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RAGE-induced changes in the proteome of alveolar epithelial cells

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ABSTRACT

The receptor for advanced glycation end-products (RAGE) is a pattern recognition receptor and member of the immunoglobulin superfamily. RAGE is constitutively expressed in the distal lung where it co-localizes with the alveolar epithelium; RAGE expression is otherwise minimal or absent, except with disease. This suggests RAGE plays a role in lung physiology and pathology. We used proteomics to identify and characterize the effects of RAGE on rat alveolar epithelial (R3/1) cells. LC-MS/MS identified 177 differentially expressed proteins and the PANTHER Classification System further segregated proteins. Proteins involved in gene transcription (RNA and mRNA splicing, mRNA processing) and transport (protein, intracellular protein) were overrepresented; genes involved in a response to stimulus were underrepresented. Immune system processes and response to stimuli were downregulated with RAGE knockdown. Western blot confirmed RAGE-dependent changes in protein expression for NFκB and NLRP3 that was functionally supported by a reduction in IL-1β and phosphorylated p65. We also assessed RAGE's effect on redox regulation and report that RAGE knockdown attenuated oxidant production, decreased protein oxidation, and increased reduced thiol pools. Collectively the data suggest that RAGE is a critical regulator of epithelial cell response and has implications for our understanding of lung disease, specifically acute lung injury.

Significance statement: In the present study, we undertook the first proteomic evaluation of RAGE-dependent processes in alveolar epithelial cells. The alveolar epithelium is a primary target during acute lung injury, and our data support a role for RAGE in gene transcription, protein transport, and response to stimuli. More over our data suggest that RAGE is a critical driver of redox regulation in the alveolar epithelium. The conclusions of the present work assist to unravel the molecular events that underlie the function of RAGE in alveolar epithelial cells and have implications for our understanding of RAGE signaling during lung injury. Our study was the first proteomic comparison showing the effects of RAGE activation from alveolar epithelial cells that constitutively express RAGE and these results can affect a wide field of lung biology, pulmonary therapeutics, and proteomics.

1. Introduction

The receptor for advanced glycation end-products (RAGE) is a pattern recognition receptor and member of the immunoglobulin superfamily whose function is to amplify and perpetuate the inflammatory response [1]. Much of our understanding of RAGE occurs within the context of diseases such as Diabetes Mellitus and Alzheimer's disease as RAGE is a known contributor to their pathogenesis. In humans, RAGE expression is notably downregulated in the absence of disease, except along the basolateral surface of the alveolar epithelium where it predominantly co-localizes with the alveolar epithelial type 1 (T1) cell [2,3]. T1 cells are large, flat squamous epithelial cells that cover 95%–98% of the alveolar surface area. T1 cells are a crucial barrier between the air filled alveolus and the blood filled capillary, and T1 cells contain a full complement of ion channels, pumps, and pores that regulate ion and water composition required for optimal gas-exchange [4]. This unique expression pattern suggests that RAGE may play an important, albeit unclear, role in the lung.

The active form of RAGE is a ~55 kDa transmembrane protein composed of a variable domain for ligand binding, two constant domains, a transmembrane domain, and a short cytoplasmic tail that is crucial for cell signaling [5]. Splice variants typically produce truncated forms of RAGE (of various sizes) that are either secreted into the extracellular space (sRAGE) or inserted into the plasma membrane (tRAGE). Both sRAGE and tRAGE serve as a decoy and reduce RAGE signaling. RAGE is a promiscuous receptor with many known ligands

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and as such RAGE functions along an axis where outcomes range from cell proliferation and regeneration to profound inflammation and injury.

Redox regulation, changes in oxidant balance that promote reversible modifications to proteins, is controlled through oxidant production, a balance between redox pairs, reduced thiol pools and reductases [6]. Redox regulation is crucial for cell signaling, and a disruption in redox regulation coupled with overwhelming oxidant production results in oxidative stress. Oxidative stress culminates in a loss cell in function and is a significant contributor to lung pathology. Targeting pathways that regulate oxidative stress during disease offer potential therapeutic points for intervention.

Acute respiratory distress syndrome (ARDS) is a severe form of lung injury that affects the alveolar epithelium, and ARDS is characterized by profound inflammation, pulmonary edema, and oxidative stress that culminate in respiratory failure [7–9]. Data from multiple studies converge to show that RAGE signaling is likely an important contributor to lung homeostasis and disease pathogenesis, particularly with lung fluid balance and inflammation [2,3,5,10–16]. For example, we have shown that RAGE regulates lung fluid balance, and that higher levels of sRAGE correlate with increased volumes of epithelial ling fluid in humans [17]. Thus, suggesting that RAGE is a contributing pathway in the regulation of lung fluid balance. Furthermore studies describe inflammatory cytokine production through NFκB and NLRP3 signaling cascades following RAGE binding [18–21]. However, the role of RAGE in the alveolar epithelium, the site of highest constitutive expression, has not been conclusively shown.

Our objective was to obtain insight into the role of RAGE in the alveolar epithelium by using proteomics coupled with complementary biochemical and molecular studies to support identified functions. We identified 177 differentially expressed proteins that were further segregated using PANTHER classification system from which we report that RAGE contributes to alveolar epithelium's response to stimuli. Using western blotting and ELISAs we found RAGE knockdown attenuated NF-κB and NLRP3 with an accompanying decrease in IL-1β, while decreasing protein ubiquitination. RAGE was also assessed for its role in redox regulation. RAGE knockdown decreased oxidant production, decreased agonist-derived protein oxidation, increased reduced (free) thiol pools, and attenuated levels of the disulfide reductase, Thioredoxin-1. Collectively, these data suggest that RAGE plays an important role the alveolar epithelium's response during the evolution and resolution of lung injury. Moreover, our cellular and molecular biological studies validate important new findings obtained via high throughput mass spectrometry analysis of alveolar cells.

2. Materials and methods

2.1. Reagents

Unless stated otherwise all reagents were obtained from Sigma-Millipore (Burlington, MA).

2.2. Cell culture and siRNA

Rat alveolar epithelial (R3/1) cells were grown in 50:50 DMEM/Hams F12 media supplemented with 10% FBS and Penicillin/Streptomycin in a humidified 5% CO_2 chamber. At 30% confluence, cells were transfected with 5pmole RAGE or scramble sequence siRNA (Santa Cruz, Dallas, TX) according to the manufacturer's protocol. 48 h after transfection, cells were treated with 2 μL/mL advanced glycation end-products (AGEs) for 30 min. Confirmation of RAGE knockdown was determined through RAGE mRNA and protein expression.

2.3. ROS measurements

For superoxide generation, R3/1 cells were seeded to 96 well plates subjected to RAGE knockdown and then treated with AGEs (2 μL/mL) for 30 min, followed by labeling with dihydroethedium (DHE, Thermo Fisher Scientific, San Jose, CA) (5 μM for 30 min at 37 °C in 5%CO_2) before being rinsed with PBS and fluorescence intensity (Ex 510, Em 615) measured using a Tecan plate reader (Basil, Switzerland). For H_2O_2 measures, the cells were seeded to 6 well plates and subjected to knock down as described above ± AGEs and supernatants removed and assayed using Amplex Red (Thermo Fischer) per the manufacturer's recommendations.

2.4. ELISA

Cleaved IL1-β concentrations were quantified from cell lysates using the IL1-β kit from R&D systems (Minneapolis, MN) per the manufacturer's recommendations. Absorbance measures were taken using a Tecan plate reader at 450 nm.

2.5. Protein biochemistry

Following treatment, R3/1 cells were rinsed with ice-cold PBS and then lysed in RIPA buffer containing 1× protease and phosphatase inhibitors (Calbiochem, Burlington, MA). R3/1 cell lysates were then electrophoresed on acrylamide gels (8%, 12%, or 15% depending on molecular weight of target) and transferred to Protran nitrocellulose membranes (Scheicher Schuell) for Western blot analysis. Membranes were incubated with either goat polyclonal anti-RAGE (Santa Cruz, sc-8229, 1:1000), anti NFκB (Abcam, ab31481, 1:1000), anti-NLRP3 (Nova Biochemi, NBP212446, 1:1000), anti-phopho-p65 (Cell Signaling, 93H1, 1:1000) or anti-ubiquitin (Cell Signaling, 39365, 1:1000) overnight. A horseradish peroxidase conjugated secondary antibody (1:20,000) was applied and incubated for 1 h at room temperature. A Chemiluminescent signal was detected using Supersignal West Dura (Thermo Fischer Scientific) and exposed using a UVP chemiluminescent imaging station and compatible software.

2.6. PCR

Total RNA was extracted using an RNeasy isolation kit (Qiagen, Grand Island, NY) following the protocol of the manufacturer, and then treated with DNaseI and reverse-transcribed using Superscript II RNaseH-reverse transcriptase (Thermo Fischer Scientific). The level of RAGE mRNA expression was determined using the following primers: RAGE (forward) ACT ACC GAG TCC GAG TCT ACC, RAGE (reverse) GTA GCT TCC CTC AGA CAC ACA. Threshold levels of mRNA expression (ΔΔCt) were normalized to rat GAPDH levels, and values represent the mean of triplicate samples ± S.E. Data are representative of 3 independent studies.

2.7. OxyBlot

Detection of carbonyl groups in proteins can be used to quantify oxidative modification of proteins. The protein carbonyl contents in R3/1 cells following RAGE knockdown were detected by the OxyBlot protein oxidation kit (Thermo Fischer Scientific) per the manufacturer's instructions. To derivatize the carbonyl group for Western blot detection, 10 μg protein was diluted with 12% SDS for a final concentration of 6% SDS, followed by the addition of 2 volumes of 20 nM 2,4-DNPH (dintrophenylhydrazine) in 10% trifluoracetic acid. The mixture was incubated at room temperature for 20 min, and a neutralization solution (1.5 volumes) from the OxyBlot kit was added to stop the reaction. Non-derivatized controls were run for each sample. A total of 10 μg protein from each sample was loaded in a 4–20% gradient gel, and SDS-PAGE and western blotting were performed according to the manufacturer's instructions. Rabbit polyclonal antibody raised against DNP (dinitrophenylhydrazone) was used at 1:150 dilution followed by an HRP-conjugated secondary antibody (1:300). Chemiluminescent signal