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Adrenomedullin deficiency potentiates hyperoxic injury in fetal human pulmonary microvascular endothelial cells

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ABSTRACT

Bronchopulmonary dysplasia (BPD) is a chronic lung disease of premature infants that is characterized by alveolar simplification and decreased lung angiogenesis. Hyperoxia-induced oxidative stress and inflammation contributes to the development of BPD in premature infants. Adrenomedullin (AM) is an endogenous peptide with potent angiogenic, anti-oxidant, and anti-inflammatory properties. Whether AM regulates hyperoxic injury in fetal primary human lung cells is unknown. Therefore, we tested the hypothesis that AM-deficient fetal primary human pulmonary microvascular endothelial cells (HPMEC) will have increased oxidative stress, inflammation, and cytotoxicity compared to AM-sufficient HPMEC upon exposure to hyperoxia. Adrenomedullin gene (*Adm*) was knocked down in HPMEC by siRNA-mediated transfection and the resultant AM-sufficient and -deficient cells were evaluated for hyperoxia-induced oxidative stress, inflammation, cytotoxicity, and Akt activation. AM-deficient HPMEC had significantly increased hyperoxia-induced reactive oxygen species (ROS) generation and cytotoxicity compared to AM-sufficient HPMEC. Additionally, AM-deficient cell culture supernatants had increased macrophage inflammatory protein 1α and 1β, indicating a heightened inflammatory state. Interestingly, AM deficiency was associated with an abrogated Akt activation upon exposure to hyperoxia. These findings support the hypothesis that AM deficiency potentiates hyperoxic injury in primary human fetal HPMEC via mechanisms entailing Akt activation.

1. Introduction

Supplemental oxygen is commonly administered as a life-saving measure in patients with impaired lung function. Although oxygen relieves the immediate life-threatening consequences transiently, it may also lead to increased reactive oxygen species (ROS) production and expression of proinflammatory cytokines and exacerbate lung injury [1]. Bronchopulmonary dysplasia (BPD) is a chronic lung disease of infancy that results from an arrested lung alveolar and vascular growth [2]. Despite improved therapies of premature infants, BPD remains the most prevalent sequelae of preterm birth [3]. Hyperoxia-induced ROS generation and lung inflammation are the major contributors in the development of BPD [4]. Infants with BPD are more likely to have long-term pulmonary problems, increased re-hospitalizations during the first year of life, and delayed neurodevelopment [3,5]. Hence, there is an urgent need for improved therapies to prevent and treat BPD.

Adrenomedullin (*Adm*, gene; AM, protein) is a 52-amino acid peptide that belongs to the calcitonin family of peptides that includes calcitonin, calcitonin gene related peptide (CGRP), amylin and intermedin [6]. AM signaling occurs by the functional receptor combination of calcitonin receptor-like receptor (CRLR) with receptor activity modifying protein 2 (RAMP-2) or RAMP-3. The RAMPs determines the responsiveness of the receptors to particular ligands [7]. AM is an ubiquitous peptide that is expressed in all tissues of the body, including blood vessels and lungs [8]. Interestingly, studies have shown that AM plays a crucial role in endothelial growth and survival [9,10]. Mice lacking the AM gene die *in utero* around embryonic day13.5 due to vascular endothelial

Abbreviations: AM, adrenomedullin; *Adm*, adrenomedullin gene; ARDS, acute respiratory distress syndrome; BPD, bronchopulmonary dysplasia; CM-H_2DCF-DA, 5-(and-6)-chloromethyl-2′, 7′-dichlorodihydrofluorescein diacetate; CRLR, calcitonin receptor-like receptor; HPMEC, human pulmonary microvascular endothelial cells; MIP-1α, macrophage inflammatory protein-1 alpha; MIP-1β, macrophage inflammatory protein-1 beta; MTT, 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide; PKB, protein kinase B; RAMP, receptor activity modifying protein; ROS, reactive oxygen species.

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disruption suggesting that AM signaling is necessary for normal endothelial development [11,12]. In addition, AM has potent anti-inflammatory [13,14], anti-oxidant [15], angiogenic [16], and vasodilatory [17] properties in the lungs. Recently, AM was found to increase pulmonary angiogenesis and attenuate alveolar simplification and pulmonary hypertension in a rat model of hyperoxia-induced BPD [18].

Akt or protein kinase B (Akt/PKB) is a serine/threonine kinase, which is activated by phosphatidylinositol (PI) 3-kinase in response to growth and survival factors [19,20]. Akt activation plays a crucial role in regulating cellular apoptosis and proliferation [21]. Moreover, Lu et al. demonstrated that a constitutively active form of Akt is sufficient to protect adult mice from hyperoxic lung injury [22]. Interestingly, AM is shown to regulate prosurvival properties of endothelial progenitor [23] and cardiac [24] cells via Akt activation.

These observations do not indicate whether AM signaling could be protective in hyperoxic lung injury in human neonates. Thus, the goal of this study was to investigate the effects of AM signaling in hyperoxia-induced oxygen toxicity in fetal human lung cells *in vitro*. Specifically, we chose the fetal human pulmonary microvascular endothelial cells (HPMEC) for our experiments because Wright et al. [25] have demonstrated the feasibility of using HPMEC to examine the mechanisms of hyperoxic injury. Using these cells, we tested the hypothesis that AM-deficient HPMEC will have increased oxidative stress, inflammation, and cytotoxicity compared to AM-sufficient HPMEC upon exposure to hyperoxia via a mechanism entailing Akt activation.

2. Materials and methods

2.1. Cell culture and hyperoxia experiments

HPMEC, the primary microvascular endothelial cells derived from the lungs of human fetus were obtained from ScienCell research laboratories (San Diego, CA; 3000) and grown according to the manufacturer's protocol. Hyperoxia experiments were conducted in a plexiglass sealed chamber [26].

2.2. Small interfering RNA (siRNA) transfections

Transfections were performed with either 50 nM control siRNA (Dharmacon, Lafayette, CO; d-001810) or 50 nM *Adm* specific siRNA (SMART pool: ON-TARGET plus siRNA, Dharmacon, Lafayette, CO; L-011199) using LipofectamineRNAiMAX (Life Technologies, Grand Island, NY; 13778030). Twenty four hours after transfection, the cells were exposed to air or hyperoxia for up to 48 h and cells were harvested and analyzed for viability, proliferation, apoptosis and necrosis, ROS generation, inflammatory gene expressions, and AM signaling.

2.3. Cell viability assay

Cell viability was determined by a colorimetric assay based on the ability of viable cells to reduce the tetrazolium salt, MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide), to formazan [26].

2.4. Cell proliferation assay

Cell proliferation was determined based on the measurement of cellular DNA content via fluorescent dye binding using the CyQUANT NF cell proliferation assay kit (Invitrogen, Carlsbad, CA; C35006) [26].

2.5. Apoptosis and necrosis assay

Apoptosis and necrosis were estimated by flow cytometry using FITC Annexin V/Dead cell apoptosis kit (Invitrogen, Carlsbad, CA; V13242) [26].

2.6. Measurement of ROS generation

Intracellular level of ROS was quantified by flow cytometry using the ROS sensitive fluorophore 5-(and-6)-chloromethyl-2′, 7′-dichlorodihydrofluorescein diacetate (CM-H_2DCF-DA) according to the manufacturer's recommendation (Invitrogen, Carlsbad, CA; C6827) [26].

2.7. Measurement of cytokine/chemokine production: multiplex luminex assay

The cell culture supernatants of the transfected cells exposed to air or hyperoxia for up to 48 h, were analyzed for cytokine/chemokine levels using Millipore Human Cytokine/Chemokine assay as per the manufacturer's recommendations. The following cytokines/chemokines were analyzed: Epidermal growth factor (EGF), Interferon (IFN) γ, interleukin (IL)-1α, IL-1β, IL-8, IL-10, monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein (MIP)-1α, MIP-1β, and vascular endothelial growth factor (VEGF).

2.8. Western blot assays

Whole-cell protein extracts from the transfected cells 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, Santa Cruz, CA; sc-47778, dilution 1:1000), Akt (Cell Signaling, Danvers, MA; 4691, dilution 1:1000), and phospho-Akt(Ser_{473}) (Cell Signaling, Danvers, MA; 4060, dilution 1:1000) antibodies.

2.9. Real-time RT-PCR assays

At 24 h of exposure, total RNA isolated from the transfected cells was reverse transcribed to cDNA [26] and real-time quantitative RT-PCR analysis was performed with 7900HT Real-Time PCR System using TaqMan Gene Expression Mastermix (Applied Biosystems Inc., Foster City, CA; 4369016) and TaqMan Gene Expression Assays (Applied Biosystems) for the following genes: ADM-Hs00969450_g1; RAMP2-Hs01006937_g1; CRLR-Hs00907738_m1; and GAPDH-Hs02758991_g1.

2.10. Analyses of data

The results were analyzed by GraphPad Prism 5 software. The effects of *Adm* gene expression, exposure, and their associated interactions for the outcome variables were assessed using ANOVA techniques. Multiple comparison testing by the posthoc Bonferroni test was performed if statistical significance of either variable or interaction was noted by ANOVA. A p value of <0.05 was considered significant.

3. Results and discussion

The present study demonstrates that AM deficiency increases the susceptibility of fetal HPMEC to hyperoxic injury via mechanism(s) entailing Akt activation. In human fetal lung-derived HPMEC *in vitro*, deficient AM-signaling mediated increase in

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