Activation of mas restores hyperoxia-induced loss of lung epithelial barrier function through inhibition of apoptosis

Abstract

Background: Neonatal therapy with a high concentration of oxygen (hyperoxia) is a known cause of bronchopulmonary dysplasia (BPD). BPD is characterized by increased pulmonary permeability and diffuse infiltration of various inflammatory cells. Disruption of the epithelial barrier may lead to altered pulmonary permeability and airways fluid accumulation. Mas receptor is a component of the renin angiotensin system and is the receptor for the protective endogenous peptide angiotensin 1-7. The activation of the Mas receptor was previously shown to have protective pulmonary responses. However, the effect of Mas receptor activation on epithelial barrier integrity has not been tested.

Objective: To determine the effects of hyperoxia with or without Mas receptor activation on epithelial cell barrier integrity.

Design/Methods: Human epithelial cell line A549 was cultured on transwell polycarbonate porous membrane to confluence and treated with 95% oxygen (hyperoxia) for 72 hours with or without the Mas receptor agonist (AVE0991), or the apoptotic inhibitors Z-VAD-FMK or aurantricarboxylic acid. The cells were then challenged with Rhodamine labeled bovine serum albumin (Rh-BSA) on one side of the membrane. Fluorescent quantitation of Rh-BSA (albumin flux) was performed on the media in the other side of the membrane 3 hours later and was compared with 21% oxygen (Normoxia) control group. A549 cells were also cultured with or without AVE0991 in hyperoxia or normoxia and used for nuclear fragmentation apoptosis assay using propidium iodide staining.

Results: Hyperoxia induced an increase in albumin flux that was significantly prevented by AVE0991 treatment and by the apoptosis inhibitors. AVE0991 also significantly decreased the hyperoxia-induced nuclear fragmentation.

Conclusion: These results suggest that hyperoxia causes a disruption in the epithelial barrier integrity, and that this disruption is inhibited by the Mas receptor agonist AVE0991 through inhibition of epithelial apoptosis. These results reveal a novel potential drug for BPD and pulmonary edema treatment.

Keywords: bronchopulmonary dysplasia, pulmonary edema, hyperoxia, alveolar epithelium, angiotensin

Introduction

Bronchopulmonary dysplasia (BPD) is a chronic lung condition that affects newborn babies who received high levels of oxygen for a long period or put on a ventilator after birth, especially those who were born prematurely. Infants develop BPD in about 1.5% of all newborn births. BPD is associated with neurodevelopmental deficits, cognitive impairments, failure to thrive, pulmonary hypertension and cor-pulmonale. High rates of in utero and perinatal exposure to infection may be causally related to preterm delivery and subsequent lung injury. Over the past two decades, the histological presentation of BPD has changed from heterogeneous pulmonary inflammation and fibrosis (Old BPD) to uniform arrest of alveolar development and variable interstitial cellularity and/or fibroproliferation (New BPD). In the new definition of BPD, lung development arrests before alveolarization, and the lungs have larger but much fewer alveoli than normal lungs. BPD is characterized by lung inflammation, airway injury secondary to interstitial and alveolar fluid overload, smooth-muscle hypertrophy, and oxidative stress. Inflammation and alveolar fluid overload is attributed to increased pulmonary permeability and diffuse infiltration of various inflammatory cells. Disruption of the epithelial barrier may lead to altered pulmonary permeability and airways fluid accumulation. The maintenance of barrier properties requires intact epithelial tight junctions. The apoptosis of alveolar epithelial cells (AECs) in vivo was shown to cause the collapse of the pulmonary epithelial barrier function.

The local renin angiotensin system (RAS) is activated after tissue injury in a variety of organs to promote repair, but abnormalities in the process promote fibrosis. Previous studies have shown that the octapeptide angiotensin II (AngII) causes alveolar epithelial injury and apoptosis through its action on AT1 receptor. The recently discovered angiotensin 1-7 (Ang1-7) heptapeptide has shown a protective effect to pulmonary cells via its action on “Mas” receptor. The non-peptide compound AVE0991 is a Mas receptor activator that stimulates the actions of Ang1-7 peptide, and has shown promise to attenuate lung injury.

In the study presented here, we investigate the effects of hyperoxia on the epithelial barrier integrity and apoptosis. We also evaluate the protective effects of AVE0991 on the epithelial barrier.
Methods

Reagents and materials

Fluorescently labeled rhodamine (tetrarmethylrhodamine) bovine serum albumin conjugate (Rh-BSA) was purchased from Molecular Probes (Invitrogen). For the apoptosis inhibitors, the caspase inhibitor Z-VAD-FMK (ZVAD) was obtained from R&D systems, and the endonuclease inhibitor aurintricarboxylic acid (ATA) was obtained from Sigma-Aldrich. Corning Costar Transwell permeable support, 6.5mm insert, 0.4μm polycarbonate membrane, 24 well plates were purchased from Sigma-Aldrich. The Mas receptor agonist AVE 0991 from MedChem Express.

Cell culture

The human lung adenocarcinoma cell line A549 was obtained from the American Type Cell Culture Collection (ATCC) and cultured in Ham's F12 medium supplemented with 10% fetal bovine serum (FBS) (Gibco). All experiments were conducted in serum-free Ham's F12 medium. For Rh-BSA flux experiments, A549 cells were plated on Transwell membranes to obtain an intact monolayer in the inner chamber membrane in the presence of complete medium. Both inner and outer chambers were washed, replaced with serum-free F12 medium and were cultured for 72 hours in either 21%O₂, 5%CO₂ (Normoxia) or in 95%O₂, 5%CO₂ (Hyperoxia) in the presence or absence of 10⁻⁵M AVE0991, 60μM ZVAD, or 10μM ATA. DMSO solvent was added in control groups for absence of AVE0991, ZVAD, or ATA. After the 72 hours, the cells on the Transwell membrane were used for the Rh-BSA flux measurement.

Albumin flux

Integrity of the cultured cells epithelial barrier was determined by albumin flux similar to described previously 11. After treating the cells with the above mentioned conditions, treatment media was removed and tetrarmethylrhodamine-bovine serum albumin (Rh-BSA, 1μg/μl) in serum free F12 media was added into the inner chambers and unlabeled albumin in serum free F12 media into the outer chambers. Cells were incubated in 21%O₂, 5%CO₂ incubator. Three hours later, 50μl aliquots were taken from outer chambers into 96 well half area black plate. Rhodamine fluorescence from outer chambers was determined on FL600 fluorescence microplate reader (BioTek Inc., Winooski, VT).

Detection of apoptosis

Cells were cultured on 24-well cell culture plates to sub-confluent density and were exposed to normoxia or hyperoxia in the presence or absence of the Mas activator AVE0991 for 72 hours then were fixed in 70% ethanol. Apoptotic cells were detected by nuclear fragmentation assay using propidium iodide (PI) as described earlier,23 after enzymatic digestion of ethanol-fixed cells with DNase-free RNase in PBS containing 5 μg/ml PI. During fixation with 70% ethanol, detached cells were retained by centrifugation of the 24-well culture plates. Cells with discrete nuclear fragments with condensed chromatin were counted as apoptotic using epifluorescence microscopy. Apoptotic cells were scored over a minimum of four separate microscopic fields from each of at least three culture vessels per treatment group.

Results

Mas activation prevents hyperoxia-induced increased albumin flux through alveolar epithelial cell monolayers

Figure 1 shows a significant increase in albumin flux through A549 cell monolayers cultured on Transwell inserts that had been exposed to 95%O₂ or normoxia (21% O₂). The increase in albumin flux was significantly decreased in the presence of the Mas activator AVE0991.

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Mas receptor activation prevents hyperoxia-induced alveolar epithelial cell apoptosis

Figure 3 Inhibitors of apoptosis block Rh-BSA flux across A549 cell monolayers in response to 95% Oxygen. A549 cells were cultured on Transwell inserts as described in Figure 1 and Methods, but the cells were exposed to 95% O2 in the presence or absence of the apoptosis inhibitors Z-VAD-fmk (ZVAD, a pan-caspase inhibitor), or the endonuclease inhibitor Aurintricarboxylic acid (ATA). Thereafter, Rh-BSA (1mg/ml) was added into the inner chambers for measurement of Rh-BSA flux as described in Figure 1. Bars are the mean +/- S.E.M.; *** p<0.001, ** p<0.01, * p<0.05 using ANOVA

Mas activation inhibits hyperoxia-induced apoptosis in A549 cells. A549 cell monolayers were exposed to 95% O2 in the presence or absence of the mas activator AVE0991. Cells were subjected to propidium iodide (PI) nuclear fragmentation assay (see Methods) after 72 hours exposure. Bars are means +/- S.E.M. normalized as percent of the normoxia control; ***P < 0.001 by ANOVA and Student-Newman-Keuls analysis, n ≥ 6.

Discussion

Hyperoxic lung injury, and BPD has disturbance of lung fluid balance that may lead to pulmonary edema or fluid overload in lung, as being called in premature X-rays in NICU as “wet lungs”, and diuretics is still a commonly used medicine to help clearing that and drying up the lung. This alveolar fluid overload and airways fluid accumulation is attributed to increased pulmonary permeability and disruption of the epithelial barrier.

Xu et al. showed that Pulmonary edema secondary to hyperoxic lung injury is secondary to injury to the pulmonary epithelial barrier, with increase in the total water Wet/Dry ratio. This Wet/Dry ratio was significant as early as the third day of hyperoxic exposure and increased on fifth then seventh day of hyperoxic exposure. Also there was significant increase in extravascular lung water content, and bronchoalveolar lavage fluid (BALF): serum FDI “fluorescein isothiocyanate-conjugated dextran 4000 ratio suggested increasing permeability with hyperoxia induced lung injury. In our study, we similarly show that hyperoxia increases the permeability of the epithelial barrier function after 72 hours of oxygen exposure (Figures 1 & 3). Other reports suggest that structure destruction of tight junction proteins occludin and ZO-1 is the mechanism of hyperoxia-induced leakage/pulmonary edema.

Tight junction proteins are necessary for the functional structure of the epithelial barrier. They also play a role in cell signaling, transcription regulation and maintaining epithelial polarity. Several studies have shown a relationship and cross-talk between the disruption of tight junction proteins and apoptosis. Beeman et al. showed that treating epithelial cells with a claudin-disrupting mimic peptide results in caspase 3 activation and apoptosis. The study also suggests a role of occludin in signaling cell death. In an animal study of rat mammary epithelia, apoptosis was found to be preceded by ZO-1 and occludin loss. Induction of apoptosis in epithelial kidney, colon, and breast cell lines caused proteolytic cleavage of tight junction proteins in a distinctive manner that correlated with the disruption of the tight junction. Notably, occludin, ZO-1 and ZO-2 were found to be fragmented by caspase cleavage and this fragmentation was blocked by caspase and/or metalloprotease inhibitors. This blockage of fragmentation of these tight junction proteins was associated with preservation of cellular morphology.

Our data presented here supports these findings where we show that inhibition of apoptosis eliminated the hyperoxia-induced epithelial permeability. This inhibition of apoptosis is presented here using three approaches; the caspase inhibitor ZVAD, the endonuclease inhibitor ATA (Figure 3), and the Mas receptor agonist AVE0991 (Figures 1 & 4).

Previous studies from our and other study groups have shown that epithelial apoptosis plays a critical role in lung edema formation. Noradrenaline administration in vivo was shown to be sufficient to invoke the collapse of the alveolar epithelial barrier via induction of apoptosis. In animal and cell line acute lung injury models, Fas activation altered the alveolar barrier integrity and function by mechanisms involving caspase-dependent apoptosis. In addition, intratracheal instillation of exogenous AngII peptide induced apoptosis and caused alveolar epithelial barrier injury through the AT1 receptor. Our data here show a role of the Ang1-7 receptor, Mas, in protection against hyperoxia-induced apoptosis (Figure 4). These emphasize the critical roles of the angiotensin system in the epithelial apoptosis in lung injury, hence epithelial barrier permeability and lung edema formation.

The angiotensin system has been reported to control tight junction proteins localization and distribution. Several studies reported that
AngII changes ZO-1 expression and cellular distribution in kidney epithelial cells, and that these changes could be blocked by AT1 receptor blockade.\(^\text{14,15}\) In diabetic retinopathy, AngII also mediated the loss of tight junction proteins in retinal endothelial cells, an effect that could be blocked by angiotensin converting enzyme (ACE) inhibition.\(^\text{16}\) While the effects of AngII on tight junction proteins in the blood brain barrier (BBB) remain controversial, AngII was shown to increase permeability of the BBB via its action on AT1 receptor.\(^\text{17,18}\)

Hyperoxia has long been known to induce AEC apoptosis.\(^\text{19,20}\) Apoptosis occurs in the alveolar epithelia of the preterm infants neonatal lungs suffering from respiratory distress and receiving supplemental oxygen and mechanical ventilation,\(^\text{21,22}\) and in animal models of BPD.\(^\text{23,24}\) The autocrine angiotensin system is well known to contribute to AEC apoptosis, whereas the pro-apoptotic arm ACE/ AngII/AT1 is opposed by the protective ACE2/Ang1-7/Mas axis.\(^\text{25-28}\) In our data the Mas receptor activator AVE0991 restored the epithelial barrier integrity and inhibited the hyperoxia-induced AECs apoptosis (Figures 1 & 4). Whether apoptosis is the cause of tight junction loss, or antagonizing apoptosis caused the restoration of tight junction, and the exact mechanisms by which AVE0991 restores the epithelial barrier integrity will be the topics of future studies.

In summary, our study suggests the involvement of the angiotensin system in the pathogenesis of hyperoxia-induced pulmonary edema in BPD and reveals the potential for utilizing AVE0991 or mas receptor activation as a candidate therapy for BPD treatment.

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**Conflicts of interest**

The authors declare that there is no conflict of interest.

**References**

1. Deakins KM. Bronchopulmonary dysplasia. Respir Care. 2009;54(9):1252–1262.
2. Northway WH, Rosan RC, Porter DY. Pulmonary disease following respirator therapy of hyaline-membrane disease. Bronchopulmonary dysplasia. N Engl J Med. 1967;276(7):357–368.
3. Payne MS, Goss KCW, Connett GI, et al. Molecular microbiological characterization of preterm neonates at risk of bronchopulmonary dysplasia. Pediatr Res. 2010;67(4):412–418.
4. Coalson JJ. Pathology of Bronchopulmonary Dysplasia. Semin Perinatol. 2006;30(4):179–184.
5. Ratner V, Slinko S, Utikina-Sosunova I, et al. Hypoxic stress exacerbates hyperoxia-induced lung injury in a neonatal mouse model of bronchopulmonary dysplasia. Neonatology. 2009;95(4):299–305.
6. Day CL, Ryan RM. Bronchopulmonary dysplasia: new becomes old again? Pediatr Res. 2016;81(1-2):210–213.
7. WARE LB, MATTHAY MA. Alveolar Fluid Clearance Is Impaired in the Majority of Patients with Acute Lung Injury and the Acute Respiratory Distress Syndrome. Am J Respir Crit Care Med. 2001;163(6):1376–1383.
8. CRANDALL ED, MATTHAY MA. Alveolar Epithelial Transport. Am J Respir Crit Care Med. 2001;163(4):1021–1029.
9. Uhal BD, Rayford H, Zhuang J, et al. Apoptosis-dependent acute lung injury and repair after intratracheal instillation of noradrenaline in rats. Exp Physiol. 2003;88(2):269–275.
10. Zhuang J-L, Li X-P, Uhal BD, et al. Apoptosis-dependent acute pulmonary injury after intratracheal instillation of angiotensin II. Sheng Li Xue Bao. 2008;60(6):715–722.
11. Ota C, Gopallawa I, Ivanov V, et al. Protection of Meconium-Induced Lung Epithelial Injury by Protease Inhibitors. J Lung Pulm Respir Res. 2017;4(5).
12. Bernasconi R, Nyström A. Balance and circumstance: The renin angiotensin system in wound healing and fibrosis. Cellular Signalling. 2018;51:34–46.
13. Abdul-Hafez A, Mohamed T, Omar H, et al. The renin angiotensin system in liver and lung: impact and therapeutic potential in organ fibrosis. J Lung Pulm Respir Res. 2018;5(1):42–47.
14. Wang R, Zagaris A, Ibarra-Sunga O, et al. Angiotensin II induces apoptosis in human and rat alveolar epithelial cells. Am J Physiol. 1999;276(5 Pt 1):L885–9.
15. Uhal BD, Gidea C, Bargout R, et al. Captopril inhibits apoptosis in human lung epithelial cells: a potential antifibrotic mechanism. Am J Physiol. 1998;275(Pt 1):L1013–7.
16. Papp M, Li X, Zhuang J, et al. Angiotensin receptor subtype AT1 mediates alveolar epithelial cell apoptosis in response to ANG II. Am J Physiol Lung Cell Mol Physiol. 2002;284(4):L713–L718.
17. Molteni A, Heffelfinger S, Moulder JE, et al. Potential deployment of angiotensin I converting enzyme inhibitors and of angiotensin II type 1 and type 2 receptor blockers in cancer chemotherapy. Anticancer Agents Med Chem. 2006;6(5):451–460.
18. Wagenaar GTM, Laghmani EH, Fidder M, et al. Agonists of MAS oncogene and angiotensin II type 2 receptors attenuate cardiopulmonary disease in rats with neonatal hyperoxia-induced lung injury. Am J Physiol Lung Cell Mol Physiol. 2013;305(5):L341–L351.
19. Gopallawa I, Uhal BD. Angiotensin-(1-7)/mas inhibits apoptosis in alveolar epithelial cells through upregulation of MAP kinase phosphatase-2. Am J Physiol Lung Cell Mol Physiol. 2016;310(3):L240–L248.
20. Wiemer G, Dobrucki LW, Louka FR, et al. AVE 0991, a nonpeptide mimic of the effects of angiotensin-(1-7) on the endothelium. Hypertens (Dallas, Tex 1979). 2002;40(6):847–852.
21. Klein N, Gembardt F, Supé S, et al. Angiotensin-(1-7) protects from experimental acute lung injury. Crit Care Med. 2013;41(11):e334–43.
22. Rodrigues-Machado MG, Magalhães GS, Cardoso JA, et al. AVE 0991, a non-peptide mimic of angiotensin-(1-7) effects, attenuates pulmonary remodelling in a model of chronic asthma. Br J Pharmacol. 2013;170(4):835–846.
23. Li X, Zhang H, Soledad-Conrad V, et al. Bleomycin-induced apoptosis of alveolar epithelial cells requires angiotensin synthesis de novo. Am J Physiol Lung Cell Mol Physiol. 2003;284(3):L501–7.
24. Xu S, Xue X, You K, et al. Caveolin-1 regulates the expression of tight junction proteins during hyperoxia-induced pulmonary epithelial barrier breakdown. Respir Res. 2016;17(1):1–14.
25. Li C, Fu J, Liu H, et al. Hyperoxia arrests pulmonary development in newborn rats via disruption of endothelial tight junctions and downregulation of Cx40. Mol Med Rep. 2014;10(1):61-67.
26. You K, Xu X, Fu J, et al. Hyperoxia disrupts pulmonary epithelial barrier in newborn rats via the deterioration of occludin and ZO-1. Respir Res. 2012;13(1):1.
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27. Niessen CM. Tight junctions/adherens junctions: Basic structure and function. J Invest Dermatol. 2007;127(1):2525–2532.

28. González-Mariscal L, Bautista P, Lechuga S, et al. tight junction scaffold protein involved in the regulation of cell proliferation and apoptosis. Ann N Y Acad Sci. 2012;1257(1):133–141.

29. Beeman N, Webb PG. Baumgartner HK. Occludin is required for apoptosis when claudin-claudin interactions are disrupted. Cell Death Dis. 2012;3(2):e273–7.

30. Phyn CVC, Stelwagen K, Davis SR, et al. Tight Junction Protein Abundance and Apoptosis During Involution of Rat Mammary Glands. J Cell Physiol. 2017;232(8):2075–2082.

31. Bojarski C, Jörg Weiske, Torsten Schöneberg, et al. The specific fates of tight junction proteins in apoptotic epithelial cells. Journal of Cell Science. 2004;117(10):2097–2107.

32. Herrero R, Tanino M, Smith LS, et al. The Fas/FasL pathway impairs the alveolar fluid clearance in mouse lungs. Am J Physiol Cell Mol Physiol. 2013;305(5):L377-L388.

33. González-Mariscal L, Raya-Sandino A, González-González L, et al. Relationship between G proteins coupled receptors and tight junctions. Tissue Barriers. 2018;6(1):e1414015.

34. Macconi D, Abbate M, Morigi M, et al. Permeselective Dysfunction of Podocyte-Podocyte Contact upon Angiotensin II Unravels the Molecular Target for Renoprotective Intervention. Am J Pathol. 2005;168(4):1073–1085.

35. Rincon-Choles H, Vaslyeva TL, Pergola PE, et al. ZO-1 expression and phosphorylation in diabetic nephropathy. Diabetologia. 2006;55(4):894–900.

36. Kim JH, Kim JH, Yu YS, et al. Blockade of Angiotensin II Attenuates phosphorylation in diabetic nephropathy. J Physiol Lung Cell Mol Physiol. 2005;55(4):894–900.

37. Fleegal-DeMotta MA, Doghu S, Banks WA. Angiotensin II Modulates Retinopathy. Am J Physiol - Lung Cell Mol Physiol. 2008;295(1).

38. Wang R, Ramos C, Joshi I, et al. Human lung myofibroblast-derived inducers of alveolar epithelial apoptosis identified as angiotensin peptides. Am J Physiol. 1999;277(6 Pt 1):L1158–64.

39. Buccellato LJ, Tso M, Akincil Ol, et al. Reactive Oxygen Species Are Required for Hyperoxia-induced Bax Activation and Cell Death in Alveolar Epithelial Cells. J Biol Chem. 2004;279(8):6753–6760.

40. Barazzoni C, White CW. Mechanisms of Cell Injury and Death in Hyperoxia. Am J Respir Cell Mol Biol. 2000;22(5):517–519.

41. May M, Ströbel P, Preisshofer T, et al. Apoptosis and proliferation in lungs of ventilated and oxygen-treated preterm infants. Eur Respir J. 2004;23(1):113–121.

42. Lukkarinen HP, Laine J, Käläpää PO. Lung epithelial cells undergo apoptosis in neonatal respiratory distress syndrome. Pediatr Res. 2003;53(2):254–259.

43. Husari AW, Dbaibo GS, Bitar H, et al. Apoptosis and the activity of ceramide, Bax and Bcl-2 in the lungs of neonatal rats exposed to limited and prolonged hyperoxia. Respir Res. 2006;7(1):100.

44. Jin Y, Peng L-Q, Zhao A-L. Hyperoxia induces the apoptosis of alveolar epithelial cells and changes of pulmonary surfactant proteins. Eur Rev Med Pharmacol Sci. 2018;22(1):492–497.

45. Uhal BD, Li X, Xue A, et al. Regulation of alveolar epithelial cell survival by the ACE-2 / angiotensin 1 – 7 / Mas axis. Am J Physiol Lung Cell Mol Physiol. 2011;301(3):L269–74.

46. Oarhe CI, Dang V, Dang M, et al. Hyperoxia downregulates angiotensin-converting enzyme-2 in human fetal lung fibroblasts. Pediatr Res. 2015;77(5):656–662.

47. Li X, Molina-Molina M, Abdul-Hafez A, et al. Angiotensin converting enzyme-2 is protective but downregulated in human and experimental lung fibrosis. Am J Physiol - Lung Cell Mol Physiol. 2008;295(1).

48. Mohamed TL, Nguyen HT, Abdul-Hafez A, et al. Prior hypoxia prevents downregulation of ACE-2 by hyperoxia in fetal human lung fibroblasts. Exp Lung Res. 2016;42(3):121-130.

49. Li X, Molina-Molina M, Abdul-Hafez A, et al. Extravascular sources of lung angiotensin peptide synthesis in idiopathic pulmonary fibrosis. Am J Physiol Lung Cell Mol Physiol. 2006;291(5):L887-L895.