Intensive Care Med (2012) 38:894–905
DOI 10.1007/s00134-012-2498-z

EXPERIMENTAL

Takeshi Oyaizu
Shan-Yu Fung
Atsushi Shiozaki
Zehong Guan
Qiao Zhang
Claudia C. dos Santos
Bing Han
Marco Mura
Shaf Keshavjee
Mingyao Liu

Src tyrosine kinase inhibition prevents pulmonary ischemia–reperfusion-induced acute lung injury

Received: 22 February 2011
Accepted: 6 December 2011
Published online: 17 February 2012
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T. Oyaizu and S.-Y. Fung contribution equally to this work.

Electronic supplementary material
The online version of this article (doi:10.1007/s00134-012-2498-z) contains supplementary material, which is available to authorized users.

T. Oyaizu · S.-Y. Fung · A. Shiozaki · Z. Guan · Q. Zhang · B. Han · M. Mura · S. Keshavjee · M. Liu
Latner Thoracic Surgery Research Laboratories, University Health Network, Toronto General Research Institute, Toronto, ON, Canada

C. C. dos Santos
Division of Critical Care Medicine, Saint Michael's Hospital, Toronto, ON, Canada

S. Keshavjee · M. Liu (✉)
Department of Surgery, Faculty of Medicine, University of Toronto, 101 College Street, Room TMDT 2-814, Toronto, ON M5G 1L7, Canada
e-mail: mingyao.liu@utoronto.ca
Tel.: +416-581-7501
Fax: +416-581-7702

Abstract *Purpose:* Pulmonary ischemia–reperfusion is a pathological process seen in several clinical conditions, including lung transplantation, cardiopulmonary bypass, resuscitation for circulatory arrest, atherosclerosis, and pulmonary embolism. A better understanding of its molecular mechanisms is very important. *Methods:* Rat left lung underwent in situ ischemia for 60 min, followed by 2 h of reperfusion. The gene expression profiles and Src protein tyrosine kinase (PTK) phosphorylation were studied over time, and PP2, an Src PTK inhibitor, was intravenously administered 10 min before lung ischemia to determine the role of Src PTK in lung injury. *Results:* Reperfusion following ischemia significantly changed the expression of 169 genes, with *Mmp8*, *Mmp9*, *S100a9*, and *S100a8* being the most upregulated genes. Ischemia alone only affected expression of 9 genes in the lung. However, Src PTK phosphorylation (activation) was increased in the ischemic lung, mainly on the alveolar wall. Src PTK inhibitor pretreatment decreased phosphorylation of Src PTKs, total protein tyrosine phosphorylation, and STAT3 phosphorylation. It increased phosphorylation of the p85α subunit of PI3 kinase, a signal pathway that can inhibit coagulation and inflammation. PP2 reduced leukocyte infiltration in the lung, apoptotic cell death, fibrin deposition, and severity of acute lung injury after reperfusion. Src inhibition also significantly reduced CXCL1 (GRO/KI) and CCL2 (MCP-1) chemokine levels in the serum. *Conclusion:* During pulmonary ischemia, Src PTK activation, rather than alteration in gene expression, may play a critical role in reperfusion-induced lung injury. Src PTK inhibition presents a new prophylactic treatment for pulmonary ischemia–reperfusion-induced acute lung injury.

Keywords Microarray · Bioinformatics · Signal transduction · Lung pathology · Molecular therapy

Introduction

Pulmonary ischemia–reperfusion (IR) is associated with a wide range of clinical events, including lung transplantation, cardiopulmonary bypass, trauma, resuscitation for circulatory arrest, atherosclerosis, and pulmonary embolism [1]. It contributes to high morbidity and mortality in these patients [2]. In the case of lung transplantation (LTx), the warm ischemic period after cardiac arrest can cause cellular and tissue damage that often leads to lung

injury [3]. Identifying molecular mechanisms associated with pulmonary IR-related lung injury may provide critical insight for the development of novel therapeutics.

Microarray-based technology provides a powerful tool to examine gene expression profiles of lungs at the genomic levels. It has been applied for gene expression profiling in IR-related tissue injury in the brain [4, 5], heart [6, 7], and kidney [8]. We have performed microarray studies on lung tissues collected during the cold IR process of rat [9] and human [10] LTx. Tyrosine phosphorylation is an important molecular mechanism related to acute inflammatory responses [11]. Particularly, activation of Src protein tyrosine kinase (PTK) has been identified as an essential mechanism for the recruitment and activation of monocytes, macrophages, and neutrophils [11]. It also plays a critical role in the regulation of vascular permeability [11, 12]. Src PTK activation is involved in ischemia- or IR-induced injury in the brain [13, 14], heart [15, 16], liver [17], and kidney [18, 19]. Src PTK inhibition prevented lipopolysaccharide (LPS)-induced acute lung injury [20, 21]. Protein tyrosine phosphorylation and Src PTK activity were altered over time during hypothermic IR processes in both clinically transplanted human lungs and experimentally transplanted rat lungs [22, 23].

In the present study, we performed gene profiling in rat lungs after pulmonary IR and investigated the effects of *warm* IR on Src PTK activation. Accordingly, a selective Src PTK inhibitor was employed to test the therapeutic potential of Src inhibition in preventing pulmonary IR-induced lung injury.

Materials and methods

Reagents and antibodies

A selective Src PTK inhibitor, 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)-pyrazolo [3,4-*d*] pyrimidine (PP2), was from Biomol International LP (Plymouth Meeting, PA). Antibodies for phosphorylated-Src (Tyr416), phoshporylated-p85 (Tyr199), STAT3, and phosphorylated-STAT3 (Tyr705) were from Cell Signaling Technology (Beverly, MA). Antibodies for p-Hck (Tyr411) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies for Src (GD11), p85α, and phosphotyrosine (4G10) were from Upstate Biotechnology Inc. (Lake Placid, NY). T6Rat IL-6 ELISA kit was from BD Biosciences (San Jose, CA). ELISA kits for CXCL1 (GRO/KC) and CCL2 (MCP-1) were from R&D Systems (Minneapolis, MN) and Invitrogen Inc. (Burlington, ON, Canada), respectively.

Animals

Male Sprague–Dawley rats, weighing from 300 to 350 g, were purchased from Charles River (Montreal, Canada). The experimental protocol was approved by the Toronto General Research Institute Animal Care and Use Committee. All animals received care in compliance with the *Principles of Laboratory Animal Care* formulated by the National Society for Medical Research, the *Guide for the Care and Use of Laboratory Animals* (NIH publication No. 85-23, Revised 1985, U.S. Government Printing Office, Washington, DC), and the *Guide to the Care and Use of Experimental Animals* formulated by the Canadian Council on Animal Care.

Rat lung IR model

Rats were anesthetized by intraperitoneal administration of ketamine (100 mg/kg) and xylazine (10 mg/kg). Atropine (0.2 mg) was injected intramuscularly. Animals were tracheotomized, intubated with a 12-gauge intravenous catheter, and ventilated using a model 683 ventilator (Harvard Apparatus Canada, Saint-Laurent, QC, Canada) with a tidal volume of 10 ml/kg with 2 cmH_2O positive end-expiratory pressure (PEEP). Fraction of inspired oxygen (FiO_2) and respiratory rate were kept at 0.6 and 70 cycles/min, respectively. A 24-gauge intravenous catheter was inserted into left jugular vein for injection. Anesthesia was maintained by administering one-third of the initial anesthetic dose in normal saline at 1 ml/h as a continuous infusion using a syringe pump (KDS100, KD Scientific Inc., Holliston, MA). Animals were placed on their right side and lateral thoracotomy was performed at the fifth left intercostal space. To achieve tissue ischemia, the left lung hilum was clamped at the end-expiration with two non-clash microclips (Sugita® Aneurysm Clip 07-940-88, Mizuho, Tokyo, Japan) 5 min after administration of an intravenous heparin bolus (50 IU/animal). The tidal volume was reduced to 6 ml/kg during the left lung hilum clamping. The lung was covered with a plastic wrap to prevent unnecessary evaporation. The clips were removed after 60 min and the thoracic cavity was sutured closed. Animals were placed in the supine position for 30 min or 120 min under mechanical ventilation in the same condition as before lung ischemia. In the Src PTK inhibition study, PP2 was dissolved with 20% dimethyl sulfoxide (DMSO) in normal saline. On the basis of literature precedent [24] and our pilot studies, PP2 (0.2 mg/kg) solution was injected into left jugular vein 10 min before lung ischemia with 20% DMSO as vehicle control.

Arterial blood gas analysis

At the end of 120 min reperfusion, animals were laparotomized to expose the thoracic and abdominal cavities. The right main bronchus and pulmonary vessels were occluded. Animals were ventilated with tidal volume of 6 ml/kg, 2 cmH_2O of PEEP, 100% FiO_2, and 70 breaths/min for 5 min. Blood samples for arterial blood gas analysis were obtained by puncturing the ascending aorta.

Histological studies

The pulmonary vasculature was flushed from the right ventricle with 20 ml of normal saline. The lung was injected with 10% formalin through the airway at 20 cmH_2O of pressure. The tissues were dehydrated, embedded in paraffin, cut into 4-μm sections, and mounted. After rehydration by paraffin removal, the tissues were stained with hematoxylin and eosin [25]. Infiltrated leukocytes were counted in a blinded fashion. Martius Scarlet Blue staining was performed for alveolar fibrin deposition [26–28]. Cell death was assessed by terminal transferase dUTP nick end labeling (TUNEL) staining [27, 29, 30]. All images were acquired with a Nikon Eclipse 80i microscope.

Immunohistochemistry staining of phosphorylated Src

The paraffin-embedded lung sections were deparaffinized, followed by antigen retrieval in boiling 0.01 M citrate buffer (pH 6) for 20 min. The slides were then blocked with 3% H_2O_2 for 5 min, avidin and biotin (Vector Labs Inc., Burlington, Canada) for 15 min, and 5% goat serum in serum-free blocking reagent (Dako Inc., Burlington, Canada) for 1 h. The slides were incubated with a polyclonal anti-phosphorylated-Src (Tyr416) antibody (Abcam, Cambridge, MA) at 1:100 dilution overnight at 4°C. After washing with PBS (containing 0.1% Tween 20), the sections were incubated with goat anti-rabbit antibody (Vector Labs Inc.) for 30 min and developed with a DAB substrate kit (Zymed Labs, San Francisco, CA), followed by hematoxylin counterstaining. Images were scanned at 40× with a ScanScope, and the positive pixels per square millimeter were quantified and normalized with a Positive Pixel Count Algorithm program (Aperio, Vista, CA).

Microarray and data analysis

Total RNA was extracted [9, 31]. Equal amounts of RNA from three animals in each group were used for microarray. Rat Gene ST 1.0 chips from Affymetrix (Santa Clara, CA) were used. Probe-based analysis, background reduction, and quantile data normalization were performed in MAS 5.0 (Affymetrix). Scaled values were analyzed in Genespring (http://www.silicongenetics.com). Gene expression data were further filtered on the basis of raw intensity values. To identify differentially expressed genes, normalized data were imported into R (Statistical Package for Microarray Analysis, http://www.statistik.lmu.de/strimmer/notes/rexpress.html), analyzed using the Linear Models for Microarray Data library (LIMMA) of the Bioconductor package (http://www.bioconductor.org), and confirmed using Significance Analysis of Microarray (SAM, http://www-stat.stanford.edu/tibs/SAM). JMP version 5.0.1J (SAS Institute Inc., Cary, NC) was used for hierarchical cluster analysis. Functional networks induced by pulmonary IR were analyzed with Ingenuity Pathway Analysis (Ingenuity Systems, Inc. Redwood City, CA) [32].

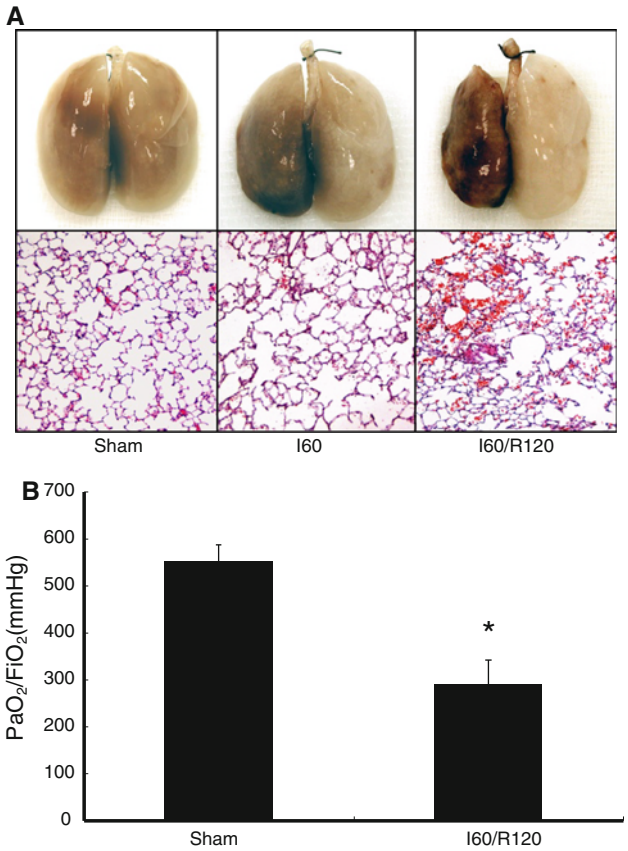

Fig. 1 Pulmonary IR-induced lung injury in rats. **a** Ischemia and IR resulted in diffuse alveolar damage in the left lung. Alveolar hemorrhage and interstitial edema were found in damaged left lung following ischemia and further enhanced after reperfusion. **b** Pulmonary IR (60 min ischemia and 120 min reperfusion) significantly reduced oxygenation function of the left lung (mean ± SEM, $n = 6$ in each group, $^*P < 0.05$)

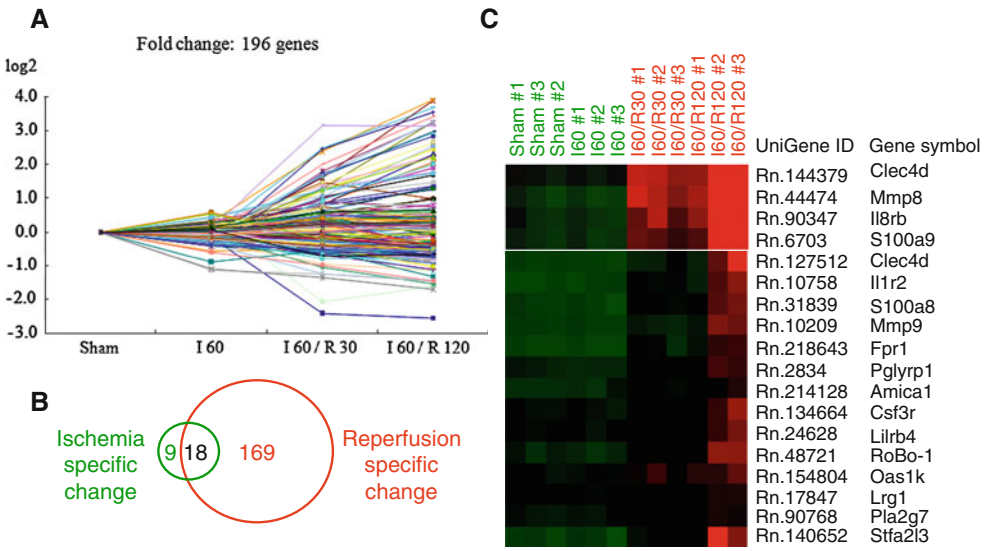

Fig. 2 Pulmonary IR-induced changes of gene expression. **a** Fold change of 196 genes expressed in log 2 scale. **b** Venn diagram shows the number of ischemia- or IR-specific genes. **c** Unsupervised hierarchical clustering analysis demonstrates gene expression patterns. *Each column* corresponds to one animal, and *each row* corresponds to a particular gene. The *matrix* represents the expression level of an individual gene in each sample, with *red* and *green* indicating gene expression levels above or below the reference RNA, respectively. *Sham* sham control animal, *I60* 60 min ischemia, *I60/R30* 60 min ischemia and 30 min reperfusion, *I60/R120* 60 min ischemia and 120 min reperfusion

Immunoblotting

Immunoblotting was performed as previously described [22, 23, 33]. In brief, 40 μg of protein was separated by SDS-PAGE and transferred to nitrocellulose membranes (Schleicher & Schuell, Whatman, Middlesex, UK). The membranes were incubated with designated antibodies. The proteins of interests were detected with an enhanced chemiluminescence detection kit (ECL) (Amersham Pharmacia Biotech, Little Chalfort, UK) on X-ray film.

Enzyme-linked immunosorbent assay (ELISA)

The inflammatory cytokine IL-6 levels in the lung lysates and serum levels of CXCL1 (GRO/KC) and CCL2 (MCP-1) were measured with ELISA, following the standard procedure.

Serum cytokine/chemokine analysis

Serum samples from 3 rats/group were tested for cytokines/chemokines levels with a multiplex magnetic beads assay (MILLIPLEX MAG Rat Cytokine/Chemokine Magnetic Bead Panel—Premixed 23 Plex, Millipore, Billerica, MA), following the company's instructions.

Statistical analyses

Statistical analyses were performed using the statistical software JMP version 5.0.1J (SAS Institute Inc., Cary, NC). Results were expressed as the mean ± standard error of the mean (SEM). A Student's *t* test was applied for comparison between two groups, whereas one-way ANOVA followed by Tukey–Kramer HSD tests were used for multiple groups. Differences are considered significant when the *P* value is no greater than 0.05.

Results

Pulmonary IR-induced lung injury

The severity of lung injury was determined by assessment of lung histology and oxygenation function. Compared to the non-ischemic right lung and the lungs in the sham group, the left lung appeared hyperemic following 60 min ischemia. Gross pathological changes were consistent with the congestion and hemorrhage. Hyperemic changes were further exacerbated after 120 min reperfusion (Fig. 1a top panel). Histological assessments confirmed the development of interstitial edema and alveolar hemorrhage in the ischemic lungs, which was further enhanced after reperfusion (Fig. 1a lower panel). Oxygenation function of the left lungs (PaO_2/FiO_2, ratio of the partial pressure of arterial oxygen to FiO_2) was

Table 1 Genes upregulated during the reperfusion period

Reperfusion-specific upregulation (top 40)			Fold change	
Gene symbol	Unigene	Gene name	I60–R30/I60	I60–R120/I60
Mmp8	Rn.44474	Matrix metallopeptidase 8	7.14	15.09
S100a8	Rn.31839	S100 calcium binding protein A8 (calgranulin A)	4.61	12.54
Il1r2	Rn.10758	Interleukin 1 receptor, type II	4.11	11.91
Fpr1	Rn.218643	Formyl peptide receptor 1	4.26	11.49
Stfa2l2	Rn.214186	Stefin A2-like 2	3.47	10.36
Mmp9	Rn.10209	Matrix metallopeptidase 9	2.55	9.98
S100a9	Rn.6703	S100 calcium binding protein A9 (calgranulin B)	4.28	9.09
Il8rb	Rn.90347	Interleukin 8 receptor, beta	3.50	8.64
Clec4d	Rn.144379	C-type lectin domain family 4, member d	3.31	7.86
LOC24906	Rn.48721	RoBo-1	2.78	6.55
Stfa2	Rn.144936	Stefin A2	2.74	6.37
Pram1	Rn.85856	PML-RAR alpha-regulated adaptor molecule 1	2.07	5.78
Pglyrp1	Rn.2834	Peptidoglycan recognition protein 1	2.49	5.23
Prok2	Rn.211872	Prokineticin 2	2.29	4.56
Il18rap	Rn.105609	Interleukin 18 receptor accessory protein	1.79	4.16
Olfm4	Rn.156265	Olfactomedin 4	1.43	4.01
Amica1	Rn.214128	Adhesion molecule, interacts with CXADR antigen 1	2.18	3.93
Csf3r	Rn.134664	Colony stimulating factor 3 receptor (granulocyte)	2.10	3.90
Fcar	Rn.123516	IgA Fc receptor	2.61	3.62
Oas1k	Rn.154804	2′–5′ oligoadenylate synthetase 1K	2.49	3.42
Lilrb4	Rn.24628	Leukocyte immunoglobulin-like receptor, subfamily B, member 4	2.07	3.24
Camp	Rn.33146	Cathelicidin antimicrobial peptide	1.12	3.09
Lrg1	Rn.17847	Leucine-rich alpha-2-glycoprotein 1	2.04	2.87
RGD1565374	Rn.22264	Similar to hypothetical protein LOC199675	1.62	2.61
Pla2g7	Rn.90768	Phospholipase A2, group VII (platelet-activating factor acetylhydrolase, plasma)	1.73	2.45
Mgam	–	Maltase-glucoamylase	1.63	2.44
F5	Rn.82910	Coagulation factor V	1.54	2.08
Eid3	Rn.206488	EP300 interacting inhibitor of differentiation 3	1.50	1.99
Nalp12	Rn.108865	NACHT, leucine-rich repeat and PYD containing 12	1.73	1.94
Pgf	Rn.6960	Placental growth factor	1.78	1.92
Gadd45a	Rn.10250	Growth arrest and DNA-damage-inducible 45 alpha	1.22	1.84
Steap2	Rn.203614	Six transmembrane epithelial antigen of the prostate 2	1.48	1.83
Jmjd3	Rn.205533	Jumonji domain containing 3	1.32	1.78
Ccr1l1	Rn.218537	Chemokine (C–C motif) receptor 1-like 1	1.24	1.69
Fn1	Rn.1604	Fibronectin 1	1.38	1.59
Fgd3	Rn.214480	FYVE, RhoGEF and PH domain containing 3	1.24	1.58
S1pr3	Rn.108119	Sphingosine-1-phosphate receptor 3	1.76	1.55
RGD1309326	Rn.145001	Similar to RIKEN cDNA 2410002F23	1.55	1.49
Map3k9	Rn.18840	Mitogen-activated protein kinase 9	1.21	1.45
Ldhc	Rn.9984	Lactate dehydrogenase C	1.18	1.42

significantly decreased after reperfusion (I60/R120, Fig. 1b). Ischemia for 60 min followed by 120 min reperfusion caused acute lung injury.

Gene expression profile after pulmonary ischemia and IR

To determine transcriptional changes associated with pulmonary ischemia and/or IR, we performed microarray analysis. Three conditions (I60, I60/R30, and I60/R120) were studied in comparison with the sham control. Of 27,342 expressed sequence tags spotted on the chip, 196 genes significantly changed during IR (Fig. 2a). Among them, 27 genes were significantly regulated after ischemia, of which only 9 genes were ischemia-specific, with 2 upregulated and 7 downregulated (Fig. 2b). In contrast, there were 169 genes differentially expressed primarily after reperfusion (Fig. 2b). Significant changes in gene expression were noted as early as with 30 min reperfusion (I60/R30). Most expression changes became more pronounced (further increased or decreased in the same direction) after 120 min reperfusion (I60/R120) (Fig. 2a). Specific trends can be seen from the top 40 upregulated (Table 1) and top 40 downregulated (Table 2) genes. In an unsupervised hierarchical cluster analysis, the sham and I60 groups were clustered together and separated from the reperfusion (I60/R30 and I60/R120) groups. A small group of genes is shown in Fig. 2c to illustrate the clustering distribution among these four groups.

Table 2 Genes downregulated during the reperfusion period

Reperfusion specific downregulation (top 40)			Fold change	
Gene symbol	Unigene	Gene name	I60–R30/I60	I60–R120/I60
Rgs13	–	Regulator of G-protein signaling 13	0.50	0.36
Slc15a2	Rn.89268	Solute carrier family 15 (H^+/peptide transporter), member 2	0.76	0.50
Kcnmb2	Rn.92476	Potassium large conductance calcium-activated channel, subfamily M, beta member 2	0.57	0.53
Acsm5	Rn.35367	Similar to Aa2-174	0.86	0.55
Igh-1a	Rn.202986	Immunoglobulin heavy chain 1a (serum IgG2a)	0.64	0.56
Sytl2	Rn.7634	Synaptotagmin-like 2	0.72	0.56
Abca8	Rn.15354	ATP-binding cassette, sub-family A (ABC1), member 8b	0.88	0.58
RGD1565033	Rn.143962	Similar to hypothetical protein LOC284018 isoform b	0.89	0.59
Akr7a3	Rn.6043	Aldo–keto reductase family 7, member A3 (aflatoxin aldehyde reductase)	0.81	0.60
Ccdc129	Rn.117897	Coiled-coil domain containing 129	0.93	0.60
Zfp462	Rn.23557	Zinc finger protein 462	0.59	0.61
LOC499980	Rn.58002	Hypothetical protein LOC499980	0.85	0.61
Kcne2	Rn.48691	Potassium voltage-gated channel, Isk-related subfamily, gene 2	0.76	0.61
Eps8l1	Rn.138370	EPS8-like 1	0.76	0.62
Gja5	Rn.88300	Gap junction membrane channel protein alpha 5	0.69	0.63
Serpinb9	Rn.95177	Serine (or cysteine) peptidase inhibitor, clade B, member 9	0.74	0.63
Lrrc1	Rn.15622	Leucine rich repeat containing 1	0.73	0.64
Zfp426l2	Rn.99479	Similar to zinc finger protein 426	0.82	0.64
Hnf4g	Rn.154763	Hepatocyte nuclear factor 4, gamma	0.80	0.65
LOC685849	Rn.214683	Hypothetical protein LOC685849	0.67	0.65
Cxcr7	Rn.12959	Chemokine (C-X-C motif) receptor 7	0.91	0.67
Pou2f3	Rn.81060	POU domain, class 2, transcription factor 3	0.66	0.67
Lmcd1	Rn.20476	LIM and cysteine-rich domains 1	0.86	0.68
Hrasls	Rn.11728	HRAS-like suppressor	0.79	0.68
Slc25a35	Rn.198873	Solute carrier family 25, member 35	0.76	0.68
Pon3	Rn.16469	Paraoxonase 3	0.85	0.69
Slco4c1	Rn.123431	Solute carrier organic anion transporter family, member 4C1	0.90	0.69
MGC72614	Rn.20514	Hypothetical LOC310540	0.86	0.69
Spata17	Rn.122794	Spermatogenesis associated 17	1.33	0.70
Cmbl	Rn.64439	Carboxymethylenebutenolidase homolog (*Pseudomonas*)	0.91	0.70
Pparg	Rn.23443	Peroxisome proliferator activated receptor gamma	0.74	0.71
Nudt2	Rn.40265	Nudix (nucleoside diphosphate linked moiety X)-type motif 2	0.87	0.71
Gpr44	Rn.198542	G protein-coupled receptor 44	0.97	0.71
RGD1564322	Rn.76400	Similar to shippo 1	1.21	0.71
Impa1	Rn.3975	Inositol (myo)-1(or 4)-monophosphatase 1	0.82	0.72
Sirt5	Rn.137920	Sirtuin 5 (silent mating type information regulation 2 homolog) 5 (*S. cerevisiae*)	0.86	0.72
Ggta1	Rn.916	Glycoprotein galactosyltransferase alpha 1,3	0.80	0.72
Ppt1	Rn.1574	Palmitoyl-protein thioesterase 1	0.79	0.73
Gemin8	Rn.18700	Gem (nuclear organelle) associated protein 8	0.87	0.73
Ltc4s	Rn.38594	Leukotriene C4 synthase	0.72	0.73

To elucidate the molecular features of IR-induced lung injury, we used Ingenuity Pathway Analysis (http://www.ingenuity.com/) to search for enrichment in predicted functions amongst genes that were significantly altered in the IR group. "inflammatory response" was the top biological function, whereas the top molecular and cellular function was "cellular movement" (Supplementary Table 1). Indeed, most of the genes in the top network (network 1) are related to "cellular movement, hematological system development and function, immune cell trafficking". These genes are connected to ERK, p38MAPK, PI3K, and PKCs, the signal transduction proteins that can be activated by and are involved in inflammatory responses (Fig. 3).

We were surprised by the fact that only nine genes changed their expression levels after ischemia. These genes are not known to play a role in inflammation or tissue damage. This suggested that the major effects of pulmonary ischemia on lung tissues are not the alteration of gene expression, thus, we further examined the inflammatory response and changes of signal transduction.

Ischemia-induced inflammatory response and Src PTK activation

The tissue levels of IL-6 increased markedly after 60 min of ischemia (Fig. 4a), even though its gene expression

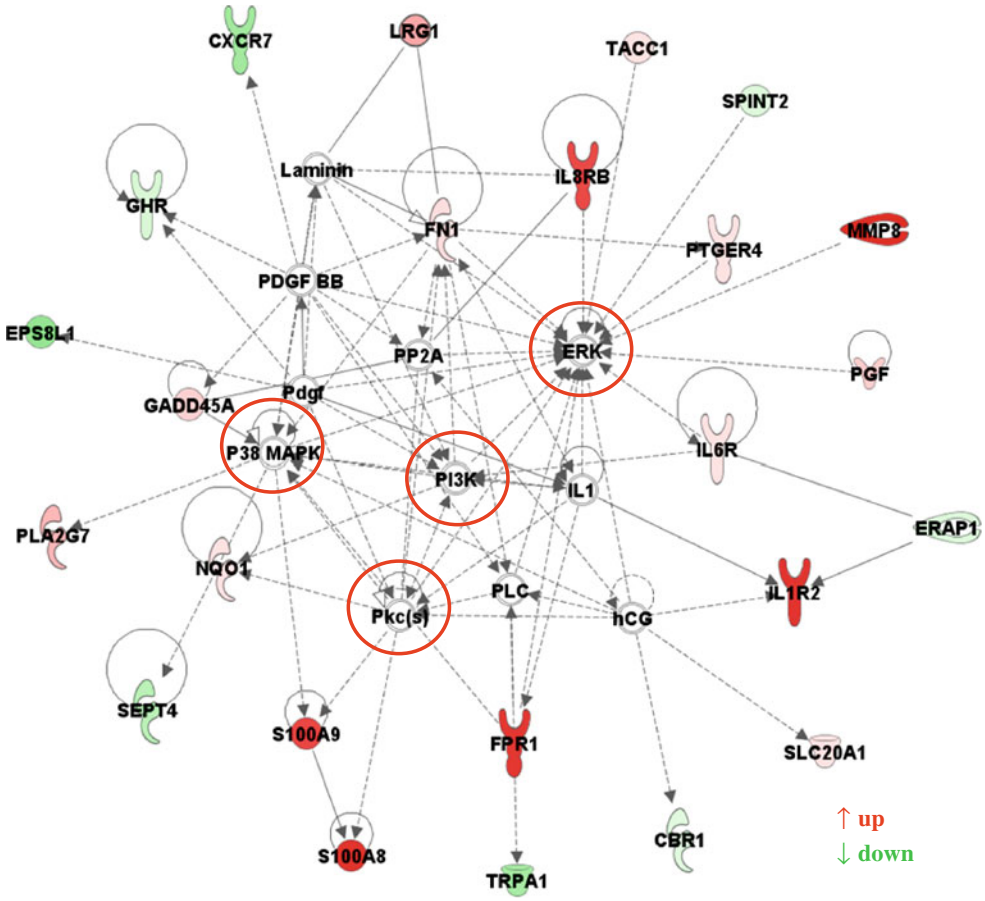

Fig. 3 The top network of genes after pulmonary IR at 2 h. Ingenuity Pathway Analysis was performed on genes significantly changed after I60/R120 (ischemia 60 min and reperfusion 120 min). Signal network related to inflammatory response shows that ERK, p38MAPK, PI3K, and PKCs are located at the *center* of the network

levels remained unchanged as determined by the microarray assay. The total Src protein levels did not change during the IR period. In contrast, the phosphorylation of Src at tyrosine 416, a sign of Src activation, was increased in the ischemic lungs and decreased in a time-dependent fashion during the reperfusion period (Fig. 4b). The total protein tyrosine phosphorylation level was lower in sham and ischemic lungs but higher after reperfusion (Fig. 4b), suggesting that other PTKs and/or protein tyrosine phosphatases may be also involved in the regulation of protein tyrosine phosphorylation during the pulmonary IR process. We then performed immunohistochemistry staining to determine the distribution of activated Src PTK with an anti-pSrcY416 antibody. In the sham control group, phosphorylated Src (brown staining) was very low in the lung (Fig. 4c, left panel). The staining clearly increased on the alveolar septa after 60 min ischemia (Fig. 4c, middle panel), whereas scattered staining was observed in the infiltrated cells in the I60R120 group (Fig. 4c, right panel). The intensity of Src phosphorylation was quantified (Fig. 4d). The increased phosphorylation (activation) of Src PTK after ischemia may prime the lung tissue cells to subsequent inflammatory responses during reperfusion.

Src PTK inhibition before ischemia reduced pulmonary IR-induced lung injury

To determine the involvement of Src PTK activation in the pulmonary IR-induced lung injury, we administrated a chemical Src inhibitor, PP2, 10 min before ischemia through the jugular vein. In comparison with 20% DMSO (the organic solvent for PP2)-treated animals, PP2 pretreatment inhibited phosphorylation of Src Tyr-416 and phosphorylation of Hck, another Src PTK family member, at Tyr-411 during the ischemia period (Fig. 5a), leading to significantly reduced total protein tyrosine phosphorylation (Fig. 5b). In LPS-induced lung injury, STAT3 has been identified as a downstream signal of Src PTK [20]. STAT3 phosphorylation in the lung tissue was significantly decreased by PP2 pretreatment (Fig. 5c). Since Src PTK family is also involved in the PI3 kinase signal pathway [34], we then examined tyrosine phosphorylation of the p85α subunit of PI3K. Surprisingly, it was increased by PP2 pretreatment (Fig. 5d), suggesting an indirect effect of Src PTK on its phosphorylation. PI3 kinase activation suppresses coagulation and inflammation in endotoxemic mice [35].

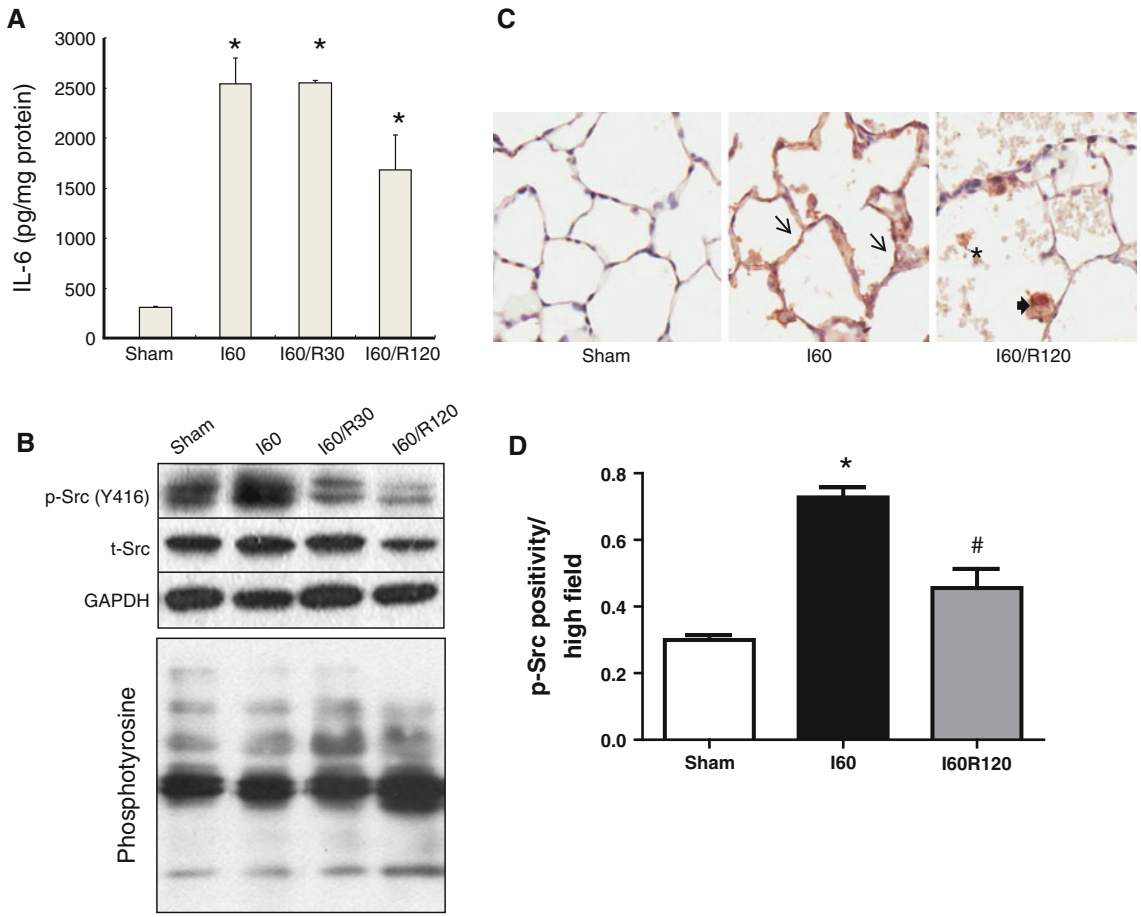

Fig. 4 Changes of IL-6 protein levels and Src PTK tyrosine phosphorylation after pulmonary ischemia and IR. **a** IL-6 in the left lung lysate was increased after pulmonary ischemia and maintained at significantly higher levels after reperfusion (mean ± SEM, $n = 3$ in each group, $^{*}P < 0.05$ compared with sham group). **b** Src Tyr 416-phosphorylation was increased after ischemia but decreased after reperfusion (*top panel*). Total Src protein levels did not change, whereas total protein tyrosine phosphorylation was higher after reperfusion (*bottom panel*). **c** Distribution of phosphorylated Src Tyr416 in the lung tissues. In sham group, pSrcTyr416 staining was very weak in the alveolar space. Ischemia increased the pSrcTyr416 in the alveolar wall (*arrows*). After 2 h reperfusion, pSrcTyr416 was seen mainly in infiltrated cells (*arrowhead*), and non-specific staining was found in the proteins that leaked into the alveolar space (*asterisk*). **d** The pSrcTyr416 staining density was quantified from four rats per group. $^{*}P < 0.01$ compared with other groups; $^{\#}P < 0.05$ compared with Sham group

Lung injury at the end of 120 min reperfusion was reduced in PP2-pretreated animals compared with 20% DMSO-treated ones. Alveolar hemorrhage and interstitial edema were reduced (Fig. 6a). The number of infiltrated leukocytes in the alveolar space was significantly decreased in the PP2-treated group (Fig. 6b). The apoptotic cell death, as determined by TUNEL staining, was significantly reduced in the PP2 group (Fig. 6c, d). Fibrin deposition (seen in the DMSO group) was clearly reduced in the PP2 group (Fig. 6e). The oxygenation function was improved by the PP2 pretreatment, as PaO_2/FiO_2 was significantly higher in PP2-pretreated animals (Fig. 6f).

We also examined the cytokine and chemokine levels in the serum. Among the 23 cytokines/chemokines tested, 8 of them showed measurable concentrations. Upon PP2 treatment, the levels of CXCL1 (GRO/KC) and CCL2 (MCP-1) were significantly decreased (Table 3). These results were validated with ELISA. The serum levels of CXCL1 (GRO/KC) decreased from 10,034 ± 1,378 to 1,724 ± 216 pg/ml ($P = 0.004$), and CCL2 (MCP-1) levels decreased from 752 ± 114 to 251 ± 34 pg/ml ($P = 0.01$). However, the mRNA levels of these two chemokines in the lung tissue lysates were not altered by PP2 treatment, indicating a systemic effect of PP2 on them. We conclude that Src PTK inhibition before ischemia is protective against pulmonary IR-induced lung injury.

Discussion

In the present study, we investigated pulmonary IR-induced gene expression profiles using a microarray/

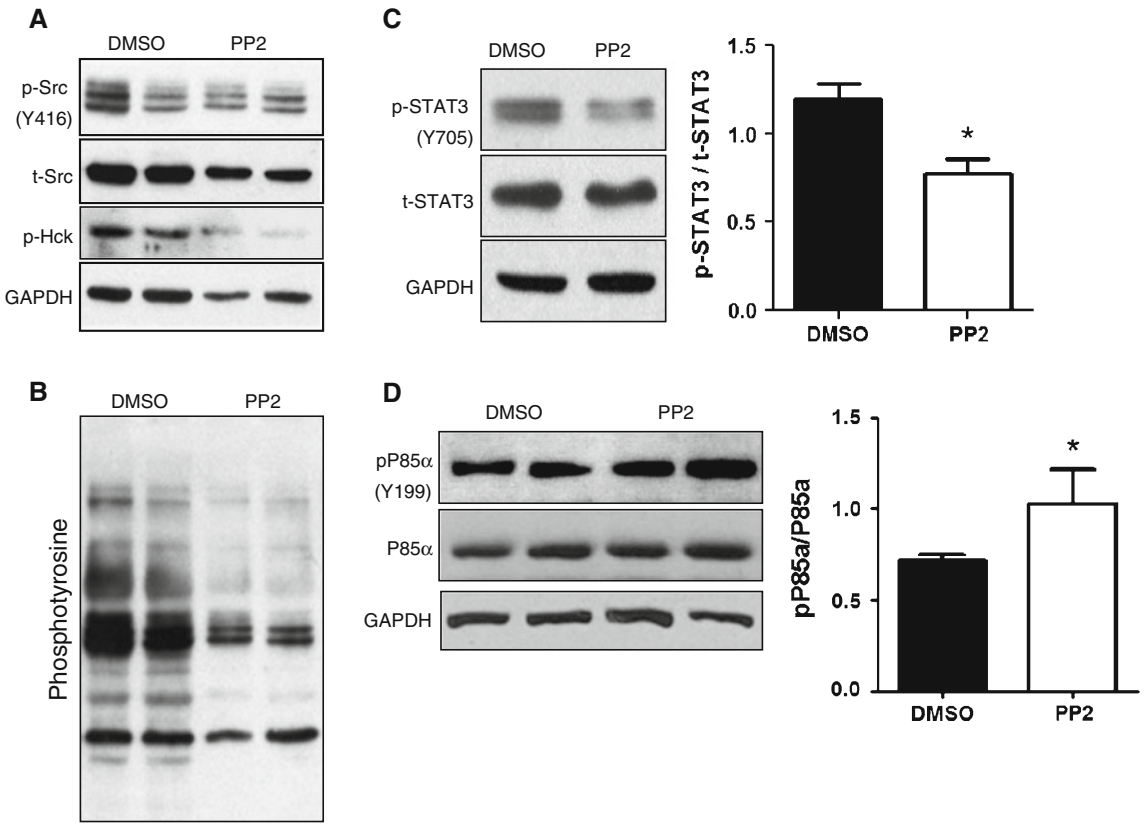

Fig. 5 Src inhibitor PP2 pretreatment reduced inflammation-related signaling. PP2 pretreatment inhibited phosphorylation of Src Tyr-416 and Hck Tyr-411 (**a**), total protein tyrosine phosphorylation (**b**), STAT3 phosphorylation (**c**), but increased tyrosine phosphorylation of PI3 kinase p85α subunit (**d**). $N = 3$ animals/group. $^*P \leq 0.05$

bioinformatics approach. Expression of many genes was significantly altered after reperfusion. Many of these upregulated genes are related to inflammatory responses (Table 1; Fig. 3). The top ranked genes include those associated with matrix metalloproteinases (*Mmp8*, *Mmp9*), chemotactic factors (*S100a8*, *S100a9*, and *Fpr1*), and inflammatory cytokine receptors (*Il1r2*, *Il8rb*). Proteins encoded by these genes are involved in inflammatory responses. *Mmp9*, *Il1r2*, *S100a8*, and *S100a9* were also found in the top listed genes after 24 h hypothermic preservation followed by 2 h reperfusion in a rat LTx model [9]. Increased expression of these genes may reflect leukocyte infiltration. However, lung stromal cells, such as lung epithelial cells, may also contribute to the inflammatory responses [36].

Although ischemia for 60 min alone did not lead to dramatic changes in gene expression, formalin fixation of the ischemic left lung showed dark color, and IL-6 levels increased significantly. Tyrosine phosphorylation (activation) of c-Src was increased. PP2 pretreatment reduced Src and Hck PTK activation. Hck is another Src PTK family member, mainly expressed in granulocytes, monocytes, and macrophages. PP2 also reduced total protein tyrosine phosphorylation, phosphorylation of STAT3, a downstream signal of cytokine receptors [37, 38]. More interestingly, PP2 increased tyrosine phosphorylation of p85α subunits of PI3 kinase, suggesting that under IR conditions, Src PTK may affect p85α tyrosine phosphorylation through both direct and indirect mechanisms. Nonetheless, PI3 kinase pathway activation suppressed coagulation and inflammation in endotoxemic mice [35]. This may explain reduced cell death, fibrin deposition, and inflammatory cytokines in the present study.

More importantly, Src inhibition reduced lung injury, reduced leukocyte infiltration in the lung, apoptotic cell death, fibrin deposition, and prevented deterioration of oxygenation function of the IR lung. CXCL1 (GRO/KC) is an important chemokine for neutrophile recruitment, whereas CCL2 (MCP-1) is important for monocyte recruitment. Their serum levels were significantly reduced by PP2 pretreatment. Therefore, Src PTK activation in ischemic lung could be a critical mechanism which contributes to IR-induced lung injury.

The finding that Src inhibition reduced pulmonary IR-induced acute lung injury implicates its clinical application. PP2 has been shown to reduce organ injury in multiple animal models for different clinical situations

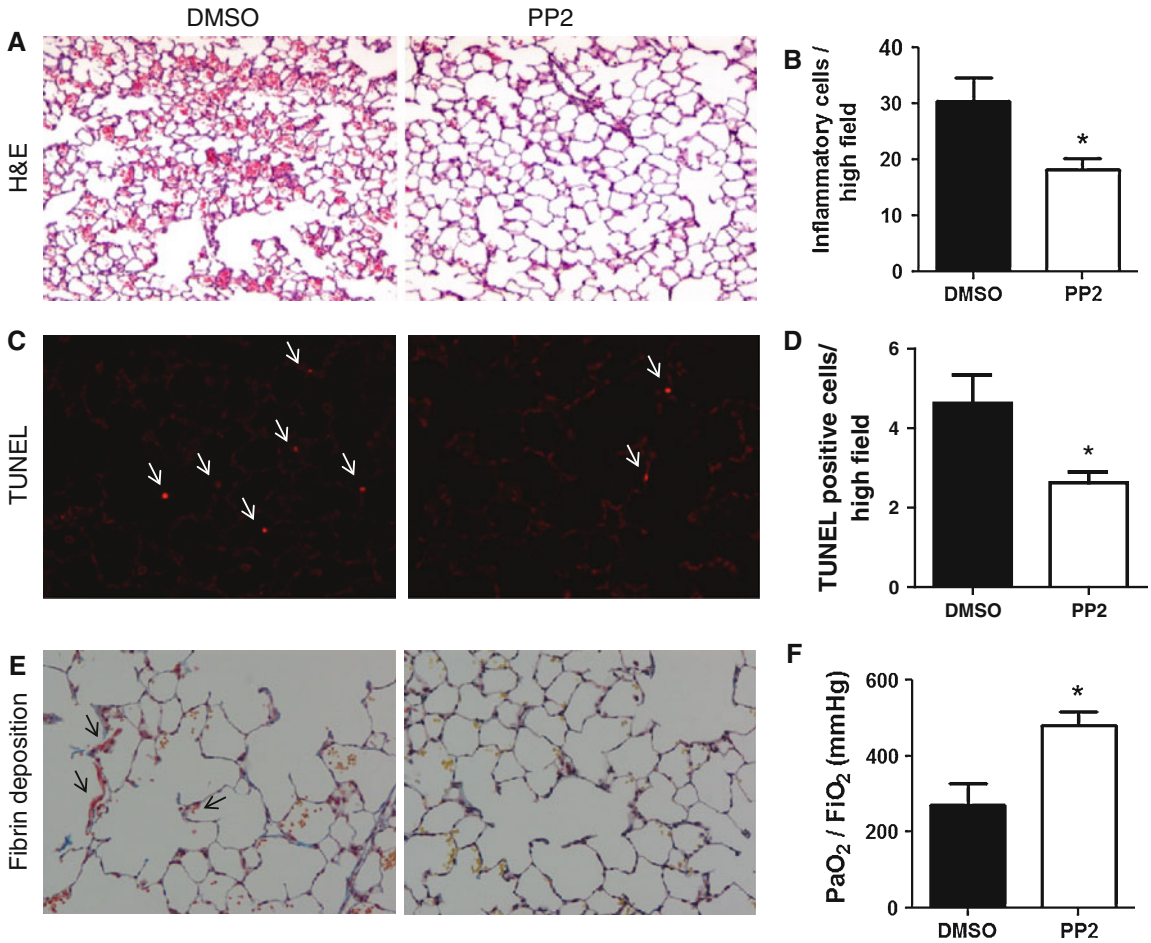

Fig. 6 Src inhibitor PP2 pretreatment reduced pulmonary IR-induced lung injury. PP2 pretreatment reduced lung injury (**a**), infiltration of leukocytes in the alveolar space (**b**), TUNEL positive cell death (**c** and **d**), alveolar fibrin deposition (revealed by Martius Scarlet Blue staining, *pink*) (**e**), and significantly improved oxygenation function of the left lungs (**f**) after 60 min ischemia and 120 min reperfusion. $N = 4$ animals/group. $^{*}P < 0.05$

Table 3 Effects of PP2 treatment on serum levels of cytokines and chemokines after pulmonary IR

Cytokine/ chemokine	DMSO (control) (pg/ml)	PP2 treatment (pg/ml)	*P* value
Leptin	31,907 ± 5,725	25,389 ± 1,934	0.341
IL-10	1,309 ± 143	1,418 ± 189	0.671
IL-12p70	54 ± 13	14 ± 14	0.101
IL-18	183 ± 39	200 ± 73	0.850
CXCL1 (GRO/KC)	38,897 ± 4,875	12,240 ± 1,012	0.006**
CCL2 (MCP-1)	1,988 ± 336	807 ± 70	0.026*
CCL3 (MIP-1α)	181 ± 22	287 ± 176	0.582
CCL5 (Rantes)	3,134 ± 464	2,484 ± 380	0.340

All values are mean ± SEM. ** $P < 0.01$; * $P < 0.05$; $n = 3$

[14, 15, 19, 20, 24]. However, PP2 is a hydrophobic chemical and cannot be dissolved directly in aqueous solution. Recently, a nano-formula has been developed for PP2, which is safe and effective in vivo to reduce LPS-induced lung injury [21]. Therefore, PP2, as well as many other hydrophobic chemicals which are effective in animal models, could be used clinically in the future, using nanoparticle-based drug delivery systems.

In conclusion, the results from the present study suggest that activation of Src PTK during the ischemic period may sensitize the lung to reperfusion-induced injury, which is associated with changes of genes related to inflammatory responses. Blocking Src activation before ischemia may represent a novel therapy to reduce pulmonary IR-induced lung injury.

Acknowledgments Drs. Takeshi Oyaizu and Atsushi Shiozaki were recipients of Research Fellowship awards from the Uehara Memorial Foundation, Japan, and the International Society for Heart and Lung Transplantation. Dr. Shan-Yu Fung was a recipient of Fellowship award from the Ministry of Research and Innovation of Ontario. This study was supported by research grants from the

Canadian Institutes of Health Research (MOP-13270, MOP-42546) and Multi Organ Transplantation Academic Enhancement Funds of University Health Network.

Conflicts of interest The authors have no financial conflict of interest.

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