Supplemental Digital Content 1

2. Materials and Methods

The study was conducted according to the guidelines for the care and use of laboratory animals of the National Institutes of Health and the Association for the Assessment and Accreditation of Laboratory Animal Care. The standard procedures required for the study were carefully revised and approved by the Animal Care and Use Committee of the University of Texas Medical Branch.

2.1. Surgical preparation

Thirty-six adult female sheep (30-40 kg; merino; ~3 years) were surgically prepared under deep isoflurane anesthesia and buprenorphine analgesia to locate various access ports for hemodynamic assessment and blood sampling. Catheters were located in the left atrium, pulmonary artery (Swan-Ganz) (7F; Edwards Lifesciences LLC, Irvine, CA) and femoral artery (16-GA, 24 inches; Becton Dickinson, Franklin Lakes, NJ), as previously described (1). Following surgical preparation, the animals were allowed to recover for 5 to 7 days (until the day of study) under buprenorphine analgesia, with free access to food and water and with a basal infusion of lactated Ringer's solution (2 mL/kg/hour).

2.2. Smoke inhalation injury model

On the day of study, following the collection of baselines for hemodynamics and blood samples, smoke inhalation injury was induced under deep anesthesia and pain control as previously shown(2). Injury consisted of

insufflation of 48 breaths of cool cotton smoke. Following injury, animals were awakened, placed on a mechanical ventilator (Servo Ventilator 900C; Siemens, Elema, Sweden), and randomized into one of four groups; Including the Control group (injured and treated with 60 mL of normal saline every 12 hours, n=7), CC10-1 group (injured and treated with rhCC10 at a dose of 1 mg/kg/day (0.5 mg/kg every 12 hours), n=8), CC10-3 group (injured and treated with rhCC10 at a dose of 3 mg/kg/day (1.5 mg/kg every 12 hours), n=7) and CC10-10 group (injured and treated with rhCC10 at a dose of 10 mg/kg/day (5 mg/kg every 12 hours), n=8). CC10 doses were selected based on efficacy observed in different models of ALI (3-5). Sham animals (n=6) were instrumented but received sham injury and saline as treatment. rhCC10 or saline was administered as intravenous infusion via the central venous catheter every 12 hours starting one hour after injury (administered at 1, 13, 25, and 37 hours post-injury). rhCC10 was provided by Therabron Therapeutics, Inc. (Rockville, MD). The rhCC10 was formulated at 5.5 mg/ml in NaCl and the volume administered depended on the body weight and dose group. Volumes injected for 1 mg/kg were about 6-7 mLs, for 3 mg/kg ~20mLs, and 10 mg/kg ~65mLs. Hemodynamics, pulmonary function, and blood gases were recorded every 6 hours. Urine and plasma samples were collected every 6 hours. Animals were monitored for 48 hours post-injury, then sacrificed, and lungs harvested as previously described (6). Criteria for euthanasia prior to 48-hour endpoint was PaO_2 < 50 mmHg or $PaCO_2$ > 90 mmHg for one hour regardless of adjustments of FiO_2, respiratory rate, and pulmonary toilet. The degree of injury was measured by comparing COHb

levels following smoke inhalation, the percent of bronchial exfoliation and the development of hypoxemia.

2.3. Fluid resuscitation, assessment of fluid balance and plasma protein

After the injury, the animals were resuscitated with Ringer's lactate using the Parkland formula (4 mL X 40 X Kg X day, with half of the daily fluid requirement delivered in the first 8 hours). The majority of patients suffering from smoke inhalation injury are affected by large cutaneous burn injuries; therefore, we provided a fluid plan close to what a burn patient receives. To accurately monitor fluid balance during the study, the sheep had free access to food, but water was restricted. (In this model, the animals are intubated in the trachea below the mouth and larynx, allowing free access to food during the study.) Urinary output was assessed with a urinary bladder catheter (Foley Catheter, 14Fr; BARDEX®, Covington, GA). Total protein concentration in fresh plasma was evaluated using a refractometer (National Instrument, Baltimore, MD), as it has shown to correlate well with colloid osmotic pressure (6).

2.4. Mechanical ventilation and lung biomechanics

All subjects were mechanically ventilated in a volume control mode with a positive end-expiratory pressure (PEEP) of 5 cm H_2O, a tidal volume (Vt) of 12 mL/kg and a respiratory rate (RR) of 20 breaths/minute. During the first 3 hours after injury, the inspired O_2 (FiO_2) concentration was maintained at 100% to induce rapid clearance of carbon monoxide after smoke inhalation. After 3 hours, FiO_2 was adjusted to keep the arterial PaO_2 between 90 and 110 mm Hg. The PCO_2 was maintained between 25 and 35 mm Hg by

adjusting the RR. The airway pressures were recorded every 6 hours and mean airway pressure (Paw) and oxygenation index (OI) were calculated with a standard equation (6).

2.5. Hemodynamics and blood sample analysis

Through the various catheters surgically placed, mean arterial pressure (MAP), pulmonary artery pressure (PAP), left atrium pressure (LAP), pulmonary capillary occlusion pressure (PCOP), and core blood temperature were continuously measured with a hemodynamic monitor (IntelliVue MP50; Philips Medizin Systeme Boeblingen, Boeblingen, Germany) and recorded every 6 hours. Simultaneously, blood was sampled from the femoral catheter every 6 hours and the PO_2, PCO_2, pH, base excess, carboxyhemoglobin (COHb), SO_2, hematocrit, hemoglobin, glucose, lactate and electrolytes were measured using a blood gas analyzer (GEM Premier 3000; Instrumentation Laboratories, Lexington, MA).

2.6. Lung tissue analysis

Immediately after euthanasia, a section of the right inferior lung was taken and stored in formalin for histopathology analysis. The samples were processed as previously described, stained with H & E and scored by two treatment-blinded pathologists(7). Samples from the same region of the right lung were also taken and frozen immediately in liquid nitrogen and stored at -80°C for biochemical analysis.

2.7. Biochemical lung analysis.

mRNA: Messenger RNA was extracted from lung tissue samples using RNeasy Mini Kit (Qiagen, Venlo, Netherlands), then CC10 mRNA was measured using q-PCR. Cyclophilin was used as the control for mRNA quantitation.

CC10 ELISA: Protein was extracted from lung tissue by grinding frozen tissue using a mortar and pestle then homogenized in PBS buffer using 3 freeze-thaw cycles at -80°C and room temperature, then sonicated at 20 kHz for 40 seconds. The PBS extract was then centrifuged at 12,000 g and the supernatant was analyzed for total protein content by BCA (Pierce-Thermo-Fisher, Waltham, MA) and total CC10 content (combination of native ovine and rhCC10) by competitive CC10 ELISA, as previously reported(3-5, 8).

MPO activity: 100 mg of frozen lung tissue was ground using a mortar and pestle, then resuspended in 1 mL cold 50 mM KPO_4, pH 6.0, vortexed, and centrifuged at 10,000g for 10 minutes. The pellet was resuspended in 0.5% hexadecyltrimethylammonium bromide (CTAB), 50 mM KPO_4, pH 6.0, then subjected to 3 freeze-thaw cycles at -80°C and room temperature, sonicated at 20 kHz for 40 seconds, then centrifuged at 10,000 g for 5 minutes. MPO activity was measured by incubating 10 μl CTAB extract in 100 μl substrate buffer (50 mM KPO_4, pH 6.0, 0.2 mg/mL o-dianisidine, 1 mM H_2O_2) in microtiter plates at room temperature for 5 hours then read at 450 nM. Standard curves were generated with MPO (Calbiochem, San Diego, CA). Blanks containing CTAB extract plus buffer were also done on each plate and all samples were analyzed in duplicate. MPO activity was normalized to total protein concentration measured by BCA assay (Pierce-Thermo-Fisher,

Waltham, MA) and data were expressed as units MPO activity per milligram of total protein.

Carbonyl ELISA: A Protein Carbonyl ELISA kit (Cell Biolabs, San Diego, CA) was used to evaluate carbonyl content in CTAB-dissolved lung tissue according to the manufacturer's instructions. Data are expressed as nmol of protein carbonyl/mg of total protein.

2.8. Recombinant ovine CC10 and anti-ovine CC10 antibody

A gene encoding ovine CC10 was synthesized using the reported mRNA sequence (Genebank accession #FJ959385). Recombinant ovine CC10 was over-expressed in bacteria as a fusion with an His-tagged ubiquitin-like protein using a T7 expression system in E. coli strain BL21/DE3, then purified to >95% purity by SDS-PAGE, using IMAC chromatography. Approximately 6 mg of recombinant ovine CC10 (roCC10) was produced. Two rabbits were immunized with roCC10 and IgG was purified from antisera. Both antibodies recognized reduced and non-reduced roCC10, and native ovine CC10 in lung tissue by Western blot. Both antibodies also recognized rhCC10 using Western blot (Data not shown). Rabbit polyclonal anti-rhCC10 antibodies were also found to cross-react with roCC10 and native ovine CC10 (not shown).

2.9. Pharmacokinetic Analysis.

To measure the concentrations of rhCC10, additional plasma samples were collected at baseline prior to injury and dosing with rhCC10, at 3 hours post-injury (2 hours after the first dose of rhCC10) and 48 hours, post-injury (11

hours after the fourth and last dose). These time points were chosen based on previous work showing the peak concentration and half-life of rhCC10 in piglets (5). All samples were stored at -80ºC until analyzed. Total CC10 (combination of native ovine CC10 and rhCC10) was measured in plasma by competitive ELISA as previously described (8).

2.10. *In vitro* modification of rhCC10 by ROS

Oxidative stress is a hallmark of ALI and ARDS(9), including SII (as measured by carbonyl content in protein extracts from lung tissue in the present study). Studies have shown that native CC10 is modified by reactive oxygen species (ROS) *in vivo*, in human premature infants experiencing severe respiratory distress, and developed bronchopulmonary dysplasia (BPD), a severe type of chronic lung disease(10, 11). Therefore, three types of reactions were used to simulate ROS modifications to rhCC10 *in vitro*: 1) Neutrophil myeloperoxidase (MPO) + H_2O_2: rhCC10 was pre-incubated for 30 minutes at 37°C in 2 mM $CaCl_2$ and 10 mM citrate buffer (pH 5.0) prior to addition of increasing amounts of MPO and 25 equivalents of H_2O_2, then incubated in the dark for 30 minutes at 37°C. After the incubation, the reaction was boosted with another aliquot of MPO and H_2O_2 and incubated for a further 30 minutes at 37°C. 2) Meta-chloroperbenzoic acid (mCPBA): Reactions were performed at room temperature (24-27°C), incubated for 15 minutes in the dark in a total volume of 0.2 mL. Reactions were initiated by adding the mCPBA. 2-100 oxidizing equivalents were used and the reactions were monitored by RP-HPLC. 3) Sodium hypochlorite (NaOCl): Reactions were performed on ice (~4°C) in the dark in a total volume of 0.2 mL. Reactions were initiated by

adding the NaOCl and quenched by adding 0.1M L-methionine. 1-100 oxidizing equivalents were used and the reactions were monitored by RP-HPLC. All reactions were quenched by adding 0.1M L-methionine.

2.11. Analysis of ROS modifications of rhCC10

Unmodified rhCC10 appears as a single peak by RP-HPLC analysis. Reaction progress was monitored by the appearance of new peaks on RP-HPLC, which was performed on an Agilent 1100 system using a VYDAC Polymeric C18 Column 300A, 5 micron, 2.1mmx250mm, (Cat #218TP52) using a mobile phase as follows: A: 0.1% in water; B: 95% acetonitrile + 0.1% TFA in water at a flow rate of 0.3 mL/min. Output was monitored by UV absorption at 214 nm. The single starting peak representing the unmodified rhCC10 homodimer turned into several peaks as the reaction progressed, and eventually became a single broad peak, except for mCPBA (the mildest of the 3 oxidants), which shifted to another single sharp peak under the conditions used (Supplemental Figure 7). Individual peaks were isolated by RP-HPLC and analyzed by electrospray mass spectrometry (Combinix, Inc., San Francisco, CA) to measure the intact mass of 17 peaks isolated from mCPBA and MPO reactions. Most but not all new mCPBA peaks represented single oxidation events, which were later mapped using mass spectrometry of proteolytic digests to methionine residues. The presence of carbonyl groups was demonstrated by reaction with dinitrophenylhydrazine (DNPH) followed by Western blot of SDS-PAGE gels using a rabbit polyclonal anti-DNP antibody (Sigma-Aldrich, Inc., St. Louis, MO). Reaction products were also analyzed by isoelectric focusing using commercially prepared pI 3-7 gels (InVitrogen, Inc.,

Waltham, MA), using ~25 mcg of each rhCC10 reaction per lane. The predicted isoelectric point (pI) of both native human CC10 protein and unreacted rhCC10 is 4.8 (Supplemental Figure 8).

2.12. Statistical analysis

Calculations were performed using SPSS Version 19.0 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism version 6 (GraphPad Software, San Diego, USA). Sampling distribution of the collected data was assessed by Shapiro-Wilk normality test. Analysis of the treatment effect of one parameter over time was evaluated using Linear Mixed Model, and adjusted pairwise comparisons by Fisher's test were used to determine which of the treatments had a different effect. Sets of data for a single time point; such as lung tissue samples, were analyzed using one-way analysis of variance followed by adjusted pairwise comparison. The survival time and mortality among groups was evaluated with log-rank test adjusted for multiple comparisons, and Fisher's exact test, respectively. Correlation coefficient between two non-normally distributed variables was assessed by Spearman's rank test. Values reported are expressed as mean ± SEM. The differences were considered significant when the p-value was smaller than 0.05.

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