Hyperoxic conditions inhibit airway smooth muscle myosin phosphatase in rat pups

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Smith PG, Dreshaj A, Chaudhuri S, Onder BM, Mhanna MJ, Martin RJ. Hyperoxic conditions inhibit airway smooth muscle myosin phosphatase in rat pups. Am J Physiol Lung Cell Mol Physiol 292: L68–L73, 2007; doi:10.1152/ajplung.00460.2005.—Exposure of rat pups to 100% oxygen is a model for studying neonatal lung injury. Airway reactivity is increased in this model, in part due to impaired airway smooth muscle (ASM) relaxation. We compared biochemical determinants of ASM contractility in rat pups exposed to 100% oxygen for 7 days vs. littersmates raised in room air. The baseline quantities of ASM contractile proteins, extent of phosphorylation of the 20-kDa myosin regulatory light chain (LC20), and amount of the myosin-binding subunit of smooth muscle myosin phosphatase (MYPT) were all comparable between the two groups. Bethachol-induced contraction increased the extent of phosphorylation of both LC20 and MYPT in the hyperoxic group (45% and 70% over control, respectively). Relaxation after electrical field stimulation demonstrated greater phosphorylation of both LC20 and MYPT in the hyperoxic group compared with controls (67% and 84%, respectively). To determine if hyperoxia induced changes in the isoforms of MYPT, isoform expression was also compared but differences were not found. To determine potential mechanisms whereby MYPT phosphorylation was increased by hyperoxia, separate tracheas were treated with the Rho kinase inhibitor Y-27632. This treatment completely eliminated differences in MYPT phosphorylation between the groups. Because phosphorylation of MYPT impairs the phosphatase activity of myosin phosphatase, these data suggest that hyperoxic conditioning during early postnatal life impairs relaxation through prolonging LC20 phosphorylation. This mechanism might contribute to increased ASM reactivity seen in bronchopulmonary dysplasia.

Airway hyperresponsiveness; myosin-binding subunit; bethanechol; trachealis muscle

A significant risk factor for the development of chronic lung disease in infants is the exposure to increased concentrations of oxygen. Such lung disease is manifest as bronchopulmonary dysplasia (BPD) with increased airway reactivity being a prominent pathophysiological consequence (9, 18). The mechanisms of this airway hyperreactivity are unknown but increased sensitivity to bronchoconstricting agents and improved airway resistance with bronchodilators implicate airway smooth muscle (ASM) in the pathophysiology. Furthermore, bronchial hyperresponsiveness persists for decades after infants develop this pattern of hyperresponsiveness (13, 19). How ASM differs from normal in such diseases as BPD or asthma is not known. There are many models of ASM explaining the increased resistance, including hypertrophy, a left shift in the agonist/response curve, decreased “tethering” of the airways, or increased maximal force production. Interestingly, impaired relaxation has been theorized to be an additional important mechanism for airway hyperresponsiveness in asthma (21). We have previously noted (15) impaired ASM relaxation in a rat pup model of hyperoxic lung damage that might reflect the hyperoxia-induced lung disease in neonates. In our previous studies (1), impaired relaxation could not be attributed to smooth muscle hypertrophy or gross structural changes.

Smooth muscle contraction is initiated when the phosphorylation of the 20-kDa regulatory light chain of myosin (LC20) allows crossbridge formation of actin and myosin. Conversely, LC20 dephosphorylation, by smooth muscle myosin phosphatase (MYPT), leads to relaxation (11). The mechanical correlates of these reactions would predict that increased maximal force is due to an increased number of cycling crossbridges. Several possibilities would explain impaired relaxation. Tissue restructuring with decreased tension of surrounding structures or hypertrophy could explain these findings. Also, either greater myosin light chain (MLC) kinase (MLCK) activity or impaired MLC phosphatase activity would result in a greater extent of LC20 phosphorylation and maintenance of more crossbridges with stiffer, less compliant smooth muscle. Inhibition of MLC phosphatase activity, in fact, appears to be the central mechanism for calcium sensitization of contraction in ASM (7).

MYPT consists of three subunits. Phosphorylation of the MYPT results in marked inhibition of phosphatase activity and calcium sensitization of force production (26). MYPT activity can also vary depending on the isoform variants expressed (25). Switching between isoform variants occurs during disease states and animal maturation in some smooth muscle tissue (20). This switching has been shown to be sufficient for changing phosphatase activity and the contractile properties of smooth muscle during maturation (10). Accordingly, we studied which of these mechanisms might account for impaired relaxation of tracheal tissue after hyperoxic conditioning using the same rat pup model we had previously reported (1, 8, 14, 15).

Materials and Methods

Animals. The Case Western Reserve University Institutional Review Board for Animal Studies approved all procedures. For each experiment, 7-day-old Sprague-Dawley rat pups from two different litters were mixed, and eight rat pups from this mixed group were selected. Four rat pups were exposed to >95% O2 (hyperoxic condition), and four of their littersmates were raised in normoxic (room air) conditions for 7 days. The rat cages were otherwise maintained at
70°F with normal day/night cycles. The rat mothers were exchanged every 24 h to ensure their survival throughout the experiments. Each experiment was repeated three times (3 different groups of 8 rat pups) so that each data point reported represents n = 12 individual animals. The rat pups were killed (inhaled CO2), and their tracheas were harvested for force production studies as previously described (14).

Experimental protocol. In all experiments, the tracheas were cleaned of external connective tissue and placed in modified Krebs-Henseleit solution (KHS) as previously described (15). The biochemical parameters were compared in three conditions, baseline (no stimulation), after exposure to bethanechol, and 10 s of electrical field stimulation (EFS). In one set of experiments, baseline conditions compared tracheas not exposed to bethanechol or EFS for contractile protein content, isoforms of MYPT, 20-kDa MLC (MLC20) phosphorylation, and MYPT phosphorylation. In separate experiments, tracheas from other rat pups were again cleaned and placed in KHS. A 3-mm segment was suspended between a glass rod and a force transducer before being incubated with bethanechol at 10−5 M. This concentration was used to replicate our previous studies (1, 14, 15) in which 50−75% maximal contraction was reliably achieved in both control and hyperoxic-conditioned rat pups. These tracheas were then frozen immediately to compare the extent of LC20 activation and MYPT phosphorylation between the two experimental groups during the peak contraction produced by this dose of bethanechol. In a separate group of experiments, after exposing the tracheal rings to the EFS30 of bethanechol for 5 min, direct-current EFS (constant voltage of 5 V for 10 s) was applied. The tracheas were immediately frozen 60 s after EFS to compare the subsequent decreases in LC20 and MYPT phosphorylation between the two groups during relaxation. This time point was chosen to replicate previous studies noting the nadir in relaxation at this time with this intensity of EFS (15). In all experiments, after exposure to the above conditions, tracheas were immediately immersed in liquid nitrogen to arrest phosphorylation/dephosphorylation reactions. Tracheas were stored at −80°C until processing.

The major mechanism responsible for calcium sensitization in ASM is through inhibition of myosin phosphatase, specifically at Thr641 of MYPT by Rho kinase (17). Inhibition of Rho kinase keeps MYPT in its active form, thereby decreasing LC20 phosphorylation and promoting smooth muscle relaxation. To determine if alterations in smooth muscle relaxation noted with EFS were due in part to impaired phosphatase activity, we performed an additional set of experiments in which the contractile stimulation with bethanechol and exposure to EFS were replicated as outlined above. In each experiment, four rat pups from each group (hyperoxia and room air littermates) were exposed to the Rho kinase inhibitor Y-27632, and four were left untreated. The tracheas were processed and studied for the proportion of LC20 and MYPT that had been phosphorylated as described above.

Determination of contractile protein content. Tracheas were transferred from −80°C storage into 500 μl of 10% trichloroacetic acid in acetonitrile/0 mM DTT on dry ice for 5 min, pulverized, washed three times with acetone/10 mM DTT, and then air-dried. The samples were rehydrated with urea sample buffer (8 M urea, 10 mM DTT, glycerol 5% vol/vol, bromophenol blue 0.1%). The samples were boiled for 3 min followed by repeated vortexing to enhance protein extraction and finally centrifugation to remove debris. Lysates (20 μg/lane) were analyzed by SDS-PAGE [12% acrylamide (19:1 acrylamide/bisacrylamide), 40% glycerol gels, 1 h at 125 mV] and then transferred to nitrocellulose membranes (Tris glycine buffer with 20% methanol, 40 min at 100 mV). After blocking (1% BSA in wash buffer), membranes were probed for the proteins described below, and these were detected by chemiluminescence (Supersignal; Pierce, Rockford, IL). Images on radiographic film were analyzed using laser densitometry (SCIscan 1000; USB, Cleveland, OH) as previously described (22).

To compare the extent of LC20 phosphorylation at each of the time points described, membranes were analyzed first for total LC20. These membranes were then stripped of antibody using 75 mM Tris HCl, pH 6.8, 2% SDS, and 100 mM 2-mercaptoethanol at 55°C for 30 min. Membranes were then washed with TBS containing 0.05% Tween 20 (TBST) and immunoblotted using the antibodies described below that were specific for phosphorylated proteins of interest. To compare extent of LC20 phosphorylation, the membranes were reprobed using an antibody specific for phosphorylated LC20 described below. Control and hyperoxic samples were placed in different lanes of the same gel and repeated in triplicate to allow normalization between samples and avoid artifact differences inherent in Western blotting. Results are reported as mean ratios of phosphorylated-to-total LC20 band intensities.

To determine if hyperoxic conditions induced increases in smooth muscle deposition (hypertrophy or hyperplasia) that might account for previously noted impaired relaxation, lysates from tracheal samples from both room air and hyperoxic conditions were compared for content of MLCK, actin, and MYPT. To determine if hyperoxic conditions might alter MYPT isoform expression, separate aliquots of the lysates were compared first for amount of MYPT by Western blotting. These membranes were then stripped and probed for the presence of the leucine zipper motif as previously described (4). For these studies, the tracheal tissue was minced and sonicated in modified RIPA buffer, pH 7.4 (1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 50 mM HEPES, 10% glycerol, 150 mM NaCl, 1.5 mM MgCl2, 1 mM EGTA, 1 mM NaVO3, 100 mM Na3PO4, 100 mM NaF, 10 μg/ml apoproteinin, 5 μg/ml leupeptin). After centrifugation (10,000 g for 5 min), a portion was removed for protein analysis and then the sample diluted in 2× sample buffer (as above) such that each sample contained 1 μg/ml and 20 μg added per lane. These samples were boiled and the proteins resolved in 4−15% Tris-HCl gels (115 V for 1 h). Proteins were transferred into nitrocellulose membranes, membranes were blocked with 5% milk in 1× TBST and then proteins were detected as described above.

Antibody sources. The primary antibody sources were as follows. Anti-actin antibody (mouse IgG) was purchased from Cell Signaling, Beverly, MA. Anti-MYPT and anti-leucine zipper antibodies (generously provided by Steven Fisher) were purchased from Covance, Denver, PA. Antibody against MLCK, MYPT phosphorylated at Thr641, LC20, and LC20-PO4 were generous gifts of Mitsuo Ikebe, Wooster, MA. Secondary antibodies (horseradish peroxidase-conjugated goat anti-mouse IgG and goat anti-rabbit IgG) were purchased from Santa Cruz Biotechnology, Santa Cruz, CA.

Statistics. Densitometry analysis of SDS gel and Western blots was done on digital images scanned from the radiographic film to a laser densitometer (600 dpi; SCIscan 5000 and Biostatistics Software, USB). Data were expressed as arbitrary optical density units normalized for protein amounts per band and expressed as percent of control. To assure reproducibility, each sample was run in triplicate lanes. Where there was greater than 5% variability in optical density measurements from artifact or obscure bands, the samples were reanalyzed by repeating the Western blotting. Data are expressed as means ± SD for 12 separate rat pups per condition (control or hyperoxic conditions) and compared by t-test with significance set at 0.05.

RESULTS

Western blot analysis of various smooth muscle proteins was used to compare differences in both content and activation state of contractile proteins between normoxic and hyperoxic groups. There were no differences between the groups in the amount of α-smooth muscle actin, MLCK, LC20, or total MYPT, and also no differences in the proportion of LC20 or MYPT phosphorylated at baseline (before activation) (Fig. 1). This is consistent with our previous studies (1) in which there were no indications of increased smooth muscle mass on
histological inspection of rat pup tracheas after exposure to hyperoxic conditions.

To detect differences in the degree of myosin phosphorylation either during bethanechol activation or subsequent EFS-induced relaxation, the extent of LC20 phosphorylation was compared using an antibody specific to LC20 phosphorylated at the Ser19 site (12). The extent of LC20 phosphorylation was greater after bethanechol in tracheas of rat pups exposed to hyperoxic condition (Fig. 2). This suggests a greater degree of activation and force generation in rat pups exposed to hyperoxic conditions and is consistent with previous data (6, 14). Furthermore, this difference persisted at the time when the nadir of relaxation had been noted after a 10-s pulse of EFS, suggesting persistent contractile activation due to impaired dephosphorylation compared with the tracheas of rat pups from normoxic conditions. Inhibition of myosin phosphatase through phosphorylation of MYPT is a potential source of impaired relaxation in smooth muscle, so we compared the ratio of phosphorylated MYPT-to-total MYPT in tracheas in each condition. There were no differences between the hyperoxic-conditioned and room air tracheas for the ratio of phosphorylated MYPT-to-total MYPT at baseline or immediately after bethanechol activation, but there was a significantly greater extent of MYPT phosphorylation in the hyperoxic-conditioned tracheas after EFS stimulation (Fig. 3). These data suggest that impaired relaxation in the hyperoxic group is due at least in part to greater inhibition of MYPT.

To study the possibility of MYPT inhibition impairing relaxation in the hyperoxic group, in separate studies, the trachealis muscle was pretreated with Y-27632 in the tissue bath and exposed to bethanechol as before. LC20 and MYPT phosphorylation both remained greater in the untreated hyperoxic group compared with the untreated normoxic group as in the earlier experiments. However, the proportions of LC20 and MYPT phosphorylation were both significantly less in the Y-27632-treated tracheas than in the untreated. Furthermore, these proportions were not different when comparing the treated hyperoxia to the treated normoxia-conditioned tracheas (Fig. 4). Because Y-27632 is a specific Rho kinase inhibitor, these data strongly suggest that RhoA is more active in the untreated hyperoxia group and likely impairs ASM relaxation through Rho kinase-mediated phosphatase inhibition. Another mechanism whereby MYPT activity might vary and influence cell relaxation is through expression of differing isoforms of MYPT. To determine MYPT isoform expression, trachealis samples were probed by Western blotting using antibodies against the leucine zipper region of MYPT1 (10). These showed no differences between the hyperoxia and the room air rat pups (Fig. 5).

**DISCUSSION**

This study explored the possible mechanisms whereby relaxation of ASM is impaired in rat pups exposed to excessive hyperoxic conditions.

Fig. 1. Comparisons of 20-kDa myosin regulatory light chain (LC20) phosphorylation and contractile proteins do not indicate baseline differences between airway smooth muscle of rat pups raised in normoxic vs. hyperoxic conditions. Comparisons of the amount of baseline actin or myosin light chain kinase (MLCK) were not different between the 2 groups. Also, the percentage of LC20 that was phosphorylated (LC20-P) and myosin-binding subunit of myosin phosphatase (MYPT) that was phosphorylated (MYPT-P) were not different at baseline. N = 12 in each group; values are means ± SE.

**Fig. 2.** LC20 phosphorylation is greater after bethanechol stimulation in hyperoxia condition and remains elevated during the relaxation induced by electrical field stimulation (EFS) in hyperoxia-conditioned rat pups. A: LC20 is phosphorylated to a greater extent after exposure to bethanechol (ED50). After incubation in bethanechol, tracheas were harvested 60 s after a pulse of EFS (10 s) had been applied. B: in the airways of rat pups exposed to hyperoxic conditions compared with those raised in room air (control). Each lane contains equal amounts of total protein from a separate animal (littermates). C: comparison of ratios of LC20-P/Total LC20 expressed as a percentage of the control animals (room air). Data are combined from all animals in the 2 groups; n = 12 per condition, *P < 0.05 t-test; values are means ± SE.
concentrations of inhaled oxygen. These data indicate that the main mechanism for impairment of relaxation is through Rho kinase-mediated MYPT phosphorylation (inhibition). Compared with normal littermates, the smooth muscle of the trachea of the hyperoxia-exposed pups undergoes a greater extent of LC20 phosphorylation when stimulated to contract with bethanechol. This signifies greater activation of the contractile apparatus. Furthermore, when preconstricted tissue is stimulated to relax by EFS, this increase in LC20 phosphorylation is maintained in the hyperoxia group. Increased phosphorylation of the myosin-binding subunit of MYPT also remained greater after EFS in the hyperoxic group of animals. Phosphorylation of MYPT inhibits the phosphatase activity, likely accounting for the observed increases in LC20 phosphorylation. Impaired phosphatase activity would result in prolonged myosin activation, so we propose that this is the proximate cause of impaired relaxation in this animal model.

The lack of evidence of ASM hypertrophy based on the content of contractile proteins is consistent with our previous studies (8, 14). However, ASM hypertrophy has been noted in other studies using similar models of hyperoxia-induced airway hyperresponsiveness (3, 6, 23). For example, in response to a 15-day exposure to 50% oxygen, an increase in tracheal smooth muscle area was observed (3). Such findings have been inconsistent, as illustrated in work with neonatal rats exposed for the observed increases in LC20 phosphorylation. Impaired phosphatase activity would result in prolonged myosin activation, so we propose that this is the proximate cause of impaired relaxation in this animal model. There was no difference between the two groups in the amount of smooth muscle present. Also, the proportion of MYPT1 leucine zipper inclusion/exclusion isoforms was the same between groups so that isoform switching of this protein could not account for differences in phosphatase activity.

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![Fig. 3. The phosphorylation of the myosin-binding subunit of myosin phosphatase maintains a higher level of phosphorylation during EFS-induced relaxation in the hyperoxic-conditioned rat pups compared with normoxic conditions. MYPT tended to be phosphorylated to a greater extent after exposure to bethanechol (ED50) but did not reach statistical significance (A), but after subsequent EFS, MYPT-P was significantly greater (B) in the airways of rat pups exposed to hyperoxic conditions compared with those raised in room air (control). Each lane contains equal amounts of total protein from a separate animal (littermates). C: comparison of ratios of MYPT-P/Total MYPT expressed as a percentage of the control animals (room air). Data are combined from all animals in the 2 groups from bethanechol and EFS studies; n = 4 pups for each group from 3 separate studies for n = 12 per condition, *P < 0.05 t-test; values are means ± SE.](http://ajplung.physiology.org/)
to 60% oxygen for 14 days that failed to show ASM hypertrophy (2). Our model differed from that of others in using younger rat pups (7 vs. 21-day-old) for shorter duration of (7 vs. 15 days) and greater concentrations of oxygen. Also, these animals were studied before being weaned from their mothers. This suggests that the duration of exposure as well as age-related differences in the model will determine the pathological changes. Age-related differences are also illustrated by the fact that adult rats fail to survive >95% oxygen conditions in contrast to immature pups. There might also be species differences in the response to high concentrations of oxygen. In our studies, Sprague-Dawley rats were used, whereas some others have used the Wistar strain. Another explanation for the differences between our findings and other investigations might be that we studied only the tracheas of these animals because of the technical challenge of measuring ASM contraction of large airways compared with smaller airways in an in vitro preparation (6). Finally, in the present study we compared biochemical data (smooth muscle specific proteins) rather than morphometric data.

In other studies, hyperoxic conditions increase inflammation of the airways. Although this might be a partial explanation, we did not find a difference in the two groups in baseline levels of LC20 phosphorylation or MYPT phosphorylation, suggesting that the duration of exposure as well as age-related differences in the model will determine the pathological changes. Age-related differences are also illustrated by the fact that adult rats fail to survive >95% oxygen conditions in contrast to immature pups. There might also be species differences in the response to high concentrations of oxygen. In our studies, Sprague-Dawley rats were used, whereas some others have used the Wistar strain. Another explanation for the differences between our findings and other investigations might be that we studied only the tracheas of these animals because of the technical challenge of measuring ASM contraction of large airways compared with smaller airways in an in vitro preparation (6). Finally, in the present study we compared biochemical data (smooth muscle specific proteins) rather than morphometric data.

In our previous studies (14) in this animal model (rat pups exposed to hyperoxia) we noted impairment of PGE2 release in the hyperoxia group concomitant with impaired relaxation. As would be expected, this was accompanied by depression of cAMP production in the smooth muscle. cAMP causes smooth muscle relaxation primarily through PKA and desensitization of the contractile apparatus to calcium activation. In that study, cGMP generation was also impaired, although to a lesser extent. Depressed cAMP and/or cGMP levels in hyperoxia conditions are consistent with the present findings, cAMP-induced relaxation occurs in part through PKA-mediated inhibition of IP3, so there is decreased release of calcium from the sarcoplasmic reticulum. Therefore decreased generation of cAMP in the hyperoxic group could ultimately result in greater calcium release, activation of MLCK, and increased LC20 phosphorylation.

Both cAMP and cGMP can also cause smooth muscle relaxation activating the nucleotide kinases PKC and PKG. These kinases interfere with the RhoA-Rho kinase pathway (16). RhoA activates Rho kinase that, in turn, directly inhibits MYPT through phosphorylation. cAMP is capable of phosphorylating RhoA interfering with its GTP activation so that the downstream effector Rho kinase is not activated and MYPT is not phosphorylated. Also, PKG can interfere directly with Rho kinase. The end result of impaired cAMP and cGMP generation then is inhibition of phosphatase activity (MYPT phosphorylation) and impaired relaxation (24). Our data are consistent with loss of cyclic nucleotide-based MYPT inhibition as a means whereby relaxation after hyperoxic conditions is impaired.

Consistent with our previous studies, we found a greater extent of LC20 phosphorylation in the hyperoxia rat pup tracheas than in the control. Several mechanisms could explain this finding. We have no evidence of ASM hypertrophy as outlined. A greater degree of activation from similar exposure to bethanechol would suggest increased sensitivity of the muscle to cholinergic stimulation. However, our earlier work (1) did not find a left shift in the dose response curve using an identical protocol. We believe impaired MYPT inhibition is an adequate explanation for the increased maximal force production as well. Because turnover of cycling crossbridges is constant in smooth muscle, the state of myosin activation is always a balance of phosphorylation/dephosphorylation reactions. Impairment of phosphatase activity would increase maximal force production as well as impair relaxation, consistent with our prior data. In fact, the same biochemical mechanisms are believed responsible for calcium sensitization of smooth muscle. Under specific conditions, smooth muscle contraction can be increased relative to the amount of cytosolic calcium, and Rho kinase-mediated inhibition of MYPT is believed the most important cause of such sensitization (26).

Other points of control that might contribute to alterations in smooth muscle relaxation include CPI7 and ZIP kinase, both of which regulate MYPT (5). We chose to compare bethanechol- and EFS-induced changes in ASM contractility in this animal model to stay consistent with previous work, but we recognize that studies of more proximate control points of LC20 phosphorylation will be important. Because a major finding in our study was that myosin dephosphorylation appeared impaired in the hyperoxic group, we also chose to examine the question of whether hyperoxic exposure led to a change in the isoform of MYPT expressed. Inclusion of a leucine zipper in the COOH terminus of MYPT has been shown to enhance calcium desensitization in response to nitric oxide or cGMP compared with MYPT that lacks the leucine zipper (10, 25). Interestingly, expression of these isoforms is tissue-specific and developmentally regulated (10) and is influenced by disease state (20). Because we did not find differences in isoform expression, the impaired ability of the hyperoxia-conditioned tracheas to relax with EFS cannot be explained by this mechanism.

We did not study the mechanisms for previously reported alterations in PGE2 and cyclic nucleotides and recognize hyperoxic conditions could cause these alterations by many mechanisms (8, 15). Certainly, hyperoxic-induced epithelial cell damage, inflammation, neutrophil recruitment, or stimulation of neuroendocrine pathways would be candidate sources. Also of interest would be the long-term consequences of hyperoxic exposure in neonatal animals and whether eventual remodeling of smooth muscle and other airway components is also an important consequence of this damage. These data also do not explore the “dose effect” or time-related effects of oxygen in this animal model.

In summary, we have employed the hyperoxic exposed rat pup model to demonstrate a prominent role for impaired ASM relaxation via inhibition of MYPT. These mechanistic findings...
provide new insight into the increased airway reactivity that characterizes preterm infants who develop BPD. Future refinements of this model employing different concentrations of inspired oxygen in conjunction with mechanical ventilation might allow us to optimize strategies for supplemental oxygen therapy and minimize the risk of subsequent airway reactivity in this infant population.

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GRANTS

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