HHS Public Access

Author manuscript

J Cell Biochem. Author manuscript; available in PMC 2024 December 11.

Published in final edited form as:

J Cell Biochem. 2018 January ; 119(1): 599–606. doi:10.1002/jcb.26219.

Hydrogen peroxide stimulates exosomal cathepsin B regulation of the receptor for advanced glycation end-products (RAGE)

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Abstract

Exosomes are nano-sized vesicles that are secreted into the extracellular environment. These vesicles contain various biological effector molecules that can regulate intracellular signaling pathways in recipient cells. The aim of this study was to examine a correlation between exosomal cathepsin B activity and the receptor for advanced glycation end-products (RAGE). Type 1 alveolar epithelial (R3/1) cells were treated with or without hydrogen peroxide and exosomes isolated from the cell conditioned media were characterized by NanoSight analysis. Lipidomic and proteomic analysis showed exosomes released from R3/1 cells exposed to oxidative stress induced by hydrogen peroxide or vehicle differ in their lipid and protein content, respectively. Cathepsin B activity was detected in exosomes isolated from hydrogen peroxide treated cells. The mRNA and protein expression of RAGE increased in cultured R3/1 cells treated with exosomes containing active cathepsin B while depletion of exosomal cathepsin B attenuated RAGE mRNA and protein expression. These results suggest exosomal cathepsin B regulates RAGE in type 1 alveolar cells under conditions of oxidative stress.

Keywords

exosomes; lipidomics; proteomics

Exosomes, membrane-bound vesicles of 30–150nm in diameter that are released into the extracellular space after fusion with the plasma membrane, are present in most biological fluids [Zeringer et al., 2015]. Exosomes serve as vehicles for cell-to-cell communication, notably spanning a wide range of distances, in which cargo (e.g. nucleic acids, proteins,

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prions, etc.…) modulate recipient cells [Jella et al., 2016]. The composition of the exosome varies depending upon cell type and context, and multiple studies converge to illustrate that exosomes affect cell function [Lang et al., 2017; Menay et al., 2017; Wang et al., 2017; Zhang et al., 2017]. The role of exosomes in health and disease is just beginning to be better understood.

Exosomes may be formed in response to a variety of stimuli, including infectious agents and oxidants. Oxidant production is elevated in the lungs of individuals with a variety of lung diseases such as chronic obstructive pulmonary disease, pneumonia, and acute respiratory distress syndrome, illustrating the importance of oxidants, and oxidative stress, in the evolution of these diseases [Liang et al., 2012; Rahman, 2005]. Exosomes may play an important role in lung disease and serve as a communication conduit between distal and proximal lung epithelium. Indeed, exosomes may play a crucial role in alerting up- or down-stream cells of regional injury, particularly to prime distant cells for a potential immunological response.

The receptor for advance glycation end-products (RAGE) is a pattern recognition receptor and member of the immunoglobulin superfamily that functions to amplify and perpetuate the inflammatory response [Reynolds et al., 2008; Schmidt et al., 2000]. RAGE is uniquely and abundantly expressed along the basolateral surface of the alveolar epithelium, localizing with differentiated alveolar type I cells [Shirasawa et al., 2004; Uchida et al., 2006]. RAGE is also expressed in differentiating alveolar type-II epithelial cells, bronchial smooth muscle cells, vascular endothelial cells, and pulmonary macrophages [Downs et al., 2015]. Deregulation of RAGE in lung tissue has been observed in pulmonary fibrosis, pneumonia, asthma, chronic obstructive pulmonary disease, acute respiratory distress syndrome, and non-small cell lung adenocarcinoma suggesting that RAGE may play a role in the pathogenesis of these diseases [Frank et al., 2007; Liliensiek et al., 2004; Lutterloh et al., 2007; Schmidt et al., 2000; Shirasawa et al., 2004; Su et al., 2009]. The effect of exosomes on RAGE expression in the lung is not known.

Cathepsin B is a constitutively expressed lysosomal cysteine protease that is involved with protein turnover in lysosomes. Cathepsin B activity is not limited to within the cell and this protease was found extracellularly where it was shown to cleave and activate the epithelial sodium channel (ENaC) [Alli et al., 2012]. Expression patterns of proteases underlie numerous pathological processes, including arthritis, inflammatory processes, and cardiovascular diseases [Ketterer et al., 2016; Weiss-Sadan et al., 2017]. In the current study we observe increased cathepsin B expression in oxidative-stress induced exosomes that promote RAGE expression in rat alveolar epithelial (R3/1) cells. However, the effect of these exosomes and their cargo on alveolar epithelial cell function is unknown, in with RAGE expression in particular. In addition, exosomes can manipulate lipids such as fatty acids, sphingomyelin, cholesterol, and thus the lipid contents of exosomes could be useful biomarkers of lipid related disease. Therefore, in the current study we sought to characterize exosomes generated by alveolar epithelial cells in response to treatment with H_2O_2 and to determine their effect on RAGE expression and further on the changes of lipid composition in exosomes treated with hydrogen peroxide.

Materials and Methods

Isolation of nano-sized vesicles from conditioned media.

Exosomes were isolated from conditioned media as described by [Jella et al., 2016] with the following modifications. The conditioned media from R3/1 cells cultured in tissue culture flasks and treated with vehicle or hydrogen peroxide was collected every other day for up to two weeks. For each of the two groups, the conditioned media was pooled. Dead cells and debris were removed from the conditioned media by centrifugation at 1,000 X g for 10 minutes. The supernatant was filtered through 0.22 μm Nalgene filters and then centrifuged at 10,000 X g for 30 minutes. A fixed-angle rotor Ti-70 (Beckman Coulter, Inc., CA) was used for ultracentrifugation of the supernatant at 118,000 X g for 70 minutes at 4°C. This step was repeated after washing the pellet with 1X phosphate buffered saline (PBS). The nano-sized vesicles were resuspended in 1X PBS and stored at −80°C.

NanoSight analysis.

Exosomes were analyzed by size distribution and concentration using a NanoSight NS300 instrument (NanoSight Ltd, Minton Park UK). A 1:1000 dilution of the reconstituted vesicles were visualized by laser light scattering and tracked as the particles moved in Brownian motion.

Protein identification by LC-MS/MS.

Exosomes were lysed in RIPA buffer (Thermo Scientific) and total protein from exosomal lysates were resolved on SDS-PAGE gels. The gels were stained with Colloidal Coomassie blue stain and individual protein bands were excised, destained, and treated with DTT and iodoacetamide. In-gel tryptic digestion was performed and the peptides were concentrated by vacuum centrifugation. Peptides were solubilized in solvent A (0.1% formic acid and 3% acetonitrile), and analyzed using an Ultimate 3000 nanoflow Ultra Performance LC system coupled to a Q-Exactive Orbitrap Plus MS (Thermo Fisher Scientific, Bremen, Germany) according to Mostafa et al., 2016 with minor modifications: The mobile phase gradient was ramped from 2–30% of solvent B (0.1% formic acid and 99.9% acetonitrile) in 114 min, then to 98 % of solvent B in 6 min and maintained for 24 min. Mass analysis was performed in positive ion mode with high collision dissociation energy. The scan range was 350–1800 m/z with full MS resolution of 70000 and 200–2000 m/z with MS2 resolution of 17500, and 445.12003 m/z (polysiloxane ion mass) was used for real-time mass calibration.

The MS data were searched using Mascot searches performed against the Swiss-Prot mouse and rat databases. The searching parameters were set to 300 and 5000 Da as minimum and maximum precursor mass filters, digestion with trypsin with two missed cleavages. Carbamidomethylation of cysteine was set as a static modification and methionine oxidation were set as a dynamic modification. Precursor mass tolerance was 10 ppm, fragment mass tolerance was 0.01 Da, and false discovery rate was 0.01 at the peptide level.

Cell culture and exosome treatment.

Rat alveolar epithelial cells (R3/1) were grown in 50:50 DMEM/Hams F12 media supplemented with 10% FBS and Penicillin/Stretpomycin in a humidified 5% CO_2 chamber.

Cells were seeded (0.5×10^6) to wells of 35mm, 6-well plates and treated with previously isolated exosomes (1μL/2mL) for up to 3 days. Cell culture media was changed every other day.

Depletion of exosomal cathepsin B.

Exo-Fect reagent (System Biosciences; Palo Alto, CA) was used to load the pharmacological inhibitor of cathepsin B, CA074 (Sigma Aldrich; St. Louis, MO) into exosomes isolated from H_2O_2 treated R3/1cells according to the manufactures instructions.

Cathepsin B fluoremetric assay.

Cathepsin B activity was determined by the fluorometric cathepsin B activity assay (Abam). Briefly, exosomes isolated from vehicle treated or H_2O_2 treated R3/1cells were resuspended in chilled lysis buffer before being incubated on ice for 20 minutes. The BCA (Thermo Fisher Scientific) assay was used to determine protein concentration. Next, 50 μg of exosomal protein made up to a total volume of 50 μl in lysis buffer was added to individual wells of a 96 well clear bottom black plate. After adding 50 μl of reaction buffer per well, 2 μL of 10 mM CB Substrate Ac-RR-AFC was added to each well. The plate was incubated at 37°C for 2 hours in the dark before reading the plate at Ex/Em = 400/505 nm.

Protein biochemistry.

Following exosome treatment, R3/1 cells were rinsed with ice-cold PBS and then lysed in RIPA buffer supplemented with 1X protease and phosphatase inhibitors (Calbiochem). R3/1 lysate was then electrophoresed on 7.5% acrylamide gels and transferred to Protran nitrocellulose membranes (Scheicher Schuell) for Western blot analysis. Membranes were incubated with goat polyclonal anti-RAGE (Santa Cruz, sc-8229) at 1:1000 overnight. For the peptide competition (Santa Cruz, sc-8229P) a 10X concentration of peptide: antibody was used. A horseradish-peroxidase conjugated secondary antibody (1:20,000) was applied and incubated for an additional hour at room temperature. Chemiluminescent signal was detected using Supersignal West Dura (Thermo Scientific) and exposed using a UVP chemiluminescent imaging station and compatible software.

Real-time PCR.

Total RNA was extracted from R3/1 cells using an RNeasy isolation kit (Qiagen) following the protocol of the manufacturer. RNA was then treated with DNaseI and reverse-transcribed using Superscript II RNaseH-reverse transcriptase (Invitrogen). 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.

Lipid extraction.

All extractions were performed in 10 mL glass screw capped tubes containing about 1–8e+011 exosomes in 5 μL buffer and 1 mL H_2O, following the Bligh and Dyer method

[Bligh and Dyer, 1959]. In brief, 2.9 mL of a mixture of methanol:dichloromethane (2:0.9 v/v) was added to the tubes followed by vortexing for 30 sec. Five μL of SPLASH Lipidomix internal standards (Avanti, Alabaster AL) including d7-phosphatidylchloline (PC) (15:0/18:1); d7-phosphatidylethanolamine (PE) (15:0/18:1); phosphatidylserine (PS) (15:0/18:1); d7-phosphatidylglycerol (PG) (15:0/18:1); d7-phosphatidylinositol (PI) (15:0/18:1); d7-phosphatidic acid (PA) (15:0/18:1); lysophosphatidylchloline (LPC) (18:1); lysophosphatidylethanolamine (LPE) (18:1); d7-cholesterol ester (CE) (18:1); d7-monoacylglycerol (MAG) (18:1); d7-diacylglycerol (DAG) (18:1); d7-triacylglycerol (TAG) (18:1); d9-sphingomyelin (SM) (18:1/18:1); and d7-cholesterol was added into the samples. The mixture was again vortexed and incubated for 30 min at room temperature. Afterwards, 1 mL H_2O and 900 μL of dichloromethane were added to each sample. The samples were gently inverted 10 times and then centrifuged at 1200 rpm for 10 min. The lower phase (dichloromethane) was collected, concentrated to dryness under a N_2 stream and reconstituted into 200 μL of methanol:dichloromethane (1:1 v/v) containing 10 mM ammonium acetate.

Sample analysis.

Lipid extracts were analyzed on the QTRAP 6500 MS/MS mass spectrometer (ABSCIEX, Redwood Shores, CA) using flow infusion. Fifty μL lipid extracts were injected into the mass spectrometer and semi-targeted scans were used to focus on the different phospholipid classes including precursor ion scan (PIS) and neutral loss scan (NL) modes. Different scan and ionization modes were used to monitor all six classes of phospholipids (PA, PS, PG, PI, PC, and PE) in a multiplexed fashion. The sphingolipid (Cer and SM) and glyceride classes (MAG, DAG, and TAG) were also included in the analysis. Lipid profile data were analyzed using LipidView software v1.3 ABSCIEX). Data were normalized to internal standards and then presented as percentage composition (relative amounts of different lipid classes within a sample). Values were mean of three replicates and differences in relative amounts for each lipid class in different groups were analyzed for statistical significance using one-way analysis of variance (ANOVA) using Graphpad Prism 5.0. Tukey post-hoc t test was used to determine the significance ($p<0.05$).

Results

Rat alveolar epithelial (R3/1) cells were treated with or without H_2O_2 in cell culture media and then exosomes were isolated from the conditioned media (Figure 1). The majority of the nano-sized particles present in the preparation were exosomes (30–150nm in in diameter). The concentration of these nanosized particles isolated from the H_2O_2 treated cells was 1.31e+012 +/− 2.68e+010 particles/ml while the concentration for nanosized particles isolated from the vehicle treated cells was 7.85e+011 +/− 9.87e+010 particles/ml. In addition to H_2O_2 treatment resulting in an increase in exosome production, it also resulted in an increase in exosome size. Exosomes isolated from the conditioned media of cells treated with H_2O_2 was 160.1 nm+/−68.5 nm while exosomes isolated the conditioned media of cells treated with vehicle was 125.1 nm+/−47.5 nm (Figure 1).

The lipid composition of exosomes was assessed using mass spectrometry as described above and data are presented as a percentage of the total lipid profile for each treatment (Figure 2). No differences were detected in the fatty acid composition between the two groups (Figure 2B). However, we observed a 40% increase in sphingolipid (SM) and a 40% reduction in select phospholipids (PC and PI) and for triacylglycerides (TAG) concentrations in H_2O_2 treated exosomes compared to controls.

In order to identify proteins present in exosomes isolated from cells treated with H_2O_2 compared to control exosomes, we performed mass spectrometry. The surrogate peptides representing unique peptides sequences for cathepsin B that were identified within exosomes from H_2O_2 treated cells but not the control exosomes are show in Figure 3.

Mass spectrometry identified cathepsin B as a differentially expressed cargo protein in the exosomes formed by the H_2O_2 treated R3/1 cells. We performed a cathepsin B fluoremetric assay to assess cathepsin B activity (Figure 4). Cathepsin B was active in control and H_2O_2 treatment conditions; however, activity was doubled in H_2O_2-generated exosomes.

Because data from multiple studies converge to illustrate the importance of RAGE expression in the pathogenesis of lung disease, we assayed epithelial cells treated with either control or H_2O_2-generated exosomes and evaluated RAGE expression (Figure 5). RAGE mRNA levels were increased after 1 day of treatment with H_2O_2-generated exosomes in alveolar epithelial cells (Figure 5A). In Figure 4B, a representative immunoblot for RAGE with a peptide competition is provided to illustrate the ~55kDa band. We also assayed for RAGE protein expression, and observed a similar effect with RAGE protein levels being elevated after 1 day of treatment with H_2O_2-generated exosomes (Figure 5C).

Mass spectrometry identified cathepsin B to be differentially expressed in the H_2O_2-generated exosomes. The activity of cathepsin B was inhibited from the H_2O_2-generated exosomes, and the cathepsin B depleted exosomes were then used to treat the alveolar epithelial cells. RAGE mRNA and protein expression were subsequently determined. Depletion of cathepsin B attenuated RAGE mRNA (Figure 6A) and protein expression (Figure 6B), suggesting that cathepsin B plays a role in RAGE expression.

Discussion

Although exosome formation is well understood, the effect of exosome cargo on recipient cell types has not been interrogated in detail. Exosome cargo and their effects on target cells is of particular interest to the study of lung diseases as exosomes formed in distal portions of the lung may affect cell function in proximal airways and vice versa. For example, exosomes formed during a bout of pneumonia may transfer critical information to more proximal airways that could exert a physiological response. In turn, these effects may influence the disease state, and ultimately, outcomes.

In the current study we report that exosomes formed in response to oxidative stress, modeled by repeated exposure to H_2O_2, generated higher concentrations of exosomes. The lipidomic profiles demonstrate that H_2O_2-generated exosomes possessed greater concentrations of sphingolipids and lower concentrations of select phospholipids such as

phosphatidylcholines (PC). Studies show that sphingolipids are important for intracellular signaling, particularly in promoting cell differentiation, proliferation and apoptosis [D'Auria and Bongarzone, 2016; Podbielska et al., 2016; Russo et al., 2016; Ueda, 2017]. In short, higher concentrations of sphingolipids may provide a "survival" or protective signaling to epithelia under conditions of oxidative stress; ceramide, the backbone of sphingolipids, has been shown to promote differentiation, proliferation, and apoptosis [Podbielska et al., 2016]. The notion that exosomes can transport protective messages under harsh conditions has been shown, specifically others have shown that exosomes generated in response to H_2O_2 communicate protective messages in mice mast cell (MC/9) [Eldh et al., 2010].

In the current study, mass spectrometry determined cathepsin B to be differentially expressed in H_2O_2-generated exosomes. Cathepsin B is highly relevant to the lung as studies show that cytokines regulate expression of cathepsins [Gerber et al., 2000], and inhibition of cathepsin B in particular, reduced lung inflammation and fibrosis in rats [Zhang et al., 2015]. Cathepsin B is a lysosomal cysteine protease that is structurally similar to the papaya enzyme papain [Rawlings et al., 2014], and cathepsin B has been observed in numerous lung diseases. Cathepsin B is a promiscuous protease with many functions including the proteolysis of extracellular matrix components and affecting intercellular communication to participate in autophagy and cannibalism [Aggarwal and Sloane, 2014]. However, a comprehensive understanding of all of cathepsin B's functions is lacking; this is complicated by redundancies with numerous other cathepsins.

In this study we provide evidence that cathepsin B generated under conditions of oxidative stress increases RAGE expression. RAGE functions along an axis where a little is believed to be beneficial and too much RAGE signaling would be detrimental [Schmidt et al., 2000]. Clinically, RAGE expression has been linked to a variety of pulmonary diseases. For example, altered RAGE expression has been reported in pulmonary fibrosis [Ding et al., 2015], acute lung injury, and chronic obstructive pulmonary disease [Hunt et al., 2016; Jabaudon et al., 2015; Shirasawa et al., 2004; Uchida et al., 2006]. Like cathepsin B, RAGE plays a central role in cell proliferation, angiogenesis, inflammation and metastasis. However, exposure to a RAGE ligand, such as advanced glycation end products has been shown to promote further RAGE expression; the net effect is to prime the affected tissue for a sustained inflammatory response upon presentation of a RAGE ligand [Reynolds et al., 2008]. Our data allude to a link between cathepsin B and RAGE during oxidative stress, which suggests that cathepsin B may be an interesting target for lung diseases, particularly those disease processes with concurrently elevated RAGE expression and activity—e.g. increased interleukin 1β expression.

Inflammation and oxidative stress promote exosome formation. Although inflammation and oxidative stress have been extensively characterized and described in the lung [Downs et al., 2013; Rahman, 2005; Zinellu et al., 2016], far less is understood regarding how exosomes impact function of the pulmonary epithelium. Here, we described the effect of H_2O_2-generated exosomes on RAGE expression in alveolar epithelial cells. Our data suggest that RAGE expression is regulated through enhanced exosomal cathepsin B expression. These findings warrant further investigation into cathepsin B and exosomal regulation of RAGE

and to determine whether therapeutic strategies such as cathepsin B inhibition can be used to block these effects under pathological conditions.

Acknowledgments.

The authors thank the ICBR Proteomics and Mass Spectrometry (PM) Core at the University of Florida, Gainesville FL for mass spectrometry services.

Contract grant sponsor: Parker B. Francis Foundation Fellowship; awarded CAD

Contract grant sponsor: NIH K01 DK099617; awarded AAA

Contract grant sponsor: NIH Shared Instrumentation Grant #1S10OD018141; awarded ND

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Bullet points:

1. H_2O_2 treatment generates exosomes with unique lipid profile
2. H_2O_2 treatment generates exosomes with increased cathepsin B
3. Cathepsin B rich exosomes promote RAGE expression in alveolar epithelial cells

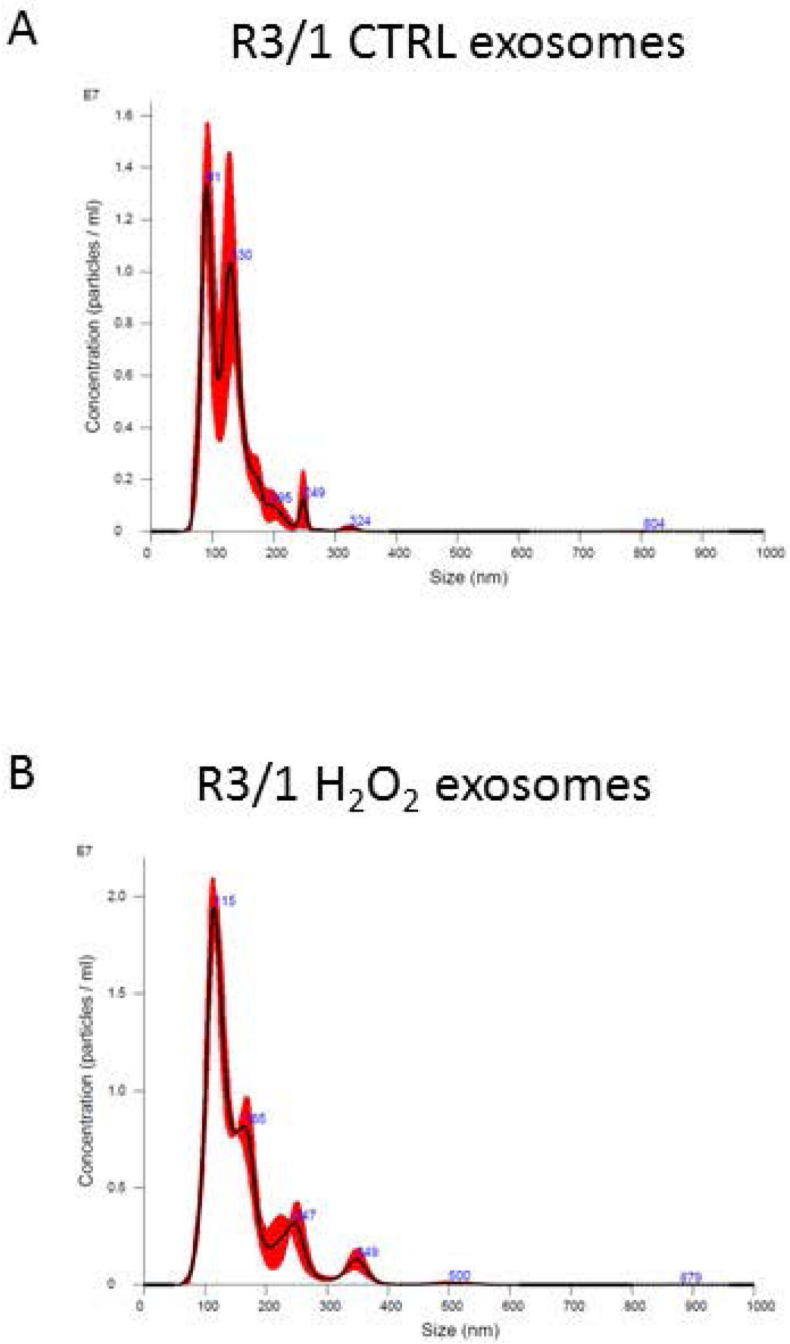

Figure 1.

Nanoparticle Tracking Analysis (NTA) of the size and concentration of exosomes isolated from R3/1 cells treated with or without H_2O_2. **A.** The concentration of exosomes isolated from 40mL of conditioned media collected from cells treated with vehicle alone (complete growth media) was 7.85e+011 +/− 9.87e+010 particles/ml. These exosomes were 125.1 nm+/−47.5 nm in diameter. **B.** The concentration of exosomes isolated from 40mL of conditioned media collected from cells treated with H_2O_2 in complete growth media was

1.31e+012 +/− 2.68e+010 particles/ml. These exosomes were 160.1+/−68.5 nm in diameter. Samples were processed from 3 × 60 second videos and analyzed using the NTA software.

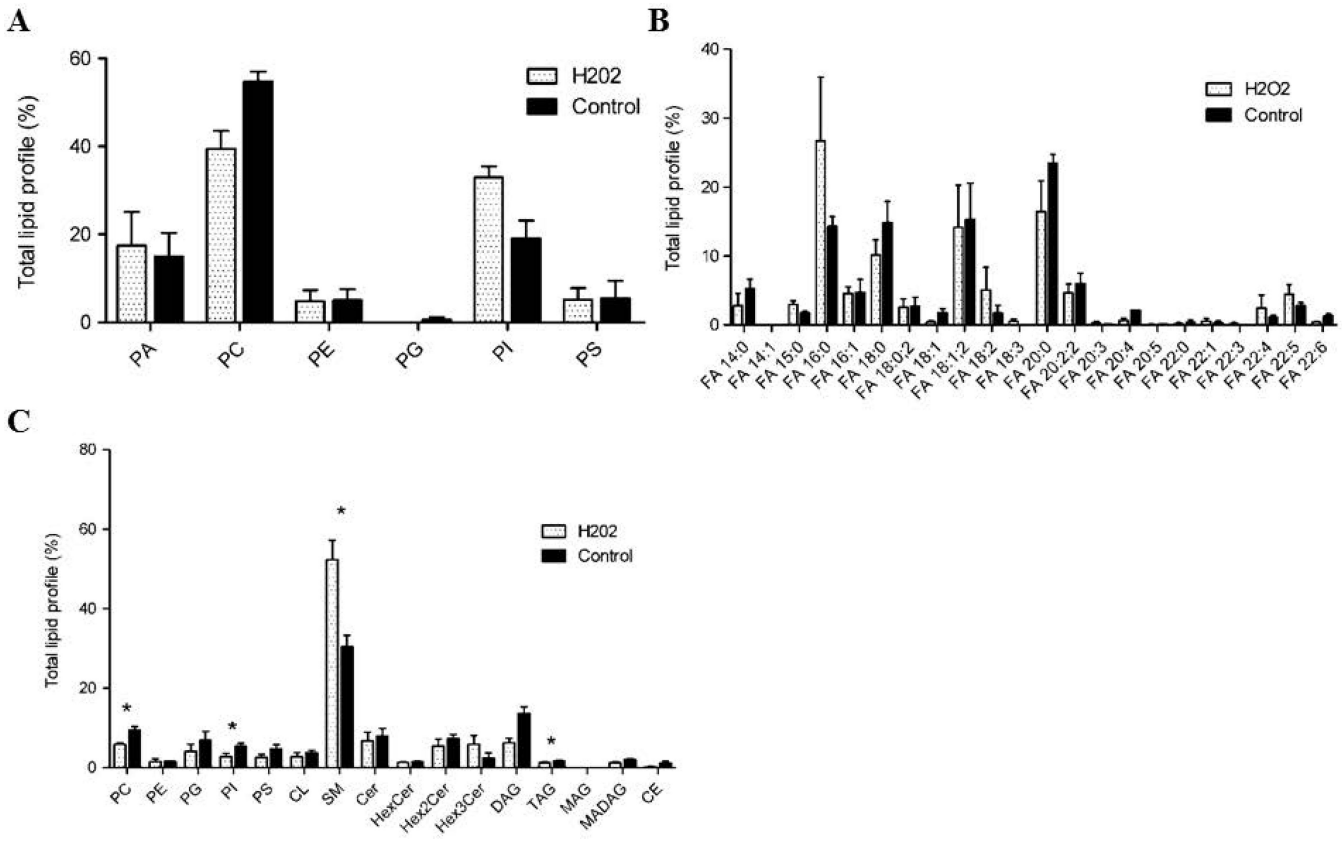

Figure 2.

Lipidomics analysis of exosomes isolated from control and H_2O_2 treated R3/1 cells. Values are mean of three independent replicates (means +/− SEM). Values were corrected with internal standards spiked into samples before extraction and then were normalized to total lipid. (A) Lipid profile of phospholipid classes analyzed in negative scan mode. (B) MS/MS spectra of the relative abundance of fatty acids in the exosomes. (C) Lipid profile analysis of exosomes under a positive scan mode. Abbreviations: PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; CL, cardiolipin; SM, sphingomyelin; Cer, Ceramide; HexCer, Hexosyl Ceremide; TAG, triacyl glycerol; DAG, diacyl glycerol; MAG, monoacyl glycerol; CE, cholesterol ester. Asterisks (*) indicates FA abundances that are significantly different (p-value ≤0.05) between the samples.

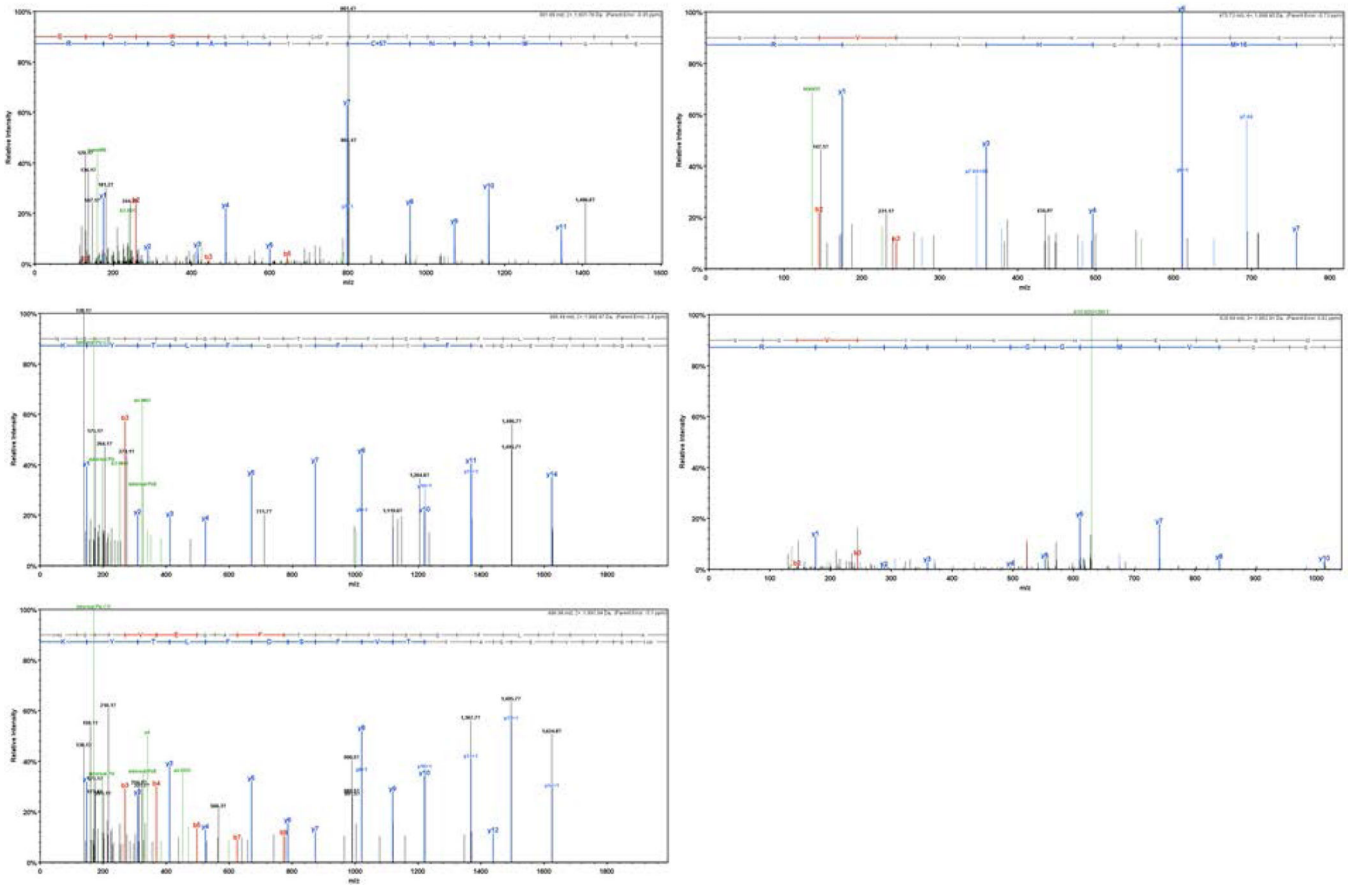

Figure 3. Identification of Cathepsin B in exosomes by mass spectrometry.

The spectrums show the relative ion intensities versus the mass-to-charge ratios (*m*/*z*) of the five surrogate peptides identified in exosomes from H2O2 treated cells that correspond to cathepsin B.

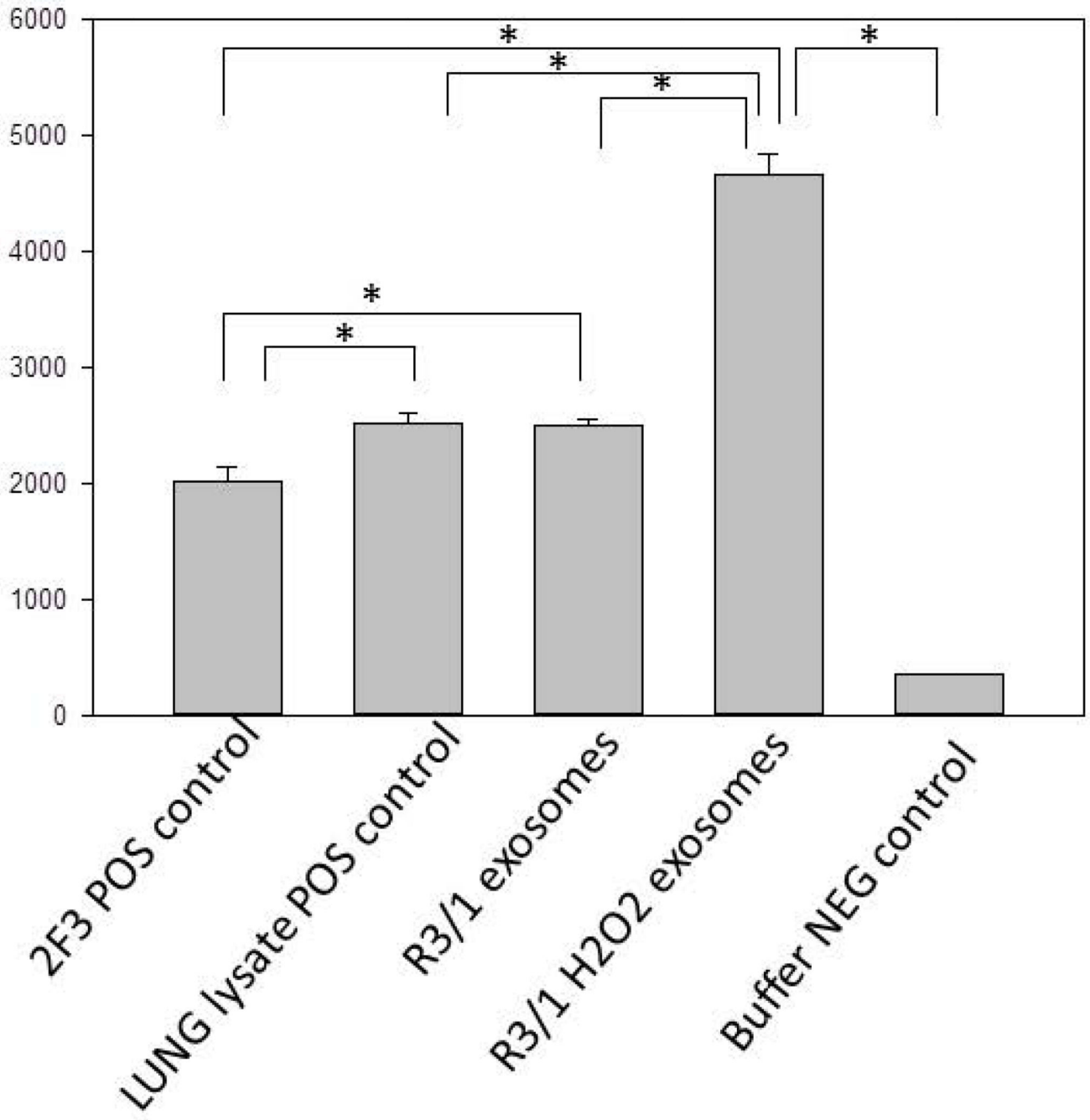

Figure 4.

Cathepsin B activity is augmented in exosomes isolated from H_2O_2 treated R3/1 cells compared to untreated cells. A cathepsin B fluorometric assay was used to quantify the relative amount of cathepsin B activity in exosomes isolated from the conditioned media of cells treated with or without H_2O_2. Cellular lysates from Xenopus 2F3 cells and mouse lung lysates were used as positive controls in the assay. The reaction buffer alone was used as a negative control. POS, represents positive control. * represents a p-value<0.05; (N=3).

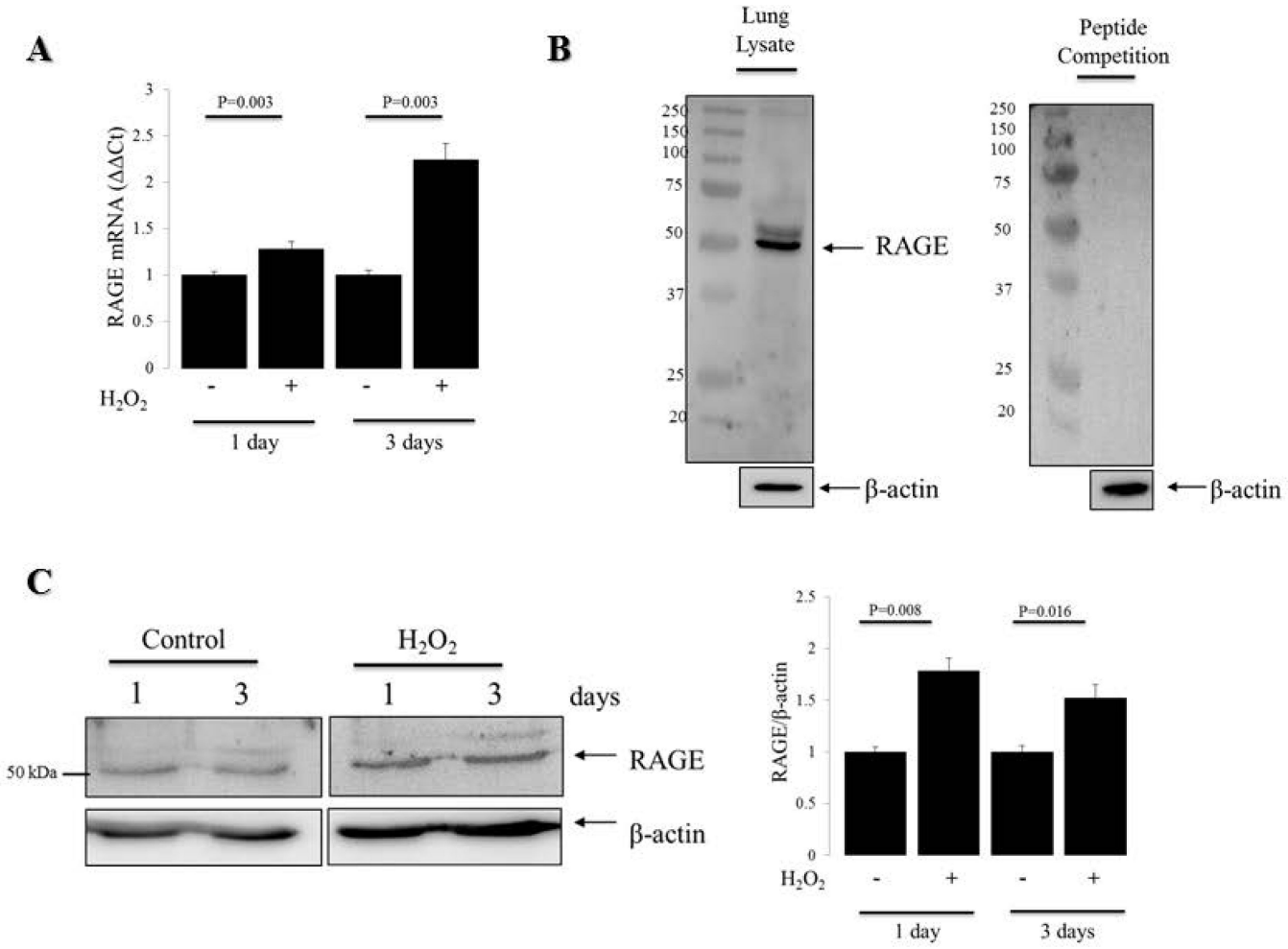

Figure 5.

Exosomes isolated from H_2O_2 treated R3/1 cells affect gene and protein expression of the receptor for advanced glycation end-products (RAGE) in a rat alveolar epithelial cell line (R3/1). **A**. R3/1 cells were exposed to exosomes for up to 3 days and RAGE mRNA levels assessed. Treatment of R3/1 cells with the exosomes isolated from cells treated with H_2O_2 increased RAGE mRNA at 1 and 3 days of exposure (N=3, P=0.003). **B**. Characterization of the anti-RAGE antibody (left panel) with a peptide competition assay (right panel) indicating antibody specificity and β-actin loading controls. **C.** Representative western blot with β-actin loading controls and quantification of RAGE protein expression after 1 day and 3 days exposure to H_2O_2-induced exosomes in R3/1 cells (N=3, P=0.008, day 1; P=0.016, day 3).

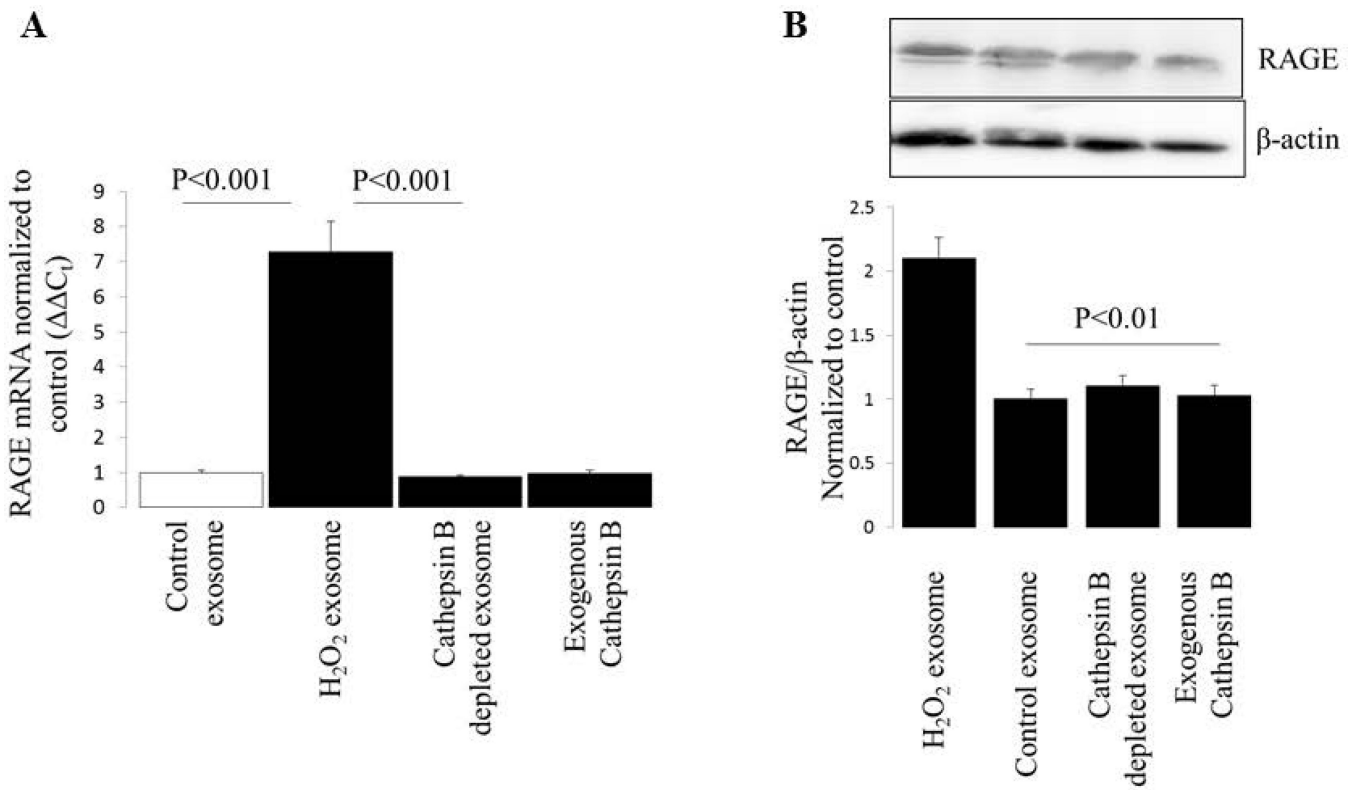

Figure 6.

Cathepsin B inhibition attenuates RAGE expression in a rat alveolar epithelial cell line (R3/1). **A.** R3/1 were exposed to exosomes for 3 days and RAGE mRNA levels assessed. Inhibition of cathepsin B from the H_2O_2-generated exosomes attenuated RAGE mRNA (N=4, p<0.001). **B.** Representative western blot of RAGE expression in R3/1 cells treated with exosomes with β-actin loading controls; inhibition of cathepsin B attenuated RAGE protein expression. (N=4, P<0.01).