Published in final edited form as:

Biochem Biophys Res Commun. 2015 September 4; 464(4): 1048–1053. doi:10.1016/j.bbrc.2015.07.067.

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 1 α and 1 β , 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.

Graphical abstract

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Keywords

Adrenomedullin; Fetal HPMEC; Hyperoxic Injury; Inflammation; Akt

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 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.

Materials and Methods

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].

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.

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].

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].

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].

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-H₂DCF-DA) according to the manufacturer's recommendation (Invitrogen, Carlsbad, CA; C6827) [26].

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)-1 α , IL-1 β , IL-8, IL-10, monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein (MIP)-1 α , MIP-1 β , and vascular endothelial growth factor (VEGF).

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(Ser₄₇₃) (Cell Signaling, Danvers, MA; 4060, dilution 1:1000) antibodies.

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.

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.

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 hyperoxic injury correlated with lack of Akt activation.

In addition to its critical role in vascular development [11, 12], AM stabilizes the endothelial barrier function [27, 28]. The latter effect may be beneficial in acute hyperoxic lung injury, which is characterized by disruption of endothelial barrier [29]. Additionally, studies suggest that AM protects against acute lung injury secondary to mechanical ventilation [13], ischemia-reperfusion [30], and administration of inflammation-inducing agents such as lipopolysaccharide [14] and carrageenan [31]. Recently, AM was also found to attenuate developmental lung injury in a rat model of hyperoxia-induced BPD [18]. However, whether AM regulates hyperoxic injury in primary human fetal lung cells is unknown. Therefore, we conducted experiments in human fetal lung derived HPMEC *in vitro*, both in the presence and absence of a functional AM, to investigate whether AM modulates hyperoxic injury.

Initially, we studied the interaction between hyperoxia and AM. Interestingly, our results suggest that hyperoxia in itself transcriptionally activates the expression of AM (Fig. 1A). Therefore, AM may play a pivotal role in hyperoxic lung injury. A similar phenomenon has been observed in newborn mice [18] and adult rats [32] exposed to hyperoxia. This phenomenon might be a protective rather than a contributory response to oxidative stress since administration of AM attenuated hyperoxic lung injury in the above studies [18, 32]. The molecular mechanisms by which hyperoxia activate pulmonary AM remain unknown at the present time.

To investigate whether the AM regulates hyperoxic injury in fetal human lung cells *in vitro*, we performed *Adm* siRNA transfection experiments to knockdown *Adm*. As expected, *Adm* siRNA significantly decreased *Adm* mRNA expression (Fig. 1B). Next, we studied the effects of AM on hyperoxia-induced cytotoxicity, cell proliferation and death. Hyperoxia results in increased generation of ROS [33], and decreased cell proliferation [34] and viability [35]. Increased ROS levels have been thought to contribute to acute and chronic lung disease in humans by inhibiting cell proliferation and increasing cell death by apoptosis or necrosis [36, 37]. Similarly, we observed increased ROS generation (Fig. 3A), decreased cell viability (Fig. 2A) and proliferation (Fig. 2B), and increased apoptotic (Fig. 2C) and late-apoptotic/necrotic cell death (Fig. 2D) upon exposure to hyperoxia. However, AM-deficiency augmented hyperoxia-induced cytotoxicity (Figs. 2A, B, and C) and ROS generation (Fig. 3A). There was no difference in hyperoxia-induced late apoptosis and necrosis between AM-sufficient and -deficient cells (Fig. 2D). These findings support the concept that AM is a crucial regulator of oxidant-injury and that it mediates its effects in part by decreasing ROS generation.

In addition to ROS, inflammation plays a key role in the pathogenesis of hyperoxia-induced lung disorders such as BPD and acute respiratory distress syndrome (ARDS) [4, 38]. Interestingly, in our model, AM-deficiency was associated with a significant increase in

MIP-1 α (Fig. 3B) and MIP-1 β (Fig. 3C) levels both in air and hyperoxic conditions. MIP-1 α and MIP-1 β are chemokines that can activate granulocytes and initiate an inflammatory response. Several other investigators have suggested that MIP-1 chemokines may be an important mediator of hyperoxia-induced acute [23] and chronic [19, 39] lung injury in mice. Thus, our result in human cells signify the beneficial anti-inflammatory role of the AM in hyperoxia-induced lung disorders in humans. Although, we did not notice significant differences in other cytokines and chemokines (Supplemental Fig. S1) that are associated with BPD and ARDS, it is possible that we might have missed the time period wherein these cytokines and chemokines levels may have been elevated.

The molecular mechanism(s) by which the pulmonary AM protects against hyperoxic lung injury remains poorly defined. Since, Akt is known to attenuate apoptosis and improve cell proliferation, and AM is known to activate Akt, we next determined whether AM protects against hyperoxic injury via Akt pathway. Interestingly, we observed that AM-deficient cells failed to activate Akt upon exposure to hyperoxia compared to AM-sufficient cells (Figs. 4A, B, and C). This suggests that AM modulates Akt activation under hyperoxic conditions. Ahmad et. al. [40] similarly observed that Akt is transiently activated in adult HPMEC exposed to hyperoxia. More importantly, they demonstrated that constitutive activation of Akt attenuates oxygen toxicity. The protective antiapoptotic and prosurvival cellular effects of Akt activation have been extensively documented, as evidenced by 1) inhibition of stress-activated kinases [41]; 2) attenuation of B-cell lymphoma 2 associated death promoter-induced cell death [39]; 3) increased transcription of cAMP response element-binding protein-regulated survival genes [42]; 4) suppression of caspase-9-induced cell death [43]; and 5) cell cycle progression [44]. Additionally, activation of Akt pathway has been demonstrated to attenuate oxidative stress and inflammation via nuclear factor, erythroid 2-like 2 pathway [45] and tumor necrosis factor- α [46]. Thus, the increased oxygen toxicity in AM-deficient cells may be attributed to a lack of Akt activation. To the best of our knowledge, this is the first study to report a novel finding that AM-deficiency potentiates hyperoxic injury in primary fetal human lung cells that is associated with lack of Akt activation upon exposure to hyperoxia. The mechanisms of this finding are unknown and deserve further investigations.

Calcitonin gene-related peptide is known to protect cardiomyocytes against reperfusion injury via CRLR/RAMP1 pathway [47], which suggests that CRLR or RAMP can confer cytoprotective properties independent of AM. Hence, we finally looked at the mRNA expression of other components of AM signaling pathway such as *CRLR* and *Ramp2*. Our results indicate that the increased oxygen toxicity observed in AM-deficient cells is directly related to AM deficiency and excludes the confounding effects of CRLR or RAMP2 deficiency since there was no difference in CRLR or RAMP2 mRNA expression between AM-sufficient and -deficient cells (Supplemental Fig. S2).

In summary, our data suggests that AM is required to decrease ROS generation and inflammation and protect fetal HPMEC against hyperoxic injury. We propose that AM deficiency increases hyperoxic injury via a mechanism that entails lack of Akt activation upon exposure to hyperoxia, which results in increased oxidative stress, inflammation, apoptosis, and decreased cell proliferation (Graphical Abstract). AM can thus be a potential

therapeutic target to improve current therapies of BPD and ARDS because of its potential to mitigate both inflammation and oxidant stress, which are significant in the development of BPD and ARDS.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Dr. Kathleen M. Caron (University of North Carolina at Chapel Hill, USA) for providing insightful comments. This work was supported by grants from National Institutes of Health HD-073323 and American Heart Association BGIA20190008 to B.S., and by the Cytometry and Cell Sorting Core at Baylor College of Medicine with funding from the NIH (AI036211, CA125123, and RR024574) and the expert assistance of Joel M. Sederstrom.

Abbreviations

AM	adrenomedullin
Adm	adrenomedullin gene
ARDS	acute respiratory distress syndrome
BPD	bronchopulmonary dysplasia
CM-H₂DCF-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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Highlights

We studied adrenomedullin signaling in hyperoxic injury in human fetal lung cells.

Hyperoxia induces adrenomedullin expression in human fetal lung cells.

Hyperoxia-induced ROS generation is augmented in adrenomedullin deficient cells.

Adrenomedullin deficiency increases hyperoxia-induced cytotoxicity and inflammation.

Hyperoxia-induced Akt activation is abrogated in adrenomedullin deficient cells.

Figure 1. Hyperoxia increases and *Adm* siRNA abrogates *Adm* mRNA expression in HPMEC
A. HPMEC were exposed to air or hyperoxia for up to 6 h, following which RNA was extracted for real-time RT-PCR analysis of *Adm* mRNA expression. B. HPMEC were transfected with either 50 nM control (SiC) or *Adm* (SiAdm) siRNA. Thirty-six hours after transfection, RNA was extracted for real-time RT-PCR analyses of *Adm* mRNA expression. Data are representative of at least three independent experiments. Values are presented as means $\pm$ SEM (n=3). *, $p < 0.05$ vs. air exposed cells (A) and vs. control siRNA (B).

Figure 2. AM deficiency potentiates hyperoxia-induced cytotoxicity in HPMEC

Control (SiC) or *Adm* (SiAdm) siRNA transfected HPMEC were exposed to air or hyperoxia for up to 48 h, following which: (A) cell viability was assessed by MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) reduction activities; (B) cell proliferation was determined based on the measurement of cellular DNA content via fluorescent dye binding using the CyQUANT NF cell proliferation assay; (C) cell apoptosis and (D) cell apoptosis and necrosis was determined by annexin V and propidium iodide staining of the cells as measured by flow cytometry. Data are representative of at least three independent experiments. Values are presented as means $\pm$ SEM (n=4). Two-way ANOVA showed an effect of hyperoxia and *Adm* gene and an interaction between them for all the dependent variables, except for late apoptosis and necrosis in this figure. Significant differences between air- and hyperoxia-exposed cells are indicated by *, $p < 0.05$. Significant differences between hyperoxia-exposed AM-sufficient and -deficient cells are indicated by †, $p < 0.05$.

Figure 3. AM deficiency potentiates hyperoxia-induced ROS generation and inflammation in HPMEC

Control (SiC) or *Adm* (SiAdm) siRNA transfected HPMEC were exposed to air or hyperoxia for up to 48 h, following which the oxidation of the fluorescent dye, CM-H₂DCF-DA, was measured by flow cytometry (A) and the cell free supernatants were analyzed for MIP-1 α (B) and MIP-1 β (C) by multiplex luminex assay. Data are representative of at least three independent experiments. Values are presented as means $\pm$ SEM (n=4). Two-way ANOVA showed an effect of hyperoxia and *Adm* gene and an interaction between them for the dependent variables in this figure. Significant differences between air- and hyperoxia-exposed cells are indicated by *, $p < 0.05$. Significant differences between hyperoxia-exposed AM-sufficient and -deficient cells are indicated by †, $p < 0.05$. Significant differences between air-exposed AM-sufficient and -deficient cells are indicated by π , $p < 0.05$.

Figure 4. AM deficiency abrogates hyperoxia-induced Akt activation

Control (SiC) or *Adm* (SiAdm) siRNA transfected HPMEC were exposed to air or hyperoxia for up to 24 h, following which the whole cell protein was extracted for total Akt (A and D) and phosphoAkt(Ser₄₇₃) (A, B, and C) protein expression. Data are representative of at least three independent experiments. Values are presented as means $\pm$ SEM (n=3). Two-way ANOVA showed an effect of hyperoxia and *Adm* gene and an interaction between them for the dependent variable, phosphoAkt (Ser₄₇₃), in this figure. Significant differences between air- and hyperoxia-exposed cells are indicated by *, $p < 0.05$. Significant differences between hyperoxia-exposed AM-sufficient and -deficient cells are indicated by †, $p < 0.05$.