Repeated β_2 -Adrenergic Receptor Agonist Therapy Attenuates the Response to Rescue Bronchodilation in a Hyperoxic Newborn Mouse Model

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Key Words

Airway hyperreactivity · Hyperoxia · Bronchodilation · β_2 -Adrenergic receptor agonist · Bronchopulmonary dysplasia · Premature lung

Abstract

Background: Preterm infants with neonatal lung injury are prone to wheezing and are often treated with β_2 -adrenergic receptor (β -AR) agonists although the benefits of β -AR agonists may be lost with chronic use. **Objective:** To investigate if repeated β -AR agonist exposure downregulates β -ARs in the immature lung resulting in a decreased response to bronchodilator rescue and whether hyperoxic exposure aggravates this response. **Methods:** Newborn mice were raised for 21 days in 60 or 21% oxygen and received daily aerosols of formoterol or saline. Respiratory system resistance (Rrs) and compliance (Crs) were measured in response to methacholine challenge and rescue bronchodilation with levalbuterol. Western blot analysis quantified the relative amount of lung β -ARs. **Results:** Hyperoxia increased the airway reactivity to methacholine. Animals raised in hyperoxia that received daily formoterol were most sensitive to methacholine and exhibited a blunted response to levalbuterol bronchodilation. Hyperoxia-exposed animals receiving daily for-

moterol versus saline showed a significant decrease in the relative amount of lung β -ARs. **Conclusions:** In this hyperoxia-exposed neonatal mouse model, repeated β -AR agonist treatments increased the airway reactivity and attenuated the response to a rescue bronchodilator. The blunted bronchodilator response could be explained by a reduced quantity of lung β -ARs. Our findings may account for the time-dependent decrease in the therapeutic benefit of β -AR agonists in preterm infants with neonatal lung injury, which may have clinical consequences for patients already prone to airway hyperreactivity.

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Introduction

Bronchopulmonary dysplasia (BPD) and wheezing disorders are major pulmonary morbidities of prematurity [1, 2]. The causes are likely multifactorial, including prematurity, oxygen toxicity/oxidative injury, volutrauma, infection, and inflammation [2]. Acute lung injury in the vulnerable infant results in airway remodeling, decreased alveolarization, alveolar simplification, and parenchymal changes [1, 2]. Increased reports of respiratory wheezing and asthma are common long-term pul-

monary consequences of preterm birth and BPD [1–3]. Indeed, a consistent observation has been that preterm infants, with or without BPD, continue to be at very high risk for airway hyperreactivity in infancy and childhood [4–6].

Although often diagnosed with asthma, infants and children with BPD are frequently less responsive to conventional asthma therapies [7]. The airway hyperreactivity observed in former preterm neonates is strongly associated with a history of prolonged supplemental oxygen and BPD compared to the airway hyperreactivity observed in term controls which instead is associated with a history of inheritance, allergy, inflammation, and cigarette exposure [5]. Wheezing preterm infants, with and without BPD, display more airway reversibility, atopy, inflammation, and exhaled nitric oxide than asthmatics [1, 6–9], yet school-age preterm infants are twice as likely to be prescribed asthma medications than their full-term peers [10]. Furthermore, β_2 -adrenergic receptor (β -AR) agonists may paradoxically increase airway resistance in some of these former premature patients [11].

There is not yet a consensus on how to manage preterm patients with developing or established BPD. Therapies include respiratory support with positive pressure and/or supplemental oxygen, diuretics, bronchodilators, inhaled and systemic corticosteroids, caffeine, and inhaled nitric oxide [12]. Clinically, β -AR agonists are both used in the neonatal intensive care unit [13] and prescribed at discharge [14]. Both short- and long-acting β -AR agonists have been shown to transiently improve the airway resistance and compliance of ventilated neonates [15, 16], but these effects have not facilitated the earlier weaning of respiratory support or decreased the rates of BPD [17]. The potential benefits of β -AR agonists may also diminish with repeated or continued use [18] since prolonged exposure can result in tolerance [19].

At the time of birth, newborn mice have developmentally immature lungs similar to the premature human lung in terms of the sequence and relative timing of alveolarization [20]. Hyperoxic exposure in newborn mice creates a lesion very similar to human BPD [21], manifesting increased airway reactivity [22]. As such, we used a neonatal mouse model to test the hypothesis that repeated exposure to a β -AR agonist will downregulate β -ARs in the immature lung, resulting in a decreased response to bronchodilator rescue, and that hyperoxic exposure will aggravate this response. These findings may provide future insight into mechanisms related to a perceived desensitized/diminished response of preterm infants to long-term β -AR agonist use.

Materials and Methods

Animal Model

The animal protocols were approved by the Institutional Animal Care and Use Committee at Case Western Reserve University. Timed pregnant C57BL/6 mice (Charles River) were purchased and housed in an AAALAC-accredited animal facility at Case Western Reserve University. The animals were maintained on standard 12-hour light-dark cycles with ad libitum standard food and water. Two or more litters of newborn pups were pooled within 24 h of birth and randomly redistributed into treatment groups. Litters, paired with a nursing dam, were placed in 60% oxygen or room air (21%). Hyperoxia-exposed animals were housed in standard cages placed in a 38-liter Plexiglas chamber with a continuous flow of oxygen (2 liters/min) for 21 days. Oxygen concentrations were monitored twice daily via an oxygen analyzer (MiniOX I; MSA Medical). To control for oxygen toxicity, nursing dams were rotated between paired litters during biweekly cage changes. The long-acting β -AR agonist formoterol was dissolved in DMSO and resuspended to a final concentration of 1 mg/10 ml formoterol in 0.1% DMSO saline (Sigma-Aldrich). Subgroups of the hyperoxic and room air-exposed animals were treated daily with either aerosolized formoterol or normal saline with 0.1% DMSO vehicle (10 ml). Solutions were delivered to caged animals in 38-liter Plexiglas chambers over 60 min using a continuous flow nebulizer (Global Medical Holdings LLC). On day 21, the animals were all weaned to room air and the aerosol treatments were discontinued. The animals were weighed and experimental procedures or tissue preparations were performed after 24–48 h had elapsed from the last aerosol treatment.

Lung Mechanics

Under anesthesia (intraperitoneal ketamine/xylazine), 3-week-old mice were placed supine on a heated surgical table, tracheostomized, and ventilated via a 19G blunt tip cannula with a commercial rodent ventilator (flexiVent; SCIREQ) at a tidal volume of 10 ml/kg, a rate of 150 breaths/min, a PEEP of 3 cm H₂O, and an FiO₂ of 50%. The animals were then paralyzed (intraperitoneal pancuronium bromide) and respiratory system resistance (Rrs) and compliance (Crs) were calculated using flexiVent software with a 1.2-second, 2.5-Hz single-frequency forced oscillation maneuver [23]. After 2 recruitment breathes of sustained inspiration up to a pressure of 30 cm H₂O for 3 s, the saline control and methacholine at 12.5, 25, 50, 100, and 200 mg/ml (Sigma-Aldrich) were aerosolized using an ultrasonic nebulizer diverted into the ventilator's inspiratory flow over 10 s (Aeroneb; SCIREQ). Measurements of the average Rrs and Crs were made every 40 s over 5 min after each dose. Following precontraction with the full methacholine dose response (200 mg/ml), the short-acting β -AR agonist lev-albuterol (2.5 mg/ml; Sigma Aldrich) was similarly aerosolized over 10 s to assess bronchodilator rescue as indicated by the average decrease in Rrs and increase in Crs 2 min after the aerosol was administered.

Western Blot

Western blot was used to quantify the relative amount of β -AR in the lung homogenates of animals from each treatment group that did not undergo lung mechanics as previously described [24]. After terminal anesthesia (intraperitoneal ketamine/xylazine), the harvested lungs were rinsed in ice-cold PBS, snap-frozen in liquid

Table 1. Body weights of animals exposed for 21 days to 60 or 21% oxygen with daily treatments of aerosolized saline or formoterol

	21% + saline	21% + formoterol	60% + saline	60% + formoterol	p value
Lung physiology	9.9±0.4 (12)	9.9±0.5 (8)	10.2±0.4 (18)	10.5±0.3 (8)	0.81
Western blot	9.5±0.4 (14)	8.7±0.5 (14)	9.2±0.4 (14)	8.6±0.4 (14)	0.44
Total	9.2±0.5 (26)	8.5±0.5 (22)	9.5±0.5 (32)	8.6±0.5 (22)	0.46

Values are presented as mean body weights (in g) ± SEM. Numbers in parentheses represent numbers of animals.

Fig. 1. Lung physiology in mechanically ventilated mice. Significant methacholine dose-dependent increases in Rrs and decreases in Crs were observed in each group. **a** Formoterol administration alone had no effect on the response of Rrs to methacholine when compared between room air cohorts. Significant increases were seen between the dose-response curves of both hyperoxic cohorts compared to room air controls (brackets). These differences were seen at the highest methacholine doses (100 and 200 mg/ml) with hyperoxia exposure alone. In contrast, combined exposure to hyperoxia and formoterol increased the sensitivity to methacholine, with a significantly higher reactivity at lower doses of methacholine (≥ 25 mg/ml) compared to room air controls. **b** Significant differences in Crs methacholine dose-response curves were not observed between the groups. * $p < 0.05$: 21% + saline vs. 60% + saline, ** $p < 0.05$: 21% + saline vs. 60% + formoterol.

nitrogen, and stored at -80°C . The tissue was resuspended in ice-cold commercial lysis buffer containing protease inhibitors (Sigma-Aldrich) and then homogenized. The protein levels of lung homogenates were determined by BCA assay (Thermo-Scientific). Samples of 15 μg protein were loaded and separated by 10% polyacrylamide gels and transferred to nitrocellulose membranes (Bio-Rad). The membranes were blocked with 5% milk/5% BSA TBS-Tween and incubated in β -AR primary antibody (55 kDa, rabbit polyclonal, 1:1,000; Abcam) overnight at 4°C and then anti-rabbit HRP-conjugated secondary antibody for 1 h at room temperature (goat polyclonal, 1:5,000; Abcam). As a loading control, β -actin primary antibody (42 kDa, mouse monoclonal, 1:5,000; Abcam) and anti-mouse HRP-conjugated secondary antibody (donkey polyclonal, 1:4,000; Abcam) were used. β -AR band intensities were quantified and normalized to β -actin using ECL (Thermo Scientific). Relative intensities were measured using densitometry software (Image J).

Statistical Analysis

Data are expressed as means $\pm$ SEM. Animal body weights were compared by one-way analysis of variance. Alterations in airway reactivity with increasing doses of methacholine were compared via two-way analysis of variance repeated-measures analysis with post hoc comparisons using a fixed-sequence method. Bronchodilatory responses were compared using Student's t test. β -AR density was compared between groups using the Kruskal-Wallis one-way analysis of variance rank test with a post hoc Student-Newman-Keuls test. $p < 0.05$ was considered statistically significant.

Results

Animal Body Weight

In total, 46 animals completed full lung mechanics testing and another 56 animals were harvested for tissue collection from 20 litters. The average body weight did not significantly differ between groups (table 1).

Airway Responsiveness to Methacholine

Following aerosolized saline, the baseline Rrs and Crs did not statistically differ between exposure groups. In each group significant increases in Rrs (fig. 1a) and decreases in Crs (fig. 1b) were observed when challenged

Fig. 2. Rescue bronchodilation with levalbuterol following pre-constriction with 200 mg/ml methacholine. **a** Significant decreases in Rrs were observed in all groups following bronchodilator administration. **b** Significant increases in Crs were observed in the 60% + saline, 21% + saline, and 21% + formoterol groups, but the Crs did not increase in the 60% + formoterol group after the administration of a bronchodilator. * $p < 0.05$: pre- vs. postbronchodilator.

with the methacholine dose response. The hyperoxic cohorts displayed significantly increased Rrs at the highest methacholine doses (100 and 200 mg/ml) when compared to the animals raised in room air ($p < 0.05$). The animals exposed to both hyperoxia and daily formoterol were particularly reactive to methacholine, displaying a significantly increased reactivity at even the lower methacholine doses (≥ 25 mg/ml; $p < 0.05$) compared to the room air controls. There was no effect of hyperoxia and/or formoterol on the Crs dose-response curves between groups.

Fig. 3. Percent change in Rrs with levalbuterol rescue following pre-constriction with 200 mg/ml methacholine. The bronchodilator response, as measured by the percent decrease in Rrs, was significantly attenuated in the hyperoxic cohort treated with daily aerosolized formoterol compared to saline. * $p < 0.05$.

Bronchodilator Rescue with Levalbuterol

Following pre-constriction with methacholine, levalbuterol was administered to assess bronchodilator rescue. All treatment groups showed a significant decrease in Rrs following bronchodilator rescue (fig. 2a). Significant changes in Crs were not observed in the group raised in hyperoxia and treated with daily formoterol (fig. 2b). To account for potential differences in maximum Rrs following pre-constriction, the percent change in Rrs was also calculated and compared between the hyperoxic cohort receiving saline and the mice receiving formoterol (fig. 3). In this manner, the hyperoxia-exposed mice receiving daily β -AR agonist treatments displayed an attenuated response to the rescue bronchodilator compared to those raised in hyperoxia receiving saline ($p < 0.05$).

Lung β -AR Expression

Since the β -AR agonist response depends, in part, on the expression of β -AR, we measured the β -AR density in the lung by Western Blot analysis (fig. 4). Treatment with daily formoterol compared to saline for 21 days significantly decreased the relative amount of β -AR: β -actin in both the room air group and the hyperoxic group (both $p < 0.05$). Compared to the room air saline controls, hyperoxic exposure with and without formoterol significantly reduced the relative amount of β -AR ($p < 0.05$ for both hyperoxic groups).

Fig. 4. Whole-lung β -AR expression assessed by Western blot. Hyperoxic exposure significantly reduced the relative quantity of receptor compared to room air controls (* $p < 0.05$). Treatment with daily formoterol for 21 days resulted in a decreased relative quantity of receptors among both the room air cohort and the hyperoxic cohort (** $p < 0.05$). $n = 14$ animals in each group.

Discussion

In this newborn mouse model, hyperoxia increased airway reactivity. Hyperoxic exposure coupled with repeated β -AR agonist treatments further increased the sensitivity to methacholine, attenuated the bronchodilator response to levalbuterol, and was associated with a reduced quantity of receptors in the lung. Taken together, these data may indicate detrimental effects of prolonged courses of β -AR agonists for preterm infants and children on supplemental oxygen therapy. Furthermore, chronic inhaled β -AR agonists may diminish the efficacy of rescue bronchodilators in a patient population already at risk for airway hyperreactivity.

Moderate hyperoxia (FiO_2 60%) as compared to 100% oxygen was used in this model because of the movement in clinical practice to utilize lower levels of oxygen in neonatal intensive care units. An increased reactivity to methacholine challenge was found in our hyperoxic cohorts compared to room air controls, which is consistent with other studies of moderate hyperoxia (FiO_2 40–70%) in newborn rodents [2, 22, 25]. Neonatal hyperoxia exposure (FiO_2 65% for just 7 days) has been shown to cause

lasting changes in the bronchiolar wall and bronchiolar-alveolar attachments in adult animals recovered in room air [26]. Further investigation of the long-term effects of early hyperoxia exposure, β -AR agonist treatment, and airway reactivity are warranted.

Although we did not observe significant differences in baseline Rrs or Crs, animals exposed to the combination of hyperoxia and chronic β -AR agonists seemed to be particularly sensitive to methacholine challenge, with observed increases in resistance at even the lower doses of methacholine. In the experimental adult asthma literature, increased airway hyperreactivity has similarly been observed with the combination of chronic β -AR agonist treatments and lung inflammation [27, 28]. Future studies targeted at the blockade of cytokine-driven lung inflammation may provide insight into some of the underlying mechanisms of this observed airway hyperreactivity.

Our animal findings revealed a relative decrease in β -AR quantity following either daily treatments with a β -AR agonist or sustained hyperoxia exposure. Agonist stimulation of β -ARs classically results in receptor inactivation and internalization with an eventual downregulation of the receptor following chronic stimulation. Under normoxic conditions, this decrease in β -ARs did not appear to have a physiological effect on the baseline Rrs or Crs or the response to a rescue bronchodilator. Hyperoxia exposure alone significantly reduced the quantity of β -ARs compared to saline room air controls. The mechanisms of hyperoxia-related decreases in β -ARs have not yet been well studied. We speculate that airway remodeling, alveolar simplification, and altered transcription may all contribute to a decreased whole-lung receptor pool and thus future studies are warranted. Despite a decreased β -AR population, a physiologic effect on baseline mechanics was not observed in these hyperoxia-exposed animals, and a marked decrease in Rrs and an increase in Crs were observed following rescue bronchodilation. We hypothesize that there likely exists an effective threshold of β -ARs required for agonist-stimulated bronchodilation. Conversely, daily treatments with formoterol in hyperoxia-exposed mice further decreased the receptor population, with these animals displaying a markedly blunted response to an equivalent rescue bronchodilator dose and no observed improvement in Crs. We speculate that the combination of sustained hyperoxic and β -AR agonist exposure may cumulatively impair the expression and/or activity of an already decreased receptor population. This may be of particular clinical concern in infants and children with underlying hyperoxic lung injury who receive regular treatments with a β -AR agonist.

Formoterol was chosen for our study design because of its ease of administration and the clinical concern that chronic formoterol use in humans may cause β -AR tachyphylaxis [29]. By utilizing a long-acting β -AR agonist, we attempted to create a phenotype of chronic stimulation of the β -AR in the developing lung. Future studies are needed to refine this model to correlate with common clinical practices, including drug selection, timing, and frequency. Although there is much practice variability, preterm infants with BPD are often maintained on scheduled β -AR agonists (such as albuterol) in the NICU and are frequently discharged home on bronchodilators [14]. Indeed, repeated stimulation of β -ARs, even with short-acting agonists, has been associated with desensitization of the receptor and a decline in the bronchodilator response in asthma [19, 30]. Similar studies are needed in the neonate with and without BPD.

Conclusions

In our immature mouse model, hyperoxia exposure resulted in an increase in airway reactivity. When hyperoxia exposure was coupled with repeated β -AR agonist treatments, animals demonstrated increased airway reactivity at lower doses of methacholine. Furthermore, animals exposed to both hyperoxia and β -AR agonists displayed an

attenuated response to rescue bronchodilation. We speculate that such an effect may be related to the associated downregulation of β -ARs in the lung. Our findings may account for the time-dependent decrease in the therapeutic benefit of β -AR agonists with prolonged or repeated use in preterm infants with and without BPD, which may have clinical consequences for patients already prone to airway hyperreactivity and bronchospasm. Additionally, chronic treatment with β -AR agonists in children with hyperoxic lung injury may further sensitize them to provoked airway bronchoconstriction. In cases where chronic bronchodilator therapies are necessary, methods for restoring receptor quantity and function in the developing lung can serve as novel therapeutic targets. Further studies are needed to investigate the effects and safety of prolonged courses of β -AR agonists in the immature human lung.

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