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Article

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Albumin Protects Lung Cells against Acrolein Cytotoxicity and Acrolein-adducted Albumin Increases Heme Oxygenase 1 Transcripts

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This manuscript has supporting information

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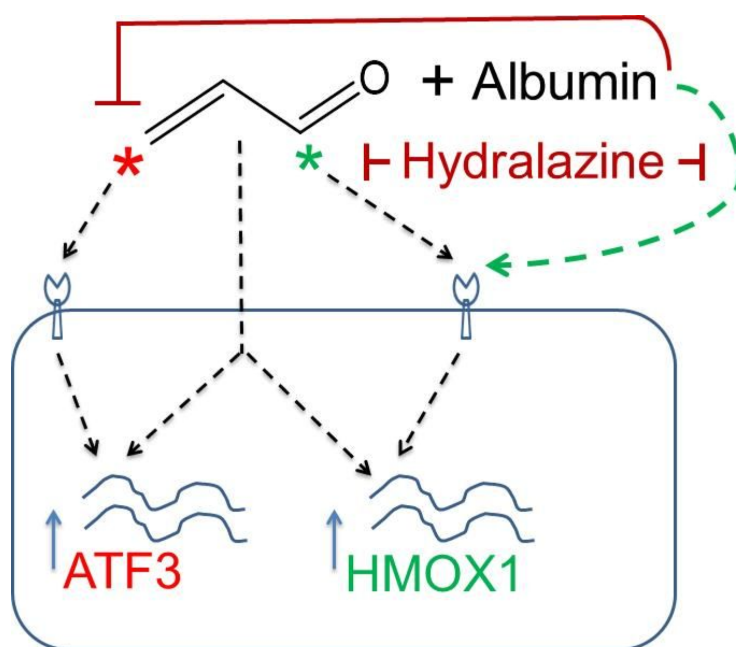

ABSTRACT

Albumin is an abundant protein in the lung lining fluid that forms an interface between lung epithelial cells and the external environment. In the lung, albumin can be targeted for adduction by inhaled acrolein. Acrolein, an α,β-unsaturated aldehyde, reacts with biomolecules via Michael addition at the β-carbon or Schiff base formation at the carbonyl carbon. To gain insight into acrolein's mode of action, we investigated *in vitro* albumin-acrolein reactivity and the consequence of albumin adduction by acrolein on cytotoxicity and transcript changes in NCI-H441 and human airway epithelial cells (HAEC). Albumin protected NCI-H441 cells from acrolein toxicity. In addition, albumin inhibited acrolein-induced increase of transcripts associated with cellular stress response, activating transcription factor 3 (ATF3), and antioxidant response, heme oxygenase 1 (HMOX1) in HAEC cells. Acrolein-adducted albumin itself increased HMOX1 transcripts but not ATF3 transcripts. The HMOX1 transcript increase was inhibited by hydralazine, a carbonyl scavenger, suggesting that the carbonyl group of acrolein-adducted albumin mediated HMOX1 transcript increase. In acutely exposed C57BL/6J mice, bronchoalveolar lavage protein carbonylation increased. Acrolein-adducted albumin Cys34 was identified by nLC-MS/MS. These findings indicate that adduction of albumin by acrolein confers a cytoprotective function by scavenging free acrolein, decreasing a cellular stress response, and inducing an antioxidant gene response. Further, these results suggest that β-carbon reactivity may be required for acrolein's cytotoxicity and ATF3 transcript increase, and the carbonyl group of acrolein-adducted albumin can induce HMOX1 transcript increase.

INTRODUCTION

Acrolein is a ubiquitous reactive aldehyde generated during cooking and is in smoke from cigarette, wood, and coal combustion. Acrolein's toxicity and other biological effects stem from its reactivity with nucleophilic proteins and other biomolecules.

Acrolein is considered a soft electrophile that preferentially undergoes 1,4-Michael type conjugate reactions targeting sulfhydryl groups [1]. Albumin, one of the major body proteins, is a nucleophilic reactant. Albumin contains 35 cysteine residues, 34 of which form 17 disulfide bridges leaving, in the reduced state, one thiol group [2-4]. Because of albumin's abundance in the plasma protein pool, it has been proposed that the free thiol group of albumin represents the principal extracellular regulator of redox balance [4,5]. Redox imbalance due to oxidative stress and α,β-unsaturated aldehydes such as acrolein can promote protein carbonylation [6] and protein carbonyl determination in biological samples has been widely used to measure oxidative stress. Indeed, acrolein-adducted albumin Cys34 has been proposed as a marker of oxidative stress in ischemia-reperfusion injury during hepatectomy [7]. However, Cys34 is not the sole residue involved in acrolein albumin interaction. In the plasma of patients with brain infarction Lys-557 and Lys-560 of albumin were conjugated with acrolein [8]. In the bronchoalveolar lavage fluid of older patients with past or ongoing smoking history but not young smokers, albumin is a major carbonylated protein [9]. Carbonyl compounds are present in high concentrations in cigarette smoke (700–800 μg/cigarette in mainstream smoke; 50–70 ppm per puff) [10,11]. Acrolein measured as FDP-lysine conjugates in human plasma ranged from 1 to greater than 300 μM [12-15]. Human diseases associated with carbonylated proteins include acute lung injury and bronchopulmonary dysplasia, Alzheimer's disease, Parkinson's disease and many others [16]. The majority of studies on acrolein reactivity have focused on acrolein toxicity or function loss of the targeted molecule. Following the initial reaction, the pathophysiological consequences of the protein carbonylation in general and acrolein adduction of albumin in particular on acrolein-induced cell death and transcript changes are not clear.

A major function of vascular albumin is regulation of colloid osmotic pressure. Other than maintenance of colloid osmotic pressure, albumin also has multiple physiological functions including binding and transportation of endogenous and exogenous molecules and ions and maintenance of vascular redox balance [17]. In lung injury, albumin extravasation due to leaky capillaries and diffuse alveolar damage may increase interstitial oncotic pressure and worsen pulmonary edema [18]. The presence of albumin in the lung may also be protective because of albumin's antioxidant properties.

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EXPERIMENTAL PROCEDURES

Materials. The chemicals, reagents, and cells obtained from commercial sources included: acrolein (Cat. No. 00016-5, Polysciences, Inc, Warrington, PA), albumin human (Cat. No. A9731), perchloric acid (Cat. No. 311421), TRI Reagent (Cat. No. T9424), 2-propanol (Cat. No. I9516), chloroform (Cat. No. C2431), 2,4-dinitrophenylhydrazine (Cat. No. D199303), hydralazine (Cat. No. H1753) from Sigma-Aldrich; Hank's Balanced Salt Solution (HBSS, Cat. No. 14-025-092), FBS (Cat. No. 26140), penicillin-streptomycin (Cat. No. 15070) from Gibco; human NCI-H441 cells (H441) (Cat. No. HTB-174), RPMI 1640 medium (Cat. No. 30-2001) from ATCC; human airway epithelial cells (HAEC) (Cat. No. SKU: FC-0016), BronchiaLife™ Basal Medium (Cat. No. LM-0007), BronchiaLife LifeFactors (Cat. No. LS-1047) from Lifeline Cell Technologies; fibronectin (Cat. No. 47743-728, VWR); CellTiter-Glo® Luminescent Cell Viability Assay (Cat. No. G7570, Promega); DNase I (Cat. No. AM1906, Ambion/Life Technologies), and iScript cDNA synthesis system (Cat. No. 170-8891, BioRad).

Acrolein Interaction with Albumin and Unreacted Acrolein Determination. Time course of acrolein interaction with albumin was spectrophotometrically determined following deproteinization with perchloric acid. Acrolein (40 μM) in HBSS was incubated for up to 2 hours alone or in the presence of 20 μM albumin at 37°C in a total volume of 200 μl. After 10, 20, 30, 60 and 120 min incubation, 200 μl of 1M perchloric acid was added, kept at 4°C for 5 min and centrifuged at 16000g for 3 min to precipitate the protein. A standard curve was generated with perchloric acid-treated acrolein alone. Free acrolein remaining in the supernatant was assessed by measuring absorbance at 210 nm (BioMate 3S-P spectrophotometer, Thermo Scientific).

Protein Carbonyl Measurement. To determine albumin carbonylation, carbonyl content of albumin incubated in the absence or presence of acrolein was determined using a 2,4-dinitrophenylhydrazine (DNPH)-based colorimetric assay (Item # 10005020, Cayman Chemical). The HBSS incubation solution containing 20 μM albumin in the absence or presence of 40 μM acrolein was incubated for 30 min or 1 h at 37°C. To remove free acrolein, the reaction solution was centrifuged (5400g, 1 h) through Amicon Ultra-2ml filter (3 kDa molecular weight limit, Cat. No. UFC200324, Millipore) followed by a washing centrifugation (5400g, 1 h) using 2 ml HBSS. The untreated control and acrolein-adducted albumin retentate samples were recovered by reverse centrifugation and diluted to 20 μM. Aliquots of the

recovered albumin samples were spectrophotometrically assessed at 370 nm for carbonyl content (Item No. 10005020, Cayman Chemical).

Cell Culture. H441 cells were used for acrolein cytotoxicity analyses because it is a lung epithelial cell line that can be maintained under low serum conditions. However, use of the H441 cell line has its limitations as it is derived from an adenocarcinoma. Thus, HAEC, which are primary human airway epithelial cells were used in this study. H441 and HAEC cells were maintained in a humidified incubator (37°C, 5% CO_2). H441 cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin G, and 100 μg/ml streptomycin. HAEC were grown in BronchiaLife™ Basal Medium supplemented with BronchiaLife LifeFactors in fibronectin-coated (5 μg/ml) flasks.

To determine pulmonary epithelial cell sensitivity to acrolein and acrolein-adducted albumin, H441 cells, which have morphological secretoglobin-producing cell-like characteristics [19], were treated with HBSS containing acrolein or acrolein plus albumin that had been pre-incubated (37°C, 30 min) to allow adduct formation before adding to cells. ATP was measured to assess cytotoxicity/viability. H441 cells were seeded (20,000 cells/well) in 96 well plates in FBS and antibiotics supplemented RPMI 1640 medium. After three days incubation (37°C, 5% CO_2), the confluent monolayer was washed two times with HBSS and 0.5% FBS-supplemented RPMI medium added. After 24 h incubation (37°C) in 0.5% FBS RPMI medium, the monolayer was washed twice with HBSS, and cells were treated with HBSS control or 10-80 μM acrolein to determine toxicity. To determine the effect of acrolein-adducted albumin on cell toxicity, 60 μM acrolein alone or 60 μM acrolein plus 10-80 μM albumin was added to the cells (100 μl/well). After 1 h incubation, FBS was added to a concentration of 0.5%, the supernatant removed and fresh RPMI containing 0.5% FBS added. The cells were incubated (37°C, 72 h) and assessed for ATP levels to determine H441 cell viability using a luminescent cell viability assay. Luminescence readings were obtained using Synergy 2 plate reader (BioTek Instruments, Inc. Winooski, VT).

HMOX1 and ATF3 Transcripts Analyses. To examine cell transcript modulation by acrolein or acrolein-adducted albumin, RNA isolated from HAEC treated with acrolein, albumin, acrolein-adducted albumin or acrolein-adducted albumin treated with hydralazine was analyzed by quantitative real time polymerase chain reaction (qRT-PCR). The aldehyde scavenger hydralazine was included to test the contribution of carbonyl groups to transcript modulation.

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HAEC cells were seeded (70,000 cells/well) in fibronectin-coated (5 µg/ml) 12 well plates in supplemented BronchiaLife™ Medium. After three days incubation (37°C, 5% CO_2), the confluent monolayer was washed two times with HBSS and glutamine (6 mM) supplemented BronchiaLife™ Basal Medium was added. After 24 h incubation (37°C) in glutamine (6 mM) supplemented BronchiaLife™ Basal Medium, the monolayer was washed twice with HBSS, and HBSS (300 µl/well) was added. After 30 min pre-incubation of cells in HBSS, 300 µl of HBSS or 160 µM albumin that had been pre-incubated in the absence or presence of 80 µM acrolein (37°C, 1 h) followed by 400 µM hydralazine treatment (37°C, 1 h) was added to the cells (300 µl/well) to a final concentration of 80 µM albumin, 200 µM hydralazine and 40 µM acrolein. After 1 h incubation, BronchiaLife™ Basal Medium (600 µl/well) was added and incubated further for 3 hours when RNA was extracted.

Total cellular RNA was isolated from control and treated HAEC cells using TRI Reagent (200 µl/well) and quantified by A_{260} absorbance determination (Take3, BioTek Instruments). The RNA was analyzed for activating transcription factor 3 (ATF3) and heme oxygenase 1 (HMOX1) transcripts by qRT-PCR. DNase I-treated RNA (0.9 µg) was reverse-transcribed using iScript cDNA synthesis system in a 30 µl reaction volume and diluted 5x using RNase-free water. An aliquot of the cDNA synthesis product (2 µl) was used in a subsequent qRT-PCR analysis (Cat. No. 4369016, TaqMan Gene Expression Master Mix, ABI/Life Technologies). The qRT-PCR analysis was performed with 7900HT Fast Real Time PCR System using the following conditions: 50°C for 2 min, 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The primers used were the internal control ribosomal protein L32 (RPL32; Cat. No. Hs00851655_g1), ATF3 (Hs00231069_m1), and HMOX1 (Hs01110250_m1) from Applied Biosystems. The comparative cycle number threshold (C_T) method ($\Delta\Delta C_T$) was used to determine transcript change.

Treatment of HAEC with Acrolein-adducted Albumin in the Absence or Presence of Hydralazine. Because acrolein preferentially reacts with target proteins via Michael-type addition reactions that retain the aldehyde functional group [20-23], we tested HAEC response to acrolein-adducted albumin. HBSS, 80 µM acrolein, 160 µM albumin, and 80 µM acrolein plus 160 µM albumin were incubated (37°C, 1 h) in a total volume of 2.2 ml. To remove unreacted acrolein, the samples were loaded in Millipore Ultra-2 ml filter, centrifuged (5400g, 1 h) and washed (5400g, 1.5 h). The retentates were recovered, diluted to 2.2 ml and filtered. The

recovered retentates were split into two sets of tubes and incubated (37°C, 1 h) in the absence or presence of hydralazine (400 μM) before adding to HAEC cells.

HAEC cells were seeded in 12-well plates and treated as described above by adding the recovered retentate samples (300 μl/well) of HBSS, acrolein, albumin and albumin plus acrolein preincubated in the absence or presence of hydralazine. After 1 h incubation, BronchiaLife™ Basal Medium (600 μl/well) was added and incubated further for 3 hours when RNA was extracted and then analyzed for ATF3 and HMOX1 transcripts by qRT-PCR.

Hydralazine Interaction with Acrolein-adducted Albumin. Hydralazine reacts with acrolein and acrolein adducts in proteins [21,24]. However, it was reported that hydralazine abolished the immunoreactivity of an acrolein-modified bovine serum albumin only if the drug was added to the protein within 30 min of commencing modification by acrolein [21]. In our system, albumin was incubated with acrolein (1 h) followed by two centrifugation steps (2.5 h duration) prior to hydralazine addition (1 h) and then cell treatment. To establish that the inhibition of acrolein-adducted albumin-induced HMOX1 transcript increase by hydralazine was accompanied by covalent bond formation, HBSS, 100 μM albumin and 100 μM albumin plus 100 μM acrolein samples were incubated at 37°C for 1 h. Hydralazine (60 μM) was added to the samples and further incubated (37°C, 1 h) followed by filtration (5400g, 1 h) and a wash (5400g, 1 h) to remove unreacted hydralazine. The absorbance of the filtrate at 263 nm and UV spectrum (240-340 nm) of the recovered retentate were spectrophotometrically determined.

SDS-PAGE and Western Blot Analysis. To detect acrolein-adducted proteins, samples were incubated with Laemmli's SDS-sample buffer and separated by 4-20% mini-protean TGX gel (Cat. No. 456-1094, Bio-Rad) electrophoresis. Because of concerns over the stability of 2,4-DNPH-derivatized protein hydrazones, samples were not heated or treated with reducing agents prior to electrophoresis [25]. To detect protein, the gel was silver-stained (Pierce Cat. No. 24612, Thermofisher Scientific). To detect carbonyl groups on acrolein-adducted proteins, samples were derivatized using 2,4-dinitrophenyl hydrazine (DNPH) [26,27]. For Western blotting, DNPH-derivatized and hydralazine conjugated acrolein-adducted albumin or DNPH-derivatized mouse bronchoalveolar lavage samples were separated by 4-20% gel electrophoresis and transferred to polyvinylidene difluoride (PVDF) membrane (Cat. No. 88518, Thermofisher Scientific). The membrane was rinsed twice (5 min each) in Tris-buffered saline (TBS; 20 mM Tris, 500 mM NaCl, pH 7.5) and blocked for 1 h with 5% nonfat milk in TBS (23°C). The membrane was then

incubated with rabbit anti-DNP antibody (Cat. No. D9656, Sigma-Aldrich) diluted 1/5,000 or rabbit anti-hydralazine antibody (Cat. No. ABIN343204, Antibodies-online Inc, Atlanta, GA) diluted 1/10,000 in 1% BSA in TBST (TBS containing 0.1% Tween 20) for 1-2 hours (23°C). After a rinse and two washes in TBST (10 min each), the membrane was incubated for 1 h (23°C) in horseradish peroxidase-conjugated anti-rabbit antibody (Cat. No. 7074, Cell Signaling, Danvers, MA) diluted 1/20,000 in 1% BSA in TBST. After three washes, the immunoblot was incubated with SuperSignal West Pico chemiluminescent substrate (Cat. No. 34080, Thermo Scientific) and the immunoreactivity signal of the carbonylated proteins was developed after exposing to x-ray film (Cat. No. F-BX57, Phenix Research Products, Candler, NC).

Densitometric quantification of the immunoreactivity signal intensity was performed using LI-COR Image Studio Lite software. Relative protein carbonylation levels were determined as fold increase compared to control samples not exposed to acrolein.

Mouse Exposure and Bronchoalveolar Lavage (BAL) Protein Analysis. This study was performed in accordance with the Institutional Animal Care and Use Committee of the University of Pittsburgh (Pittsburgh, PA) and mice were housed under pathogen-free conditions. Nine-week-old C57BL/6J mice (n = 3-5 mice/group; Jackson Laboratories, Bar Harbor, ME) were exposed to filtered air (control) or acrolein (75 ppm, 30 min). Acrolein vapor was generated by blowing breathing air into a flask containing liquid acrolein and introduced into a 0.32-m^3 stainless steel chamber. The acrolein exposure concentration was continuously monitored using an in-line Chemgard infrared monitor (MSA, Cranberry Township, PA).

After exposure, mice were euthanized by intraperitoneally injecting pentobarbital sodium followed by severing the posterior abdominal aorta. To obtain BAL, a cannula was inserted in the trachea and the lungs were lavaged initially with 0.8 ml and then with 0.5 ml of PBS containing 0.4 mM EDTA. The collected lavage was centrifuged (500g, 10 min, 4°C) to pellet cells and the supernatant was used for carbonylation analysis of acrolein-adducted proteins. To examine overall protein carbonylation, BAL was DNPH-derivatized and assessed by Western blotting. To identify acrolein-adducted proteins, BAL from control and acrolein exposed mice were analyzed by NanoLC-MS/MS (nLC-MS/MS).

Site-specific Identification of Protein Adduction by Tandem Mass Spectrometry. To identify acrolein-adducted proteins in acrolein exposed mouse pulmonary epithelial lining fluid,

BAL was collected within 2 hours following exposure from control air- and acrolein-exposed (75 ppm, 30 min) C57BL/6J mice (5 mice/group). The BAL supernatant was treated with 5 mM sodium borohydride to reduce the acrolein adduct as previously described [28]. BAL aliquots from the control or acrolein exposed mice were combined to create a pooled sample. Trypsin (Cat. No. V5111, Promega Corporation, Madison, WI) was added to BAL aliquots containing 15 µg of protein and in-solution digestion was carried out at 37°C overnight in ammonium bicarbonate (pH 8.0) [9]. The tryptic peptides were desalted with C18 Spin Columns (Cat. No. 89873, Thermo Fisher Scientific, Waltham, MA), lyophilized, and resuspended in 15 µL 0.1% formic acid. An LTQ-XL linear ion trap mass spectrometer (Thermo Fisher Scientific, Waltham, MA) coupled to a Dionex Ultimate 3000 RSLC nanosystem chromatography system (Thermo Fisher Scientific, Waltham, MA) and operated in a data-dependent acquisition mode was used to acquire low-resolution tandem mass spectra for BAL tryptic peptides. Tandem mass spectra were analyzed via a database search using the MASCOT search engine (Version 2.4.0., Matrix Science Ltd) [29] and the Uniprot mouse database (downloaded 1/31/2019, 209,404 entries) with precursor and product ion mass tolerances of 1.4Da and 0.8Da, respectively. To search for site-specific carbonylation at cysteine, histidine, and lysine residues a dynamic modification of +58Da was considered. High confidence sequence identifications were selected using the PeptideProphet™ and ProteinProphet® algorithms [30] with a protein threshold cutoff of 99% and a peptide threshold cutoff of 90%, implemented in Scaffold™ (Proteome Software, Portland, OR). Final sequence assignments acrolein-adducted peptides were confirmed by manual inspection of the tandem mass spectra.

Statistical analysis: One-way ANOVA was performed and significance ($p \leq 0.05$) determined by Holm-Sidak all pairwise multiple comparison procedures (SigmaStat Program; SPSS, Inc., Chicago, IL).

RESULTS

Acrolein Interaction with Albumin. The reactivity of commercial albumin can vary [31,32]. To assess albumin reactivity with acrolein, albumin was incubated at 37°C in the presence of acrolein up to 2 hours and unreacted acrolein remaining was determined. After 30 min incubation, the starting 40 µM acrolein concentration decreased to 21 µM in the presence of albumin compared with 37 µM in the absence of albumin (Figure 1A). To verify that the acrolein decrease observed reflected acrolein's reactivity with albumin, the carbonyl content of albumin

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incubated alone or in the presence of acrolein was determined. The carbonyl level of acrolein treated albumin was higher compared with untreated albumin - evidence of acrolein-adducted albumin formation (Figure 1B).

Albumin Cytoprotection against Acrolein-Induced Cytotoxicity. To determine H441 cell sensitivity to acrolein, ATP levels were measured 72 h after a 1 h exposure to acrolein (0 to 80 μM). Acrolein reduced ATP levels by 10% to 99 % in a concentration dependent manner (Figure 2A). In contrast, when H441 cells were treated with 60 μM acrolein that had been pre-treated with albumin to form adducts, albumin inhibited acrolein-induced cellular ATP loss in a concentration dependent manner (Figure 2B). The results indicate that adduction of albumin by acrolein is cytoprotective against acrolein-induced lung cell toxicity. It is noteworthy that albumin displayed protective effect even when used at a low concentration of 10 μM against 60 μM acrolein.

Albumin-Acrolein Interaction and Transcript Changes in HAEC Cells. Acrolein has been associated with increased oxidative stress [33-35]. Transcripts associated with major cellular pathways including cell death and NFE2L2 mediated oxidative stress response are increased in acrolein exposed mouse lungs *in vivo* and in A549 cells in culture [36,37]. To determine the consequence of acrolein-adducted albumin formation on acrolein-induced transcript changes, ATF3 and HMOX1 transcripts were selected for analysis. Acrolein treatment of HAEC cells induced ATF3 and HMOX1 transcripts (Figure 3). In support of the HAEC cell response, RNA analysis of mouse lungs collected six hours after acrolein exposure demonstrated that ATF3 and HMOX1 transcripts increased. ATF3 and HMOX1 in acrolein exposed lungs increased 7-fold and 22-fold, respectively, compared to air exposed control lungs. In the presence of albumin, acrolein-induced ATF3 and HMOX1 transcripts decreased in HAEC. Interestingly, the presence of albumin, which decreased basal ATF3 and HMOX1 transcripts, displayed complete inhibition of acrolein-induced ATF3 (Figure 3A) but partial inhibition of HMOX1 (Figure 3B) transcripts. Because HMOX1 transcripts remained high compared with ATF3 transcripts following albumin treatment, we hypothesized that the incomplete inhibition of HMOX1 transcripts induction may be due to HMOX1 transcripts increase by the carbonyl group in the acrolein-adducted albumin rather than albumin's inefficient inhibitory capacity.

Albumin-Acrolein Adducts Induce HMOX1 Transcripts. To test HAEC response to acrolein-adducted albumin, acrolein, albumin and albumin plus acrolein were incubated (37°C, 1 h)

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followed by centrifugation through a filter to remove free acrolein. The retentate was recovered and further incubated in the absence or presence of hydralazine before adding to the cells. Albumin-acrolein adducts induced HMOX1 transcripts. Treatment of HAEC cells with the retentate recovered after centrifugation of the acrolein sample did not induce HMOX1 transcripts, indicative of the efficacy of the filtration procedure in removing residual free acrolein from the retentate (Figure 4). To test that the aldehyde group contributed to the HMOX1 transcripts increasing action of the acrolein-adducted albumin, the retentate samples were incubated with hydralazine. Acrolein-adducted albumin-induced HMOX1 transcript increase was inhibited by hydralazine (Figure 4).

Biomolecular Interaction between Hydralazine and Acrolein-adducted Albumin. To establish that conjugation of the carbonyl groups in the acrolein-adducted albumin by hydralazine mediated the HMOX1 transcript inhibition observed, albumin and acrolein-adducted albumin (37°C, 1 h) were further incubated (37°C, 1 h) in the absence or presence of hydralazine. To remove free hydralazine and acrolein from adducted albumin molecules the samples were filtered. The absorbance of the filtrate of HBSS plus hydralazine and albumin plus hydralazine was comparable (A=0.457 vs 0.434, respectively) whereas the absorbance of the filtrate of albumin plus acrolein plus hydralazine decreased greatly; Nearly 80% of the hydralazine was retained by the albumin-acrolein adducts whereas less than 5% of the hydralazine was retained by control albumin not treated with acrolein (Supporting Figure S1A). Further, UV-VIS spectroscopy scanning of the retentate samples demonstrated that the UV scan peak of the acrolein-adducted albumin plus hydralazine was greater compared to albumin in the absence or presence of hydralazine (Amax=0.972 vs 0.778 and 0.713, respectively, Supporting Figure S1B). The decreased absorbance in the filtrate and increased absorbance of the hydralazine treated acrolein-adducted albumin sample support the conclusion that hydralazine conjugates to acrolein-adducted albumin and thus traps carbonyl groups.

One consequence of acrolein adduction of biomolecules is intermolecular and intramolecular cross-linking [38]. Incubation of albumin with increasing acrolein concentration (40-320 μM) resulted in correspondingly higher molecular weight forms as demonstrated by SDS-PAGE separation and silver-staining of the protein gel (Figure 5). Similarly, carbonyl and hydralazine signal of higher molecular weight forms increased in a concentration-dependent manner as demonstrated by immunoblotting of DNPH-derivatized and hydralazine reacted samples,

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respectively (Figure 5). Thus, the monomeric albumin and crosslinked albumin contain carbonyl groups that are reactive with hydralazine.

BAL protein carbonylation. To examine protein carbonylation during acute exposure, BAL was collected from acrolein exposed mice. The lavage was performed immediately after exposure (within 90 min). Under these conditions, average protein concentration in control samples was 0.220 $\pm$ 0.016 mg/mL and protein concentration in acrolein exposed samples was 0.319 $\pm$ 0.056 mg/mL. Acrolein increased BAL protein carbonylation as detected in DNPH-derivatized samples from acrolein exposed C57BL/6J mice compared with control air-exposed mice (Figure 6). Acrolein-adducted albumin in BAL from acrolein exposed mice was confirmed by nLC-MS/MS spectra of the tryptic digest peptide (C*SYDEHAK) (Figure 7). Albumin adduction by acrolein in the epithelial lining fluid occurs *in vivo* on Cys34.

DISCUSSION

Albumin is an abundant body protein with numerous functions including colloid osmotic pressure regulation and transportation of exogenous and endogenous molecules such as drugs, toxins, nutrients, hormones and metabolites. Because of its inherent structural and biochemical features, albumin binds covalently and non-covalently to diverse ligands [39]. Acrolein is a highly reactive aldehyde [40]. The heart-shaped structure of albumin is composed of three domains (I, II, III), each of which has two subdomains (A, B). The free cysteine (Cys34) and histidine (His39) residues in domain 1, the lysine residue (Lys351) in domain II, and the lysine residues (Lys 541, Lys 545, Lys557, Lys560) in domain III can act as nucleophilic reactants with the α,β-unsaturated aldehyde acrolein generating carbonylated albumin [8,9,20,33,41].

Protein carbonylation determination has long been used to assess the degree of oxidative stress in a variety of diseases including Alzheimer's disease, chronic lung disease, chronic renal failure, diabetes, sepsis, amyotrophic lateral sclerosis, cataractogenesis, cystic fibrosis, rheumatoid arthritis, and ischemia-reperfusion injury [42-44]. Because of its abundance and nucleophilic residues, which can interact with α,β-unsaturated aldehydes such as acrolein, carbonylated albumin has been investigated as a biomarker of oxidative stress in uremia, liver disease, stroke, and diabetes [45-49]. While acrolein interaction with cytoskeletal proteins and enzymes has been implicated in cell and tissue damage [50-53], the causative or consequential contribution of acrolein-mediated albumin carbonylation in disease onset and/or progression is unclear.

To investigate the role of acrolein-adducted albumin on lung cell toxicity/survival and transcript regulation, acrolein-adducted albumin was added to epithelial cells. Acrolein-adducted albumin formation was evidenced by higher carbonyl content in acrolein treated albumin compared with untreated albumin. Adduction of albumin by acrolein was cytoprotective against acrolein-induced cytotoxicity in H441 cells. It should be noted that albumin is protective from acrolein-induced toxicity in H441 human lung cells (this work) and in mouse mammary FM3A cells [8]. Albumin has also been reported to be toxic to renal proximal tubular epithelial cells [54-56]. Acrolein's key molecular feature underlying its chemical reactivity is the presence of two electrophilic centers: the β-carbon and the carbonyl carbon [22,23]. Acrolein can react with nucleophilic biomolecules via the β- carbon forming Michael-type adducts or the aldehyde group forming Schiff base products; the preferred mode of attack being the β-carbon. Our interpretation is that consequent to albumin reaction with the β-carbon of acrolein, acrolein-adducted albumin reduces the concentration of free acrolein rendering acrolein less cytotoxic. These observations suggest that the conjugate complex formed is less toxic in agreement with previous findings that aliphatic aldehydes and substituted unsaturated aldehydes are less potent enzyme inactivators compared with acrolein [51].

The effects of acrolein are not limited to cytotoxicity and interaction with target biomolecules. Transcriptomic and metabolomics studies have demonstrated that acrolein exposure alters a wide variety of gene transcripts and small molecule metabolites [36,37,57]. To investigate the influence of acrolein-adducted albumin on acrolein-induced transcript changes, ATF3 and HMOX1 transcripts were selected for analysis. The transcription factor ATF3 and the antioxidant HMOX1 gene products are induced in response to tissue injury, oxidative stress, toxic insults, and acrolein [58-61]. Acrolein treatment of HAEC induced ATF3 and HMOX1 transcripts. Interestingly, the presence of albumin inhibited acrolein-induced HMOX1 transcript increase only partially. In contrast, albumin inhibited strongly acrolein-induced ATF3 transcript increase. Further analysis demonstrated that acrolein-adducted albumin decreased basal ATF3 transcripts but increased HMOX1 transcripts.

One limitation of this study is that the mechanism by which acrolein-adducted albumin increased HMOX1 transcripts was not determined. *HMOX1* gene expression can be triggered through extracellular and intracellular effector molecules. Acrolein-adducted albumin contains a reactive carbonyl which may interact with a membrane receptor(s) that triggers intracellular signaling. The inhibition of this activity by hydralazine indicates that the carbonyl group is critical to the response. Alternatively, acrolein-adducted albumin could enter the cell and trigger

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signaling by an additional mechanism. Albumin levels in the lung are regulated tightly to maintain osmotic gradients. Albumin binds to scavenger receptors (mainly CD36) on the apical membrane of alveolar epithelial cells, and when intact, transverses to the basolateral membrane and is released by transcytosis [62]. However, chemically modified albumin binds other scavenger receptors (gp18 and gp30 likely OLR1) and is directed to lysosomes for degradation [63]. It is unknown whether this latter pathway could induce cell signaling that could selectively initiate *HOMX1* and not *ATF3* transcription. Transcriptional regulation of *HMOX1* is mediated via multiple signaling pathways elicited by different pathophysiological stimuli and transcriptional activators and repressors that bind to cis-regulatory elements at the proximal and distal 5' promoter regions of *HMOX1* gene [64]. Acrolein induces *HMOX1* promoter activity through PKC-delta and PI3K signaling that leads to NRF2-dependent and NRF2-independent activation, respectively, in human bronchial epithelial cells [65]. Further studies are needed to determine the role of extracellular and intracellular interactions of acrolein-adducted albumin and the mechanism mediating HMOX1 transcripts increase.

Albumin binds to a variety of ligands, including pharmaceutical drugs [39]. The anti-hypertensive drug hydralazine, which is also an aldehyde scavenger, binds to albumin [66-68]. Spectroscopic studies suggested that the noncovalent binding of hydralazine with human serum albumin occurs through subdomain IIIA [68]. Hydralazine covalently interacted with acrolein-adducted albumin and inhibited acrolein-adducted albumin-induced HMOX1 transcript increase. Acrolein binding to albumin can result in cross-linking and protein oligomerization. Incubation of albumin with increasing acrolein concentration resulted in correspondingly higher molecular weight forms. Albumin was incubated with increasing acrolein concentration for 2 hours before further incubation in the absence or presence of hydralazine. Although hydralazine is an efficient acrolein-trapping reagent, the results indicate that acrolein-induced cross-linking occurred within 2 hours of incubation prior to hydralazine addition. Nonetheless, crosslinking was likely not complete, and a portion of acrolein-adducted albumin contained an unreacted carbonyl that was "trapped" following hydralazine addition. In addition, acrolein adduction may alter albumin's structure [69,70]. Oxidized albumin exhibits altered protease susceptibility, ligand-binding affinity and antioxidant activity compared to reduced albumin [71]. Thus, we cannot exclude the possibility that structural or conformational changes in acrolein-adducted albumin contribute to HMOX1 transcript increase. However, the inhibitory effect of hydralazine conjugation suggests that hydralazine traps the carbonyl group forming a hydrazone and thus inhibits acrolein-adducted albumin-induced HMOX1 transcript increase. Because acrolein preferentially reacts with target proteins via Michael-type addition reactions [20-23], the acrolein-adducted albumin is

produced primarily due to acrolein's reactivity at the β-carbon. Acrolein-adducted albumin, which retains the aldehyde functional group, increased HMOX1 transcripts but not ATF3 transcripts. The HMOX1 transcript increase by acrolein-adducted albumin was inhibited by hydralazine. The inhibition of ATF3 increase consequent to albumin addition to the β-carbon and the inhibition of HMOX1 transcript increase by the carbonyl scavenger suggest that acrolein's two reactive centers may activate distinct downstream gene targets. Because our interpretation is that carbonyl groups of acrolein-adducted albumin mediate HMOX1 transcript increase, we predict that other carbonylated proteins can induce HMOX1 transcripts increase.

In healthy humans, the Cys34 residue of plasma albumin exists predominantly (70% of total albumin) in the reduced form [72]. Although not fast-reacting with oxidants, the plasma albumin thiol is considered an important scavenger because of its considerable concentration (0.4-0.5 mM) [73]. Although albumin is the most abundant protein in the pulmonary epithelial lining fluid, the albumin concentration in the pulmonary epithelial lining fluid is about 10% of the plasma albumin concentration [74]. In marked contrast, the concentration of glutathione, taken as an example of lung antioxidants, is about 140-fold higher in the pulmonary lining fluid compared to that of plasma [75]. Despite the presence of high concentrations of glutathione and other small molecular weight antioxidants in the lung, nLC-MS/MS analysis of mouse BAL protein carbonylation in acutely acrolein-exposed C57BL/6J mice identified an acrolein adduct on albumin Cys34. This suggests the thiol group of albumin contributes to the extracellular acrolein scavenging capacity of the pulmonary epithelial lining fluid. Further, it has long been thought that although the cysteine thiol group is the most readily reactive group with acrolein, no acrolein-cysteine adducts have been identified *in vivo* due to instability [76-78]. Our work provides evidence of identification of *in vivo* formed acrolein-cysteine adducts in mouse BAL.

In conclusion, acrolein exposure increases albumin carbonylation *in vitro* and *in vivo* in mice and consistent with carbonylation use as a marker, the carbonylation increase is associated with an oxidative insult. We cannot exclude the possibility that the adduction decreases the antioxidant capacity of albumin and induces conformational changes that may negatively impact its physiological functions. Yet, our work suggests that albumin adduction is cytoprotective due to its reactivity with the alkene group of acrolein, but the albumin carbonylation also suppresses stress associated gene responses such as ATF3 while inducing anti-oxidant responses such as HMOX1 transcript increase. We also report identification of an *in vivo* formed acrolein-cysteine adduct.

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Supporting Information. Hydralazine interacted with acrolein-adducted albumin and increased the UV absorbance peak of acrolein-adducted albumin (S1).

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Figure Legends

Figure 1. Acrolein reactivity with albumin. (A) Acrolein (40 μM) was incubated at 37°C in the presence of 20 μM albumin. At the indicated times albumin was precipitated using perchloric acid and the unreacted acrolein remaining in the supernatant spectrophotometrically determined at 210 nm (n = 1-3/group). (B) Albumin (20 μM) was incubated (37°C) in the absence (no acrolein) or presence (acrolein, 40 μM) for 30 or 60 min. Following centrifugation through Millipore Ultra-2ml filter to remove excess unreacted acrolein, the filter-retained control and acrolein-adducted albumin samples were derivatized with DNPH (RT, 1 h). To measure carbonyl content, hydrazone formation was spectrophotometrically determined at 370 nm. Values are mean ± SEM (n = 6/group). *Values were significantly increased vs control albumin ($p < 0.05$) as determined by one-way analysis of variance with Holm-Sidak all pairwise multiple comparison procedure.

Figure 2. Albumin protected H441 cells from acrolein cytotoxicity. (A) H441 cells were exposed to increasing acrolein doses. HBSS control or 10-80 μM acrolein was added to H441 cells and incubated (37°C, 1 h). (B) H441 cells were incubated (37°C, 1 h) in 60 μM acrolein that had been preincubated (37°C, 30 min) in the absence or presence of 10-80 μM albumin to generate acrolein-adducted albumin. After addition of 0.5% FBS-supplemented RPMI1640 medium, treated cells were incubated for 72 h and ATP level was determined. Values are mean ± SEM (n = 6-12/group). *Values were significantly decreased vs untreated control (A) or increased vs acrolein treated with no albumin added (B) ($p < 0.001$) as determined by one-way analysis of variance with Holm-Sidak all pairwise multiple comparison procedure.

Figure 3: Albumin inhibited acrolein-induced activating transcription factor 3 (ATF3) transcript increase. HAEC were treated (37°C, 1 h) with HBSS or 80 μM acrolein that had been preincubated in the absence or presence of 80 μM albumin (37°C, 1 h) to generate acrolein-adducted albumin. The cells were further incubated for 3 h following addition of epithelial cell basal medium. After 4 h incubation, RNA was extracted and analyzed for ATF3 (A) and HMOX1 (B) transcripts by qRT-PCR. Values are mean ± SEM (n = 6/group). Values were: *significantly different vs HBSS control; †significantly decreased vs HBSS acrolein ($p < 0.001$) as determined by one-way analysis of variance with Holm-Sidak all pairwise multiple comparison procedure.

Figure 4. Hydralazine inhibited acrolein-adducted albumin-induced heme oxygenase 1 (HMOX1) transcript increase. HBSS, 80 μM acrolein, 160 μM albumin and 80 μM acrolein plus 160 μM albumin samples were incubated (37°C, 1 h) to generate acrolein-adducted albumin. Following filtration to remove free acrolein, the recovered retentate samples were incubated in the absence or presence of 400 μM hydralazine (37°C, 1 h) to trap carbonyl groups and then added to wells containing HAEC (37°C, 1 h). The cells were further incubated for 3 h following addition of epithelial cell basal medium. After 4 h incubation, RNA was extracted and analyzed for HMOX1 transcripts by qRT-PCR. Values are mean ± SEM (n = 6/group). Values were: *significantly different vs HBSS control; †significantly different vs no hydralazine ($p < 0.001$) as determined by one-way analysis of variance with Holm-Sidak all pairwise multiple comparison procedure.

Figure 5. Increased acrolein dose enhanced acrolein-adducted albumin cross-linking and hydralazine reacted with the monomeric and cross-linked forms. Albumin (20 μM) was incubated with 0 and 40-320 μM acrolein (37°C, 2 h) followed by incubation without or with 400

μM hydralazine (37°C, 1 h). Samples were separated by SDS-PAGE (4-20%). (A) Western blot detection of albumin carbonylation. Acrolein-adducted albumin samples were DNPH-derivatized and blotted (anti-DNP) using anti-DNP antibodies (1 μg protein/lane). (B) Western blot detection of hydralazine conjugated to acrolein-adducted albumin. The blot of acrolein-adducted albumin-hydralazine reaction samples (anti-HYD) was probed using anti-hydralazine antibodies (2 μg protein/lane). (C) Silver staining of acrolein-adducted albumin samples (0.1 μg protein/lane).

Figure 6. Acrolein exposure of C57BL/6J mice increased carbonylation of pulmonary epithelial lining fluid proteins. Bronchoalveolar lavage (BAL) collected from control and acrolein exposed (75 ppm, 35 min) mice was DNPH-derivatized and separated using SDS-PAGE. BAL from control and acrolein exposed mice not derivatized with DNPH was included as a negative control for specificity of anti-DNP antibody. (A) Western blot detection of BAL protein carbonylation. BAL samples were DNPH-derivatized and blotted using anti-DNP antibodies. (B) Quantification of BAL protein carbonylation signal in (A) lanes. Values are mean $\pm$ SEM (n = 3/group). *Values were significantly increased vs control ($p < 0.05$) as determined by one-way analysis of variance with Holm-Sidak all pairwise multiple comparison procedure.

Figure 7. Tandem mass spectrum of acrolein-adducted mouse albumin tryptic peptide C*SYDEHAK (m/z = 505.7). Acrolein-adducted albumin was detected in mouse BAL fluid following acrolein treatment. Note that product ions y_7^+ and b_2^+ confirm Cys34 as the site of adduction.

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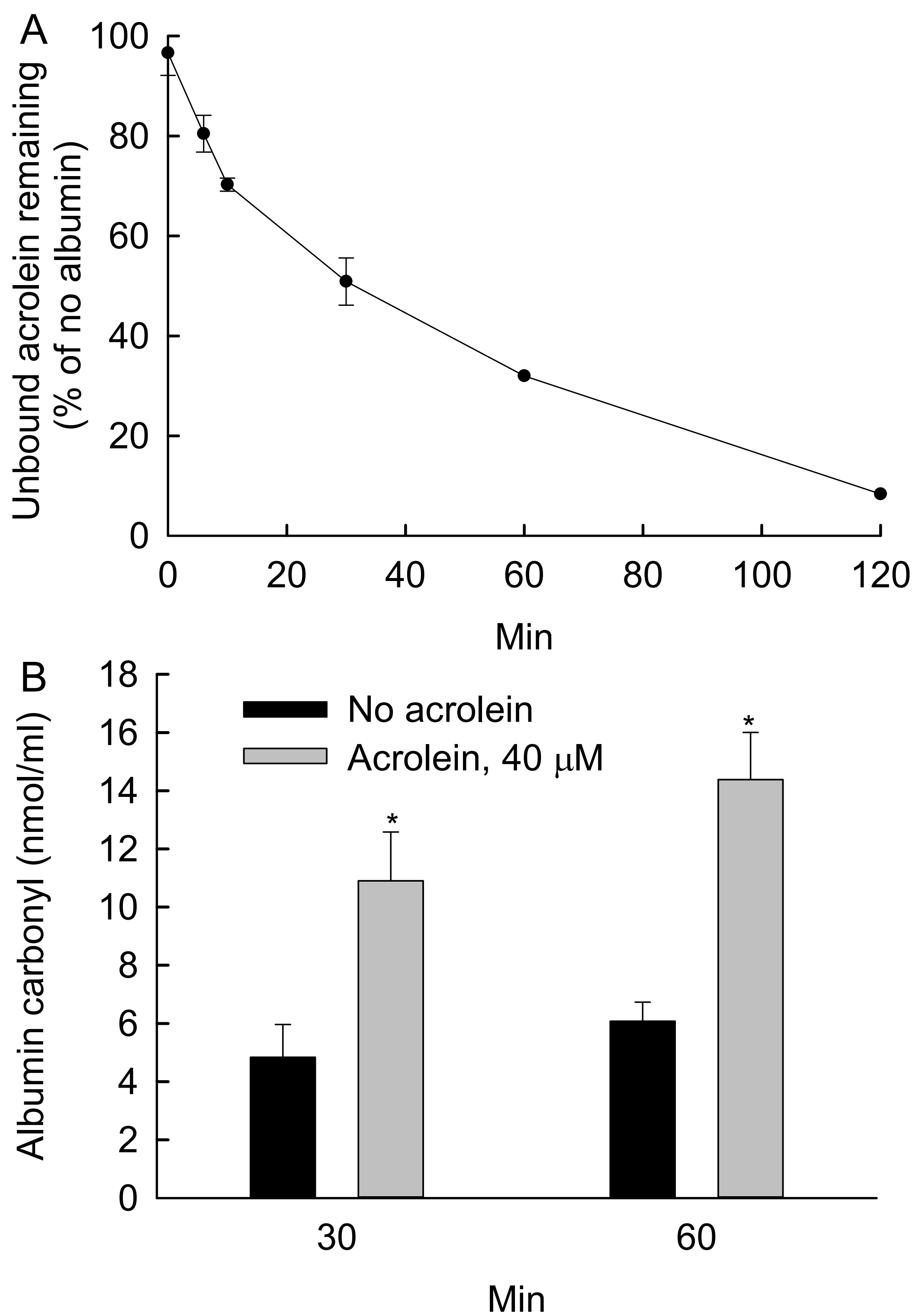

Figure 1

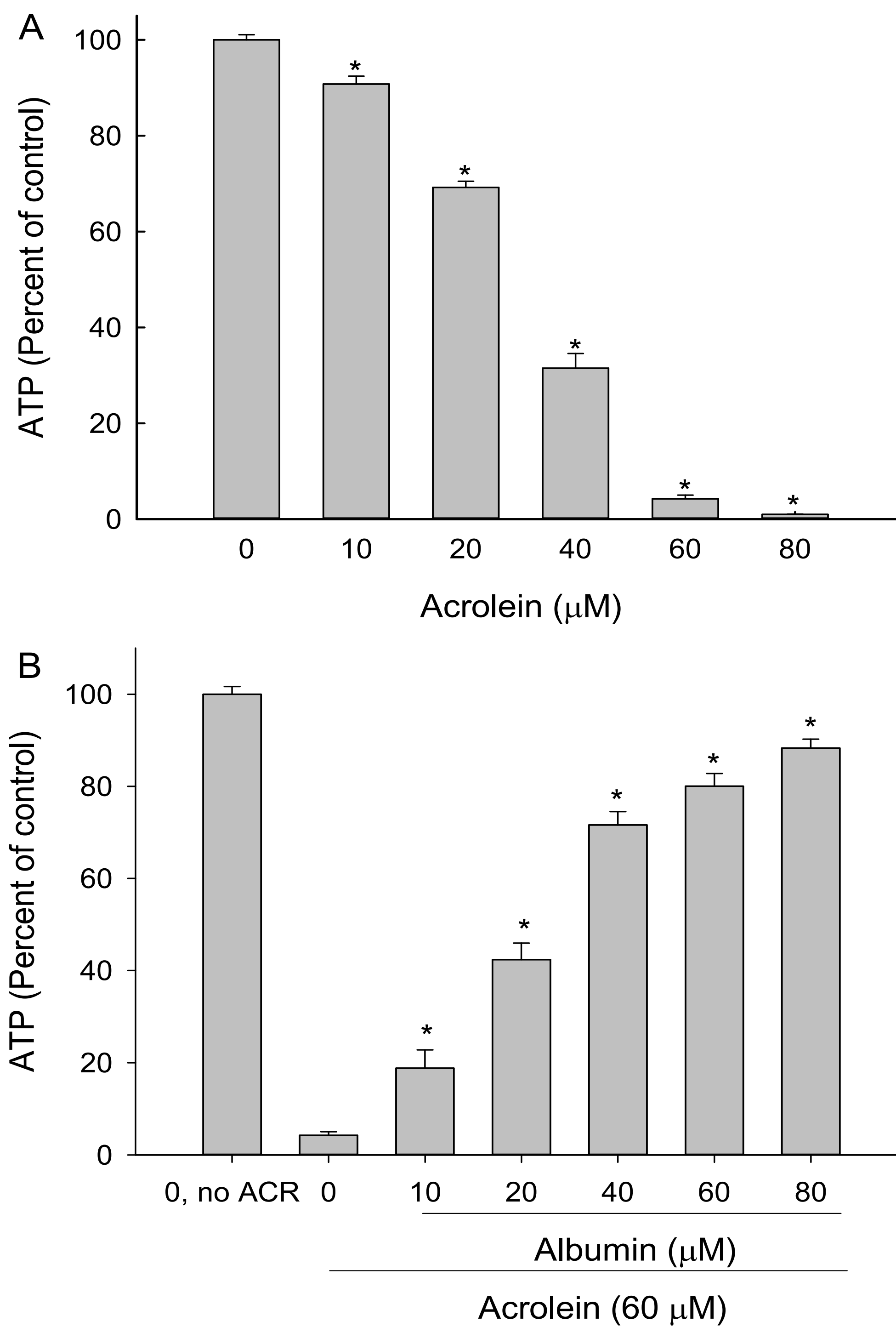

Figure 2

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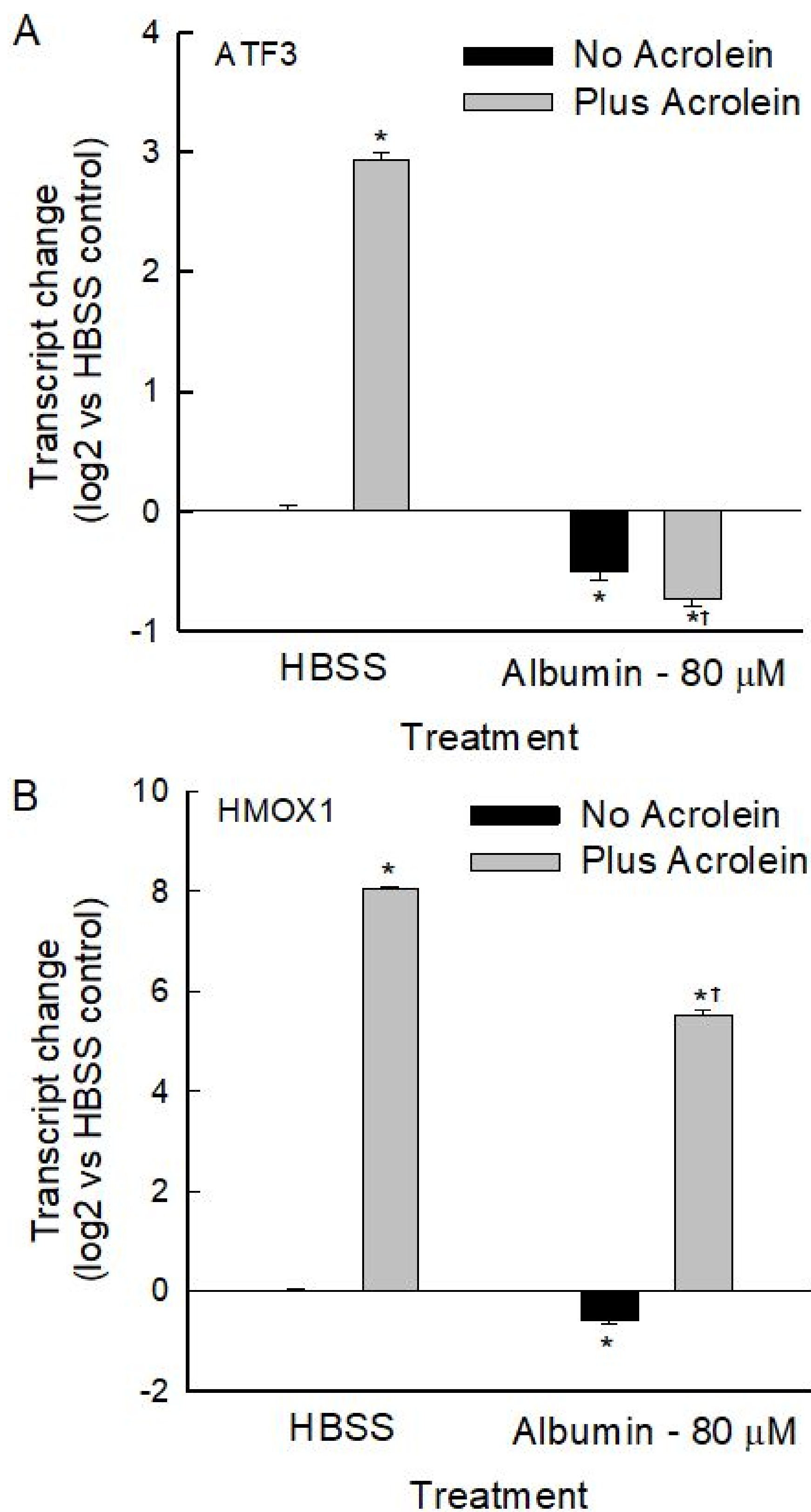

Figure 3

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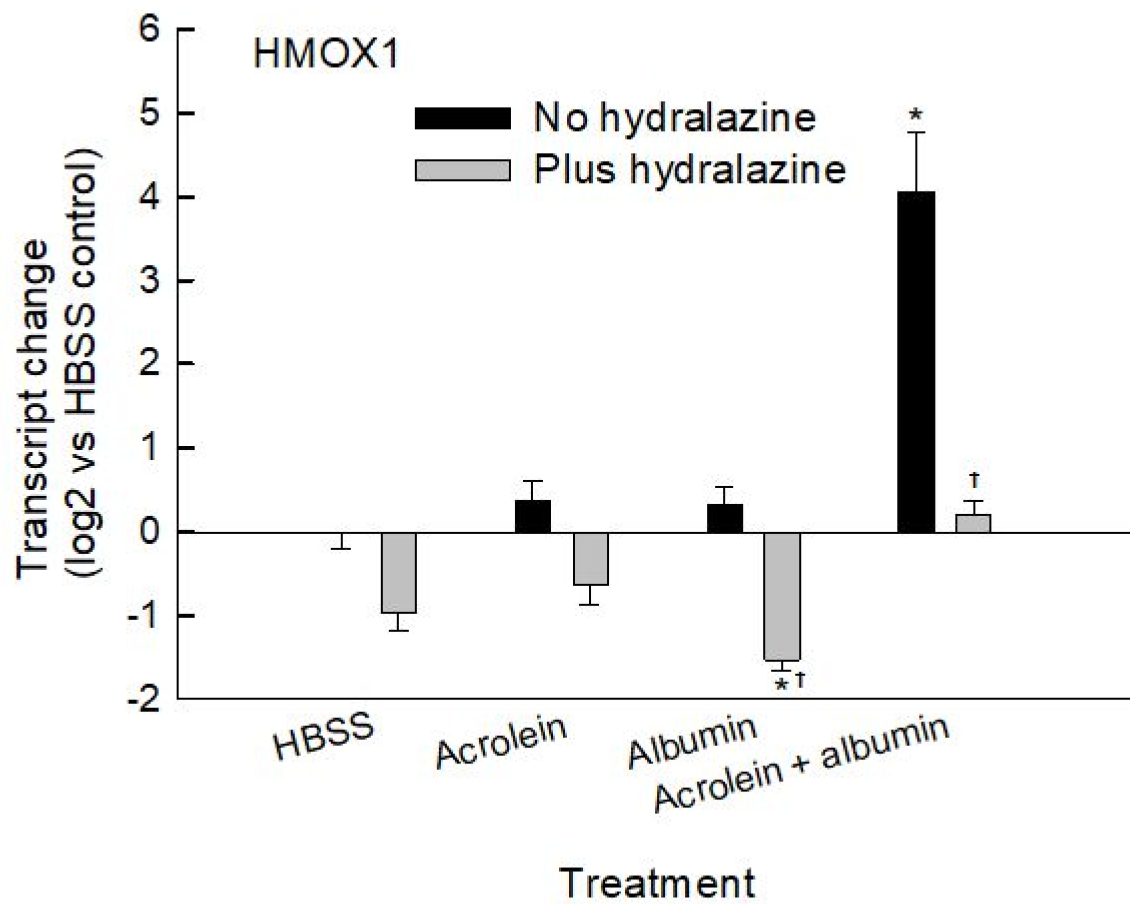

Figure 4

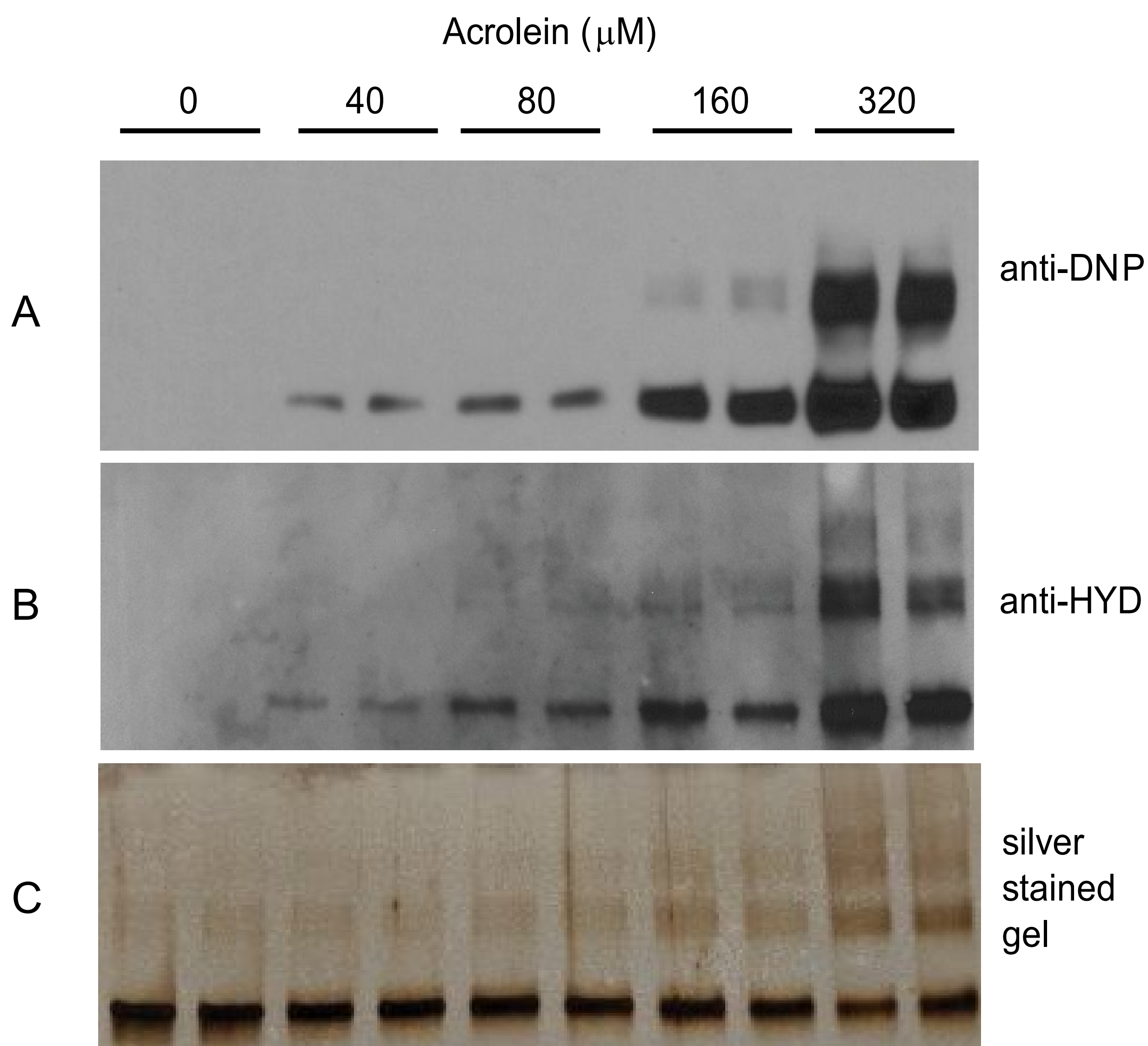

Figure 5

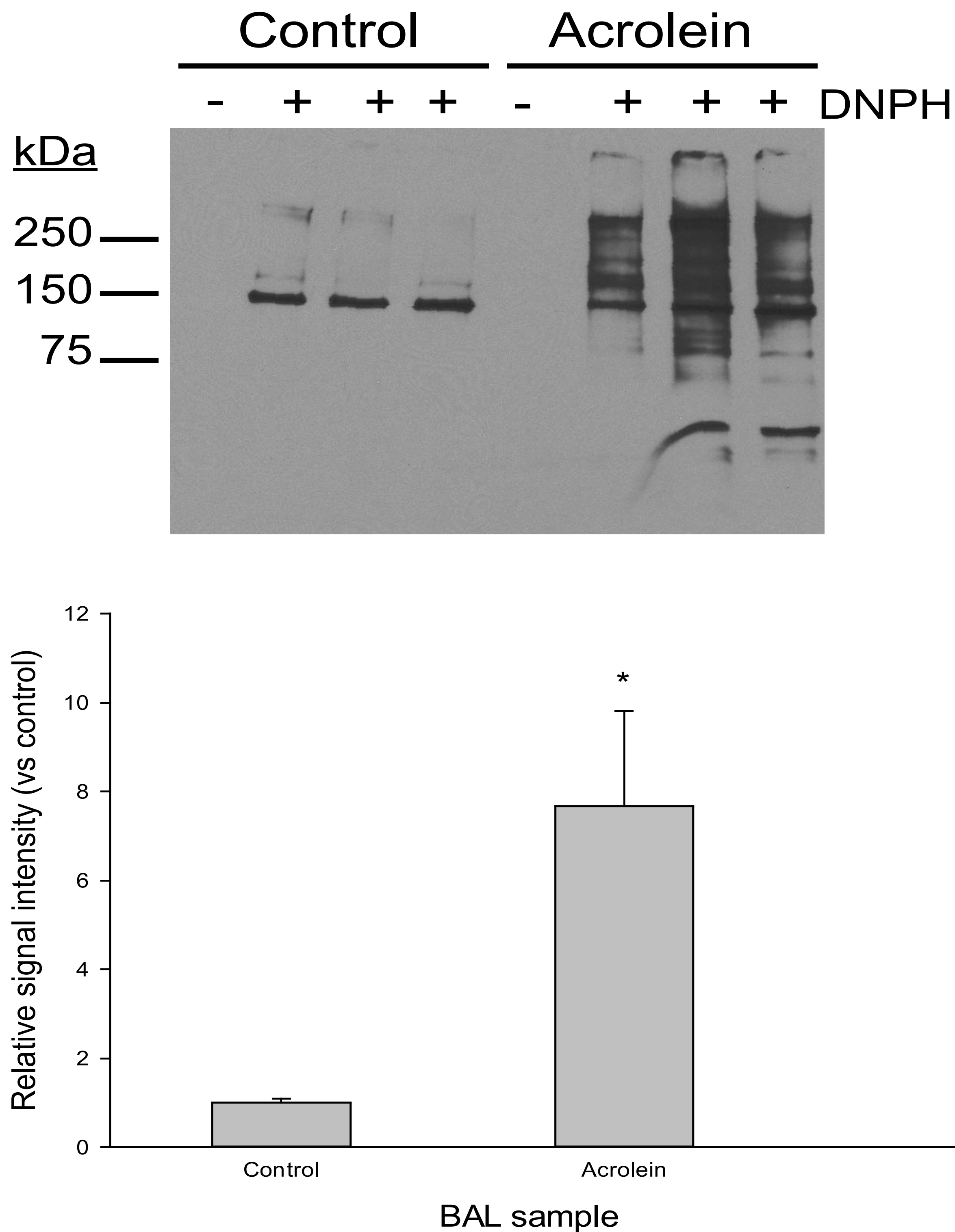

Figure 6

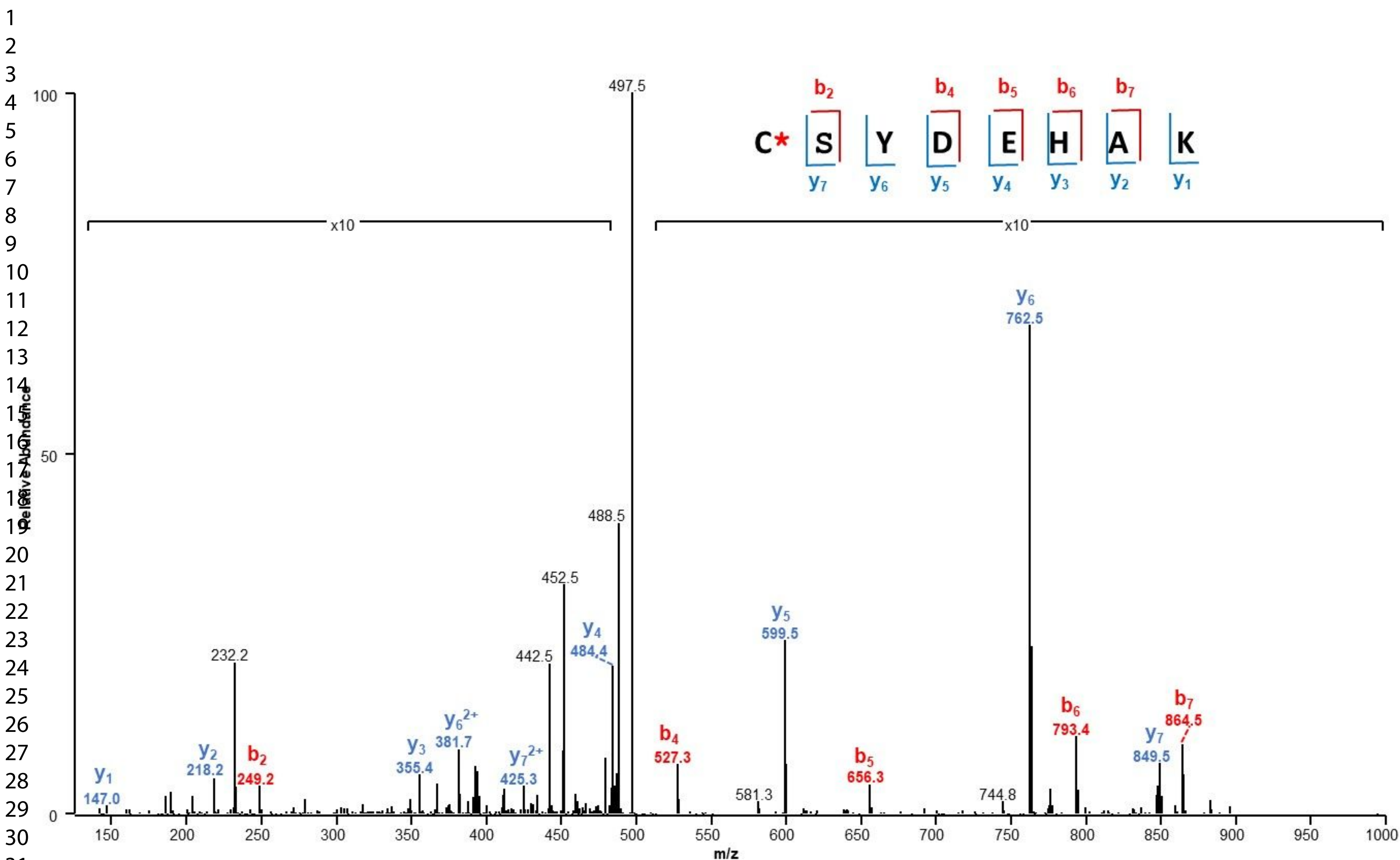

Figure 7