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Selexipag improves Lipopolysaccharide-induced ARDS on C57BL/6 mice by modulating the cAMP/PKA and cAMP/Epac1 signaling pathways

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SUMMARY

Selexipag, a long-acting and selective prostacyclin (PGI$_2$) IP receptor agonist, has in aged rats with stroke revealed effects of inhibiting inflammation, ameliorating damage to the blood-brain barrier, and alleviating oxidative stress. However, in the case of acute respiratory distress syndrome (ARDS) characterized by diffuse alveolar damage and lung capillary endothelial injury, its effects yet remain unknown. In this study, we investigated effects of the prophylaxis by Selexipag on a mouse model of ARDS established by the Lipopolysaccharide (LPS) challenge and potential mechanism. Compared to the LPS-challenged mice, the LPS-challenged mice with the prophylaxis by 0.5 mg/kg or 1 mg/kg of Selexipag exhibited significantly alleviated lung histological manifestations, reduced protein leakage, decreased levels of interleukin (IL)-1β, IL-6, and monocyte chemotactic protein-1 (MCP-1), diminished expressions of E-selectin and vascular cell adhesion molecule-1 (VCAM-1) mRNA, noticeably increased expressions of zonula occludens-1 (ZO-1) and vascular endothelial cadherin (VE-cadherin) protein, escalated lung cyclic adenosine monophosphate (cAMP) levels, and raised levels of lung relative phosphorylated-protein kinase A catalytic subunit (p-PKA C) at Thr197 and exchange protein activated by cAMP 1 (Epac1) protein. These results suggest that, through suppressing inflammation and reducing vascular endothelial damage, Selexipag can effectively ameliorate the LPS-induced ARDS on mice. The lung cAMP and its downstream signaling modules, PKA and Epac1, possibly constitute the main regulative molecular mechanism. Selexipag appears to hold promise to become a new potential therapeutic option for ARDS.

Keywords: Selexipag; Acute respiratory distress syndrome; Epac 1; PKA; C57BL/6
INTRODUCTION

Acute respiratory distress syndrome (ARDS) is a common and lethal clinical condition with high mortality ranging from 35% to 46%.\(^1\)\(^,\)\(^2\) It pathophysiologically features diffuse alveolar damage (DAD) and lung capillary endothelial injury. A variety of pulmonary and extrapulmonary insults resulting in injuries either to vascular endothelium or to alveolar epithelium bring about an increase in the permeability of alveolar-capillary barrier, generate fluid accumulation in alveolar septa and alveoli, and hence lead to ARDS.\(^3\)\(^,\)\(^4\) Early ARDS usually presents exudative lesions, whereas the late phase frequently exhibits fibroproliferative changes in character. Presentations of ARDS in the early phase catalyzed by pulmonary insults differ from extrapulmonary insults. Pathologically and radiologically, the former prominently reveals injured alveolar epithelial cells plus lung consolidation, while the latter dominantly indicates damaged pulmonary vascular endothelial cells as well as lung ground glass-like lesions.\(^5\)\(^,\)\(^6\)

The lung capillary endothelium is the main site of injuries in early ARDS associated with extrapulmonary insults. In a routine physiological environment, the permeability of pulmonary capillary endothelial barrier is regulated through adherens junction, tight junction, and gap junction.\(^7\)\(^,\)\(^8\) In a process maintaining normal barrier function, the adherens junctions formed by vascular endothelial cadherin (VE-cadherin), the major endothelial adhesion molecule, and catenins, operate in a dynamic manner and actively participate in junctions between endothelial cells, actin cytoskeleton remodeling, and intracellular signal transduction; the tight junctions constituted by occludins, claudins, zonula occludens (ZO) and tight junction adhesion molecules build a highly polarized barrier that selectively passes water, solutes and macromolecules through paracellular osmosis.\(^9\)\(^,\)\(^10\) Furthermore, cytoskeleton remodeling, cell contact reorganization, relaxation and contraction of actin are essential mechanisms to dynamically regulate the endothelial permeability. Some protein kinases, for instance, myosin light chain kinase, protein kinase C, calmodulin-dependent kinase II, protein kinase A (PKA), and protein tyrosine kinase, function as major mediators in these above regulative mechanisms.\(^11\)
Activation of inflammatory cells is widely considered to play a key role in the pathogenesis of ARDS. Activated inflammatory cells give rise to damage to pulmonary vascular endothelial cells and alveolar epithelial cells by substantially elevating production of proinflammatory and inflammatory cytokines, and levels of oxygen free radicals and proteolytic enzymes. In turn, injured vascular endothelial cells promote secretions of inflammatory mediators and adhesion molecules, and further aggravate inflammatory responses. The vicious circle formed between damaged vascular endothelium and inflammatory mediators ultimately leads to progression of ARDS. A range of proinflammatory and inflammatory cytokines, such as interleukin (IL)-1β, IL-6, and tumour necrosis factor alpha (TNF-α) are associated with the occurrence and development of ARDS caused by infections (eg, sepsis), and levels of these cytokines frequently reflect the severity of inflammation in the lung.

Inhibiting inflammation, reducing damage to vascular endothelial cells, and enhancing the vascular endothelial barrier are among key strategies for the therapy of ARDS. According to recent studies, prostacyclin (PGI₂) and its receptors have been implicated in regulations of inflammation and vascular endothelial permeability, and have shown positive effects of anti-inflammatory response and endothelium-dependent anti-edema in acute lung injury (ALI). However, current PGI₂ and PGI₂ analogues possess limited values to ARDS therapy in clinical settings due to their unstable chemical metabolism, short half-life, and low selectivity for PGI₂ receptor, all which result in a series of adverse effects: noticeable hypotension, rapid heartbeat, impaired gastrointestinal function, and pulmonary artery contraction.

Selexipag is a novel prostacyclin IP receptor agonist that can cross the blood-brain barrier. It shows a long half-life lasting 6-8 hours, high selectivity for prostacyclin IP receptor even at low dose, and few incidence of hypotension when applied to the treatment of pulmonary artery hypertension. It is a diphenyl pyrazine derivative and possesses a chemical structure distinguished from PGI₂ and PGI₂ analogues. As exhibited in a number of previous experiments, Selexipag, through its active constituent, MRE269, reduced levels of inflammatory cytokines including IL-1β, TNF-α, monocyte chemotactic protein-1 (MCP-1), and interferon-gamma, lowered the expression of NADPH oxidase (NOX) 2, one of products of oxidative stress, and...
the level of matrix metalloproteinase 9 in mice brain, promoted the secretion of insulin by raising the level of cyclic adenosine monophosphate (cAMP) in MIN6 cells, mitigated damage to pancreatic β cells in streptozotocin-induced diabetes mice by the cAMP/PKA/nephrin signaling pathway, decreased expressions of human skin-derived fibroblasts, like alpha-smooth muscle actin, S100 calcium binding protein A4, collagen type 1, and fibronectin protein, by downregulating phosphorylation of extracellular signal-regulated kinase 1/2 (Erk1/2) and protein kinase B (PKB/Akt), and reduced the production of transforming growth factor-β1 through an increase in the level of cAMP within human lung fibroblasts.

In light of these abovementioned effects displayed by Selexipag, consisting of anti-inflammatory response, alleviating oxidative stress, ameliorating damage to the blood-brain barrier, and anti-fibrosis, we assumed that Selexipag might also be able to produce protection against ARDS. To the best of our knowledge, this study is among the first attempts in the world to reveal what role Selexipag can play in the treatment of ARDS. In this study, we built a mouse model of ARDS by the intraperitoneal injection of Lipopolysaccharide (LPS) and investigated a range of parameters relevant to inflammation, endothelial permeability, damage to vascular endothelial cells, and molecular signal pathway in a bid to explore the effect of Selexipag on ARDS and associated mechanism, as well as to offer some insights into the search for novel therapies of ARDS.

MATERIALS AND METHODS

Drugs and agents

Selexipag was bought from Selleck Chemicals (Houston, TX, the USA), and LPS, Dimethyl Sulfoxide (DMSO), Evans blue, and Formamide from Sigma-Aldrich Corp. (St. Louis, MO, the USA). Bicinchoninic Acid (BCA) Protein Assay Kit was obtained from Beyotime Institute of Biotechnology (shanghai, China), and IL-1β, IL-6, and MCP-1 enzyme-linked immunosorbent assay (ELISA) kits from Jianglai Biotechnology (shanghai, China). TRIzol reagent was acquired from Invitrogen (Carlsbad, CA, the USA) and cDNA Reverse Transcription Kit from Takara Bio Inc (Kusatsu, Shiga, Japan). Primer sequences of IL-1β, IL-6, MCP-1, E-selectin,
VCAM-1, and β-actin were purchased from Sangon Biotech (shanghai, China), and Rabbit anti-VE-cadherin monoclonal antibody, Rabbit anti-ZO-1 monoclonal antibody, Rabbit anti-Epaca1 monoclonal antibody, and cAMP ELISA kit from Abcam (Cambridge, the UK). Rabbit anti-PKA C monoclonal antibody, Rabbit anti-phospho-PKA C (Thr197) monoclonal antibody, Rabbit anti-β-actin monoclonal antibody, and Anti-rabbit IgG (H+L) were obtained from Cell Signaling Technology (Danvers, MA, the USA).

**Animal model establishment**

A total of 72 C57BL/6 male mice, 8-10 weeks, weighing 20-24 g, were purchased from the Animal Experiment Center of Guangxi Medical University and were raised in cages inside an experimental environment with 45-60% of indoor humidity, 22-25 °C of temperature, and light imitative of natural light accompanied by day and night alternation. Food and drink were given ad libitum.

The 72 mice were randomly and equally assigned into four groups: the control (Con) group, the LPS (LPS) group, the LPS plus the prophylaxis by low-dose of Selexipag (LPS+LS) group, and the LPS plus the prophylaxis by high-dose of Selexipag (LPS+HS) group. Each group included 18 mice. The Con group was treated by gavage with 0.9% sodium chloride solution containing 1% of DMSO, twice a day, for 3 days straight, and 2 hours after the last gavage, was intraperitoneally injected (I.P.) with the same solution as the previous gavages; the LPS group was coped with in a similar way as the Con group except for being I.P. with LPS of 20 mg/kg 2 hours after the last gavage; both the LPS+LS group and the LPS+HS group were I.P. with LPS of 20 mg/kg 2 hours after the last gavage following a 3-day of gavage with either 0.5 mg/kg or 1 mg/kg of Selexipag containing 1% DMSO, twice a day. The selected dosages of Selexipag (0.5 and 1 mg/kg) were determined based on a preliminary study, in which we, according to relevant literature21-23), had tried 5 different doses of Selexipag (0.1, 0.25, 0.5, 1, 2 mg/kg) on LPS-challenged mice. The results found that only two doses of 0.5 and 1 mg/kg showed protections against the LPS-induced ARDS (data not shown).
Specimens

All the mice were anesthetized with 1% sodium pentobarbital at 50 mg/g 24 hours after the I.P., then lung tissues and bronchoalveolar lavage fluid (BALF) were collected. Left lungs were washed with PBS and fixed with 4% paraformaldehyde for hematoxylin-eosin (HE) staining. The upper right lungs were measured for Wet/Dry (W/D) weight ratio and the remaining lungs were detected by ELISA, western blotting, and Polymerase Chain Reaction (PCR). The alveolar lavage fluid was centrifuged at 4 °C and 1500 g for 15 minutes and the supernatant was refrigerated in a -80 °C refrigerator for later use. Some lung tissues were mixed with PBS proportional to tissue weight (1 ml PBS vs 100 mg lung tissue), ground, ultrasonically lysed, and centrifuged at 5000 g and 4 °C for 10 min. The supernatant was refrigerated in a -80 °C refrigerator for later use.

H&E Staining and Lung injury score (LIS)

The mice lung tissues were washed with PBS and fixed in 4% paraformaldehyde for 48-72 hours. A series of routine operations, including dehydration, transparency, paraffin embedding, wax block and section preparation, deparaffinization, hydration, HE staining, dehydration, and mounting, were performed. The Olympus microscope was employed to collect images of lung tissue sections and to assess the LIS.

Lung injury were scored based on the following four pathological manifestations:①thickened Alveolar septum; ②Alveolar hemorrhage; ③inflammatory infiltrate in the alveoli; ④fibrin deposition in the alveoli. Each item was scored from 0 to 3 according to the severity of lesions demonstrated in pathological slides. 0: not apparent or very mild; 1: mild; 2: moderate; 3: severe. The LIS amounted to sum of the above four scores.

Lung W/D Ratio Measurement

Lung tissues were washed with PBS, drained by absorbent paper, and weighed as soon as possible for the wet weight. Once the measurement of wet weight finished, the lung tissues were dehydrated in a 70 °C electronic thermostat for 72 hours until a consistent weight was gained, and then the dry weight was scaled.
Protein content in BALF Measurement

A BCA working solution was prepared by blending BCA reagent A with reagent B at the ratio of 50:1. Standards of different concentration gradients (0, 0.025, 0.05, 0.1, 0.2, 0.3, 0.4, 0.5 mg/ml) were produced according to the manufacture instruction. Each standard of specific concentration in 20 μl and the supernatant out of BALF in 20 μl were transferred separately into different wells of a 96-well plate, then both were mixed with the BCA working solution in 200 μl. Successively, the plate was incubated in an electronic thermostat at 37 °C for 30 minutes. Optical density (OD) of each well was detected by a microplate reader at 562 nm of absorbency wavelength. An equation obtained from a standard curve that was built based on various concentration gradients of the standards and their corresponding OD values, was used to calculate the protein concentration in the supernatant.

Lung capillary endothelium permeability Measurement

The mice were injected with Evans blue at 30 μg/g (Evans blue/mouse weight) into the tail veins and two hours later, anesthetized and executed. Lung tissues were cleaned, separated, mixed with the formamide solution in proportion to tissues weight (100 mg lung tissue/1 ml formamide), homogenized, ultrasonically lysed, and incubated in a thermostatic water bath box at 37 °C for 24 hours. The homogenate was centrifuged at 12000 g and 20 °C for 15 minutes, and then the supernatant was collected. Evans blue/formamide standards of different concentration gradients (0, 5, 10, 20, 40, 80, 160 μg/ml) were prepared. Every standard of specific concentration in 200 μl and the supernatant in 200 μl were added respectively in different wells of a 96-well plate. Each well was measured for OD at 620 nm of absorbency wavelength by a fluorescence microplate reader. The concentration of the supernatant was calculated through an equation.

ELISA

ELISA was carried out following the manufacture instruction. Standards of various concentrations in 50 μl and the sample in 50 μl were placed separately in different wells of a 96-well plate. Then, each well was added 100 μl of HRP-labeled antibody. The plate was incubated in an electronic thermostat at 37 °C for 60 minutes, then washed, added with 50 μl of Substrate A and B solutions in each well, and incubated
in the dark inside an electronic incubator at 37 °C for 15 minutes. Each well was added 50 μl of stop reagent and examined for OD at 450 nm of absorbency wavelength by a fluorescence microplate reader within 15 minutes. A standard curve was produced based on various concentrations of the standards and the corresponding OD values. The concentration of the sample was calculated by an equation.

**RT-PCR**

The extraction of total RNA was performed through a TRIzol RNA extraction kit. The reverse transcriptions were done by the SYBR Green analysis with a cDNA Reverse Transcription Kit and the reaction conditions were 37 °C for 15 minutes, 85 °C for 5 seconds, 4 °C for the end. Real Time PCR reaction (20 μl system, ABI 7500, primer sequences see Table 1) were conducted under conditions of 95 °C for 10 minutes combined with 40 cycles of 95 °C for 10 seconds and 60 °C for 30 seconds. The 2^{−ΔΔCt} was calculated according to the CT value, and then relative expression of mRNA on various samples were obtained.

**Western Blotting**

Lung tissues were mixed proportionally with the protein lysate, the protease inhibitor, and the phosphatase inhibitor. Then, a set of operations, including routine grind, ultrasonic lysis, and centrifugation were conducted. The supernatant was collected, treated with the loading buffer, denatured, and preserved. Separating gel and concentrated gel, both with various concentrations, are prepared on the basis of the molecular weights of different target proteins. Protein samples went through the following processes: electrophoresis, transmembrane, stripping, and blocking. The polyvinylidene fluoride (PVDF) membrane was incubated at 4 °C with various primary antibody solutions in a refrigerator overnight, then washed with TBST, and incubated at room temperature with a secondary antibody solution in the dark on a table concentrator for one hour. The PVDF membrane was scanned by a LI-COR Odyssey infrared fluorescence scanner and protein strips gained from the scan were analyzed by the Image J software.

**Statistics**

SPSS 19.0 and Graphpad Prism 5.0 software were used to analyze experimental data and to draw figures. The data that conformed to a normal distribution are
described as the mean ± standard deviation, and comparisons between groups were performed by one-way analysis of variance and Dunnett’s post hoc analyses. The data of LIS are represented as scatter dot plots with medians, and its comparisons between groups were performed by Mann–Whitney U-test. $P < 0.05$ was regarded as statistically significant in all comparisons.

**Ethical Approval**

All procedures involved in animal management were carried out in accordance with the *Guiding Opinions on the Treatment of Laboratory Animals* issued by the Ministry of Science and Technology of the People’s Republic of China and the *Laboratory Animal-Guideline for Ethical Review of Animal Welfare* by the National Standard GB/T35982-2018 of the People’s Republic of China. The study was approved by the Ethics Committee of Animal Experiment of Guangxi Medical University with the approval number as 201908004.

**RESULTS**

**Selexipag histologically reduced lung injuries in the LPS-induced ARDS mice**

The histological manifestations in lungs were assessed by H&E staining. As indicated in Fig. 1A and 1E, the mice from the Con group showed clear integrity of lung structures, including alveoli, alveolar septa, and capillaries, and no signs of inflammatory infiltrates or leakage either in the alveolar spaces or in the septa; All the mice treated with LPS without the prophylaxis by Selexipag exhibited thickened alveolar septa, congested capillaries, severe infiltrates of inflammatory cells and red blood cells in the alveoli, alveolar septa and around pulmonary capillaries, as well as noticeably increased LIS (Fig. 1B and 1E). The mice preconditioned with Selexipag of 0.5 mg/kg or 1 mg/kg showed significantly improved histological images and reduced LIS, such as decreased swellings in the alveoli septa, mild inflammatory cell infiltrates in the alveolar spaces and alveolar septa (Fig. 1C, 1D, and 1E), compared to the LPS-challenged mice without the prophylaxis by Selexipag.

**Selexipag decreased protein leakage in lungs of the LPS-induced ARDS mice**

The effect of Selexipag against leakage in lungs were measured through lung W/D ratio (Fig. 2A), protein content in BALF (Fig. 2B), and Evans blue content (Fig. 2C).
Levels of all the three indicators above were significantly higher in the LPS group than in the control group. However, all of them apparently diminished both in the LPS+LS group and in the LPS+HS group, compared to the LPS group.

**Selexipag ameliorated inflammatory responses in the LPS-induced ARDS mice**

IL-1β, IL-6, and MCP-1 are recognized as important proinflammatory cytokines leading to ARDS. Quantified changes on the three cytokines above and their mRNAs were detected by ELISA and RT-PCR. Compared to the control group, the LPS group indicated significantly increased levels of IL-1β, IL-6, and MCP-1 in BALF (Fig. 3 A1-A3) as well as in lung homogenates (Fig. 3 B1-B3), and evidently escalated expressions of their mRNAs in the lung (Fig. 3 C1-C3). Both levels of all the three cytokines above and expressions of their mRNAs were distinctly lower in the two groups with the prophylaxis by Selexipag than in the LPS group.

**Selexipag relieved vascular endothelial injuries and enhanced endothelial barrier in the LPS-induced ARDS mice**

E-selectin and VCAM-1 are key markers for the presence of endothelial cell injury. VE-cadherin, the major adhesion junction protein, and ZO-1, a tight junction protein, are associated with endothelial barrier function. The LPS group exhibited significantly increased expressions of lung E-selectin mRNA and VCAM-1 mRNA (Fig. 4A and 4B), both quantified by RT-PCR, while showed noticeably reduced relative protein expressions of VE-cadherin and ZO-1(Fig. 4C and 4D), both detected by Western Blotting, in contrast to the control group. The trends of the above four indicators in the two groups administered with Selexipag opposed that in the LPS group with evidently reduced expressions of lung E-selectin mRNA and VCAM-1 mRNA, and escalated relative protein expressions of VE-cadherin and ZO-1.

**Selexipag raised the intracellular level of lung cAMP in the LPS-induced ARDS mice**

The cAMP is a vital second messenger and has been implicated in a wide range of physiological process: metabolism, inflammation, apoptosis, cell growth and differentiation, and smooth muscle contraction. Compared with the control group, the intracellular level of lung cAMP measured by ELISA in the LPS group was significantly decreased. However, the identical indicator in the two groups with the
prophylactic administration of Selexipag evidently rebounded in contrast to the LPS group. (See Fig. 5)

**Selexipag upregulated levels of lung relative p-PKA C (Thr197) and Epac1 in the LPS-induced ARDS mice**

PKA and Epac are two key mediators for cAMP actions. Compared with the Con group, Western Blotting revealed that the levels of relative p-PKA C (Thr197) (Fig. 6A, C) and Epac1 (Fig. 6A, B) were dramatically decreased in lungs of the LPS group. The levels of the two molecules in the two groups preconditioned with Selexipag obviously bounced, drawing a contrast to the LPS group (Fig. 6A-C).

**DISCUSSION**

The most important discovery in our study was that the prophylaxis by Selexipag of either 0.5 mg/kg or 1 mg/kg revealed significant protection against the LPS-induced ARDS. Compared to the LPS-challenged mice, the mice with the prophylaxis by Selexipag exhibited evidently attenuated histological changes, including mildly thicken alveolar septa, reduced inflammatory infiltration, and diminished LIS. They also showed trends in a variety of indicators concerning leakage, inflammation, and endothelial cell injury as opposed to their counterparts in the LPS group, which were obvious decreases amid lung W/D ratio, protein content in BALF, lung Evans blue content, levels of IL-1β, IL-6, and MCP-1, expression levels of IL-1β mRNA, IL-6 mRNA, MCP-1 mRNA, E-selectin mRNA, and VCAM-1 mRNA, and noticeable elevations in expressions of VE-cadherin and ZO-1 protein. Furthermore, these protective effects of Selexipag could be classified as reducing lung protein leakage, refraining excessive inflammation, alleviating damage to vascular endothelial cells, and enhancing vascular endothelial barrier.

Selexipag is not a PGI2 analogue, but in our study, it apparently displayed effects of anti-inflammatory response and enhancement of vascular endothelial barrier as did PGI2 analogues and their receptors in some previous researches. Toki S, et al.17) through both in vivo and in vitro animal experiments, proved that Cicaprost, an analogue of PGI2, could upregulate the expression of NOX4 by the cAMP/PKA/cyclic AMP response-element binding protein (CREB) pathway, and then produce...
protection for vascular endothelial cells in the LPS-challenged mice. The same experiment also found, compared with wild type mice, PGI2 IP receptor knockout mice exhibited significant increases amid neutrophils in BALF, proteins of KC, LIX, and TNF-α in lung homogenates after the LPS challenge, and further concluded that defects in the PGI2-IP signaling pathway might genetically increase risk of the endotoxin-induced ARDS. Vicil S and Erdogan S, et al. reported in an LPS-induced A549 cell injury model that a precondition with Beraprost, a PGI2 analogue, could downregulate intracellular levels of IL-1β, TNF-α, and malondialdehyde (MDA), and upregulate levels of glutathione (GSH) and catalase, suggesting a protective effect upon damaged A549 cells by depressing inflammation and oxidative stress. Beraprost also mitigated chronic brain injuries in aluminum gluconate-overload rats by refraining oxidative stress. Birukova AA et al. revealed that Iloprost, a PGI2 analogue, increased cAMP in pulmonary vascular endothelial cells, upregulated the expression of cell adhesion junction protein VE-cadherin, and enhanced pulmonary vascular endothelial cell barrier. Birukova AA et al. found in the LPS-induced ALI mice that Beraprost inhibited the p38 mitogen-activated protein kinase (MAPK) and nuclear factor-kappaB (NF-κB) signaling pathways and relieved lung injuries. Olave N, et al. reported that Iloprost downregulated levels of inflammatory factors, including myeloperoxidase (MPO), IL-1β and TNF-α, increased the expression of elastin in alveolar septa, and mitigated bronchopulmonary dysplasia in newborn mice. Deng J et al. discovered that Beraprost reduced phosphorylations of P38 and c-Jun N-terminal kinase (JNK), inhibited inflammation, apoptosis and autophagy, and mitigated liver ischemia-reperfusion injuries in mice. In addition, Misawa H et al. experimentally proved that PGI2 mimetics ONO-1301 and Beraprost alleviated mice liver damage resulting from lipopolysaccharide/D-galactosamine through inhibiting liver cell apoptosis activated by signal transducer and activator of transcription 3 (STAT3), and reduced liver oxidative stress. Based on these abovementioned researches and our study, it appears safe to conclude that either of two different mechanisms, strengthening the binding between PGI2 and IP receptor or boosting the level of PGI2, actively involves in several processes: inhibiting inflammatory...
responses, ameliorating vascular endothelial cells injuries, and enhancing vascular endothelial barrier.

This study further explored the molecular mechanism of these positive impacts that Selexipag exerted against the LPS-induced ARDS. It is well established that PGI₂ can raise the intracellular level of cAMP through binding the IP receptor, and through downstream signaling pathways, the increased cAMP generates a series of physiological impacts, including anti-inflammation, vasodilatation, enhancement of vascular endothelial cell barrier, and anticoagulation.³⁴,³⁵ The PKA and Epac signaling pathways have been widely acknowledged as two main molecular mechanisms for cAMP actions. PKA can be activated by increased intracellular cAMP and its activation reduces endothelial myosin light chain kinase (MLCK) activity, suppressing phosphorylation of myosin light chain (MLC), relaxing actomyosin complex, and reinforcing cell-matrix adhesions.³⁷,³⁸ Besides, PKA can phosphorylate Ras homology-guanosine diphosphate (Rho-GDP) dissociation inhibitor, which negatively regulates small Guanosine-Triphosphate hydrolase (GTPase) Rho, resulting in inactivation of GTPase Rho and blockage of endothelial cell hyper-permeability mediated by GTPase Rho.³⁷,⁴⁰ PKA exists in mammalian cells as an inactive tetrameric holoenzyme composed of two regulatory (R) subunits and two catalytic (C) subunits. There are multiple phosphorylation sites on each C subunit, and a threonine residue in the activation loop (Thr197) is the key phosphorylation site affecting the activity of the C subunit and converting PKA from an inactive to an active state.⁴² Epac, also known as cAMP-guanine nucleotide exchange factor (GEF), is implicated in a cAMP-mediated and PKA-independent regulation of small GTPase Ras-related protein (Rap).⁴³ Epac proteins comprise two members, Epac1 and Epac2, and the two are present in most tissues with different expression levels. Epac1 is frequently found in blood vessels, central nervous system, adipose tissue, kidney, uterus, and ovary whereas Epac2 is mostly expressed in adrenal gland, pancreas and central nervous system.⁴⁴-⁴⁶ Epac1 and Epac2 are directly activated by increased level of intracellular cAMP and both are able to activate small GTPase Rap1 in a cAMP-dependent and PAK-independent manner. Activated GTPase Rap1 engages in diverse processes, including cell adhesion, cell-cell junction
formation, regulation of endothelial barrier. Apart from enhancement of endothelial barrier, both PKA and Epac1/2 also participate in the resolution of inflammation. Activated PKA causes phosphorylation of the cAMP-CREB that moves to the nucleus and propels the production of pro-resolving mediators and anti-inflammatory cytokines, stimulation of macrophage polarization, granulocyte apoptosis and efferocytosis. PKA also can lower the expressions of inflammatory genes by decreasing the transcription of NF-κB and by inhibiting phosphoinositide 3-kinase (PI3K)/Akt. Epac1/2 take part in restraining the production of pro-inflammatory cytokines and NF-κB is an important mediator involved.

As demonstrated in the results, the levels of lung intracellular cAMP, relative phosphorylated PKA C at Thr197 and Epac1 protein expression were significantly higher in the mice with the prophylaxis by Selexipag than in the mice with the LPS-challenge. The gaps in the levels of cAMP, relative p-PKA C (Thr197) and Epac1 also echoed between the control group and the LPS group. Given these interactions amid cAMP, PKA, and Epac1, the cAMP mediated PKA and Epac1 signaling pathways possibly are leading mechanisms to modulate protective effects of Selexipag against the LPS-challenged ARDS, including strengthening pulmonary vascular endothelial barrier and inhibiting inflammation. However, in the case of Selexipag, further in-depth researches are required to explore detailed molecular pathways between PKA or Epac1 and its ultimate protection against ARDS.

Furthermore, our study successfully produced a mouse model of ARDS with LPS. The LPS-challenged mice indicated typical histological manifestations of ARDS and a range of molecular changes that highly suggested increased protein leakage, excessive inflammation and impaired vascular endothelial barrier — three features shaping the early stage of ARDS. The LPS-induced ARDS model built in our study reflected the main characteristics of ARDS at both the histological and the molecular levels. LPS, found on the outer membrane of most gram-negative bacteria, is an endotoxin and the essential component of gram-negative bacteria responsible for occurrence of infections. Therefore, it is frequently used to build the animal model of ARDS caused by infections. Multiple patterns of LPS application include intranasal or airway instillation, tail vein injection, and intraperitoneal injection. Among them, the
intraperitoneal injection is widely considered as a simple and repeatable pattern, and an ARDS model established based on this pattern often achieves consistent experiment outcomes.

In conclusion, Selexipag, a PGI$_2$ IP receptor agonist, showed significant protective effects against the LPS-induced ARDS, which present as inhibiting inflammatory responses, mitigating damage to pulmonary vascular endothelial cells, and strengthening pulmonary capillary endothelial barrier. The cAMP mediated PAK and Epac1 signaling pathways are relevant molecular mechanisms to modulate these positive impacts. Selexipag appears to hold promise to become a new therapeutic option for ARDS.

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Author contributions
Chaoqian Li: Conceptualization, Supervision, Project administration, and Funding acquisition; Hongliu Chen: Conceptualization, Methodology, Investigation, Formal analysis, Data curation, Writing-Original Draft, and Visualization; Ying Shen: Investigation, Formal analysis, Data Curation, Writing-Original Draft, Writing-Review and Editing, and Visualization; Yi Liang: Investigation and Formal analysis; Ying Qiu: Formal analysis; Meili Xu: Investigation.

Conflict of Interest
The authors declare no conflict of interest.
REFERENCES


Table 1 Primer sequences for RT-PCR

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</table>
Fig.1. Lung histological manifestations and lung injury scores in the four groups
A-D: lung histological manifestations of mice in each group; E: lung injury scores of mice in the four groups (n=6, each group). Con: the control group; LPS: the LPS group, mice in the group were administered with LPS; LPS+LS: the LPS+LS group, mice in the group were administered with 0.5 mg/kg of Selexipag plus LPS; LPS+HS: the LPS+HS group, mice in the group were administered with 1 mg/kg of Selexipag plus LPS. The data are represented in scatter plots with the bars indicating the median. *: <0.05, compared to LPS, **: <0.01, compared to LPS.
Fig. 2. Lung W/D ratio, Protein content in BALF, and Evans blue content in the lung in the four groups
A: lung W/D ratio in the four groups (n=6, each group); B: Protein content in BALF in the four groups (n=6, each group); C: Evans blue content in the lung in the four groups (n=6, each group); The data are expressed as the mean ± standard deviation. *: <0.05, compared to LPS, **: <0.01, compared to LPS, ***: ≤0.001, compared to LPS.
Fig. 3. Levels of IL-1β, IL-6, and MCP-1 as well as expressions of their mRNAs in the four groups

A1-A3: levels of IL-1β, IL-6, and MCP-1 in BALF in the four groups respectively (n=6, each group); B1-B3: levels of IL-1β, IL-6, and MCP-1 in lung homogenates in the four groups respectively (n=6, each group); C1-C3: expressions of IL-1β mRNA, IL-6 mRNA, and MCP-1 mRNA in the lung in the four groups respectively (n=6, each group). The data are expressed as the mean ± standard deviation. *: <0.05, compared to LPS, **: <0.01, compared to LPS, ***: ≤0.001, compared to LPS.
Fig.4. Expression levels of E-selectin mRNA, VCAM-1 mRNA, VE-cadherin, and ZO-1 in the four groups

A-B: Expression levels of E-selectin mRNA and VCAM-1 mRNA in the four groups (n=6, each group); C-D: protein relative expressions levels of VE-cadherin and ZO-1 in the four groups (n=6, each group). The data are presented as the mean ± standard deviation. *: <0.05, compared to LPS, **: <0.01, compared to LPS, ***: ≤0.001, compared to LPS.
Fig. 5. Levels of cAMP in the four groups

The intracellular level of lung cAMP are presented as the mean ± standard deviation. n=6 in each group; *: <0.05, compared to LPS. ***: ≤0.001, compared to LPS.
Fig. 6. Levels of lung p-PKA C and lung Epac1 in the four groups
A: The protein levels of Epac1, β-actin, p-PKA C (Thr197), and PKA C in the four groups. (n=6, each group); B: The protein relative expression of lung Epac1/β-actin in the four groups (n=6, each group); C: the relative phosphorylated PKA C (Thr197) level in the four groups (n=6, each group). The Data are expressed as the mean ± standard deviation. *: <0.05, compared to LPS, ***: ≤0.001, compared to LPS.