. This could counteract the effect of the expected reduced production of ROS on ASM proliferation. Further studies will be necessary to elucidate these points that are at the present substantially speculative, but nonetheless highlight a potential crucial role for NAD(P)H oxidase in airway structural development and remodeling.

Despite the tracheal structural alterations in p47^{phox}^{-/-} mice, the ASM contractile response was essentially unaffected, as shown by our data on active stress generation, shortening velocity, *in vitro* sensitivity to ACh and *in vivo* airway responsiveness to MCh. Nonetheless,

our FV study *in vitro* as well as the PV loops and baseline compliance *in vivo* concordantly indicate greater tissue compliance in null mice and further suggest that ROS may be involved in the structural development of the airway wall. ROS have been shown to promote extracellular matrix synthesis in kidney (19) and vascular smooth muscle cells (40). A reduced synthesis of extracellular matrix in the airway walls of p47^{phox}^{-/-} mice would likely account for the increased compliance we report in this study. However, at least in our experimental setting, this modification of the mechanical properties of the airway wall was not sufficient to produce quantitative changes in the ASM contractile response and in the narrowing capability of the airways. Controversial results about the role of ROS in ASM have been reported in the literature. Some studies show that ROS may directly produce bronchoconstriction and airway hyperresponsiveness (26,42) and others provide evidence of a relaxing effect of oxidants on ASM (6,18,32,41). These discrepancies could originate from different concentrations or species of oxidants employed in those studies. It is also possible that exogenous ROS target cellular components not affected by endogenously generated ROS. Endogenous generation of superoxide by ASM has been shown to occur in response to both proliferating (8) and contractile stimuli (9). In particular, stimulation with acetylcholine determined a substantial superoxide release from guinea pig bronchial smooth muscle, which was not affected by the epithelium (9). Whether ASM from p47^{phox}^{-/-} mice produces less superoxide in response to contractile stimulation is not known. Indeed, the results of the present study could be explained by constitutively low levels of ROS, which may affect the redox state of the cell and in turn the regulation of the signaling pathways controlling ASM relaxation. Based on these considerations, we suggest that endogenous basal generation of oxidants in ASM has a relaxing role. A contractile effect of ROS could occur at high concentrations, attained by stimulation of NAD(P)H oxidase activity, for example due to inflammatory events. This scenario would be consistent with the findings of increase in ASM contractile response and myosin light chain phosphorylation induced by tumor necrosis factor- α , which was prevented by antioxidants (48). In the present work, we only studied *in vitro* and *in vivo* responsiveness in healthy animals without stimulating NAD(P)H oxidase. To test the possibility that p47^{phox}^{-/-} mice would be protected from airway hyperresponsiveness secondary to inflammation, studies should be performed in p47^{phox}^{-/-} mice presenting airway inflammation either following allergic-sensitization and challenge or administration of inflammatory agents.

Our results provide the first evidence that endogenous ROS generated by NAD(P)H oxidase under physiological conditions play a role in ASM relaxation and in the structural and mechanical arrangement of airway wall. An impaired ASM relaxation may contribute to the severity of bronchospasm by substantially delaying its removal. Although our measurement of airway responsiveness did not show differences between wild type and p47^{phox}^{-/-} mice, when conditions arise that may bring about airway inflammation and release of mediators, the alterations in structure, tissue compliance, and intrinsic ability of ASM to reverse its contractile state could become critical factors contributing to the severity of airway narrowing. In conclusion, NAD(P)H oxidase activity and the consequent generation of ROS are involved in the regulation of airway structure and ASM function. Our results support the concept that NAD(P)H oxidase may play a role in airway remodeling and hyperresponsiveness.

Acknowledgments

Supported by National Heart, Lung, and Blood Institute grants HL-075307, HL-67281, and HL-067021, and by the Duke Neonatal Perinatal Research Institute.

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Fig. 1. Histological images illustrating the cross section of the paries membranaceus in transverse sections of a mouse trachea. Sections shown in panel A, B, and C are respectively from the proximal, median, and distal portion of the trachea and illustrate the progressive changes in structural organization along the trachea.

Fig. 2.

Typical recordings of force generation in response to 10s electric field stimulation (EFS) in tracheal strips from wild type (C57BL/6J) and p47^{phox} -/- mice. An impaired spontaneous relaxation can be seen in the p47^{phox} -/- strip.

Fig. 3. Maximal tissue stress generated by electric field stimulation in tracheal strips from wild type (C57BL/6J) and p47^{phox-/-} mice. Panel A shows stress normalized per strip cross sectional area. Panel B shows that the greater stress produced by p47^{phox-/-} strips was cancelled out when corrected for the percent of airway smooth muscle in the airway wall. Means and standard errors are shown, n=16 for both groups.

Fig. 4.

A. Force-Velocity curves calculated as average of the single curves obtained for each strip by fitting experimental data with a modification of the Hill's equation for airway smooth muscle (see text and reference 41). Dashed and solid lines represent data obtained in tracheal strips from wild type and $p47^{phox-/-}$ mice, respectively. B. Maximal power in tracheal strips from wild type and $p47^{phox-/-}$ mice. P_o is the maximal stress generated by electric field stimulation at the moment of the force clamp, l_{ref} is reference length. Means and standard errors are shown, $n=6$ for both groups.

Fig. 5.

The maximal rate of tension relaxation (RTR_{end}) and the time required to reduce tension by half (t_{1/2}) after 10 s electric field stimulation in strips from wild type (C57BL/6J) and p47^{phox-/-} mice are shown respectively in panel A and B. RTR_{end} was significantly reduced (p<0.01 by t-test), whereas t_{1/2} was significantly increased (p<0.01 by t-test) in p47^{phox-/-} mice compared with wild type. Means and standard errors are shown, n=16 for both groups.

Fig. 6.

Typical recordings of force generation in response to 20 s electric field stimulation (EFS) in tracheal strips from wild type (C57BL/6J) and p47^{phox-/-} mice. No spontaneous relaxation was present during the electric field stimulation in this p47^{phox-/-} strip.

Fig. 7.

The maximal rate of tension relaxation that took place during a 20 s electric field stimulation (RTR_{st}) and the amount of tension relaxation at 20 s (TR) in strips from wild type (C57BL/6J) and p47^{phox-/-} mice are reported respectively in panel A and B. Both parameters were significantly reduced ($p < 0.01$ by t-test) in p47^{phox-/-} mice compared with wild type. Means and standard errors are shown, $n=16$ for both groups.

Fig. 8. Cumulative concentration-response curves to acetylcholine (ACh) in tracheal rings from wild type (C57BL/6J, circles) and p47^{phox}^{-/-} mice (triangles). Means and standard errors are shown, n=5 for both groups.

Fig. 9. Increase in total lung resistance (R_T) induced by aerosolized methacholine (MCh) above baseline R_T (panel A) and baseline pressure-volume loops before MCh challenge (panel B) in wild type (C57BL/6J) and $p47^{phox-/-}$ mice. Means and standard errors are shown, $n=6$ for both groups.

Table 1

Tracheal smooth muscle content in wild type (C57BL/6J) and p47^{phox}^{-/-} mice.

		C57BL/6J	p47 ^{phox} ^{-/-}
Smooth muscle thickness (μm)	Proximal	31.3 ± 2.3	36.1 ± 2.8
	Median	37.5 ± 5.1	39.9 ± 2.2
	Distal	64.9 ± 3.1	57.3 ± 4.9
Smooth muscle length (μm)	Proximal	940.6 ± 47.8	1003.1 ± 61.0
	Median	963.0 ± 43.4	1110.3 ± 23.3
	Distal	846.8 ± 70.2	1139.9 ± 72.1
Smooth muscle area (% section total area)	Proximal	19.3 ± 0.8	24.4 ± 2.1
	Median	25.6 ± 2.3	27.3 ± 0.5
	Distal	31.9 ± 3.3	37.7 ± 2.6
Tracheal wall thickness (μm)	Proximal	259.8 ± 14.5	227.8 ± 9.3
	Median	265.6 ± 8.2	201.9 ± 20.5
	Distal	277.8 ± 9.4	222.9 ± 15.0

Data obtained from tracheal cross sections of 5 wild type and 5 p47^{phox}^{-/-} mice. Means and standard errors are shown.