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Hyperoxia Exposure Disrupts Adrenomedullin Signaling in Newborn Mice: Implications for Lung Development in Premature Infants

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Abstract

Hyperoxia contributes to the development of bronchopulmonary dysplasia (BPD), a chronic lung disease of human infants that is characterized by disrupted lung angiogenesis. Adrenomedullin (AM) is a multifunctional peptide with angiogenic and vasoprotective properties. AM signals via its cognate receptors, calcitonin receptor-like receptor (Calcrl) and receptor activity-modifying protein 2 (RAMP2). Whether hyperoxia affects the pulmonary AM signaling pathway in neonatal mice and whether AM promotes lung angiogenesis in human infants are unknown. Therefore, we tested the following hypotheses: (1) hyperoxia exposure will disrupt AM signaling during the lung development period in neonatal mice; and (2) AM will promote angiogenesis in fetal human pulmonary artery endothelial cells (HPAECs) via extracellular signal-regulated kinases (ERK) 1/2 activation. We initially determined AM, Calcrl, and RAMP2 mRNA levels in mouse lungs on postnatal days (PND) 3, 7, 14, and 28. Next we determined the mRNA expression of these genes in neonatal mice exposed to hyperoxia (70% O₂) for up to 14 d. Finally, using HPAECs, we evaluated if AM activates ERK1/2 and promotes tubule formation and cell migration. Lung AM, Calcrl, and RAMP2 mRNA expression increased from PND 3 and peaked at PND 14, a time period during which lung development occurs in mice. Interestingly, hyperoxia exposure blunted this peak expression in neonatal mice. In HPAECs, AM activated ERK1/2 and promoted tubule formation and cell migration. These findings support our hypotheses, emphasizing that AM signaling axis is a potential therapeutic target for human infants with BPD.

Keywords

Adrenomedullin; Calcitonin receptor-like receptor; Receptor activity-modifying protein 2; Hyperoxia; Bronchopulmonary dysplasia

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Introduction

Bronchopulmonary dysplasia (BPD) is a chronic lung disease of premature infants that results from an imbalance between lung injury and repair in the developing lung [1]. Despite improved respiratory care management of premature infants, BPD remains the most common morbidity in these infants [2]. In addition, BPD increases the economic burden with an estimated cost of \$2.4 billion per year in United States, making it the second most expensive childhood disease after asthma. Thus, there is a need for improved therapies to prevent and/or treat BPD in human infants. Lung blood vessels play a crucial role in lung health. Disrupted lung angiogenesis is a hallmark of developmental lung disorders such as BPD [3]. Lung angiogenesis actively contributes to alveolarization (lung development), and disruption of angiogenesis in the developing lungs causes arrested alveolarization [4]. Thus, understanding the mechanisms that promote the development and function of the lung vascular system is vital to prevent and treat this disease in human infants.

Supplemental oxygen is frequently used as a life-saving therapy in human infants with hypoxic respiratory failure; however, excessive oxygen exposure or hyperoxia leads to BPD. In alignment with other studies [5, 6], we recently demonstrated that hyperoxia-induced lung parenchymal and vascular injury in newborn mice leads to a phenotype that is similar to human BPD [7]. So, we used this model to investigate whether AM signaling is disrupted in experimental BPD.

Adrenomedullin (AM) is a 52-amino acid peptide that is particularly enriched in highly vascularized organs, including lungs, heart, kidneys, and adrenal glands [8]. AM signaling occurs by the functional receptor combination of calcitonin receptor-like receptor (Calcrl) with receptor activity-modifying protein (RAMP)-2 and -3 [9]. AM plays a crucial role in endothelial cell growth and survival [10–12], mainly via extracellular signal-regulated kinases (ERK) 1/2 pathway [13, 14]. AM^{-/-} mice die *in utero* due to vascular endothelial disruption [13, 15, 16]. Calcrl^{-/-} and RAMP2^{-/-} mice have a phenotype identical to that of AM^{-/-} mice [13, 15, 17], whereas RAMP3^{-/-} mice survive with few phenotypic defects [18]. These data demonstrate that AM, Calcrl, and RAMP2 genes are critical for vascular development; therefore, our studies focused on these genes.

AM is shown to regenerate alveoli and vasculature in a pulmonary emphysema mouse model [19]. However, several factors remain poorly understood, including: (1) the ontogeny of pulmonary AM, Calcrl, and RAMP2 in mice; (2) the effects of hyperoxia on the AM signaling axis in the developing lungs of mice; and (3) the effects of AM on lung angiogenesis in human preterm infants. Therefore, using neonatal C57BL/6J wild type (WT) and fetal human lung cells, we tested the following hypotheses: (1) hyperoxia exposure will disrupt AM signaling during the alveolarization period in neonatal mice; and (2) AM will promote angiogenesis of fetal human pulmonary artery endothelial cells (HPAECs) via activation of ERK1/2 pathway.

Materials and Methods

In Vivo Experiments

Animals—This study was approved and conducted in strict accordance with the federal guidelines for the humane care and use of laboratory animals by the Institutional Animal Care and Use Committee of Baylor College of Medicine. The C57BL/6J wild type (WT) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Timed-pregnant mice raised in our animal facility were used for the experiments.

Analyses of AM signaling pathway

The lungs were harvested from both male and female mice on postnatal days (PND) 3, 7, 14, and 28 (n=9/time point) for gene expression analyses. Real-time quantitative RT-PCR analysis was performed with 7900HT Real-Time PCR System using TaqMan gene expression master mix (Grand Island, NY; 4369016) and TaqMan Gene Expression Assays (Applied Biosystems) for the following genes: AM, RAMP2, Calcrl, and GAPDH.

Hyperoxia Exposure—Within 24 h of birth, male and female pups were exposed to normoxia (21% O₂, n=36) or hyperoxia (70% O₂, n=36) for up to 14 d. The dams were rotated between normoxia- and hyperoxia-exposed litters every 48 h to prevent oxygen toxicity in the dams [7]. Following hyperoxia exposure, mice were allowed to recover in air for 14 d as described previously [20]. The lung tissues were harvested on PND 3, 7, and 14 (n=9/time point/exposure) for the analyses of the AM signaling pathway. Additionally, the lung tissues were harvested for lung morphometry and immunohistochemistry on PND 28 (n=9/exposure).

Alveolar and pulmonary vascular morphometry—Alveolar development on selected mice was evaluated by radial alveolar counts (RAC) and mean linear intercepts (MLI) [7]. Pulmonary vessel density was determined based on immunohistochemical staining for von Willebrand factor (vWF), which is an endothelial specific marker. At least 10 counts from 10 random non overlapping fields (20x magnification) was performed for each animal (n=9/exposure group).

In Vitro Experiments

Cell culture and treatment—The fetal human pulmonary artery endothelial cells (HPAECs) were obtained from ScienCell research laboratories (San Diego, CA; 3100) and grown according to the manufacturer's protocol. Cells were treated with various concentrations (0.1 – 1000 nM) of AM for 15 min, after which whole-cell protein was harvested to determine if AM phosphorylates ERK1/2. For tubule formation and scratch assays, cells were treated with 10 nM AM.

Western Blot Assays—Whole-cell protein extracts were obtained by using radio immunoprecipitation assay lysis buffer system (Santa Cruz Biotechnologies, Santa Cruz, CA; sc-24948) and subjected to western blotting with the following antibodies: β -actin (Santa Cruz Biotechnologies; sc-47778, dilution 1:1000), total ERK1/2 (Cell Signaling,

Danvers, MA; 4695, dilution 1:1000), and phospho-ERK1/2 (Cell Signaling; 9106, dilution 1:1000) antibodies.

Tubule formation and scratch assays—Matrigel and scratch assays were used to determine tubule formation and cell migration, respectively [21, 22]. Briefly, HPAECs grown in reduced serum medium were harvested for the assays. The cells were pretreated with AM and loaded on top of growth factor-reduced Matrigel (BD Bioscience). Following an incubation period of 18 h, tubule formation was quantified. For the scratch assay, the cells were wounded with a pipette tip before they were treated with AM. The wound closure or cell migration area was estimated using Image J software by comparing the wounded areas at 0 h and 16 h.

Statistical Analyses

The results were analyzed by GraphPad Prism 5 software. Data are expressed as mean $\pm$ SD. *In vivo experiments*: At least three separate experiments were performed for each measurement (n = total animals from the 3 experiments). The effects of age and exposure for the outcome variables (AM, Calcr1, and RAMP2) were assessed using ANOVA, whereas the effect of exposure on lung development was assessed by t-test. *In vitro experiments*: At least three separate experiments were performed for each measurement. The dose dependent effects of AM on ERK1/2 phosphorylation were assessed by ANOVA, whereas the effects of AM on angiogenesis were assessed by t-test. A *p* value of <0.05 was considered significant.

Results and Discussion

The present study demonstrates that AM and its signaling receptors, Calcr1 and RAMP2, are increasingly expressed during lung development in mice and exposure of neonatal mice to hyperoxia disrupts the AM signaling pathway in the lungs of these animals. Further, our study demonstrates that AM activates ERK1/2 pathway and promotes angiogenesis in fetal HPAECs.

Lung angiogenesis actively contributes to alveologenesis during development and healthy lung blood vessels are necessary to maintain structural and functional integrity of alveolar structures later in life. Recently, AM signaling is increasingly been recognized as a necessary pathway for vascular remodeling and immune response in physiological states such as pregnancy [23], and disrupted AM signaling is shown to contribute to several pathophysiological states such as preeclampsia [24], systemic and pulmonary hypertension [25, 26], myocardial infarction [27], tumors [28], and lymphedema [29]. Further, Calcr1-RAMP complexes are being explored as important drug targets to treat acute myocardial infarction, pulmonary hypertension, migraine headache, cancer, and several other disorders [30–32], indicating that AM signaling axis can be targeted to develop therapies. However, the effects of hyperoxia on the AM signaling axis in the developing lungs is poorly understood. Additionally, whether AM modulates lung angiogenesis in human infants is unknown. Our studies were designed to address these knowledge gaps.

Initially we determined the developmental profile of AM and its receptor components in the lungs of neonatal and adolescent mice by real-time RT-PCR analyses. The mRNA levels of

AM (Fig. 1A), *Calcrl* (Fig. 1B), and *RAMP2* (Fig. 1C) progressively increased from PND 3 and peaked on PND 14, before decreasing to the baseline levels on PND 28. Interestingly, the peak expression of AM and its signaling receptors occurred in the time period during which alveolarization occurs in mice. Ramos and colleagues demonstrated a similar finding in the developing human fetal lung [33]. In the lungs, AM and its receptors are co-expressed mainly in endothelial cells and epithelial cells of the airway and alveoli [34–36], suggesting that the effects of AM occur via autocrine and paracrine mechanisms. Increased expression of AM signaling components in the cells that modulate proliferation and differentiation combined with increased AM expression during the alveolarization period indicate that there is a mechanistic link between AM signaling and lung development.

We demonstrated that our mouse model of hyperoxia-induced lung injury meets the requirements needed to model BPD with pulmonary hypertension (PH) experimentally [7]. In that study, we observed that hyperoxia exposure disrupted lung development in mice at PND 14. However, human preterm infants with BPD have anatomical and functional lung abnormalities that persist into adolescence. Therefore, we exposed one-day-old WT mice to normoxia (21% O₂) or hyperoxia (70% O₂) for 14 d. Hyperoxia-exposed mice were allowed to recover in air for 14 d [20]. Following this recovery period, alveolarization and pulmonary vascularization were determined on PND 28. Hyperoxia-exposed mice continued to have significant decreases in RAC (Figs. 2A–C) and increases in MLI (Figs. 2A,B, and D) indicating that their alveoli were fewer in number and larger in diameter, respectively, when compared with normoxia-exposed mice. Further, hyperoxia-induced decrease in vWF stained lung blood vessels (Figs. 2E–G) persisted following the recovery period. These findings indicate that neonatal mice with hyperoxia-induced lung injury have interrupted lung development that persist to adolescence (PND 28). Our mouse model thus recapitulates the long-term lung morbidity seen in human infants with BPD, making our data clinically relevant. Therefore, we used this mouse model to interrogate the effects of hyperoxia on the AM signaling axis in the developing lungs. Interestingly, hyperoxia exposure in neonatal mice attenuated the increase in AM (Fig. 3A), *Calcrl* (Fig. 3B), and *RAMP2* (Fig. 3C) mRNA expression during the alveolarization period, indicating that AM signaling plays a mechanistic role in hyperoxia-induced developmental lung injury. Our findings are contradictory to those of Vadivel and colleagues, who showed that hyperoxia exposure increased the pulmonary AM expression in neonatal rats [37]. This discrepancy may be due to differences in animal species and the oxygen concentration used for the experiments. However, they demonstrated that the increase in AM expression was a compensatory rather than a contributory response to hyperoxic injury because AM treatment attenuated the lung injury in their experimental animals, a finding which signifies the protective effects of AM against hyperoxic injury. Future studies with AM transgenic mice are needed to determine if AM signaling is both necessary and sufficient to protect against hyperoxia-induced developmental lung injury.

To examine the clinical significance of our animal studies, we investigated the effects of AM treatment in fetal HPAECs. HPAECs were selected because: (1) their proliferation and maturation are crucial for alveolarization and lung growth; (2) their dysfunction contributes to BPD pathogenesis; and (3) AM and its signaling receptors are highly enriched in arterial endothelial cells. ERK1/2 primarily mediates proliferation and differentiation in many cell

types [38] and regulate lung morphogenesis in rats [39]. Further, AM signaling induces proliferation and promotes growth and survival of endothelial cells, mainly via ERK1/2 pathway [13, 14, 40]. Hence, we treated HPAECs with various concentrations of AM for 15 min, after which we determined if AM phosphorylates ERK1/2. The treatment time was based on studies showing that AM-induced phosphorylation of this protein peaks 15 min after treatment [13]. AM-induced ERK1/2 phosphorylation was pronounced when treated with 10 nM AM (Figs. 4A–B), a finding that is consistent with the above mentioned studies. Consequently, 10 nM AM was used to examine if AM promotes lung angiogenesis in human infants. AM-treated fetal HPAECs were subjected to Matrigel [21] and scratch [22] assays to determine tubule formation and cell migration, respectively. AM increased HPAEC tubule formation (Figs. 4C–E) and migration (Figs. 4F–H). Our results are in agreement with the pro-angiogenic properties of AM observed in other endothelial cell types [11–13, 41, 42]. While the observed beneficial effects of AM are seen at concentration that is 10-fold higher than its normal plasma levels, it is possible that the normal cell-specific AM concentration could be possibly much higher than its plasma concentration. Our findings in HPAECs complement our previous study, which showed that AM is necessary to protect human pulmonary microvascular endothelial cells against hyperoxic injury [43]. These findings indicate that AM signaling does play a pivotal role in the lung angiogenesis and development in human preterm infants.

In summary, our data suggests that hyperoxia exposure disrupts lung AM signaling in a clinically relevant neonatal mouse model of experimental BPD. Further, our *in vitro* experiments with fetal human lung cells indicate that AM promotes HPAEC tubule formation and migration mostly via ERK1/2, thereby adding a clinical significance to our *in vivo* experiments in neonatal mice. Interrupted lung angiogenesis is a major risk factor for BPD and our study suggests that AM has the potential to promote lung angiogenesis; therefore, we propose that AM signaling axis is a potential target to develop meaningful therapies for BPD in human infants.

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Abbreviations

AM	adrenomedullin
BPD	bronchopulmonary dysplasia
Calcr1	calcitonin receptor-like receptor
ERK	extracellular signal-regulated kinases
HPAECs	human pulmonary artery endothelial cells
MLI	mean linear intercepts

PH	pulmonary hypertension
PND	postnatal day
RAC	radial alveolar counts
RAMP	receptor activity-modifying protein
WT	wild type
vWF	von Willebrand factor

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Highlights

We studied the ontogeny of adrenomedullin (AM) signaling in mouse lungs.

Expression of AM and its receptors peak during lung development in mice.

Hyperoxia downregulates the expression of AM and its receptors in mouse lungs.

AM activates ERK1/2 in human fetal pulmonary artery endothelial cells (HPAEC).

AM promotes HPAEC migration and tubule formation.

Figure 1. Ontogeny of AM and its signaling receptors in mouse lungs

The lung tissues of air-breathing (normoxia) mice were extracted on postnatal days (PND) 3, 7, 14, and 28, after which RNA was extracted for real-time RT-PCR analysis of AM (A), Calcr1 (B), and RAMP2 (C) mRNA expression. Data are representative of at least three independent experiments. Values are presented as mean $\pm$ SD (n=3/time point). Significant difference compared to 3-d-old mice is indicated by *, $p < 0.05$ (ANOVA).

Figure 2. Quantification of alveolarization and pulmonary vascularization on postnatal day 28
A–B. Representative hematoxylin and eosin-stained lung sections, C. Radial alveolar count, D. Mean linear intercepts, E–F. Representative vWF stained lung blood vessels, and G. Quantitative analysis of vWF-stained lung blood vessels per high power field (HPF) of neonatal WT mice that were exposed to normoxia (air) or hyperoxia (70 % O₂) for 14 d and allowed to recover in air for 14 d. Data are representative of at least three independent experiments. Values are presented as mean $\pm$ SD (n=3/exposure group). Significant differences between normoxia and hyperoxia-exposed mice are indicated by *, $p < 0.05$ (t-test). **Scale bar = 100 μ m.**

Figure 3. Expression of AM and its signaling receptors in neonatal mouse lungs exposed to hyperoxia

Real-time RT-PCR analysis of AM (A), Calcr1 (B), and RAMP2 (C) mRNA expression in the lungs of neonatal WT mice exposed to normoxia (air) or hyperoxia (70% O₂) for up to 14 d. Data are representative of at least three independent experiments. Values are presented as mean $\pm$ SD (n=3/time point/exposure group). Significant difference between normoxia- and hyperoxia-exposed mice is indicated by *, $p < 0.05$ (ANOVA).

Figure 4. Expression of phosphorylated (p) ERK1/2 and quantification of angiogenesis in AM-treated fetal HPAECs

Primary fetal HPAECs were treated with 0.1, 10, 100, or 1000 nM AM for 15 min, following which whole-cell protein was extracted to determine p- and total (t)-ERK1/2 protein expression (A) by immunoblotting. p-ERK1/2 band intensities were quantified and normalized to t-ERK1/2 band intensities (B). Representative photographs showing tubule formation in growth factor-reduced Matrigel (C–D) and cell migration (F–G), and quantitative analyses of tubule formation (E) and cell migration (H) in HPAECs treated with 10 nM AM. Data are representative of at least three independent experiments. Values are presented as mean $\pm$ SD. Significant differences between untreated- and AM-treated cells are indicated by *, $p < 0.05$ (ANOVA (p-ERK1/2), t-test (tubule formation and scratch assays)). Scale bar = 100 μ M.