E-type prostanoid receptor 1 (EP1) agonist, a novel regulator of endothelial barrier function

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STATUTORY DECLARATION

I declare that I have authored this thesis independently, that I have not used other than the declared sources / resources, and that I have explicitly marked all material which has been quoted either literally or by content from the used sources.

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Abstract

Endothelial cells build a strict barrier between blood flow and interstitium. Dysfunction of the endothelial barrier leads to increased vascular permeability and edema formation. Agents, which are involved in inflammatory processes, such as prostaglandin E_2 (PGE_2) might counteract the vascular leakage and reduce tissue damage. PGE_2, the most abundant prostanoid in humans, is released by various cell types including endothelial cells, alveolar macrophages and lung fibroblasts. PGE_2 acts through four different E-type prostanoid receptors (EP1-4), which are G-protein coupled receptors. It was recently shown that PGE_2 exerts enhancing effects on the endothelial barrier function of human microvascular endothelial cells. This work aimed to investigate the role of the EP1 receptor agonist 17-phenyl trinor (pt)-PGE_2 on the endothelial barrier function and the underlying molecular mechanism.

Expression of EP receptors on human lung microvascular endothelial cells (HMVEC-L) and human pulmonary artery endothelial cells (HPAEC) was determined using flow cytometry. The cellular localization of EP1 receptor was investigated by immune fluorescence staining. As functional readout of the EP receptor expression in endothelial cells, intracellular Ca^{2+} release was determined using selective EP1-4 receptor agonists and PGE_2. In order to prove involvement of certain EP receptors, selective EP receptor antagonists (EP1: SC-51089, SC-51322 and ONO-8711; EP4: ONO AE3-208, GW 627368X and L-161,982) and non-selective EP1-2-3/DP antagonist (AH 6809) were applied. Moreover, selective Gαi- and Gαq-protein inhibitors (Pertussis toxin and MH-362-63-8) were used to identify the activated signaling pathway. In order to determine the origin of the 17-pt-PGE_2 induced Ca^{2+} elevation, the Ca^{2+} chelator EGTA was utilized. Furthermore, the EP1 receptor agonist ONO-DI-004 and the IP/EP1 receptor agonist iloprost were applied to investigate whether they could mimic the effect of 17-pt-PGE_2 on HMVEC-Ls. Endothelial electrical resistance indicating the endothelial barrier function was performed by using an ECIS device. Ca^{2+} chelators (EGTA, EGTA-AM and BAPTA-AM) were used to determine the role of Ca^{2+} elevation in the endothelial barrier function. Moreover, we assessed the regulatory role of 17-phenyl trinor-PGE_2 on VE-cadherin expression in the endothelial junctions and F-actin polymerization by using immunofluorescence microscopy.

We could show that EP1, EP3 and EP4 receptors are expressed on the pulmonary microvascular and macrovascular endothelial cells. Furthermore, EP2 receptors are

expressed on HMVEC-Ls, whereas HPAECs show small amounts of EP2 receptor expression. In microvascular endothelial cells, EP1 receptor is localized in the cytoplasm and the perinuclear space and the selective EP1 agonist 17-pt-PGE_2 significantly elevated the intracellular Ca^{2+} levels. PGE_2 and EP4 receptor agonist CAY10598 induced a moderate Ca^{2+} signal, whereas EP2 agonist butaprost and EP3/EP1 receptor agonist sulprostone had no significant impact on intracellular Ca^{2+} release. In contrast, pulmonary artery endothelial cells did not show a significant increase of intracellular Ca^{2+} levels after stimulation with specific EP1-4 agonists or PGE_2. In microvascular endothelial cells, 17-pt-PGE_2 concentration-dependently increased the endothelial electrical resistance. Interestingly, the 17-pt-PGE_2 induced Ca^{2+} release as well as the endothelial barrier enhancement could not be inhibited by selective EP1 receptor antagonists but by EP4 receptor antagonists. However, another EP1 agonist ONO DI-004 did not cause intracellular Ca^{2+} release or endothelial barrier promotion. Regarding the G-protein coupling of the activated EP receptor, Gαi- and Gαq-protein inhibitors markedly reduced the Ca^{2+} signal stimulated by 17-pt-PGE_2.

These data show that the effects of 17-pt-PGE_2 exerted on the pulmonary microvascular endothelial cells were mediated by EP4 and not by EP1 receptors; however, the endothelial barrier enhancement was independent of the intracellular Ca^{2+} mobilization. Additionally, 17-pt-PGE_2 treatment enhanced the expression of VE-cadherin in the endothelial junctions and the peripheral actin-ring formation, while the thrombin-induced disintegration of endothelial monolayers was markedly reversed by 17-pt-PGE_2.

Since endothelial dysfunction is a hallmark of various pathological processes including inflammation, sepsis and acute lung injury the EP1 agonist 17-pt-PGE_2 as a hypothesized biased ligand of EP4 receptor might be a promising target for novel therapeutic approaches.

Zusammenfassung

Endothelzellen bilden eine dichte Barriere zwischen dem Blutstrom und dem Interstitium. Endotheliale Dysfunktion führt zu einer erhöhten Permiabilität der Blutgefäße und kann in der Bildung von Ödemen resultieren. Substanzen, wie Prostaglandin E_2 (PGE_2), die eine Rolle in inflammatorischen Prozessen spielen, könnten der endothelialen Dysfunktion entgegenwirken und dadurch den Gewebeschaden reduzieren. PGE_2 ist das häufigste Prostaglandin im menschlichen Körper und wird von vielen verschiedenen Zelltypen, wie Endothelzellen, Alveorlarmakrophagen und Fibroblasten aus der Lunge gebildet. PGE_2 bindet und aktiviert vier verschiedene Rezeptoren (EP1-4), die zu der Familie der G-Protein gekoppelten Rezeptoren gehören. Es würde kürzlich gezeigt, dass PGE_2 die endotheliale Barriere Funktion erhöhen kann. Diese Arbeit hatte das Ziel, den Einfluss des EP1 Rezeptor Agonisten 17-phenyl trinor (pt)-PGE_2 auf die endotheliale Barriere Funktion zu untersuchen und den zugrundeliegenden Signaltransduktionsweg zu bestimmen.

Die Expression der EP1-4 Rezeptoren auf pulmonalen, humanen, mikrovaskulären Endothelzellen (HMVEC-L) und Endothelzellen aus der Arteria pulmonalis wurden mittels Durchflusszytometrie bestimmt. Außerdem wurde die Lokalisierung des EP1 Rezeptors durch Immunofluoreszenzfärbung bestimmt. Als funktionelle Studie der EP Rezeptor Expression wurde die intrazelluläre Ca^{2+} Freisetzung unter Einfluss von selektiven EP1-4 Rezeptor Agonisten und PGE_2 bestimmt. Spezifische EP Rezeptor Antagonisten (EP1: SC-51089, SC-51322 und ONO-8711; EP4: ONO AE3-208, GW 627368X and L-161,982), sowie der unspezifische EP1-2-3/DP Antagonist (AH-6809) sollten Aufschluss über die Beteiligung der EP Rezeptor an der intrazellulären Ca^{2+} Freisetzung geben. Außerdem sollten die Gαi- und Gαq-Protein Inhibitoren Pertussis-Toxin und MH-362-63-8 Auskunft über den beteiligten Signaltransduktionsweg geben. Der Ca^{2+} Chelator EGTA wurde verwendet um die Herkunft der 17-pt-PGE_2 induzierten Ca^{2+} Freisetzung zu bestimmen. Darüber hinaus sollte festgestellt werden, ob der EP1 Agonist ONO_DI-004 und der IP/EP1 Rezeptor Agonist denselben Effekt auf HMVEC-Ls aufweisen wie 17-pt-PGE_2. Die endotheliale Barriere Funktion, die durch den endothelialen Widerstand angegeben wird, wurde mittels einem ECIS Gerät bestimmt. Um den Einfluss der Freisetzung von Ca^{2+} auf die endotheliale Barriere Funktion zu ermitteln, wurden Ca^{2+} Chelatoren (EGTA, EGTA-AM und BAPTA-AM) verwendet. Außerdem wollten wir den Einfluss von 17-pt-PGE_2 auf die Expression von VE-Cadherin

in den endothelialen Adhäsionsverbindungen und weiters auf die Polymerisierung von F-Aktin mittels Immunofluoreszenz-Mikroskopie bestimmen.

Wir konnten zeigen, dass EP1, EP3 und EP4 Rezeptoren sowohl auf HMVEC-L, als auch auf HPAEC exprimiert werden. Außerdem werden EP2 Rezeptoren auf HMVEC-Ls exprimiert, wohingegen HPAECs nur geringe Expression von EP2 Rezeptoren aufwiesen. Der EP1 Rezeptor ist im Zytoplasma und im perinuklären Raum lokalisiert. PGE_2 und 17-phenyl trinor-PGE_2 erhöhten signifikant die intrazelluläre Ca^{2+} Freisetzung in HMVEC-L. Der EP4 Rezeptor Agonist CAY10598 erhöhte den intrzelluläre Ca^{2+} Spiegel, wohingegen der EP2 Rezeptor-Agonist Butprost und der EP2 Rezeptor-Agonist Sulproston keinen Einfluss hatten. In HPAEC hingegen konnte kein signifikanter Anstieg des Ca^{2+} Spiegels nach der Behandlung mit spezifischen EP1-4 Rezeptor-Agonisten detektiert werden. Weiters konnten wir zeigen, dass 17-pt-PGE_2 die endotheliale Barriere Funktion konzentrationsabhängig erhöht. Interessanterweise konnte weder die durch 17-pt-PGE_2 induzierte intrazelluläre Ca^{2+} Freisetzung, noch die Erhöhung der endothelialen Barriere-Funktion durch selektive EP1 Rezeptor-Antagonisten inhibiert werden, wohingegen spezifische EP4 Rezeptor-Antagonisten beide Effekte hemmten. Zusätzlich konnten die Gαi- und Gαq-Protein-Inhibitoren Pertussis-Toxin und MH-362-63-8 den intrazellulären Ca^{2+} Einstrom merklich reduzieren. Diese Ergebnisse lassen darauf schließen, dass die von 17-pt-PGE_2 induzierten Effekte auf Endothelzellen eher durch EP4 Rezeptoren als durch EP1 Rezeptoren vermittelt werden. Die Erhöhung der endothelialen Barriere-Funktion durch 17-pt-PGE_2 war unabhängig vom intrazellulären Ca^{2+} Einstrom. Außerdem konnten wir zeigen, dass die Behandlung von konfluenten Endothelmonolayern mit Thrombin zur Ausbildung von Actin-Stressfasern und weiters zur Bildung von parazellulären Lücken zwischen den benachbarten Zellen führte. Dieser Effekt konnte durch 17-pt-PGE_2 aufgehoben werden.

Nachdem verschiedene pathologische Prozesse wie Entzündung, Sepsis und akutes progressives Lungenversagen durch eine endotheliale Dysfunktion charakterisiert sind, könnte sich 17-pt-PGE_2 als potenzieller EP4 Rezeptorligand als vielversprechendes Ziel für neue Behandlungsmethoden herausstellen.

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Abbreviations

17-pt-PGE_2	17-phenyl trinor-PGE_2
Ab	antibody
AC	adenylyl cylcase
AJ	adherens junctions
ATP	adenosine triphosphate
BALF	broncho-alveolar lavage fluid
BAPTA-AM	1,2-bis(o-Aminophenoxy)ethane-N,N,N′,N′-tetraacetic Acid Tetra(acetoxymethyl) Ester
Ca^{2+}	Calcium ion
cAMP	cyclic adenosine monophosphate
Cdc42	cell division control protein 42 homolog
COX	cyclooxygenase
CREB	cAMP response element binding protein
DAG	diacylglycerol
DAPI	4',6-diamidino-2-phenylindole
DP	D-type prostanoid receptor
DSS	dextran sulfate sodium
EBM-2	endothelial basal medium-2
ECIS	Electric Cell-substrate Impedance Sensing System
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol tetraacetic acid
EGTA-AM	Ethyleneglycol-bis(β-aminoethyl)-N,N,N′,N′-tetraacetoxymethyl ester
EP1-4	E-type prostanoid receptor 1-4
EPRAP	EP4 receptor-associated protein
FACS	fluorescence activated cell sorting
F-actin	filamentous actin
FLEX	Fluid-Excitation
H1	histamine receptor 1
HBSS	HEPES buffered saline solution
HIF-1α	hypoxia-inducible factor-1α
HMVEC-L	human lung microvascular endothelial cell
HPAEC	human pulmonary artery endothelial cell
hsp27	heatshock protein 27
IFN-γ	Interferon-γ

IL-12	Interleukin 12
IP	I-type prostanoid receptor
IP_3	inositol 1,4,5-trisphosphate
JAM	junctional adhesion molecules
LPS	lipopolysaccharide
MDRP-4	multidrug resistance protein-4
MEK	mitogen-activated protein kinase
MFI	mean fluorescence intensity
MHC II	major histocompatibility complex class II molecules
mPGES	microsomal PGE synthase
mRNA	messenger RNA
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
Nurr1	nuclear receptor related 1 protein
PBS	phosphate buffered saline
PGE_2	prostaglandin E2
PGI_2	prostaglandin I_2
PI3K	phosphatidylinositol 3-kinase
PIP_2	phosphatidylinositol 4,5-bisphosphate
PKA	protein kinase A
PLA_2	phospholipase A_2
PLC	phosopholipase C
Rac1	Ras-related C3 botulinum toxin substrate 1
RFU	relative fluorescent unit
Rho	Ras homology
RhoA	Ras homolog gene family, member A
ROI	region of interest
S1P	sphingosine-1-phosphate
S1PR1-5	sphingosin-1-phosphate receptor 1-5
TNF-α	Tumor necrosis factor-α
TP	thromboxane receptor
VE-cadherin	vascular endothelial cadherin
VEGF	vascular endothelial growth factors

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1 Introduction

1.1 Prostaglandins

Prostaglandins are vasoactive substances, which are derived from arachidonic acid via the cyclooxygenase pathway. They are synthesized in various tissues, for instance by vascular endothelial cells, alveolar macrophages, and lung fibroblasts (Birukova et al., 2007; Li et al., 2011). Arachidonic acid is released by phospholipase A_2 (PLA_2) out of membrane phospholipids and then presented to cyclooxygenase (COX) enzymes, which convert it into PGH_2 (Funk, 2001; Alfranca et al., 2006; Legler et al., 2010). Two isoforms of COX enzymes are known: under normal physiological conditions prostanoids are synthesized by the constitutively expressed COX-1 enzyme, which maintains basal levels of prostaglandins (Funk, 2001). The expression of the second isoform COX-2 is induced under cellular stress, such as inflammation, shear stress, hypoxia and mechanical stress. Moreover, cytokines, angiotensin II, certain growth factors and endothelin-1 can induce the release of prostaglandin E_2 (PGE_2) and prostaglandin I_2 (PGI_2) (Norel 2007). PGE_2 is the most abundant prostanoid in humans. It is synthesized by cytosolic or by membrane-associated microsomal PGE synthase (mPGES) out of prostaglandin H_2 (PGH_2) (Samuelsson et al., 2007). mPGES-1 is constitutively expressed and couples to COX-1 enzyme. Correspondingly to COX-2 enzyme, mPGES-2 expression is induced by cytokines and growth factors (Legler et al., 2010). Finally synthesized, PGE_2 is actively transported across the cell membrane by the adenosine triphosphate (ATP)-dependent multidrug resistance protein-4 (MDRP-4) and acts as an autocrine or paracrine substance (Legler et al., 2010). PGE_2 is broadly involved in different physiological processes including regulation of metabolism, immune and neuronal functions (Woodward et al., 2011). Besides its classical pro-inflammatory character PGE_2 also shows anti-inflammatory functions (Tang et al., 2012). PGE_2 exerts these variety of biological functions via four different G-protein coupled receptors, depending on the amount of PGE_2, which is available and furthermore on the gene expression patterns of EP receptors in a distinct tissue (Legler et al., 2010).

1.2 Prostaglandin E_2 receptors

PGE_2 acts in autocrine or paracrine fashion by binding to transmembrane G-protein coupled receptors. Four E-type prostanoid receptor subtypes (EP1-4) are known for PGE_2 (Norel, 2007). E-type prostanoid receptors are classified into three groups,

depending on the activated G-protein. Activation of EP1 receptor leads to increase of intracellular Ca^{2+} level, assumed of being coupled to a Gαq-protein. EP2 and EP4 receptors are coupled to Gαs protein, which leads to production of cyclic adenosine monophosphate (cAMP) and further to the activation of protein kinase A (PKA). There are several isoforms of EP3 receptors known, which can be generated by mRNA splicing. EP3 receptor couples to a Gαi-protein and inhibits cAMP synthesis (Alfranca et al., 2006).

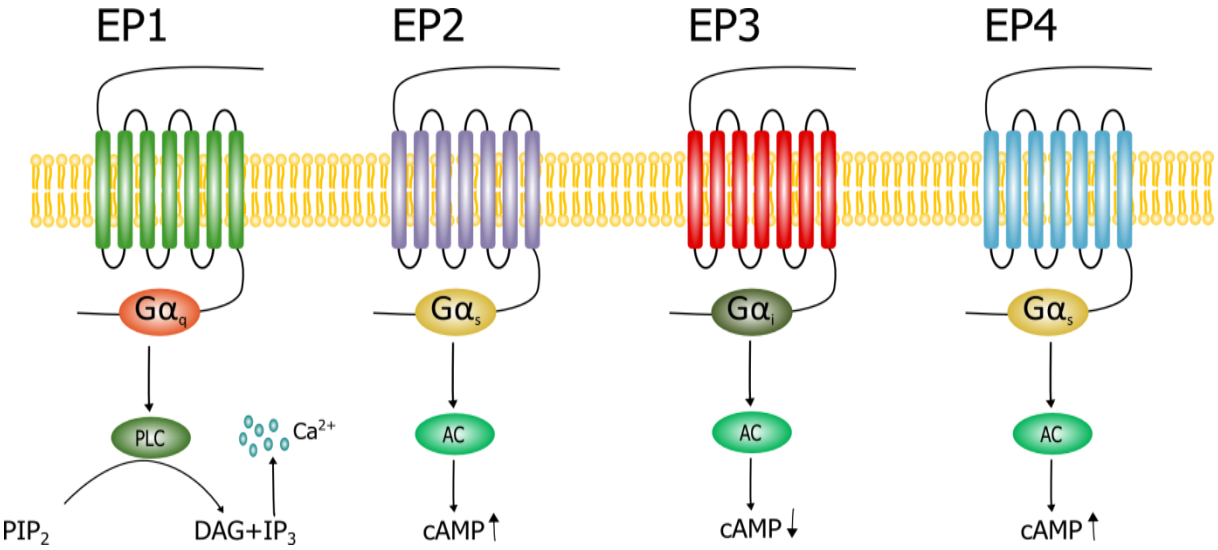

Figure 1. PGE_2 signaling through E-type prostanoid (EP) receptors. *Prostanoid receptors are G-protein coupled transmembrane receptors. PGE_2 activates 4 different E-type prostanoid receptors (EP1-4). EP1 is coupled to a Gαq-protein. Receptor binding activates the phosopholipase C (PLC)/diacylglycerol (DAG) pathway and is characterized by an increase of intracellular Ca^{2+} levels. EP2 and EP4 are both coupled to Gαs. Receptor binding leads to an activation of adenylyl cylcase (AC) and results in increase of cAMP levels. In contrast, EP3 receptor is coupled to Gαi-protein and inhibits AC.*

1.2.1 EP1 receptor

It is generally assumed that the EP1 receptor is coupled to a Gαq-protein which triggers the release of Ca^{2+} from the endoplasmic reticulum via the PLC/inositol 1,4,5-trisphosphate (IP_3) pathway and further activation of protein kinase C (PKC) (Alfranca et al., 2006). However, linkage of EP1 receptor to Gαq-protein has been questioned, since receptor activation leads only to a slight increase of IP_3, which cannot be responsible for the strong intracellular Ca^{2+} release (Watabe et al., 1993). Therefore it

remains controversial, which type of G-proteins couples to EP1 receptors. It was shown that PGE_2 can promote the bone formation in rat osteoblasts via the EP1/PLC/PKC signaling pathway, which indicates coupling to Gαq-proteins (Tang, 2005). In contrast, EP1 receptor induced upregulation of hypoxia-inducible factor-1α (HIF-1α) seems to be mediated by a pertussis toxin-sensitive G-protein, indicating Gαi-protein coupling (Ji et al., 2010). Moreover it was shown, that EP1 receptor activation is able to upregulate the orphan receptor nuclear receptor related 1 protein (Nurr1) via cAMP independent activation of PKA, cAMP response element binding protein (CREB) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (Ji et al., 2012).

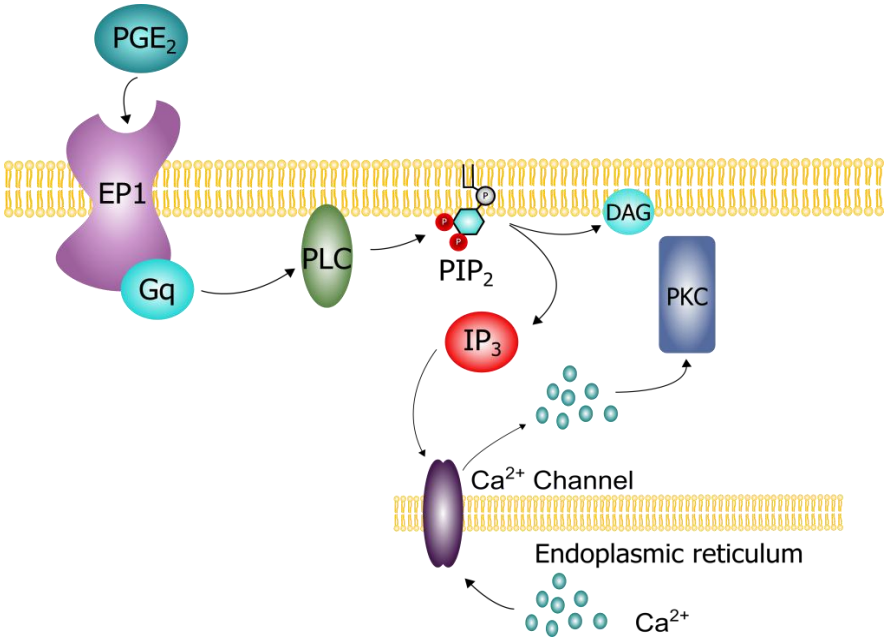

Figure 2. EP1 receptor signaling. *Stimulation of EP1 receptor, which is coupled to Gαq-protein leads to the activation of PLC. IP_3 and DAG are released from phosphatidylinositol 4,5-bisphosphate (PIP_2) PLC. IP_3 binds to Ca^{2+} channels in the endoplasmic reticulum membrane and triggers the release of Ca^{2+} into the cytosol. Further on Ca^{2+} activates PKC.*

In humans, EP1 receptors are expressed on keratinocytes, in the myometrium, mast cells, longitudinal muscle cell layer of the colon, in the pulmonary vein and in micro- and macrovascular pulmonary endothelial cells (Woodward et al., 2011; Konya et al., 2012). EP1 receptor seems to be involved in various processes in the body including cardiovascular homeostasis, regulation of neuronal functions, inflammation and is also involved in neoplasia(Audoly et al., 1999; Matsuoka et al., 2005; Jones et al., 2009; Konger et al., 2009). Deletion studies of EP1 receptor revealed that the resting systolic blood pressure was markedly reduced in these animals (Stock et al., 2001).

Mice which lacked of EP1 receptors showed social dysfunction and stress-induced impulsive aggression, indicating that the EP1 receptor has major function in the central nervous system (Matsuoka et al., 2005). As mentioned above, the EP1 receptor shows pro-tumorigenic properties in colon and skin cancer (Watanabe et al., 2000; Rundhaug et al., 2011). Inhibition of EP1 receptor by the selective antagonist ONO-8711 markedly reduced the formation of polyps and inhibited the formation of colon crypts (Watanabe et al., 1999). Correspondingly to these results, in an azoxymethane induced colon cancer model, the EP1 knock-out mice showed reduced formation of aberrant crypt foci (Watanabe et al., 2000). In addition, EP1 receptors are expressed in the epidermis and furthermore seem to be involved in the malignant expansion of keratinocytes (Woodward et al., 2011).

EP1 receptor agonists and antagonists

The early characterization of EP1 receptor has been achieved by the development of EP1 receptor agonist and antagonists (Woodward et al., 2011).

The substance 17-phenyl trinor-PGE_2 exerts moderate agonistic functions on EP1 and EP3 receptors (Lawrence et al., 1992). ONO-DI-004, which was developed by Ono Pharmaceuticals, seems to be a more specific EP1 receptor agonist (Okada et al., 2000; Suzawa et al., 2000). Interestingly, the prostaglandin I_2 analogs carbacyclin and iloprost also show partial EP1 receptor agonism (Lawrence et al., 1992; Woodward et al., 2011).

EP1 receptor antagonists are diverse in structure and selectivity. Some of them belong to the group of prostanoids, but none of them has a similar structure to PGE_2 (Woodward et al., 2011). The first EP1 antagonist was SC-51220, structurally characterized as a dibenzoxazepine hydrazide (Woodward et al., 2011). Despite its low affinity, it contributed substantially to the early pharmacological studies of EP receptors (Woodward et al., 2011). Two further members of this series are SC-51089 and SC-51322 (Hallinan et al., 2001; Jones et al., 2009). Another commonly used EP1 receptor antagonist is AH 6809 (Lawrence et al., 1992). Depending on the used concentration, AH 6809 shows inhibition of EP1,2 and 3 receptor and moreover DP receptors. The antagonist ONO-8711, developed by Ono Pharmaceuticals, exerts EP1/EP3 antagonism (Jones et al., 2009). The non-prostanoid antagonists ONO-8713 shows high affinity for EP1 receptors (Watanabe et al., 2000). Further EP1 receptor antagonists were developed by Merck-Frosst (Jones et al., 2009).

1.2.2 17-phenyl trinor (pt)-PGE_2

Besides ONO-DI-004, 17-pt-PGE_2 is frequently used as an EP1 receptor specific ligand (Takeuchi et al., 2001; Rutkai et al., 2009; Woodward et al., 2011; Liu et al., 2012). Because of its moderate selectivity for the EP1 receptor, it has been designated to be the prototype for more potent EP1 receptor agonists (Lawrence et al., 1992).

17-phenyl trinor-PGE_2 and PGE_2 were shown to be able to induce an intracellular Ca^{2+} release in cultured rat mircroglia cells, whereas the EP3 recpetor agonist sulprostone had no impact on intracellular Ca^{2+} levels (Caggiano and Kraig, 1999). Moreover, it was shown that 17-pt-PGE_2 can enhance the constriction of arterioles in diabetic mice. This effect was abolished by AH 6809 (Rutkai et al., 2009). In rat hypoxia treated cortical neurons, 17-pt-PGE_2 was able to elevated expression of caspase-3, which indicates an involvement of 17-pt-PGE_2 in the regulation of apoptosis. This effect was significantly reduced by the EP1 receptor antagonist SC-51322 (Liu et al., 2012). Moreover, it was shown that, 17-phenyl trinor-PGE_2 is able to prevent from hydrochloric acid/ethanol induced gastric mucosa disruption, which could be inhibited by EP1 receptor antagonist ONO-AE-829 in rats (Takeuchi et al., 2001). Additionally, ligand binding assays have revealed that 17-pt-PGE_2 exerts affinity for the EP4 receptor (Davis and Sharif, 2000) and furthermore is able to activate a pertussis-toxin sensitive G-protein via the EP4 receptor, which might indicate 17-pt-PGE_2 as biased ligand for the EP4 receptor (Leduc et al., 2009).

1.2.3 EP4 receptor

The EP4 receptor is generally described of being a Gαs-protein coupled receptor. EP4 receptor binding leads to transient increase of cAMP and the subsequent activation of PKA and further the phosphorylation of CREB (Tang et al., 2012). Interestingly it was shown that activation of EP4 leads to significantly less cAMP compared to EP2 receptor (Fujino, 2005a). This can be explained by the fact that EP4 receptors also exert other signaling properties (Woodward et al., 2011). It was shown that stimulation of the EP4 receptor can result in activation of phosphatidylinositol 3-kinase (PI3K) and further activation of Akt, which might be mediated by the Pertussis-toxin sensitive Gαi-protein (Fujino, 2005a, 2005b). The selective activation of more than one signaling pathway by a distinct GPCR agonist has been referred to as functional selectivity (Galandrin et al., 2007). This model has also been verified for natural and synthetic prostaglandin EP4 receptor agonists (Leduc et al., 2009). This study characterized the properties of EP4

receptor agonist to activate other signaling pathways besides the classical activation of Gαs-protein (Leduc et al., 2009).Moreover, EP4 initiates anti-inflammatory signaling through the EP4 receptor-associated protein (EPRAP). EPRAP possesses binding sites at the long, cytoplasmic C-terminal domain of the EP4 receptor and inhibits NF-κB and the mitogen-activated protein kinase (MEK), resulting in inhibition of macrophage activation and, furthermore, in the prevention of immature B-cell expansion (Takayama, 2006; Minami et al., 2008; Prijatelj et al., 2012).

The EP4 receptor exerts various biological features including pro- and anti-inflammatory functions, it is involved in the intestinal homeostasis and also in pathological states like arteriosclerosis and cardiovascular diseases (Tang et al., 2012). It was shown that the anti-inflammatory properties of PGE_2 are majorly mediated by EP4 receptors. In human macrophages PGE_2 inhibits the expression of pro-inflammatory cytokines (TNF-α, IL-12, IFN-γ) and chemokines via EP4 receptors (Tang et al., 2012). Moreover, EP4 receptor signaling impairs the expression of major histocompatibility complex class II molecules (MHC II) and thereby reduces antigen presenting properties of macrophages (Takayama et al., 2002). Interestingly, PGE_2 could not inhibit lipopolysaccharide (LPS) induced upregulation of pro-inflammatory cytokines in EP4-deficient mouse macrophages (Minami et al., 2008). Furthermore, EP4 receptor impairs the differentiation of $CD4^+$ T-helper cells and the activation and expansion of $CD8^+$ cytotoxic T-cells (Tang et al., 2012). Moreover, EP4 receptors suppress the production of TNF-α in mouse neutrophils (Yamane et al., 2000). Beside its anti-inflammatory functions, EP4 might induce the immune response. PGE_2 promotes the proliferation of Th1 and the IL-23-induced expansion of Th17 cells (Yao et al., 2009). Corresponding to these *in vitro* data, it was shown in a contact hypersensitivity mouse model that a selective EP4 receptor antagonist ONO AE-208 could inhibit the recruitment of Th1 and Th17 cells into the regional lymph node (Yao et al., 2009).

Moreover, EP4 receptors are involved in maintenance of intestinal homeostasis (Tang et al., 2012). EP4 receptor knock-out mice showed severe colitis after application of 3% dextran sulfate sodium (DSS), a dose which induced mild colitis in wild-type mice (Kabashima et al., 2002). Wild type mice treated with the EP4 receptor antagonist ONO AE3-208 developed severe colitis after exposure to 3% DSS (Kabashima et al., 2002). Moreover, the EP4 receptor promotes the survival of mucous epithelial cells and thereby augments ulcer healing (Jiang et al., 2009).

EP4 receptors are likewise involved in cardiovascular disease (Tang et al., 2012). EP4 receptor activation reduces the ischemic damage in reperfused rat myocardium (Hishikari et al., 2008). Treatment with an EP4 receptor agonist reduces the size of the infarct. This cardioprotective effect is mediated by impairing the recruitment of inflammatory cells, majorly macrophages, to the infarcted side and by reducing the production of pro-inflammatory cytokines and reactive oxygen species (Tang et al., 2012).

EP4 receptor activation promotes angiogenesis and endothelial cell migration (Woodward et al., 2011). It was recently shown that PGE_2 can promote the endothelial barrier function via the EP4 receptor. This effect was mimicked by an EP4 receptor agonist and abolished by an EP4 receptor antagonist. This barrier promoting effect was independent of the classical cAMP/PKA pathway. Moreover, EP4 receptor activation impaired the adhesion of neutrophils on endothelial cells, which could account for the reduced expression of E-selectin on EP4 receptor agonist pre-treated endothelial cells (Konya et al., 2012).

EP4 receptor agonists and antagonists

As mention earlier, selective agonists as well as their counterparts, selective antagonists have contributed excessively to the better understanding of receptors (Jones et al., 2009).

EP4 receptor agonists are characterized by containing a 16-phenyl group (Woodward et al., 2011). ONO-AE1-329 is a full EP4 receptor agonist (Cao et al., 2002). Further receptor agonists are L-902688, CP-734432 and compound 12 (Woodward et al., 2011).

The first developed EP4 receptor antagonist was AH 23848 (Coleman et al., 1994). However, AH-23848 possesses low selectivity for EP receptors and was the pharmacophore for more potent substances like L-161,982 and GW-627368 (Jones et al., 2009).

1.3 Endothelial barrier function

Endothelial cells build the inner lining of the vasculature. They control the passage of plasma components into the interstitial space of surrounding tissues. The endothelial barrier function is affected in several pathological states, including inflammation, sepsis and ischemia (Dejana et al., 2008).

Endothelial dysfunction might lead to edema formation and to an increase of the interstitial pressure (Dejana et al., 2009). The integrity of the endothelial barrier function is partially controlled by the coordinated opening and closure of the endothelial adherens junctions (AJ) resulting in paracellular exchange of fluid and substrates through the endothelium (Weis and Nelson, 2006; Dejana et al., 2008). Besides AJ, tight junctions are involved in the formation of cell-cell contacts (Dejana and Giampietro, 2012). Transcellular pathways are characterized by the transport either through fenestrated endothelium or engaging a laborious complex of transport vesicles (Yuan and Rigor, 2010). In contrast to that, the paracellular passage involves the rearrangement of adhesion proteins and the cytoskeleton and is regulated by the dynamic opening and closure of endothelial junction molecules. To maintain this sophisticated system several control mechanism are required to avoid thrombus formation for instance. Substances, which might introduce vascular permeability transiently, include histamine, thrombin and vascular endothelial growth factors (VEGF) (Dejana et al., 2008) .

1.3.1 Adherens junctions

In endothelial cells, adherens junctions contain the vasculature specific vascular endothelial (VE-) cadherin. Moreover, endothelial cells express N-cadherin, but it plays a minor role in the formation of adherent junctions (Giampietro et al., 2012). It is thought to be important for the anchorage of endothelial cells to mesenchymal stem cells. VE-cadherin is associated with various intracellular proteins, which mediate the anchorage to the actin cytoskeleton (Weis and Nelson, 2006). VE-cadherin molecules form dimers, and each molecule consists of five homologous extracellular domains. The intracellular domain is associated with intracellular binding proteins, including p120, plakoglobin and β-catenin (Dejana et al., 2008). Plakoglobin and β-catenin are directly connected to α-catenin, which mediates the contact to actin filaments. Interaction of VE-cadherin is essential for the integrity of the adherent junctions and further allows their dynamical opening and closure (Dejana et al., 2008).

1.3.2 Tight junctions

The second group of junctions, which are involved in regulation of cell-cell contacts are tight junctions. Tight junctions consist of different proteins than adherens junctions. Major component is the claudin family. In endothelial cells tight junctions are formed by the endothelial specific claudin-5. Further proteins, which compose tight junctions,

include occluding, nectins and junctional adhesion molecules (JAM) (Dejana and Giampietro, 2012).

1.3.3 Actin cytoskeleton

The actin cytoskeleton is responsible for the dynamic opening and closure of the endothelial junctions. Inactivated endothelial monolayers are characterized by a prominent cortical actin ring and furthermore, lack of stress fibers. In contrast to that, the activated monolayers appear with a thin cortical actin ring and abundant stress fiber formation. The transition between these two states is regulated by the rearrangement of actin filaments (Bogatcheva and Verin, 2008).

The rearrangement of the actin cytoskeleton is regulated by various proteins, such as gelsolin, cofilin and heatshock protein 27 (hsp27). Moreover, the small Ras homology (Rho) familiy GTPases ras-related C3 botulinum toxin substrate 1 (Rac1), Ras homolog gene family, member A (RhoA) and cell division control protein 42 homolog (Cdc42) are involved in the regulation of the actin skeleton. Furthermore, the actin-mysion machinery is strongly linked with the regulation of the actin cytoskeleton (Yuan and Rigor, 2010).

1.3.4 Barrier promoting factors

The integrity of the endothelial barrier is compromised by various blood circulating factors, including thrombin and histamine (Yuan and Rigor, 2010). Barrier promoting factors might be released in response to barrier compromising agents and thereby restore the endothelial barrier function (Weis, 2008).

It was shown, that PGI_2 exerts barrier promoting effects in pulmonary endothelial cells, via the PKA dependent activation of (Birukova et al., 2007). Furthermore, PGI_2 abated the disruption of endothelial monolayers which was induced by LPS. In an LPS-induced acute lung injury mouse model, PGI_2 reduced the number of neutrophils in the broncho-alveolar lavage fluid (BALF) (Birukova et al., 2012).

Not only PGI_2, but also PGE_2 exerts endothelial barrier promoting effects. It was recently shown that PGE_2 enhances the endothelial barrier function via the EP4 receptor. This was independent of cAMP/PKA signaling but seems to be mediated by PI3K and PKC (Konya et al., 2012).

Besides prostanoids, phospholipids also show barrier promoting properties (Yuan and Rigor, 2010). Sphingosine-1-phosphate (S1P), which is derived from platelets was shown, to promote the endothelial barrier function (Garcia et al., 2001). S1P acts

through five receptor subtypes (S1P1-5), which belong to the G-protein coupled receptor family (Wang and Dudek, 2009). Generally, S1P is kept at low blood concentrations. At low concentrations (<1 μM), S1P exerts its barrier promoting character via the activation of an Gαi-protein and the subsequent activation of Rac-1 and thereby strengthens the cell-cell contacts (Yuan and Rigor, 2010). At concentrations which exceed 5 μM, S1P binds to a lower affinity receptor, resulting in formation of stress fibers and increasing vascular permeability via the activation of Gαq and Rho GTPase (Mehta et al., 2001; Wang and Dudek, 2009).

Angiopoietins (Ang1-4) represent another family of proteins, which alter the functions of the endothelium (Augustin et al., 2009). Two members, Ang-1 and Ang-2 modulate the endothelial barrier function and permeability (Fiedler, 2004), through the activation of endothelial tyrosine kinase with Immunoglobin (Ig) and Epidermal Growth Factor (EGF)-like homology domain receptors (TIE-1 and TIE-2) (Augustin et al., 2009). Generally, activation of TIE-2 by Ang-1 promotes the endothelial barrier function and sustains the quiescent phenotype of endothelial cells (Augustin et al., 2009). Activation of TIE-2 leads to the subsequent activation of PI3K/Akt pathway, resulting in reduction of endothelial hyperpermeability, and also stimulates the production of S1P (Yuan and Rigor, 2010). In contrast to that, Ang-2 antagonizes the effects of Ang-1 (Fiedler et al., 2006). Elevated Ang-2 blood levels are associated with several pathological states, including inflammatory diseases, sepsis and inflammatory lung disease (Yuan and Rigor, 2010).

1.3.5 Determination of transendothelial resistance

The increased permeability of a confluent endothelial monolayer is dependent on the amount of open pores at endothelial junctions. Pores allow water and other hydrophilic molecules to pass through the cell membranes. Endothelial membranes can be seen as an electrical insulator, since they consist of lipophilic molecules like phospholipids and cholesterol (Yuan and Rigor, 2010). Through this insulating character they are able to maintain physiological electrical membrane potential (Yuan and Rigor, 2010). Therefore, appearance of pores in the endothelial membrane will result in a decrease of endothelial resistance, which is dependent on the amount of pores. Electric Cell-substrate Impedance Sensing System (ECIS) represents a quantitative method to determine the electrical transendothelial resistance. For this purpose endothelial cells are grown to confluent monolayers on small arrays including gold electrodes. ECIS

experiments are performed by the application of alternating current directly on the electrodes and determination of the impedance, which is equal to the endothelial resistance. Changes in the integrity of endothelial junctions will result in changes of endothelial resistance (Yuan and Rigor, 2010).

1.3.6 Pulmonary endothelial cells

Endothelial cells build the barrier between the blood flow and the interstitium. They are involved in exchange of ions, water and other macromolecules between the blood and the interstitial space. Moreover they provide access for circulating cells in the blood, such as leukocytes, to the tissue. Endothelial cells also produce vasoactive substances and are able to adapt rapidly to changes in blood pressure (Aird, 2012).

Morphological differences are known among different endothelial cells. Endothelium has been classified as being continuous, discontinuous or fenestrated type. The subtypes differ based on the presence of basal membrane, the distance between neighboring cells and the presence of fenestrations. In the pulmonary vasculature the continuous endothelium can be found (Stevens, 2011).

The microvascular system differs from the macrovascular system in size of the blood vessels and in the height of the blood pressure, the microvascular system being exposed to higher blood pressure (Stevens, 2011). Moreover, microvascular endothelial cells form a far more restrictive barrier than the macrovascular endothelial cells, which is supported by high basal cAMP concentration (Kelly et al., 1998; Stevens, 2011). Additionally, differences between pulmonary microvascular and macrovascular endothelial cells appear in proliferation, organelle distribution, apoptosis, gene expression patterns and the organization of signaling transduction pathways (Stevens, 2011).

1.4 Aim of the study

It was recently shown that PGE_2 exerts enhancing effects on the endothelial barrier function of human microvascular endothelial cells. Thus, this work aimed to investigate the role of the EP1 receptor agonist 17-phenyl trinor (pt) PGE_2 on the endothelial barrier function and the underlying signaling pathway.

First, EP receptor expression on human microvascular endothelial cells from the lung (HMVEC-L) and human pulmonary artery endothelial cells (HPAEC) was determined using flow cytometry. Localization of EP1 receptors in endothelial cells was investigated by immunofluorescence microscopy. As functional read out, intracellular Ca^{2+} release induced by PGE_2 and specific EP1-4 receptor agonists was recorded. Selective EP receptor antagonists, specific G-protein inhibitors and Ca^{2+} chelators were applied to determine the underlying signaling pathway. The impact of 17-pt-PGE_2 on the endothelial barrier function regulation was analyzed by electrical resistance experiments. Furthermore, specific EP receptor antagonists and Ca^{2+} chelators were used to investigate a potential involvement of intracellular Ca^{2+} mobilization and endothelial barrier function.

2 Materials and Methods

2.1 Chemicals

Laboratory chemicals were from Sigma-Aldrich (Vienna, Austria) unless specified. Media for cell culture were purchased from Lonza (Verviers, Belgium). Dulbecco's PBS was from PAA. PGE_2 and the selective agonists and antagonists for EP receptors and primary polyclonal EP receptor antibodies were from Cayman Chemical (Ann Arbor, MI, USA). The EP4 mouse monoclonal antibody and the VE-cadherin mouse monoclonal antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). EP1 receptor agonist ONO-DI-004 and EP4 receptor antagonist ONO AE3-208 were gifts from ONO Pharmaceuticals (Osaka, Japan). MH-362-63-8 was a gift from Unigen Inc. (Lacey, Washington, USA. 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 was from Fisher Scientific (Vienna, Austria). FACSflow and CellFix were from BD (San Jose, CA, USA). FLIPR Calcium 4 Assay Kit was from Molecular Devices (Molecular Devices, Sunnyvale, CA, USA). Vectashield/DAPI mounting medium was obtained from Vector Laboratories (Vector Laboratories, Burlingam, CA, USA).

Fixative solution was prepared dissolving 1ml CellFix in 10 ml distilled water and 30ml FACSflow. Pharmacological substances and Ca^{2+} chelators were dissolved in ethanol, dimethyl sulfoxid or distilled water. Further dilutions were made in FLEX assay buffer or EBM-2 basal medium.

2.2 Cell culture

Human lung microvascular endothelial cells (HMVEC-L) and human pulmonary artery endothelial cells (HPAEC) were purchased from Lonza as tertiary cultures and were cultivated in EGM-2 MV bullet kit media supplemented with 5% FCS (HMVEC-L) or EGM-2 SingleQuot Kit Supplemental and Growth Factors (HPAEC) supplemented with 2% FCS, respectively.

Media was changed every second day and cells were grown to 90% confluence and passaged. To this end, cells were washed with HBSS and incubated with trypsin/EDTA for 5 min until the cells were detached from the cell culture flasks. The reaction was stopped with equal amount of trypsin neutralizing solution. The cell solution was transferred into a 50 ml tube and then centrifuged at 220 x g for 5 min. Afterwards, the

supernatant was removed and the cell pellet was resuspended in EGM-2 MV or EGM-2 medium. Cells were grown at 37°C at 5% CO_2 in a humidified incubator. Cells from passage 5-9 were used for experiments (Konya et al., 2012).

2.3 EP receptor staining by flow cytometry

For EP receptor staining, HMVEC-L and HPAEC were cultured in 48-well plates. 30,000 cells were seeded per well and cultured for three days. On the assay day cells were washed once with pre-warmed HBSS and fixed with 3.7% formaldehyde at room temperature for 10 min and then rinsed once with PBS. Afterwards 0.1% Triton X-100 was used for permeabilization of the cells at room temperature for 10 min. Cells were harvested using Trypsin/EDTA for 5 min, transferred into FACS tubes and centrifuged at 400 x g for 5 min.

After removal of the supernatant, non-specific binding sites were blocked using Ultra V blocking solution at room temperature for 30 min. Afterwards, the cells were washed with PBS followed by incubation with specific EP1, EP2, EP3 or EP4 antibodies or isotype control antibodies at 4°C for 1 hour. Specific EP receptor antibodies and isotype control antibodies were diluted in antibody diluent. The used antibodies (Ab) are shown in *Table 1*.

Table 1. Antibodies used for the detection of EP receptors

Antibody	**Concentration**
EP1 rabbit polyclonal Ab	1 µg/ml
EP2 rabbit polyclonal Ab	1 µg/ml
EP3 rabbit polyclonal Ab	1 µg/ml
Rabbit isotype control Ab	1 µg/ml
EP4 mouse monoclonal Ab	1 µg/ml
Mouse isotype control Ab	1 µg/ml
2nd goat-anti rabbit AF488	4 µg/ml
2nd goat-anti mouse AF488	4 µg/ml

After incubation with the first antibody cells were washed with PBS and centrifuged with 400 x g for 5 min. Supernatants were removed and cells were finally incubated with goat-anti-rabbit or goat-anti-mouse secondary antibody, conjugated with Alexa Fluor

488 in the dark at 4°C for 30 min. Afterwards cells were washed with PBS and centrifuged at 400 x g for 5 min. Cell pellets were resuspended in fixative solution for measuring fluorescence intensity by flow cytometry (Konya et al., 2012). Fold increase over control antibody was determined.

2.4 Intracellular Ca^{2+} measurement using FLEX

Endothelial cells (12,000/well) seeded in 1% gelatine pre-coated 96-well plate were grown for 48 h. On the assay day media was exchanged to EBM-2 basal medium and cells were incubated at 37°C for 1 h. Afterwards, half of the volume was replaced by a Ca^{2+} dye-containing buffer: Calcium dye was solved in 12 ml assay buffer and well mixed in the dark (FLIPR Calcium 4 assay kit, Molecular Devices). 100 µl Ca^{2+} dye-containing buffer was added per well and incubated at 37°C for 1 h.

To determine the intracellular Ca^{2+} release, cells were treated with different EP receptor agonists, PGE_2 and histamine as positive control or vehicle. Used concentrations of the agonists are shown in *Table 2*. To determine receptor involvement and signaling pathways, specific EP1 and EP4 receptor antagonists as well as G-protein inhibitors, adenylate cyclase inhibitor and Ca^{2+} chelators were used. Changes in the intracellular Ca^{2+} concentration were recorded for 2 min using FLEXII station (Molecular Devices) (Sedej et al. 2012). Normalized relative fluorescent unit (RFU) was calculated as:

$$Normalized\ RFU = \frac{relative\ maximum\ Ca^{2+}peak}{number\ of\ cells} \cdot 100$$

2.5 Immunofluorescence staining

Endothelial cells (60,000/chamber) were seeded in 1% gelatine pre-coated Permanox chamber slides, and cells were grown to confluence for 48 h. On the assay day cells were washed with warm HBSS, followed by incubation with 3.7% formaldehyde for 10 min to fix the cells. Further, the cells were washed three times with PBS and were incubated with the Ultra V blocking solution for 30 min. All incubation steps were performed at room temperature. Further steps are documented in the following sections.

2.5.1 EP1 receptor staining

Endothelial cells were pre-treated as described in section 2.5. Afterwards, fixed cells were washed with PBS and incubated with the specific EP1 receptor rabbit antibody or an isotype control antibody (1 µg/ml each) at room temperature for 1.5 h (Konya et al., 2011).

Specific EP1 rabbit polyclonal antibody and isotype control antibody were diluted in antibody diluent. Cells were then washed three times with PBS followed by incubation with an Alexa Fluor 488-conjugated goat anti-rabbit antibody in the dark for 30 min. The slide was rinsed with PBS three times and mounted using Vectashield/DAPI mounting medium. Images were taken utilising the Olympus IX70 fluorescence microscope and an Olympus UPlanApo-60x/14.2 oil immersion lens.

2.5.2 VE-Cadherin and F-actin staining

Endothelial cells, grown to confluence on gelatine coated chamber slides were incubated with 100 nM 17-pheny trinor-PGE_2 or vehicle at 37°C for 10 min followed by thrombin at 37°C (0.5 U/ml) for 15 min.

After preparation as stated in Section 2.5 endothelial cells were incubated with primary VE-Cadherin antibody (1µg/ml, Santa Cruz Biotechnology) for an hour followed by secondary goat anti-mouse-AF 488 conjugated antibody (4 µg/ml) and Texas Red-X Phalloidin conjugate 5 U/ml for half an hour in the dark. All antibodies were diluted in antibody diluent. The slide was rinsed with PBS three times and mounted using Vectashield/DAPI mounting medium. Images were taken utilizing the Olympus IX70 fluorescence microscope and an Olympus UPlanApo-60x/14.2 oil immersion lens. Mean fluorescence intensity (MFI) of VE-cadherin and F-actin was determined out of the fluorescence micrographs using ImageJ software. For this purpose, a region of interest (ROI) was selected and MFI of all endothelial cells per image was determined. MFI of vehicle treated cells was assumed as 100% (Konya et al., 2012).

2.6 Endothelial resistance measurement

Endothelial cells (80,000/well) were grown to confluence on 1% gelatine pre-coated 8W10E+ polycarbonate arrays including 40 gold microelectrodes over 48 h. On the assay day medium was changed to EMB-2 basal medium and endothelial cells were serum starved for one hour before electrical resistance measurement. Electrical resistance measurement was performed at multiple frequencies with an Electrical Cell-substrate Impedance Sensing System (ECIS) (Applied Biophysics, Troy, NY) (Konya et al., 2012). At first, a stable baseline was recorded for one hour. Afterwards EP receptor antagonist, Ca^{2+} chelators or vehicle were added 15 or 30 min prior to addition of 17-phenyl trinor-PGE_2, CAY10598 or vehicle. Used drugs are stated in *Table 2* and *Table 3*. Data analysis was performed at 4000 Hz. Normalized resistance was determined from the endothelial resistance raw data.

2.7 Pharmacological substances and Ca^{2+} chelators

Table 2. Used agonists, antagonists and inhibitors.

	Specificity	Concentration	Company
Agonists			
PGE_2	EP1-4	10 – 1000 nM	Cayman Chemicals
17-pheny ltrinor-PGE_2	EP1	10 – 1000 nM	Cayman Chemicals
Butaprost	EP2	10 – 1000 nM	Cayman Chemicals
Sulprostone	EP3/EP1	10 – 1000 nM	Cayman Chemicals
CAY10598	EP4	10 – 1000 nM	Cayman Chemicals
Iloprost	IP/EP1	10 – 1000 nM	Cayman Chemicals
ONO DI-004	EP1	10 – 1000 nM	ONO Pharmaceuticals
Histamine	H1	10 – 1000 nM	Sigma-Aldrich
Antagonists			
SC-51322	EP1	1 – 10 µM	Cayman Chemicals
SC-51089	EP1	10 µM	Cayman Chemicals
ONO-8711	EP1/TP	1 µM	Cayman Chemicals
AH 6809	EP1-2-3/DP	30 µM	Cayman Chemicals
ONO AE3-208	EP4	1-10 µM	ONO Pharmaceuticals
GW 627368X	EP4>TP	10 µM	Cayman Chemicals
L-161,982	EP4	1-10 µM	Cayman Chemicals
Inhibitors			
MH-362-63-8	$G_{\alpha s}$	1 µM	Unigen Inc.
Pertussis toxin	$G_{\alpha i}$	100 µg/ml	Sigma-Aldrich
SQ22536	Adenylate cyclase	10 µM	Sigma-Aldrich

Table 3. Used Ca^{2+} chelators.

	Concentration	Company
Calcium chelators		
EGTA	2 mM	Sigma-Aldrich
EGTA-AM	10 µM	Sigma-Aldrich
BAPTA-AM	10 µM	Sigma-Aldrich

2.8 Statistical analysis

All data are shown as mean + SEM for n observations. Statistical analyses were performed by SigmaPlot 12.1 software using one-way repeated measurement ANOVA with Bonferroni post-hoc test. $P < 0.05$ was considered as statistically significant.

3 Results

3.1 EP receptors are expressed on pulmonary endothelial cells

The EP receptor expression on human pulmonary endothelial cells was determined by using specific EP receptor antibodies and the expression profiles were detected by flow cytometry. Therefore pulmonary endothelial cells were incubated with specific primary EP receptor antibodies followed by incubation with fluorescently labeled secondary antibodies. Data are shown as fold increase over the corresponding isotype control antibodies.

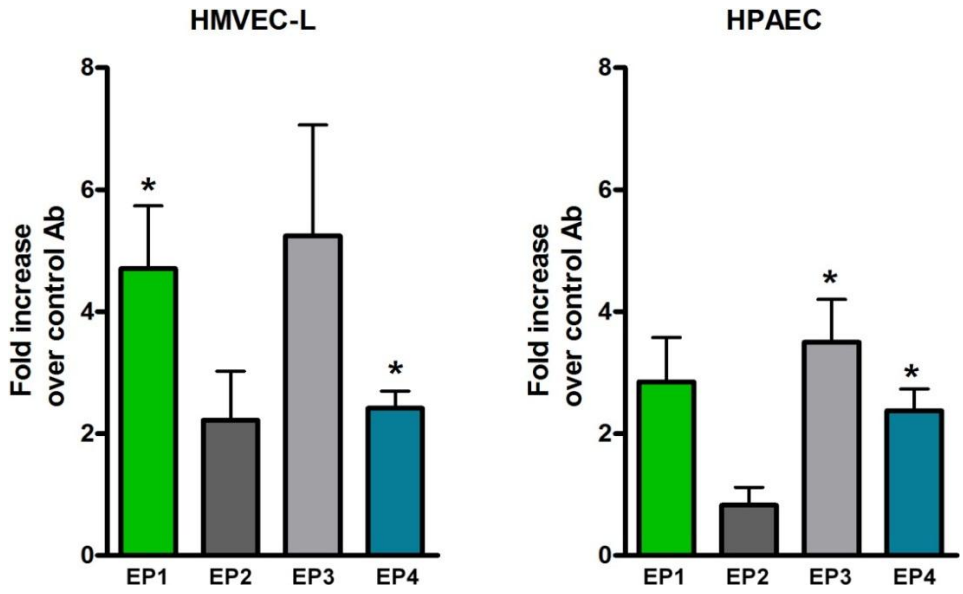

Figure 3. EP receptor expression was investigated by flow cytometry. *HMVEC-L and HPAEC were incubated with specific primary EP receptor antibodies or isotype control antibodies followed by Alexa Fluor 488-conjugated secondary antibodies. Data are shown as mean + SEM of fold increase over control antibody out of n independent observations,* indicates P<0.05 versus isotype control antibody, n=4-5.*

In detail, we found that pulmonary microvascular and macrovascular endothelial cells express different amounts of E-type prostanoid receptors. The pulmonary microvascular endothelial cells express more EP1, EP2 and EP3 receptors compared to the pulmonary artery endothelial cells. EP4 receptor was expressed equally on the two cell types. HPAECs show only very small amounts of EP2 receptor expression (**Figure 3**).

3.2 Visualizing EP1 receptor expression

Immunofluorescence staining for EP1 receptor was performed on HMVEC-Ls which were grown until confluence on chamber slides. Next step was to investigate the cellular localization of EP1 receptor. Therefore HMVEC-Ls were incubated with specific polyclonal primary anti-EP1 antibody or an irrelevant isotype control antibody.

Immunofluorescence staining indicates that EP1 receptor is located in the cytoplasm and perinuclearly (*Figure 4*, right panel) in pulmonary microvascular endothelial cells, whereas incubation with the isotype control antibody did not show specific staining on the endothelial cells (*Figure 4*, left panel).

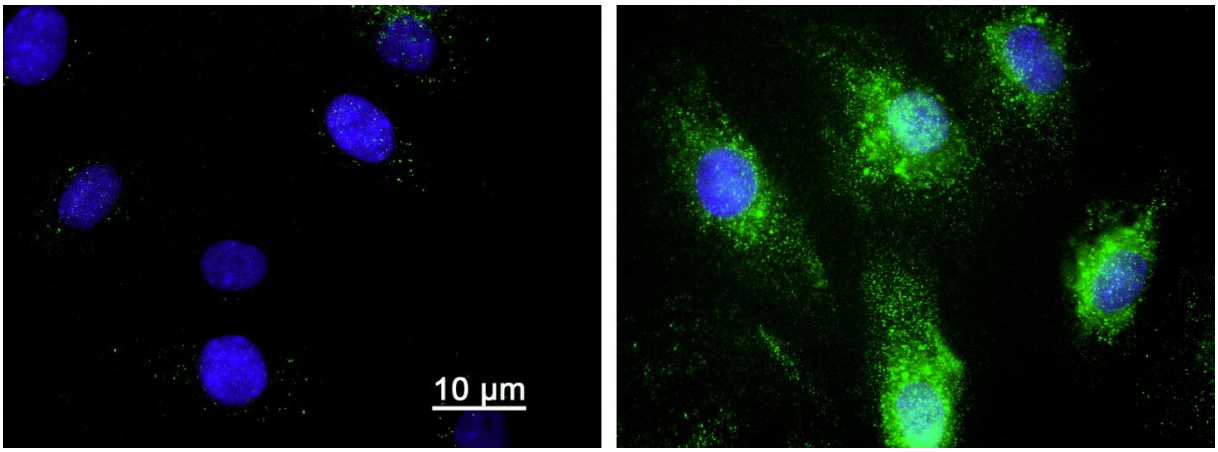

Figure 4. Fluorescence staining of EP1 receptor in HMVEC-L. *Endothelial cells were cultured in chamber slides until confluence and stained with an irrelevant isotype control antibody (left) or specific anti-EP1 antibody (green) and the nuclear stain DAPI (blue). Pictures were taken with an Olympus IX70 fluorescence microscope and Olympus UPlanApo-60x/14.2 oil immersion objective. Images are representative of 3 independent staining experiments.*

3.3 Stimulation of EP1 receptor induces intracellular Ca^{2+} release in endothelial cells

Next we investigated the intracellular Ca^{2+} release in pulmonary endothelial cells induced by specific EP receptor agonists. For this purpose HMVEC-L and HPAEC were grown in 96-well plates for 48 hours. Endothelial cells were treated with PGE_2, the specific EP1 agonist 17-phenyl trinor-PGE_2, the EP2 specific agonist butaprost, the EP3 agonist sulprostone and the EP4 agonist CAY10598 (10, 100, 1000 nM each) or vehicle. Ca^{2+} release was measured over 120s. Relative fluorescent unit was determined out of

the maximum Ca^{2+} release and the number of cells. Histamine, a known inducer of intracellular Ca^{2+} release, was used as positive control.

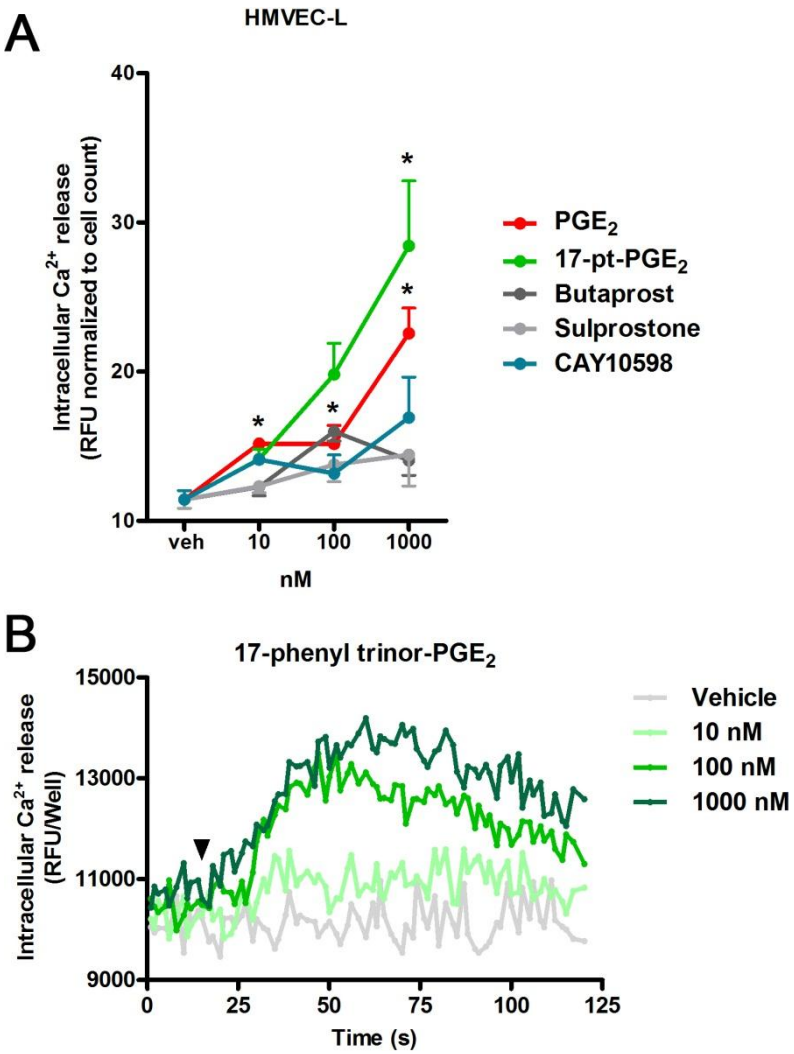

Figure 5. Intracellular Ca^{2+} release induced by EP receptor agonists and PGE_2 in the pulmonary microvascular endothelial cells. *(**A**) HMVEC-L were treated with vehicle, PGE_2, EP1 agonist 17-phenyl trinor-PGE_2, EP2 agonist butaprost, EP3 receptor agonist sulprostone or EP4 receptor agonist CAY10598 (10, 100, 1000 nM each). Agonist-induced Ca^{2+} release is shown as mean + SEM of relative fluorescent unit, * indicates P<0.05 versus vehicle, n=3. (**B**) Real-time intracellular Ca^{2+} release induced by 17-phenyl trinor-PGE_2. The original tracings are representative of 3 independent experiments. The arrowhead indicates the addition of EP1 receptor agonist.*

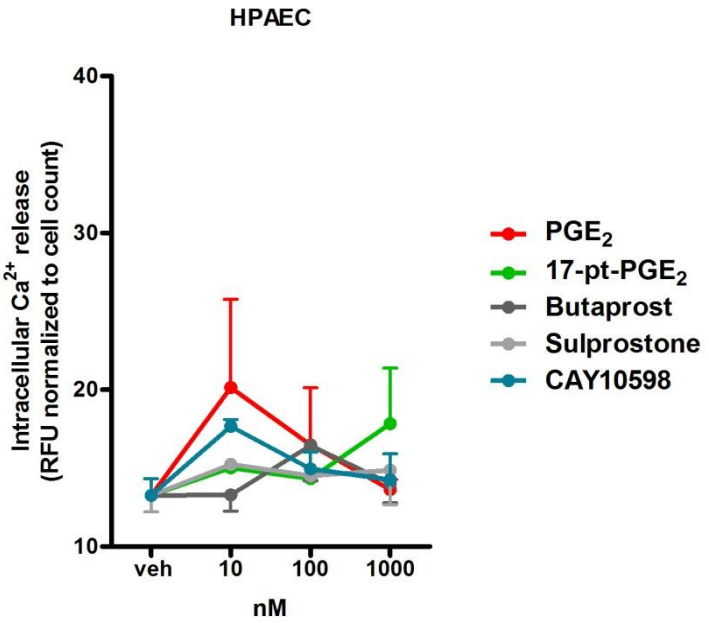

Figure 6. Intracellular Ca^{2+} release induced by EP receptor agonists and PGE_2 in pulmonary artery endothelial cells. *HPAECs were treated with vehicle, PGE_2, EP1 agonist 17-phenyl trinor-PGE_2, EP2 agonist butaprost, EP3 receptor agonist sulprostone or EP4 receptor agonist CAY10598 (10, 100, 1000 nM each). Agonist-induced Ca^{2+} release is shown as mean + SEM of relative fluorescent unit n=3.*

PGE_2 and 17-phenyl trinor-PGE_2 induced concentration-dependent intracellular Ca^{2+} release in HMVEC-L. Highest concentration of 17-phenyl trinor-PGE_2 caused a Ca^{2+} release up to 2.5 fold elevated from the vehicle. Interestingly, the 17-phenyl trinor-PGE_2 induced Ca^{2+} release was 1.2 fold higher than that induced by PGE_2. Moreover, the EP4 receptor agonist Cay10598 also induced a modest Ca^{2+} release, which was up to 1.5 fold higher as compared with vehicle. The EP2 and EP3 receptor agonists butaprost and sulprostone, respectively, had no effect on the Ca^{2+} release (***Figure 5A***). In contrast to the findings in HMVEC-L, we did not detect a specific Ca^{2+} signal in human pulmonary artery cells (*Figure 6*). These findings correspond to the results of EP receptor staining, since HPAECs express lower amounts of EP1,2 and 3 receptors (***Figure 3***). 1 µM histamine caused in both cell types a Ca^{2+} release up to 20-fold higher than the vehicle (data not shown).

3.4 17-phenyl trinor-PGE_2 induced Ca^{2+} release is mediated by EP4 receptors and not EP1 receptors

To prove EP1 receptor involvement in 17-pt-PGE_2 induced Ca^{2+} release, we assessed three different EP1 antagonists. HMVEC-Ls were pre-treated with the EP1 receptor antagonist SC-51322, SC-51089 and ONO-8711 (10 µM, 10 µM and 1 µM) for 15 min. Thereafter, cells were stimulated with 17-pt-PGE_2 (10, 100, 1000, each) or vehicle. Histamine was used as a positive control. Intracellular Ca^{2+} release assay was performed.

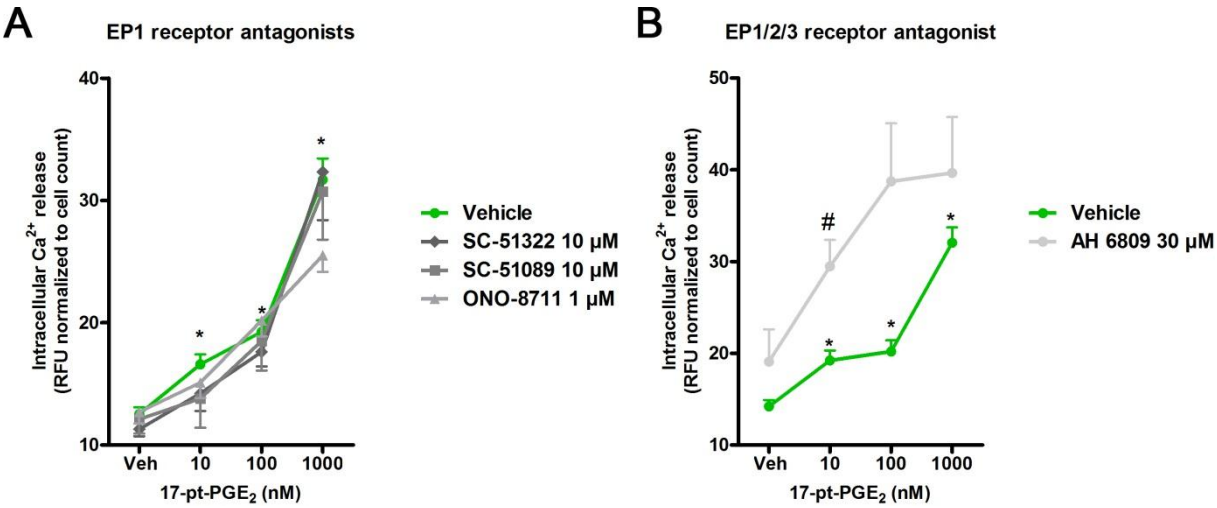

Figure 7. Intracellular Ca^{2+} release is not inhibited by specific EP1 receptor antagonist or AH 6809. *HMVEC-L were pre-treated with selective EP1 receptor agonist (**A**) or unselective EP1/2/3 antagonist AH 6809 (**B**) or vehicle followed by treatment with 17-pt PGE_2 (10, 100, 1000 nM each). Agonist-induced Ca^{2+} release is shown as mean + SEM of relative fluorescent unit, * indicates P<0.05 versus vehicle, n=3-5.*

Our data showed that none of the selective EP1 receptor antagonists was able to inhibit the 17-pt-PGE_2 induced Ca^{2+} release (***Figure 7A***). Therefore, we decided to use a nonselective EP1/2/3 and DP antagonist, AH 6809, to probe receptor involvement. But like all EP1 receptor antagonist AH 6809 was not able to abolish the 17-pt-PGE_2 induced Ca^{2+} release, but even elevated the signal (***Figure 7B***). As we used AH 6809 in a relatively high concentration (30 µM) unspecific fluorescent reaction might have occurred,hich was suggested by an elevation of the fluorescent signal at baseline.

Since we could show that neither specific EP1 receptor antagonists nor an EP1/2/3 antagonist had an impact on the intracellular Ca^{2+} release and since CAY10598 could

induce a modest Ca^{2+} signal, we speculated of a potential involvement of the EP4 receptor.

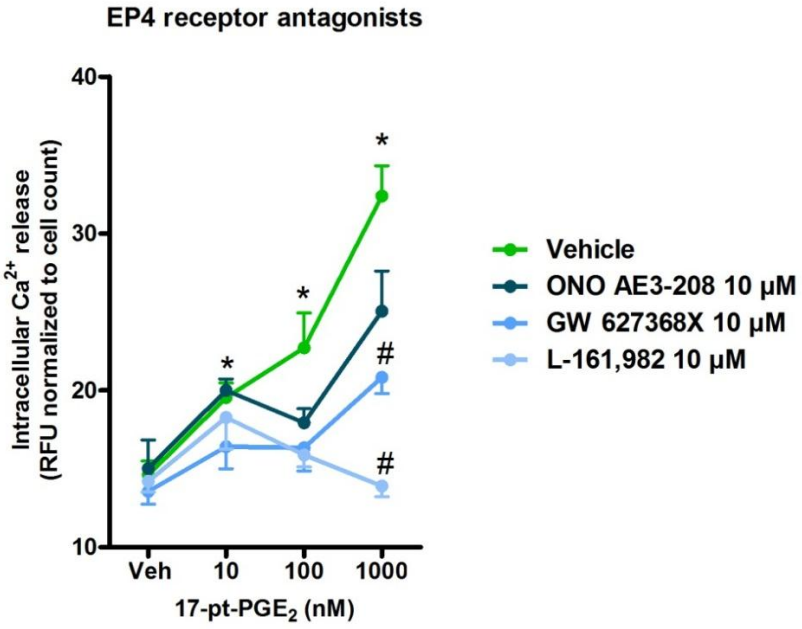

Figure 8. Inhibition of intracellular Ca^{2+} release in HMVEC-L by specific EP4 receptor antagonists. *HMVEC-L were pre-treated with selective EP4 receptor agonist or vehicle for 15 min followed by treatment with 17-pt-PGE_2 (10, 100, 1000 nM each). Agonist-induced Ca^{2+} release is shown as mean + SEM of relative fluorescent unit, * and # indicate P<0.05 versus vehicle, n=5.*

Therefore, we pretreated HMVEC-Ls for 15 min with the EP4 receptor antagonists ONO AE3-208, GW 627368X and L-161,982 (10 µM each) prior to incubation with 17-pt-PGE_2. EP4 receptor antagonists GW 627368X and L-161,982 significantly abolished the 17-pt-PGE_2 induced Ca^{2+} release. ONO AE3-208 slightly reversed the effect (***Figure 8***). Two EP4 antagonists (GW 627368X and L-161,982) completely reversed the 17-pt-PGE_2-induced intracellular Ca^{2+} mobilization while suprisingly, the more potent EP4 antagonist ONO AE3-208 did not significantly inhibit the Ca^{2+} release.

3.5 Gαi and Gαq-proteins but not adenylyl cyclase are involved in 17-pt-PGE_2-induced Ca^{2+} release

As EP receptors belong to the family of G-protein coupled receptors, we further investigated which downstream signaling pathway is activated by 17-pt-PGE_2. Therefore, endothelial cells were pre-treated with Gαi-protein inhibitor Pertussis toxin (PTx, 100 µg/ml) or Gαq-protein inhibitor MH-362-63-8 *(1µM).* We wanted to exclude

the possibility that 17-pt-PGE_2 activates the cAMP pathway, therefore we blocked adenylyl cyclase to see the effect on the Ca^{2+} release induced by 17ptPGE$_2$. For this purpose, we treated HMVEC-L with the adenylate cyclase inhibitor SQ22536 (10µM) before addition of 17-pt-PGE_2.

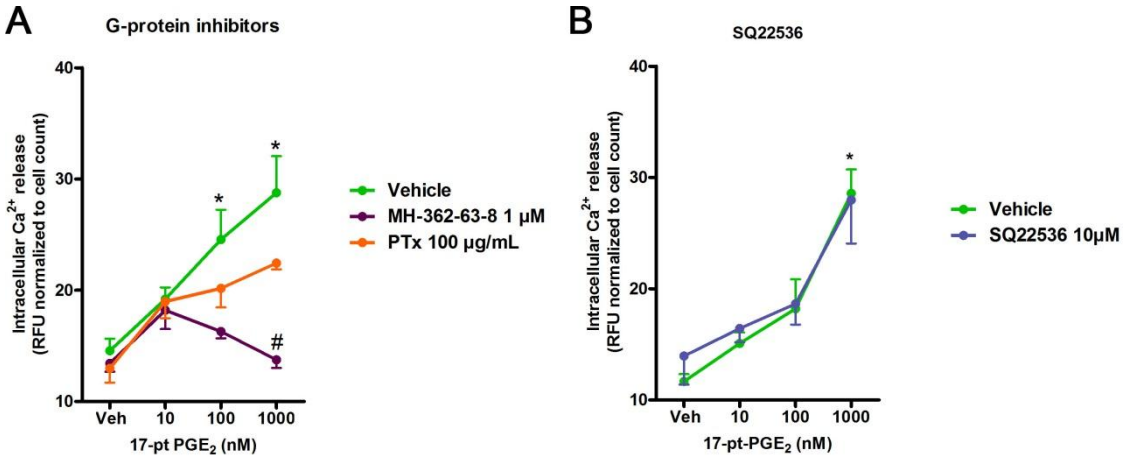

Figure 9. Gαi and Gαq-proteins but not adenylyl cyclase are involved in 17-pt-PGE_2-induced Ca^{2+} release. *HMVEC-L were pre-treated with Gαi-(Pertussis toxin, 100 µg/ml), Gαq-protein inhibitor (MH-362-63-8 1µM) (**A**), adenylate cyclase inhibitor (SQ22536, 10 µM) (**B**) or vehicle for 60 min followed by treatment with 17-pt-PGE_2 (10, 100, 1000 nM each). Agonist-induced Ca^{2+} release is shown as mean + SEM of relative fluorescent unit, * and # indicate P<0.05 versus vehicle, n=3-5.*

After pre-treatment with pertussis toxin and MH-362-63-8 for one hour and SQ22536 or vehicle for 30 min, endothelial cells were treated with 17-pt-PGE_2 (10, 100, 1000 nM) or vehicle. We could show that Gαi-protein inhibitor pertussis toxin partially, while the Gαq-protein inhibitor MH-362-63-8 completely reversed the effect of 17-pt-PGE_2 in endothelial cells, whereas SQ22536 had no effect on the intracellular Ca^{2+} release *(**Figure 9**).*

3.6 EP1 receptor agonist ONO-DI-004 and IP/EP1 receptor agonist iloprost do not induce intracellular Ca^{2+} release in endothelial cells

In order to investigate whether other EP1 receptor agonists have a similar effect on HMVEC-Ls like 17-pt-PGE_2 we treated endothelial cells with the specific EP1 receptor agonist ONO-DI-004, the IP/EP1 receptor agonist iloprost (10, 100, 1000 nM each) or

vehicle. It is evident in ***Figure 10*** that neither ONO-DI-004 nor iloprost could mimic the intracellular Ca^{2+} mobilization stimulated by 17-pt-PGE_2.

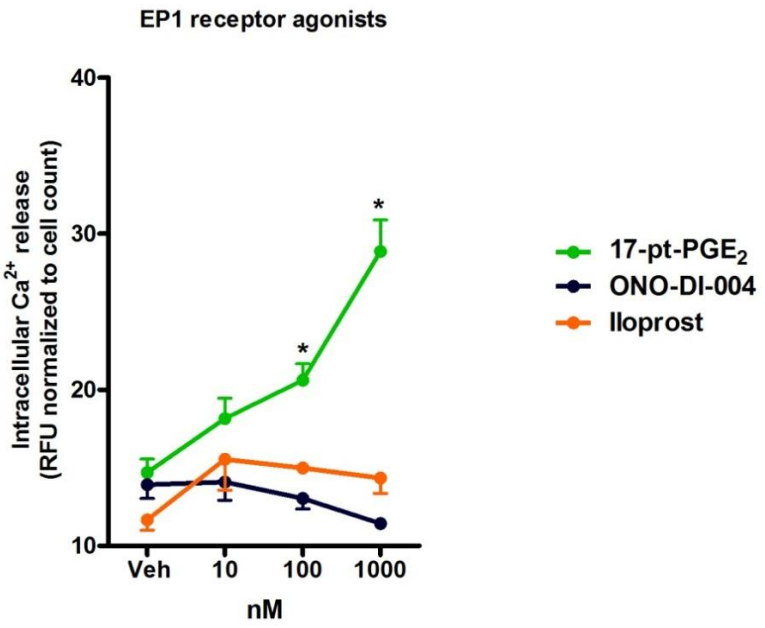

Figure 10. Intracellular Ca^{2+} release induced by EP1 receptor agonists in the pulmonary microvascular endothelial cells*. HMVEC-L were treated with vehicle, EP1 agonist 17-phenyl trinor PGE_2, EP1 receptor agonist ONO-DI-004 or IP/EP1 receptor agonist iloprost (10, 100, 1000 nM each). Agonist-induced Ca^{2+} release is shown as mean + SEM of relative fluorescent unit, * indicates P<0.05 versus vehicle, n=3.*

As we could show that the 17-pt-PGE_2 induced Ca^{2+} release is mediated by EP4 receptors (***Figure 8***) we were eager to investigate the possible impact of EP4 receptor agonist CAY10598 on the17-pt-PGE_2-induced Ca^{2+} mobilization and for this purpose we co-incubated endothelial with 17-pt-PGE_2 and CAY10598 (10, 100, 100 nM).
It is evident in ***Figure 11*** that co-incubation of 17-pt-PGE_2 and CAY10598 did not alter the elevation of Ca^{2+} levels elevated by 17-pt-PGE_2 on HMVEC-Ls, compared to the Ca^{2+} increase, induced by 17-pt-PGE_2 exclusively.

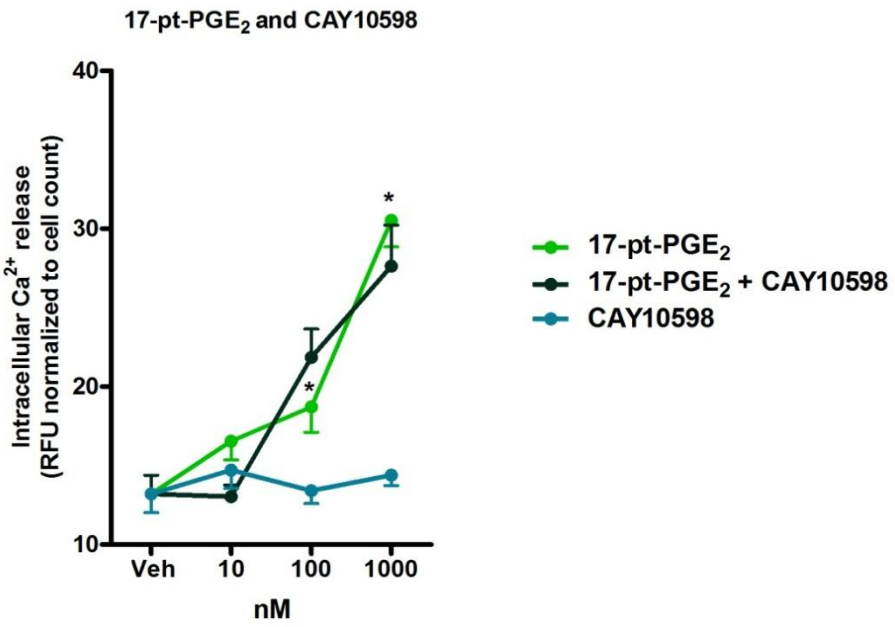

Figure 11. CAY10598 does not influence the 17-pt-PGE$_2$ induced Ca^{2+} release in pulmonary microvascular endothelial cells. *HMVEC-L were treated with vehicle, EP1 agonist 17-phenyl trinor-PGE$_2$, EP4 receptor agonist CAY10598 or with the two agonists together (10, 100, 1000 nM each). Agonist-induced Ca^{2+} release is shown as mean +SEM of relative fluorescent unit, * indicates P<0.05 versus vehicle, n=5.*

3.7 17-phenyl trinor-PGE$_2$ and PGE$_2$ mediate Ca^{2+} influx from the extracellular space

Since we could show that PGE$_2$ and 17-pt-PGE$_2$ are able to enhance intracellular Ca^{2+} levels in HMVEC-Ls, we were interested to see whether the released Ca^{2+} is derived from the endoplasmic stores or from the extracellular space. To address this issue we incubated endothelial cells with ethylene glycol tetraacetic acid (EGTA), a Ca^{2+} chelating substance which binds extracellular Ca^{2+}. Endothelial cells were treated with EGTA (2 mM) 15 min prior to addition of PGE$_2$, 17-pt-PGE$_2$ (10, 100, 1000 nM each) or vehicle.

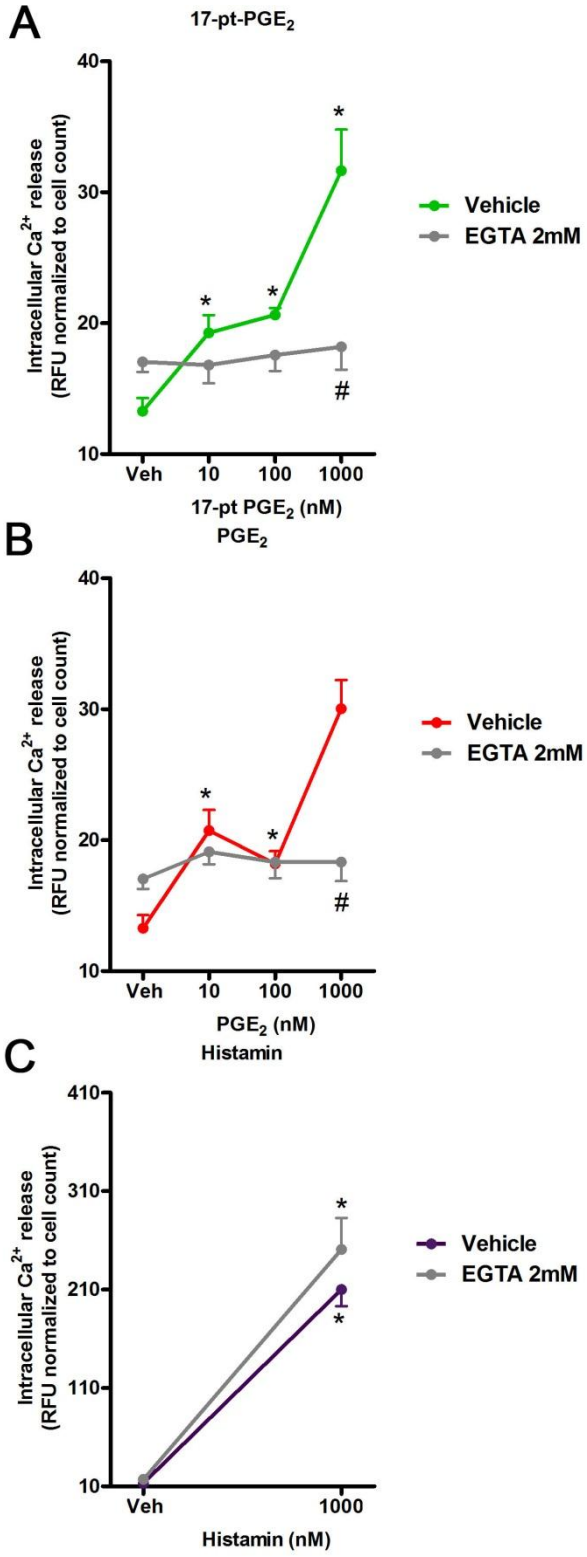

Figure 12. The intracellular Ca^{2+} elevation induced by 17-pt-PGE_2 and PGE_2 is derived from the extracellular space. *HMVEC-L were pre-treated with vehicle or 2 mM EGTA for 15 min followed by (A) 17-phenyl trinor-PGE_2 (B) PGE_2 (10, 100, 1000 nM each), (C) histamine (1000 nM) or vehicle. Agonist-induced Ca^{2+} release is shown as mean + SEM of relative fluorescent unit, * indicates $P<0.05$ versus vehicle, n=4.*

EGTA completely abolished the intracellular Ca^{2+} release induced by PGE_2 and 17-pt-PGE_2 in HMVEC-L (***Figure 12 A and B***). However, it had no effect on the on the changes in intracellular Ca^{2+} levels triggered by histamine ***(Figure 12C***).

3.8 17-phenyl trinor-PGE_2 promotes endothelial barrier function

It was previously shown that PGE_2 and EP4 receptor agonist CAY10598 can enhance endothelial barrier function (Konya et al., 2012). Therefore, we hypothesized that the EP1 agonist 17-pt-PGE_2 might also have an impact on endothelial barrier function. For this purpose endothelial cells were grown to confluence on polycarbonate arrays, containing 40 gold electrodes. One hour prior to resistance measurement endothelial cells were serum starved. Electrical endothelial resistance measurement was performed at multiple frequencies using Electric Cell-substrate Impedance Sensing (ECIS) device. After recording a stable baseline for one hour, cell were treated with 17-pt-PGE_2 (10, 100, 1000 nM) or vehicle in order to measure changes in endothelial resistance.

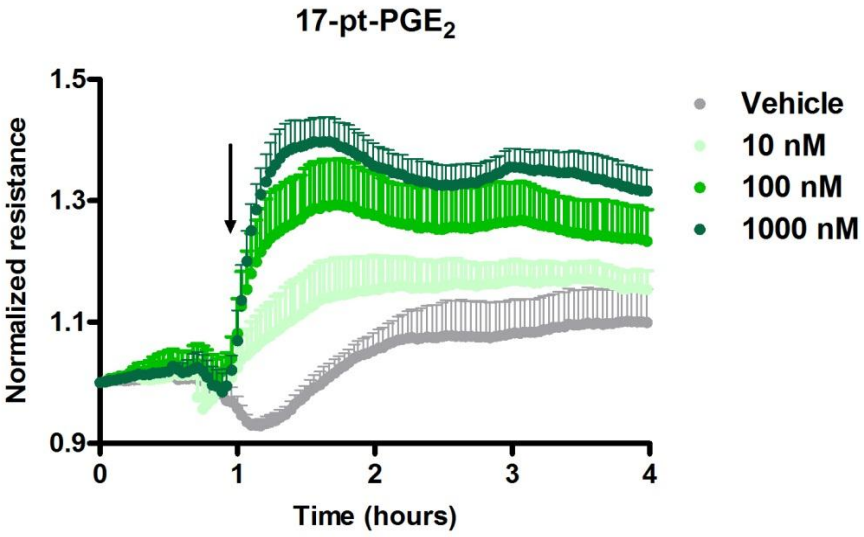

Figure 13. 17-phenyl trinor-PGE_2 increases endothelial barrier function of human lung microvascular endothelial cells. *Endothelial cells were grown on gold microelectrodes. Changes of endothelial resistance were monitored by electric cell substrate impedance sensing (ECIS). At first, baseline resistance was determined followed by treatment with 17-pt-PGE_2 (10, 100, 1000 nM each) or vehicle (arrow). Endothelial resistance is shown as mean + SEM of normalized resistance of 3 independent experiments.*

17-pt-PGE_2 concentration-dependently increased endothelial electrical resistance (***Figure 13***). Highest used concentration (1000 nM) of 17-pt-PGE_2 elevated the resistance of HMVEC-Ls up to 40% of baseline. We observed approximately 30% barrier enhancement upon 100 nM 17-pt-PGE_2 treatment; in comparison, 100 nM EP4 agonist CAY10598 induced 50% increase in the endothelial resistance relative to the baseline (***Figure 14***).

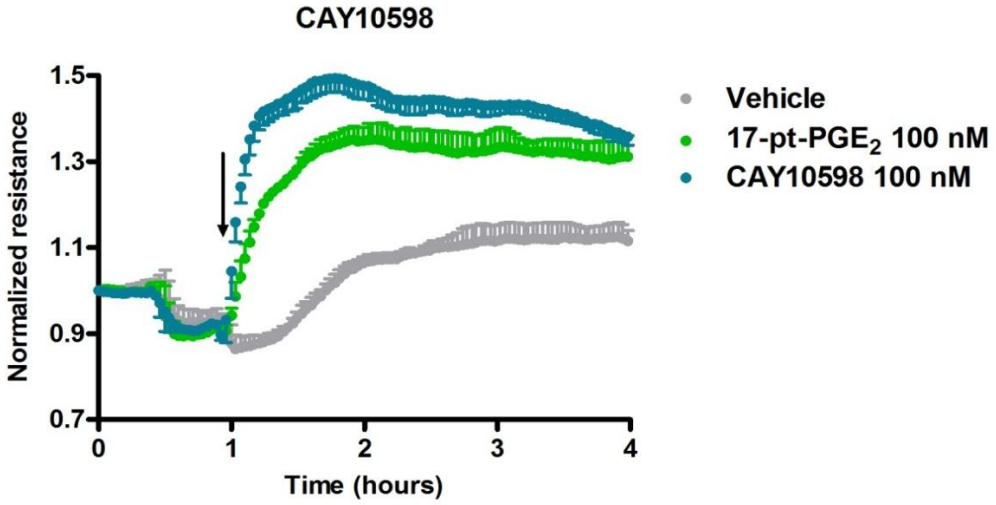

Figure 14. 17-phenyl trinor PGE_2 and CAY10598 increase endothelial barrier function of human lung microvascular endothelial cells. *HMVEC-Ls grown on gold microelectrodes were treated with 17-pt-PGE_2, CAY10598 (100 nM each) or vehicle (arrow). Endothelial resistance is shown as mean + SEM of normalized resistance of 3 independent experiments*

3.9 The endothelial barrier-promoting effect of 17-phenyl trinor-PGE_2 is mediated by EP4 receptors

Further on, we tested the specific involvement of EP1 receptor in the endothelial barrier function by using EP1 specific antagonists (SC-51322 and ONO-8711). The EP1 antagonists were applied 15 min before the 17-pt-PGE_2 treatment. Specific inhibition of EP1 receptor had no effect on the resistance promoting effect of 17-pt-PGE_2 (***Figure 15***). As we could previously show that the intracellular Ca^{2+} release induced by 17-pt -PGE_2 is mediated by EP4 receptors (**Figure 8**) and an EP4 receptor agonist CAY10589 has been already shown to exert endothelial barrier promoting effects *(**Figure 14***) (Konya et al., 2012) we speculated that 17-pt -PGE_2 might promote barrier function by engaging EP4 receptors.

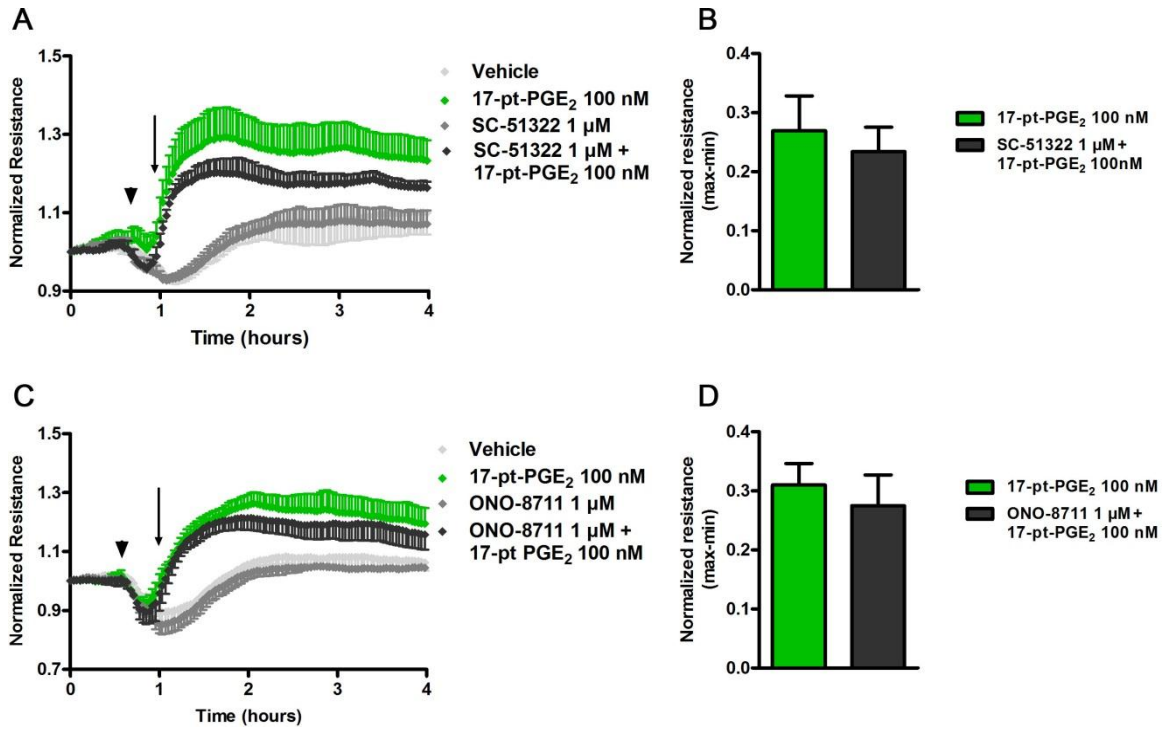

Figure 15. The 17-phenyl trinor-PGE$_2$ induced elevation in endothelial barrier function is not mediated by EP1 receptors. *Endothelial cells grown on gold microelectrodes were pre-treated with EP1 receptor antagonists (**A**)SC-51322 or (**C**) ONO-8711 for 15 min (arrowheads) followed by treatment with 17-pt-PGE$_2$ or vehicle (100 nM each) (arrow). Normalized maximal resistance after treatments is shown in (**B**) and (**D**). Endothelial resistance is shown as mean + SEM of normalized resistance of 3 independent experiments, or as mean + SEM of normalized resistance max-min, which was determined out of the difference between the maximal normalized resistance and the minimal normalized resistance.*

Endothelial cells were incubated with selective EP4 receptor antagonists (ONO AE3-208 and L-161,982, 1 µM each) for 15 min before adding 17-pt-PGE$_2$ or vehicle (100 nM). It is shown in ***Figure 16*** that both EP4 receptor antagonists reversed the endothelial promoting effect of 17-pt-PGE$_2$.

These data suggest that 17-pt-PGE$_2$ enhances the endothelial electrical resistance via activating EP4 receptors and not EP1 receptors.

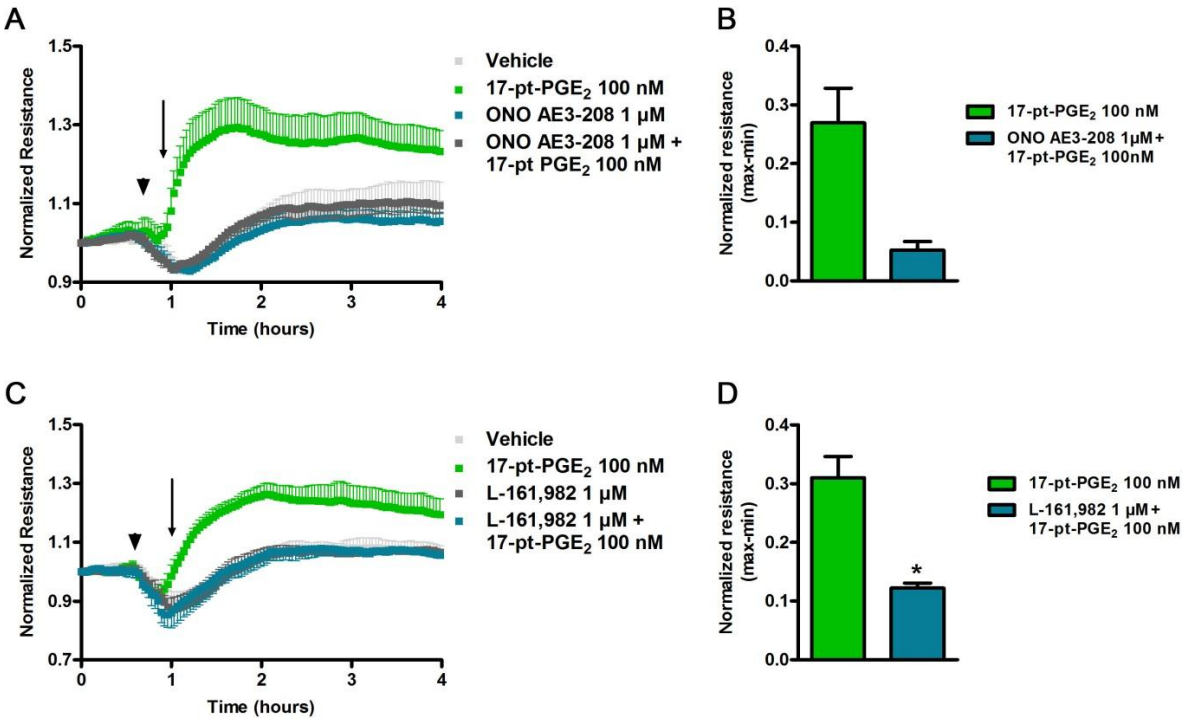

Figure 16. The 17-phenyl trinor-PGE$_2$-induced endothelial barrier enhancement is mediated by EP4 receptors. *Endothelial cells grown on gold microelectrodes were pre-treated with EP4 receptor antagonist ONO AE3-208 or L-161,982 for 15 min (arrowheads) followed by treatment with 17-pt-PGE$_2$ or vehicle (100 nM each) (arrow). Normalized maximal resistance after treatments is shown in (B) and (D). Endothelial resistance is shown as mean + SEM of normalized resistance of 3 independent experiments, or as mean + SEM of normalized resistance max-min, which was determined out of the difference between the maximal normalized resistance and the minimal normalized resistance, * indicates P<0.05 versus 17-pt-PGE$_2$.*

3.10 The barrier-promoting effect of EP receptor agonists is not dependent on intracellular Ca^{2+} mobilization

We investigated the possible involvement of intracellular Ca^{2+} level elevation upon 17-pt-PGE$_2$ treatment of endothelial cells in the endothelial barrier promoting function of 17-pt-PGE$_2$. For this purpose we used different Ca^{2+} chelating agents. Endothelial cells were pre-incubated for 15 min with EGTA (2 mM) or 30 min with EGTA-AM or BAPTA-AM (10 µM each) before addition of 17-pt-PGE$_2$ or CAY10598. We have previously shown that EGTA completely blocked the intracellular Ca^{2+} release induced by 17-pt-PGE$_2$ (Figure 10). However, EGTA had no impact on the increase of endothelial resistance induced by 17-pt-PGE$_2$. EGTA itself induced a dramatic drop in the

endothelial resistance which was quickly reversed (***Figure 17A)***. Additionally, we used the membrane-permeable forms of the Ca^{2+} chelators EGTA and 1,2-Bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetrakis(acetoxymethyl ester) BAPTA, *i.e.* EGTA-AM and BAPTA-AM, respectively. Our data suggest (***Figure 17)*** that Ca^{2+} mobilization is not involved in the barrier promoting effect of 17-pt-PGE_2 or CAY10598, since none of the Ca^{2+} chelators was able to reverse the barrier promoting effect of these two agonists. EGTA and BAPTA-AM had even an endothelial barrier-disrupting effect on the endothelial monolayer (***Figure* 17BCD**).

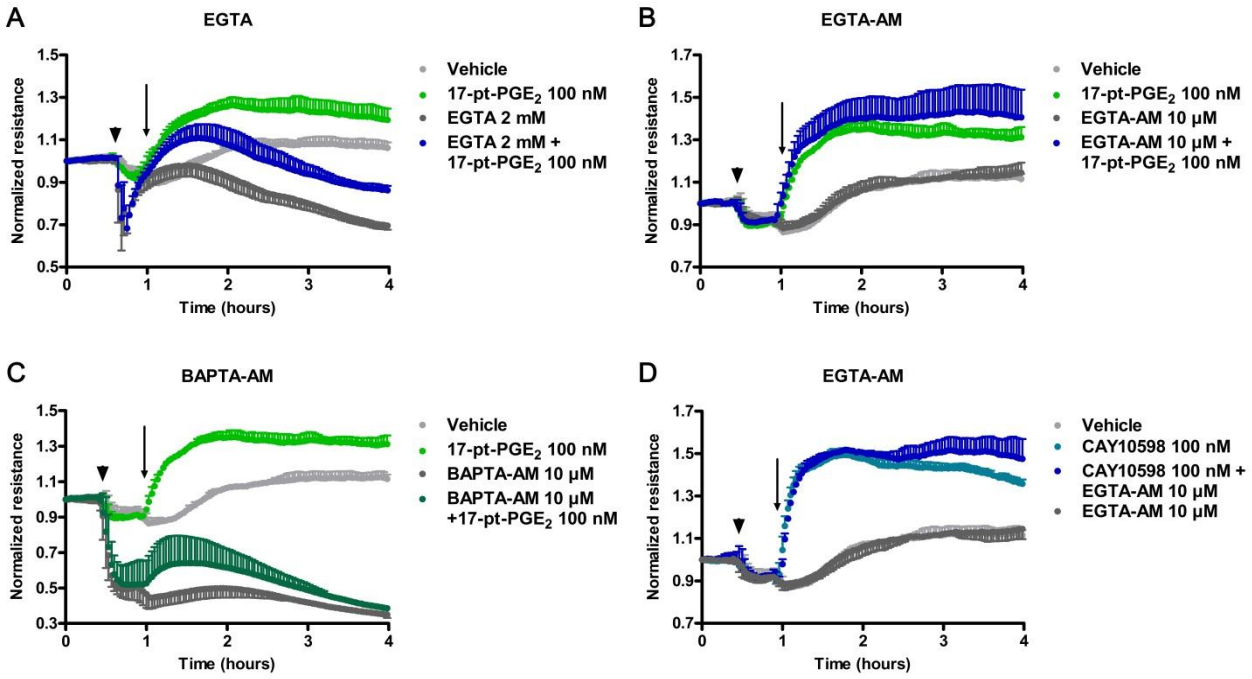

Figure 17. The protective effect of 17-phenyl trinor-PGE_2 and CAY10598 on the endothelial barrier function is not mediated by calcium ions. *Endothelial cells grown on gold microelectrodes were pre-treated by (**A**) EGTA (2mM, for 15 min) or by (**B**) EGTA-AM, (C) BAPTA-AM (10 µM each, for 30 min) or vehicle (arrowheads) before addition of 17-pt-PGE_2, (D) CAY10598 (100 nM each) or vehicle (arrows). Endothelial resistance is shown as mean + SEM of normalized resistance of 2 or 3 independent experiments.*

3.11 The EP1 receptor agonist ONO-DI-004 does not influence the endothelial barrier function

The possible involvement of EP1 receptors in the endothelial barrier enhancement was further challenged by using another EP1 receptor agonist ONO-DI-004. Similar concentrations (10, 100, 1000 nM) were used for ONO-DI-004 as from 17-pt-PGE_2.

It is evident in **Figure 18** that ONO-DI-004 had no appreciable effect promoting endothelial barrier function.

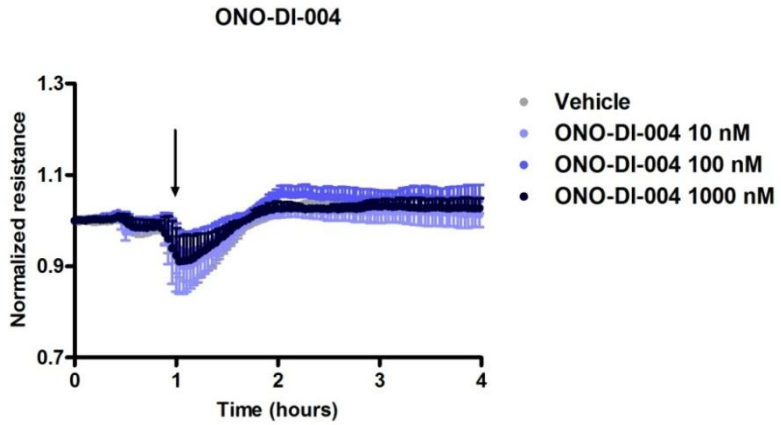

Figure 18. The EP1 agonist ONO-DI-004 does not promote barrier function in human lung microvascular endothelial cells. *Endothelial cells grown on gold microelectrodes were treated with ONO-DI-004 (10, 100, 1000 nM) or vehicle (arrow). Endothelial resistance is shown as mean + SEM of normalized resistance of 3 independent experiments.*

3.12 17-phenyl trinor-PGE_2 prevents from thrombin induced endothelial junction disruption

The integrity of endothelial cell layer is maintained by endothelial adherens junctions connected to cytoskeletal filamentous-actin (F-actin) network. We assessed the regulatory role of 17-phenyl trinor-PGE_2 on VE-cadherin expression in the endothelial junctions and changes of F-actin polymerization by using immunofluorescence microscopy. HMVEC-L were grown to confluence on chamber slides and were pre-incubated with 17-pt-PGE_2 (100 nM) or vehicle followed by thrombin (0.5 U/ml) or vehicle to mimic inflammatory processes.

Generally, vehicle treated cells showed tight adherens junction complexes between adjacent cells and peripheral F-actin polymerization (***Figure 19***). After 15 min treatment with 17-phenyl trinor-PGE_2 VE-cadherin expression in the adherens junctions appeared to be more intensive and we observed a more pronounced F-actin network in the paracellular regions. Incubation with thrombin (0.5 U/ml) caused disruption of endothelial adherens junctions, remodeling of the F-actin network into stress fibers, and formation of paracellular gaps. Treatment of HMVEC-L with 17-phenyl trinor-PGE_2 prior to thrombin significantly prevented from junctional disruption and reduced gap

formation and F-actin redistribution. This suggests that 17-phenyl trinor-PGE_2 plays protective roles against thrombin-induced junctional disruption.

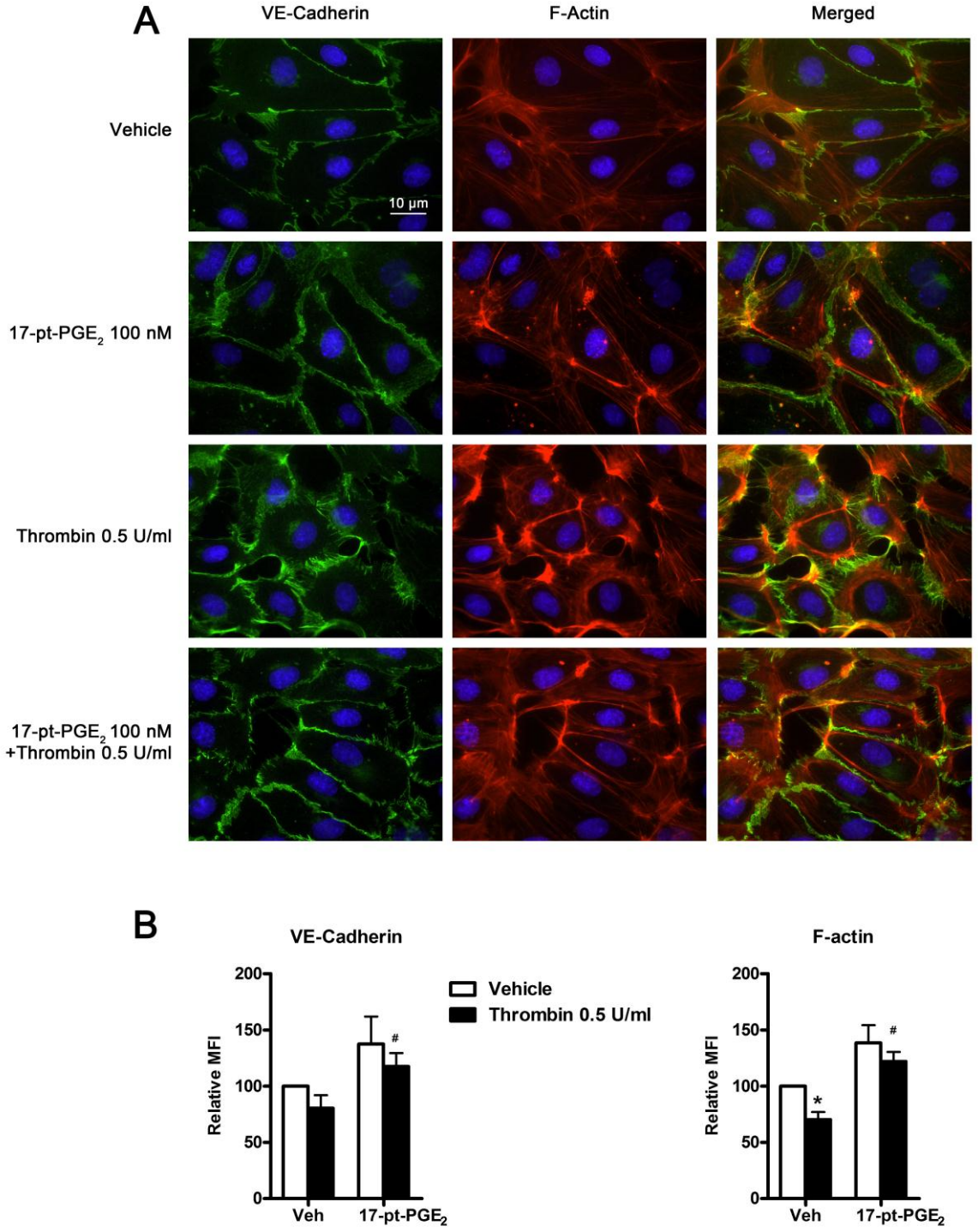

Figure 19. 17-phenyl trinor-PGE_2 prevents the thrombin-induced disruption of endothelial junctions. *HMVEC-Ls were cultured in chamber slides until confluence. Endothelial cells were pre-incubated with 100 nM 17-pt-PGE_2 for 15 min followed by exposure to vehicle or 0.5 U/ml thrombin and then (**A**) stained with anti-VE-cadherin antibody (green) or phalloidin-Texas Red-X conjugate (red) and DAPI (blue). (**B**) A region*

of interest (ROI) was selected on the fluorescence micrographs and mean fluorescence intensity (MFI) of all endothelial cells per image was determined. MFI of vehicle treated cells was assumed as 100%. MFI + SEM relative to control treatment (n=4). P and # < 0.05 versus vehicle /thrombin.*

4 Discussion

Endothelial dysfunction is a hallmark of various pathological conditions including inflammation, sepsis and acute lung injury. Substances which are involved in inflammatory processes might counteract the vascular leakage and reduce tissue damage. PGE_2 is the most abundant prostanoid in humans (Samuelsson et al., 2007). It acts through four different receptors (EP1-4), which are transmembrane G-protein coupled receptors (Norel, 2007). It was recently shown that PGE_2 can promote the endothelial barrier function via a signaling mechanism involving EP4 receptor. (Konya et al., 2012). EP4 receptor activation increases the cAMP level transiently and subsequently activates PKA (Tang et al., 2012). Interestingly, the barrier promoting effect of PGE_2 was independent of cAMP and PKA, and rather seemed to involve PKC/PI3K activation (Konya et al., 2012).

In this study we characterized the role of the EP1 receptor agonist 17-pt-PGE_2 on human pulmonary endothelial cells. For this purpose, we performed experiments with two different cells types, human pulmonary microvascular and pulmonary artery endothelial cells.

Expression of EP receptors in the endothelial cells was assessed by using flow cytometry. Our data suggest that pulmonary microvascular endothelial cells show higher expression of EP1, EP2 and EP3 receptors than pulmonary artery endothelial cells. However, both cell types express equal amounts of EP4 receptors. As a functional readout of EP receptor expression, we determined the intracellular Ca^{2+} release induced by specific EP1-4 receptor agonists and PGE_2 itself. It has been reported that the EP1 receptor agonist 17-pt-PGE_2 (Nicola et al., 2005), the EP3/EP1 receptor sulprostone (Ankorina-Stark et al., 1997) and PGE_2 itself (Watabe et al., 1993) are able to induce Ca^{2+} elevations in isolated rat cortical collecting duct seqments, human trophoblasts and HEK-293 cell, stably expressing EP1. Therefore, we were expecting similar results in our study. In pulmonary microvascular endothelial cells, 17-phenyl trinor PGE_2 elevated the intracellular Ca^{2+} release significantly, which indicated the importance of EP1 receptors. PGE_2 and EP4 receptor agonist CAY10598 induced a moderate Ca^{2+} signal, whereas EP2 agonist butaprost and notably, the EP3/EP1 receptor agonist sulprostone had no significant impact on intracellular Ca^{2+} release. Surprisingly, pulmonary artery endothelial cells did not show significant increase of intracellular Ca^{2+} levels after

stimulation with any of the selective EP1-4 agonists or PGE_2. This might be explained by the differences in EP receptor expression of the two cell types. In further experiments, the cellular localization of EP1 receptor in human pulmonary microvascular endothelial cells was visualized by using immunofluorescence microscopy. In pulmonary microvascular endothelial cells we could show that EP1 receptor is localized to the perinuclear space and the cytoplasm, while we would have expected EP1 expression in the cell membrane. Further experiments addressed the underlying signaling mechanism of the PGE_2 and 17-pt-PGE_2 induced Ca^{2+} release. Three EP1 receptor antagonists, which differ in structure (SC-51089, SC-51322 and ONO-8711) were used to prove EP1 receptor engagement. Unexpectedly, none of these selective EP1 receptor antagonists was able to prevent the 17-pt-PGE_2-induced intracellular Ca^{2+} release in the pulmonary microvascular endothelial cells.

A further interesting point was to determine if other available EP1 receptor agonists were able to mimic the intracellular Ca^{2+} elevation induced by 17-pt-PGE_2. For this purpose we used ONO-DI-004, which is known to be a more selective EP1 receptor agonist than 17-pt-PGE_2, and iloprost, an IP receptor agonist, which also shows EP1 receptor binding affinity (Woodward et al., 2011). In our hands, neither ONO-DI-004 nor iloprost appeared to have intracellular Ca^{2+} elevating activity. In contrast to our findings, 1 µM iloprost was shown to trigger intracellular Ca^{2+} release in another cell type, i.e. human immature megakaryocytes (Den Dekker et al., 2001). This might indicate that iloprost activates cell-specific signaling pathways, which are different in megakaryocytes and endothelial cells.

Since we could show that all specific EP1 receptor antagonists were not able to abate the 17-pt-PGE_2-induced Ca^{2+} release, we utilized a non-selective antagonist of EP1/EP2/EP3/DP receptors, AH 6809, which enabled us to clarify possible involvement of all these receptors at a time. AH 6809 did not inhibit the 17-pt-PGE_2-induced intracellular Ca^{2+} elevation but even increased the basal Ca^{2+} level. So far it is unclear, whether this was due to unspecific effects of the antagonist or blockade of an inhibitory receptor. Since neither selective EP1 receptor antagonists nor the non-selective antagonist inhibited the 17-pt-PGE_2-induced Ca^{2+} increase, and the EP4 receptor agonist CAY10598 induced also modest Ca^{2+} signal, we speculated for the possible role of EP4 receptor activation. The selective EP4 receptor antagonists GW 627368X and L-161,982 significantly reversed the 17-pt-PGE_2 induced intracellular Ca^{2+} release. Interestingly, a third EP4 antagonist, ONO AE3-208, was less effective at inhibiting this response which

could be explained by different binding sites of the antagonists. The EP4 antagonist L-161,982 also exerts affinity for other prostanoid receptors at the concentration we used. However, we can exclude this possibility, since the non-selective EP1/EP2/EP3/DP antagonist AH 6809 did not affect the 17-pt-PGE_2 induced Ca^{2+} release. Considering that GW 627368X also possesses affinity for the TP receptor, it can be argued that TP receptors are involved in the 17-pt-PGE_2 induced Ca^{2+} release. As we have not studied this issue, this point remains to be elucidated.

These data clearly suggest a novel pharmacological function for the purported EP1 agonist 17-pt-PGE_2. In detail, the intracellular Ca^{2+}-elevating activity of 17-pt- PGE_2 seems to depend on EP4 receptors but not on EP1 receptors in human pulmonary microvascular endothelial cells.

Since EP receptors belong to the family of the G-protein coupled receptors, we were eager to investigate the activated downstream signaling pathway (Norel, 2007). EP1 receptors were generally accepted to couple to Gαq-proteins. However, this fact has been seriously challenged, since the receptor activation leads to low IP_3 mobilization, which cannot account for the induced Ca^{2+} response, as determined via digital imaging microscopy in CHO cells stably expressing the EP1 receptor (Watabe et al., 1993). More recent studies have revealed that the EP1 receptor couples to an Pertussis toxin sensitive G protein and is also able to activate PKA independently from cAMP formation (Ji et al., 2010, 2012). Therefore, it remains controversial which G-protein is couples to the EP1 receptor. To address this issue we used the Gαq-protein inhibitor MH-362-63-8. EP4 receptors couple to Gαs-proteins which results a transient increase of cAMP and activation of PKA (Tang et al., 2012). Besides this classical signaling pathway, EP4 receptors show other signaling properties including the engagement of the Pertussis toxin-sensitive Gαi-protein (Fujino, 2005b). Pertussis toxin and the adenylate cyclase inhibitor SQ22536 were used to investigate which type of G-protein is involved. MH-362-63-8 completely and Pertussis toxin partially abolished the 17-pt-PGE_2 induced Ca^{2+} release, whereas the adenylate cyclase inhibitor SQ22536 had no impact on intracellular Ca^{2+} levels. These data suggest that 17-pt-PGE_2-induced Ca^{2+}release is dependent on Gαq- and possibly Gαi-protein signaling pathways. To determine the impact of PLC on the 17-pt-PGE_2-induced Ca^{2+} increase, we applied the PLC inhibitor U 73122, but in our hands, U 73122 showed unspecific effects in the Ca^{2+} assay, such as increase of Ca^{2+} in the absence of an agonist (data not shown). In order to investigate the role of IP_3 in the

17-pt-PGE_2-induced Ca^{2+} elevation, IP_3 receptor antagonists (2-APB) can be utilized in future experiments.

Since we found that the 17-pt-PGE_2-stimulated intracellular Ca^{2+} mobilization is dependent on EP4 receptors, we wanted to investigate whether co-incubation of 17-pt-PGE_2 and the EP4 receptor agonist CAY10598 has an additional effect on the intracellular Ca^{2+} elevation in terms of receptor internalization or receptor competition. It is evident in *Figure 11* that co-incubation of 17-pt-PGE_2 and CAY10598 had no effect on the Ca^{2+} levels elevated by 17-pt-PGE_2 on pulmonary endothelial cells.

We next decided to identify the source of intracellular Ca^{2+}-elevation stimulated by 17-pt-PGE_2 and PGE_2 in the microvascular endothelial cells. Intracellular free Ca^{2+} is generally mobilized from endoplasmic reticulum or owes to influx from extracellular space. To address this issue the endothelial cells were incubated with EGTA, a Ca^{2+} chelating substance which binds extracellular Ca^{2+}. Histamine, which is known to be able to induce Ca^{2+} release from the endoplasmic reticulum, was used as positive control. Our data indicate that the intracellular Ca^{2+} increase induced by 17-pt-PGE_2 and PGE_2 is derived from extracellular space, since it was completely abolished by EGTA. In contrast to that, intracellular Ca^{2+} increase induced by histamine was hardly affected by EGTA, indicating involvement of the endoplasmic reticulum. In summary, out data indicate that 17-pt-PGE_2 can significantly increase intracellular Ca^{2+} levels in microvascular endothelial cells, which effect seems to be mediated by EP4 receptors and not by EP1 receptors. Gαi- and Gαq-proteins are most probably involved, since Pertussis toxin and MH-362-63-8 could abate the increase of Ca^{2+}, whereas inhibition of adenlyate cyclase had no impact. Restriction of extracellular Ca^{2+} by EGTA completely abolished the intracellular Ca^{2+} mobilization. We succeeded in revealing some parts of the 17-pt-PGE_2 signaling on microvascular endothelial cells, while the exact molecular mechanism awaits further investigations.

In the second part of my thesis, I analyzed the regulatory role of 17-pt-PGE_2 on the endothelial barrier function, since PGE_2 has been recently described as enhancer of endothelial barrier via activating EP4 receptors (Konya et al., 2012). Endothelial electrical resistance was recorded by an Electric Cell-substrate Impedance Sensing (ECIS) device, as a readout of endothelial barrier function. 17-pt-PGE_2, similar to PGE_2 (Konya et al., 2012), enhanced the endothelial resistance concentration-dependently. Highest concentration (1000 nM) of 17-pt-PGE_2 resulted in an increase by approximately 40% of baseline resistance. We observed approximately 30% barrier

enhancement upon 100 nM 17-pt-PGE_2 treatment; in comparison, 100 nM of the EP4 agonist CAY10598 induced 50% increase in endothelial resistance relative to baseline. Application of specific antagonists for EP1 (SC-51322 and ONO-8711) and EP4 receptors (ONO AE3-208 and L-161,982) revealed that the 17-pt-PGE_2-induced promotion of endothelial barrier function is mediated by EP4 receptors and not by EP1 receptors. In 2001, Takeuchi and colleagues have shown that, 17-phenyl trinor-PGE_2 is able to prevent hydrochloric acid/ethanol-induced gastric mucosal disruption, which could be inhibited by EP1 receptor antagonist ONO-AE-829 in rats (Takeuchi et al., 2001). This study supports our findings that 17-pt-PGE_2 might serve as barrier protective substance, however, for epithelial cells. In contrast to our results this study proved that the cytoprotective effect of 17-pt-PGE_2 was mediated by EP1 receptors. Therefore, it seems likely that 17-pt-PGE_2 exerts its effects through different receptors in different tissues and cell types. Another study showed that 17-pt-PGE_2 did not promote skin healing after scratch-induced epithelial barrier disruption (Honma et al., 2005).

Our next aim was to establish a possible connection between the 17-pt-PGE_2-induced Ca^{2+} mobilization and the protective effect on the endothelial barrier function. Ca^{2+} mobilization is a two-edged sword in context with endothelial barrier function. In quiescent endothelial cells Ca^{2+} is kept in low concentrations due to specific Ca^{2+} ATPases, which remove Ca^{2+} from the cytosol (Moccia, 2012). Both, histamine and thrombin activate Gαq protein-coupled receptors, which leads to IP_3-mediated release of Ca^{2+} (Yuan and Rigor, 2010). Likewise, S1P induces an increase of intracellular Ca^{2+} levels, relying on PLC activation and IP_3 formation (Thennes and Mehta, 2012). Altough these two signalling pathway result in an increase of intracellular Ca^{2+} levels, they show a complete contrary outcome with regard to endothelial barrier function (Thennes and Mehta, 2012). Intracellular Ca^{2+} increase induced by histamine or thrombin results in hyperpermeability of blood vessels, due to the activation of RhoA, whereas S1P induced Ca^{2+} elevation exerts endothelial barrier promoting features via Rac-1 (Yuan and Rigor, 2010). Further studies are needed to clarify the distinct role of Ca^{2+} elevation in these two completely opposite processes (Thennes and Mehta, 2012). Therefore, we were interested to see whether the intracellular Ca^{2+} mobilization, induced by 17-pt-PGE_2 has any impact on the 17-pt-PGE_2 mediated promotion of the endothelial barrier function. To this end, we applied the Ca^{2+} chelating substance EGTA, since we could show that EGTA completely abolished the 17-pt-PGE_2 induced Ca^{2+} elevation. Although EGTA pre-incubation resulted in a dramatic drop of endothelial baseline resistance, it had no

impact on the barrier enhancement induced by 17-pt-PGE_2. Additionally, we used membrane permeable forms of EGTA and BAPTA, i.e. EGTA-AM and BAPTA-AM, respectively, which were likewise unable to prevent the barrier-promoting effect of 17-pt-PGE_2 or CAY10598. Hence, we could show that Ca^{2+} mobilization is not involved in the barrier-promoting effect of 17-pt-PGE_2 or CAY10598.

We further tested whether the EP1 receptor agonist ONO-DI-004 can mimic the 17-pt-PGE_2 induced enhancement of endothelial barrier function. Although, we used similar concentrations of ONO-DI-004 as for 17-pt-PGE_2, we observed no increase in the endothelial resistance after ONO-DI-004 treatment.

Additionally, we visualized the morphological changes of microvascular endothelial cells upon treatment with 17-pt-PGE_2. In immunofluorescence staining experiments, pharmacological disruption of endothelial cell junctions by thrombin was assessed to mimic inflammatory conditions. Treatment of endothelial cells with 0.5 U/ml thrombin led to formation of paracellular gaps and furthermore the appearance of actin stress fibers and disruption of endothelial adherens junctions. This effect could be effectively prevented by the addition of 17-pt-PGE_2 suggesting a protective role of 17-phenyl trinor PGE_2 in thrombin-induced junctional disruption, which effect was comparable to that of PGE_2 and EP4 receptor agonist ONO AE1-329 (Konya et al., 2012).

In conclusion, we could show that 17-pt-PGE_2 exerts endothelial barrier promoting effects which were reversed by EP4 but not EP1 receptor antagonists. Although, the EP4 receptor seems to mediate 17-pt-PGE_2 induced Ca^{2+} influx and the promotion of endothelial barrier function, we were unable to reveal a link between these two responses. The biological function of the 17-pt-PGE_2-mediated elevation of intracellular Ca^{2+} levels remain to be elucidated. Further, studies should compare the binding properties of 17-pt-PGE_2 to EP1 and EP4 receptors, in order to substantiate our findings of EP4-mediated effects of 17-pt-PGE_2 in HMVEC-L. It was shown, that 17-pt-PGE_2 exhibits considerable affinity for the EP4 receptor in HEK-293 cells, with Ki values of 34.5 nM (Davis and Sharif, 2000) and furthermore is able to activate a pertussis-toxin sensitive G-protein via the EP4 receptor, which might indicate that 17-pt-PGE_2 as biased ligand for the EP4 receptor (Leduc et al., 2009). Hypothetically, the structure of 17-phenyl trinor-PGE_2 appears to be quite similar to PGE_2 and the binding affinity of PGE_2 to EP4 receptor is 10 times higher than for EP1 (Alfranca et al., 2006; Woodward et al., 2011).

Endothelial cells build a strict barrier between the blood flow and the interstitial space, in order to maintain the electrolyte and fluid homoeostasis and thereby sustain the physiological functions of the surrounding tissues. The integrity of the endothelial barrier function is regulated by the coordinated opening and closure of adherens junctions and, furthermore, tight junctions are involved in the preservation of cell-cell contacts. Endothelial dysfunction is a hallmark of various pathological states, including inflammation, sepsis and acute lung injury. Research from the last years contributed enormously to our knowledge about the pathological mechanisms underlying endothelial dysfunction. Substances, which are released during inflammatory processes, such as S1P, PGI_2 and PGE_2 exert barrier promoting effects. In this study, we could show that the purported EP1 receptor agonist 17-pt-PGE_2 possesses barrier enhancing properties in microvascular endothelial cell, which are mediated by the EP4 receptor. The dual action of 17-pt-PGE_2 as an EP1/EP4 agonist might be a promising novel therapeutic approach in vascular inflammatory diseases.

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