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Arterioscler Thromb Vasc Biol. Author manuscript; available in PMC 2019 May 01.

Published in final edited form as:

Arterioscler Thromb Vasc Biol. 2018 May ; 38(5): 1115–1124. doi:10.1161/ATVBAHA.118.310713.

A Protective Role of Microsomal Prostaglandin E Synthase-1 Derived PGE_2 and the Endothelial EP4 Receptor in Vascular Responses to Injury

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Abstract

Objective—Deletion of microsomal (m) prostaglandin (PG) E synthase (S)-1, an anti-inflammatory target alternative to cyclooxygenase-2, attenuates injury-induced neointima formation in mice. This is attributable to the augmented levels of PGI_2, a known restraint of the vascular response to injury, acting via I prostanoid receptor (IP). To examine the role of mPGES-1 derived PGE_2 in vascular remodeling without the IP.

Approach and Results—Mice deficient in both IP and mPGES-1 (double knockout, DKO) and littermate controls (IP KO) were subjected to angioplasty-wire injury. Compared with the deletion of IP alone, coincident deletion of IP and mPGES-1 increased neointima formation, without affecting media area. Early pathological changes include impaired reendothelialization and increased leukocyte invasion in neointima. Endothelial cells (ECs), but not vascular smooth

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Conflict of Interest Disclosures

Patent application (CN201710293360.9) is pending.

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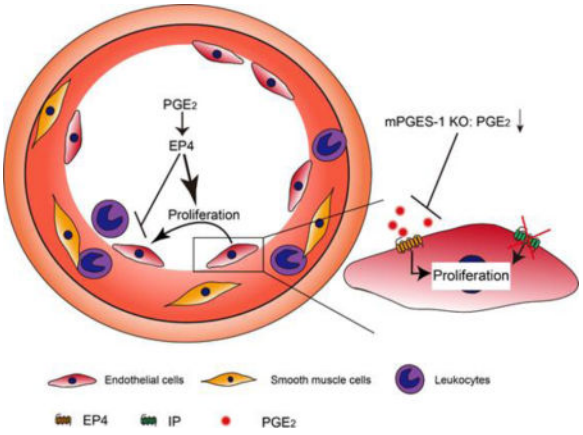
muscle cells, isolated from DKOs exhibited impaired cell proliferation. Activation of PGE_2 receptor (EP)4 (and EP2, to a lesser extent), but not of EP1 or EP3, promoted EC proliferation. EP4 antagonism inhibited proliferation of mPGES-1-competent ECs, but not of mPGES-1-deficient ECs, which showed suppressed PGE_2 production. EP4 activation inhibited leukocyte adhesion to ECs in vitro, promoted reendothelialization and limited neointima formation post injury in the mouse. Endothelium-restricted deletion of EP4 in mice suppressed reendothelialization, increased neointimal leukocytes, and exacerbated neointimal formation.

Conclusions—Removal of the IP receptors unmasks a protective role of mPGES-1 derived PGE_2 in limiting injury-induced vascular hyperplasia. EP4, in the endothelial compartment, is essential to promote reendothelialization and restrain neointimal formation after injury. Activating EP4 bears therapeutic potential to prevent restenosis after percutaneous coronary intervention.

Graphical abstract



Keywords

prostaglandin E synthase-1; PGE_2; EP4; endothelium; vascular remodeling

Subject Codes

Vascular Biology; Remodeling; Coronary Artery Disease; Endothelium; Stenosis

Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs) ameliorate pain, fever and inflammation by inhibiting cyclooxygenase (COX, two major isomers identified as COX-1 and COX-2), the rate-limiting enzyme in the synthetic cascade of prostanoids[1]. Clinical evidence shows that NSAIDs selective for COX-2 inhibition increase cardiovascular thrombotic events[2-4]. This is attributable to suppression of COX-2 derived prostaglandin (PG) I_2[5]. Microsomal (m) PGE synthase(S)-1[6] has emerged as a therapeutic target that is downstream of COX-2[7, 8]. Deletion of mPGES-1, unlike COX-2 inhibition, does not predispose to thrombosis, due to augmented PGI_2 (redirection of PGH_2, the COX product, to PGI synthase), and retards atherogenesis independent of IP (the receptor for PGI_2) deficiency[9-11]. Targeting mPGES-1 may avoid the cardiovascular risk associated with COX-2 inhibitors while preserving their

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analgesic and anti-inflammatory efficacy[12]. Accumulative evidence suggests that COX pathway components differentially modulate vascular responses to injury.

Celecoxib, a COX-2 selective NSAID, reduces in-stent late luminal loss in patients with coronary artery disease treated with aspirin plus clopidogrel after percutaneous coronary intervention (PCI)[13]. However, a follow-up trial confirmed an increased thrombotic risk with celecoxib despite the dual anti-platelet therapy[14], which limits its clinical application. Tissue-specific deletion of mPGES-1, the underlying alternative target for COX-2, in endothelial cells (ECs) or vascular smooth muscle cells enhances neointima formation, whereas myeloid cell mPGES-1 deletion reduces vascular hyperplasia response to injury[15]. Our previous study shows that deletion of mPGES-1 attenuates injury-induced neointimal formation in mice, with sustained reduction in PGE_2 and augmentation in PGI_2[16]. PGI_2 signaling via the IP is known to restrain the neointimal formation[17]. In contrast to IP deletion, deletion of the thromboxane A_2 receptor depresses this response[17]. The role of mPGES-1 derived PGE_2 in the vascular remodeling that is independent of the IP signaling remains unknown.

PGE_2 has four G-protein coupled receptors, EP1 through EP4, which mediates differential downstream signaling[18]. EP3 mediates vascular smooth muscle cell migration facilitating neointimal hyperplasia in mice[19]. EP4 protects against ischemia-reperfusion injury[20, 21], hypertension[22] or atherosclerosis[23]. EP4 promotes endothelial migration and angiogenesis[24, 25]. However, whether EP4 regulates injury-induced neointimal formation is unknown.

Here, we report that removal of the IP receptor uncovers a protective role of mPGES-1 derived PGE_2 in endothelial repair and the vascular response to injury. Furthermore, deletion of endothelial EP4 exacerbates, whereas pharmacological activation of EP4 limits neointimal hyperplasia after wire injury.

Materials and Methods

Animal study

Mice deficient in mPGES-1 (gene: Ptges)[26] and IP (gene: Ptgir)[17] were obtained from Pfizer and the FitzGerald lab at the University of Pennsylvania, respectively. Both strains had been backcrossed to a C57BL/6 background for over 10 generations, and were used to derive IP/mPGES-1 double knockout (DKO) mice and littermate controls (IP KO) by intercrossing *Ptgir*$^{-/-}$ *Ptges*$^{-/-}$ with *Ptgir*$^{-/-}$ *Ptges*$^{+/-}$. DKO mice develop normally without overt abnormalities. Global deletion of EP4 is perinatally lethal due to patent ductus arteriosus[27]. To circumvent this defect, endothelial-specific EP4 (gene: Ptger4) knockout mice were generated using a tamoxifen-*CreERT2* strategy[28]. Briefly, C57BL/6 *Ptger4*$^{flox/flox}$ mice[29] were intercrossed with *Cdh5*-promoter driven *CreERT2* (*Cdh5 (PAC)-CreERT2*$^{+}$) mice[30], kindly provided by Ralf Adams. The resulting *Ptger4*$^{f/f}$ *Cdh5-CreERT2*$^{+}$ and *Ptger4*$^{f/f}$ *Cdh5-CreERT2*$^{-}$ mice were then intercrossed to generate the animals used in this study—endothelial EP4 conditional-knockout (*Ptger4*$^{f/f}$ *Cdh5-CreERT2*$^{+}$: abbreviated, cKO) and littermate controls (*Ptger4*$^{f/f}$ *Cdh5-CreERT2*$^{-}$: abbreviated, Ctl). To induce endothelial EP4 deletion, tamoxifen (37.5 mg/ml dissolved in sunflower seed oil) was intraperitoneally

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injected into the experimental mice and littermate controls at a dose of 150 mg/kg/day for six days which were interrupted for three days after the third dose. Gene-modified mice used in this study were gender matched. The pooled data from both gender were used to show the gene specific effects in each study, and gender-specific sub-group analyses were provided in Supplemental Table I and II. Male C57BL/6 mice aged 6-8 weeks from the National Institutes for Food and Drug Control (Beijing, China) were used to determine the effect of AE1-329 (Gifted by ONO Pharmaceutical Co., Ltd., Osaka, Japan) or misoprostol (410004; purity, 98.9%; National Institutes for Food and Drug Control, Beijing, China) in vascular remodeling. All animal protocols were performed following the guidelines of the Institutional Animal Care and Use Committee, the Experimental Animal Center, Fuwai Hospital, National Center for Cardiovascular Diseases, China.

Femoral artery injury model

Femoral arteries were injured using a protocol as we described previously[28]. Briefly, a groin incision was made on one side of the anesthetized mouse. The femoral artery and its small branch between the rectus femoris and vastus medialis muscles were then carefully exposed and separated from the accompanying nerve and vein via blunt dissection. A 6-0 silk suture was then looped round the proximal femoral artery to stop the blood flow during the surgery. Another 6-0 silk suture was placed under the branch. A transverse arteriotomy was then made in the branch, and a flexible angioplasty wire (0.35 mm diameter; Cook Inc., IN, USA) was inserted into the femoral artery from the branch for a length no less than 5 mm toward the iliac artery. The wire was left in place for 3 min to dilate and denude the artery. Then, the wire was removed, the branch was ligated proximally with the 6-0 silk suture, and the blood flow in the femoral artery was restored by releasing the sutures for blood flow control. The skin incision was then closed with a 5-0 silk suture. Seven or twenty-eight days after injury, the arteries were harvested, embedded in paraffin and stained with haematoxylin and eosin (H&E) for determination of the severity of hyperplasia. In detail, cross-sections of the arteries were serially obtained for 10-13 levels at 150 μm intervals, and the sections with the most severe hyperplasia were used for comparisons. To evaluate reendothelialization, the sections from the arteries 7 days post-injury were immunostained with von Wllebrand Factor (vWF), an endothelial cell marker, and photographed with a CCD camera coupled to a microscope system (AXI0; Zeiss, Oberkochen, Germany). The segments along the vascular inner wall that were positive in vWF and the peripheral length of the vascular inner wall were measured using Image-Pro Plus 6.0 software (Media Cybernetics, MD, USA). The ratio of the total length of the vWF positive segments to the peripheral length was calculated to represent the extent of reendothelialization. To evaluate leukocytes infiltration/migration, representative sections from the arteries injured for 7 days were analyzed with H&E staining. Briefly, after capturing the images, intima leukocytes were objectively determined using a Hue (H) Saturation (S) Intensity (I)-based color selection strategy using Image-Pro Plus 6.0 software (Media Cybernetics, MD, USA).

Cell study

Endothelial cells—Mouse aorta endothelial cells (MAECs) were isolated as previously described[28, 31]. Briefly, aortas were harvested and cut into 1-2 mm^2 sections. The aortic segments were attached to a petri dish by their luminal side, and were then cultured in a

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DMEM medium containing 20 % fetal bovine serum (FBS) and 100 ug/mL endothelial cell growth supplement (ECGS) for 5-7 days, to allow the outgrowth of endothelial cells. Then, the endothelial cells were passaged and cultured. MAECs in passage 2-6 were used in this study. Endothelial cell proliferation was compared in MAECs isolated from IP KO or DKO, in passage 2. Primary human microvascular endothelial cells (HMECs) were brought from ScienCell (6000; Carlsbad, CA, USA), and cultured in the same medium as that for MAEC culture.

Smooth muscle cells—Mouse aorta smooth muscle cells (MASMCs) were isolated from IP KOs and DKOs. Briefly, aortas were isolated, scratched for three times on the inner surfaces, and cut into 1-2 mm^2 sections. The aortic segments were then attached to a petri dish by their luminal side, covered by a cover slid, and cultured in a DMEM medium containing 10 % FBS for 5-7 days. The MASMCs were then passaged and cultured. Proliferation of MASMCs in passage 2 was compared.

Cell proliferation—Cell growth was determined with a cell counting kit-8 (CCK-8; 40203ES60; Yeasen, Shanghai, China), following the manufacturer's instructions, as previously described[28]. The assay allows multiple detection without obvious cell toxicity. Briefly, the cells were seeded onto a 96-well flat-bottomed plate. After the cells were attached, ECs were cultured in the medium containing 3% FBS for 6-8 hours. Afterwards, the culture medium was replaced with a 3% FBS medium-CCK-8 mixture (10:1 in volume). Thereafter, the cells were cultured in the mixture for no longer than 4 hours; absorbance at 450 nm was determined as the baseline. Then the cells were cultured in fresh 3% FBS medium with the indicated reagents for another 48 hours. The culture medium was then replaced by the medium-CCK-8 mixture, and the culture continued for the same time as above for determination of the absorbance at 450 nm. The change in absorbance between the two measurements was used to define the cell growth. To determine MASMC proliferation, cells were pre-starved in FBS-free serum for 24 hours, then cultured in a medium containing 1% FBS, with two sequencial incubations with CCK-8. The reagents and their concentrations used in the proliferation studies were as follows: AE1-329 (0.1-1 μmol/L; Gifted by ONO Pharmaceutical Co., Ltd., Osaka, Japan), Butaprost (1 μmol/L; 13740; Cayman Chemical, MI, USA), Sulprostone (1 μmol/L; 14765; Cayman), GW627368X (0.1-1 μmol/L; HY-16963; MedChemExpress, NJ, USA), L-798106 (1 μmol/L; 11129; Cayman), iloprost (1 μmol/L; 18215; Cayman), Cay10441 (10 μmol/L; 10005186; Cayman), PF-04418948 (1 μmol/L; S7211; Selleck), ONO-8130 (1 μmol/L; 19118; Cayman), SQ22536 (200 μmol/L; S8283; Selleck), H89 2HCl (10 μmol/L; S1582; Selleck), ESI-09 (10 μmol/L; 19130; Cayman), db-cAMP (3-100 μmol/L; D0260; Sigma, Darmstadt, Germany) and misoprostol (10 μmol/L; 410004; National Institutes for Food and Drug Control, Beijing, China).

Endothelium-leukocytes adhesion study—For endothelium-leukocyte adhesion assay, MAECs were seeded into the 96-well plate, pre-starved in the DMEM containing 3% FBS for 6-8 hours, and incubated with tested reagents for 2 hours. Leukocytes were gathered from mouse peritoneal. Briefly, 4% Brewer Modified Thioglycollate Medium (211716; BD Biosciences, NJ, USA) was injected into the mouse peritoneal (1 mL per mouse). Four to

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five hours later, the peritoneal leukocytes were washed out with 0.1% bovine serum albumin, centrifuged, and re-suspended in the 1640 medium containing 10% FBS. When the preparation of endothelial cells was completed, the culture medium with indicated drugs was replaced by the 1640 medium containing leukocytes (3×10^4/well). Endothelial cells and leukocytes were then co-cultured for 30 minutes. Later, the cells were rinsed once with 1640 medium containing Rhodamine 6G (200 μg/mL; 252433; Sigma, Darmstadt, Germany), followed by 3 washes with fresh 1640 medium. The fluorescent signals were finally detected by a microplate reader (Excitation wavelength: 560 nm, emitter wavelength: 560 nm; Infinite M200, Tecan, Hombrechtikon, Switzerland). The reagents and their concentrations used in this study were as follows: misoprostol (10 μmol/L; 410004; National Institutes for Food and Drug Control, Beijing, China), AE1-329 (1 μmol/L; Gifted by ONO Pharmaceutical Co., Ltd., Osaka, Japan), GW627368X (1 μmol/L; HY-16963; MedChemExpress, NJ, USA) and db-cAMP (30 μmol/L; D0260; Sigma, Darmstadt, Germany).

Immunofluorescence staining

Immunofluorescence staining was performed following the same protocol as we described previously[28]. Briefly, the paraffin sections (5 μm) were deparaffinized, rehydrated, and subjected to antigen retrieval using an EDTA antigen-retrieval water (PH 9.0; ZSGB-BIO, Beijing, China). Following 1-hour incubation with normal goat serum at room temperature, the samples were incubated with primary antibodies overnight at 4 °C, and subsequently stained with Alexa Fluor-488-coupled or Alexa Fluor-594-coupled secondary antibodies for 3 hours at room temperature. The sections were then mounted with a VectaShield medium containing DAPI to stain nuclei, and imaged using a Zeiss microscope system (AXI0; Zeiss) or a laser-scanning confocal microscope system (SP8; Leica). For determination of reendothelialization, images were analyzed using Image-Pro Plus 6.0 software (Media Cybernetics, Inc. Rockville, MD, USA). The primary antibodies and dilution fold used in the study were polyclonal anti-EP4 antibody (2.5 μg/mL; 101775; Cayman Chemical, MI, USA), rabbit polyclonal anti-vWF antibody (1 μg/mL; F3520; Sigma, Darmstadt, Germany), sheep polyclonal anti-vWF antibody (10 μg/mL; Ab11713; Abcam, Cambridge, UK), monoclonal anti-α-SMA antibody (5 μg/mL; A5228; Sigma, Darmstadt, Germany) and polyclonal anti-F4/80 antibody (2 μg/mL; Ab16911; Abcam, Cambridge, UK).

Western blot analysis

For Western blot analysis, the cells were lysed in a RIPA buffer that contained protease inhibitors (4693116001, Roche, Basel, Switzerland). After centrifugation (15800 g, 10 min), cell lysates were mixed with a loading buffer, fractionated with 10% SDS-PAGE, and transferred onto polyvinylidene fluoride membranes. The membranes were then probed with the primary poly-clonal antibodies against EP4 (2.5 μg/mL; 101775; Cayman, Michigan, USA) overnight at 4 °C, followed by incubation with goat anti-rabbit secondary antibody for 1 hour at room temperature. Finally, the membranes were incubated with ECL luminous liquid (P1020; PPLYGEN, Beijing, China), and signals from immunoreactive bands were visualized using a FluorChem System (ProteinSimple, CA, USA).

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Prostanoid determination

PGE_2 and PGI_2 levels were determined via measuring their major urinary metabolites, tetranor-PGEM and 2,3-dinor-6-keto-PGF1α by liquid (L) chromatography (C)–tandem mass (M) spectrometry (S), as previously described[32].

Statistical analysis

Statistical analysis was performed using GraphPad Prism 5 software (GraphPad Software Inc., San Diego, California, USA). When only two groups were involved, Student's two-tailed unpaired t-test was used for comparison. Comparisons of multiple groups were made using a one-way ANOVA analysis. Tukey's or Bonferroni's post hoc tests were performed for the data with equal variances, while Dunnett's post hoc tests were used for data with unequal variances. And Bartlett's tests were used for analyzing the variances of data. Results are expressed as mean ± SEM. Differences were considered statistically significant at $p<0.05$.

Results

1. mPGES-1 derived PGE_2 protects against wire injury-induced neointimal formation in IP-deficient mice

Deletion of mPGES-1 in mice enhances PGI_2 production, which leads to the attenuated vascular remodeling[16]. To elucidate a role of mPGES-1 derived PGE_2 in vascular remodeling, we crossed mPGES-1 KOs with IP KOs to generate double KO (DKO) mice and littermate IP KO mice as control. The mice were subjected to wire-injury (endothelial denudation) of the femoral artery for 28 days. On this IP deficient background, mPGES-1 deletion increased neointima area by ~84%, and also increased the ratio of intimal to medial area, with media thickness unaltered (Figure 1A-D). This reveals a protective role of mPGES-1 derived PGE_2 in the vascular response to injury. Urinary metabolite of PGE_2 was reduced in DKO mice, while PGI_2 metabolite was enhanced (Figure 1E & F).

To explore the underlying mechanism, vessels were harvested at 7 days after surgery for histology examination. Again, the neointimal area and the ratio of intima to media, but not the medial area, were significantly enhanced in DKO (Figure 2A & B). Intima leukocytes, mainly macrophages (positive for F4/80 staining), were increased in the DKOs (Figure 2C, D and Supplemental Figure I). Expression of α-SMA was mainly detected in smooth muscle cells (SMCs) in the media, but rarely detected in the neointima area regardless genotypes (Figure 2E). When von Wllebrand Factor (vWF, an endothelial marker) was stained, a striking reduction in the number of endothelial cells (ECs) was observed in the DKOs (Figure 2D & F), reflecting suppressed reendothelialization after the wire denudation injury.

To pursue a potential impact on endothelial repair, ECs were isolated from IP KOs and DKOs, and their capacity for proliferation was evaluated in vitro (Figure 2G). The EC proliferation was significantly impaired in the DKOs (Figure 2H), revealing a proliferative effect of PGE_2 signaling in ECs. Further, treatment of ECs with misoprostol, a PGE analogue, promoted EC proliferation in vitro (Figure 2I), and also decreased leukocyte adhesion to endothelial monolayer in vitro (Figure 2J).

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Primary aortic smooth muscle cells were also isolated from IP KOs and DKOs. No difference in cell proliferation was detected between the two groups (Supplemental Figure II).

2. EP4 activation promotes endothelial cell proliferation and reduces endothelium-leukocyte adhesion

Receptors that might mediate the effect of PGE_2 on EC proliferation were then studied in vitro. ECs from DKO mice were treated with agonists[33, 34] for the EP1/3 (sulprostone, EC50=0.42 nmol/L for EP3, also a weak agonist for EP1), EP2 (butaprost, EC50=32 nmol/L) or EP4 (AE1-329, EC50=3.1 nmol/L) receptors, all at a concentration of 1 μmol/L. AE1-329, but not sulprostone or butaprostpromoted proliferation in the DKO ECs (Figure 3A). Similar effects were observed with wild type ECs (Figure 3B). We further tested each drug at mutiple concentrations for cell proliferating activity (Supplemental Figure III). AE1-329 dose-dependently promoted EC proliferation, while butaprost showed a pro-proliferative effect at a higher concentration, i.e., 10 μmol/L. AE1-329 enhanced EC proliferation in either absence or presence of IL-1β, a stimulus to PGE_2 production (Figure 3C & Supplemental Figure IVA). Conversely, GW627368X (a selective EP4 antagonist) markedly inhibited EC proliferation under IL-1β stimulation (Figure 3D). When mPGES-1 KO ECs were used, where PGE_2 production was depressed (Supplemental Figure IVB), the effect of EP4 agonism, but not that of EP4 antagonism, remained significant (Figure 3E). Thus, endogenous PGE_2 derived from EC mPGES-1 plays an active role in promoting proliferation of the IL-1β stimulated ECs, which can be blocked by antagonism of EP4, but not of other PGE_2 receptors (Figure 3F). This further confirms the proliferative effect of EP4 activation in ECs (Figure 3A & B).

To elucidate EP4 downstream signaling, SQ22536 [an adenylyl cyclase (AC) inhibitor], H 89 2HCl (a PKA inhibitor) and ESI-09 (an EPAC inhibitor) were used. Treatment with SQ22536 or H 89 2HCl, but not with ESI-09, abrogated the pro-proliferation effect of AE1-329 (Figure 3G) and the anti-proliferation effect of GW627368X (Figure 3H), indicating that cAMP-PKA axis underlies the enhanced EC proliferation driven by EP4 activation. Consistent with this finding, Db-cAMP (a cell permeable cAMP analogue) and forskolin (a potent AC activator) both promoted EC proliferation, and such proliferative effects were blunted by PKA inhibition with H89 2HCl or PKI (Figure 3I, and Supplemental Figure V & VI). PGI_2 is known to elevate cAMP signaling via IP receptor[1]. Indeed, iloprost (1 μmol/L), an IP agonist, promoted endothelial proliferation, whereas Cay10441 (10 μmol/L), an IP antagonist inhibited this response (Figure 3J), indicating a proliferative effect of PGI_2 on ECs.

The effect of EP4 on endothelium-leukocyte adhesion was also studied in vitro. Incubation of a monolayer of ECs with AE1-329, the EP4 selective agonist, significantly suppressed endothelium-leukocyte adhesion, while inhibition of endothelial EP4 by GW627368X enhanced the leukocyte adhesion to ECs (Figure 3K). This is consistent with a cAMP mediated effect, since Db-cAMP (a cell permeable cAMP analogue, 30 μmol/L) treatment similarly reduced the leukocyte adhesion (Figure 3L).

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3. Endothelium-restricted deletion of EP4 impairs reendothelization and exacerbates neointimal formation

The role of endothelial EP4 in vascular remodeling was then examined in mice deficient in this receptor only in ECs (Figure 4A) as confirmed by significant suppression of EP4 protein expression in ECs (Figure 4B & C). The mice were subjected to the wire-injury and studied 28 days later. The neointimal area and the ratio of intimal to medial area were both enhanced in the mice lacking endothelial EP4, while the medial area was unchanged (Figure 4D-G). Seven days after vascular injury, reendothelialization was significantly suppressed in mice lacking EP4 only in ECs (Figure 5A & B). This coincided with an increase in the number of intimal leukocytes and enhanced neointimal formation in cKOs (Figure 5C-E).

4. EP4 activation protects against wire-injury induced neointimal formation

Systemic administration of AE1-329, the EP4 agonist, also ameliorated neointimal formation without affecting media thickness (Figure 6A & B). Misoprostol, which promotes endothelial proliferation in vitro (Figure 2I), attenuated neointimal formation (Figure 6A & C) and promoted reendothelialization at 7 days after vascular injury (Figure 6D & E) in vivo. Misoprostol also decreased leukocyte infiltration (Supplemental Figure VII).

5. EP4 critically involves in human endothelial cell proliferation

Activation of EP4 by AE1-329, but not of other PGE_2 receptors, promoted proliferation of human primary endothelial cells (Figure 7A). Conversely, among antagonists of the four EPs, only EP4 blockade by GW627368X inhibited the EC proliferation (Figure 7B).

Discussion

Here we report a novel mechanism that regulates vascular responses to injury in mice: PGE_2 acts via endothelial EP4 to promote reendothelialization and restrain neointimal formation after wire induced vascular injury. A similar mechanism promotes proliferation of human endothelial cells in vitro. These observations raise the possibility that EP4 agonists may have value as adjunctive therapy in patients undergoing percutaneous revascularization.

We have previously reported that augmented biosynthesis of PGI_2 explains the reduced neointimal response to injury in mPGES-1 KO, raising the possibility that inhibitors of this enzyme will have a more favorable cardiovascular profile than NSAIDs targeting COX-2 that depress PGI_2 biosynthesis[16]. Here, removal of the PGI_2 receptor reveals a second therapeutic opportunity as mPGES-1 derived PGE_2 can limit neointimal formation after vascular injury (Figures 1&2). Indeed, we show in mice that pharmacological activation of EP4 confers protection against the injury-induced intima hyperplasia (Figure 6). Mechanistically, these beneficial effects are at least mediated through a support of EC repair by mPGES-1 derived PGE_2 and EP4 (Figures 2&6). We also observed an increased leukocytes infiltration (Figure 2C). This is consistent with a suppressing effect of EP4 activation on leukocyte adhesion (Figure 2J and 3K). Previous studies[22, 35-39] indicate that the PGE_2/EP4 axis inhibits mobilization of inflammatory cells and their response to inflammation. These studies highlights a possible contribution of the PGE_2/EP4 axis on bone marrow microenvironment and on inflammatory cells per se in mediating the increased

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leukocyte infiltration to the injured vessels observed in the present study. Here, we discovered that EP4 in the endothelial compartment also mediates endothelial-leukocytes adhesion (Figure 5). There was no discernible difference in the neointimal SMCs (Figure 2E) or in the proliferation of VSMCs isolated from the mPGES-1 deficient mice (Supplemental Figure II). In cultured ECs, activation of EP4 (and perhaps EP2, but not EP1 and EP3), via cAMP-PKA pathway, promotes endothelial proliferation in vitro (Figure 3), consistent with the observed suppression of neointimal formation after injury by such ligands in vivo. In line with this mechanistic hypothesis, deletion of the endothelial EP4 impaired reendothelialization and exacerbated neointimal formation after injury (Figure 4&5). Furthermore, EP4 activation promoted reendothelialization and inhibited neointima formation in mice (Figure 6). Therefore, EC EP4 mediated endothelial repair underscores a key mechanism that restrains the vascular hyperplasia after denudation injury. Promotion of endothelialization by activating endothelial EP4 represents a novel strategy to limit restenosis after PCI[40]. The mechanism of mPGES-1 derived PGE_2 in vascular remodeling is schematically illustrated in Supplemental Figure VIII.

Development of mPGES-1 inhibitors has now reached clinical stage. Interestingly, an augmented production of PGI_2 was observed with suppression of mPGES-1 derived PGE_2[7]. This is consistent with our initial observation with mPGES-1 KO mice[11]. Potential elevation of PG products due to substrate rediversion may depend on specific tissue/cell or disease pathology[10]. Their significance in mediating the cardiovascular profile of mPGES-1 inhibition relative to COX-2 inhibition, warrants future study. Our study here suggests that augmented PGI_2 may balance a vascular risk of suppressing PGE_2-EP4 axis when mPGES-1 inhibition is pursued under pathologic insult.

In conclusion, we have previously provided evidence that inhibition of mPGES-1 may offer an approach adjunctive to PCI to limit restenosis due to its capacity to augment PGI_2. Here, we provide evidence that PGE_2 formation might be targeted to achieve the same objective, here by activating the EP4 receptor in endothelial cells. Given that restenosis remains a restraint on the clinical effectiveness of PCI[40, 41], the provision of pre-clinical data consistent with two distinct strategies by which this might be mitigated is timely.

Study limitations

The mechanistic exploration of this study was mainly focused on endothelial proliferation/repair by mPGES-1 derived PGE_2 and EP receptors. Whether impaired reendothelialization process might allow enhanced exposure to certain thrombotic components that may also contribute to the vascular responses, warrants future study. This study was conducted with mice of C57BL/6 background. Confirming this study with other mouse strains or other species more relevant to human pathophysiology would help increase the translational potential of the mechanism discovered here.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Acknowledgments

We thank Yuuki Nagao from ONO Pharmaceutical Co., Ltd. for supplying the research materials and for reviewing the manuscript. We thank Xu Zhang from Tianjin Medical University for measuring prostaglandins.

Funding Sources: This work was supported by the National Natural Science Foundation of China (81570269 and 81370222), Chinese Academy of Medical Sciences Innovation Fund for Medical Sciences (2017-12M-1-008, 2016-I2M-1-003/005), a research award from the National 1000-Talent Plan of China and research funds from Fuwai Hospital, Peking Union Medical College and Chinese Academy of Medical Sciences, with all to MW, by the fund from National Heart, Lung, and Blood Institute (HL117798) to G.A.F., and by the National Natural Science Foundation of China (81703517) to HH.

Abbreviations

AE1	AE1-329
Buta	Butaprost
CCK-8	Cell counting kit-8
cKO	Endothelial EP4 conditional-knockout mice
COX	Cyclooxygenase
Ctl	Littermate controls for cKO
DKO	Double knockout
ECGS	Endothelial cell growth supplement
ECs	Endothelial cells
EP1, 2, 3, 4	Prostaglandin E receptor 1, 2, 3, 4
FBS	Fetal bovine serum
GW	GW627368X
H&E	Haematoxylin and Eosin
H 89	H 89 2HCl
HMECs	Human microvascular endothelial cells
IL-1β	Interleukin 1β
i.p.	Intraperitoneal
IP	I prostanoid receptor
IP KO	I prostanoid receptor knockout
MAECs	Mouse aorta endothelial cells
Miso	Misoprostol
mPGES-1	Microsomal prostaglandin E synthase-1

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NSAIDs	Nonsteroidal anti-inflammatory drugs
PCI	Percutaneous coronary intervention
PG	Prostaglandin
PGI_2	Prostacyclin
SMA	Smooth muscle actin
SQ	SQ22536
Sul	Sulprostone
Veh	Vehicle
VSMC	Vascular smooth muscle cell
vWF	von Willebrand Factor

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Highlights

- Deletion of mPGES-1, on a background of IP depletion, exacerbates wire-injury induced neointima formation, with early impairment of reendothelialization.
- Activation of EP4 or EP2 promotes endothelial cell proliferation, and endothelium-restricted deletion of EP4 impairs reendothelialization and increases neointima formation.
- EP4 agonism promotes reendothelialization and attenuates neointima formation, raising a possibility that EP4 agonism may prevent restenosis after percutaneous coronary intervention.

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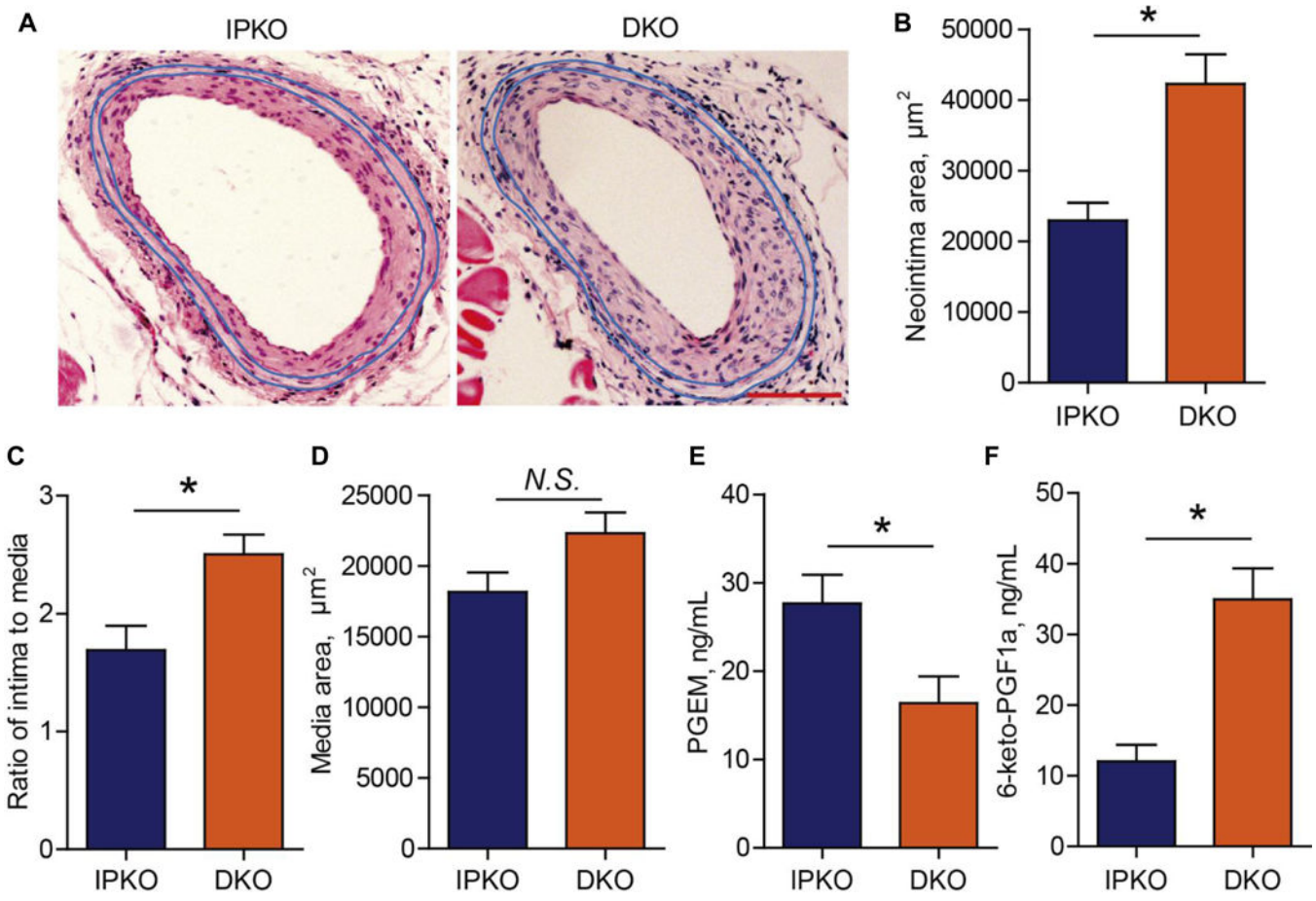

Figure 1. IP deletion reveals a protective role of mPGES-1 derived PGE_2 against wire-injury-induced neointimal formation

DKO (*Ptgir* $^{-/-}$ *Ptges* $^{-/-}$) and littermate IPKO (*Ptgir* $^{-/-}$) mice were subjected to wire-injury at femoral arteries, and the vessels were harvested at 28 days after injury and quantified for neointima formation. Representative images of hematoxylin and eosin (H&E) staining are shown (**A**; Bar= 100 μm). Neointimal area (**B**), ratio of intima to media (**C**) and media thickness (**D**) were determined (n= 14 IPKO, 16 DKO). PGE_2 (**E**) and PGI_2 (**F**) metabolites in urine were determined by HPLC-MS/MS as detailed in the Method (n= 8). *P<0.05; Student's unpaired t-test.

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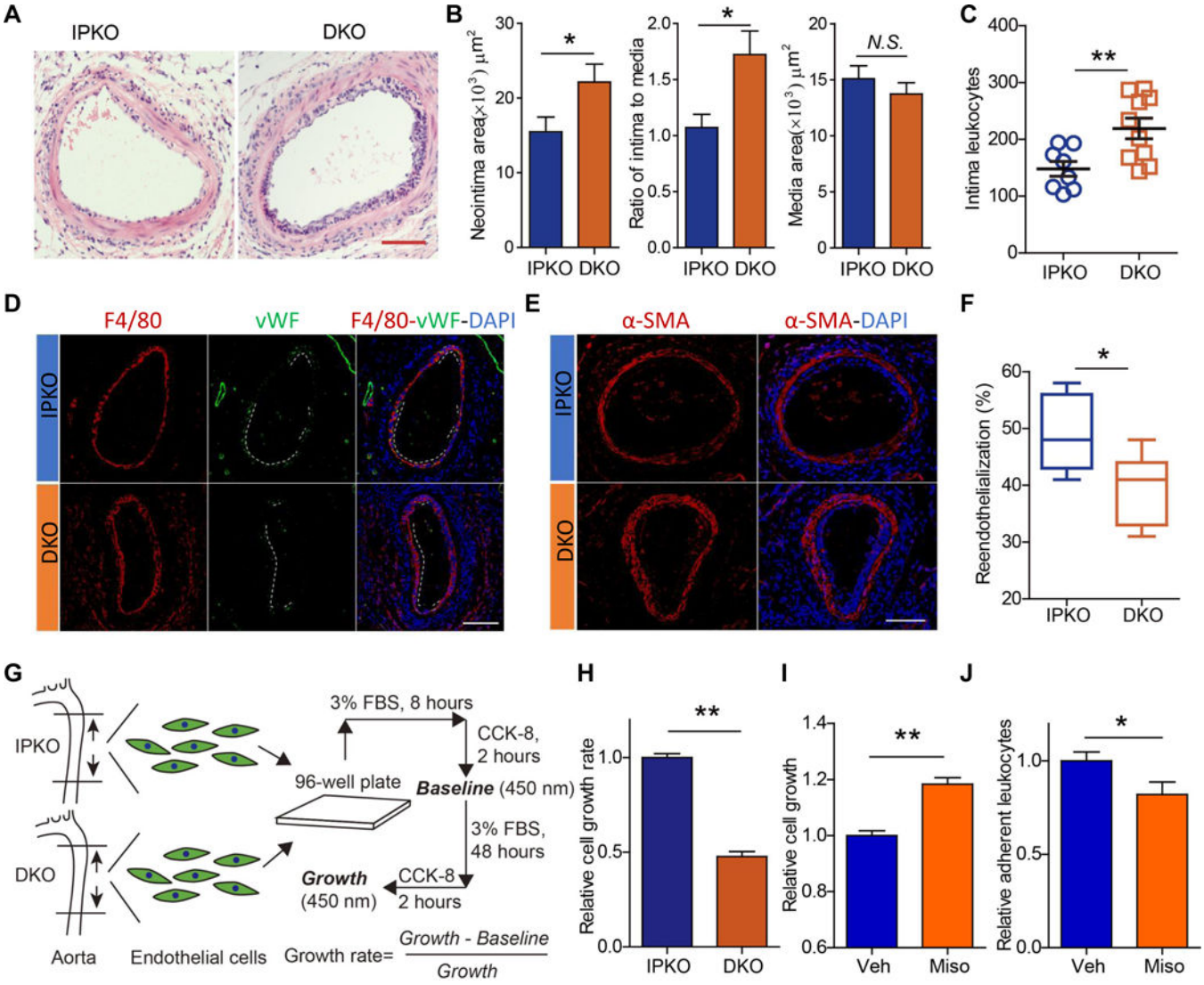

Figure 2. Deletion of mPGES-1 suppresses reendothelialization after endothelial denudation injury in IP deficient mice

Injured femoral arteries of DKO and IPKO were harvested at 7 days after surgery. Neointima formation and leukocytes infiltration were evaluated on H&E stained sections, with representative H&E images shown (**A**; Bar= 100 μm). Neointimal area, ratio of intima to media and media thickness were quantified (**B**; n= 14 IPKO, 16 DKO). Number of neointima leukocytes was quantified by analyzing the H&E stained tissue sections with IPP software (**C**; n= 8 IPKO, 10 DKO). Injured vessels were immunostained for F4/80 (a marker of macrophage, red) and vWF (an EC marker, green) (**D**; Bar= 100 μm) and for α-SMA (a SMCs marker) (**E**; Bar= 100 μm). DAPI stains nuclei in blue. The number of vWF-positive cells was quantified to determine reendothelialization (**F**; n=7). ECs were isolated from the descending aortae of IPKO and DKO mice, and their cell proliferation was analyzed in vitro (**G**) and compared (**H**). Calculation of the EC proliferation rate was based on the difference in cell number before vs after treatment with 3% FBS for 48 hours. Misoprostol, a PGE analogue at 10 μmol/L, promoted endothelial proliferation (**I**, n=9 from two independent

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experiments) and inhibited endothelium-leukocytes adhesion (**J**, n=9 from three independent experiments). *P<0.05, **P<0.01; Student's unpaired t-test.

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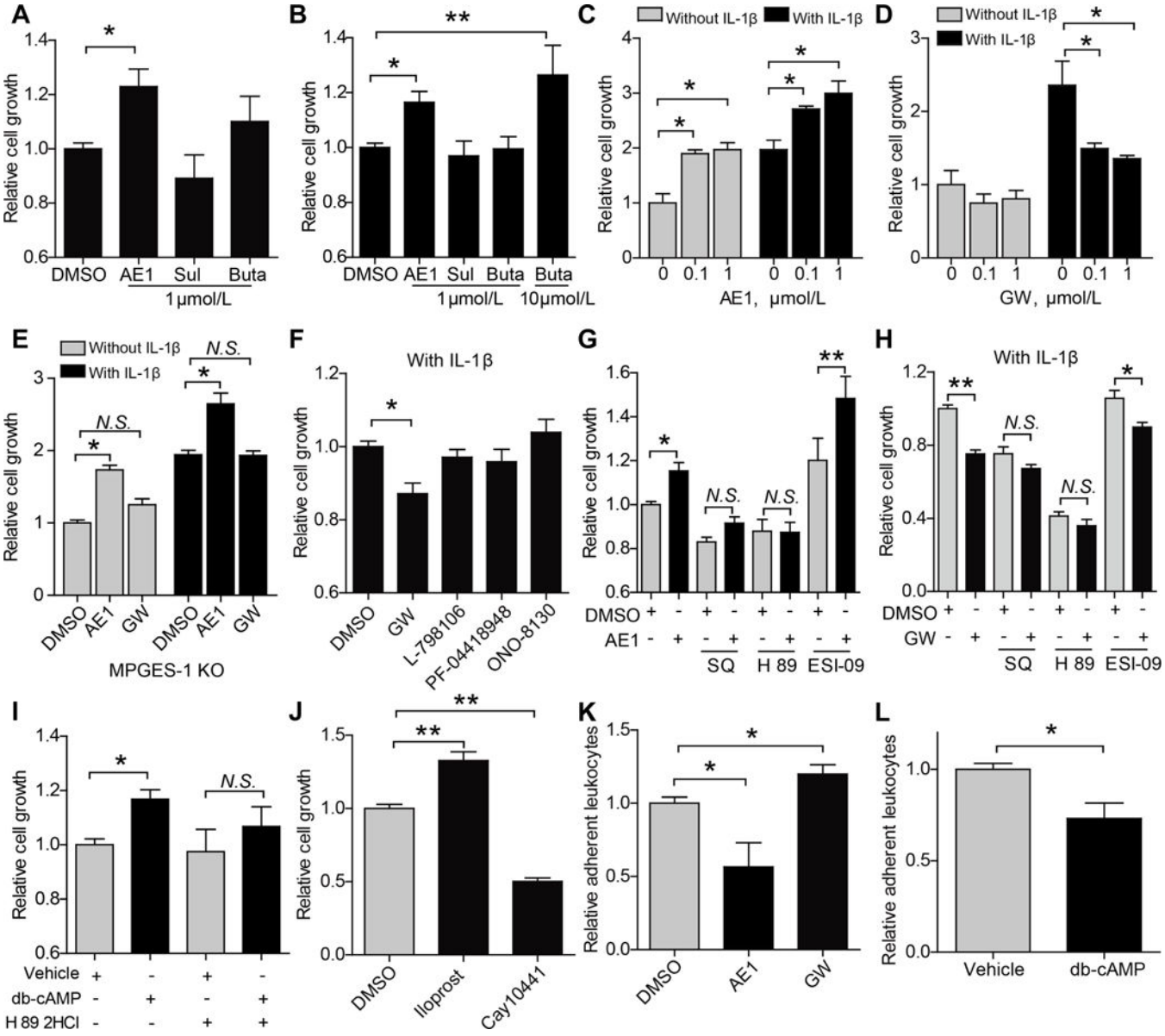

Figure 3. PGE_2 promoted in vitro endothelial cell proliferation via EP4/cAMP/PKA signaling

Mouse aortic endothelial cells (MAECs) were used for the study of cell proliferation in vitro. Cells from DKO (**A**) and wildtype (WT) mice (**B**) were stimulated with agonists for PGE_2 receptors [AE1-329 (AE1), an EP4 agonist; Sulprostone (Sul), an EP1/3 agonist; Butaprost (Buta), an EP2 agonist], and relative proliferation is shown. WT ECs were treated with AE1 of different concentrations, with or without IL-1β (10 ng/mL), and proliferation was determined (**C**). GW627368X (GW, an EP4 antagonist) suppressed proliferation of IL-1β-stimulated MAECs (**D**). Effects of AE1 or GW on the proliferation of *Ptges* $^{-/-}$ ECs, stimulated with IL-1β (10 ng/mL) or not, were shown (**E**). Under IL-1β (10 ng/mL) stimulation, GW (an EP4 antagonist) but not L-798106 (an EP3 antagonist), PF-04418948 (an EP2 antagonist) or ONO-8130 (an EP1 antagonist), all at a concentration of 1 μmol/L, inhibited endothelial cell proliferation (**F**). The pro-proliferative effect of AE1 (**G**) and the

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anti-proliferative effect of GW (**H**) was prevented by SQ (SQ22536, an adenylate cyclase inhibitor; 200 μmol/L) or H 89 (H 89 2HCl, a PKA inhibitor; 10 μmol/L), but not by ESI-09 (an EPAC inhibitor; 10 μmol/L). Db-cAMP (**I**; a cell permeable cAMP analogue, 30 μmol/L) promoted endothelial proliferation, and this was also blunted by H89 2HCl (a PKA inhibitor). Endothelial proliferation was stimulated by iloprost (an IP agonist, 1 μmol/L) and inhibited by Cay10441 (an IP antagonist, 10 μmol/L) (**J**). The adhesion of leukocytes to ECs was inhibited by the EP4 agonist, AE1-329, and was promoted by GW, an EP4 antagonist (**K**). Db-cAMP treatment reduced leukocyte adhesion to ECs (**L**). All results are from at least three independent data sets. *P<0.05. **P<0.01; One-way ANOVA was used for data comparisons with Bonferroni's (A, G-K), Dunnett's (B) or Turkey's (C-F) post-test. Student's unpaired t-test was used in L.

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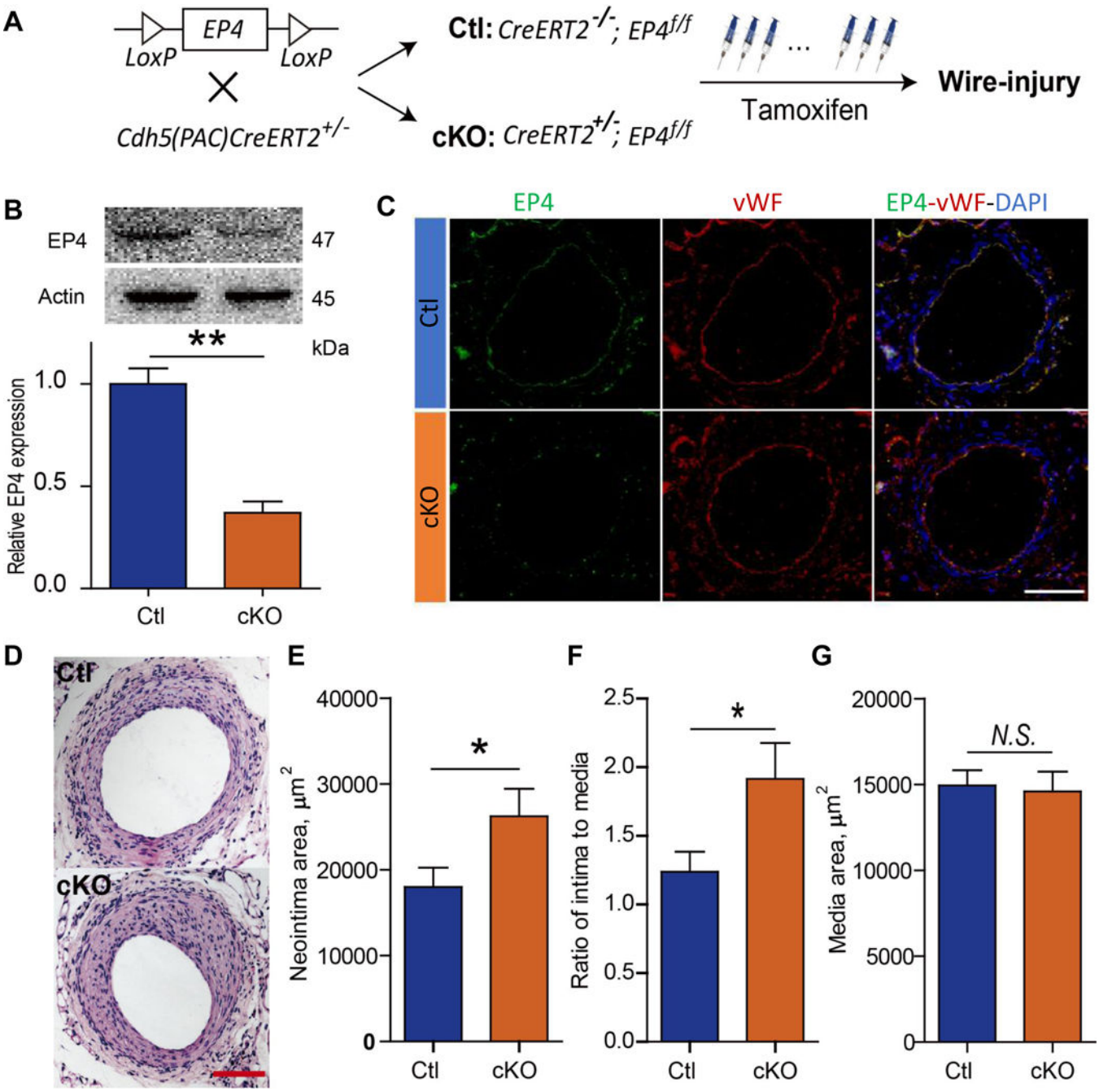

Figure 4. Induced deletion of endothelial EP4 promotes neointimal formation

Postnatal deletion of endothelial EP4 gene was induced in mice by tamoxifen treatment (**A**). Expression of EP4 in the primary ECs isolated from cKO and Ctl mice was detected with Western blot (**B**), and its expression in femoral arteries was determined by immunofluorescent staining (**C**; Bar= 100 μm). Representative images are shown (EP4, green; vWF, red). The injured vessels, harvested at 28 days after surgery, were stained with H&E, and representative images are shown (**D**; Bar= 100 μm). Neointimal area (**E**), ratio of

intima to media (**F**) and medial thickness (**G**) were quantified. n= 14 Ctl, 10 cKO. *P<0.05. **P<0.01; Student's unpaired t-test.

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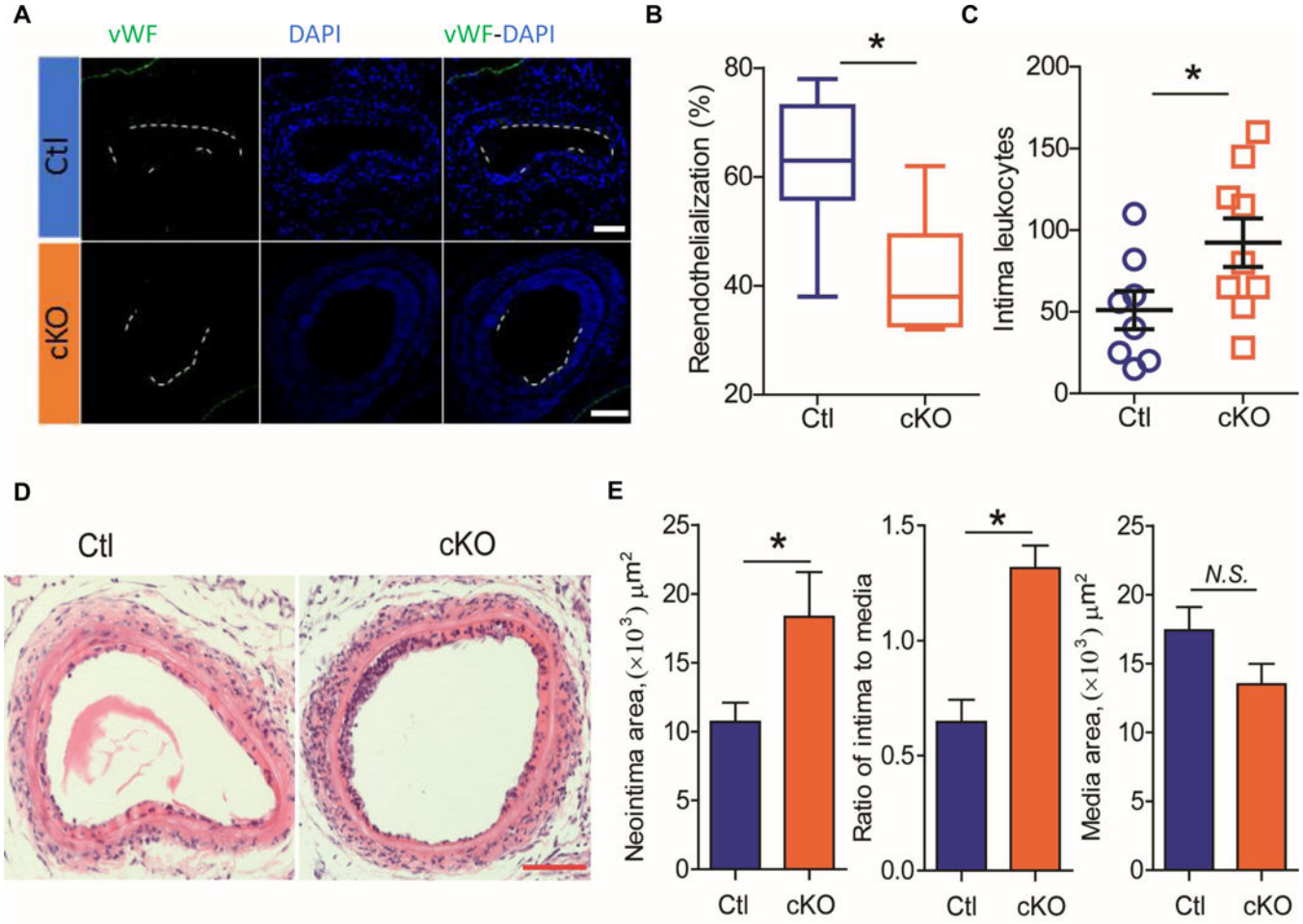

Figure 5. Induced deletion of endothelial EP4 impaired reendothelialization

Injured femoral arteries of Ctl and cKO mice were harvested at 7 days after wire-injury. Endothelial cells were immunostained for vWF (green) and quantified for vascular coverage (reendothelialization) (**A** & **B**, respectively; n= 7 Ctl, 6 cKO). Neointimal formation and leukocyte infiltration were evaluated with H&E staining. The number of neointimal leukocytes was quantified (**C**; n= 8 Ctl, 9 cKO). Representative H&E images are shown (**D**; Bar= 100 μm). Neointimal area, ratio of intima to media and media thickness were statistically quantified (**E**; n= 8). DAPI stains nuclei in blue. *P<0.05; Student's unpaired t-test.

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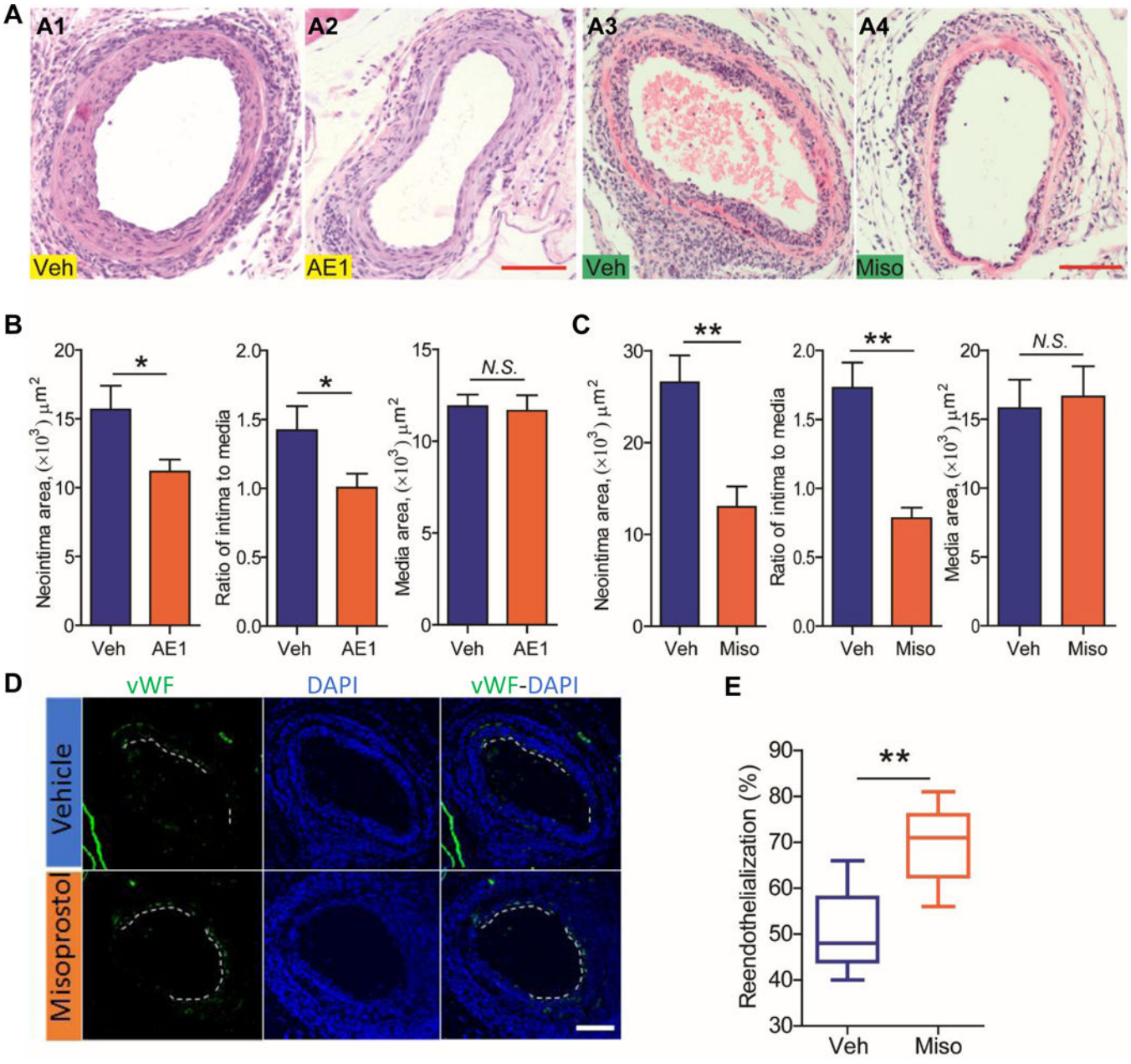

Figure 6. Pharmacological activation of EP4 promoted endothelial repair and protected against neointimal formation

Following vascular injury, C57BL/6 mice were i.p. injected with vehicle (Veh), or with AE1-329 (AE1, an EP4 selective agonist) at a dose of 0.3 mg/kg/day for 28 days (n= 12). Injured vessels were stained with H&E, with representative images shown (**A;** A1, A2). Neointimal area, ratio of intima to media, media thickness was quantified (**B**). Another batch of mice, after vascular injury, were treated via i.p. injection with vehicle (Veh), or with misoprostol (Miso, a PGE analogue) at a dose of 100 μg/Kg, three times a day, for a total of 7 days (n= 7 Veh, 5 Miso). Injured vessels were stained with H&E, with representative images shown (**A;** A3, A4). Neointimal area, ratio of intima to media, media thickness was

quantified (**C**). Reendothelialization was examined by immunostaining of vWF and quantified (**D** & **E**) as detailed previously. *P<0.05, **P<0.01; Student's unpaired t-test.

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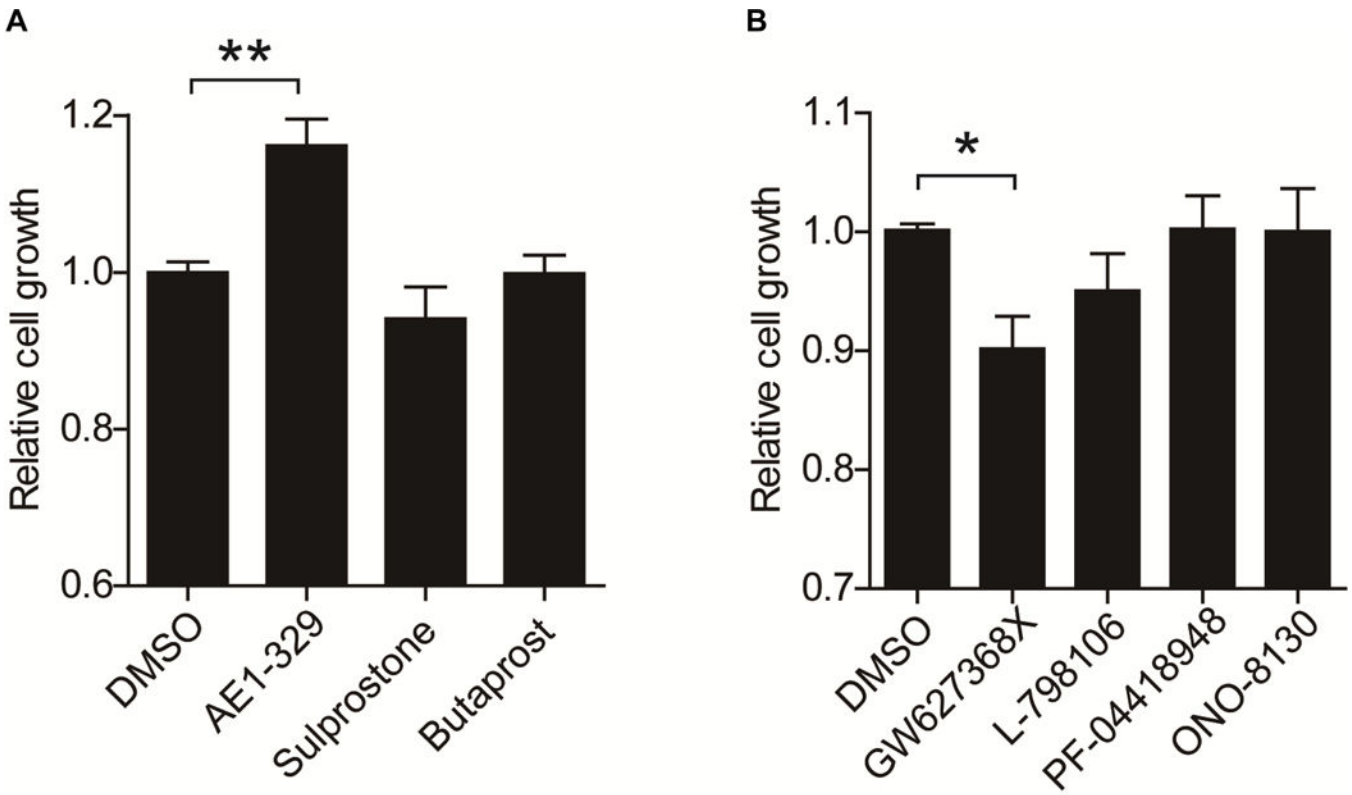

Figure 7. EP4 signaling participated in the proliferation of human endothelial cells

Human microvascular endothelial cells were treated with 1 μmol/L of agonists (**A**) or antagonist (**B**) of the receptors of PGE_2, and relative proliferation was determined. Agonists: AE1-329 (EP4), sulprostone (EP1/3) and butaprost (EP2). Antagonist: GW627368X (EP4), L-798106 (EP3), PF-04418948 (EP2) and ONO-8130 (EP1). Both results were from three independent data sets. *P<0.05, **P<0.01; One-way ANOVA with Dunnett's post-test was used for data comparisons.