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The EP1/EP3 receptor agonist 17-pt-PGE_2 acts as an EP4 receptor agonist on endothelial barrier function and in a model of LPS-induced pulmonary inflammation

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ABSTRACT

Endothelial dysfunction is a hallmark of inflammatory conditions. We recently demonstrated that prostaglandin (PG)E_2 enhances the resistance of pulmonary endothelium in vitro and counteracts lipopolysaccharide (LPS)-induced pulmonary inflammation in vivo via EP4 receptors. The aim of this study was to investigate the role of the EP1/EP3 receptor agonist 17-phenyl-trinor-(pt)-PGE_2 on acute lung inflammation in a mouse model. In LPS-induced pulmonary inflammation in mice, 17-pt-PGE_2 reduced neutrophil infiltration and inhibited vascular leakage. These effects were unaltered by an EP1 antagonist, but reversed by EP4 receptor antagonists. 17-pt-PGE_2 increased the resistance of pulmonary microvascular endothelial cells and prevented thrombin-induced disruption of endothelial junctions. Again, these effects were not mediated via EP1 or EP3 but through activation of the EP4 receptor, as demonstrated by the lack of effect of more selective EP1 and EP3 receptor agonists, prevention of these effects by EP4 antagonists and EP4 receptor knock-down by siRNA. In contrast, the aggregation enhancing effect of 17-pt-PGE_2 in human platelets was mediated via EP3 receptors. Our results demonstrate that 17-pt-PGE_2 enhances the endothelial barrier in vitro on pulmonary microvascular endothelial cells, and accordingly ameliorates the recruitment of neutrophils, via EP4 receptors in vivo. This suggests a beneficial effect of 17-pt-PGE_2 on pulmonary inflammatory diseases.

1. Introduction

The vascular endothelium forms the barrier between blood circulation and the interstitial space and regulates the exchange of plasma components, adhesion and extravasation of leukocytes, and haemostasis [1]. During inflammatory processes endothelial leakage occurs resulting in plasma extravasation and edema formation [2]. The integrity of the endothelial barrier is tightly regulated by cell-to-cell contacts like adherent and tight junctions between adjacent cells and connection to the actin cytoskeleton [1,3,4]. Prostanoids and phospholipids such as sphingosin-1-phosphate are involved in the regulation of endothelial barrier [5,6].

Prostaglandin (PG)E_2 is the most abundant prostanoid in humans [7] and exerts a variety of biological functions through four different receptors (EP1–4), which differ in tissue specific gene expression [8]. These receptors activate different signaling pathways: EP1 receptor binding leads to an increase of intracellular Ca^{2+} levels, assumed of being coupled to a Gαq-protein. EP2 and EP4 receptors induce cyclic AMP (cAMP) production, whereas EP3 receptors couple to a Gαi-protein and inhibit cAMP synthesis [9]. PGE_2 is mainly regarded as a potent pro-inflammatory mediator due to its effects on vasodilation, vascular permeability and nociception [10]. However, the role of PGE_2 in the regulation of immune responses is more complex. Notably the lung represents a privileged organ with regard to PGE_2 actions [11]. In the airways, PGE_2 shows an anti-inflammatory mode of action as it was demonstrated to inhibit the release of a number of cytokines and chemokines via activation of the EP4 receptor [10–12] and inhibition of mast cell-induced bronchoconstriction via the EP2 receptors [13]. However, EP1 and EP3 receptors play a minor role in regulation of inflammatory processes in the lung [12]. PGE_2 was shown to enhance the endothelial barrier function of human pulmonary artery endothelial cells via PKA and Epac/Rap activation leading to Rac activation and cytoskeletal remodeling [6]. We recently revealed that PGE_2 promotes barrier

Abbreviations: 17-pt-PGE_2, 17-phenyl trinor prostaglandin E_2; BAL, bronchoalveolar lavage; EP, E-type prostanoid receptor; HMVEC-L, human pulmonary microvascular endothelial cells; HUVEC, human umbilical vein endothelial cells; LPS, lipopolysaccharide; MMVEC-L, murine pulmonary microvascular endothelial cells.

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function in human pulmonary microvascular endothelial cells (HMVEC-L) via EP4 receptor-induced strengthening of the junction and reduces endothelial trafficking of neutrophils [14]. Moreover, we recently demonstrated that PGE_2, via activation of the EP4 receptor, also shows barrier promoting effects in vivo in a mouse model of lipopolysaccharide (LPS)-induced acute lung inflammation [15].

In this study we investigated the role 17-phenyl tinor (pt)-PGE_2 on endothelial barrier function and the underlying molecular mechanism in HMVEC-Ls as well as in a murine model of LPS-induced acute pulmonary inflammation. We found that 17-pt-PGE_2 concentration-dependently enhanced endothelial barrier function, whereas a more specific EP1 receptor agonist ONO DI-004 [16] or the EP3 receptor agonist sulprostone did not mimic this effect. Surprisingly, the effect induced by 17-pt-PGE_2 was mediated by the EP4 receptor and not by EP1 or EP3 receptors. Furthermore, we show that 17-pt-PGE_2 strengthens the endothelial junctions of HMVEC-Ls and reduces stress fiber formation upon treatment with thrombin. Conversely to our findings in endothelial cells, 17-pt-PGE_2 promotes platelet aggregation via EP3 receptors. In a murine model of acute pulmonary inflammation, 17-pt-PGE_2 caused a decrease in pulmonary extravasation and a reduction of infiltrating neutrophils, which was mediated by EP4 receptor activation.

Our results demonstrate that 17-pt-PGE_2 - in addition to its described effects on EP1 and EP3 receptors - also acts as an EP4 agonist and thereby enhances vascular barrier function.

2. Materials and methods

2.1. Reagents

Laboratory chemicals were from Sigma-Aldrich (Vienna, Austria) unless specified. The EP1 receptor agonist ONO DI-004, the EP4 receptor agonist ONO AE1-329 and the EP4 receptor antagonist ONO AE3-208 were kind gifts from ONO Pharmaceuticals (Osaka, Japan). PGE_2, 17-pt-PGE_2, SC51089, GW627368X, L-161,982, ONO-8711, iloprost, and isobutylmethylxanthine were from Cayman Chemical (Ann Arbor, MI, USA). L-161,798 was purchased from Tocris Biosciences (Bristol, UK) and SC51322 from Biomol (Hamburg, Germany). The VE-cadherin mouse monoclonal antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and secondary fluorescently-labeled antibodies and Texas Red-X Phalloidin were purchased from Invitrogen (Invitrogen, Lofer, Austria). Antibody diluent was from Dako (Glostrup, Denmark), Ultra V Block from Fisher Scientific (Vienna, Austria). Vectashield/DAPI mounting medium was obtained from Vector Laboratories (Vector Laboratories, Burlingam, CA, USA).

2.2. Animals

Male BALB/c mice, 6–8 week old (body weight 22–25 g), were obtained from Charles River (Sulzfeld, Germany). Mice were housed in individually ventilated cages (4 per cage) under controlled conditions of temperature (set point 21 °C), air humidity (set point 50%) and a 12 h light/dark cycle (lights on at 6:00 a.m.) and habituated to the environment for at least one week. Standard chow and water was provided ad libitum. Mice were randomly assigned before treatment. The experimental procedure used in this study was approved by the Austrian Federal Ministry of Science, Research and Economy (BMWF 66.010/032-II/10b/2012 and BMWF 66.010/0094_II/3b/2013) and performed in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and comply with the ARRIVE guidelines [17]. Experiments were performed as humanely as possible to minimize all suffering.

2.3. LPS-induced acute pulmonary inflammation

Pulmonary inflammation was induced as described [15]. Briefly, mice were slightly anaesthetized with ketamine/xylazine (50 mg/5 mg/kg i.p.). LPS (*Escherichia coli* O55:B5; 20 μg per mouse in 50 μL PBS,) or vehicle or LPS in combination with 17-pt-PGE_2 (20 μg per mouse), the EP1 agonist ONO DI-004 (20 μg per mouse) or the respective vehicle were applied intranasally in a volume of 25 μL to each snare. Pretreatment with the EP1 receptor antagonist SC51089 (25 mg/kg) [18] and the EP4 receptor antagonists ONO AE3-208 (10 mg/kg) [19] and GW627368X (10 mg/kg) [20] was performed subcutaneously, 30 min prior to intranasal application. Mice were sacrificed 4 h post intranasal treatment with an overdose of pentobarbital (100 mg/kg i.p), followed by bronchoalveolar lavage (BAL) fluid sampling as previously described [21]. Leukocytes in BAL fluid were analyzed by flow cytometry as described below.

2.4. Leukocyte analysis by flow cytometry

Leukocytes were analyzed as described [15]. Briefly, BAL fluid was centrifuged at 400 ×*g* for 7 min at 4 °C. Following two washing steps with Ca^{2+} and Mg^{2+} free PBS, cells were incubated for 30 min at 4 °C with the following monoclonal antibodies: FITC-conjugated MHC-II PE conjugated CCR3 AB, PE-Cy5.5 conjugated CD3e Ab PE-Cy7 conjugated B220 Ab APC conjugated CD11c Ab and CD16 Block (all Ab from BD Pharmingen). Samples were washed, fixed and measured on a FACSCalibur flow cytometer.

2.5. Pulmonary vascular permeability

Mice were intranasally treated with LPS (20 μg per mouse), vehicle or LPS in combination with 17-pt-PGE_2 (20 μg or 60 μg per mouse) and in additional experiments mice were pretreated with the EP4 antagonist ONO AE3-208 (10 mg/kg) as described above and Evans blue leakage was determined [15]. Briefly, 3 h post treatment mice were injected with Evans blue (60 mg/kg in saline) in the tail vein, 1 h later mice were sacrificed with pentobarbital (100 mg/kg, i.p) and exsanguinated by cutting the abdominal aorta. The lungs were perfused via the right ventricle with 10 mL of phosphate buffered saline (PBS) containing 5 mM EDTA. Thereafter lungs were excised and weighed, then homogenized and incubated with formamide (18 h, 60 °C). After centrifugation (5000 ×*g* for 20 min) the absorbance at 620 nm and 740 nm was measured. The Evans blue content of tissue was calculated and corrected for the presence of heme pigments as described [22].

2.6. Culture of endothelial cells and electrical resistance measurements

HMVEC-L were purchased from Lonza (Verviers, Belgium) as tertiary cultures and were cultivated in EGM-2 MV bullet kit media supplemented with 5% FCS and cultivated as previously described [14]. For resistance measurements cells were grown to confluence on 1% gelatin-coated polycarbonate biochips with gold microelectrodes (ECIS 8W10E+) for 48 h. Cells were serum starved for 1 h and endothelial resistance was measured with an Electrical Cell-substrate Impedance Sensing System (ECIS; Applied Biophysics, Troy, NY, USA), as described [14]. 3–4 independent experiments were performed in duplicates.

2.7. Transfection of endothelial cells and real time RT-PCR

HMVEC-L were transfected with Lipofectamine RNAiMAX (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Briefly, HMVEC-L were seeded in 6-well plates and transfected when reaching 50–70% confluence with PTGER4 FlexiTube – GeneSolution siRNA or a non-targeting control siRNA (Qiagen, Hilden, Germany). Knock-down of PTGER4 was achieved by application of 50 nM of specific PTGER4 siRNA. For relative quantification of mRNA real time PCR was performed (CDX Connect™ Real-Time PCR detection system with CFX Manager™ software 3.1 (Bio-Rad, Hercules, CA, USA)). 48 h after transfection, RNA isolation was performed using RNA easy kit (Qiagen) and DNA removal was performed with Ambion DNA removal