Original Paper

ITRAQ-Based Proteomics Analysis of Acute Lung Injury Induced by Oleic Acid in Mice

Xiaotao Xu^a Qingwei Zhu^b Rong Zhang^a Yan Wang^a Fangfang Niu^a
Wenying Wang^a Dawei Sun^a Aizhong Wang^a

^aDepartment of Anesthesiology, Shanghai Jiao Tong University Affiliated Sixth People's Hospital, Shanghai, ^bDepartment of Neurosurgery, Shanghai Jiao Tong University Affiliated Sixth People's Hospital, Shanghai, China

Key Words

Lung injury • Oleic acid • Proteomics • ITRAQ • Antithrombin III

Abstract

Background/Aims: This study was conducted to investigate the relationship between differentially expressed proteins (DEPs) and the pathogenesis of oleic acid (OA)-induced acute lung injury (ALI) in mice. **Methods:** Eight-week-old male C57BL/6 mice were injected with OA through the tail vein and sacrificed 6 hours after OA administration to identify protein expression levels in lung tissue using isobaric tags for relative and absolute quantification (iTRAQ) technology. Then, DEPs such as antithrombin III (AT III), 12-lipoxygenase (12-LO), dedicator of cytokinesis 2 (DOCK2), polycystin-2 and plasminogen were identified by western blotting. Subsequently, we focused on investigating the effect of AT III on endothelial integrity using siRNA interference technology. The levels of IL-6, IL-1 β , TNF- α and TGF- β expression were detected using an enzyme-linked immunosorbent assay (ELISA). Alterations in the tight junction component ZO-1 and the phosphorylation of myosin light chain (pMLC) were determined by western blotting. The stress fiber F-actin were also detected by immunofluorescence staining. In addition, endothelial permeability was determined via a transwell permeability assay. **Results:** A total of 5152 proteins were found to be expressed in lung tissues from the OA-treated and saline-treated mice. Among these proteins, 849 were differentially expressed between the two groups, including 545 upregulated and 304 downregulated proteins. After AT III knockdown, the levels of inflammatory factors and endothelial permeability were elevated, the expression of ZO-1 was decreased, and the expression of F-actin and pMLC was increased. All these results illustrated that AT III knockdown exaggerated the disruption of endothelial integrity mediated by OA. **Conclusion:** These findings using iTRAQ technology demonstrate, for the first time, differences in the lung tissue expression levels of proteins between OA-treated mice and saline-treated mice. This study reveals that 12-LO, DOCK2 and especially AT III may be candidate biomarkers for OA-induced acute lung injury.

© 2017 The Author(s)
Published by S. Karger AG, Basel

X.-T. Xu and Q.-W. Zhu contributed equally to this work.

Dr. Ai-Zhong Wang

Department of Anesthesiology, Shanghai Jiao Tong University
Affiliated Sixth People's Hospital, No. 600 Yishan Road, Shanghai, (China)
Tel. +86-21-3829-7723, E-Mail w19680420@sohu.com

Introduction

In 2001, the acute respiratory distress syndrome (ARDS) was redefined by experts convened in Berlin. The Berlin definition of ARDS included three categories based on degree of hypoxemia: mild ($200 \text{ mm Hg} < \text{PaO}_2/\text{FiO}_2 \leq 300 \text{ mm Hg}$), moderate ($100 \text{ mm Hg} < \text{PaO}_2/\text{FiO}_2 \leq 200 \text{ mm Hg}$), and severe ($\text{PaO}_2/\text{FiO}_2 \leq 100 \text{ mm Hg}$). In addition, four ancillary variables were also used to assess the severe ARDS: radiographic severity, respiratory system compliance ($\leq 40 \text{ mL/cm H}_2\text{O}$), positive end-expiratory pressure ($\geq 10 \text{ cm H}_2\text{O}$), and corrected expired volume per minute ($\geq 10 \text{ L/min}$) [1, 2]. ARDS is characterized by massive inflammatory cell infiltration, increased capillary permeability, severe alveolar damage and the formation of hyaline membranes [3-6]. The primary insults leading to ARDS include microbial infection, pollutants, toxic gasses, gastric acids, autoantibodies, fatty emboli, free fatty acids and transfusion [7, 8].

Within the past several years, isobaric tags for relative and absolute quantification (iTRAQ) technology has been used to investigate idiopathic pulmonary fibrosis [9] and hepatic ischemia-reperfusion-induced lung injury [10]. However, to our knowledge, no previous studies have used quantitative proteomics technologies to identify DEPs and their associated signaling pathways in OA-induced ALI.

To detect the potential biomarkers that could be likely implicated in the development of ARDS in this study, we present the first proteomics analysis of lung tissues using the iTRAQ approach, in which we identified a set of DEPs from the lungs of OA-treated and saline-treated mice. One study has demonstrated that intravenous injection of OA disrupts the endothelial barrier and causes severe lung edema [11]. In addition, the degree of abnormalities in coagulation and fibrinolysis proteins may strongly impact the outcomes of patients with ARDS [12]. Interestingly, the coagulation and complement pathways were among the top signaling pathways identified in this study. Moreover, it has been reported that antithrombin III (AT III), one upregulated component of the coagulation and complement pathways in our study, exhibits anti-inflammatory effect via decreasing the endothelial hyperpermeability of ALI in the animal models of burn and smoke inhalation [13]. Accordingly, we first validated the lung tissue expression levels of AT III by western blotting in OA-induced ALI, and then further confirmed the effect of AT III on OA-induced endothelial injury in human lung microvascular endothelial cells (HMVECs) by means of RNA interference technology. To further confirm the effect of AT III on OA-induced endothelial injury, human lung microvascular endothelial cells (HMVECs) were transfected with a specific siRNA against AT III. In our work, we found that the expression of AT III demonstrated by western blotting analysis were in accordance with that obtained using iTRAQ in the lung tissue, and knockdown of AT III exaggerated OA-induced endothelial cell integrity damage in HMVECs, suggesting that the expression levels and functional changes of AT III is related to OA-induced ALI. Our data showed that AT III may be a potential biomarker that could be likely implicated in the development of ARDS.

Materials and Methods

Cell culture

HMVECs were obtained from the Lonza (Walkersville, MD, USA). The HMVECs were grown in endothelial basal medium 2 (EBM 2) (Lonza) supplemented with endothelial growth medium SingleQuots and 5% FBS (Gibco BRL, MO). HMVECs were maintained at 37°C in a humidified 5% CO_2 incubator.

Animals

Mice were purchased from the Shanghai SLAC Laboratory Animal Co. Ltd. and housed in a specific pathogen-free facility with 12-h light/dark cycles and free access to food and water. All animal experiments and procedures were approved by the Institutional Animal Care and Use Committee of the Sixth People's Hospital Affiliated with Shanghai Jiao Tong University (SYXK [Shanghai, China] 2011-0128, 1 January 2011) and were conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (No. 85-23, revised 1996, 1 January 1996).

Animal model and grouping

For OA-induced ALI, mice were injected with OA (200 μ L/g, O1383, Sigma) via the tail vein. Mice treated with equal volumes of sterile saline served as the control group. The mice were killed 6 hours after OA administration. The perfused left lungs were cut into 5- μ m paraffin sections, followed by staining with hematoxylin-eosin (H&E) for histological examination.

Protein lysis, SDS-PAGE separation, digestion and iTRAQ labeling

Protein lysis and SDS-PAGE separation. Lung tissues were frozen in liquid nitrogen and ground into powder using a glass homogenizer. The powder was resuspended and mixed with 5 volumes of TCA/acetone (1:9). The mixture was stored at -20 °C for 4 h and centrifuged at 6000 g for 30 min at 4 °C. The supernatant was discarded, and the precipitate was washed three times with pre-cooled acetone. SDT buffer was added to the precipitate, and the sample was mixed and boiled for 5 min. The lysate was collected, sonicated and boiled for an additional 15 min. The supernatant was then centrifuged at 14000 g for 40 min, followed by filtration through a 0.22- μ m filter. The filtrate was quantified using a BCA Protein Assay Kit (P0012, Beyotime), and the samples were stored at -80 °C until further use. Next, 20 μ g of protein from each sample was mixed with loading buffer, boiled for 5 min and then separated on a 12.5% SDS-PAGE gel. Protein bands were then visualized with Coomassie Blue R-250 staining.

Filter-aided sample preparation (FASP digestion). A 200- μ g aliquot of protein from each sample was added to 30 μ L of SDT buffer (4% SDS, 100 mM DTT, and 150 mM Tris-HCl, pH 8.0). The detergent, DTT and other low-molecular-weight components were then removed using UA buffer (8 M urea and 150 mM Tris-HCl, pH 8.5) with repeated ultrafiltration (Sartorius, 30 kD). To block reduced cysteine residues, 100 μ L of iodoacetamide (100 mM IAA in UA buffer) was added, and the samples were incubated in the dark for 30 min. The filters were washed three times with 100 μ L of UA buffer, followed by two washes with 100 μ L of dissolution buffer (DS buffer). Finally, the protein suspensions were digested with 4 μ g of trypsin (Promega) in 40 μ L of DS buffer overnight at 37 °C, and the resulting peptides were collected as filtrate samples. Using UV light spectral density at 280 nm with an extinction coefficient of 1.1, the peptide content of a 0.1% (g/L) solution was calculated based on the frequencies of tryptophan and tyrosine in vertebrate proteins.

iTRAQ labeling. A 100- μ g aliquot of peptide mixture from each sample was labeled using the iTRAQ reagent according to the manufacturer's instructions (Applied Biosystems, Milan, Italy). Briefly, pooled samples from the saline-treated mice were labeled with the 113, 114, and 115 iTRAQ tags, whereas those from the OA-treated mice were labeled with the 116, 117, and 118 tags. The labeled peptide samples were incubated at room temperature for 1 h and then mixed at equivalent ratios. One machine repeat was added to control for the randomness of the experiment. The iTRAQ procedure was conducted twice for protein screening.

Peptide fractionation with reversed-phase (RP) chromatography

RP chromatography was performed to remove all interfering substances using the Agilent 1260 Infinity II HPLC Pump system. The vacuum-dried peptide mixture was reconstituted with buffer A (10 mM HCOONH₄, 5% ACN, pH 10.0) and loaded onto a 4.6 mm X 100 mm XBridge Peptide BEH C18 column containing 5- μ m particles. The peptides were eluted at a flow rate of 1 mL/min with a gradient of 0%–7% buffer A for 5 min, 7–40% buffer B (10 mM HCOONH₄, 85% ACN, pH 10.0) for 5–40 min, 40%–100% buffer B for 45–50 min, and then 100% buffer B for 50–65 min. The elution was monitored by measuring the absorbance at 214 nm, and fractions were collected every 1 min during the 5- to 50-min run period. The collected fractions were then dried via vacuum centrifugation.

LC-MS/MS and data analysis

LC-MS/MS analysis was performed on a Q Exactive mass spectrometer (Thermo Fisher Scientific) coupled to an Easy Nano Liquid Chromatography apparatus (nLC, Thermo Fisher Scientific). The peptide mixture was loaded onto a 15-cm Acclaim PepMap RSLC C18 reversed-phase analytical column (P/N164943; Thermo Fisher Scientific, San Jose, CA) in buffer A (0.1% formic acid). The sample was loaded at 8 μ L/min for 4 min and then was subjected to the following gradients: 300 nL/min buffer B (80% acetonitrile, 0.1% formic acid), a linear gradient to buffer B for 5 min, 6–38% buffer B for 45 min, 38–100% buffer B for 5 min and a final hold in 100% buffer B for 5 min. The mass spectrometer was operated in positive ion mode, and the MS data were obtained using a data-dependent top10 method that dynamically selected the most

abundant precursor ions from the survey scan (350–1800 m/z) for HCD fragmentation. The automatic gain control (AGC) target was set to 3e6, and the maximum injection time was set to 50 ms. Survey scans were acquired at a resolution of 70,000 at 200 m/z, the HCD spectra resolution was set to 17,500 at 200 m/z, the isolation width was 2 m/z, and the normalized collision energy was 30 eV.

Data analysis. The MS/MS spectra were searched using the MASCOT engine (Matrix Science, London, UK; version 2.5) embedded in Proteome Discoverer 2.1. Next, we performed a bioinformatics analysis of the identified proteins, and DEPs were defined in the iTRAQ experiment according to the following criteria: unique peptides ≥ 1 , P-value < 0.05 , fold change > 1.2 (or < 0.8333). The DEPs were annotated using the Gene Ontology (GO) database (<http://www.geneontology.org/>), and protein classification was performed based on functional annotations using the GO terms for cellular components, biological processes and molecular functions. A Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of the DEPs was conducted using the KEGG database (<http://www.genome.jp/kegg/>).

Verification of proteins by western blot analysis

The perfused lung tissues were homogenized in lysis buffer with a glass homogenizer. The lysates were centrifuged at 12,000 rpm for 10 min at 4 °C, and the supernatant was collected. The total protein concentration in the supernatant was quantified using a Pierce BCA protein assay kit. The protein extracts (50 µg/sample) were loaded on 10% gels for electrophoresis before transfer to PVDF membranes. The membranes were blocked with 5% dry milk in Tris-buffered saline with 0.1% Tween-20 for 2 h and incubated with specific primary antibodies against AT III (1:200; sc-365194, Santa Cruz), 12-LO (1:200; sc-365194, Santa Cruz), DOCK2 (1:200; sc-365242, Santa Cruz), polycystin-2 (1:200; sc-47734, Santa Cruz), plasminogen (1:200; sc-376405, Santa Cruz) and ZO-1 (1:200, ab59720, Abcam) overnight at 4 °C, followed by incubation with a horseradish peroxidase-conjugated secondary antibody for 1 h. The loading control was the constitutively expressed β -actin protein (1:5000; Hangzhou HuaAn Biotechnology Co. Ltd.). The blots were visualized using an enhanced chemiluminescence system.

RNA interference and transfection

AT III siRNA oligonucleotides were synthesized by GenePharma (Shanghai, China). The target sequences were as follows: siRNA-802: 5'-CGAAUCACCGAUGUCAUUCTT-3'; siRNA-854: 5'-UGCUGGUAAACAC-CAUUUATT-3'; and siRNA-919: 5'-AAGGAACUGUUCUACAAGGTT-3'. The sequence 5'-UUCUCCGAACGUGU-CACGUTT-3' was used as a negative control siRNA. HMVECs were transfected with 100 nM siRNA using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. siRNA knockdown was confirmed by western blot 72 h after transfection, and only the most effective siRNA species was used in the following study.

Enzyme-linked immunosorbent assay (ELISA)

HMVECs were transfected with AT III siRNA and control siRNA for 72 h and then treated with OA (0.5 mmol/L) for an additional 24 h. The supernatants were collected for the measurement of IL-6, IL-1 β , TNF- α and TGF- β concentrations by ELISA using commercially available ELISA kits (ExCell Biology, Inc., Shanghai, China). ELISA was performed according to the manufacturer's instructions. All samples from the AT III siRNA group and the control siRNA group were tested in triplicate.

Immunofluorescence staining

First, endothelial cells were plated on cover slips in 24-well plates and pretreated with 0.5 mmol/L OA for 24 h. The cells were then fixed with 4% paraformaldehyde for 10 min at room temperature (RT). The cells were blocked in 2% BSA for 2 h at RT and incubated overnight with a primary antibody against ZO-1 (1:100; ab59720, Abcam). Secondary antibodies (1:200; goat anti-rabbit IgG [Alexa Fluor 594], ab150080) were from Abcam. F-actin was stained with phalloidin labeled with rhodamine (Sigma, USA), and nuclei were stained with the DNA-binding fluorescent dye DAPI (Beyotime) at RT. After staining, the cells were washed three times with PBST and analyzed using a fluorescence microscope (Olympus, Tokyo, Japan).

FITC-dextran transwell permeability assay

A previously described transwell permeability assay was used to determine the degree of HMEC monolayer permeability [14]. Briefly, after transfection with AT III siRNA or control siRNA, HMVECs were

grown to confluency on matrigel-coated transwell inserts containing 3- μ m pores (EMD Millipore) in a 24-well plate. Twenty-four hours after plating, FITC-labeled dextran (3 kDa, Invitrogen) and OA were added to the lower chamber to final concentrations of 10 μ g/mL and 0.5 mmol/mL, respectively. Ten-microliter aliquots of medium were removed from the upper inserts, diluted in 90 μ L of water/well in a 96-well plate 60 min after treatment, and then analyzed for fluorescence intensity by fluorometry.

Statistical analysis

The data are expressed as the means $\pm$ SD. A two-tailed Student's t-test was used to analyze differences between the two groups. P-values less than 0.05 were considered to indicate statistical significance.

Results

Successful establishment of OA-induced ALI model and volcano plots of DEPs

The histologic results indicated that lung tissues from OA-treated mice had massive inflammatory cell infiltration and fibrin deposition in the air space, thickened alveolar walls, edema, and formation of hyaline membranes compared with lung tissues from saline-treated mice. Based on the histologic and pathologic results, we determined that the OA-induced ALI model was well established (Fig. 1A).

Volcano plots were created using P-values ($-\log_{10}$ P-value) versus the $\log_2$ fold change in each protein to compare the level of significance and magnitude of change across the global proteomes of the lung tissues between two groups. The volcano plots illustrated that a large proportion of proteins were remarkably upregulated or downregulated in the lung tissues of the OA-treatment group compared with those in the lung tissues of the saline-treatment group (Fig. 1B).

Identification of DEPs

To verify the DEPs in OA-induced lung injury, total proteins from the lung tissues of the OA-treatment group and the saline-treatment group were extracted for iTRAQ analysis. In total, 5152 proteins were detected using iTRAQ. Compared with the saline group, the expression levels of 849 proteins were significantly different (upregulated ≥ 1.2 -fold or downregulated ≤ 0.84 -fold; $P \leq 0.05$) in the OA group. Specifically, 545 of the 849 proteins were upregulated (Table 1), whereas the others were downregulated (Table 2). A hierarchical clustering heat map of the DEPs is shown in Fig. 2.

GO analysis of DEPs

The above DEPs were classified according to their biological processes, cellular components and molecular functions using the David database (Fig.

Fig. 1. Hematoxylin-eosin staining of lung tissues and volcano plots of differentially expressed proteins (DEPs). (A) Representative pathological and histological analyses of lung tissues from mice treated with OA and saline. (B) Volcano plot of quantified lung tissue proteins expressed in the OA-treatment group compared with those expressed in the saline-treatment (control) group. The X-axis indicates $\log_2$ -fold changes in up- (positive values) and downregulated (negative values) proteins. The Y-axis represents P-values in the form of $-\log_{10}$; smaller numerical values indicate higher P-values and lower credibility. Therefore, the pink dots in the top left quadrants represent downregulated proteins, and the pink dots in the top right quadrants represent upregulated proteins.

Table 1. The list of up-regulated differentially expressed proteins between OA group and control group by iTRAQ analysis

Accession	Fold Change	Accession	Fold Change	Accession	Fold Change	Accession	Fold Change	Accession	Fold Change	Accession	Fold Change	Accession	Fold Change
P98086	6.4419	H7BX99	2.7917	Q61247	2.3468	Q8D8E6	2.1609	Q9D8U6	1.9959	P07309	1.8363		
E9PZJ8	6.2833	Q00623	2.7658	P07724	2.3412	Q9Z1R9	2.1549	Q8R121	1.9845	P41731	1.8327		
Q8CDG1	4.5453	Q9CZW2	2.7512	Q9JHH6	2.3268	P29788	2.1476	P15508	1.9766	Q2WFW2	1.8293		
A8DUP7	4.4040	P11672	2.7413	B2RPR7	2.3091	P01027	2.1250	P15072	1.9732	P32261	1.8286		
Q6P8R3	4.3547	Q9Z284	2.7145	P61939	2.3082	Q00897	2.1109	Q3UKU5	1.9710	B2RWZ2	1.8242		
Q9QWK4	4.1336	Q3TR40	2.6165	P29918	2.3067	Q8CAG6	2.1068	P21614	1.9580	Q3V3V2	1.8211		
P09813	4.0120	Q01339	2.6151	E9Q604	2.2992	A0A0R410X5	2.1061	P29699	1.9552	A0A0N4SVW8	1.8088		
Q9EQI5	3.8961	Q8VC79	2.6024	P06683	2.2970	Q61599	2.1058	Q61704	1.9545	A0A0J9YTR2	1.7957		
Q61878	3.8766	E9Q1Y3	2.6015	P04919	2.2958	P31725	2.1027	Q3V3W7	1.9525	P01898	1.7912		
Q7TM98	3.8688	E9PV24	2.5928	Q88593	2.2951	B6ZCE5	2.1014	Q91WP0	1.9508	Q80ZU1	1.7911		
Q8CC67	3.8451	Q61838	2.5871	Q61093	2.2945	A0T1G3	2.0968	Q99KR7	1.9468	Q9D211	1.7902		
Q88299	3.6778	Q01488	2.5763	Q08692	2.2818	P11247	2.0958	A0A0R410I1	1.9390	Q8R2S8	1.7898		
B7ZN25	3.6261	K7TRL3	2.5159	A2A520	2.2799	G5E8I2	2.0930	Q70145	1.9155	E9PUM5	1.7892		
Q88947	3.5384	Q8VCM7	2.4897	A2MY50	2.2725	A0A0G21FK7	2.0875	P32037	1.9067	Q9CVI6	1.7889		
F7BGN3	3.4570	P28665	2.4688	O54890	2.2504	A0A0G21GD2	2.0826	A2AQ07	1.8919	A2AWF8	1.7866		
A0A140LH16	3.3962	P08226	2.4547	Q91VE7	2.2466	Q61702	2.0662	G3UYC6	1.8847	P11859	1.7819		
Q7TRP4	3.3914	P97290	2.4375	P26262	2.2437	Q06770	2.0656	P01872	1.8815	Q3UR44	1.7683		
F7CVM6	3.3772	Q35744	2.4053	Q06890	2.2358	A2AKM2	2.0635	A0A0J9YUP	1.8810	S4R293	1.7639		
Q03734	3.1992	Q3KQ04	2.3991	P23953	2.2317	A0A0U1RPM2	2.0604	Q3TSU8	1.8751	F6XR20	1.7612		
P04940	3.1521	P18528	2.3979	P08032	2.2231	B2RY51	2.0586	P41245	1.8741	A2NHM3	1.7542		
Q80SX2	3.1477	Q3TYT5	2.3930	B7ZWM9	2.2216	Q70138	2.0520	Q3U0C4	1.8665	G3XA09	1.7498		
Q61268	3.0259	A11L0U3	2.3824	Q9DBB9	2.2079	D3YM084	2.0454	Q8VB76	1.8587	Q9DAC2	1.7421		
B2RUF9	2.9846	O89020	2.3756	Q3UV74	2.1848	D3YX36	2.0443	Q1X79	1.8517	Q3TTT2	1.7412		
A0A075B5V	2.9333	D6RCQ0	2.3686	P49182	2.1816	Q64726	2.0321	P39655	1.8457	Q99P30	1.7366		
P28293	2.9318	P51910	2.3647	Q8CJ96	2.1811	Q8KOW0	2.0321	E9PDV2	1.8451	Q9D8W4	1.7310		
Q8VHL0	2.9206	Q54AH9	2.3646	Q91X72	2.1763	P97369	2.0316	O89053	1.8426	Q8K1B8	1.7226		
Q9ESB3	2.8889	B2RWX2	2.3623	B2RXM6	2.1753	P04186	2.0238	Q91YU9	1.8423	P40240	1.7188		
Q3UTF0	2.8349	Q8BSM7	2.3499	P08071	2.1752	P15327	2.0042	P56400	1.8402	A0A075B5M7	1.7107		
Q8BH35	2.8279	Q61703	2.3472	P41317	2.1715	Q54218	1.9988	P49290	1.8397	Q9CQI6	1.7084		
Accession	Fold Change	Accession	Fold Change	Accession	Fold Change	Accession	Fold Change	Accession	Fold Change	Accession	Fold Change	Accession	Fold Change
G3UZ60	1.7064	Q6ZQ14	1.6143	E9Q3Z4	1.5326	D3YU83	1.4757	Q61493	1.4398	Q3UVM5	1.3881		
G3UY13	1.7042	P06684	1.6106	Q8C6Q7	1.5296	Q61649	1.4755	Q00612	1.4361	Q8K215	1.3866		
P14106	1.7030	Q9D365	1.6060	Q8R5A3	1.5291	E9Q1P5	1.4741	Q9CVI3	1.4343	F6UIS1	1.3859		
Q91WP9	1.7018	O55042	1.6060	Q9D6K8	1.5248	A2A9N9	1.4725	E0CY03	1.4336	Q8BPY1	1.3814		
A0A0R41N3	1.7004	P27005	1.6057	Q3TV93	1.5177	A0A075B5K2	1.4722	Q8BJD1	1.4312	Q99JH8	1.3793		
D3YXK2	1.6943	Q3YXK2	1.6057	P39039	1.5123	F6SL78	1.4705	Q1WVWK3	1.4263	Q9DBT5	1.3781		
A2A614	1.6933	E9PXX9	1.6040	A2A191	1.5118	Q3TYU3	1.4704	Q9JW9	1.4251	Q3UZZ6	1.3780		
D6RET4	1.6917	A0A0R410B2	1.6033	Q3TN61	1.5093	P49710	1.4691	F7B9M9	1.4226	Q92IM7	1.3779		
A2NW55	1.6859	F7B276	1.6019	Q3UAZ7	1.5061	F6THL5	1.4689	Q80TG5	1.4210	P29351	1.3735		
Q70469	1.6688	Q3TXV7	1.6010	Q811E7	1.5048	G3UXX9	1.4677	Q62422	1.4200	Q8BM72	1.3729		
Q91X70	1.6687	Q9DBX5	1.5985	Q8BND5	1.5047	H3BJN8	1.4661	Q78T54	1.4187	Q9CYL5	1.3711		
I6I966	1.6655	Q3YYP5	1.5977	Q8BP55	1.5037	Q3U7R2	1.4653	P14069	1.4160	Q9ES46	1.3710		
F6QY25	1.6637	Q8BV79	1.5866	A2ASM1	1.5037	Q505D7	1.4635	Q9DCF9	1.4150	A0A0R41138	1.3709		
Q9D154	1.6625	A8Y5N8	1.5857	Q8BMG7	1.5001	H7BWZ1	1.4632	Q8R1S0	1.4132	A0A0A6YWU2	1.3688		
Q8R3M1	1.6611	P17665	1.5850	Q3V1L4	1.4974	Q80X85	1.4622	Q3TC98	1.4123	Q99KW9	1.3673		

continued Table 1

[illegible]

Table 2. The list of down-regulated differentially expressed proteins between OA group and control group by iTRAQ analysis

Accession	Fold Change	Accession	Fold Change	Accession	Fold Change	Accession	Fold Change	Accession	Fold Change	Accession	Fold Change	Accession	Fold Change
O88207	0.2920	O9D574	0.6362	O91XA9	0.6936	A0A0N4SVT9	0.7260	O50117	0.7508	E90394	0.7721		
B2RSH2	0.3634	O8BT62	0.6377	F8WHS8	0.6950	O9D6F9	0.7267	A0A087WOF8	0.7509	O3TFP8	0.7723		
A0A02315Y9	0.4165	O3UTB8	0.6405	O6P3D8	0.6954	O9D757	0.7267	A0A1B0GR64	0.7509	P69566	0.7724		
O9DCI1	0.4228	O9D719	0.6464	O8OV42	0.6961	O8OZT6	0.7270	A2AVZ9	0.7524	A2AWI9	0.7727		
A0A097BW18	0.4699	O8CAY6	0.6474	A0A1B0GSB1	0.6973	O9EPB5	0.7274	E9PWB1	0.7543	O9CX60	0.7732		
O62009	0.4798	A0A1B0GR84	0.6492	O8B009	0.7003	O56119	0.7319	O92019	0.7558	A2AI08	0.7740		
F6VPB5	0.4835	A0A0A6YWP9	0.6509	P59222	0.7007	D6RDC2	0.7325	A2AKA9	0.7561	G5E874	0.7742		
O9IL30	0.5002	O9D7E4	0.6524	E9O214	0.7017	O8CHU3	0.7329	P28828	0.7562	O9D172	0.7752		
O6P616	0.5132	O3U7F6	0.6539	O8BG11	0.7026	O8CGF7	0.7331	O91Y08	0.7567	O8CG79	0.7757		
F7BK11	0.5157	O6RFS9	0.6545	E9O933	0.7069	O922F4	0.7332	A0A171EBK9	0.7572	O61087	0.7758		
E9O9T1	0.5251	O91641	0.6546	A0A0D4ET98	0.7069	O9C7C8	0.7335	O61553	0.7576	O8BKR5	0.7759		
A0A1B0GTA0	0.5392	O8BHA3	0.6566	P21841	0.7081	E9O907	0.7351	O3TM31	0.7584	O8C4D5	0.7769		
O8CDZ6	0.5650	O8BK72	0.6568	O3TIN8	0.7091	O6G0S1	0.7355	O8BP06	0.7585	P26516	0.7780		
O9N9B8	0.5691	O3TPA5	0.6612	O3TPA5	0.7099	O91MA9	0.7357	D3YWT1	0.7586	E9PZ16	0.7781		
O8BGW1	0.5791	O6PAM1	0.6616	A2AU66	0.7114	O3U747	0.7365	B2RUG6	0.7595	O6PCW0	0.7784		
O3V231	0.5870	O7TMG7	0.6688	O3U6A2	0.7114	O3USX2	0.7371	O8BZB2	0.7604	E9OIS0	0.7785		
O91YE3	0.5942	O3TXH6	0.6698	O9CSX4	0.7140	G5E8C3	0.7384	O8VCA8	0.7621	O91YP0	0.7788		
O8C8L6	0.5946	P84444	0.6703	E0CYG4	0.7142	O5SVF8	0.7384	D6RIN4	0.7630	D6RIN4	0.7790		
O80YR4	0.5974	O80Y14	0.6720	A0A0N4SVZ5	0.7162	A0A0G2IDW7	0.7410	O8R5F8	0.7643	D3Z2X5	0.7790		
O80Y95	0.5978	O3UH68	0.6753	O9ES29	0.7163	O3U7Z0	0.7415	O571M9	0.7672	O6VN19	0.7790		
O3UWK4	0.6079	O8K209	0.6754	P28659	0.7174	P52479	0.7418	O6OMT3	0.7676	O3UVG3	0.7792		
A0A1B0GR85	0.6095	D3Z3F8	0.6842	O8BI05	0.7189	O6NZN9	0.7425	O3U763	0.7677	O9DC07	0.7798		
O3U613	0.6267	O3UYC0	0.6868	O3U605	0.7211	O61206	0.7432	O3TRG2	0.7684	O9Z2H7	0.7800		
O9ET22	0.6281	O8C586	0.6881	O9OZ26	0.7217	O80XC3	0.7468	P51175	0.7693	F6RK07	0.7801		
A2A025	0.6291	O3U3H2	0.6886	O8OSZ7	0.7229	O8BLN5	0.7476	P52503	0.7699	O9Z2S5	0.7806		
A2AI10	0.6301	O3UWP1	0.6896	O06318	0.7239	A2AAU5	0.7485	O991J0	0.7700	H7BWY4	0.7818		
F6RI01	0.6330	A2AKD7	0.6903	O3U210	0.7254	O8CH02	0.7485	D3YWS3	0.7701	O80Y24	0.7819		
P63024	0.6340	O9D6S7	0.6907	B2ZRS8	0.7254	O62093	0.7490	F6SMY7	0.7718	B1AYL1	0.7823		
O3V3U1	0.6349	O9IMK2	0.6911	A2A7S8	0.7257	P17897	0.7490	O3UGU1	0.7719	O9EP82	0.7831		
O80T06	0.6361	D5MCW4	0.6912	O7M092	0.7259	O61425	0.7496	O66L71	0.7720	O6P9R4	0.7834		
O9CWN7	0.7839	O91VW5	0.7926	O8R3Y8	0.8021	O5XG73	0.8139	H3BIC4	0.8220	O3TVB5	0.8293		
E9O1M1	0.7842	O8BK63	0.7929	D3Z6G3	0.8027	Z4YKC4	0.8156	O9ESY9	0.8225	O05CR3	0.8294		
O9ESU6	0.7847	F8WHP8	0.7938	O3UGR5	0.8036	O3TCN2	0.8163	P00184	0.8229	O3TDN4	0.8297		
P62137	0.7851	O8BHC9	0.7942	P56213	0.8047	A0A0A1HAM8	0.8164	O8CGB6	0.8233	O8BI08	0.8303		
O9OY59	0.7858	G3CE24	0.7947	O91VC3	0.8048	D3Z5R2	0.8174	O8R3F5	0.8233	O9OXT0	0.8304		
O8C5W3	0.7863	O61789	0.7958	O99K13	0.8052	D3Z5T2	0.8175	D3Z1G7	0.8235	E9O057	0.8304		
O8VCFO	0.7868	O920L1	0.7960	O9ERL9	0.8055	O61292	0.8177	P27546	0.8247	O3U935	0.8305		
E9PPY1	0.7870	F8WGT1	0.7962	O3UW66	0.8062	O8CFR5	0.8182	F6VMV6	0.8249	O9Z0R4	0.8306		
O8BG95	0.7873	O6PEV3	0.7965	A0A1B0GS13	0.8067	O640N1	0.8189	O3T121	0.8257	A2AU89	0.8308		
O3T5T8	0.7876	O8C1F9	0.7967	O02788	0.8081	F8W1J3	0.8190	O8BL36	0.8259	A0A087WNM1	0.8309		
O54784	0.7880	D3YTX1	0.7971	B1AZ15	0.8087	P97361	0.8191	G5E896	0.8260	O3TLI6	0.8312		
O9DCT2	0.7883	O9O8K8	0.7974	D3YU22	0.8092	P52633	0.8194	E9O811	0.8265	F6W8Y8	0.8318		
G3UD26	0.7887	P05532	0.7987	O8K3H0	0.8093	O8BG60	0.8194	O3TVI0	0.8266	O09167	0.8319		
P01831	0.7899	V9CX50	0.7987	O3TDT0	0.8094	E9PWK7	0.8195	Z4YIV4	0.8266	O8OX02	0.8320		
O04857	0.7899	O8K0M3	0.7995	O9D8C4	0.8099	O99K30	0.8201	H9KV01	0.8268	O9CZD3	0.8320		
A0A087W089	0.7903	O8K2H2	0.7995	A0A1B0GR19	0.8108	O3TG92	0.8210	P61148	0.8272	O8R3B1	0.8327		
O9CRA5	0.7904	O3THL5	0.7999	O6NVF0	0.8118	O3UER5	0.8213	O3URF1	0.8272	O8R5G7	0.8332		
A0A0R410S1	0.7910	O3V038	0.8001	A0A0R41023	0.8119	O9CX86	0.8213	P62915	0.8278	O7TS70	0.8333		
O3TU49	0.7911	G3UXH4	0.8003	O91X52	0.8127	I3OMM7	0.8214	A0A0N4SVE6	0.8279				
O3V3X9	0.7918	O9ONA7	0.8009	O5SRX1	0.8129	O8BGD4	0.8214	O08915	0.8280				
D3Z7D2	0.7920	F9O0P6	0.8013	A0A087WPF8	0.8132	O8K2C6	0.8218	O64105	0.8283				

3). As shown in Fig. 4, the enriched GO terms (top 20) revealed that the proteins of interest were involved in the following areas or processes: extracellular region, extracellular space, extracellular region part, immune system, protein activation cascade, blood microparticle system, overall immune response, humoral immune response, protein-lipid complexes, lipoprotein particles, plasma lipoprotein particles, regulation of plasma lipoprotein particle levels, defense response, wound healing response, regulation of wound healing, positive regulation of responses to stimuli, plasma lipoprotein particle organization, protein-lipid complex subunit organization, and peptidase inhibitor activity.

Fig. 2. Heat map of differentially expressed proteins (DEPs) in lung tissue from OA-treated and control mice. Cluster map comparing the protein expression patterns of OA-treatment and control groups. Blue represents lower protein expression levels than, red represents higher expression levels than, and white indicates the same expression levels as those in the control. The color scale shown at the lower right indicates the fold changes in protein expression of all the samples.

KEGG pathway analysis of DEPs

A KEGG pathway map was applied to determine the pathways related to these DEPs. The top 20 KEGG pathways are listed in Fig. 5. The results of the KEGG analysis showed that the

Fig. 3. Gene ontology (GO) annotation of differentially expressed proteins (DEPs). All identified proteins were functionally annotated in the GO database according to their cellular component (A), molecular function (B) and biological process (C). Most of the proteins with differential abundance levels analyzed for cellular component, molecular function and biological process were associated with cell parts, binding, and cell processes, respectively.

complement and coagulation cascade pathways were the predominant processes associated with the DEPs. The cytokine-cytokine receptor interaction and ECM-receptor interaction pathways were also identified.

Western blot validation

Based on the KEGG pathway and fold change analyses, the lung expression levels of AT III, 12-LO, DOCK2, polycystin-2 and plasminogen were selected for further validation. The results showed that the expression levels of these DEPs were remarkably increased in the OA-treatment group compared with those in the control group (Fig. 6A). In addition, in HMVECs exposed to 0.5 mmol/L OA, AT III expression was significantly enhanced in the OA-treatment group compared with that in the control group (Fig. 6B). The quantification of the verified proteins was highly inconsistent with the proteomic results. However, the roles and mechanisms of these upregulated proteins in OA-induced lung injury remain poorly understood. Previous studies have shown that the primary pathogenesis of sepsis involves the initiation of the coagulation cascade, followed by pro-inflammatory cytokine production [15]. Satoshi Hagiwara also reported that AT III suppressed LPS-induced activation of the nuclear factor (NF)- κ B pathway by preventing phosphorylation of the IKK pathway, and this may possibly inhibit the secretion of HMGB1 and improve endotoxin-induced ALI in rats [16].

AT III knockdown increases cytokine secretion

To further elucidate the role of AT III in OA-induced endothelial inflammation and integrity, we utilized a specific siRNA to silence AT III expression. AT III knockdown was validated by western blot analysis of whole-cell lysates from non-specific (control) siRNA- and AT III-specific siRNA-transfected endothelial cells. As shown in Fig. 7A, the knockdown effect of AT III siRNA-919 was the most effective; therefore, this siRNA was used in the following study. To detect the effects of AT III on the release of inflammatory factors in OA-

Fig. 4. Gene ontology (GO) enrichment analysis. The GO terms of most significantly differentially expressed proteins (DEPs) identified from the comparison between the OA-treatment and control groups were mainly enriched in the extracellular region, followed by the extracellular region part and immune system processes.

treated endothelial cells, culture supernatants from HMVECs that had been transfected with AT III siRNA or control siRNA and then treated with OA were collected, centrifuged and subjected to IL-6, IL-1 β , TNF- α and TGF- β ELISA. Compared with the control siRNA group, the levels of IL-6, IL-1 β , TNF- α and TGF- β in the culture supernatants were significantly increased 24 h after the administration of OA in the AT III siRNA group (Fig. 7B, 7C, 7D and 7E; $P < 0.05$), which suggested that knockdown of AT III increased cytokine secretion in OA-stimulated endothelial cells.

AT III knockdown impairs endothelial integrity

Endothelium intracellular junctions, such as endothelial tight junctions and adherent junctions, maintain the barrier function of the endothelium and regulate endothelial cell contraction [17, 18]. Endothelial hyperpermeability is specifically caused by cell junction breakdown, which ultimately leads to the formation of intracellular gaps [19]. Endothelial tight junctions are composed of the tetraspan transmembrane proteins claudin and occludin, the latter of which maintains the integrity of the endothelium in the blood-brain barrier [20]. Adherens junctions maintain the mechanical strength of intracellular adhesion by homophilic Ca^{2+} -dependent trans-interactions with VE-cadherin [21]. Both endothelial tight junctions and adherens junctions interact with the cortical actin cytoskeleton through adaptor molecules, such as the tight junction components ZO-1, -2, and -3 and the catenin family members α -, β -, and γ -catenin [22]. In this study, the expression of the tight junction protein ZO-1 was markedly decreased (Fig. 8A), whereas the expression of the stress fiber F-actin (Fig. 8B) was significantly increased in the AT III siRNA group compared with that in the control siRNA group. Additionally, endothelial cell integrity was examined using a FITC-dextran transwell permeability assay. (Fig. 8C) In this assay, which monitors the passage of a small molecule, FITC-labeled dextran, through endothelial cells on matrigel-coated transwell inserts containing 3- μm pores, we found that FITC-labeled dextran permeability

Fig. 5. KEGG pathway analysis of differentially expressed proteins (DEPs). The most highly enriched pathways were the complement and coagulation cascade pathways, followed by Fc gamma R-mediated phagocytosis and amoebiasis.

was markedly increased in the AT III siRNA group compared with that in the control siRNA group ($P < 0.05$), suggesting that AT III knockdown increased endothelial cell permeability. In addition, compared with the control group, the expression of phosphorylated myosin light chain (pMLC) was notably increased in the AT III siRNA group (Fig. 8D).

Discussion

This study represents the first-ever investigation of lung tissue proteomic profiles from mice with OA-induced ALI using iTRAQ and nano-LC-MS/MS techniques to detect potential biomarkers and elucidate the molecular alterations that occur following lung injury. Interestingly, we identified and quantified lung tissue proteins that were differentially expressed in the OA-treatment group mice compared with those in the saline-treatment group.

Although the exact mechanisms by which abnormalities in coagulation and fibrinolysis proteins contribute to patient outcomes in ALI are not well established, a previous study indicated that coagulation and the inflammatory cascade may affect the pathogenesis of ALI and ARDS [12]. Some experimental evidence has revealed that abnormalities in the pro-coagulation and fibrinolysis protein pathways are features of ALI [23, 24]. ALI is accompanied by fibrin deposition in the lung and increased plasminogen activator inhibitor expression in bronchoalveolar lavage (BAL) fluid. Intriguingly, by applying the iTRAQ approach to identify

Fig. 6. Confirmation of differentially expressed proteins (DEPs) by western blot. (A) Immunoblotting analysis of proteins (AT III, 12-LO, DOCK2, polycystin-2 and plasminogen) in OA-treated and control mice. (B) Immunoblotting analysis of AT III protein in HMVECs. The β -actin protein was used as a control.

Fig. 7. Changes in inflammatory factor levels determined by ELISA. (A) AT III knockdown was validated by western blotting of whole-cell lysates from non-specific (control) siRNA- and AT III-specific siRNA-transfected endothelial cells. HMVECs were transfected with non-specific RNA or AT III-specific siRNA duplexes, followed by treatment with OA or saline for 24 h. (B, C, D, E) After 24 h of OA stimulation, the supernatants from siRNA-transfected HMVECs were collected and centrifuged to remove cell debris. The concentrations of IL-6, IL-1 β , TNF- α and TGF- β were detected via ELISA ($P < 0.05$).

Fig. 8. Effects of AT III knockdown on endothelial integrity. (A) The expression of ZO-1 in endothelial cells treated with control siRNA and AT III-specific siRNA, followed by stimulation with 0.5 mmol/L OA, was confirmed by western blotting. Equal protein loading was detected by membrane re-probing with a β -actin antibody. (B) After siRNA transfection, followed by stimulation with 0.5 mmol/L OA, endothelial cells cultured on 24-well glass coverslips were subjected to immunofluorescence staining for F-actin. Bar = 50 μ m. (C) FITC-dextran transwell permeability assay to observe the degree

of HMVECs monolayer permeability ($P < 0.05$ compared with the control group). (D) The expression of pMLC in HMVECs treated with control siRNA and AT III-specific siRNA, followed by stimulation with OA, was validated by western blotting. Equal loading was confirmed by re-probing with anti-total MLC antibody.

relevant KEGG pathways, we found that the coagulation and complement pathways were among the top signaling pathways identified in the present study.

One upregulated component of the coagulation and complement pathways was AT III. AT III, encoded by *Serpinc1*, is an important anti-coagulation molecule and serine protease inhibitor in the coagulation cascade [25, 26]. Moreover, there is evidence demonstrating that AT III exerts anti-inflammatory effects, at least partially, by increasing the level of PGI₂, suppressing NF- κ B, decreasing leucocyte-endothelial interactions, and inhibiting microvascular leakage [27-31]. Wang et al. [32] noted that *Serpinc1* insufficiency aggravated renal ischemia/reperfusion injury by increasing renal oxidative stress, tubular apoptosis, and macrophage infiltration. Nevertheless, continuous infusion of rhAT III attenuated neutrophil activation, adhesion and transendothelial migration and thereby reduced endothelial hyperpermeability of ALI following burn and smoke inhalation in animal models [13]. The potential mechanism of action may be the interaction between rhAT III with the syndecan-4 receptor on endothelial cells and neutrophils. Using *in vivo* and *in vitro* experiments, we confirmed that AT III was upregulated in the OA-treatment group compared with that in the saline-treatment group. To further identify the role of AT III in OA-induced inflammatory responses, we utilized AT III-targeted siRNA. We found that AT III knockdown exaggerated OA-induced inflammatory responses, as demonstrated by increased production of inflammatory cytokines, such as IL-6, IL-1 β , TNF- α and TGF- β .

From an analysis of the coagulation and complement signaling pathways, we observed that AT III is associated with not only inflammatory responses but also endothelial permeability. It has been reported that intravenous injection of OA has a direct toxic effect on vascular endothelium within minutes, which leads to permeability edema [33, 34]. In this study, we also found that FITC-labeled dextran permeability markedly increased in the AT III knockdown group compared with that in the control siRNA group. It is well known that cellular tight junctions are critical for the stabilization of endothelial barrier function. ZO-1, which plays a crucial role in forming tight junctions, also regulates angiogenesis and endothelial barrier formation [35-38]. Using *in vitro* experiments, we determined that when endothelial cells were exposed to OA, the expression of ZO-1 was decreased in the control siRNA group, and this effect was further exacerbated in the AT III siRNA group. In addition, cytoskeletal F-actin stress fibers possibly lead to pulmonary endothelial hyperpermeability. Some evidence has indicated that regulation of F-actin stress fiber formation is partially

associated with endothelial barrier function, but the exact mechanism underlying this process is poorly understood [39]. Our results illustrated that AT III knockdown enhanced the formation of F-actin stress fibers in endothelial cells exposed to OA. These findings suggest that AT III has a protective role in endothelial cell permeability, possibly via interactions with ZO-1 and the F-actin cytoskeleton.

Dudek et al. [40, 41] showed that thrombin induced disruption of the endothelial barrier, which caused physiological dysfunction such as hypoxemia, atherosclerosis, and pulmonary edema, a typical characteristic of ALI. Van Hinsbergh et al. [42] noted that the possible mechanism of histamine-induced transient short-term hyperpermeability involved Ca^{2+} /calmodulin-dependent activation of the myosin light chain (MLC) kinase. The cause of thrombin-induced endothelial hyperpermeability involved activation of the small GTPase RhoA and Rho kinase, which increased the phosphorylation of MLC. In addition, Parker et al. [43] demonstrated that thrombin induced endothelial barrier destabilization, at least partly, by inhibiting the activation of Rap1 and Rac1 and increasing the phosphorylation and distribution of VE-cadherin, the phosphorylation of MLC, and actin polymerization. Consistent with the results of previous studies, we observed that after endothelial cells were exposed to 0.5 mmol/L OA, the expression of pMLC was increased in the AT III siRNA group compared with that in the control siRNA group.

In short, AT III knockdown exaggerated OA-induced inflammatory responses, as demonstrated by increased production of inflammatory cytokines, such as IL-6, IL-1 β , TNF- α and TGF- β ; enhancement of endothelial hyperpermeability, as indicated by increased passage of FITC-labeled dextran and phosphorylation of MLC; decreased expression of ZO-1; and increased expression of F-actin stress fibers.

The present study demonstrated that most of the verified DEPs may serve as therapeutic targets for ALI. For instance, after endothelial cells were exposed to OA, we observed that the expression of AT III increased *in vivo* and *in vitro*, whereas AT III knockdown exaggerated inflammatory responses and endothelial integrity dysfunction. We think that, to some degree, AT III may attenuate endothelial hyperpermeability and further maintain endothelial integrity during thrombin-induced disruption of endothelial barrier function. In future studies, we will focus on identifying the relationship between these proteins and the pathogenesis of OA-induced ALI to provide guidelines for treating patients with ALI.

Acknowledgements

This work was supported by grants from the National Natural Science Foundation of China (81272147 to AZ Wang) and the Suitable Technology Extension Program of Shanghai Health and Family Planning Commission (2013SY031 to AZ Wang).

Disclosure Statement

We declare that we have no financial or commercial conflicts of interest.

References

- 1 Ferguson ND, Fan E, Camporota L, Antonelli M, Anzueto A, Beale R, Brochard L, Brower R, Esteban A, Gattinoni L, Rhodes A, Slutsky AS, Vincent JL, Rubenfeld GD, Thompson BT, Ranieri VM: The berlin definition of ards: An expanded rationale, justification, and supplementary material. *Intensive Care Med* 2012;38:1573-1582.
- 2 Ji Y, Gao F, Sun B, Hao J, Liu Z: Angiotensin-converting enzyme 2 inhibits apoptosis of pulmonary endothelial cells during acute lung injury through suppressing smad2 phosphorylation. *Cell Physiol Biochem* 2015;35:2203-2212.

- 3 Yin X, Liang Z, Yun Y, Pei L: Intravenous transplantation of bmp2-transduced endothelial progenitor cells attenuates lipopolysaccharide-induced acute lung injury in rats. *Cell Physiol Biochem* 2015;35:2149-2158.
- 4 Yu X, Lin Q, Qin X, Ruan Z, Zhou J, Lin Z, Su Y, Zheng J, Liu Z: Ace2 antagonizes vegfa to reduce vascular permeability during acute lung injury. *Cell Physiol Biochem* 2016;38:1055-1062.
- 5 Xu S, Xu M, Li GG, Wang C, Song H, Bai J: Early recruitment of il-10-producing b cells into alveoli improved the resolution of acute lung injury. *Cell Physiol Biochem* 2016;38:1752-1760.
- 6 Karmaliotis D, Kosmidou I, Ingenito EP, Hong K, Malhotra A, Sunday ME, Haley KJ: Angiogenic growth factors in the pathophysiology of a murine model of acute lung injury. *Am J Physiol Lung Cell Mol Physiol* 2002;283:L585-595.
- 7 Zhang HX, Liu SJ, Tang XL, Duan GL, Ni X, Zhu XY, Liu YJ, Wang CN: H2s attenuates lps-induced acute lung injury by reducing oxidative/nitrative stress and inflammation. *Cell Physiol Biochem* 2016;40:1603-1612.
- 8 Chen Y, Wang L, Kang Q, Zhang X, Yu G, Wan X, Wang J, Zhu K: Heat shock protein a12b protects vascular endothelial cells against sepsis-induced acute lung injury in mice. *Cell Physiol Biochem* 2017;42:156-168.
- 9 Niu R, Liu Y, Zhang Y, Zhang Y, Wang H, Wang Y, Wang W, Li X: Itraq-based proteomics reveals novel biomarkers for idiopathic pulmonary fibrosis. *PloS one* 2017;12:e0170741.
- 10 Hirsch J, Niemann CU, Hansen KC, Choi S, Su X, Frank JA, Fang X, Hirose R, Theodore P, Sapru A, Burlingame AL, Matthay MA: Alterations in the proteome of pulmonary alveolar type ii cells in the rat after hepatic ischemia-reperfusion. *Crit Care Med* 2008;36:1846-1854.
- 11 Tao W, Miao QB, Zhu YB, Shu YS: Inhaled neutrophil elastase inhibitor reduces oleic acid-induced acute lung injury in rats. *Pulm Pharmacol Ther* 2012;25:99-103.
- 12 Ware LB, Matthay MA, Parsons PE, Thompson BT, Januzzi JL, Eisner MD: Pathogenetic and prognostic significance of altered coagulation and fibrinolysis in acute lung injury/acute respiratory distress syndrome. *Crit Care Med* 2007;35:1821-1828.
- 13 Rehberg S, Yamamoto Y, Sousse LE, Jonkam C, Zhu Y, Traber LD, Cox RA, Prough DS, Traber DL, Enkhbaatar P: Antithrombin attenuates vascular leakage via inhibiting neutrophil activation in acute lung injury. *Crit Care Med* 2013;41:e439-446.
- 14 Martins-Green M, Petreaca M, Yao M: An assay system for *in vitro* detection of permeability in human "endothelium". *Methods Enzymol* 2008;443:137-153.
- 15 Iba T, Kidokoro A, Yagi Y: The role of the endothelium in changes in procoagulant activity in sepsis. *J Am Coll Surg* 1998;187:321-329.
- 16 Hagiwara S, Iwasaka H, Matsumoto S, Noguchi T: High dose antithrombin iii inhibits HMGB1 and improves endotoxin-induced acute lung injury in rats. *Intensive Care Med* 2008;34:361-367.
- 17 Franke WW: Discovering the molecular components of intercellular junctions--a historical view. *Cold Spring Harb Perspect Biol* 2009;1:a003061.
- 18 Bazzoni G, Dejana E: Endothelial cell-to-cell junctions: Molecular organization and role in vascular homeostasis. *Physiol Rev* 2004;84:869-901.
- 19 Aird WC: Phenotypic heterogeneity of the endothelium: I. Structure, function, and mechanisms. *Circ Res* 2007;100:158-173.
- 20 Lee WLA, Michael-Titus AT, Shah DK: Hypoxic-ischaemic encephalopathy and the blood-brain barrier in neonates. *Dev Neurosci* 2017;39:4-58.
- 21 Giannotta M, Trani M, Dejana E: VE-cadherin and endothelial adherens junctions: Active guardians of vascular integrity. *Dev Cell* 2013;26:441-454.
- 22 Schnittler H: Contraction of endothelial cells: 40 years of research, but the debate still lives. *Histochem Cell Biol* 2016;146:651-656.
- 23 Frantzeskaki F, Armaganidis A, Orfanos SE: Immunothrombosis in acute respiratory distress syndrome: Cross talks between inflammation and coagulation. *Respiration* 2017;93:212-225.
- 24 Ozolina A, Sarkele M, Sabelnikovs O, Skesters A, Jaunalksne I, Serova J, Ievins T, Bjertnaes LJ, Vanags I: Activation of coagulation and fibrinolysis in acute respiratory distress syndrome: A prospective pilot study. *Front Med* 2016;3:64-73.
- 25 Zhao Q, Yin J, Lu Z, Kong Y, Zhang G, Zhao B, Wang F: Sulodexide protects contrast-induced nephropathy in sprague-dawley rats. *Cell Physiol Biochem* 2016;40:621-632.
- 26 Lu Z, Wang F, Liang M: Serpinc1/antithrombin iii in kidney-related diseases. *Clin Sci (Lond)* 2017;131:823-831.

- 27 Mizutani A, Okajima K, Uchiba M, Isobe H, Harada N, Mizutani S, Noguchi T: Antithrombin reduces ischemia/reperfusion-induced renal injury in rats by inhibiting leukocyte activation through promotion of prostacyclin production. *Blood* 2003;101:3029-3036.
- 28 Oelschlager C, Romisch J, Staubitz A, Stauss H, Leithauser B, Tillmanns H, Holschermann H: Antithrombin III inhibits nuclear factor kappaB activation in human monocytes and vascular endothelial cells. *Blood* 2002;99:4015-4020.
- 29 Mansell A, Reinicke A, Worrall DM, O'Neill LA: The serine protease inhibitor antithrombin III inhibits LPS-mediated NF-κB activation by TLR-4. *FEBS Lett* 2001;508:313-317.
- 30 Souter PJ, Thomas S, Hubbard AR, Poole S, Romisch J, Gray E: Antithrombin inhibits lipopolysaccharide-induced tissue factor and interleukin-6 production by mononuclear cells, human umbilical vein endothelial cells, and whole blood. *Crit Care Med* 2001;29:134-139.
- 31 Dunzendorfer S, Kaneider N, Rabensteiner A, Meierhofer C, Reinisch C, Romisch J, Wiedermann CJ: Cell-surface heparan sulfate proteoglycan-mediated regulation of human neutrophil migration by the serpin antithrombin III. *Blood* 2001;97:1079-1085.
- 32 Wang F, Zhang G, Lu Z, Geurts AM, Usa K, Jacob HJ, Cowley AW, Wang N, Liang M: Antithrombin III/Serpinc1 insufficiency exacerbates renal ischemia/reperfusion injury. *Kidney Int* 2015;88:796-803.
- 33 Gil Cano A, Gracia Romero M, Monge Garcia MI, Guijo Gonzalez P, Ruiz Campos J: Preemptive hemodynamic intervention restricting the administration of fluids attenuates lung edema progression in oleic acid-induced lung injury. *Med Intensiva* 2017;41:135-142.
- 34 Haque A, Scultetus AH, Arnaud F, Dickson LJ, Chun S, McNamee G, Auken CR, McCarron RM, Mahon RT: The emulsified PFC oxybyte® improved oxygen content and lung injury score in a swine model of oleic acid lung injury (OALI). *Lung* 2016;194:945-957.
- 35 Zhou W, Fong MY, Min Y, Somlo G, Liu L, Palomares MR, Yu Y, Chow A, O'Connor ST, Chin AR, Yen Y, Wang Y, Marcusson EG, Chu P, Wu J, Wu X, Li AX, Li Z, Gao H, Ren X, Boldin MP, Lin PC, Wang SE: Cancer-secreted miR-105 destroys vascular endothelial barriers to promote metastasis. *Cancer Cell* 2014;25:501-515.
- 36 Tornavaca O, Chia M, Dufton N, Almagro LO, Conway DE, Randi AM, Schwartz MA, Matter K, Balda MS: ZO-1 controls endothelial adherens junctions, cell-cell tension, angiogenesis, and barrier formation. *J Cell Biol* 2015;208:821-838.
- 37 Qiu AW, Liu QH, Wang JL: Blocking IL-17a alleviates diabetic retinopathy in rodents. *Cell Physiol Biochem* 2017;41:960-972.
- 38 Watari A, Sakamoto Y, Hisaie K, Iwamoto K, Fueta M, Yagi K, Kondoh M: Rebeccamycin attenuates TNF-α-induced intestinal epithelial barrier dysfunction by inhibiting myosin light chain kinase production. *Cell Physiol Biochem* 2017;41:1924-1934.
- 39 Gabrys D, Greco O, Patel G, Prise KM, Kanthou C: Radiation effects on the cytoskeleton of endothelial cells and endothelial monolayer permeability. *Int J Radiat Oncol Biol Phys* 2007;69:1553-1562.
- 40 Dudek SM, Camp SM, Chiang ET, Singleton PA, Usatyuk PV, Zhao Y, Natarajan V, Garcia JG: Pulmonary endothelial cell barrier enhancement by FTY720 does not require the S1P1 receptor. *Cell Signal* 2007;19:1754-1764.
- 41 Dudek SM, Garcia JG: Cytoskeletal regulation of pulmonary vascular permeability. *J Appl Physiol* 2001;91:1487-1500.
- 42 van Hinsbergh VW, van Nieuw Amerongen GP: Intracellular signalling involved in modulating human endothelial barrier function. *J Anat* 2002;200:549-560.
- 43 Parker WH, Qu ZC, May JM: Intracellular ascorbate prevents endothelial barrier permeabilization by thrombin. *J Biol Chem* 2015;290:21486-21497.