Mechanism of 1,25(OH)₂D₃ in the Treatment of Acute Lung Injury Based on UHPLC/Q-TOF MS-Based Metabolomics and Network Pharmacology

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Abstract

Background: Sepsis-induced acute lung injury (ALI), a high morbidity and mortality disease, still has no effective therapies. $1,25(\text{OH})_2\text{D}_3$ is one of the indispensable nutrients in our body. The regulation mechanism of $1,25(\text{OH})_2\text{D}_3$ in inflammation has been recognized gradually. Network pharmacology was used widely to broaden the understanding of diseases and advance drug discovery. In this study, we used network pharmacology and metabolomics to generate the potential mechanism of $1,25(\text{OH})_2\text{D}_3$ on acute lung injury.

Methods: We used metabolomics and network pharmacology to elucidate the therapeutic mechanism of $1,25(\text{OH})_2\text{D}_3$ on acute lung injury. Serum samples, collected from mice with LPS-induced acute lung injury, were detected by UHPLC/Q-TOF MS to evaluate the differential metabolites from multiple metabolic pathways. Meanwhile, the H&E staining, ELISA and QPCR were used to estimate the efficacy of $1,25(\text{OH})_2\text{D}_3$ on acute lung injury.

Results: The results of animal experiments showed that $1,25(\text{OH})_2\text{D}_3$ could mitigate severe pulmonary edema and inflammatory infiltration caused by LPS, and the treatment of $1,25(\text{OH})_2\text{D}_3$ reduced the levels of inflammatory cytokines, interacted 25 related proteins and TNF signaling pathway, Toll-like receptor signaling pathway, PI3K-Akt signaling pathway.

Conclusions: The integrated methods coupled with UHPLC/Q-TOF MS and network pharmacology provided a new way to study the potential mechanism of $1,25(\text{OH})_2\text{D}_3$ on acute lung injury, which may provide a possible solution for patients with clinical acute lung injury.

1. Background:

Acute lung injury (ALI) and its severe form acute respiratory distress syndrome (ARDS) are two kinds of severe respiratory disorders clinically, which caused by many conditions, such as sepsis, severe trauma with shock, pancreatitis or inhalation of poisonous gases[1]. With a mortality over 40%, ALI and ARDS remain a leading cause of morbidity and mortality[2, 3]. ALI is characterized with the disruption of the alveolar endothelial and epithelial barrier[4, 5], which in turn leads to pulmonary alveolar and interstitial edema, impaired gas exchange and hypoxemia[6]. Although many efforts have been made, the mechanism of ALI is still unclear and there are no effective pharmacotherapies available that can improve survival of patients with ALI/ARDS. Therefore, an effective treatment of ALI must consider all these factors and the molecular networks behind them.

$1,25$ -dihydroxyvitamin D ($1,25(\text{OH})_2\text{D}_3$) is a fat-soluble steroid hormone which regulates the calcium-phosphorus homeostasis. Growing evidences from epidemiological and basic researches reveal that $1,25(\text{OH})_2\text{D}_3$ can modulate immune responses and many acute and chronic disorders[7]. $1,25(\text{OH})_2\text{D}_3$, the active form of vitamin D binds to vitamin D receptor (VDR) and retinoid X receptor (RXR) and the trimeric complex translocates into the nucleus, regulating the expression of more than 900 genes[8, 9], regulating cell proliferation, differentiation and survival[10, 11]. Recent studies have shown that $1,25(\text{OH})_2\text{D}_3$ plays an important role in anti-infection and anti-inflammation[12]. In children and adults with asthma and chronic obstructive pulmonary disease, the levels of serum 25 -hydroxyvitamin D are associated with impaired lung function, airway hyperresponsiveness and increased exacerbation frequency[13–15]. Besides, $1,25(\text{OH})_2\text{D}_3$ deficiency has been found to be associated with lung injury, high-dose preoperative treatment with cholecalciferol reduced changes in postoperative pulmonary vascular permeability index effectively[16]. Despite the evidence linking $1,25(\text{OH})_2\text{D}_3$ to lung diseases, there is little information concerning $1,25(\text{OH})_2\text{D}_3$ and ALI and the potential targets are still unclear.

Because of the complex mechanisms of ALI, the traditional reductionist ‘one drug, one target’ cannot investigate all the mechanisms. With the rapid development of bioinformatics, metabolomics and network pharmacology have become effective methods to detect the molecular and pharmacological mechanisms of diseases and drugs, focusing on the multiple targets[17]. Network pharmacology can reveal the action mechanisms of the drug through the combination of computational biology, systems biology and “omics” related to target drugs[18]. Metabolomics, a new omics technique developed after

genomics, transcriptomics, and proteomics, has developed rapidly. It focuses on the holistic investigation of multi-parametric metabolite responses of living systems and can be used to find new biomarkers, gain a better understanding of metabolic pathways[19]. What's more, metabolomics combined with network pharmacology can be used to construct a network, containing drug-targets-pathways-disease, to detect the potential therapeutic mechanisms of drugs.

In this study, we sought to determine the potential therapeutic mechanisms of $1,25(\text{OH})_2\text{D}_3$ on acute lung injury by metabolomics combining with network pharmacology. First, we identified the differential metabolites among mice in different groups and predicted the potential targets of these metabolites. Then we detected the GEO database for microarray data related to ALI. Since VDR is the receptor of $1,25(\text{OH})_2\text{D}_3$, we also detected the potential proteins related to VDR and examined the overlapping targets shared by above three target lists. Our work highlights the mechanisms of $1,25(\text{OH})_2\text{D}_3$ treating ALI and facilitate the development of novel drugs.

2. Methods:

2.1 Animal Models and Sample Collection

24 Balb/C mice (22–24 g) were purchased from Cavens Animal Corp (Changzhou, Jiangsu, China), and housed in specific pathogen-free conditions (24–26°C, 50 ± 5% humidity) with a 12 h light/dark cycle. Animals had free access to standard diet and water. All animals were randomly divided into three groups: the control group (n = 8), LPS group (n = 8) and LPS + VD group (n = 8). The LPS group was established by injecting lipopolysaccharide (LPS, Sigma-Aldrich, St. Louis, MO, USA) into peritoneal cavity (10 mg/kg). The LPS + VD group was established by treating calcitriol (2ug/kg) at 48, 24 and 1 h before LPS injection[20]. 24 h after LPS injection, all animals were euthanized and the lung tissues were collected for further analysis. All studies were reviewed and approved by the Animal Experiment Administration Committee of the Shanghai Pulmonary Hospital (Shanghai, China).

2.2 Hematoxylin and Eosin (H&E) Staining

Lung tissues were fixed with 4% paraformaldehyde for 24 hours, embedded in paraffin, stained with hematoxylin-eosin (H&E) using the standard protocol. Then, images were captured with a digital camera (Nikon, Tokyo, Japan) by pathologists blinded to the groups.

2.3 RNA Extraction and Real-Time Quantitative PCR

Total RNA from the lung tissues were isolated by TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions and reverse-transcribed into cDNA at 37°C for 15 minutes using a commercial kit from Takara according to the standard protocol. The expression levels of target genes were detected by the SYBR Premix Ex Taq™ II (RR820A, Takara, Japan) with an ABI 7500 PCR system (Life Technologies, USA). Primers for RT-qPCR analysis synthesized by Shanghai Sangon Biotech Co., Ltd (Shanghai, China) and listed in Supplementary Table 1. Expression levels were calculated using the $2^{-\Delta\Delta C_t}$ method and normalized with the level of β -actin gene in the same sample.

2.4 ELISA Analysis

The quantification of sera cytokines (IL-1 β , IL-6 and TNF- α) were assessed with Mouse ELISA MAX™ Standard Sets (BioLegend, San Diego, CA) according to the manufacturer's instructions using serum samples.

2.5 Metabolomic Analysis

Serum samples were collected at room temperature, ice-cold methanol containing 25ug/ml 2-chloro-L-phenylalanine was added to 100 ul serum, prepared as the internal standard to precipitate the protein and extract the metabolites. After vortexing for 1 min, the serum samples were mixed with the internal standard for 5 minutes, tranquilized for 10 minutes and centrifuged at 13000 rpm, 4°C for 15 minutes. The supernatant was transferred into a new vial. The QC sample was prepared from all samples collected in this study for monitoring the data acquisition performance during analysis.

UHPLC-Q-TOF/MS analysis was performed using an Agilent 1290 Infinity LC system equipped with an Agilent 6538 Accurate-Mass Quadrupole Time-of-Flight (Q-TOF) mass spectrometer (Agilent, USA). Chromatographic separations were performed at 40°C using an Acquity UPLC HSS T3 column (2.1 mm × 100 mm, 1.8 µm; Waters, Milford, MA, USA). The flow rate was 0.4 mL/min and the injection volume was 3 µL. 0.1% formic acid (A) and ACN modified with 0.1% formic acid (B) were composed in the mobile phase. The total run time for equilibration and the optimized UHPLC elution conditions were set as follows: 5%B at 0–2 min, 5–95%B at 2–17 min, 95%B at 17–19 min. The flow rate was 0.4 mL/min and the injection volume was 3 µL.

Mass spectrometry was operated in both positive and negative ion modes of operation. The optimized conditions used set as follows: capillary voltage, 4 kV for the positive mode and 3.5 kV for the negative mode; drying gas flow, 11 L/min; gas temperature, 350°C; fragment or voltage, 120 V; nebulizer pressure, 45 psig; and skimmer voltage, 60 V. Data were collected in the profile mode and the mass range was set at 50 to 1,100 m/z. The biomarkers were further analyzed by MS/MS, and the collision energy was set at 10 to 40 eV.

The raw data in the instrument specific format (.d) were collected and converted into a common (mz.data) format using the Agilent Masshunter Qualitative Analysis B.04.00 software (Agilent Technologies, USA), in which the filtration threshold of the high of the absolute peak was set to 500, and the isotope interferences were excluded. We used the XCMS program[21] (<https://xcmsonline.scripps.edu>) to identify peak extraction, retention time correction, RT alignment, and integration, in order to generate a visualization matrix. After 80% based on the principle of selection, frequency of more than 80% of the ions present in each group retained samples[22], and to correct the MS response shift, all detected ions in each sample were normalized to total intensity. The sample names, RT, and m/z pairs, were imported to SIMCA-P software (version 13.0, Umetrics, Umea, Sweden) for principal component analysis (PCA) and partial least squares discriminate analysis (PLS-DA). Variable importance plot (VIP) was used to select metabolites with the threshold value of 1. The statistical significant differences were analyzed by SPSS 17.0. $P < 0.05$ was considered statistically significant.

To identify the discovered biomarkers, the exact masses of ion were input into databases such as Metlin (<http://metlin.scripps.edu>), Human Metabolome Database (<http://www.hmdb.ca/>) and PubChem (<http://pubchem.ncbi.nlm.nih.gov>). Moreover, these metabolites were imported into metaboanalyst database (<https://www.metaboanalyst.ca/>) for metabolic pathway analysis.

2.6 Network Pharmacology

The target proteins of the differential metabolites were searched for in the Search Tool for Interactions of Chemicals database (STITCH, <http://stitch.embl.de>). The gene expression data of LPS-induced ALI were searched from the GEO database (GSE1871), the differential gene was analyzed by GEO2R. $P < 0.05$, $FC > 2$ or $FC < -2$ were considered as significant differential genes. The proteins related to VDR were obtained from the Search Tool for the Retrieval of Interacting Genes database (STRING, <https://string-db.org/>). A VDR-metabolites-target-disease network was constructed using Cytoscape (<http://cytoscape.org>) software.

2.7 Protein-protein Interaction Data

The protein-protein interaction (PPI) data was obtained from STRING database (<https://string-db.org/>)[23]. STRING database provides information regarding the experimental and predicted interactions of these proteins.

2.8 Gene Ontology and Pathway Enrichment

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis were performed with the STRING Database (string-db.org/). GO enrichment analysis and pathways with False Discovery Rate (FDR) < 0.01 were defined as enriched terms and pathways. R software (R 3.6.0 for Windows) was used to chart the bubble plot in KEGG enrichment analysis.

2.9 Statistical analysis

Data were expressed as mean $\pm$ standard error of the mean (SEM) and were assessed by one-way ANOVA followed by a Tukey's post-test using SPSS 17.0 statistical software (SPSS Inc., Chicago, IL, USA). Values of $P < 0.05$ were considered statistically significant.

3. Results:

3.1 Scheme for precise investigation of calcitriol against acute lung injury

The pharmacologic effect of calcitriol against ALI was evaluated in vivo (Fig. 1). Firstly, the physiopathological changes of mice lung tissues and the changes of pro- and anti-inflammatory cytokines were measured. Then, the changes of the differential metabolites in mice were measured by metabolic profiling analysis. Differential metabolites-related proteins, disease-related proteins from GSE1871 and vitamin D receptor-related proteins were obtained from databases. Finally, signatures biomarkers corresponding to the specific efficacy of calcitriol can be obtained.

3.2 Effects of calcitriol on lung injury and production of pro- and anti-inflammatory cytokines

The representative images of hematoxylin and eosin (H&E) staining for each group are illustrated in Fig. 2A. In LPS group, treated with 10 mg/kg LPS, severe pulmonary edema and inflammatory infiltration were detected. It is noteworthy that when treated with calcitriol (2ug/kg), the lung injury was attenuated significantly.

The levels of pro-inflammatory cytokines in serum such as IL-1 β , IL-6 and TNF- α were significantly higher than control group after LPS administration, while calcitriol treatment effectively decreased these pro-inflammatory cytokines in serum (Fig. 2B-D). The pro-inflammatory cytokines in the lung tissues were detected by RT-qPCR analysis, just as the trends in serum, the levels of pro-inflammatory cytokines were up-regulated and anti-inflammatory cytokine (IL-10) were down-regulated after LPS administration. Conversely, calcitriol pretreatment attenuated the changes in LPS group. Compared with LPS group, lower levels of pro-inflammatory cytokines and higher levels of anti-inflammatory cytokine were shown after calcitriol pretreatment (Fig. 2E-H).

3.3 Metabolic profiling analysis

The PLS-DA model was used to investigate the general interrelation among control group, LPS group and LPS + VD group to observe the clear separation in positive and negative ion models. In the PLS-DA model, R^2Y and Q^2 [cumulative (cum)] were parameters used for evaluation of the models, indicating the prediction ability. R^2Y and Q^2 (cum) in the PLS-DA model were 0.804 and 0.96 in the positive mode and 0.548 and 0.693 in the negative mode, respectively. The result of the permutation test is shown in Figure S1. As shown in Fig. 3A-F, S-plot and variable importance plots (VIPs) were used to select the potential biomarkers in control group, LPS group and LPS + VD group. The points farther away from the origin point were selected as potential biomarkers with higher possibility.

3.4 Identification of the potential biomarkers

Among these significant differential metabolite ions related to ALI, we identified 49 differential metabolites altered in LPS group, and the trends of 16 metabolites were reversed by calcitriol intervention. These reversed metabolites were regarded as the biomarkers related to the protective effect of calcitriol (Table 1). As shown in Fig. 4A-B, all identified differential metabolites in three groups were presented in the heatmaps both in positive (A) and negative (B) modes. Moreover, metabolic pathway analysis of these metabolites were shown in Fig. 4C.

Table 1

Identification of significantly differential metabolites for serum from control, LPS, and LPS + VD groups by UHPLC/Q-TOF MS.

NO	RT/min	MZ	VIP	Ion	Formula	Metabolites	Fold change	
							LPS/Control	LPS + VD/LPS
1	0.696268	61.03356	0.983097	M + H	C2H4O2	Glycolaldehyde	2.754402127	1.59926289
2	1.250586	86.09663	1.6358	M + H	C5H11N	Piperidine*	0.963904131	1.39014743
3	0.993188	111.0095	1.18601	M - H	C4H4N2O2	Uracil*	0.438642696	3.67222609
4	2.055882	120.081	1.85554	M + H	C4H9NO3	L-Threonine	1.309282899	1.37004179
5	0.705338	126.0217	1.15636	M + H	C2H7NO3S	Taurine	1.87481513	2.65370823
	0.667629	147.9987	1.18648	M + Na	C2H7NO3S	Taurine	1.761525587	2.50380626
6	0.996105	129.0171	0.87722	M + Na	C3H6O4	Glyceric acid*	0.451512997	3.13561109
7	1.250776	132.1033	4.14766	M + H	C6H13NO2	L-Leucine*	0.952893982	1.41128624
8	0.98994	150.0583	1.05242	M + H	C5H11NO2S	L-Methionine	0.674615886	0.57873851
9	1.620905	160.0756	1.22108	M + H	C10H9NO	Indoleacetaldehyde	0.647751563	0.5449116
10	0.720671	160.1306	1.08727	M + H	C7H13NO3	3-Dehydrocarnitine*	0.699145004	1.24129545
11	0.68031	162.1024	1.19521	M + H	C7H15NO3	L-Carnitine	0.542209405	0.65133356
12	2.055615	166.0877	2.97873	M + H	C9H11NO2	L-Phenylalanine	1.356721357	1.41046288
13	0.992655	167.0224	2.0763	M - H	C5H4N4O3	Uric acid*	1.200870188	0.5048149
14	0.659433	169.9812	1.25207	M + H	C3H7NO2Se	Selenocysteine	1.800485889	2.20630299
15	1.621185	177.1022	0.892941	M + H	C10H12N2O	Serotonin	0.657505546	0.53921093

NO	RT/min	MZ	VIP	Ion	Formula	Metabolites	Fold change	
							LPS/Control	LPS + VD/LPS
16	0.679864	181.9677	1.27828	M-H	C4H9NO5S	L-Homocysteic acid	1.914035459	2.49345592
17	1.086303	182.0813	0.823781	M+H	C9H11NO3	L-Tyrosine	0.776547569	0.93915227
18	5.02191	194.0812	1.34835	M+H	C10H11NO3	Phenylacetylglutamine*	0.863155554	4.01770682
19	0.666578	203.0478	2.32927	M+Na	C6H12O6	alpha-D-Glucose	0.48326983	0.73344698
20	0.998042	210.061	0.911296	M+Na	C11H9NO2	Indoleacrylic acid*	0.354370923	2.68831631
21	0.999036	215.0163	1.09932	M+Na	C6H8O7	Citric acid*	0.390267741	2.90480586
22	0.679991	215.035	2.43108	M+FA-H	C3H7O6P	Glyceraldehyde 3-phosphate	0.587480231	0.80876195
23	0.693568	249.1469	0.889754	M+H	C13H16N2O3	6-Hydroxymelatonin	2.18840512	1.71031366
24	14.00614	281.2484	0.820619	M-H	C18H34O2	Oleic acid	2.218271566	1.79832587
25	11.36941	303.2319	0.979612	M+Na	C18H32O2	Linoleic acid	0.503425461	0.90872121
26	11.3527	319.2286	1.53245	M-H	C20H32O3	12 Hydroxy arachidonic acid	0.508691578	0.92111293
27	13.07992	327.2328	1.31749	M-H	C22H32O2	Retinol acetate*	0.965109071	1.76896173
28	11.26451	343.2283	0.835149	M-H	C22H32O3	7-HDoHE*	0.465790864	1.27157518
29	10.67054	400.3423	2.05668	M+H	C23H45NO4	L-Palmitoylcarnitine	1.97163726	2.10745436
30	10.69522	452.2781	1.67362	M-H	C21H44NO7P	LysoPE(16:0/0:0)*	1.724115601	0.74454549
	10.72116	454.2927	1.56769	M+H	C21H44NO7P	LysoPE(16:0/0:0)*	1.621746354	0.72598017
31	10.33063	476.2781	1.0134	M-H	C23H44NO7P	LysoPE(18:2/0:0)	1.469777981	1.03154655
32	11.85894	480.3089	1.44114	M-H	C23H48NO7P	LysoPC(15:0/0:0)	1.618409062	0.73223248

NO	RT/min	MZ	VIP	Ion	Formula	Metabolites	Fold change	
							LPS/Control	LPS + VD/LPS
33	10.22379	482.3238	1.30551	M + H	C23H48NO7P	LysoPE(18:0/0:0)	0.485193827	0.86372619
34	10.03739	494.3244	2.13156	M + H	C24H48NO7P	LysoPC(16:1/0:0)*	0.478558134	1.13361137
	10.0086	538.3146	1.79425	M + FA-H	C24H48NO7P	LysoPC(16:1/0:0)*	0.55425296	1.14306529
35	10.37779	500.2778	0.812714	M-H	C25H44NO7P	LysoPE(20:4/0:0)*	0.873090995	1.08055551
36	11.34217	510.3554	0.974645	M + H	C25H52NO7P	LysoPE(20:0/0:0)	0.543726803	0.65707045
37	10.40312	520.3447	4.92177	M + H	C26H50NO7P	LysoPC(18:2/0:0)	0.744997405	0.68678646
38	11.08722	522.3595	4.55548	M + H	C26H52NO7P	LysoPC(18:1/0:0)	0.546745532	0.73788905
39	11.05837	566.3466	2.49128	M + FA-H	C26H52NO7P	LysoPC(18:1/0:0)	0.638709042	0.78526408
40	10.35312	524.2778	0.990594	M-H	C27H44NO7P	LysoPE(22:6/0:0)	1.349455838	1.12424067
41	11.81125	524.3753	8.31843	M + H	C26H54NO7P	LysoPC(18:0/0:0)*	0.328487311	1.8948763
42	10.3764	526.2934	1.12899	M + H	C27H44NO7P	LysoPE(22:6/0:0)	1.381892745	1.13231358
43	12.51895	538.3861	0.824555	M + H	C27H56NO7P	LysoPE(22:0/0:0)	0.44120658	0.50416438
44	10.44247	544.342	4.55657	M + H	C28H50NO7P	LysoPC(20:4/0:0)	0.370007621	0.67010627
	10.4198	588.3304	2.74979	M + FA-H	C28H50NO7P	LysoPC(20:4/0:0)	0.440875805	0.70382592
45	10.82393	546.3552	2.12407	M + H	C28H52NO7P	LysoPC(20:3/0:0)	0.295259573	0.46945333
	10.79584	590.3453	1.29514	M + FA-H	C28H52NO7P	LysoPC(20:3/0:0)	0.363299824	0.51203885

NO	RT/min	MZ	VIP	Ion	Formula	Metabolites	Fold change	
							LPS/Control	LPS + VD/LPS
46	11.3922	548.3705	0.975057	M + H	C28H54NO7P	LysoPC(20:2/0:0)	0.327371202	0.65154209
47	12.15332	550.3864	1.33528	M + H	C28H56NO7P	LysoPC(20:1/0:0)	0.319489064	0.69898131
	12.12674	594.3762	0.850176	M + FA-H	C28H56NO7P	LysoPC(20:1/0:0)	0.378870595	0.72996296
48	13.10649	552.4018	1.20628	M + H	C28H58NO7P	LysoPC(20:0/0:0)	0.379335542	0.78102387
49	10.41946	568.3414	2.54023	M + H	C30H50NO7P	LysoPC(22:6/0:0)	0.614535868	0.93920062
	10.396	612.3301	1.44529	M + FA-H	C30H50NO7P	LysoPC(22:6/0:0)*	0.732949942	1.01360236
* metabolites with different trends in LPS/Control and LPS + VD/LPS								

3.5 Network pharmacology analysis

Reversed differential metabolites were selected to do the further analysis. The protocol of the integrated systems pharmacology approach is listed in Fig. 1. The predicted proteins targeted by reversed differential metabolites was detected in STITCH database (<http://stitch.embl.de>) and 2202 proteins were obtained (Supplementary table 2). GEO database mining was used to search genes related to LPS-induced ALI. We screened the database of LPS-induced acute lung injury as the sample and GSE1871 were selected finally. We used the GEO2R to analysis the differential genes between vehicle group and LPS group and genes with significant difference ($P < 0.05$, $FC > 2$ or $FC < -2$) were compared with the metabolites-related proteins. Since calcitriol is the active ligand of vitamin D receptor (VDR) and the VD_3 -VDR axis has been shown to regulate both innate and adaptive immune responses, we used STRING database (<https://string-db.org/>) to detect the protein targets of VDR. In Fig. 5A, after combining these target proteins together and take their intersection, 25 proteins were obtained (Fig. 5A and Table 2): Akt1, Arf2, Arf3, Aspn, Creb1, Cxcl10, Cyp11a1, Cyp1a1, Cyp27a1, Cyp27b1, Cyp7b1, Dpep1, Ldlr, Myc, NFKBIA, Ptgs2, SERPINB2, SERPINB8, SERPINB9, SERPINE1, Sod2, Sost, Sox9, Tlr4 and Trp53. A visual network about the three groups and protein-protein interaction (PPI) network are shown in Fig. 5B–C and helpful to explore the protective effect of calcitriol on ALI by acting on these targets and their related pathways.

Table 2
Target proteins with potentially critical roles in calcitriol treatment of acute lung injury.

No.	Gene abbreviation	UniProt ID	Degree	BetweennessCentrality
1	Trp53	P02340	6	0.02037845
2	Tlr4	Q9QUK6	3	0.0572075
3	Sox9	Q04887	3	0.00782159
4	Sost	Q99P68	3	0.00782159
5	Sod2	P09671	3	0.0572075
6	SERPINB1	Q9D154	3	0.01014867
7	SERPINB9	O08797	3	0.01014867
8	SERPINB8	O08800	3	0.01014867
9	SERPINB2	Q542A3	3	0.01014867
10	Ptgs2	Q05769	3	0.00782159
11	NFKBIA	Q9Z1E3	3	0.01014867
12	Myc	P01108	3	0.01799181
13	Ldlr	P35951	3	0.0572075
14	Dpep1	P31428	3	0.00965975
15	Cyp7b1	Q60991	3	0.01014867
16	Cyp27b1	O35084	3	0.01014867
17	Cyp27a1	Q9DBG1	3	0.01014867
18	Cyp1a1	P00184	3	0.01014867
19	Cyp11a1	Q9QZ82	3	0.01014867
20	Cxcl10	P17515	3	0.01799181
21	Creb1	Q01147	3	0.00782159
22	Aspn	Q99MQ4	3	0.00965975
23	Arf3	P61205	3	0.00965975
24	Arf2	Q8BSL7	3	0.00965975
25	Akt1	P31750	3	0.01799181

3.6 Functional enrichment analysis of the target proteins

To further understand the function and role of calcitriol in treating ALI, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis were performed by STRING database (<https://string-db.org/>). For GO term, the top ten enriched biological process terms, molecular function terms and cellular component terms were shown in Fig. 6A-C. KEGG pathway analysis further showed that the proteins involved in TNF signaling pathway, Toll-like receptor signaling pathway, PI3K-Akt signaling pathway and so on. The top 20 pathways were shown in Fig. 6D in the form of bubble plot.

4. Discussion

The diseases of acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) still keep a poor prognosis, which leads to high mortality every year worldwide. It is important to figure out the potential mechanisms of ALI and find better treatments for patients. LPS can regulate the lung injury involving biomacromolecule synthesis, hepatocyte apoptosis stimulation, free radical generation, lipid peroxidation and other various mechanisms. In this study, high levels of pro-inflammatory cytokines were found in serum and lung tissues from animal model of acute lung injury induced by LPS. When treated with calcitriol, the expression of these pro-inflammatory cytokines were decreased and anti-inflammatory cytokines were increased significantly.

The role of calcitriol on ALI has not been clear till now. It is generally accepted that vitamin D has an effective therapeutic role in sepsis and calcitriol, the active form of vitamin D, has been reported to induce host defense[24]. Since ALI is characterized with the disruption of the alveolar endothelial and epithelial barrier[4, 5], vitamin D has been reported to attenuate lung edema via blocking the Ang-2-Tie-2-MLC kinase cascade and the renin-angiotensin system to repair alveolar endothelial and epithelial barrier[25]. Meanwhile, several researches have demonstrated that $1,25(\text{OH})_2\text{D}_3$, the hormonal form of vitamin D, has been shown to reduce vascular permeability and ameliorate pulmonary edema[25–27]. Nie et al. showed that vitamin D augments transalveolar fluid clearance via unregulating the expression of a-ENaC[28]. Moreover, vitamin D has been reported to alleviate seawater aspiration-induced lung injury via inhibiting nuclear translocation of NF- κ B and membrane translocation of RhoA and protecting alveolar epithelial and pulmonary microvascular endothelial barrier[27].

In the current study, metabolomics was used to detect the changes of differential metabolites in the three groups. A total of 49 metabolites were identified as the differential metabolites and the trends of 16 metabolites were opposite in LPS group and LPS + VD group. These metabolites were selected to detect their target proteins via STITCH database. We integrated these target proteins with differential genes in GSE1871 and target proteins of VDR. Finally, a total of 25 proteins were selected and considered to be closely related to the mechanism of calcitriol ameliorates ALI. STRING database was used to demonstrate the GO analysis and KEGG pathway analysis.

From the KEGG pathways, TNF signaling pathway, PI3K-Akt signaling pathway and Toll-like receptor signaling pathway are closely related to the mechanism of calcitriol ameliorates ALI.

TNF signaling pathway is one of the critical pathways in the development and maintenance of inflammation[29] and it has been found to provoke the release of inflammatory cytokines in ALI and mediate the following innate immune response and inflammatory process[30]. Previous study demonstrated that TNF is initiated by TNF- α receptor 1 (TNFR1), its activation induces the increased expression of nuclear factor kappa B (NF- κ B)[31]. Zhang et. al. has indicated that $1,25(\text{OH})_2\text{D}_3$ down-regulated TNF signaling pathway to alleviate osteoarthritis[32].

Toll-like receptor signaling pathway functions as molecular patterns in adaptive or innate immunity and the activation of toll-like receptor signaling pathway leads to the production of costimulatory factors and cytokines, which is one important reasons contributing to ALI[33]. The normal activation of TLRs is critical to defense molecules in the recognition of pathogens, including M. tuberculosis[34–36]. In HIV-seropositive individuals, vitamin D rescues impaired Mycobacterium tuberculosis-mediated tumor necrosis factor release in macrophages by enhancing toll-like receptor signaling pathway[37]. However, uncontrolled excessive TLR activation may lead to serious infection[38]. Multiple lines of evidence showed that ARDS is associated with the vigorous activation of TLR2, TLR3, TLR4 and TLR9[39] and noninfectious ALI is associated with the activation of TLR2 and TLR4[40]. In addition, the survival rates of ARDS patients were correlated with downregulation of TLR1, TLR4 and TLR5[41]. One pharmacologic strategy to manage the excessive inflammation in ALI is to regulate over activation of Toll-like receptors (TLRs)[42].

PI3K-Akt signaling pathway is expressed in eukaryotes widely, which plays critical roles in growth, differentiation, proliferation, and survival[43, 44]. Several groups have documented that $1,25(\text{OH})_2\text{D}_3$ activates PI3K/Akt in vitro in a variety of cell types, like squamous cell carcinoma cells, osteoblasts and podocytes[45–47]. Xiao et al. has demonstrated that $1,25(\text{OH})_2\text{D}_3$ activates PI3K-Akt signaling pathway to protect podocytes from apoptosis[47]. Studies showed that the phosphorylation of

Akt downregulates the downstream pro-apoptotic factor Bax and upregulates the anti-apoptotic factor Bcl-2, which inhibits cell apoptosis[48]. LPS-induced ALI is highly associated with cell apoptosis[49] and thus in the treatment of calcitriol, PI3K-Akt signaling pathway plays a critical role.

In conclusion, in this study, the pharmacologic effect of calcitriol against LPS-induced ALI was evaluated in vivo and with the combination of metabolomics, GEO database and network pharmacology, we found out 25 related proteins which were associated to the protective effects of calcitriol. GO and KEGG analysis were used to classify these identified proteins and we selected TNF signaling pathway, PI3K-Akt signaling pathway and Toll-like receptor signaling pathway to be the potential pathway which are closely related to the mechanism of calcitriol ameliorates ALI. Since the network pharmacology strategy produces many possible targets, these differential targets should be thoroughly tested in our further studies.

5. Conclusions:

In this study, the pharmacologic effect of $1,25(\text{OH})_2\text{D}_3$ against ALI was evaluated in vivo. Then we performed metabolomics to find out 16 differential metabolites which were associated with the protective effects of $1,25(\text{OH})_2\text{D}_3$ against ALI. Meanwhile, STRING database and GEO database was used to find out the differential metabolites-related proteins and disease-related proteins. GO and KEGG analysis were used to classify these identified proteins. Further studies will be performed about the differential proteins to understand the mechanism of $1,25(\text{OH})_2\text{D}_3$ against ALI and the potential clinical applications.

Abbreviations

ALI: acute lung injury; ARDS: acute respiratory distress syndrome; VDR: vitamin D receptor; RXR: retinoid X receptor; LPS: lipopolysaccharide; H&E: hematoxylin-eosin; PCA: principal component analysis; PLS-DA: partial least squares discriminate analysis; VIP: Variable importance plot; GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; $1,25(\text{OH})_2\text{D}_3$: 1,25-dihydroxyvitamin D.

Declarations

Ethics approval and consent to participate:

All studies were reviewed and approved by the Animal Experiment Administration Committee of the Shanghai Pulmonary Hospital (Shanghai, China).

Consent for publication:

Not applicable.

Availability of data and materials:

The data and materials in this study are available from the corresponding author on reasonable request.

Competing interests:

The authors report no conflicts of interest.

Funding:

Authors' contributions:

XS, YLC and YGZ planned and did experiments including animal experiments, QPCR and ELISA. XHJ, HPZ and PCZ analyzed the data from metabolomics, JW, DF, QQZ and YG used the database to obtain the related proteins, YGZ and XHJ conceived the data, XS and YLC wrote the manuscript, XL planned the project and conceived the experiments. All authors read and approved the final manuscript.

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Figures

Figure 1

Scheme for signature-oriented precise investigation of calcitriol against sepsis-induced ALI. Efficacy evaluation of calcitriol was measured by H&E and inflammatory cytokines. Signatures of sepsis-induced ALI and calcitriol pretreatment were measured by metabolomics and network pharmacology. Shared proteins, targets and pathways were obtained by combining these target proteins together.

Figure 2

Calcitriol ameliorated the sepsis-induced ALI. (A) Hematoxylin and eosin staining of lung tissues (upper panel: Magnification, $\times 100$; lower panel: Magnification, $\times 200$). (B-D) IL-1 β , IL-6 and TNF- α were measured by ELISA in serum samples. (E-H) The mRNA expression of IL-1 β , IL-6, TNF- α and IL-10 were measured by RT-qPCR analysis.

Figure 3

PLS-DA and VIP-score plot in positive and negative modes. (A-B) Score plots of the partial least squares discriminant analysis (PLS-DA) scores performed on the UHPLC/Q-TOF MS profile of serum samples obtained from the control group (black squares), LPS group (red round) and LPS+VD group (blue rhombus) in the positive (A) and negative (B) modes. (C-D) S-plot based on PLS-DA analysis from ESI positive ion mode (C) and negative mode (D). (E-F) The combination of S-plot and VIP-score plot based on PLS-DA analysis from ESI positive ion mode (E) and negative mode (F).

Figure 4

Heatmaps showed the expression levels of differential metabolites. (A-B) Heatmaps visualization of the differential metabolites in the three groups in positive (A) and negative (B) groups. Each row represents a metabolite and each column represents the expression level (red: upregulation, blue: downregulation). (C) Enriched pathways of these differential metabolites.

Figure 5

Topological network schematic of differential metabolites-related proteins, disease-related proteins and VDR-related proteins. (A) A venn diagram of proteins in these three groups and 25 proteins were shown in all three groups. (B) Protein-protein interactions networks of proteins targeted by differential metabolites, genes associated with acute lung injury and genes associated with VDR. (C) Metabolites-targets-disease network. The hexagon outside represent the differential metabolites; the nodes in the middle part represent the proteins overlapping proteins. Nodes with deeper color means they have more interactions with other nodes.

Figure 6

GO and KEGG analysis of overlapping proteins. (A-C) GO enrichment analysis identified genes involved in biological processes (A), cellular components (B) and molecular functions (C). (D) KEGG pathway enrichment analysis. The color scales indicate different thresholds of adjusted p-values, and the sizes of the dots represent the gene counts of each term.

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