

RESEARCH ARTICLE

Effects of prostaglandin lipid mediators on agonist-induced lung endothelial permeability and inflammation

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²Department of Pharmaceutical Chemistry, University of Graz, Graz, Austria; ³Division of Pulmonary and Critical Care Medicine, Department of Medicine, University of Maryland School of Medicine, Baltimore, Maryland; and ⁴Department of Anesthesiology, University of Maryland School of Medicine, Baltimore, Maryland

Submitted 22 November 2016; accepted in final form 19 June 2017

Ke Y, Oskolkova OV, Sarich N, Tian Y, Sitikov A, Tulapurkar ME, Son S, Birukova AA, Birukov KG. Effects of prostaglandin lipid mediators on agonist-induced lung endothelial permeability and inflammation. *Am J Physiol Lung Cell Mol Physiol* 313: L710–L721, 2017. First published June 29, 2017; doi:10.1152/ajplung.00519.2016.—Prostaglandins (PG), the products of cyclooxygenase-mediated conversion of arachidonic acid, become upregulated in many situations including allergic response, inflammation, and injury, and exhibit a variety of biological activities. Previous studies described barrier-enhancing and anti-inflammatory effects of PGE₂ and PGI₂ on vascular endothelial cells (EC). Yet, the effects of other PG members on EC barrier and inflammatory activation have not been systematically analyzed. This study compared effects of PGE₂, PGI₂, PGF_{2α}, PGA₂, PGJ₂, and PGD₂ on human pulmonary EC. EC permeability was assessed by measurements of transendothelial electrical resistance and cell monolayer permeability for FITC-labeled tracer. Anti-inflammatory effects of PGs were evaluated by analysis of expression of adhesion molecule ICAM1 and secretion of soluble ICAM1 and cytokines by EC. PGE₂, PGI₂, and PGA₂ exhibited the most potent barrier-enhancing effects and most efficient attenuation of thrombin-induced EC permeability and contractile response, whereas PGI₂ effectively suppressed thrombin-induced permeability but was less efficient in the attenuation of prolonged EC hyperpermeability caused by interleukin-6 or bacterial wall lipopolysaccharide, LPS. PGD₂ showed a modest protective effect on the EC inflammatory response, whereas PGF_{2α} and PGJ₂ were without effect on agonist-induced EC barrier dysfunction. In vivo, PGE₂, PGI₂, and PGA₂ attenuated LPS-induced lung inflammation, whereas PGF_{2α} and PGJ₂ were without effect. Interestingly, PGD₂ exhibited a protective effect in the in vivo model of LPS-induced lung injury. This study provides a comprehensive analysis of barrier-protective and anti-inflammatory effects of different prostaglandins on lung EC in vitro and in vivo and identifies PGE₂, PGI₂, and PGA₂ as prostaglandins with the most potent protective properties.

pulmonary endothelial cells; permeability; inflammation; prostaglandins; acute lung injury

PRESERVATION of the endothelial cell (EC) vascular barrier integrity is important for the maintenance of homeostasis and lung fluid balance. In pathological settings, additional stimulation of vascular barrier-enhancing mechanisms is a key

mechanism to prevent catastrophic consequences of uncontrolled vascular leakiness in the lung or other organs caused by bacterial pathogens, excessive mechanical forces, or cytokine storm associated with infection, sepsis, or trauma (3, 29).

Prostaglandins (PG) are products of the arachidonic acid metabolic pathway and are synthesized by many tissues, including vascular endothelial cells. The first step involves intracellular release of arachidonic acid from plasma membrane phospholipids via the action of phospholipase A₂. Arachidonic acid is then converted sequentially to PGG₂ and PGH₂ by the cyclooxygenase and peroxidase activities of a single enzyme, PGH synthase (also known as cyclooxygenase or COX). There are two forms of COX: a constitutive form (COX-1) and an inducible form (COX-2), whose expression is induced by proinflammatory cytokines. Specific prostaglandins are then produced via the action of specific synthases including PGE₂, PGD₂, and PGF_{2α} synthase. Alternatively, PGH₂ may be converted to thromboxane (TxA₂) or prostacyclin (PGI₂). The cyclopentenone prostaglandins PGA₂ and PGJ₂ are formed by dehydration within the cyclopentane ring of PGE₂ and PGD₂, respectively (reviewed in 28).

Prostaglandins exert their biological activities by interacting with specific receptors. PGE₂ activates four distinct G protein-coupled receptors, EP1–EP4. Of them, EP1, couples to G_q and stimulates phospholipase C; EP2 and EP4 are coupled with G_s and stimulate adenylate cyclase (AC), leading to an increase in intracellular cAMP; and EP3 is coupled with G_i and inhibits AC, which suppresses the cAMP formation. PGI₂ activates the IP receptor, which is coupled to G_s similarly to EP2 and EP4. PGF_{2α} acts via receptor FP, which is coupled to G_q similarly to EP1. PGD₂ receptors, DP1 and DP2, activate and inhibit AC via G_s and G_i, respectively (reviewed in 32). 15d-PGJ₂ has been shown to activate two receptors: a membrane receptor, chemoattractant receptor homologous molecule expressed on Th2 cells (CRTH2) (18) and a nuclear receptor, peroxisome proliferator-activated receptor γ (PPAR γ) (14). The primary receptor for PGA₂ is not well defined.

The role of PGs and their receptors in inflammation and regulation of vascular permeability is complicated. Previous studies by our and other groups described potent anti-inflammatory and barrier-protective effects of PGE₂ and PGI₂ on pulmonary vascular endothelium in vitro and in animal models of lung injury caused by bacterial pathogens and excessive

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mechanical ventilation (6, 7, 21). Barrier-enhancing effects of PGE₂ and PGI₂ are mediated by PG-induced elevation of intracellular cAMP levels, activation of cAMP-dependent protein kinase A (PKA), and the PKA-independent signaling pathway, which involves cAMP-activated guanine nucleotide exchange factor Epac1, its effector GTPase Rap1, and downstream GTPase Rac1 (16). Anti-inflammatory effects by PGI₂ and PGE₂ are in large part driven by cAMP-mediated inhibition of RhoA GTPase activity and suppression of the NFκB inflammatory signaling cascade (7). However, effects of many other PG products generated under inflammatory and other pathological conditions remain to be precisely characterized.

This study performed a comprehensive analysis of functional effects of PGA₂, PGE₂, PGI₂, PGD₂, PGF_{2α}, and PGJ₂ on lung endothelial cell barrier under basal conditions, in EC stimulated with barrier disruptive agonist thrombin, and in cells exposed to proinflammatory agents: IL-6 and Gram-negative bacterial wall lipopolysaccharide (LPS). Functional effects of PGs in these models were assessed by analysis of EC barrier function and associated Rho signaling, expression of EC adhesion molecule ICAM1, and IL-8 and soluble ICAM1 release as parameters of EC inflammatory activation. Protective effects of PGs were further tested in the mouse model of acute lung injury.

MATERIALS AND METHODS

Reagents and cell culture. PGE₂, PGI₂, PGA₂, PGD₂, PGF_{2α}, and PGJ₂ were obtained from Cayman Chemical (Ann Arbor, MI). Human IL-6 and IL-6 soluble receptor were obtained from R&D Systems (Minneapolis, MN); human thrombin was obtained from Sigma (St. Louis, MO). Phospho-MLC antibody was obtained from Cell Signaling (Beverly, MA); ICAM1 antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). Unless otherwise specified, all biochemical reagents including antibody to β-actin and bacterial wall lipopolysaccharide (LPS, *Escherichia coli* O55:B5) were obtained from Sigma (St. Louis, MO). Human pulmonary artery endothelial cells (HPAEC) were obtained from Lonza (East Rutherford, NJ) and used for experiments at passages 5–7.

Endothelial permeability assays. Measurements of transendothelial electrical resistance (TER) across human lung EC monolayers were performed using the electrical cell-substrate impedance sensing system (ECIS) (Applied Biophysics, Troy, NY) as described (2, 4). Endothelial permeability to macromolecules was monitored by permeability visualization assay (XPerT) (12) available from Millipore (cat. no. 17–10398). Binding of FITC-avidin fluorescent tracer to the matrix-coated culture dish bottoms increases if the EC barrier is compromised. After cell stimulation with agonists, FITC-avidin solution at a final concentration of 25 μg/ml was added directly to the culture medium for 3 min before termination of the experiment. Unbound FITC-avidin was washed out with PBS, pH 7.4, 37°C (2 cycles, 10 s each). Visualization of FITC-avidin labeled permeability sites in EC monolayers was performed using inverted microscope Nikon Eclipse TE300 with epifluorescence module and SPOT RT monochrome digital camera and image processor (Diagnostic Instruments, Sterling Heights, MI). For the 96-well format assays, the fluorescence of matrix-bound FITC-avidin was measured on Victor X5 Multilabel Plate Reader (Perkin Elmer, Waltham, MA) using an excitation wavelength of 485 nm and emission wavelength of 535 nm.

RT-PCR analysis of PG receptors. HPAEC and SAEC cell monolayers grown in 35-mm dishes were lysed in 350 μl RLT Plus Buffer (Qiagen) containing beta mercaptoethanol. The RNA was isolated using the RNeasy plus mini kit (Qiagen) as per manufacturer's instructions. Five-hundred nanograms of RNA was reverse transcribed to cDNA using iScript cDNA synthesis kit (Bio-Rad). The

expression levels of the different prostaglandin receptors were quantified by qRT-PCR using PerfeCTa SYBRGreen Fastmix (Quanta Bio cat. no. 95072-012) on a Bio-Rad CFX96 Real-Time PCR System as per the manufacturer's instructions. The following primer sequences were used for RT-PCR analysis: EP1 receptor, forward 5'-ACCTTCTTTGGCGGCTCTC-3', reverse 5'-GCACGACACCACCATGATAC-3'; EP2 receptor, forward 5'-CAGTCTCCCTGCTCTTCTGC-3', reverse 5'-GCACCGAGACAATGAGAAGC-3'; EP3 receptor, 5'-TCATCGTCGTGTACCTGTCC-3', reverse 5'-CGATGACAAACGAGAGAGC-3'; EP4 receptor, forward 5'-TGCTCTTCTTTCAGCCTGTCC-3', reverse 5'-AGACTGCAAAGAGCGTGAAG-3'; DP1 receptor, forward 5'-TGATGACCGTGCTCTTCACT-3', reverse 5'-CCA AGG GTC CAC AATTGAAA-3', DP2 receptor, forward 5'-TCTGCGCGCTACCTTTCATG-3', reverse 5'-TCTCTCGTGGACCATCTGGATA-3'; FP receptor, forward 5'-GCA-CATTGATGGGCAACTAGAA-3', reverse 5'-GCACCTATCAT-TGGCATGTAGCT-3'; IP receptor, forward 5'-GCCGATCAGCT-GCTGTTTCT-3', reverse 5'-TTTCTCTGTCCCTCACTCTCTTC-3'. Ct values were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) using the delta-delta method.

Western blot analysis. Immunoblot detection of proteins of interest was performed as described previously (5, 8). In brief, protein extracts were subjected to 15% or 7.5% SDS-PAGE, transferred to nitrocellulose membrane in semidry transfer apparatus (Bio-Rad, Hercules, CA) 10 V for 1 h, and probed with the antibody of interest. Immunoreactive proteins were visualized by SuperSignal West Dura chemiluminescence reagent according to the manufacturer's protocol (Pierce, Rockford, IL).

Measurement of cytokines and chemokines. For cytokine measurements in preconditioned medium of human pulmonary EC cultures, supernatants from treated EC were collected and centrifuged to remove debris. Levels of IL-8 and soluble ICAM1 (sICAM1) levels were determined by ELISA (R&D Systems, Minneapolis, MN) following manufacturer's protocol. Absorbance was read at 450 nm within 30 min in a Victor X5 Multilabel Plate Reader (Perkin Elmer, Waltham, MA).

Animal studies. All animal care and treatment procedures were approved by the University of Chicago Institutional Animal Care and Use Committee. Animals were handled according to the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. C57BL/6J mice were purchased from Jackson Laboratories (Bar Harbor, ME). Bacterial lipopolysaccharide (LPS, 1 mg/kg body wt; *Escherichia coli* O55:B5) or sterile water was injected intratracheally in a small volume (20–30 μl) using a 20-gauge catheter (Exelint International, Los Angeles, CA). Prostaglandins of choice (20 μg/kg) or sterile saline solution was injected concurrently with LPS instillation and 5 h after LPS challenge by intravenous injection in the external jugular vein. After 24 h of LPS challenge, animals were euthanized by exsanguination under anesthesia. BAL was performed using 1 ml of sterile Hanks' balanced salt buffer and measurements of cell count and protein concentration were conducted as previously described (15).

In vivo optical imaging. Control and agonist-treated mice were injected with 100 μl of 2 nmol vascular fluorescent blood pool imaging agent Angiosense 680 EX (Perkin Elmer, Boston, MA), intravenously via tail vein 24 h before imaging sessions. Fluorescent optical imaging was performed in the Integrated Small Animal Imaging Research Resource (iSAIRR) at the University of Chicago using Xenogen IVIS 200 Spectrum (Caliper Life Sciences, Alameda, CA). Mice were exposed to isoflurane anesthesia with O₂ through the gas anesthesia manifold and placed on the imaging stage. Acquisition and image analysis were performed with Living Image 4.3.1 Software.

Statistical analysis. Results are expressed as means ± SD of three to eight independent experiments. Stimulated samples were compared with controls by unpaired Student's *t*-test. For multiple-group comparisons, one-way ANOVA and Tukey's post hoc multiple-comparison test were used. *P* < 0.05 was considered statistically significant.

RESULTS

Effects of PGA_2 , PGE_2 , PGD_2 , $\text{PGF}_{2\alpha}$, PGJ_2 , and PGI_2 on lung endothelial cell barrier. Effects of PGs on human pulmonary EC barrier properties were examined by the measurements of the changes in transendothelial electrical resistance (TER) of cell monolayers grown on gold microelectrodes and stimulated with various concentrations of PGs. Among six PG types, PGA_2 , PGE_2 , and PGI_2 caused the most pronounced dose-dependent TER increases with maximal response to 0.5 μM of each PG compound (Fig. 1A). Maximal barrier-protective response was observed by 15 min of stimulation. TER levels remained elevated for up to 4–6 h in PGA_2 - and PGE_2 -stimulated cells, but returned to baseline in PGI_2 -stimulated cells by 1 h after treatment. In turn, stimulation with PGD_2 caused a modest TER increase at 15 min which gradually declined over time, whereas $\text{PGF}_{2\alpha}$ and PGJ_2 did not affect TER levels in EC monolayers (Fig. 1, A and B). An RT-PCR analysis showed expression of all tested prostaglandin receptors in HPAEC (Fig. 1C).

Effects of PGA_2 , PGE_2 , PGD_2 , $\text{PGF}_{2\alpha}$, PGJ_2 , and PGI_2 on thrombin-induced EC permeability. We evaluated the protective effects of PGs on thrombin-induced human lung EC hyperpermeability using TER measurements. First, EC pretreated with various PGs were challenged with low thrombin dose (0.2 U/ml), which causes a submaximal permeability response. Pretreatment with PGA_2 , PGE_2 , and PGI_2 suppressed thrombin-induced TER decline. PGI_2 completely abolished the TER decrease caused by thrombin, whereas PGA_2 and PGE_2 only partially attenuated the rapid phase of thrombin-induced EC permeability (Fig. 2A). Overall, all these three prostaglandins exhibited barrier-protective effects. In contrast, pretreatment with PGD_2 , $\text{PGF}_{2\alpha}$, or PGJ_2 was without effect on thrombin-induced permeability. Similar results were obtained in quantitative assays of FITC-avidin permeability using a 96-well plate format (Fig. 2B).

Thrombin-induced EC barrier dysfunction is associated with activation of RhoA GTPase pathway leading to increased phosphorylation of regulatory myosin light chains (MLC) and actomyosin contraction of pulmonary EC (31). We next examined effects of PGs on the thrombin-induced MLC phosphorylation. The extent of PG-induced attenuation of thrombin-induced EC permeability directly correlated with inhibition of thrombin-induced MLC phosphorylation caused by each PG compound (Fig. 2C).

PGs also showed similar relative potency to attenuate EC permeability caused by high dose of thrombin (0.5 U/ml). PGI_2 exhibited the most pronounced barrier-protective effect followed by PGE_2 and PGA_2 effects, whereas PGD_2 , PGF_2 , and PGJ_2 were without effect at all (Fig. 3A).

In addition to TER measurements, PG's protective effects against thrombin-induced EC hyperpermeability were examined using fluorimetry-based quantitative permeability assay and permeability visualization by microscopy. The bar graph provides comparative analysis of the potency of all six PGs in attenuation of thrombin-induced EC permeability (Fig. 3B). In visualization assays, thrombin caused a massive disruption of the EC barrier manifested by a robust accumulation of FITC-labeled tracer on the substrate under EC monolayers (Fig. 3C). Experiments using endothelial permeability imaging assay showed that pretreatment with PGA_2 , PGE_2 , and PGI_2 (15 min)

strongly attenuated thrombin-induced accumulation of fluorescent FITC-avidin tracer on the biotinylated gelatin coating of culture plates underneath EC monolayers after 5 min of thrombin stimulation. These results further substantiated protective effects of PGA_2 , PGE_2 , and PGI_2 against thrombin-induced EC permeability. In contrast, PGD_2 , $\text{PGF}_{2\alpha}$, and PGJ_2 did not affect thrombin-induced permeability for FITC-avidin tracer (Fig. 3B).

Effects of PGA_2 , PGE_2 , PGD_2 , $\text{PGF}_{2\alpha}$, PGJ_2 , and PGI_2 on EC barrier dysfunction caused by inflammatory agonists. Because increased vascular leak is a distinctive feature of vascular response to bacterial pathogens and inflammatory cytokines, we evaluated effects of PGs on EC permeability caused by IL-6. Treatment with PGA_2 and PGE_2 caused significant and sustained attenuation of TER decline caused by IL-6 challenge. Protective effects of PGI_2 were less expressed and markedly diminished after 5 h of treatment, although TER levels did not reach TER levels registered in EC stimulated with IL-6 alone (Fig. 4A). Treatment with PGD_2 , $\text{PGF}_{2\alpha}$, and PGJ_2 did not protect human pulmonary EC from IL-6-induced barrier compromise (Fig. 4A). The results of quantitative fluorimetric permeability assay are presented in Fig. 4B.

This group of PGs also exhibited similar effects on EC barrier disruption caused by another inflammatory agent, bacterial wall lipopolysaccharide (LPS). PGA_2 , PGE_2 , and PGI_2 attenuated the LPS-induced TER decline (Fig. 5A) and FITC-avidin penetration through EC monolayer (Fig. 5B). PGA_2 exhibited the most pronounced protective effect and PGI_2 showed the weakest protective effect among these three PG products. Interestingly, whereas PGJ_2 and $\text{PGF}_{2\alpha}$ did not affect LPS-induced barrier dysfunction, PGD_2 showed modest improvement of LPS-induced EC barrier compromise (Fig. 5, A and B); this effect was not observed in thrombin- or IL-6-challenged EC.

In addition to disruption of EC monolayer integrity, exposure of vascular EC to bacterial compounds activates an inflammatory response characterized by upregulation of proinflammatory cell adhesion molecules ICAM1 and VCAM1 required for leukocyte adhesion and PMN infiltration into inflamed organs, and by increased production of soluble ICAM1 and inflammatory cytokines including $\text{TNF}\alpha$ and IL-8 (7, 25). Treatment of LPS-challenged EC with PGA_2 , PGE_2 , or PGI_2 suppressed the LPS-induced ICAM1 expression, and PGD_2 exhibited a modest effect, whereas $\text{PGF}_{2\alpha}$ and PGJ_2 were without effect (Fig. 5C). Analysis of soluble ICAM1 and IL-8 levels in conditioned medium from LPS-challenged human pulmonary EC showed marked elevation of these markers of inflammation upon LPS challenge, which was significantly attenuated by PGA_2 , PGE_2 , and PGI_2 (Fig. 6). In addition, LPS-induced elevation of cytokines and sICAM1 was partially attenuated by PGD_2 . In agreement with other results described above, $\text{PGF}_{2\alpha}$ and PGJ_2 did not significantly affect the pulmonary EC inflammatory response to LPS.

Protective effects of PGs in mouse models of acute lung injury. Effects of different prostaglandins were further tested in the murine model of acute lung injury (ALI) caused by intratracheal administration of LPS. Experimental animals were treated with PGA_2 , PGE_2 , PGD_2 , $\text{PGF}_{2\alpha}$, PGJ_2 twice, concurrently with LPS administration and 5 h later. After 24 h, bronchoalveolar lavage was performed, and protein concentration and cell counts were analyzed as described in MATERIALS

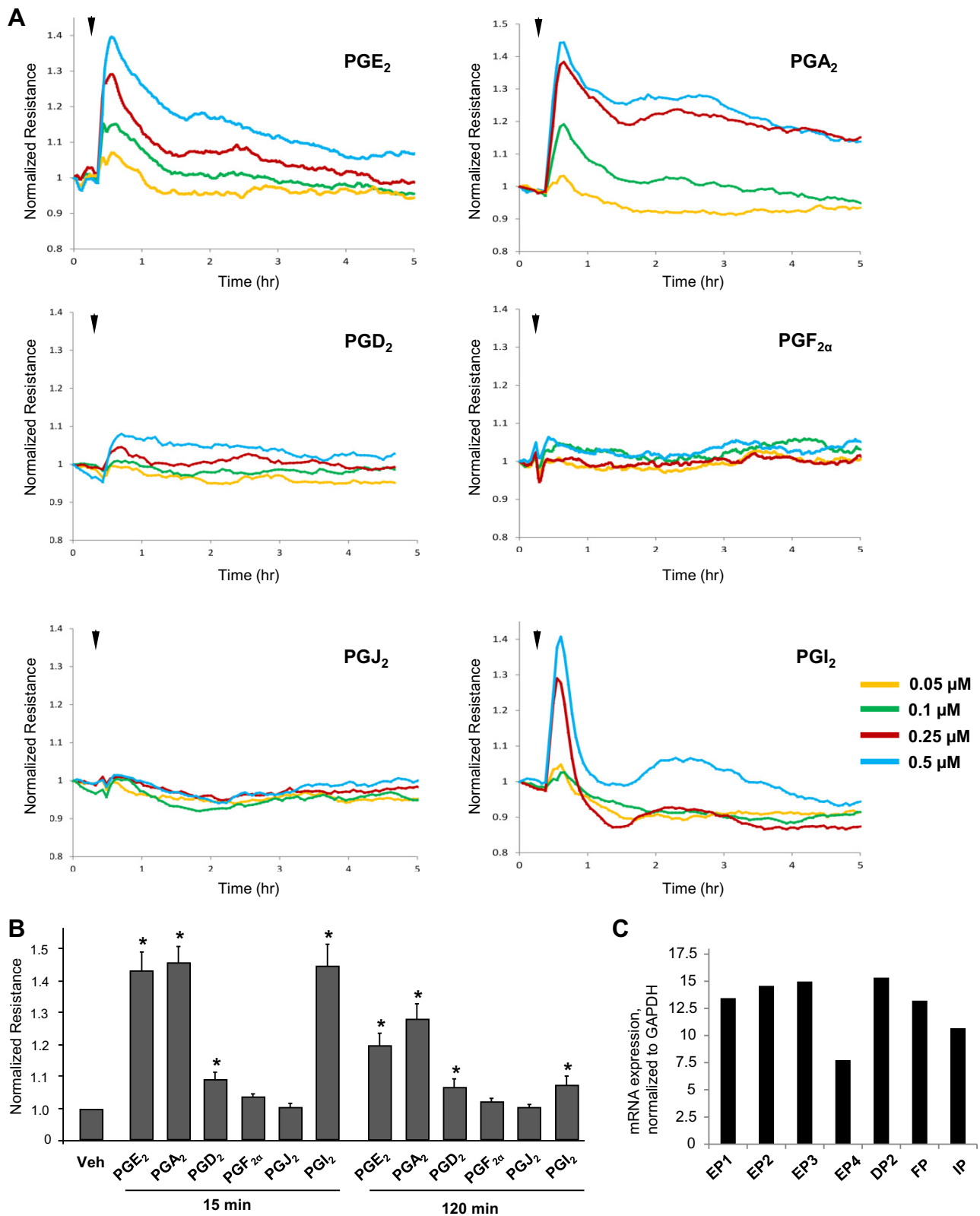


Fig. 1. Effects of PGA₂, PGE₂, PGD₂, PGF_{2α}, PGJ₂, and PGI₂ on basal lung endothelial cell barrier properties. **A**: pulmonary EC grown on microelectrodes were stimulated with vehicle or 0.05 μM, 0.1 μM, 0.25 μM, or 0.5 μM of PGE₂, PGA₂, PGD₂, PGF_{2α}, PGJ₂, or PGI₂. Changes in transendothelial resistance (TER) were monitored over 5-h time period. **B**: summary of TER measurements performed at 15 min and 120 min after agonist stimulation; $n = 5$. * $P < 0.05$ vs. vehicle. **C**: RT-PCR analysis of prostaglandin receptor mRNA levels in human pulmonary EC. Receptor expression was normalized to GAPDH levels.

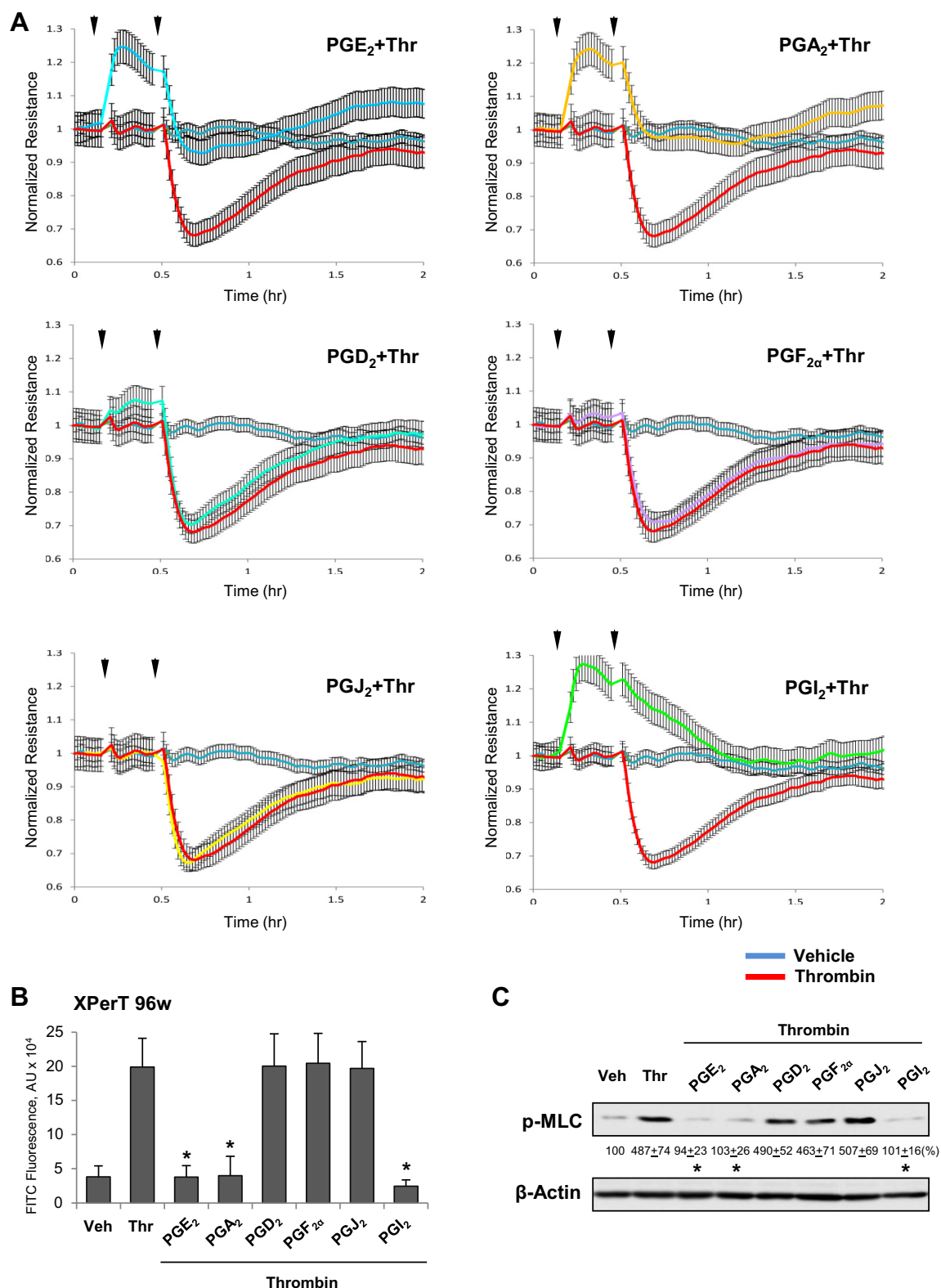


Fig. 2. Effects of PGA₂, PGE₂, PGD₂, PGF_{2α}, PGJ₂, and PGI₂ on EC permeability caused by low dose of thrombin. **A**: pulmonary EC were treated with indicated PG (0.5 μM, first arrow) for 15 min followed by addition of low dose of thrombin (Thr; 0.2 U/ml, second arrow). Changes in TER were monitored over 2-h time period. **B**: analysis of EC permeability for macromolecules using XPerT assay. Bar graph depicts permeability changes after 5 min of thrombin treatment; $n = 4$. * $P < 0.05$ vs. thrombin. **C**: cells treated with indicated PG (0.5 μM, 15 min) or vehicle were stimulated with thrombin for 30 min, and the levels of MLC phosphorylation were evaluated by Western blot with diphospho-MLC antibody. Probing with β-actin antibody was used as a normalization control. Results of densitometry are shown as means ± SD; $n = 3$. * $P < 0.05$.

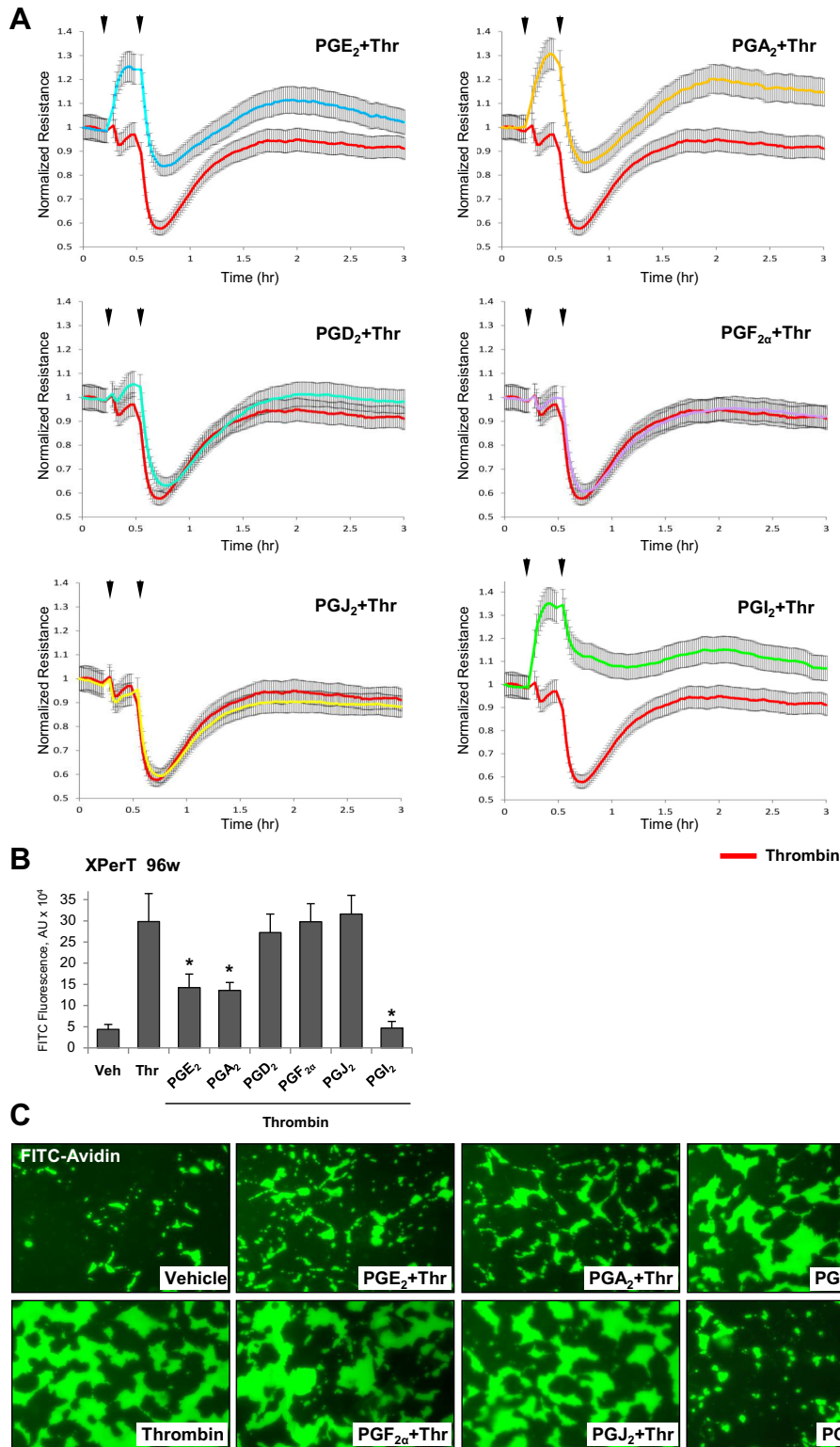


Fig. 3. Effects of PGA₂, PGE₂, PGD₂, PGF_{2α}, PGJ₂, and PGI₂ on EC permeability caused by high dose of thrombin. **A**: pulmonary EC were treated with indicated PG (0.5 μM, first arrow) for 15 min followed by addition of thrombin (0.5 U/ml, second arrow). Changes in TER were monitored over 3-h time period. **B**: analysis of EC permeability for macromolecules using XPerT assay. Bar graph depicts permeability changes after 5 min of thrombin treatment; *n* = 5, **P* < 0.05 vs. thrombin. **C**: visualization of agonist-induced permeability in endothelial monolayers. HPAEC monolayers grown on coverslips coated with biotinylated gelatin were treated with corresponding PG (0.5 μM, 15 min) or vehicle followed by stimulation with thrombin (0.5 U/ml, 5 min) and addition of FITC-avidin tracer as described MATERIALS AND METHODS. Green fluorescence depicts areas permeable for FITC-labeled avidin; bar = 20 μm.

AND METHODS. LPS induced a prominent acute inflammatory response, characterized by increase in BAL cell counts and BAL protein (Fig. 7A). Treatment with different PGs caused different levels of attenuation of parameters of LPS-induced ALI. PGA₂, PGE₂, and PGI₂ significantly inhibited the LPS-induced increase in cell counts and protein content in BAL.

PGD₂ partially attenuated the LPS-induced increase in BAL cell count and protein content, whereas treatment with PGJ₂ or PGF_{2α} failed to significantly attenuate parameters of LPS-induced lung dysfunction (Fig. 7A). There was no significant difference in BAL cell counts and BAL protein between PG-treated animals and controls in the absence of LPS chal-

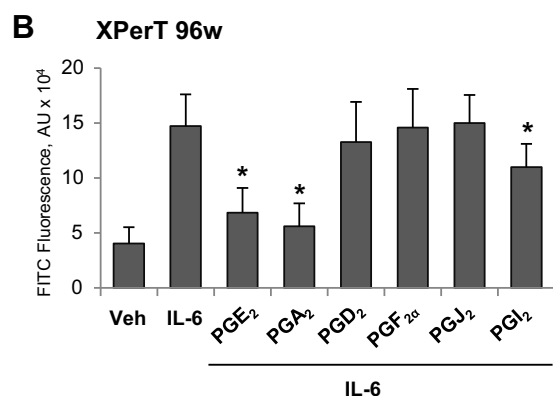
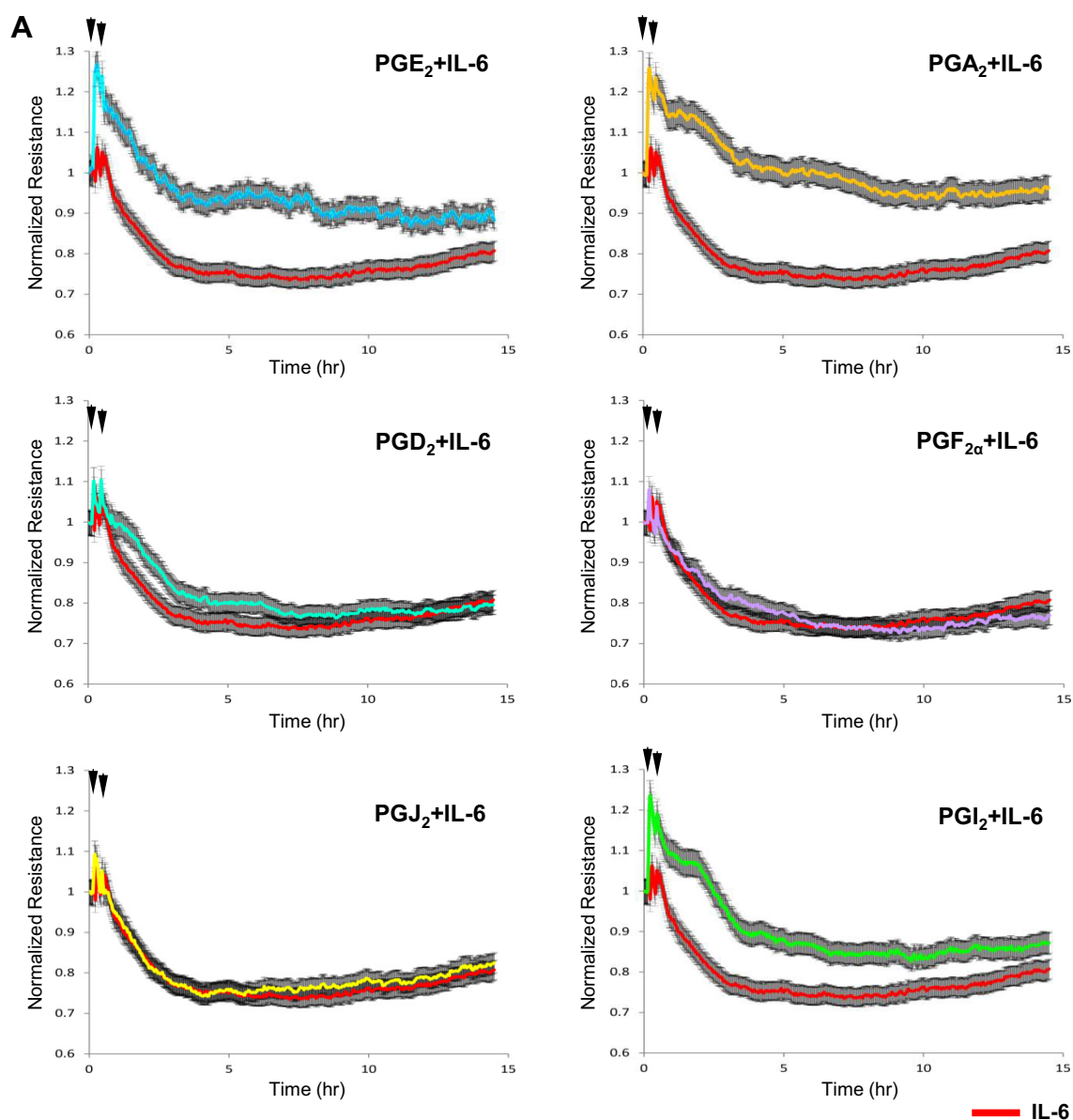


Fig. 4. Effects of PGA₂, PGE₂, PGD₂, PGF_{2α}, PGJ₂, and PGI₂ on EC barrier dysfunction caused by IL-6. **A:** pulmonary EC pretreated with indicated PG (0.5 μM, first arrow) were stimulated with a combination of IL-6 (20 ng/ml) and IL-6 soluble receptor (SR, 40 ng/ml) as shown by second arrow. Changes in TER were monitored over 15-h time period. **B:** analysis of EC permeability using XPerT assay. Bar graph depicts permeability changes after 6 h of IL-6/SR treatment; *n* = 5. **P* < 0.05 vs. IL-6/SR.

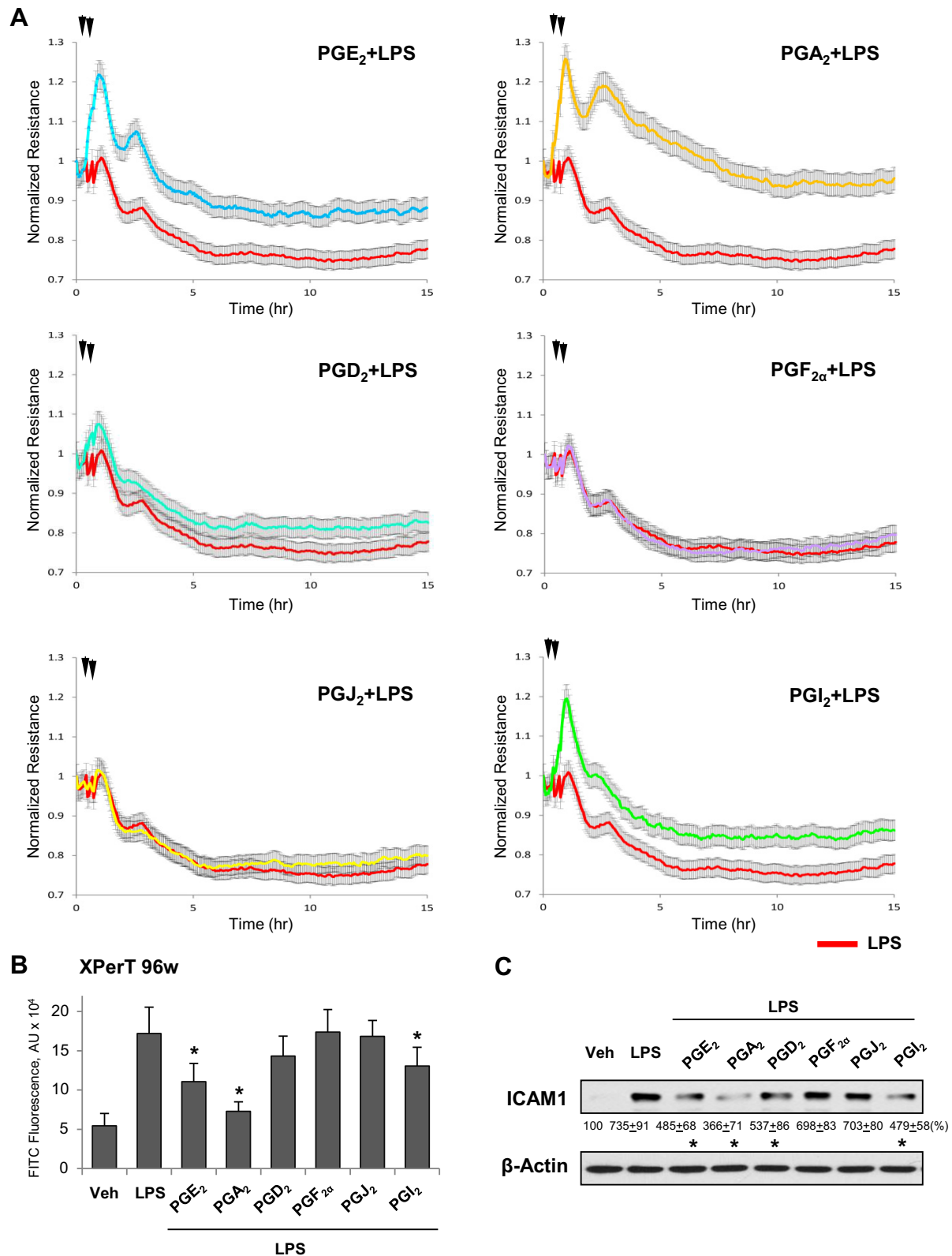
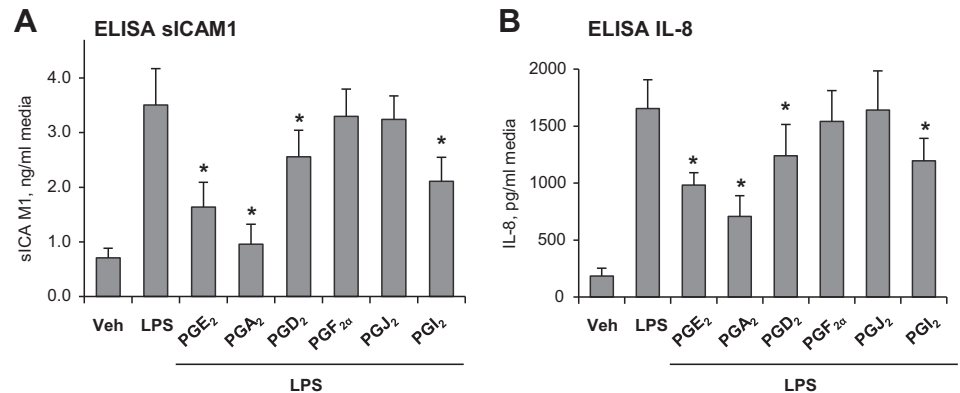


Fig. 5. Effects of PGA₂, PGE₂, PGD₂, PGF_{2α}, PGJ₂, and PGI₂ on LPS-induced EC permeability and ICAM1 expression. **A**: pulmonary EC were treated with indicated PG (0.5 μM, first arrow) followed by addition of LPS (300 ng/ml, second arrow). Changes in TER were monitored over 15-h time period. **B**: analysis of EC permeability using XPerT assay. Bar graph depicts permeability changes after 6 h of LPS treatment; *n* = 5. **P* < 0.05 vs LPS alone. **C**: cells treated with indicated PG (0.5 μM, 15 min) or vehicle were stimulated with LPS (300 ng/ml, 6 h), and ICAM1 protein expression was evaluated by Western blot. Probing with β-actin antibody was used as a normalization control. Results of densitometry are shown as means ± SD; *n* = 4. **P* < 0.05.

Fig. 6. Effects of PGA_2 , PGE_2 , PGD_2 , $\text{PGF}_{2\alpha}$, PGI_2 , and PGJ_2 on LPS-induced sICAM1 and IL-8 production. Pulmonary EC were treated with indicated PG (0.5 μM , 15 min) followed by addition of LPS (300 ng/ml, 6 h). Secretion of inflammation markers into the culture medium was detected by ELISA assays. A: sICAM1. B: IL-8. $n = 3$. * $P < 0.05$ vs. LPS.



lence (data not shown). In agreement with cell count and protein content data, PGA_2 , PGE_2 , PGI_2 , and PGD_2 attenuated the LPS-induced increase of soluble ICAM1 detected in BAL samples (Fig. 7B).

Lung vascular leak in mice challenged with LPS with and without PG treatment was further monitored using a noninvasive fluorescence optical imaging approach described in MATERIALS AND METHODS. Lung accumulation of the fluorescent tracer Angiosense 680 EX tracer injected intravenously reflects lung vascular barrier dysfunction and lung injury. Effects of PG treatment on LPS-induced lung injury were analyzed 24 and 72 h after the treatment. LPS-induced lung dysfunction was significantly reduced in mice treated with PGA_2 , PGE_2 , or PGI_2 and PGD_2 during LPS challenge (Fig. 7C). In contrast, treatment with PGJ_2 and $\text{PGF}_{2\alpha}$ the compounds which did not exhibit protective effects in EC culture, did not show significant improvement of lung function in the animal model of LPS-induced ALI.

DISCUSSION

Beneficial effects of prostaglandins extend beyond their vasodilating effects and regulation of local circulation and involve direct protective effects on the vascular endothelium (1, 9, 16). However, the complexity of tissue-specific patterns of PG receptor expression and PG functional effects highlights the importance of understanding the role of specific PG in control of pulmonary vascular endothelium as a critical therapeutic target in ALI. This study performed a systematic analysis of barrier-enhancing, barrier-protective, and anti-inflammatory effects exhibited by different members of prostaglandin family on human pulmonary endothelial cells.

Our results show that PGE_2 and PGA_2 exhibited the most pronounced and sustained barrier enhancing effects in pulmonary EC. PGI_2 also caused EC barrier enhancement response, which was more transient and diminished by 1–2 h of treatment. Other PGs— PGD_2 , $\text{PGF}_{2\alpha}$ and PGJ_2 —did not considerably affect the EC barrier.

PGE_2 , PGA_2 , and PGI_2 were also protective against thrombin-induced EC hyperpermeability, whereas three other PGs— PGD_2 , $\text{PGF}_{2\alpha}$, and PGJ_2 —did not affect barrier-disruptive effects of thrombin. PG barrier-protective effect against thrombin was directly associated with an extent of PG-induced inhibition of thrombin-induced RhoA GTPase signaling, which leads to increased MLC phosphorylation (13). This trend also remained in the ability of PGs to attenuate a more sustained EC

barrier dysfunction and inflammatory response caused by inflammatory mediators, IL-6 and LPS, which caused development of EC barrier dysfunction within 5 h. PGA_2 and PGE_2 were the most efficient in protecting EC barrier and suppressing the LPS-induced EC inflammatory activation monitored by increased expression of ICAM1 and secretion of soluble ICAM1 and IL-8 into the culture medium. In contrast to potent protective effects on thrombin-induced permeability, PGI_2 exhibited a less potent effect on LPS-induced TER decline. Modest, although statistically significant, attenuation of IL-6- and LPS-induced TER decline and inflammatory molecules expression was also observed in EC treated with PGD_2 , whereas $\text{PGF}_{2\alpha}$ and PGJ_2 were without effect in this model as well.

One potential explanation of the less potent PGI_2 effects is relatively short PGI_2 half-life [6–8 min (24)] vs. PGE_2 [8–11 h (20)], which reduces its EC protective potential in settings of more sustained EC barrier dysfunction caused by inflammatory agents. In contrast, PGI_2 's more stable analog, iloprost [half-life 30–40 min (19)], exhibited more potent and sustained protective and anti-inflammatory effects in vitro and in an animal model of LPS-induced ALI (7).

The differences in the PGs effects on pulmonary vascular inflammatory and permeability responses were consistent with PG effects on parameters of lung inflammation and vascular leak in vivo, as reflected by analysis of BAL samples, cytokine levels, and optical imaging of lung barrier dysfunction in live animals.

Despite the fact that PGA_2 , PGE_2 , and PGI_2 engage different receptors, they all exhibited a protective effect on vascular EC in the settings of agonist-induced barrier disruption or inflammation. These findings suggest common protective mechanisms activated by these PGs in pulmonary EC. We have previously reported that activation of protein kinase A and Epac-Rap1-Rac1 signaling cascades by PGE_2 and PGI_2 stimulated peripheral actin cytoskeletal dynamics leading to enhancement of endothelial barrier and inhibition of RhoA GTPase-mediated cell contraction and EC barrier compromise (6, 9). The results of this study suggest that this signaling may be a unifying mechanism of barrier-protective effects observed in PGA_2 -, PGE_2 -, and PGI_2 -treated pulmonary EC. In addition to Epac- and PKA-dependent signaling, each receptor stimulates additional pathways (10, 17) which may affect the magnitude and duration of protective response to specific PG observed in this study. It is important to note that, in contrast

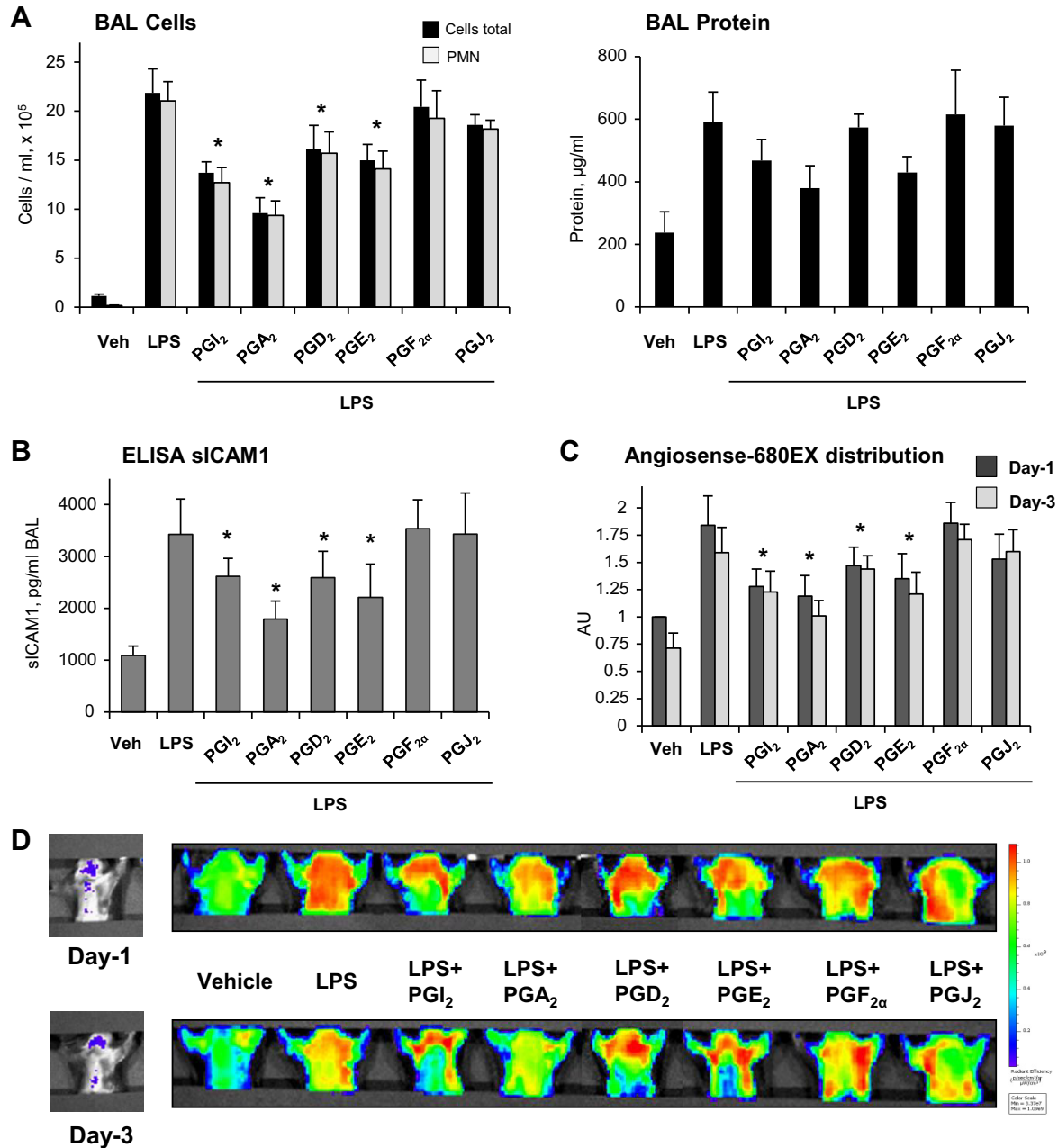


Fig. 7. Effects of PGA₂, PGE₂, PGD₂, PGF_{2α}, PGJ₂, and PGI₂ on LPS-induced ALI. Indicated PGs (20 μg/kg iv) were injected with indicated PG intravenously twice, concurrently and 5 h after LPS challenge (0.7 mg/kg it). Shown are cumulative data from 3 independent experiments, with 2 animals per experimental condition in each experiment. **A:** analyses of protein concentration and total cell and PNM counts were performed in BAL samples obtained from control and experimental groups 24 h after LPS challenge. **B:** levels of soluble ICAM1 in BAL samples were measured using ELISA assay; $n = 4$. * $P < 0.05$. **C:** live imaging analysis of lung vascular barrier dysfunction after LPS intratracheal injection with and without indicated PGs. LPS-induced accumulation of fluorescent Angiosense 680 EX imaging agent in the lungs of same animals was detected and quantified using Xenogen IVIS 200 Spectrum imaging system on *day 1* and *day 3* after LPS challenge; $n = 3$. * $P < 0.05$. **D:** visualization of fluorescent tracer intensity and distribution in the injured lungs is presented in arbitrary colors.

to better understood mechanisms of PG barrier-protective effects, specific anti-inflammatory signaling cascades triggered by particular PG remain to be more precisely elucidated.

Interestingly, PGD₂ did not noticeably affect thrombin-induced EC permeability but caused modest attenuation of LPS-induced EC inflammation and parameters of lung injury. In addition, PGJ₂ did not affect the inflammatory response in EC experiments but showed a trend to decrease of LPS-induced ALI, although it did not reach statistical significance.

This result is somewhat surprising given the anti-inflammatory effects of 15d-PGJ₂, which prevented the release of proinflammatory cytokines by macrophages (26), neutrophils (30), and monocytes (22). The effects of 15d-PGJ₂ were attributed to activation of peroxisome proliferator-activated receptor-γ (PPAR-γ) (23). Taken together with published studies, our results suggest that EC barrier-protective responses mediated by PGs are less dependent on PPAR-γ than inflammatory signaling in myeloid cells. These mechanisms may also explain

the low efficiency of PGJ₂ on lung vascular barrier dysfunction in vivo. Based on these results, it is tempting to speculate that cell type-specific or even vascular bed-specific differences in PG receptors expression patterns may define the overall response to specific PG in particular pathological conditions.

In summary, this study performed a comprehensive analysis of functional effects of prostaglandin family members on pulmonary vascular endothelial EC barrier properties and inflammatory response to clinically relevant insults. We identified PGE₂, PGA₂, and PGI₂ as prostaglandins with the most pronounced barrier-protective and anti-inflammatory effects on lung vascular endothelium and potential to attenuate lung injury caused by bacterial pathogens and vasoactive agonists.

GRANTS

This work was supported by the National Heart, Lung, and Blood Institute Grants HL-076259, HL-087823, and HL-107920 and National Institute of General Medical Sciences Grant GM-114171.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Y.K., O.O., N.S., Y.T., A.S., M.E.T., and S.S. performed experiments; Y.K., O.O., Y.T., A.S., M.E.T., A.A.B., and K.G.B. analyzed data; Y.K., O.O., A.S., M.E.T., A.A.B., and K.G.B. interpreted results of experiments; Y.K. and K.G.B. drafted manuscript; Y.K., O.O., N.S., Y.T., A.S., M.E.T., S.S., A.A.B., and K.G.B. approved final version of manuscript; O.O., A.A.B., and K.G.B. conceived and designed research; N.S., Y.T., S.S., and A.A.B. prepared figures; A.A.B. and K.G.B. edited and revised manuscript.

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