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Bronchopulmonary Dysplasia Impairs L-Type Amino Acid Transporter-1 Expression in Human and Baboon Lung

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Summary

Bronchopulmonary dysplasia (BPD) is an inflammatory lung disorder common in premature infants who undergo mechanical ventilation with supplemental oxygen. Inhaled nitric oxide (iNO) has been used to prevent experimental and clinical BPD. Earlier studies showed that NO effects in alveolar epithelial cells (AEC) are mediated by S-nitrosothiol uptake via L-type amino acid transporter-1 (LAT1). Because LAT1 expression could influence the efficacy of iNO therapy, we sought to determine whether pulmonary LAT1 expression is altered in preterm baboons with experimental BPD and in human newborns susceptible to developing BPD. Using fixed lung obtained from 125 d to 140 d gestation baboon models of BPD, LAT1 immunostaining was measured in control and BPD animals. In adult controls and in 140 d gestational controls (GC), LAT1 was expressed in both type I and type II AECs. In 140 d BPD lungs, LAT1 expression density in alveolar tissue was decreased. In 125 d GC baboons, LAT1 immunostaining was largely confined to cuboidal AECs, whereas animals given 14 d of mechanical ventilation exhibited diminished alveolar septal LAT1 Labeling. The pattern in adult human donor lung was comparable to that observed in adult baboons. LAT1 was expressed in lungs obtained from some but not all very premature newborns at autopsy. In human and baboon lung, adult and newborn, pulmonary vascular cells expressed LAT1. In summary, LAT1 is expressed in AECs and pulmonary vascular cells in baboons and humans. Experimental BPD in premature baboons decreases pulmonary LAT1 expression and alters its spatial localization. Heterogeneity of functional LAT1 could affect S-nitrosothiol importation, which could impair iNO therapy.

Keywords

pulmonary medicine; neonatology; lung diseases

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INTRODUCTION

Inhaled nitric oxide (iNO) therapy is a widely used treatment for infants with persistent pulmonary hypertension,^{1,2} and it is also currently being investigated for clinical use in the prevention and treatment of bronchopulmonary dysplasia (BPD).³ Despite the efficacy of iNO in experimental lung injury and experimental BPD, it has not been uniformly successful in clinical trials to prevent BPD.⁴ Therefore, previously undescribed host factors may affect the ability of iNO to achieve optimal pharmacologic effects. Recent studies have shown that NO pharmacologic effects in alveolar epithelium are partly dependent on uptake of *S*-nitrosothiols (SNO) through the L-type amino acid transporter 1 protein (LAT1/SLC7A5).⁵ Similar LAT1-system dependence of NO signaling has been reported in other cell types such as human skin cells, *Xenopus* vascular cells, and *Xenopus* oocytes.^{6–8}

Conditions that impair the LAT1 system could theoretically affect iNO efficacy through alterations in SNO uptake in the lung. In particular, hyperoxia has recently been shown to impair LAT1 expression in vitro and in vivo and inhibit pharmacologic actions of NO gas in alveolar epithelium in a LAT1-dependent manner.⁵ These findings suggest that hyperoxia impairs LAT1 function and expression in the lung, potentially limiting SNO transport into the lung. Clinical conditions for which iNO would be prescribed are typically accompanied by oxidative stress.⁹ Thus, oxidative stress-induced impairment of the LAT1 system could contribute to the heterogeneous effects of iNO observed in clinical therapy.

We therefore tested the hypothesis that experimental BPD impairs pulmonary LAT1 expression in baboons. In addition, we assessed pulmonary LAT1 spatial expression in adult human donor lung and in autopsy tissues obtained from patients with pathologic diagnoses of extreme prematurity.

MATERIALS AND METHODS

Normal adult and experimental BPD preterm baboon lung were fixed in 4% paraformaldehyde and embedded in paraffin. Sections were obtained from the Southwest Foundation for Biomedical Research, San Antonio TX.¹⁰ The 140d BPD and 125d PRN (*pro re nata*) BPD models were developed by Jacqueline Coalson and colleagues and have previously been described in detail.^{11–13} Adult human donor lung was obtained at pneumonectomy as previously described,¹⁴ and newborn human lungs were obtained at autopsies using a standardized protocol designed to preserve morphologic and molecular integrity, as previously described in detail.¹⁵ Group sizes were between N = 3–6 animals per group. All procedures were approved by the relevant institutional animal care and use committees and institutional review boards.

140d Baboon Model

In the 140d BPD model, post-delivery, non-BPD control animals (N = 4) were placed on positive-pressure ventilation and only administered PRN oxygen support for the survival of the animal. 140d gestational controls (GCs) correspond approximately to human infants at 32 weeks gestation.¹⁰ To model severe hyperoxic BPD, premature baboons (N = 5) were mechanically ventilated for 10 days with fraction of inspired oxygen (FiO₂) = 1 (100% O₂).

This original 140d/100% O₂ regimen sought to model the effects of hyperoxia combined with sustained mechanical ventilation in the pre-surfactant era. Clinical features of BPD, including atelectasis, pulmonary fibrosis, and squamous metaplasia of the airways were observed by days 8–9 of life.

125d Baboon Model

In the 125d model, BPD animals were exposed to PRN oxygen for either 6 days (N = 6) or 14 days (N = 5) postnatally. In these disease animals, management included antenatal steroids, a single dose of early surfactant replacement, low tidal volume ventilation, and PRN oxygen targeted to achieve P_aO₂ 55–70 mm Hg as monitored by arterial blood gas measurements.¹² In the control group, 125d GCs (N = 4) were used to assess normal intrauterine development during the time that the BPD animals were undergoing hyperoxic conditions. 125d GCs correspond approximately to human infants at 26 weeks gestation.¹⁰

Adult Human Lung

Adult human lung sections were obtained from a clinical lung transplant donor at Duke University Medical Center.¹⁴ In addition, fixed, paraffin-embedded normal lung tissue from two autopsy cases were purchased from a commercial vendor (Amsbio LLC, Cambridge, MA; See Table 1).

Human Newborn Lung

Human newborn lung sections were obtained from infant autopsy samples obtained from the University of Rochester Medical Center.¹⁵ Preterm human lungs were obtained from infants who were at risk to develop BPD due to respiratory distress syndrome and extreme prematurity, with exposure to either brief or lengthy mechanical ventilatory support. Full-term newborn lungs were obtained from an infant born at 41 weeks gestational age and had no diagnosed lung pathology at the time of death (See table).

Immunohistochemistry Reagents

Tissues were labeled using a mouse monoclonal anti-human LAT1 at a 1:800 dilution (gift of H. Endou, J Pharma Ltd., Yokohama, Japan). Alveolar type 1 epithelium was identified using rabbit monoclonal anti-human aquaporin 5 (AQP5) at a 1:500 dilution, (Abcam, Cambridge MA, catalog # 92320) and alveolar type II epithelium was identified using rabbit polyclonal anti-human pro-surfactant protein-C (pro-SPC) at a 1:1,000 dilution (Millipore, Temecula CA, catalog # AB3786).

Enzyme Detection Immunohistochemistry

Paraffin-embedded baboon lung sections obtained from the Southwest National Primate Research Center were dewaxed, rehydrated through serial gradation of ethanol: water, and subjected to antigen retrieval in 10 mM Tris 1.0 mM EDTA 0.05% Tween™ 20 at pH 9 for 3.0 min using a pressure cooker. Sections were blocked with 5% bovine album serum (BSA) in phosphate-buffered saline with Tween™ 20 in 0.05% (PBS-T) for 1.0 hr and incubated overnight at 4°C with mouse anti-human LAT1 (1:800) or 5% BSA (no primary antibody) as negative control, then detected with rabbit anti-mouse alkaline phosphatase (Jackson

Immunoresearch Laboratories, West Grove PA) (1:500). Cellular sites of expression were determined in sections that were co-incubated with anti-AQP5 or anti-pro-SPC, then detected with ImmPRESS anti-rabbit horseradish peroxidase (Vector Laboratories, Burlingame, CA). LAT1 labeling was achieved by reacting nitro-blue tetrazolium chloride and 5-bromo-4-chloro-3'-indolylphosphate *p*-toluidine salt (NBT/BCIP) (Roche Life Sciences, Indianapolis IN) with alkaline phosphatase conjugated to the secondary antibody (Immunoresearch Laboratories). Pro-SPC or AQP5 labeling was achieved by reacting 3,3'-diamino-benzidine (DAB) solution (Vector) with horseradish peroxidase conjugated to the secondary antibody (Vector). Images were obtained by light microscopy using a digital camera (DP11, Olympus, Center Valley, PA).

Image Analysis

MetaMorph (version 6.1, Molecular Devices, Sunnyvale CA) was used to estimate the expression of LAT1 in stained lung tissue. Ten random non-overlapping high-power fields from each sample per treatment group were imaged at 20× magnification via light microscopy under identical illumination conditions. Each image had a total pixel dimension of $1,712 \times 1,368$ pixels. To perform semi-quantification of LAT1 labeling, a color threshold was set to include only NBT/BCIP labeling. Each image was overlaid with 12 boxed regions, each with dimensions of 200×200 pixels, in a 4×3 column-row arrangement. For each region, the LAT1 positive area and alveolar tissue area were determined by two different color thresholds. Only regions that overlaid alveolar lung tissue were used for analysis. Sample regions that overlaid conducting airways or blood vessels were not included. Digital images were corrected for stained matter within airspaces prior to thresholding. The mean LAT1 positive area, the mean alveolar tissue area, and the normalized ratio of these two measurements were calculated for each subject for statistical comparisons.

Statistical Analysis

Grouped data were expressed as mean $\pm$ standard error (SE). Intergroup differences were compared by one-way ANOVA with Tukey's multiple comparison test, assuming a type I error of 0.05 and type II error of 0.10. Significance was accepted at $P < 0.05$.

RESULTS

Pulmonary LAT1 Expression in Adult and Premature (140d) Baboon Models

In adult baboon lung, LAT1 co-localized with AQP5 and pro-SPC, consistent with expression in type 1 and 2 AECs, respectively (Fig. 1). LAT1 expression was observed along the outer surface of AQP5+ AECs and in discrete pro-SPC+ AECs of the lung alveoli. LAT1 was also widely expressed in bronchiolar epithelium and pulmonary vascular endothelium (Fig. 2).

The pattern of LAT1 expression density in 140d GCs was similar to that of the adult baboon lungs, but characterized by decreased overall LAT1 labeling proportional to similarly decreased total alveolar tissue area (Fig. 4A and B). In 140d/100% O₂ baboons following 10 days of experimental BPD, LAT1 expression was significantly diminished in alveolar tissue,

with loss of labeling on the surfaces of the alveolar septal cells (Figs. 2 and 4A, C). Labeling in pulmonary vascular tissue was less uniform, although this was not quantified (Fig. 2). Compared to gestational controls, the 140d BPD baboons also exhibited thicker alveolar interstitial spaces (Fig. 2) and greater alveolar tissue per unit area (Fig. 4B).

Pulmonary LAT1 Expression in 125d Baboon Model

In contrast to LAT1 expression in the AECs of adult and 140d GC baboons, LAT1 expression in 125d GC, 6d PRN oxygen, and 14d PRN lungs was most prominent in cuboidal epithelial cells, with diminished apical labeling in cells lining distal air spaces (Fig. 3). In the 14d PRN lungs, distal air spaces often lacked cells expressing LAT1. In the 6d PRN and 14d PRN groups, a greater proportion of LAT1 was expressed ectopically in the interstitial regions of the lung, rather than the apical alveolar epithelium as seen in the 125d GC and adult lungs. The proportions of LAT1-positive alveolar lung tissue from all treatment groups of the 125d model were significantly decreased compared to that of the 140d GC baboons (Fig. 4). Compared to the 125d GC, the 6d PRN showed a significantly higher proportion of LAT1-positive alveolar lung tissue. The lung tissues of 125d GC, 6d PRN, and 14d PRN animals all exhibited some atelectasis.

Pulmonary LAT1 Expression in Humans

Normal adult human lung obtained from a donor showed septal and pulmonary vascular endothelial LAT1 labeling (Fig. 5) comparable to that seen in adult baboon (Fig. 1). In full term newborn lung obtained at autopsy from a patient with neuromuscular disease, the LAT1 expression pattern was comparable to that of normal adult human lung (Fig. 5C and D). In a very premature newborn (27 weeks gestation) that died at 4d of age due to respiratory distress syndrome and sepsis, LAT1 expression was much less prominent in alveolar septal tissue, largely confined to the interstitium, although expression in bronchiolar epithelium was robust. However, in a newborn at similar gestational age at birth but who died after 3 months of mechanical ventilation, LAT1 expression was widespread (data not shown). Qualitative LAT1 expression in human lung samples is described in the table.

DISCUSSION

Since therapeutic NO effects appear to depend in part on *S*-nitrosothiol uptake through LAT1, we sought to determine if experimental BPD, which models a condition for which NO treatment has been used, was associated with impaired LAT1 expression. Alveolar LAT1 expression has not been previously reported in primates. We found that the pulmonary LAT1 expression pattern in baboons and humans without lung injury was comparable to that which we have previously reported in healthy rodents.^{5,16,17} In comparison, baboons and humans with lung injury exhibited decreased and ectopic pulmonary LAT1 expression. The current study is the first, to our knowledge, to demonstrate impaired LAT1 under BPD conditions in primate lung.

We utilized an existing sample set from the baboon BPD models developed by Jacqueline Coalson and colleagues.^{11,12} Baboons are well suited for modeling human respiratory disease because their lung anatomy closely approximates that of humans, and they have

similar pathological responses to hyperoxia.^{18,19} The previously established experimental BPD baboon models represent so-called “old” and “new” BPD,¹² using higher supplemental oxygen and mechanical ventilation in the 140d BPD model and more tightly managed oxygen and mechanical ventilation in the less mature 125d BPD model. The 125d model used prenatal steroids prior to elective delivery of baboon infants at the canalicular stage of development, along with treatment with postnatal exogenous surfactant as described in detail elsewhere.¹²

The effects of experimental BPD on pulmonary LAT1 expression are consistent with our recent report of in vitro hyperoxia effects on alveolar epithelial LAT1 expression. We previously demonstrated that hyperoxia impairs SNO uptake, downstream NO effects on soluble guanylyl cyclase activity, and LAT1 function and expression in rat and human alveolar epithelial cell culture.⁵ Since oxidative stress was a key component of inducing experimental BPD in the baboon models, we expected that alveolar LAT1 expression would be impaired in the BPD baboons, as we observed.

Expression of LAT1 in human lungs obtained at autopsy suggests that suppression of LAT1 expression may be temporary, since we found low expression in very premature newborn lung obtained at 4 days of age, but relatively robust expression in specimens obtained at later chronological ages. Any conclusions about the effects of gestation and duration of post-natal intensive care on LAT1 expression must be limited because of the inherent limitations of using autopsy material.

We compared the absolute LAT1 labeling area and the alveolar tissue area of the two groups separately. We found that the 140d BPD group exhibited lower absolute LAT1 labeling (Fig. 4A), and greater alveolar tissue area due to a thickening of interstitial tissue secondary to lung injury (Fig. 4B), both of which contributed to the decrease in normalized LAT1/alveolar tissue ratio.

Adult baboons had significantly higher absolute LAT1 expression and overall alveolar tissue area than any other group (Fig. 4A and B). However, the higher absolute LAT1 expression was outweighed by a proportionally greater increase in alveolar tissue area, leading to a lower normalized LAT1 expression in adult baboons compared to the 140d GC group (Fig. 4C). The greater normalized LAT1 expression in the 140d GC group could be due to heightened cell growth and proliferation that occurs during the perinatal period. LAT1 transports large neutral amino acids that are critical carbon and nitrogen sources for the synthesis of proteins, nucleotides, and glutathione. Previous studies have found that LAT1 expression correlates with cell proliferation and angiogenesis,²⁰ thus providing a potential explanation for the observed increase in normalized alveolar LAT1 in 140d GC compared to adult baboons.

Image analysis indicated that baboons with experimental BPD exhibited decreased LAT1 expression specifically in alveolar lung tissue, with a greater proportion of LAT1 in interstitial lung regions (Fig. 4C). Ectopic expression of LAT1 has been previously shown to impair LAT1 transport function. Site-directed mutagenesis of two specific Cys sites in rabbit LAT1, C88S and C439S, impaired the transporter activity and its expression on the cell

membrane.²¹ This was consistent with our recent finding of diminished membrane LAT1 in hyperoxia-exposed alveolar epithelial cells.⁵ Alteration of Cys residues in LAT1 could impair folding and transporter insertion into the membrane. Because the induction of experimental BPD was associated with partial displacement of LAT1 expression from the alveolar epithelium, BPD pathophysiology may be associated with protein modifications that impair LAT1 transportation to the membrane, as well as potential effects on LAT1 synthesis.

There are important limitations to these observations. Because of the significant costs inherent to baboon studies, the sample size of each experimental group was small, ranging from N = 3–6 animals per group. In addition, the assessments could only be semi-quantitative and do not measure LAT1 expression on a cell-specific level because of the limitations of immunohistochemistry and our inability to perform whole organ stereological sampling with the archived materials available. This study does not measure how the changes in alveolar LAT1 expression during BPD are distributed across different cell types; further research is needed to measure LAT1 expression with greater cellular specificity.

Our study demonstrates evidence of altered pulmonary LAT1 expression in BPD, but does not assess functional effects on LAT1. We could not test whether impaired LAT1 expression results in reduced SNO transport in baboons or humans. Further work is required to determine whether impaired LAT1 expression reduces LAT1 function, decreases SNO transport, or impairs NO pharmacologic effects in vivo.

CONCLUSIONS

We found decreased alveolar septal LAT1 expression in experimental baboon BPD model systems compared to adult and gestational controls. Pulmonary LAT1 expression in human autopsy and donor lung without lung disease was similar to the baboon expression pattern. Combining these translational data with previous studies that identify LAT1 as the major transporter for SNO, we conclude that experimental BPD is associated with altered LAT1 expression in the alveolar epithelium, which could contribute to reduced SNO transport. By developing a more complete understanding of LAT1 function in primates, LAT-dependent SNO transport could potentially be optimized to improve the efficacy of NO-based therapies.

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Fig. 1. LAT1 is expressed in type I AECs (A, *arrows*), with co-localization with AQP5 (B, *arrow*, brown labeling) to the alveolar septum. Co-localization with pro-SPC was seen in type II AECs (C, *arrow*). There was no labeling in sections incubated without primary antibody (D). Bar = 50 μ m.

Fig. 2. LAT1 expression in adult and premature (140d GC and 140d BPD) baboon lung. A, C, E: Adult and 140d gestation control (140d GC) demonstrate prominent alveolar septal labeling (A, C) that is markedly diminished in the 140d BPD lung (E). B, D, F: Pulmonary vascular endothelial labeling was only seen routinely in adult baboons (B) with heterogeneous, mostly negative pulmonary vascular smooth muscle labeling in both 140d GC (D) and 140d BPD (F) groups. Bar = 50 μ m.

Fig. 3. LAT1 expression in premature (125d GC and 125d BPD) baboon lung. A, C, E: Air saccules in the 125d GC (A), 125d BPD 6d PRN (C), and 14d PRN (E) show non-uniform LAT1 labeling at apical surfaces of epithelial lining cells, with intense labeling in some cuboidal cells lining the air spaces. B, D, F: Pulmonary vascular endothelial and vascular smooth muscle labeling was heterogeneous across the 125d GC (B), 6d PRN (D), and 14d PRN (F) treatment groups.

Fig. 4.

Proportion of LAT1-positive alveolar tissue in premature and adult baboon lung. A: Total LAT1 expression per field is lower in the 140d BPD group compared to the 140d GC group ($P = 0.24$). Total LAT1 expression was higher in the 125d 6d PRN BPD group than the 125d GC and 125d 14d PRN BPD groups. Adult baboons had significantly greater total LAT1 expression compared to all newborn groups (mean $\pm$ SEM, $*P < .05$ vs. adult). B: The average alveolar tissue area is greater in the 140d BPD group compared to the 140d GC group ($P = 0.30$). There were no significant differences in alveolar tissue area between the 125d groups. The adult group had significantly increased alveolar tissue area compared to all newborn groups (mean $\pm$ SEM, $*P < .05$ vs. adult). C: The proportion of LAT1-positive alveolar tissue is decreased in the 140d BPD baboons ($N = 5$) compared to the adult control ($N = 3$) and 140d GC animals ($N = 4$). Adult control animals also exhibited significantly decreased LAT1-positive alveolar tissue compared to 140d GC animals. The proportions of LAT1-positive alveolar tissue in 125d GC ($N = 4$), 6d PRN BPD ($N = 6$), and 125d 14d PRN

BPD (N = 5) showed no significant pair-wise differences, but were all significantly lower than that of the 140d GC animals (mean $\pm$ SEM, * P < .01 vs. 140d GC).

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Fig. 5. Qualitative LAT1 expression in human lung. Adult donor lung obtained at transplant shows (A) alveolar septal and (B) pulmonary vascular endothelial LAT1 expression (*arrows*). (C) Alveolar septal and (D) pulmonary vascular LAT1 expression seen in term newborn lung in the absence of diagnosed lung pathology. (E) Septal LAT1 expression is not in AECs and confined to the interstitium in lung obtained from a preterm newborn at 27 weeks gestation with sepsis, but is preserved in the bronchiolar epithelium (F). Bar = 50 μ m.

TABLE 1

Source Material and LAT1 Expression Patterns in Human Lung

Clinical diagnosis	Pathologic diagnosis	GA at birth (wk)	Age at death (wk)	LAT1 expression
Donor lung	Normal lung	Adult	n/a	Prominent in alveolar septa most arteries
Autopsy, adenocarcinoma lung	Normal lung	Adult	n/a	Prominent in alveolar septa most arteries
Autopsy, myocardial infarction	Normal lung	Adult	n/a	Prominent in alveolar septa most arteries
Neuromuscular disease	Normal lung	41	0.5	Prominent in alveolar septa most arteries
RDS, sepsis	Hyaline membranes, irregular inflation, hemorrhage	27	0.6	Only interstitial alveolar septal labeling few arteries labeled
BPD, respiratory failure	Interstitial fibrosis, cuboidal alveolar epithelium	25	9.0	Prominent in alveolar septa most arteries