Superoxide mediates tight junction complex dissociation in cyclically stretched lung slices

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ABSTRACT

We found that stretching Type I rat alveolar epithelial cell (RAEC) monolayers at magnitudes that correspond to high tidal-volume mechanical ventilation results in the production of reactive oxygen species, including nitric oxide and superoxide. Scavenging superoxide with Tiron eliminated the stretch-induced increase in cell monolayer permeability, and similar results were reported for rats ventilated at large tidal volumes, suggesting that oxidative stress plays an important role in barrier impairment in ventilator-induced lung injury associated with large stretch and tidal volumes. In this communication we show that mechanisms that involve oxidative injury are also present in a novel precision cut lung slices (PCLS) model under identical mechanical loads. PCLSs from healthy rats were stretched cyclically to 37% change in surface area for 1 hour. Superoxide was visualized using MitoSOX. To evaluate functional relationships, in separate stretch studies superoxide was scavenged using Tiron or mito-Tempo. PCLS and RAEC permeability was assessed as tight junction (TJ) protein (occludin, claudin-4 and claudin-7) dissociation from zona occludins-1 (ZO-1) via co-immunoprecipitation and Western blot, after 1 h (PCLS) or 10 min (RAEC) of stretch. Superoxide was increased significantly in PCLS, and Tiron and mito-Tempo dramatically attenuated the response, preventing claudin-4 and claudin-7 dissociation from ZO-1. Using a novel PCLS model for ventilator-induced lung injury studies, we have shown that uniform, biaxial, cyclic stretch generates ROS in the slices, and that superoxide scavenging that can protect the lung tissue under stretch conditions. We conclude that PCLS offer a valuable platform for investigating antioxidant treatments to prevent ventilation-induced lung injury.

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1. Introduction

Mechanical ventilator-induced lung injury (VILI) occurs in 5% to 15% of all patients who require mechanical ventilation, or 200,000 annually in the US (Parker et al., 1993; Ware and Matthay, 2000; Johnson and Matthay, 2010), with a mortality rate of 34–60% in ventilated patients with adult respiratory distress syndrome (ARDS) (Haake et al., 1987). VILI is characterized by acute respiratory failure, alveolar cell dysfunction, and profound changes in barrier permeability (Wirtz and Dobbs, 1990; Parker et al., 1993; Lecuona et al., 1999; Waters et al., 1999; Dos Santos and Slutsky, 2000; Slutsky and Ranieri, 2000; Ware and Matthay, 2000; Ricard et al., 2003). Human and animal studies have demonstrated that VILI is associated with mechanical ventilation with high regional lung volumes (Egan, 1982; Kim and Crandall, 1982; Tsuno et al., 1991; Dreyfuss and Saumon, 1998), and may also be related to reopening of collapsed lung regions (Muscedere et al., 1994; Matthay et al., 2002). Consequently, the biomechanical environmental determinants of lung injury (inspired tidal volume, frequency, and use of positive end-expiratory pressure to ensure more uniform lung expansion) are central to designing injury mitigation strategies. ARDS results in diffuse pulmonary endothelial and epithelial damage, neutrophil accumulation, transudation of proteins into the interstitial and alveolar spaces and a loss of Type I pneumocytes (Ware and Matthay, 2000). Current management recommendations to limit acute lung injury in patients with ARDS include use of low tidal volumes (Brower et al., 2000; Brower and Rubenfeld, 2003) and fluid conservative protocols (Johnson and Matthay, 2010). There is a paucity of options available for reducing the morbidity and mortality during ventilation when small tidal volumes fail to achieve sufficient gas exchange. Consequently, in this communication we focus on mechanisms associated with altered lung barrier properties during moderate tidal volumes and stretch, to identify opportunities to intervene and ameliorate lung injury.

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The alveolar epithelium provides nearly all of the barrier properties to protein passage and over 90% of the resistance to the transport of nonpolar and charged solutes (Lubman et al., 1997), such that extra-alveolar fluid is excluded from the alveoli by the active and passive barrier properties of the alveolar epithelial lining (Bai et al., 1999; Ma et al., 2000; Borok and Verkman, 2002), even in the presence of impaired endothelial barrier properties and interstitial edema. The tight junctions (TJ) located between adjacent Type I epithelial cells are the primary barrier to paracellular transport (Mitic and Anderson, 1998). Occludin (Chen et al., 1997; Saitou et al., 1997) and claudins (4, 5, 7 and 18) are the principal TJ proteins regulating paracellular barrier resistance and charge selectivity in the Type I lung epithelium, as well as zona occludens (ZO-1, located between TJ proteins and cytoskeletal proteins) (Stevenson et al., 1989; Tsukamoto and Nigam, 1997; Denker and Nigam, 1998; Wang et al., 2003). TJ permeability is correlated with actin reorganization (Bacallao et al. 1994; Fanning, 2001), actin-bound pools of TJ proteins (Basuroy et al., 2006), and quantities of TJ proteins (Tsukamoto and Nigam, 1997; Balda and Matter, 2000; Fanning, 2001). Previously we reported stretch-magnitude dependent changes in TJ proteins, actin arrangement and barrier dysfunction in pulmonary epithelial monolayers (Cavanaugh et al., 2001, 2006; Cohen et al., 2008; Cohen et al., 2010; DiPaolo et al., 2010; Cohen et al., 2012; Davidovich et al., 2013; Dipaolo et al., 2013). We hypothesize that reactive oxygen species (ROS) mediate TJ rearrangement and barrier dysfunction during stretch.

Exposure of *unstretched* cells and tissues to ROS has been shown to increase permeability (Shasby et al., 1982; Chapman et al., 2002; Tasaka et al., 2008). In the lung, oxidative stress studies in unstretched cells are primarily performed with either cell lines or Type II alveolar epithelial cells. Recently we demonstrated the cyclic stretch of primary epithelial cells with Type I properties increased monolayer permeability, mediated via stretch-associated superoxide release (Davidovich et al., 2013). Although we have reported cell morbidity and mortality during "sighs" and sustained cyclic stretch of isolated primary Type I-like alveolar epithelial cells are comparable to intact in vivo lungs (Mitra et al., 2011; Tschumperlin and Margulies, 1998; Fisher and Margulies, 2002), when we co-cultured Type I and Type II RAECs, we reported interactions between resident cells, such that stretch-induced surfactant release was mediated by paracrine ATP signaling between Type I and Type II cells (Patel et al., 2005). This evidence suggests that our established monoculture preparation may not capture all elements of the intact lung milieu with its spectrum of cell types. In this communication, we determine if our previous results in monolayers relating stretch-induced ROS mediated increases in permeability are relevant to the intact lung using a novel preparation, cyclically stretched lung slices (Davidovich et al., 2013; Davidovich et al., 2013), and we hypothesize that like alveolar epithelial monolayers, stretch induces ROS release, which in turn potentiates tight junction protein dissociation.

2. Methods

All protocols for isolating rat alveolar epithelial cells (RAECs) and rat precision cut lung slices (PCLSs) were approved by the University Animal Care and Use Committee. Permeability of RAEC monolayers was evaluated with and without cyclic stretch to 37% change in surface area (%SA) for 10 min, and with and without superoxide scavenger Tiron or MitoTempo. At least 2 monolayers from every rat were evaluated for each experimental group, at least 3 rats per group. All remaining studies for ROS release and TJ protein association were performed in unstretched or stretched (37%ΔSA for 1 h) PCLSs, and with and without superoxide scavenger Tiron or MitoTempo. We used 2 or 3 slices per rat, and at least 6 rats per group. For each study, the number of rats or PCLSs per group (n) is provided.

2.1. Rat alveolar epithelial cell (RAEC) monolayer permeability with stretch

Previously, we used permeable deformable substrates to measure permeability across RAEC monolayers, and observed that large deformations within the physiological range increased tracer transport (0.15–0.55 nm) dramatically (Cavanaugh et al., 2006). Using the data from a spectrum of tracers we modeled the monolayer as a theoretical population of large and small radii. Unstretched monolayers were nearly entirely (99.9986%) composed of small pores (0.4 nm), with the remaining large pores (4.3 nm) occupying only 0.15% of the total transport area. After cyclic stretch to 37% ΔSA for 1 h, permeability to tracers of all sizes increased significantly, both the small and large theoretical pore radii increased (0.9 and 6.3 nm, respectively), the number of large pores increased 10-fold, and number of small pores decreased (Cavanaugh et al., 2006). To measure transport in cells cultured on impermeable silastic membranes we developed several novel methods (Cavanaugh and Margulies, 2002; Song et al., in press), and recently showed that our method whereby trans-monolayer transport of FITC-streptavidin (60 kD, likely through the expanded large pores) to bind at the biotinylated fibronectin-coated silastic membrane was more sensitive for measuring RAEC monolayer permeability than our previously published method utilizing BODIPY-ouabain binding to basolateral cell surfaces (Song et al., in press). Because interpretation of the BODIPY-ouabain method developed in our laboratory (Cavanaugh and Margulies, 2002) is complicated by reporter binding (as well as permeability) that is sensitive to stretch (Davidovich et al., 2013), in this communication, we also sought to confirm our previous report in which we found ROS mediated stretch-induced permeability increases in RAEC permeability (Davidovich et al., 2013). To confirm those previous findings using our superior method, we isolated Type II RAECs from Sprague Dawley rats (250–350 g, n=3–5) as previously described (Tschumperlin and Margulies, 1998; Tschumperlin et al., 2000), and cultured on plasma-treated biotinylated fibronectin-coated silastic membranes (3 wells per rat, per group) as reported previously (Song et al., in press) for 4 days, until they expressed Type I-specific TJ proteins and barrier properties (Cavanaugh et al., 2001; Oswari et al., 2001; Cavanaugh et al., 2006). Using our improved method to measure permeability, on the day of study, RAEC monolayers were designated as vehicle controls or treated with superoxide scavengers prior to stretch for 2 h after 1hr serum deprivation in DMEM+HEPES: 500 μM of mitoTempo (n=5, Santa Cruz Biotech, Dallas, USA) or 10 mM of Tiron (n=3) in DMEM+HEPES. The concentration of mitoTempo was derived from the literature (Nakahira et al., 2011; Iyer et al., 2013), and the concentration of Tiron was used by us previously and shown to completely reduce superoxide to unstretched levels (Davidovich et al., 2013). Vehicle control (VC; n=5) wells were provided 1 μl of DMSO in DMEM+HEPES. Using our custom-designed multi-well device which imparts equi-biaxial uniform stretch to cells seeded on flexible membranes, cyclic stretch of 37% ΔSA (17% engineering strain in radial and azimuthal directions) was applied to RAEC monolayers for 10 min at a rate of 0.25 Hz, equivalent to inflation to total lung capacity at a human-like respiratory rate (Tschumperlin and Margulies, 1998; Cohen et al., 2010). Previously, we reported changes in permeability at this stretch magnitude after only 10 min, and that these barrier disruptions were significantly mitigated with superoxide scavenger Tiron (Davidovich et al., 2013). During cyclic stretch, FITC-streptavidin (25 μg/ml in DMEM+HEPES; Invitrogen, Carlsbad, USA) was added, which bound to any exposed biotinylated fibronectin via intercellular breaches in the TJs. After stretch, each monolayer was washed with DMEM+HEPES three times, and three fluorescent fields were captured with a Nikon TE-300 inverted epifluorescence microcope. Monolayer permeability was evaluated from the proportion of the field that was above a FITC-positive threshold obtained from unstretched VC wells via a custom image analysis program, described elsewhere (Song et al., in press). Image acquisition settings remained constant for data from each rat. Data from the 3 image fields were averaged.

2.2. Precision cut lung slices (PCLSs) and mechanical stretch

Isolated lungs from Sprague Dawley rats (n=4–12) were inflated with a low-melting point agarose, and 250 μm thick slices which contained no major airways or blood vessels were cut using VT 1000S tissue slicer (Leica Microsystems, Buffalo Grove, USA), incubated floating in serum-free minimal essential medium (MEM) supplemented with 0.5 μl/ml gentamicin and 1 μl/ml amphotericin B and kept in a CO_2 incubator at 37 °C for at least 24 h prior to stretch. To remove residual agarose and cellular debris, the medium was changed every 30 min for first 2 h, every 1 h for the next 2 h, and every 24 h thereafter. Immediately before stretching, slices were stitched using a 5–0 non-absorbable silk suture in a star-shaped pattern onto a silastic membrane mounted in custom wells (Davidovich et al., 2013). PCLSs were stretched in the same device as the RAECs in a uniform biaxial manner to 37% ΔSA for 1hr at a frequency of 0.25 Hz. As with RAECs, prior to stretch, each PCLS was designated as a vehicle control (VC), or treated with superoxide scavenger mito-Tempo or Tiron for 2 h after serum deprivation. Medium was collected for ROS release, PCLSs were used to image ROS or TJ proteins, or were homogenized for Western blots or immunoprecipitation (IP), all described below.