Biochemical and Biophysical Research Communications 487 (2017) 666–671

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Hyperoxia exposure disrupts adrenomedullin signaling in newborn mice: Implications for lung development in premature infants

Renuka T. Menon, Amrit Kumar Shrestha, Binoy Shivanna*

Section of Neonatology, Department of Pediatrics, Texas Children's Hospital, Baylor College of Medicine, Houston, TX 77030, United States

ARTICLE INFO

Article history:
Received 16 April 2017
Accepted 20 April 2017
Available online 21 April 2017

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

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% O_2) 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.

1. 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

* Corresponding author. Division of Neonatal-Perinatal Medicine, Texas Children's Hospital, Baylor College of Medicine, 1102 Bates Avenue, MC: FC530.01, Houston, TX 77030, United States.
E-mail address: shivanna@bcm.edu (B. Shivanna).

http://dx.doi.org/10.1016/j.bbrc.2017.04.112
0006-291X/

Abbreviations

AM	adrenomedullin
BPD	bronchopulmonary dysplasia
Calcrl	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

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.

2. Materials and methods

2.1. In vivo experiments

2.1.1. 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.

2.2. 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.

2.2.1. Hyperoxia exposure

Within 24 h of birth, male and female pups were exposed to normoxia (21% O_2, n = 36) or hyperoxia (70% O_2, 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).

2.2.2. 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 (20× magnification) was performed for each animal (n = 9/exposure group).

2.3. In vitro experiments

2.3.1. 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.

2.3.2. 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.

2.3.3. 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.

2.4. Statistical analyses

The results were analyzed by GraphPad Prism 5 software. Data are expressed as mean ± 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, Calcrl, 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.