19 Chronic Intermittent Hypoxia in Premature Infants: The Link Between Low Fat Stores, Adiponectin Receptor Signaling and Lung Injury

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Abstract

Premature infants have chronic intermittent hypoxia (CIH) that increases morbidity, and the youngest and the smallest premature infants are at the greatest risk. The combination of lung injury from inflammation/oxidative stress causing low functional residual capacity combined with frequent short apneas leads to CIH. Adiponectin (APN) is an adipose-derived adipokine that protects the lung from inflammation and oxidative stress. Premature and small for gestational age (SGA) infants have minimal body fat and low levels of circulating APN. To begin to understand the potential role of APN in lung protection during lung development, we characterized the developmental profile of APN and APN receptors (AdipoR1 and AdipoR2) protein and mRNA expression in the newborn rat lung at fetal day (FD) 19, and postnatal days (PD) 1, 4, 7, 10, 14, 21, and 28. Protein levels in lung homogenates were measured by western blot analyses; relative mRNA expression was detected by quantitative PCR (qPCR); and serum high molecular weight (HMW) APN was measured using enzyme-linked immunosorbent assay (ELISA). Results: APN protein and mRNA levels were lowest at FD19 and PD1, increased 2.2-fold at PD4, decreased at PD10, and then increased again at PD21. AdipoR1 protein and mRNA levels peaked at PD1, followed by a threefold drop by PD4, and remained low until PD21. AdipoR2 protein and mRNA levels also peaked at PD1, but remained high at PD4, followed by a 1.7-fold drop by PD10 that remained low by PD21. Serum APN levels detected by ELISA did not differ from PD4 to PD28. To date, this is the first report characterizing APN and APN receptor protein and mRNA expression in the rat lung during development. The developmental stage of the newborn rat lung models that of the premature human infant; both are in the saccular stage of lung development. In the newborn rat lung, alveolarization begins at PD4, peaks at PD10, and ends at PD21. Importantly, we found that AdipoR1 receptor protein and mRNA expression is lowest during lung alveolarization (PD4 to PD21). Thus, we speculate that low levels of AdipoR1 during lung alveolarization contributes to the increased susceptibility to developing acute

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lung edema and chronic lung injury such as bronchopulmonary dysplasia (BPD) in premature human infants.

Keywords

Adiponectin · Alveolarization · Bronchopulmonary dysplasia · Developmental profile · Lung development

19.1 Introduction

CIH that occurs in premature infants is the combination of several biological and physiological factors that come together to create the perfect storm (Di Fiore et al. 2013; Gauda and McMaster 2017). These factors include (1) the influence of the carotid body on baseline breathing and (2) low functional residual capacity decreasing oxygen stores. The carotid body contributes 40% to baseline breathing in premature infants, and thus their breathing is characterized by short apneas and periodic breathing. Because of lung injury resulting in low functional residual capacity, rapid oxygen desaturations occurs with even short apneas. Adiponectin (APN) is a pleiotropic adipokine, produced mainly by adipocytes. It is best known for its role in metabolism, promoting insulin-sensitivity and improved glucose uptake. Relevant to our work, APN also has potent anti-inflammatory and antioxidant effects (Sliman et al. 2013). APN mediates its effects via APN receptors, AdipoR1 and AdipoR2. AdipoR1 and AdipoR2 are ubiquitously expressed throughout the body including the lung, with the highest level of AdipoR1 expression in skeletal muscle, while AdipoR2 is predominately expressed in the liver. Premature and small for gestational age (SGA) infants have minimal body fat, and therefore, low levels of circulating APN. Moreover, premature infants have immature lungs and are susceptible to acute lung injury from exposure to inflammation and oxidative stress. We speculate that the low levels of APN may cause or at least contribute to their increased risk for early lung edema and later bronchopulmonary dysplasia (BPD). In adult models, APN protects the lung from inflammatory and oxidative injury (Sliman et al. 2013; Xu et al. 2013). However, the biological role of APN in the lung during early development, and how the lack of endogenous APN contributes to an increased susceptibility to acute and chronic lung injury, has yet to be explored. Thus, we studied the developmental profile of APN and APN receptor (AdipoR1 and AdipoR2) protein and mRNA expression in the newborn rat, in which lung development during the first 2 weeks after birth parallels that of premature human infants born between 23 and 25 weeks of gestation.

19.2 Methods

19.2.1 Animals

Experiments involving animals were conducted in accordance with the Canadian Council on Animal Care Guidelines, and the use and care of animals were in accordance with the Declaration of Helsinki Conventions. Animal studies were approved by the Animal Care Committee of The Hospital for Sick Children. Sprague-Dawley rat pups from fetal day (FD) 19 to postnatal day (PD) 21 were used. At different time-points (FD19, PD1, PD4, PD7, PD10, PD14, PD21, and PD28) 8 male pups were euthanized. Lung and heart tissues were then collected and flash frozen in liquid nitrogen. Blood was also taken, and serum was separated and frozen. A total of 12 litters (n = 8 male pups per litter) were studied for each time point.

19.2.2 Western Blot Analyses

Tissues previously stored at –80 °C were homogenized in RIPA cell lysis buffer (0.1 M $NaPO_4$, 1% (wt/vol) sodium deoxycholate, 1% (vol/vol) Nonidet P-40, 20% SDS, 0.877% (wt/vol) NaCl, 0.074% (wt/vol) EDTA, pH 7.2) containing protease and phosphatase inhibitors. The homogenates were incubated for 20 min on ice and then

Table 19.1 Rat (Rattus Norvegicus) primer sequences for quantitative PCR (qPCR)

Gene (RefSeq accession No.)	Forward 5′–3′	Reverse 5′–3′
Rat adiponectin (NM_144744.3)	GAGACGCAGGTGTTCTTGGT	GGAACATTGGGGACAGTGAC
Rat ADIPOR1 (NM_207587.1)	GTTGTACCCACCATGCACTTT	GGTTGGACACCCCATAGAAGT
Rat ADIPOR2 (NM_001037979.1)	GCCATTATCGTCTCTCAGTGG	GTCACATTTGCCAGGAAAGAA

sonicated for 20 s, vortexed and incubated for 10 min on ice. Samples were then centrifuged at 12,000 RPM for 10 min. Protein concentrations were quantified by Bradford spectrophotometry assay, using known concentrations of bovine serum albumin (BSA) as a standard. Lung tissue lysates containing 50 μg of total protein were boiled for 5 min in SDS sample buffer (0.125 M Tris·HCl, 4% SDS, 20% glycerol, and 10% 2-mercaptoethanol, 0.004% bromphenol blue, pH 6.8). Equal amounts of protein were then loaded onto a gel, subjected to SDS polyacrylamide gel electrophoresis for 1 h at 150 V, and transferred onto polyvinylidene difluoride membranes. The membranes were blocked in TBST (20 mM Tris base, 150 mM NaCl, pH 8.0, with 0.1% Tween 20) for 1 h and then incubated with anti-adiponectin antibody (26 kDa, Novus Biologicals), anti-AdipoR1 antibody (43 kDa, Novus Biologicals), or anti-AdipoR2 antibody (44 kDa, Novus Biologicals) overnight at 4°C. To compensate for differences in protein loading, each membrane was incubated with the anti-beta-actin (β-actin) antibody (42 kDa, Santa Cruz Biotechnologies) for 2 h at room temperature. The membrane was washed and incubated with anti-rabbit IgG secondary antibody conjugated to horseradish peroxidase for 1 h at room temperature. After several washes with TBST, protein bands were visualized using an enhanced chemiluminescence method. Images were digitally captured using a MicroChemi chemiluminescent system, and band intensities were quantified using ImageJ software.

19.2.3 qPCR

RNA was extracted and reverse transcribed from tissue samples stored in RNAlater®. qPCR was performed on a Stratagene MX3000P qPCR system using SYBR Green qPCR Master Mix (Qiagen). Cycling conditions were 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. A standard curve for each primer set was performed using rat lung cDNA standard (Agilent), to ensure that reaction efficiency was equivalent to primers for the housekeeping gene, β-actin. The expression of β-actin was unaffected by the experimental conditions. Primer sequences are listed in Table 19.1. Following each standard curve reaction, molecular weight standards (O-GeneRuler; Fermentas, Burlington, Ontario, Canada) and PCR products underwent DNA gel electrophoresis to confirm the presence of a single product of predicted length. A dissociation (melt) curve and a no-template control were run for each set of samples to exclude nonspecific product formation and reaction contamination. Samples were run in duplicate, and expression of the gene of interest was normalized to β-actin. Change in expression relative to the calibrator samples was calculated by the 2Δct method using Stratagene MxPro software (version 4.01).

19.2.4 Elisa

Rat HMW APN ELISA kits from Amsbio (Cambridge, MA, USA) were used to detect the serum concentrations of HMW APN. Frozen serum samples were thawed and all reagents were allowed to reach room temperature. Samples and HMW APN standards were applied to an antibody-precoated microplate, and then the conjugate was added. After incubating and washing, samples were incubated with substrate and the reaction was stopped using stop solution. Optical density was determined using a microplate reader at 450 nm.

19.2.5 Statistical Analysis

All values are expressed as means ± SE (n = 7–8 per group). Data were subjected to one-way analysis of variance (ANOVA) followed by pairwise multiple comparisons using the Holm-Sidak method with $p < 0.05$ indicating significance.

19.3 Results

19.3.1 Adiponectin Protein Developmental Profile

As shown in Table 19.2, total lung APN protein levels were lowest at FD19 and PD1 which then increased fourfold by PD7, followed by a twofold decrease from PD7 to PD10. Thereafter, APN protein levels increased from PD10 by PD21 and then decreased slightly at PD28.

19.3.2 AdipoR1 Protein Developmental Profile

As shown in Table 19.3, total lung AdipoR1 protein levels peaked at PD1, decreased threefold by PD4, and remained low until PD14. AdipoR1 protein levels then increased at PD14 to levels comparable to levels at PD1. There was a subsequent fall in AdipoR1 at PD21 and levels remained low thereafter.

Table 19.2 Western blot analysis for total lung expression of APN (26 kDa) normalized to β-actin (43 kDa) in the newborn rat at fetal day (FD) 19, and postnatal days (PD) 1, 4, 7, 10, 14, 21 and 28

Age	Mean ± SE
FD19	0.43 ± 0.03
PD1	0.57 ± 0.06
PD4	1.27 ± 0.09*
PD7	1.61 ± 0.07
PD10	0.82 ± 0.09**
PD14	0.94 ± 0.06
PD21	1.25 ± 0.11***
PD28	0.98 ± 0.07

Note. Values represent means ± SE for n = 7–8 samples per group analyzed by one-way ANOVA. *$p < 0.05$ compared to all groups except PD21 and PD28. **$p < 0.05$ compared to FD19, PD4, PD7, and PD21. ***$p < 0.05$ compared to FD19, PD1, PD7, PD10, and PD14

Table 19.3 Western blot analysis for total lung expression of AdipoR1 (43 kDa) normalized to β-actin (43 kDa) in the newborn rat at fetal day (FD) 19, and postnatal days (PD) 1, 4, 7, 10, 14, 21 and 28

Age	Mean ± SE
FD19	0.74 ± 0.10
PD1	1.33 ± 0.31
PD4	0.45 ± 0.07*
PD7	0.73 ± 0.04
PD10	0.57 ± 0.12*
PD14	1.30 ± 0.12
PD21	0.69 ± 0.09*
PD28	0.89 ± 0.05

Note. Values represent means ± SE for n = 7–8 samples per group analyzed by one-way ANOVA. *$p < 0.05$ compared to PD1 and PD14

Table 19.4 Western blot analysis for total lung expression of AdipoR2 (44 kDa) normalized to β-actin (43 kDa) in the newborn rat at fetal day (FD) 19, and postnatal days (PD) 1, 4, 7, 10, 14, 21 and 28

Age.	Mean ± SE
FD19	0.90 ± 0.08
PD1	1.23 ± 0.08
PD4	1.02 ± 0.10*
PD7	0.77 ± 0.04
PD10	0.60 ± 0.06**
PD14	0.96 ± 0.15
PD21	0.61 ± 0.07**
PD28	1.22 ± 0.05

Note. Values represent means ± SE for n = 7–8 samples per group analyzed by one-way ANOVA. *$p < 0.05$ compared to PD10 and PD21. **$p < 0.05$ compared to PD1, PD4, PD14, and PD28

19.3.3 AdipoR2 Protein Developmental Profile

As shown in Table 19.4, total lung AdipoR2 protein levels increased by 1.8-fold from FD19 to PD1 and peaked at PD1. There was a threefold drop from PD4 to PD10, and then an increase at PD14 to levels comparable to levels at PD1. AdipoR2 protein levels subsequently dropped at PD21, and then increased again at PD28 to levels comparable to levels at PD1.

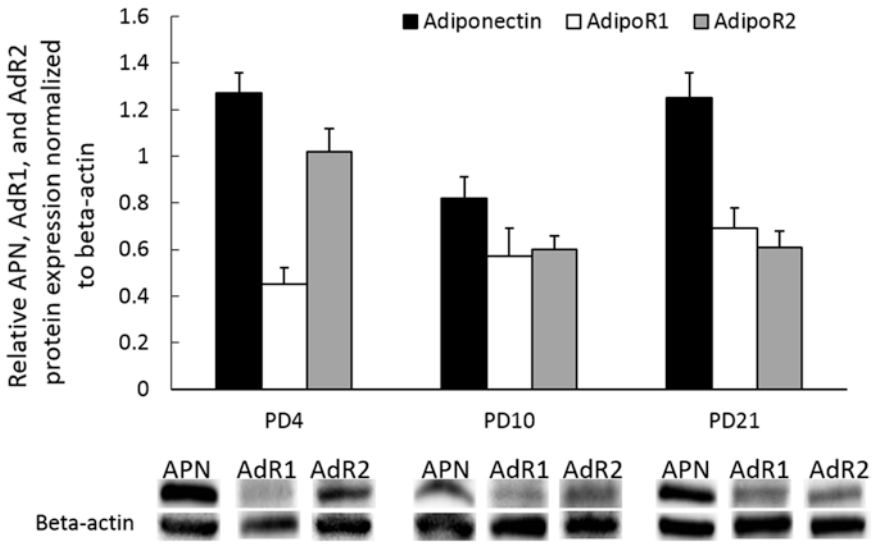

Fig. 19.1 Western blot analysis for total lung expression of adiponectin (26 kDa), AdipoR1 (43 kDa), and AdipoR2 (44 kDa) normalized to β-actin in the newborn rat during alveolarization (postnatal days (PD) 4, 10 and 21). APN is adiponectin, AdR1 and AdR2 are adiponectin receptor 1 and 2, respectively. See Tables 19.1, 19.2 and 19.3 for a more complete expression profile

19.3.4 Adiponectin, AdipoR1, and AdipoR2 Western Blot Analyses During Alveolarization

As shown in Fig. 19.1, APN protein levels are highest at PD4, decreased at PD10, and then at PD21 increased to levels comparable to PD4 levels. AdipoR1 protein levels are lowest at PD4 and increased slightly at PD10 and again at PD21. AdipoR2 protein levels are highest at PD4, subsequently decreased at PD10, and remained low at PD21.

19.3.5 Adiponectin, AdipoR1, and AdipoR2 mRNA Developmental Profile

The pattern of mRNA relative expression for lung APN, AdipoR1, and AdipoR2 mirrored the protein expression (data not shown).

19.4 Discussion

The major finding from this preliminary study is that APN and AdipoR1 and AdipoR2 protein expression in the lung undergo significant changes during the first 3 weeks of development in the newborn rat. Specifically, we found that total lung (1) APN protein expression does not significantly increase until PD4, (2) AdipoR1 receptors remain low throughout the phase of alveolarization (PD4-PD21), while AdipoR2 expression is consistently low from PD7-PD21. At birth, the newborn rat is in the saccular stage of lung development, alveolarization starts at PD4, peaks at PD10, and is complete by PD21. Infants who are born preterm at 23–26 weeks of gestation are in the saccular stage of lung development while alveolarization continues throughout the first week of life.

Infants born prematurely sustain interruptions in lung development and alveolarization and are more susceptible to developing chronic lung disease, such as BPD (Jobe and Ikegami 2000). This

increased susceptibility can be attributed to the immaturity of lung development at birth and an imbalance between anti-inflammatory and pro-inflammatory mediators, favoring the latter (reviewed in Speer 2003). As a result of this imbalance, the inflammatory responses that occur in the lung can overwhelm the system and create acute lung injury presenting as lung edema. This initial damage then leads to further injury affecting alveolar development and abnormal development of the pulmonary vasculature leading to BPD (reviewed in Speer 2003). Infants who are SGA from intrauterine growth restriction are at particular risk for developing BPD and have the lowest levels of plasma APN at birth (Hansen-Pupp et al. 2015). APN signaling is complex but appears to mediate lung protection in multiple ways: decreases endothelial leak, decreases inflammation, and decreases oxidative stress. Although the mechanism has not been elucidated, APN signaling also does appear to be involved in normal lung development since APN knockout mice have abnormal alveolarization (Bianco et al. 2013). Zana-Taieb et al. (2015), using postnatal genome-wide analysis, reported that in a newborn rat model of growth restriction alveolarization is impaired, and the peroxisome proliferator-activated receptor (PPAR) pathway is affected. PPARs are ligand-activated nuclear transcription factors that regulate the expression of genes encoding APN, AdipoR1, and AdipoR2 (Zana-Taieb et al. 2015). Moreover, giving PPAR agonists increased the expression of APN, and nebulization of the agonist blocked hyperoxic lung injury in a newborn rat model of BPD (Morales et al. 2014).

We found that total lung APN levels are lowest immediately before birth and during the first week of life in our animal model. APN modulates innate immunity, decreasing inflammation by binding to receptors on macrophages (M1 and M2) and neutrophils. APN suppresses M1 activation which produces pro-inflammatory cytokines, and promotes M2 macrophage proliferation which is involved in tissue repair and produces anti-inflammatory cytokines (Luo and Liu 2016). APN also reduces the production of reactive oxygen species (ROS) from activated neutrophils. Neutrophil infiltration from the blood into the lung produces a significant inflammatory response and contributes to the development of acute and chronic lung injury. Low levels of APN in the lung during the saccular stage of lung development may increase the vulnerability to inflammatory injury causing abnormal alveolarization, which occurs in infants with BPD. Moreover, we detected the lowest level of APN expression at FD19, suggesting that the lungs are at risk during inflammation that may occur prior to birth.

AdipoR1s are expressed on human airway epithelial cells (Miller et al. 2009) and both AdipoR1s and AdipoR2s are expressed on alveolar epithelial cells (A549 cells) (Nigro et al. 2013). However, it appears that AdipoR1 is the primary receptor protecting alveolar epithelial cells from inflammatory injury and apoptosis. More specifically, the AdipoR1 receptor blocked TNF-α-mediated translocation of NF-Kappa β in alveolar epithelial cells and increased the production of IL-10, an anti-inflammatory cytokine (Nigro et al. 2013). We found that AdipoR1 levels were lowest throughout the stage of alveolarization in the rat lung. Less is known about the function of AdipoR2 receptors on cells and tissues other than the liver, although they are present on cells in multiple tissues including the lung. Globular APN activates AdipoR1 while the full length HMW APN binds AdipoR2. From the available literature, AdipoR1 appears to be the most functional receptor on lung cells, including airway and alveolar epithelial cells (Miller et al. 2009), pulmonary artery smooth muscle cells (Weng et al. 2011), and vascular endothelial cells (Chen et al. 2015). We speculate that AdipoR1 receptors are expressed on both airway and alveolar epithelial cells during lung development in our model. The significance of our findings that AdipoR2 are also present in the lung during early development requires additional studies to determine the localization and function of these receptors. Of note, at PD4, the start of alveolarization, AdipoR1 protein expression is lower than AdipoR2.

Interpretation of our data is limited by the fact that we measured gene and protein expression for

APN, AdipoR1, and AdipoR2 in lung homogenates and thus, we are unable to know the specific cells that are expressing these proteins and their function. Neither do we know whether APN might have anti-inflammatory or pro-inflammatory properties since APN can, in fact, have pro-inflammatory effects, by stimulating the expression of cytosolic phospholipase A_2, cyclooxygenase-2, and ROS such as it does in human alveolar type II cells (Chen et al. 2016). Nevertheless, we observed significant changes in total lung APN and APN receptor protein and mRNA expression, especially during alveolarization. Although we are the first to characterize the pattern of expression of this important adipokine and its receptors during early lung development, we know that key experiments will need to be done to determine APN and APN receptor localization and its role in lung protection in a model of lung injury.

In conclusion, the youngest and the smallest infants are at risk for significant lung injury that contributes to the expression of CIH. We know that plasma and serum APN are low in these infants during the first weeks of life. If the developmental profile of the expression of APN, AdipoR1, and AdipoR2 protein in the human infant is similar to what we described for the rat, low levels of APN protein expression during the first 3 days of life and low levels of AdipoR1 receptors throughout alveolarization could increase the risk of developing inflammatory and oxidative lung injury.

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