Published in final edited form as:

Prostaglandins Other Lipid Mediat. 2018 May ; 136: 33–43. doi:10.1016/j.prostaglandins.2018.04.001.

Endogenous PGI₂ signaling through IP inhibits neutrophilic lung inflammation in LPS-induced acute lung injury mice model

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Abstract

Endogenous prostaglandin I₂ (PGI₂) has inhibitory effects on immune responses against pathogens or allergens; however, the immunomodulatory activity of endogenous PGI₂ signaling in endotoxin-induced inflammation is unknown. To test the hypothesis that endogenous PGI₂ down-regulates endotoxin-induced lung inflammation, C57BL/6 wild type (WT) and PGI₂ receptor (IP) KO mice were challenged intranasally with LPS. Urine 6-keto-PGF_{1α}, a stable metabolite of PGI₂, was significantly increased following the LPS-challenge, suggesting that endogenous PGI₂ signaling modulates the host response to LPS-challenge. IPKO mice had a significant increase in neutrophils in the BAL fluid as well as increased proteins of KC, LIX, and TNF-α in lung homogenates compared with WT mice. In contrast, IL-10 was decreased in LPS-challenged IPKO mice compared with WT mice. The PGI₂ analog cicaprost significantly decreased LPS-induced KC, and TNF-α, but increased IL-10 and AREG in bone marrow-derived dendritic cells (BMDCs) and bone marrow-derived macrophages (BMMs) compared with vehicle-treatment. These results indicated that endogenous PGI₂ signaling attenuated neutrophilic lung inflammation through the reduced inflammatory cytokine and chemokine and enhanced IL-10.

Keywords

Acute lung injury; Prostaglandin I₂; cicaprost; neutrophil

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S.T. designed and performed experiments, analyzed data and wrote the manuscript. W.Z., S.R. and K.G. contributed to acquisition of data. W.Z., D.C.N., and D.E.D. advised and contributed to the overall development of this study and helped finalize the manuscript. W.E.L. contributed to murine type II epithelial cell study and helped finalize the manuscript. R.S.P. designed and supervised the research, and edited the manuscript

Author Disclosures

The authors declare no conflict of interest.

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Introduction

Acute lung injury (ALI) is triggered by respiratory bacterial infections, trauma, aspiration of gastric contents, inhalation injury, sepsis, and other causes [1], resulting in significant morbidity and mortality in critically ill patients. Features of ALI include enhanced vascular permeability, increased expression of inflammatory cytokines in the lung tissue, and accumulation of both protein-rich fluid and activated neutrophils in the air spaces [2, 3]. These inflammatory events result in the clinical manifestations of poor lung compliance, severe hypoxemia, and bilateral infiltrates on chest radiograph. Over the past several decades, despite increasing insight into ALI or acute respiratory distress syndrome (ARDS) pathobiology, the mortality rate still remains approximately 25–40% [4–6]. Recent intervention trials in ALI or ARDS patients have incorporated therapies such as corticosteroids, nitric oxide, and ketoconazole [7–9]; however, the results of these trials have not been promising for improving mortality and there is a great need for understanding the mechanisms that lead to severe ALI.

Airway neutrophilia is an important component of the inflammatory response in ALI. Although neutrophil activation is vital for host defense, excess activation leads to tissue damage by the release of proinflammatory agents such as proteinases, cationic polypeptides, cytokines, and reactive oxygen species (ROS) [10, 11]. Previous studies have demonstrated the importance of neutrophils in ALI/ARDS pathophysiology. In clinical studies, the number of neutrophils in the bronchoalveolar lavage (BAL) fluid correlated with the severity of ALI/ARDS and poor outcomes [12, 13]; and a selective neutrophil elastase inhibitor, sivelestat improved oxygenation and multiple organ dysfunction score in patients with ALI/ARDS after surgery for abdominal sepsis [14]. In mice, severe lung injury has been attenuated by neutrophil depletion [15]. These studies suggest that decreasing airway and lung neutrophilia may prevent respiratory deterioration in ALI.

Prostanoids are important mediators of various pathologic processes, not only in amplifying inflammation, but also in suppressing immune responses [16, 17]. Prostanoids are products of the arachidonic acid metabolic pathway and are synthesized by the sequential action of cyclooxygenase (COX) and prostanoid-specific synthases. Prostaglandin I₂ (PGI₂) is a potent endogenous inhibitor of platelet aggregation, and a strong vasodilator that also inhibits the growth of vascular smooth muscle cells [18]. In addition, previous studies reported that PGI₂ inhibits inflammatory responses. PGI₂ analogs inhibited the expression of the proinflammatory cytokine TNF- α by LPS-stimulation of a human monocyte cell line, THP-1 [19], and a murine monocyte macrophage cell line, J774 cells [20]. Our group previously reported that PGI₂ analogs inhibited LPS-induced proinflammatory cytokine and chemokine production, and enhanced IL-10 production by LPS-stimulated bone marrow-derived dendritic cells (BMDCs) [21]. Further, our group published that mice with epithelial cell overexpression of PGI synthase, the terminal enzyme responsible for PGI₂ production, were protected against mortality in a bleomycin-induced lung toxicity [22]. In that study, we showed that PGI₂ induced the expression of NADPH:quinone oxidoreductase 1 (Nqo1), an enzyme that prevents the generation of ROS. ROS are mainly generated in macrophages and neutrophils to kill bacteria that are engulfed by phagocytosis [23]. The PGI₂ analog iloprost decreased the production of superoxide anion radical (O₂^{•-}), a precursor of most ROS, by

canine neutrophils activated with opsonized zymosan[24]. In addition, the PGI₂ analogs iloprost and beraprost decreased LPS-induced ALI and improved endothelial barrier recovery [25–27]; however, the effect of endogenous PGI₂ on the pathogenesis of LPS-induced lung injury is unknown. The previous studies suggest that a deficiency in the ability to signal through IP may be a risk factor for severe ALI. We therefore hypothesized that endogenous PGI₂ signaling through the PGI₂ receptor (IP) down-regulates LPS-induced lung inflammation in a mouse model of ALI. To test this hypothesis, we challenged wild type (WT) or mice deficient in the PGI₂ receptor IP (IPKO) with LPS intranasally and measured the number of airway neutrophils and proinflammatory cytokine/chemokine production. We further investigated the effect of the PGI₂ analog cicaprost on the expression of proinflammatory cytokines and chemokines in LPS-stimulated BMDCs, bone marrow derived macrophages (BMMs), and murine immortalized type II alveolar epithelial cells (AECs) *in vitro* to determine whether PGI₂ regulates proinflammatory, or anti-inflammatory mediator expression from lung resident cells. Determining the role of endogenous PGI₂ signaling in the mouse model of LPS challenge may provide insight into whether defects in this pathway may be a genetic susceptibility factor for worsened outcomes in the setting of endotoxin-induced ALI in humans.

Materials and Methods

Mice

IPKO mice, the kind gift of Dr. Garret FitzGerald, were previously generated by homologous recombination in embryonic stem cells and were backcrossed to the C57BL/6 background for over 10 generations [28]. Age- and sex-matched WT C57BL/6 mice were obtained from The Jackson Laboratory (Bar Harbor, ME). All mice were female and 9–13 weeks of age. All experiments were approved by the Vanderbilt Institutional Animal Care and Utilization Committee.

Detection of LPS-induced stable PGI₂metabolite (6 -keto-PGF_{1α}) in urine

Three WT or IPKO mice were placed in separate metabolic cages to collect urine. The mice were anesthetized with ketamine and xylazine, and then intranasally challenged with 10 µg LPS (*Escherichia coli* Serotype O111:B4, Sigma-Aldrich, St. Louis, MO) in 80 µl saline, or 80 µl saline as the vehicle on day 0. The urine was collected once per day from day -2 to day 4. The specimens were centrifuged to remove debris, and stored at –80°C. The stable PGI₂ metabolite, 6-keto-prostaglandin F_{1α} (PGF_{1α}), and creatinine were measured by ELISA (Cayman Chemical; Ann Arbor, MI) according to the manufacturer's protocols.

Collection of BAL fluid and cell differential counting after LPS challenge

WT and IPKO mice were anesthetized with ketamine and xylazine. The mice were intranasally challenged with 10 µg LPS in 80 µl saline and were sacrificed 0, 6, 12, 24, 48, and 72 h after the challenge to evaluate lung inflammation in a time-course dependent manner.

BAL fluid was harvested following instillation of 1 ml saline. The total cell number in the BAL fluid samples was determined using a hemocytometer after Red blood cell lysis. The

supernatant was collected and stored at -80°C for protein assays. Cytospin preparations of BAL cells were performed for each sample. The slides were stained with Richard-Allan Scientific^{TM} Three Step Stain Kit (Thermo Fisher Scientific, Waltham, MA) to determine cell differential. Differential counts were based on counting 200 cells per slide.

Measurement of the wet lung weight

Whole lung tissues from WT and IPKO mice were harvested 48 h after LPS or saline intranasal challenge; and the wet lung was weighed immediately. The lung weight was normalized by the mouse body weight before sacrifice.

Quantitative real-time RT-PCR

Whole lung tissues from WT and IPKO mice were harvested 48 h after LPS or saline intranasal challenge; and the lungs were preserved in RNA^{later}^{TM} solution (Invitrogen, Carlsbad, CA). Total RNA was isolated from the left lung tissue using the RNeasy Kit (Qiagen, Valencia, CA); and cDNA was synthesized using SuperScript IV (Invitrogen). Quantitative real-time PCR analyses were performed using the QuantStudio^{TM} 12K Real-Time PCR System (ThermoFisher Scientific) with iTaq^{TM} Universal SYBR® Green Supermix (Bio-Rad) and QuantiTect Primer Assays (Mm_Gapdh_3_SG, Mm_Ptger_1_SG, Mm_Ptger2_1_SG, Mm_Ptger1_1_SG, Mm_Ptger2_1_SG, Mm_Ptger3_1_SG, Mm_Ptger4_2_SG, Mm_Ptger1_1_SG, and Mm_Tbxa2r_1_SG) according to manufacturer's instructions. The following primers were used for *Ptgir* detection; 5' - GCCTATGCTCGAAACAGCTC-3', and 5' - GCGGTACATGTGATAGAGGC-3'. The relative gene expression was analyzed by using delta delta Ct ($\Delta\Delta\text{Ct}$) method.

Preparation of BMDCs and treatment with PGI₂ analog

Immature BMDCs were generated using a previously described method with modifications [29]. Bone marrow (BM) cells from femurs and tibias were cultured in complete RPMI 1640 medium (Mediatech Inc, Manassas, VA) containing 5% heat-inactivated FBS (HyClone, Logan, UT), 50 $\mu\text{g}/\text{ml}$ gentamicin, 55 nM 2-ME, and 20 ng/ml GM-CSF (PeproTech, Rocky Hill, NJ). On day 3, fresh complete medium containing 20 ng/ml GM-CSF was added. On day 6, non-adherent and loosely adherent cells were harvested. More than 80% of the harvested cells were CD11c⁺ cells. These cells were treated with 0.1, 1, 10, 100 nM cicaprost (Cayman Chemical) or methyl acetate as vehicle in complete medium without GM-CSF in the presence or absence of LPS (100 ng/ml) for 24 h, and then cell-free culture supernatant was harvested for cytokine and chemokine measurements.

Preparation of BMMs and treatment with LPS and PGI₂ analog

BMMs were generated using a previously described method with modifications [30]. BM cells from femurs and tibias were cultured in complete RPMI 1640 medium (Mediatech Inc) containing 10% heat-inactivated FBS (HyClone), 100 U/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, and 20 ng/ml M-CSF (PeproTech). The cells were cultured in 6-well plates and maintained at 37°C in humidified air containing 5% CO₂. On day 3 and 6, half of the culture medium was changed to fresh complete medium. On day 7, adherent cells were dissociated by 5 mM EDTA/PBS on ice for 10 min. The cells were treated by adding 0.1, 1,

10, 100 nM cicaprost or the vehicle to the complete medium without M-CSF in the presence or absence of LPS (20 ng/ml) for 24 h, and then culture supernatant was harvested for cytokine and chemokine measurements.

Immortalized murine type II alveolar epithelial cell culture and treatment with LPS and PGI₂ analog

Immortalized murine type II alveolar epithelial cells (AECs) were obtained from immortomouse, with temperature-sensitive expression of simian virus 40 large T antigen [31]. The cells were cultured in DMEM (Corning) containing 10% heat-inactivated FBS (HyClone), 100 U/ml penicillin, and 100 µg/ml streptomycin with IFN- γ at 33°C for proliferation, and then the cells were seeded into 6 well plates and cultured without IFN- γ at 37°C to lose expression of the SV40 antigen and revert to the primary phenotype. A monolayer of the cells was treated by increasing doses of cicaprost or the vehicle in the presence or absence of LPS (100 ng/ml) for 24 h, and then culture supernatant was harvested for cytokine and chemokine measurements.

ELISA for lung cytokine and chemokine detection and protein assay

Lungs were homogenized with ground glass in 2 ml of MEM media by grinding with a mortar and pestle. After centrifugation at 2000×g for 15 min, the supernatant was collected to detect proteins. DuoSet ELISA kits (KC, LIX, TNF- α , IL-1 α , IL-1 β , IL-6, IL-10, and amphiregulin (AREG)) from R&D Inc. (Minneapolis, MN) and Mouse Myeloperoxidase ELISA Kit from Thermo Fisher Scientific were used to measure the concentration of cytokines, chemokines, and myeloperoxidase in lung homogenates according to manufacturer's instructions. Values below the detection limit were assigned a half of the lower limit of detection value. Total protein levels in the BAL fluid were measured by Pierce BCA Protein Assay Kit (Thermo Fisher Scientific).

Flow cytometry

BAL fluid from WT and IPKO mice were harvested 48 h after LPS or saline intranasal challenge. The BAL cells were stained with fluorescently labeled antibodies; and then the cells were measured on BD LSR II Flow Cytometer (BD Biosciences, San Jose, CA), and analyzed using FlowJo Software. The following antibodies were used: anti-CD45-red Fluor 710 (F30-11, TONBO Biosciences, San Diego, CA), anti-CD11c-PE (N418, eBioscience, Waltham, MA), anti F4/80-APC (BM8, Biolegend, San Diego, CA), anti-Gr-1-FITC (R86-8C5, BD Biosciences), anti-CD80-Pacific Blue (16-10A1, Biolegend), anti-CD86-PE-Cy7 (PO3, Biolegend), anti-I-A/I-E-APC-Cy7 (M5/114.15.2, Biolegend). Alveolar macrophages were identified as CD45⁺ CD11c⁺ F4/80⁺ Gr-1⁻ cells.

Statistical analysis

The *p* values were calculated by one-way, or two-way analysis of variance (ANOVA) with Bonferroni-multiple comparisons test using GraphPad Prism 7 (GraphPad Software, La Jolla, CA). Values of *p* < 0.05 were considered significant.

Results

Intranasal LPS challenge induced 6-keto-PGF_{1α} in mouse urine

While PGI₂ is unstable and the half-life is approximately 3–5 min [32], 6-keto-PGF_{1α} is a stable metabolite of PGI₂. To determine endogenous PGI₂ induction following an LPS airway challenge that mimics ALI, we measured urine 6-keto-PGF_{1α} before and after LPS challenge. The values of 6-keto-PGF_{1α} were normalized by urine creatinine concentration. LPS challenge significantly increased urine 6-keto-PGF_{1α} within 24 h compared with saline challenge, and the induction continued through 72 h after LPS challenge (Fig. 1). By 96 hours, the LPS-induced urine 6-keto-PGF_{1α} concentration was the same as in the saline-challenged group. This result revealed that PGI₂ synthesis was modulated by LPS challenge, suggesting that endogenous PGI₂ may regulate disease pathogenesis.

Endogenous PGI₂ decreases neutrophil infiltration and activation in the airway following LPS-challenge

A previous study reported that intranasal LPS-challenge in mice induces neutrophil infiltration into the lung in a time course-dependent manner with a peak at 48 h [33], but the effect of endogenous PGI₂ on neutrophil infiltration is unknown. To determine whether endogenous PGI₂ regulates LPS-induced neutrophilic lung inflammation, WT and IPKO mice were intranasally challenged with LPS, and the BAL fluid was harvested 0, 6, 12, 24, 48, and 72 h after the LPS-challenge (Fig. 2A). There were no neutrophils in the BAL fluid of both WT and IPKO mice at baseline (0 h). The number of neutrophils in the BAL fluid in both WT and IP KO mice was increased in a time dependent fashion by 48h after the LPS-challenge (Fig. 2B). IPKO mice had significantly greater number of neutrophils in the BAL fluid compared with WT mice 24 h after the LPS-challenge (Fig. 2B). In contrast there were no significant differences in the number of macrophages (Fig. 2C) and lymphocytes (Fig. 2D) in the BAL fluid between WT and IPKO mice at any time point. Next, we measured myeloperoxidase (MPO) level in the lung homogenates to evaluate the accumulated lung neutrophil activation. IPKO mice had significantly greater MPO concentration in the lung homogenates 6, 12, 48, and 72 h after LPS challenge compared with WT mice (Fig. 2E). To further evaluate the effect of endogenous PGI₂ signaling on LPS-induced lung damage, we measured total protein concentration in BAL fluid following the LPS-challenge. The total protein concentration in the BAL fluid was significantly increased time-dependently after LPS challenge by 48 h; and IPKO mice had significantly greater protein concentration in the BAL fluid compared with WT mice 48 h after the LPS-challenge (Fig. 2F). To evaluate the severity of pulmonary edema in WT and IPKO mice, we measured wet lung weight 48 h after LPS or saline challenge. Although there was a significant difference in BAL total protein concentration 48 h after LPS challenge between WT and IPKO mice (Fig. 2F), the weight of wet lung normalized by bodyweight was not changed between WT and IPKO mice after LPS challenge (Fig. 2G). These results indicated that the inability of endogenous PGI₂ to signal through IP increased LPS-induced airway neutrophilia, but the severity of pulmonary edema was not changed.

LPS challenge decreased mRNA expression on prostaglandin receptors

We measured mRNA expression of prostaglandin receptors in the lung from WT and IPKO mice 48 h after LPS or saline challenge. We confirmed that IPKO mice had no mRNA expression of *Ptgir* (Fig. 3). LPS challenge significantly decreased mRNA expression of *Ptgdr*, *Ptgdr2*, *Ptger1*, *Ptger3*, *Ptgir*, *Ptgfr* and *Tbxa2r* compared to saline challenge in WT mice. However, there is no significant difference in the mRNA expression of prostaglandin receptors after LPS challenge between WT and IPKO mice, except for *Ptgir* expression (Fig. 3). These results showed that WT and IPKO mice have approximately same mRNA expression of prostaglandin receptors except for *Ptgir* in IPKO mice during LPS-induced airway inflammation.

Endogenous PGI₂ decreases LPS-induced KC, LIX and TNF- α production, and increases IL-10 in the lung

Next, we measured lung cytokines and chemokines in a time course-dependent manner following LPS-challenge. IPKO mice had significantly greater lung protein expression of the neutrophil chemoattractant KC 6 h and 24 h after LPS-challenge compared with WT mice (Fig. 4). An alternative neutrophil chemoattractant, LIX, and the inflammatory cytokine TNF- α were significantly greater in the lung of IPKO mice compared with WT mice 6 h after the LPS-challenge. In contrast, IPKO mice had significantly lower lung protein expression of the anti-inflammatory cytokine IL-10 compared with WT mice 6 h, 24 h, and 48 h after the LPS-challenge (Fig. 4). While lung IL-6 protein expression was significantly greater in the IPKO mice compared with WT mice 6 h after the LPS-challenge, the IL-6 expression was lower in the IPKO mice compared with WT mice 48 h after the LPS-challenge (Fig. 4). Further, there was no difference in the lung protein expression of IL-1 α , IL-1 β , and AREG between WT and IPKO mice at any time point. These results suggest that endogenous PGI₂ signaling may inhibit LPS-induced inflammation through the suppression of proinflammatory cytokines and chemokines for neutrophil recruitment at an early time point, and results in the induction of anti-inflammatory cytokine IL-10.

PGI₂ analog treatment decreases proinflammatory cytokines and chemokines, and increases IL-10 and AREG in LPS-stimulated BMMs and BMDCs

Alveolar macrophages are the first phagocytes to contact inhaled pathogens or particulate matter and are important for innate immune responses [34]. To determine whether PGI₂ signaling regulates proinflammatory cytokine or chemokine expression in LPS-stimulated macrophages, cultured BMMs from WT mice were treated with increasing doses of the PGI₂ analog cicaprost or vehicle in the presence or absence of LPS for 24 h. LPS stimulation increased the expression of KC, LIX, TNF- α , IL-6, and IL-10, but not AREG (Fig. 5). Cicaprost treatment significantly decreased LPS-induced KC, TNF- α , and IL-6 in a dose-dependent manner; but cicaprost did not change LPS-induced LIX from BMMs at any dose. In contrast, cicaprost treatment significantly increased IL-10 and AREG in LPS-stimulated BMMs in a dose-dependent manner (Fig. 5).

DCs exist in mucosal tissues to survey their local environment and to mediate various types of inflammatory immune responses. We therefore investigated the effect of cicaprost treatment on LPS-stimulated BMDCs. LPS stimulation increased the expression of KC,

LIX, TNF- α , IL-6, IL-10, and AREG from BMDCs (Fig. 6). Cicaprost treatment decreased LPS-induced KC, LIX, TNF- α , and IL-6, while it increased IL-10 and AREG in a dose-dependent manner (Fig. 6). These results suggest that PGI₂ signaling decreased the expression of proinflammatory cytokines and chemokines, but increased IL-10 and AREG in LPS-stimulated macrophages and DCs, possibly to prevent lung damage caused by LPS.

Endogenous PGI₂ did not change the expression of MHC class II, CD80, and CD86 in alveolar macrophages

We found that PGI₂ analog treatment decreased LPS-induced proinflammatory cytokine expression from BMMs *in vitro* (Fig. 5). To evaluate the effect of endogenous PGI₂ on alveolar macrophages *in vivo*, we further measured cell surface expression of major histocompatibility complex (MHC) class II, CD80, and CD86 on alveolar macrophages from WT and IPKO mice 48 h after LPS or saline challenge. Fig. 7A shows the gating strategy of alveolar macrophages. LPS challenge increased the intensity of CD11c and F4/80 expression on alveolar macrophages. Fig. 7B shows the representative histogram of MHC class II, CD80, and CD86 expression on alveolar macrophages. LPS challenge significantly increased MHC class II expression on alveolar macrophages. However, there was a trend for an increase in the MFI of MHC class II expression in the positive gate of IPKO mice compared to WT mice, but the difference was not statistically significant ($p=0.0514$) (Fig. 7C). There is no difference in CD80 and CD86 expression between WT and IPKO mice after LPS challenge (Fig. 7C).

PGI₂ analog treatment increases IL-6 in murine type II AECs

We found that the lung IL-6 protein expression was greater in WT mice compared with IPKO mice 48 h after LPS challenge (Fig. 4), suggesting that endogenous PGI₂ signaling increases LPS-induced IL-6 at later phase of the lung inflammation. However, *in vitro*, LPS-induced IL-6 expression in BMMs and BMDCs was decreased by cicaprost treatment (Fig. 5, 6). Therefore, this discrepancy suggests that PGI₂ signaling increases IL-6 production by another cell type in the lung. Type II AECs are another important source of IL-6 [35], therefore we hypothesized that PGI₂ signaling increases IL-6 production by type II AECs. To test this hypothesis, immortalized murine type II AECs were cultured with different doses of cicaprost with 100 ng/ml of LPS or saline for 24 h. LPS stimulation significantly increased the expression of IL-6, KC, and LIX in the culture medium (Fig. 8), but TNF- α , IL-10, and AREG were not detected (data not shown). The 100 nM of cicaprost treatment significantly increased the LPS-induced IL-6, but KC and LIX expression levels were not changed (Fig. 8). These results revealed that the biological effects of IP signaling is cell type-specific. Our *in vivo* results reveal that there was greater lung IL-6 protein expression in IPKO compared to WT mice at 6 h after LPS challenge; however, WT mice had greater lung IL-6 protein expression than IPKO mice at 48 h after LPS challenge (Fig. 4). The *in vitro* data using type II AECs suggest that the main source of IL-6 may change from macrophages at 6 h time point to type II AECs at 48 h time point.

Discussion

In this study, we first found that LPS induced a significant increase in the stable urinary metabolite of PGI₂ that reflects body PGI₂ production. This result suggests the possibility that endogenous PGI₂ regulates the host immune response to LPS and this concept is supported by our finding that endogenous PGI₂ signaling down-regulated neutrophilic inflammation after LPS-intranasal challenge. The number of neutrophils in BAL fluid, lung MPO level, and the expression of KC, LIX, and TNF- α that support neutrophil recruitment and neutrophilic inflammation, were increased in IPKO mice compared with WT mice. In contrast, the anti-inflammatory cytokine IL-10 was increased in WT mice compared with IPKO. In *in vitro* experiments, PGI₂ analog cicaprost-treatment decreased protein levels of KC, LIX, IL-6 and TNF- α , and increased IL-10 and AREG by LPS-stimulated BMMs and BMDCs. Meanwhile, lung IL-6 expression 48 h after LPS-challenge *in vivo* was increased in WT mice compared with IPKO mice; and cicaprost-treatment enhanced IL-6 protein by LPS-stimulated murine type II AECs. The *in vivo* and *in vitro* findings suggest that PGI₂-IP signaling induces substantially more IL-6 in type II alveolar cells than PGI₂ signaling reduces IL-6 in macrophages and dendritic cells. Taken together, our findings suggest that endogenous PGI₂ signaling through IP decreases neutrophil infiltration in LPS-challenged lungs via reduction of chemokines involved in neutrophil chemotaxis and potentially protects the lung via induction of anti-inflammatory cytokine IL-10.

Höcherl et al. previously reported that intravenous injection of LPS to rats significantly induced 6-keto-PGF_{1 α} in the plasma at 3–12 h after LPS administration [36]. This report and our findings suggest that body PGI₂ is promptly induced by LPS stimulation, and that endogenous PGI₂ signaling down-regulates LPS-induced KC and LIX at the 6 h time point, and subsequent neutrophil accumulation. Birukova et al. reported that exogenous administration of a PGI₂ analog iloprost, attenuated the disruption of the endothelial monolayer structure by LPS stimulation, and post-treatment of the PGI₂ analog beraprost following LPS challenge inhibited lung vascular leak of Angiosence 680 EX [25–27]. These studies and our finding suggest that PGI₂ signaling may prevent lung damage by LPS stimulation. Serhan et al. reported that specialized pro-resolving mediators decreased neutrophilic inflammation in ALI caused by bacterial infection or pathogen injection [37]. For instance, lipoxin A4 receptor deficient mice had delayed resolution of *E.coli*-induced lung inflammation [38]. Therefore, other lipid mediators, in addition to PGI₂, may be involved in the resolution process.

We found that LPS challenge decreased mRNA expression of prostaglandin receptors except for *Ptger2* and *Ptger4* in the lung. The ratio of immune cells to stromal cells in the lung was increased after LPS challenge. Therefore, it is not clear whether the mRNA expression was decreased per cell basis, or the mRNA expression in total lung was decreased by accumulation of immune cells that have no expression of prostaglandin receptors. However, mRNA expression of prostaglandin receptors was not changed between WT and IPKO mice after LPS challenge except *Ptgir*. These results suggest that the effects of prostaglandin receptors except *Ptgir* are presumably equal in WT and IPKO mice.

Although PGI₂ analog itself did not induce IL-10 and AREG expression in BMMs and BMDCs, PGI₂ analog increased the LPS-induced anti-inflammatory cytokine IL-10, as well as the epithelial growth factor AREG. In contrast, PGI₂ suppressed expression of the proinflammatory chemokines and cytokines KC, LIX, and TNF- α by LPS-stimulated BMMs and BMDCs. This may be one of the mechanisms by which endogenous PGI₂ suppressed LPS-induced neutrophilic lung inflammation. In addition, we found that PGI₂-IP signaling increased LPS-induced IL-6 expression in the lung 48 h after LPS-challenge. This *in vivo* result with our *in vitro* results implies that the PGI₂-driven increase in IL-6 produced by type II AEC offset the PGI₂-mediated reduction in IL-6 produced by BMM and BMDC. Previous studies reported that IL-6 deficient mice had more severe colitis score, and less Ki-67 positive crypt cells in the colon following dextran sodium sulfate treatment compared with WT mice [39], and IL-6 treatment suppressed H₂O₂ induced cell death in A549, human lung epithelial cell line [40]. Taken together, PGI₂-IP signaling-enhanced IL-6 may promote epithelial cell survival or proliferation to recover from lung injury. However, more *in vivo* studies are needed to determine the protective effect of PGI₂-IP signaling on the healing phase following LPS-induced lung inflammation. Further, we confirmed alveolar macrophage activation 48 h after LPS challenge in WT and IPKO mice *in vivo*. However, we could not find statistical difference in the macrophage activation markers between WT and IPKO mice. LPS challenge increased intensity of CD11c and F4/80 expression on alveolar macrophages. The alveolar macrophages may change from lung resident alveolar macrophages to monocyte derived alveolar macrophages during LPS-induced lung inflammation as well as bleomycin-induced lung fibrosis model [41]. This alternation in alveolar macrophage phenotype could be a factor for our inconsistent results in macrophage activation between *in vivo* and *in vitro* experiments.

PGI₂ analogs are currently approved by the Food and Drug Administration (FDA) for the treatment of pulmonary hypertension (PH) [42], and its analogs are used in the clinical management of the patients with PH [43–45]. The magnitude of PH correlated with the severity of lung injury in patients with ALI/ARDS and the onset of acute right heart failure as a complication of ALI [46]. Therefore, PGI₂ may protect against lung injury in ALI/ARDS as well as PH by vasodilatation and improving vascular flow in the lung. Meanwhile, genetic studies revealed that there is polymorphism on the promotor region of PGI₂ synthase (PGIS) gene and IP gene. A variable-number tandem repeat (VNTR) polymorphism in the promoter region of the PGIS gene altered 6-keto-PGF_{1 α} concentration in plasma or urine [47, 48]; and the genotype of the repeat number of VNTR correlated with urine 6-keto-PGF_{1 α} levels, and inversely correlated with respiratory syncytial virus (RSV) lower respiratory tract infection severity score [48]. In addition, single-nucleotide polymorphism (SNP) of IP gene correlated with PGI₂ analog binding affinity, cAMP induction [49], and platelet activation level [50]. These studies suggest that a polymorphism of PGI₂ related gene may have therapeutic relevance in ALI. Flores and colleagues performed a quality assessment of genetic association studies supporting susceptibility and outcome in ALI and reported that the genes encoding for IL-6, mannose binding lectin, surfactant protein B, and angiotensin converting enzyme were the most replicated across studies [51]. To our knowledge, we are the first to find that the inability to signal through the PGI₂ receptor IP regulates endotoxin-induced ALI. Although the endotoxin LPS-induced ALI model mimics

the features of ALI in human, bacterial infectious process was not reflected. Therefore, further studies using bacterial pneumonia model are required. In addition, we propose that future genetic susceptibility studies of ALI in humans should include variants in either the IP or PGIS genes as potential risk factors for severity of illness.

Conclusion

We found that endogenous PGI₂ significantly decreased the number of BAL neutrophils and MPO level in the lung through the reduction of KC and LIX expression following intranasal LPS-challenge. Further, IL-10 and IL-6 expression at the peak time of lung neutrophil accumulation was greater in WT mice than IPKO mice. These results suggest that endogenous PGI₂ may improve LPS-induced acute lung injury by inhibiting neutrophil accumulation in the lung and by enhancing anti-inflammatory mediators, such as IL-10. These findings may have important clinical implication in patients with ALI.

Acknowledgments

We thank Dr. Garret A. FitzGerald (Philadelphia, Pennsylvania, USA) for providing us the PGI₂ receptor deficient mouse.

Funding

This work was supported by NIH R01 AI 111820, Department of Veterans Affairs 2I01BX000624, NIH U19 AI 095227-02 and NIH R01 HL 090664-04.

Abbreviations

ALI	acute lung injury
ARDS	acute respiratory distress syndrome
ROS	reactive oxygen species
BAL	bronchoalveolar lavage
COX	cyclooxygenase
PGI₂	prostaglandin I ₂
IP	prostaglandin I ₂ receptor
MPO	myeloperoxidase
BMDCs	bone marrow-derived dendritic cells
BMMs	bone marrow-derived macrophages
6-keto-PGF_{1α}	6-keto-prostaglandin F _{1α}
PH	pulmonary hypertension

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Highlights

- LPS intranasal challenge significantly increased urine 6-keto-PGF_{1α}, a stable metabolite of PGI₂.
- Endogenous PGI₂-IP signaling reduced LPS-induced neutrophilic inflammation in the lung.
- Endogenous PGI₂-IP signaling increased LPS-induced IL-10 and IL-6 expression in the lung.
- The defects in the PGI₂-IP signaling pathway may be a genetic risk factor in endotoxin-mediated ALI.

Fig. 1. 6-keto-PGF_{1α} in mouse urine following LPS-challenge. WT mice were challenged with LPS or saline, and urine specimens were collected daily from 3 mice per one metabolic cage. The urinary 6-keto-PGF_{1α} concentration was corrected by urine creatinine concentration. The results are combined with 2 independent experiments, and shown as mean $\pm$ SEM of 8 specimens. * $p < 0.05$.

Fig. 2.

Effects of endogenous PGI_2 signaling on BAL cell differentials, lung MPO production, and total protein level in BAL fluid following LPS-challenge. (A) WT and IPKO mice were challenged with 10 μ g LPS in 80 μ l saline, and BAL fluid was harvested 0, 6, 12, 24, 48, and 72 h after the challenge. (B) Neutrophil, (C) macrophage, and (D) lymphocyte numbers were counted after staining using Three-Step Stain kit. (E) MPO in the lung homogenates and (F) total protein in the BAL fluid are measured by ELISA or BCA protein assay. (G) Wet lung weight was measured 48 h after LPS or saline challenge. The results are combined with 2 or 3 independent experiments, and shown as mean $\pm$ SEM of 5 mice in 0 h, and 9–13 mice in 6–72 h. * $p < 0.05$.

Fig. 3. mRNA expression of prostaglandin receptors in the lung 48 h after LPS or saline challenge. Relative mRNA expression was calculated using the $2^{-(Ct)}$ method using *Gapdh* mRNA expression as housekeeping gene and the average of WT-saline group as the control sample. The results are combined with 2 independent experiments, and shown as mean $\pm$ SEM of 7 mice.

Fig. 4.

Effects of endogenous PGI₂ signaling in cytokine and chemokine expression in the lung following LPS-challenge. Lungs were harvested 0, 6, 12, 24, 48, and 72 h following the LPS challenge. Cytokine/chemokine protein expression in the lung homogenates was measured by ELISA. The results are representative of three separate experiments. The results are combined with 3 independent experiments, and shown as mean $\pm$ SEM of 5 mice in 0 h, and 9–13 mice in 6–72 h. * $p < 0.05$.

Fig. 5.

Proinflammatory cytokine and chemokine expression from BMMs. BMMs were treated with cicaprost (0.1–100 nM) in the presence or absence of LPS (20 ng/ml) for 24 h. The levels of cytokine and chemokine in the culture supernatants were measured by ELISA. The results are shown as mean $\pm$ SEM of 4 independent experiments. * $p < 0.05$ compared with vehicle treated cells.

Fig. 6.

Proinflammatory cytokine and chemokine expression from BMDCs. BMDCs were treated with cicaprost (0.1–100 nM) in the presence or absence of LPS (100 ng/ml) for 24 h. The levels of cytokine and chemokine in the culture supernatants were measured by ELISA. The results are shown as mean $\pm$ SEM of 4 independent experiments. * $p < 0.05$ compared with vehicle treated cells.

Fig. 7.

Alveolar macrophage activation 48 h after LPS or saline challenge. (A) The gating strategy of alveolar macrophages. The alveolar macrophages were identified by flow cytometry as CD45⁺ CD11c⁺ F4/80⁺ Gr-1⁻ cells. (B) Representative histograms of MHC class II, CD80, and CD86 on alveolar macrophages. (C) MFI of MHC class II was calculated in positive gate. Ratio of MFI in CD80 and CD86 was normalized with MFI of respective isotype staining control. The results are shown as mean $\pm$ SEM (n=4–5).

Fig. 8.

Proinflammatory cytokine and chemokine expression from murine type II AECs.

Immortalized murine type II AECs were treated with cicaprost (0.1–100 nM) in the presence or absence of LPS (100 ng/ml) for 24 h. The levels of IL-6 in the culture supernatants were measured by ELISA. The results are shown as mean $\pm$ SEM of 5 independent experiments using different passage of the cells. * $p < 0.05$ compared with vehicle treated cells.