Dissertation

PGI_2 and the PGE_2-EP4 signaling control the transendothelial trafficking of eosinophils

submitted by

M.Sc.

Viktoria KONYA

for the Academic Degree of

Doctor of Philosophy

(Ph.D.)

at the

Medical University of Graz

Institute of Experimental and Clinical Pharmacology

under Supervision of

Univ. Prof. Dr. Akos HEINEMANN

2010

Table of contents

ABSTRACT

Enhanced eosinophil extravasation into the tissue is a characteristic feature of bronchial asthma and other allergic inflammatory diseases. The process of eosinophil extravasation involves sequential interaction steps with the vessel wall forming endothelial cells. The vascular endothelial cells assure the barrier between the circulating blood cells and the surrounding tissue. However, during asthma, various inflammatory mediators can induce a transient vascular leakage, which is prolonged by the contribution of activated leukocytes at later stages of the inflammatory response and eventually results in dramatic remodeling of the microvasculature and the tissue. Prostaglandin I_2 (PGI_2, prostacyclin) is the major prostanoid released by endothelial cells. Prostaglandin E_2 (PGE_2) is the predominant cyclooxygenase (COX) product of airway macrophages, epithelial cells and smooth muscle cells. Earlier studies showed that COX-1 or COX-2 deficiency in mice significantly increased lung inflammation and airway hyperresponsiveness in ovalbumin-induced asthma model. I-type-prostanoid receptor (IP)-deficient mice showed the enhancement of allergic inflammation in the airway and skin associated with the increases in vascular permeability and augmentation of Th2 response. Conversely, PGE_2 was found to exert bronchoprotective effect in patients with bronchial asthma. Thus, the aim of this thesis was to define the role of PGI_2 and PGE_2 in the regulation of eosinophil trafficking across endothelial monolayers, respectively the inflammatory functions of eosinophils and the barrier functions of endothelial cells.

The first part of the thesis describes the findings on the regulatory role of PGI_2 in endothelial-eosinophil interactions, starting with the inhibitory effect of PGI_2 on eosinophil functions. The *in vitro* chemotaxis of human peripheral blood eosinophils was markedly attenuated by exogenous PGI_2. This effect was prevented by the IP receptor antagonist Cay10441 and the adenylyl cyclase inhibitor SQ29548. Expression of IP receptor on eosinophils was demonstrated by indirect flow cytometry and also by Western blot. PGI_2 reduced eosinophil adhesion to fibronectin, inhibited the activation and the up-regulation of the CD11b/CD18 adhesion molecule, and blocked podosome formation in response to eotaxin. In terms of interaction with lung microvascular endothelial cells, eosinophil adhesion to endothelial cells was enhanced upon blocking the endogenous PGI_2 release of endothelial cells using a non-selective COX inhibitor,

diclofenac. Transendothelial migration of eosinophils was similarly augmented by diclofenac. Furthermore, abolished PGI_2 production itself decreased the electrical resistance of endothelial monolayers and disrupted the intercellular junctions as visualized by VE-cadherin and F-actin staining.

In the second part I focused on the endothelial barrier-protective role of PGE_2. It was found that the effects of PGE_2 on the endothelium were mainly mediated by the activation of the EP4 receptor subtype. Correspondingly, I found that EP4 receptors are expressed on human pulmonary microvascular cells. PGE_2 and the selective EP4 receptor agonist (ONO-AE1-329) prevented the barrier-disrupting effect of thrombin on the endothelial monolayer, as visualized by VE-Cadherin staining. Selective blocking of EP4 receptors by an EP4 antagonist (ONO-AE3-208) abolished the protective effect of PGE_2 on endothelial monolayer. PGE_2 and the EP4 agonist accelerated the regeneration of electrically wounded endothelial monolayers. Specificity of EP4 receptor involvement was proved by using the EP4 receptor antagonist, which prevented the protective effect of PGE_2, and by selective agonists for EP2 and EP3 receptors. butaprost and sulprostone, respectively, which lacked any effect on endothelial cells. PGE_2 and the EP4 receptor agonist attenuated the TNFα-induced up-regulation of E-selectin. Surprisingly, this effect was not rescued by an adenylyl cyclase inhibitor, however, inhibition of PKC activity reversed the effect of the EP4 receptor agonist and PGE_2, at reducing E-selectin expression. Eosinophil transmigration across the thrombin-stimulated endothelial cells was also effectively reduced by pre-treatment of the endothelial monolayers with the EP4 agonist. In further experiments I could demonstrate that eosinophils themselves express EP4 receptors. PGE_2 and the selective stimulation of EP4 receptor attenuated eosinophil chemotaxis, prevented the activation and cell surface clustering of CD11b/18 integrins on eosinphils. Morphological studies revealed that formation of eosinophil adhesive structures was also significantly reduced by PGE_2 and EP4 agonist treatment of eosinophils.

Based on these observations, PGI_2 and the PGE_2 – EP4 signaling axis might be important protective factors in keeping inappropriate eosinophil infiltration under control and might modulate allergic responses by inhibiting eosinophil responsiveness to chemoattractants in terms of adhesion and migration. Furthermore, IP and EP4 receptor activation strengthens the barrier function of the endothelium. Therefore, IP

and EP4 agonists might be useful therapeutic options for otherwise inadequately controlled inflammation in eosinophilic diseases.

ZUSAMMENFASSUNG

Ein charakteristisches Merkmal von Asthma bronchiale und anderen allergisch-entzündlichen Reaktionen ist eine verstärkte Einwanderung von Eosinophilen in das Gewebe. Die Auswanderung der Zellen aus dem Blut bedingt eine Interaktion mit den Endothelzellen der Blutgefäße, die eine natürliche Barriere zwischen zirkulierenden Blutzellen und dem umgebenden Gewebe bilden. Bei Asthma bronchiale können jedoch verschiedene inflammatorische Mediatoren einen transienten vaskulären "Leck" bewirken, der durch Beteiligung aktivierter Leukozyten in späteren Stadien der Entzündungsreaktion verstärkt wird. Schließlich kommt es zu einer drastischen Umgestaltung der Gefäß- und Gewebestrukturen. Ein hauptsächlicher Mediator, der von Endothelzellen freigesetzt wird, ist Prostaglandin I_2 (PGI_2, Prostacyclin). Prostaglandin E_2 (PGE_2) wird von Cyclooxygenasen (COX) in Makrophagen der Luftwege, Epitethelzellen und glatten Muskelzellen gebildet. Frühere Studien zeigten, dass COX-1- oder COX-2-defiziente Mäuse mit Ovalbumin-induziertem Asthma eine signifikant verstärkte Entzündung der Lunge sowie eine Überempfindlichkeit der Luftwege aufwiesen (20). IP-defiziente Mäuse zeigten eine verstärkte allergische Enzündung der Luftwege und der Haut, begleitet von einer erhöhten vaskulären Permeabilität und einer gesteigerte Th2 Antwort. Im Gegensatz dazu wurde gezeigt, dass PGE_2 einen bronchoprotektiven Effekt in Patienten mit bronchialem Asthma ausübt. Demzufolge war das Ziel dieser Arbeit, die Rolle von PGI_2 and PGE_2 in der Regulation der Migration von Eosinophilen durch die Endothelschicht, bzw. die inflammatorische Funktion von Eosinophilen sowie die Barriere Funktion von Endothelzellen zu definieren.

Der erste Teil der Arbeit beschreibt die Befunde über die regulatorische Rolle von PGI_2 in Endothel-Eosinophil Interaktionen, beginnend mit dem inhibitorischen Effekt von PGI_2 auf die Funktion von Eosinophilen. Chemotaxis von humanen Eosinophilen im peripheren Blut *in vitro* wurde durch exogenes PGI_2 deutlich geschwächt. Dieser hemmende Effekt wurde durch den IP Rezeptor-Antagonisten Cay10441 und den Adenylatcyclase Inhibitor SQ29548 aufgehoben. Die Expression des IP Rezeptors in Eosinophilen wurde mittels indirekter Immunfärbung in der Durchflusszytometrie und Western Blot nachgewiesen. PGI_2 reduzierte die Adhäsion von Eosinophilen an Fibronection, inhibierte die Aktivierung und Aufregulation des Adhäsionsmoleküls

CD11b/CD18 und blockte die Eotaxin-induzierte Formation von Podosomen. Hinsichtlich der Interaktion mit mikrovaskulären Lungenendothelzellen wurde die Adhäsion von Eosinophilen an das Endothel durch das Fehlen von PGI_2 verstärkt, hervorgerufen durch Hemmung der endogenen PGI_2 Synthese in Endothelzellen mittels des unselektiven COX Inhibitors Diclofenac. Transendotheliale Migration von Eosinophilen wurde durch Diclofenac ähnlich verstärkt. Darüber hinaus verminderte eine gehemmte PGI_2 Synthese *per se* den elektrischen Widerstand der Endothelschicht und zerstörte die interzellulären Verbindungen, wie mit VE-Cadherin und F-Actin Färbungen gezeigt werden konnte.

Im zweiten Teil fokussierte ich den protektiven Effekt von PGE_2 auf die Barrierefunktion des Endothels. Es wurde gezeigt, dass die Effekte von PGE_2 auf das Endothel hauptsächlich durch Aktivierung des EP4 Rezeptor Subtyps vermittelt werden. Dementsprechend konnte ich die Expression von EP4 Rezeptoren in humanen pulmonalen mikrovaskulären Zellen nachweisen. PGE_2 und der selektive EP4 Rezeptor-Agonist (ONO-AE1-329) verhinderten den Barriere-zerstörenden Effekt von Thrombin auf die Endothelschicht, wie mit VE-Cadherin Färbungen gezeigt werden konnte. Eine selektive Hemmung des EP4 Rezeptors durch einen EP4 Antagonisten (ONO-AE3-208) hob den protektiven Effekt von PGE_2 auf das Endothel auf. PGE_2 und der EP4 Agonist beschleunigten die Regeneration einer mittels Elektroschock ge störten Endothelschichten. Die Spezifität der EP4 Rezeptorbeteiligung wurde mit einem EP4 Rezeptor-Antagonisten bewiesen, der den protektiven Effekt von PGE_2 verhinderte, sowie durch selektive Agonisten der EP2 and EP3 Rezeptoren, Butaprost und Sulprostone, die keinerlei Effekt auf Endothelzellen zeigten. PGE_2 und der EP4 Rezeptor-Antagonist verminderten die TNFα-induzierte Aufregulation von E-Selectin. Überraschenderweise wurde dieser Effekt nicht durch einen Adenylatcyclase-Inhibitor wiederhergestellt. Im Gegensatz kehrte die Inhibierung der PKC Aktivität den inhibitorischen Effekt des EP4 Rezeptor-Agonisten und PGE_2 auf die E-Selectin Expression um. Transmigration von Eosinophilen durch Thrombin-stimulierte Endothelzellen wurde außerdem durch Vorbehandlung des Endothels mit dem EP4 Agonist reduziert. In weiteren Versuchen konnte ich die Expression des EP4 Rezeptors in Eosinophilen und Endothelzellen nachweisen. PGE_2 und die selektive Stimulierung des EP4 Rezeptors verhinderten die Aktivierung sowie das Clustering der CD11b/CD18 Integrine in Eosinophilen. Morphologische Studien belegten, dass die Bildung von

Adhäsionsstrukturen in Eosinophilen durch PGE_2 und EP4 Agonisten signifikant vermindert wurde.

Diese Befunde stützen die Annahme, dass PGI_2 und PGE_2 – EP4 Signalwege wichtige protektive Faktoren sind, die die Infiltration von Eosinophilen in das Gewebe kontrollieren und dadurch allergische Reaktionen modulieren. Dies geschieht indem sie die Reaktivität von Eosinophilen gegenüber chemotaktisch aktiven Substanzen eingrenzen und somit Adhesion and Migration dieser Blutzellen inhibieren. Darüber hinaus stärkt die Aktivierung der IP und EP4 Rezeptoren die Barrierefunktion des Endothels. Demzufolge könnten IP and EP4 Agonisten sinnvoll für die Therapie bisher unzureichend kontrollierter Entzündungsreaktionen mit Beteiligung von Eosinophilen eingesetzt werden.

I. INTRODUCTION

1. PROSTANOIDS

The large group of eicosanoids, including prostaglandins (PGs) and thromboxanes (TXs), and leukotrienes (LTs), are derived from 20 carbon unsaturated fatty acids, the most prominent of which is arachidonic acid (AA). AA is usually found in phosphoglycerides of mammalian cell membranes and is released by phospholipases. Prostanoid synthesis starts with the formation of cyclic peroxides named endoperoxides from poly-unsaturated fatty acids. PGH_2 is the common endoperoxide intermediate during biosynthesis of prostanoids derived from AA. Conversion of AA into PGH_2 is a reaction catalyzed by COX (cyclooxygenase, also called PGH-synthase) in two steps. First, AA is converted to the endoperoxide PGG_2 (cyclooxygenase reaction) wich is then reduced to PGH_2 (peroxidase reaction). After synthesis of PGH_2, it is converted into the other prostanoids by specific synthases. In general, this process is cell specific with different cells producing only one of the major prostanoids in abundance. PGE_2 and PGD_2 are position isomers, which are formed by simple nonoxidative rearrangements (isomerizations) of PGH_2. These reactions may occur either spontaneously, at least *in vitro*, or enzymatically catalyzed by specific synthases. PGF_{2a} is formed through a two-electron reduction of PGH_2 by PGF synthase The enzyme catalyzing the conversion of PGH_2 into TXA_2 is TXA synthase (TXAS). PGI_2 is formed from PGH_2 by means of PGI_2 synthase (PGIS, prostacyclin synthase). Two types of PGD synthase, hematopoietic and lipocalin-type PGD synthase, have been described and can be found in high amounts in mast cells and macrophages, and the central nervous system, respectively. Microsomal PGE synthase (mPGES) appears in most cell types, but is particularly abundant in airway epithelial cells, and is responsible for PGE_2 synthesis. PGF synthase is expressed mostly in the uterus, and thromboxane synthase is present in platelets, endothelial cells and macrophages (Smith, 1992).

Two COX isozymes, called COX-1 and COX-2, are encoded by separate genes located in human chromosome 9 and 1, respectively. In general COX-1 can be viewed as a constitutive enzyme whose expression appears to be regulated developmentally. Prostanoids formed through the action of COX-1 mediate so-called ‘‘housekeeping’’ functions, such as regulation of renal function, and maintain integrity

of gastric mucosa and hemostasis. In contrast, COX-2 is an inducible enzyme that is normally absent from cells but is expressed transiently in response to different stimuli. The best-studied inducers of COX-2 are bacterial LPS and proinflammatory cytokines, such as TNF-α and IL-1β. Growth factors and some tumor promoters, such as PMA, also stimulate COX-2 expression in various cell types .

1.1. Prostanoid receptors

Prostanoids normally act in an autocrine or a paracrine fashion, by binding to specific receptors on the target cells. Most bind to and activate seven transmembrane G protein-coupled receptors: TP for TXA_2; EP1-4 for PGE_2; IP for PGI_2; DP_1 and $CRTH_2$ for PGD_2; and FP for PGF_{2a}. Some prostanoids or their metabolites also recognize nuclear receptors of the PPAR family. Transmembrane prostanoid receptors have been classically grouped into three classes, depending on the type of G protein they bind to: the contractile receptors EP1, FP and TP are coupled to Gq and activate PLC, leading to free intracellular Ca^{2+} increases; the relaxant type, which includes IP, DP1, EP2 and EP4, signal through Gs to induce adenylyl cyclase activity; and the inhibitory receptor EP3 couples to Gi, leading to a decrease in intracellular cAMP content (Funk, 2001). This preferential coupling of each prostanoid receptor to a specific G-protein, with the subsequent activation of a definite signal transduction pathway, creates an additional regulatory level for the biological activities of these molecules in any given environment. This is particularly important for those prostanoids that can bind to multiple receptors or receptor isoforms, and are therefore able to activate diverse G-proteins and signal transduction pathways. The differential expression of these receptors in several cell types, or the modulation of their expression in a particular cell, can give rise to a wide array of complex biological responses to a single prostanoid.

Signals triggered by prostanoids are mostly terminated by agonist-induced homologous desensitisation and internalisation of their receptors. Homologous desensitisation is achieved through receptor phosphorylation by Ser/Thr kinases activated by ligand binding, such as GRKs, PKC or PKA. This phosphorylation leads to the uncoupling of G protein subunits from the receptor, and in some cases the phosphorylated receptor binds to proteins of the arrestin family, and is then internalised via chlathrin-coated pits . This process has been suggested to be a means by which

receptors are dephosporylated and recycled to the membrane. In addition to homologous desensitisation, some prostanoid receptors can induce heterologous desensitisation of other receptors through the activation of second-messenger kinases such as PKA and PKC .

Prostanoids are involved in a variety of clinically important areas such as inflammation, fever, thrombosis, cancer and allergic responses.

Figure 1.

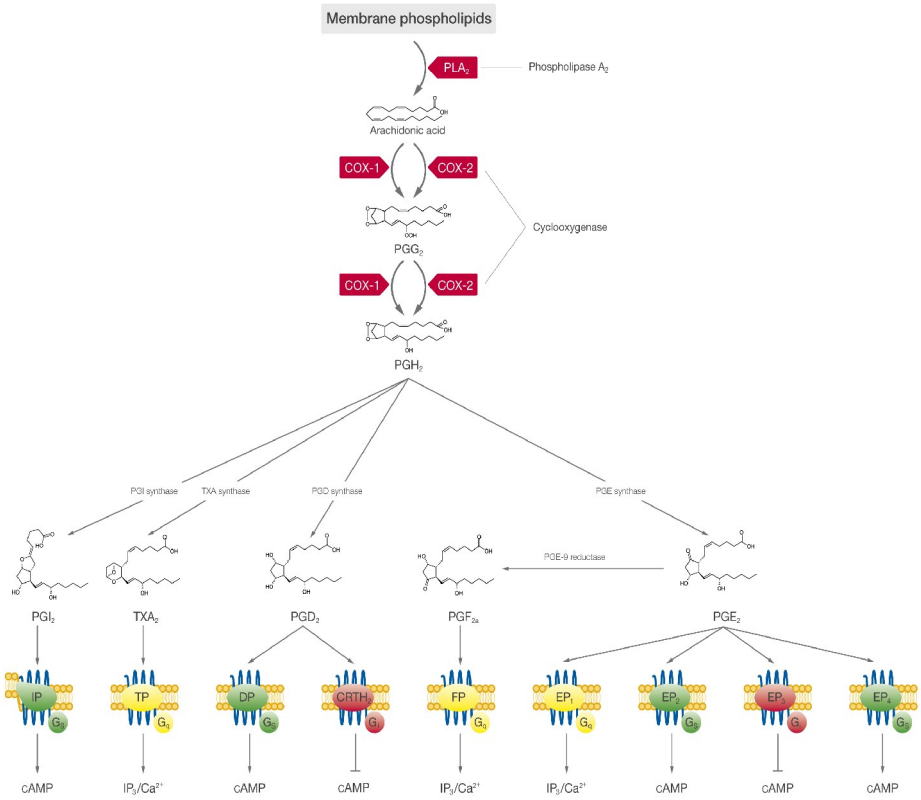

Figure 1. Prostanoid biosynthesis, receptors and signaling pathways. *Arachidonic acid is liberated from membrane phospholipids by phospholipase A_2 enzyme. Arachidonic acid as the substrate of cyclooxygenase 1 and 2 enzymes is converted to the endoperoxide PGG_2 and reduced to PGH_2. Finally, prostanoids are formed from PGH_2 by specific terminal synthases and activate their corresponding receptors: I-type prostanoid receptor (IP), TP, FP, DP, chemoattractant receptor homologous molecule expressed on Th2 cells (CRTH2) and four isotypes of EP receptors. These receptors are coupled to different G proteins, which lead to subsequent activation of a specific signal transduction pathway.*

2. BRONCHIAL ASTHMA

2.1 Pathology of asthma

Asthma, an inflammatory disorder that was first described by Hippocrates in the fourth century B.C. as "to breathe hard or pant", has now become a devastating social and economic burden to most countries' health care systems, with millions of asthma-afflicted individuals worldwide. Bronchial asthma is a disease which is both polygenic and multi-factorial in nature. Various genetic factors, coupled with environmental stimuli interact in susceptible individuals to produce the phenotype. Asthma is a disease of chronic airway inflammation characterized by reversible airway obstruction, airway hyperresponsiveness (AHR), infiltration of eosinophils and T-helper type 2 (Th2) lymphocytes into the airway submucosa, mucus hypersecretion, and airway remodelling (Broide, 2001). Allergic asthma is classified as a type 1 hypersensitivity reaction. Locally produced chemokines stimulate the recruitment of eosinophils, macrophages, neutrophils, and T lymphocytes (Broide, 2001). Once present, effector cells such as eosinophils release a collection of toxic granules that in turn cause prolonged bronchoconstriction and damage epithelial layers. Cytokines such as IL-4, IL-5, IL-6, and IL-13 ensure that this cycle of allergic inflammation persists. Additionally, asthma can develop in the absence of allergic sensitization, following viral or bacterial infections of the respiratory tract, smoking, air pollution, emotional disstress or physical exercise. These forms of asthma have been referred to as “intrinsic” asthma, as they share both pathophysiological and clinical features of allergic asthma, but without being tied to allergen exposure.

2.2. Prostaglandins in allergic inflammation, asthma

In asthmatic airways, COX-2 gene expression is increased, which suggests involvement of COX products in the pathogenesis of this disease . Interestingly, there is an exaggeration of airway eosinophilia, IgE production, and airway hyperresponsiveness in both COX-1- and COX-2-deficient mice . Moreover, inhibition of COX with the nonselective COX inhibitor indomethacin augments ovalbumin (OVA)-induced allergen airway eosinophilia, Th2 type cytokine production, and airway hyperresponsiveness in a mouse model of allergic asthma). All of these data suggest

that endogenous PGs play a suppressive role in the regulation of asthmatic response by COX-2 expression and production of key products. However, the involvement of COX in the pathognesis of asthma is controversial, since some PGs have pro-allergic inflammatory activity, whereas others have anti-allergic activities, and little is understood about the relationship between pro- and anti-asthmatic prostanoids. Most likely, there is a regulated balance between the bronchoconstriction and bronchodilation action of various PG that contributes to bronchial tone, possibly mediated by a balance between PGD_2 and PGE_2.

2.3. Role of PGI_2 in the pathogenesis of asthma

PGI_2 is produced during the allergic reaction in human lung and in murine airway after OVA inhalation . PGI2 inhibits allergic mediator release and eosinophil recruitment in experimental animals (Burka and Garland, 1977). In OVA-induced asthma model, selective inhibition of COX-2 specifically reduces PGI_2 synthesis and results in a marked increase in Th2-mediated lung inflammation. The elevated Th2-mediated inflammatory response elicited by selective COX-2 inhibitors is associated with enhanced airway hyperreactivity and is coincident with a marked increase in the levels of Th2 type of cytokine, including IL-4, IL-5, and IL-13 in the airways . In contrast to the proinflammatory effects of prostacyclin, which are important for the generation of edema and pain accompanying inflammation, these findings suggest that PGI_2 may play a role in inhibiting Th2 inflammatory response. Interestingly, IP receptor mRNA is up-regulated in $CD4^+$ Th2 cells , and IP-deficient mice showed the augmentation of allergic inflammation in the airway and skin, associated with the increases in vascular permeability and enhancement of Th2 response . Recently, IP receptor has been shown to be involved in airway remodeling in chronic allergen challenge model. Additionally, IP-deficient mice have more goblet cell hyperplasia and subepithelial fibrosis compared with wild-type mice .

2.4. Role of PGE_2 in the pathogenesis of asthma

There is a controversy regarding the role of PGE_2 in asthma because of contradicting outcomes of *in vitro* and *in vivo* studies. In *in vitro* studies, PGE_2 appears to polarize cellular response toward a Th2 phenotype enhancing IL-4 and IL-5 production and inhibiting of macrophage IL-12 production. Despite of these *in vitro* data, it has been suggested that PGE_2 has a bronchoprotective effect in patients with bronchial asthma. PGE_2 has been shown to protect against exercise-induced , allergen-induced , and aspirin-induced bronchoconstriction as well as bronchoconstrictor agents such as methacholine and histamine . PGE_2 not only prevents allergen-induced bronchoconstriction but also ameliorates allergen-induced airway inflammation, including decreased airway eosinophilia in asthma patients and Th2 cytokine production in the OVA-induced murine model . COX-1 deficient mice exhibited significantly increased lung inflammation and airway hyperresponsiveness in OVA-induced asthma model, which is correlated with abrogation of PGE_2 biosynthesis . More specifically, PGE2 modulates the function of T cells and macrophages, which are critical for the immune response. There is little known about interaction between anti-inflammatory PGE_2 and pro-inflammatory PGD_2. Both PGs seem to have an opposite role in terms of allergic inflammation. It is suggested that PGE_2 overrides the pro-asthmatic properties of PGD2. Nebulized PGE_2 administered before allergen challenge attenuates the early asthmatic reaction, which may be via its action on down-regulation of PGD_2 in bronchoalveolar lavage fluid (BAL-fluid) .

3. ENDOTHELIUM BARRIER FUNCTION AND INFLAMMATION

The endothelium is the thin layer of cells that form the inner surface of blood vessels, providing a barrier between the enclosed circulating blood in the lumen and the surrounding tissue. Endothelial cells line the whole circulatory system, from the heart to the smallest capillary. Endothelial tissue is a specialized type of epithelium tissue; more specifically, it is a simple squamous epithelium with the increased presence of vimentin rather than keratin filaments which are typical in epithelial cells. Endothelium is hence a thin membranous tissue that consists almost exclusively of cells. It appears in its most isolated form in the capillary blood vessels, the walls of which are simply tubes of endothelium. The endothelium does not form a passive barrier but actively controls the extravasation of blood components and blood cells to the surrounding tissues. Even though endothelial cells from different vascular beds have many features in common and originate from the same embryonic precursor cells, the hemangioblasts, variations in permeability have been reported in experiments focusing on different regions of the endothelium, where microvessels showed 10-fold less permeability in comparison to macrovascular endothelial cells . Under pathological conditions, vascular permeability can increase. Many diseases share the common feature of vascular leakage. Vascular leakage is often a negative side effect of a disease worsening the patient's condition. Increased vascular leakage contributes to the pathogenesis of numerous, often life-threatening disorders. Excessive plasma extravasation often contributes to acute obstruction of respiratory airways during asthma and other pulmonary diseases (Groeneveld, 2002).

3.1. Vascular leakage in inflammation

Depending on the severity and duration of an inflammatory reaction, three major steps contribute to initial and prolonged vascular leakage. At an early stage of inflammation, various inflammatory mediators induce a transient vascular leakage. Intracellular actin–myosin interaction and loss of junctional integrity are crucial processes in enhancing endothelial permeability and cause the formation of small gaps between the endothelial cells, in particular, in postcapillary venules. Subsequently, activated leukocytes contribute to prolongation of vascular leakage. Both their

interactions with the endothelium and their released products act further on the endothelium. If the inflammatory stimuli persist, the microvasculature may become subject to remodeling initiated by angiogenic factors. Angiogenic growth factors affect the integrity of the cell junctions by induction of endothelial migration. Moreover, they are capable of inducing a hyperpermeable status, increased permeability of the endothelium by themselves. Vascular endothelial growth factor (VEGF) has been suggested to particularly induce vascular hyperpermeability. Angiogenesis has been described as a major factor in airway remodeling (Aceves and Broide, 2008).

3.2. Regulation of endothelial permeability via junctions and matrix interactions

The interendothelial junctional proteins and integrin receptors provide the endothelial monolayer with adhesive strength to resist separation of cells from the substrate and adjacent cells through their link with the actin cytoskeleton. Endothelial cells are connected to each other by a complex set of junctional proteins that comprise adherens junctions (AJs), tight junctions (TJs), and gap junctions (GJs). While GJs form transmembrane channels between adjacent cells, TJs and AJs form pericellular zipper-like structures along the cell border through their transmembrane homophilic interaction.

3.2.1. Adherens junctions (AJs)

Endothelial adheres junction s (AJs) contain vascular endothelial (VE)-cadherin as the major structural protein that mediates homophilic binding and adhesion of adjacent cells in a Ca^{2+}-dependent manner. The extracellular domain of VE-cadherin contains five cadherin-like repeats that oligomerize to form *cis*-oligomers (on the same cell) and *trans*-oligomers (homophilic interaction with the neighboring cell). The cadherin cytoplasmic tail contains two functional domains: the juxtamembrane domain (JMD) and the COOH-terminal domain (CTD). These domains interact with three related proteins: β-catenin, plakoglobin (λ-catenin), and p120-catenin. JMD of VE-cadherin binds p120-catenin, whereas CTD binds β-catenin or plakoglobin in a mutually

exclusive fashion. β-catenin or plakoglobin then binds α-catenin, which links the cadherin-catenin complex to the actin cytoskeleton (Bazzoni and Dejana, 2004). VE-cadherin is required for the proper assembly of AJs and development of normal endothelial barrier function. Deletion of VE-cadherin in mice is embryonically lethal due to immature vascular development .

3.2.2. Tight junctions

Endothelial cells of the central nervous system that form the blood-brain barrier are connected by tight junctions (TJ). TJs were identified by electron microscopy as points where the outer leaflets of lateral membranes between adjacent endothelial cells were fused (Predescu and Palade, 1993). TJs are composed of occludin, claudins, and junctional adhesion molecules (JAMs). Occludin through its interaction with the zonula occludens 1 protein (ZO-1) and the actin cytoskeleton stabilizes TJs, and presumably transduces information between AJs and TJs. Claudins bind to each other in a homotypic and heterotypic manner to form TJs . The cytoplasmic end of claudin binds ZO-1, which in turn links claudin with the actin cytoskeleton and ZO-1-associated proteins. JAMs, belonging to the immunoglobulin superfamily, are single-pass membrane proteins with a long extracellular “domain” . JAMs also play a similar role in regulating the integrity of the endothelial barrier. ZO-1, -2 and -3 are known to interact directly or indirectly via bridging proteins with claudins, JAMs, and occluding , thus orchestrating intercellular adhesion and thereby controlling endothelial permeability.

3.2.3 Gap junctions

Gap junctions (GJs) provide electrotonic and metabolic pathways for direct cell-to-cell transfer of signaling molecules (i.e. inositol triphosphate) and ions (i.e. Ca^{2+}). Each GJ is made up of two connexons (one on each of the two cells), where one connexon is made up of six connexin subunits to form a hexamer. Endothelial cells express connexins Cx37, Cx40, and Cx43 . The connexin composition of the connexons determines the size and the charge of the molecules that are allowed to pass.

3.2.4. Endothelial junction-associated proteins

A group of junction-associated proteins can also contribute to the regulation of interendothelial permeability. The platelet-endothelial cell adhesion molecule (PECAM-1; CD31), a member of the immunoglobulin superfamily of transmembrane proteins, is concentrated in endothelial junctions . PECAM-1 is a ligand for $\alpha_v\beta_3$-integrin , $\alpha_4\beta_1$- and β_2-integrins. It is likely that the interaction of PECAM-1 with leukocyte integrins is important in mediating transendothelial migration of leukocytes, rather than endothelial permeability. Leukocyte migration requires the binding of endothelial PECAM-1, mobilized from a surface-connected membranous "subjunctional reticulum" recycling pool, to the β_3-integrins expressed on leukocytes .

The actin-polymerizing proteins, vasodilator-stimulated phosphoprotein (VASP) , formin, α-actinin and vinculin are also localized along the AJs where they form links with β-catenin. Their propitious localization suggests that they could play a role in stabilizing the endothelial junctions.

3.2.5. Barrier disruptive mediators

Permeability-increasing mediators induce the disintegration of endothelial junctions and promote gap formation between contiguous endothelial cells. It has been found that various mediators act via different signaling pathways to result in the same end, junctional gaps between endothelial cells. Clinically, disruption of the endothelial barrier results in tissue edema, plasma extravasation and enhanced leukocyte infiltration, which subserve the inflammatory response. Pharmacological modulation of endothelial barrier function hence represents an important anti-inflammatory strategy.

Thrombin, a pro-coagulant serine protease, is well known to increase endothelial permeability (Lum and Malik, 1996). Thrombin mediates increased endothelial permeability by binding to its receptors, PAR-1, protease activated receptor-1, on endothelial cells (Coughlin, 2000). PAR-1 is activated by its ligation, which induces thrombin-dependent proteolysis of the PAR-1 extracellular extension. Data from endothelial cells show that thrombin increases transendothelial permeability within

minutes in a reversible manner, where recovery occurs within 2 h . PAR-1 is coupled to Gq protein and activation of the receptor leads to a rapid release of sequestered Ca^{2+} into the cytosol. Extracellular Ca^{2+} entry makes an important contribution to the endothelial permeability response to thrombin. Furthermore, endothelial myosin light chain kinase (MLCK) is required because thrombin fails to increase lung microvessel permeability in mice deficient in endothelial cell *mlck.*

Histamine, another classical mediator of increased endothelial permeability is stored in cytoplasmic granules of mast cells and basophil granulocytes. Histamine acts locally to activate endothelial cells, fibroblasts, and smooth muscle cells following its release from mast cells. Histamine mediates its effect by binding to one of four receptor subtypes, H1, H2, H3 and H4 (Hill, 1990). Both H1 and H2 receptors are present in large-vessel and microvessel endothelial cells (Hill, 1990); however, the histamine effect on vascular permeability occurs through the activation of H1 receptors . Histamine likewise induced junctional gap formations by increasing intracellular Ca^{2+} and myosin light chain phosphorylation in endothelial cells, but to a lesser extent than that reported for thrombin , which may account for the short-lived histamine-induced permeability responses. Histamine also disrupts endothelial junctions by activating the phosphorylation and recruitment of AJ and TJ components . In addition, histamine induced actin stress fiber formation in a RhoA- and Rac-dependent manner.

VEGF, Vascular endothelial growth factor induces angiogenesis by stimulating endothelial cell migration and proliferation primarily through the VEGF type 2 receptor (VEGFR2, KDR/Flk-1) which are phosphotyrosine-containing transmembrane receptors (Matsumoto and Claesson-Welsh, 2001). VEGF binding initiates autophosphorylation of VEGFR2, which is followed by activation of diverse key angiogenic enzymes such as MAP kinases and Akt (Matsumoto and Claesson-Welsh, 2001). One of the initial responses of quiescent endothelial cells to induce angiogenesis is the loosening of cell-to-cell contacts, which is followed by migration of ECs to form capillary tube networks that eventually become functional capillaries (Ausprunk and Folkman, 1977). VEGF increases permeability in intact vessels (Bates, 1998) as well as cultured endothelial cell

monolayers , implicating VEGF as an important mediator regulating endothelial permeability.

TNF-α is a crucial mediator of increased endothelial permeability *in vivo* , isolated lungs, and cultured endothelial cells . TNF-α is an inflammatory cytokine released from activated monocytes and macrophages sequestered into the interstitial environment and the microcirculation . TNF-α is believed to contribute to disruption of lung vascular barrier function upon engaging TNF-α receptor-1, leading to increased permeability of proteins (Lum and Malik, 1996). In addition, TNF-α results in the up-regulation of endothelial adhesion molecules ICAM-1, VCAM-1 and E-selectin , thereby promoting neutrophil and eosinophil adhesion to endothelium and ROS generation, which in turn can increase endothelial permeability. Evidence indicated that PKC-α by inducing RhoA activity mediates the TNF-α-induced increase in endothelial permeability . TNF-α can affect endothelial permeability by multiple mechanisms, increasing permeability under specific instances and also modulating it by transcriptionally regulating the expression of receptors of proinflammatory agonist (e.g., PAR-1) and the Ca^{2+} channel TRPC1.

LPS (lipopolysacchrides), also known as bacterial endotoxin, is a structural component of the outer membrane of Gram-negative bacteria that triggers a systemic inflammatory response, which includes vasomotor dysfunction resulting in vasodilation and hypotension, endothelial cell apoptosis, and coagulation activation with fibrin deposition (Cohen, 2002). Administration of LPS in various models induced profound vascular leakage *in vivo* (Penn and Chisolm, 1991) and increased permeability of cultured endothelial cells *in vitro* . Studies showed that in cultured endothelial cells, an LPS-induced increase in endothelial permeability occurred as the result of endothelial contraction caused by RhoA-dependent increase in MLC phosphorylation, reorganization of actin filaments, and protein tyrosine phosphorylation . LPS effects on the endothelium can also occur secondary to LPS-induced release of inflammatory mediators such as TNF-α, IL-1, and IL-8 (Cohen, 2002). LPS activates the release of these cytokines by a transcriptional mechanism involving the activation of NFκ-B (Cohen, 2002). LPS binds to LPS binding protein (LBP) and this complex interacts

with soluble opsonic receptor CD14 (sCD14). sCD14 is found in serum of healthy individuals. LPS-LBP-sCD41 in turn associates with the Toll-like receptor 4 (TLR4), which requires MD-2 (lymphocyte antigen 96) for inducing the activation of NFκ-B, and the subsequent transcription/translation of cytokines (Cohen, 2002).

3.2.6. Barrier promoting mediators

The endothelial barrier stabilizing mediators may be released in response to permeability-increasing mediators and could serve to restore endothelial barrier function. Their role has the potential for providing novel therapeutic strategies in inflammatory diseases that would reverse the defects in vessel wall permeability (van Nieuw Amerongen and van Hinsbergh, 2002).

Sphingosine-1-phosphate (S1P) has been shown to have unique barrier-protective properties in cultured endothelial cells and intact microvessels. Sphingosine kinase, phosphatase, and lyase regulate S1P plasma levels . Sphingosine kinase by phosphorylating sphingosine leads to the formation of S1P . Degradation of S1P is mediated by either sphingosine phosphatase or sphingosine lyase that catalyzes the phosphate-dependent lysis of S1P to phosphoethanolamine . Platelets lack sphingosine-1-phosphate lyase and thus are the primary storage sites of S1P. S1P released from platelets binds to serum albumin. Therefore, endothelial cells may be continuously exposed to S1P from circulating platelets *in vivo*. S1P effects in the endothelium mainly depend on the Gi-coupled receptor S1PR1, formerly known as endothelial differentiation gene (*edg*)-1 (300). A key effector of this pathway is activated Rac, which leads to endothelial cell cortical actin assembly and assembly of AJ . S1P induces AJ and assembly, which may contribute to its barrier promoting effect. *Edg-1* knockout mice die prenatally due to vascular leakage as a consequence of incomplete vascular maturation . S1P also binds to four additional receptors termed S1PR2 to S1PR5, which have a more specific distribution in tissues .

Hepatocyte growth factor (HGF) was the first angiogenic that was found to enhance endothelial barrier function, as observed by transendothelial electrical resistance . At that time this was surprising, since all other known angiogenic factors, both under physiological and pathological conditions, were characterized by increase in microvascular permeability (Dvorak, 2002). HGF induces an increase in endothelial cortical F-actin modulated by multiple and complex signaling pathways, which include PI-3K, p38 MAP kinase and PKC activation, with linkage to barrier-regulation. Interestingly, there is one potential and common target for each of these signaling pathways, namely glycogen synthase kianse-β (GSK-3β). Phosphorylation of GSK-β regulates cell-to-cell adhesion through the modulation of the association of β-catenin with VE-Cadherin. However, the clinical use of HGF in the treatment of vascular leakage is not considered feasible due to its angiogenic properties.

cAMP (cyclic adenosine monophosphate) is one of the few signaling molecules that generally and ubiquitously improves endothelial barrier function in the circulation. Elevation of endothelial cAMP appears effective in reducing the permeability of endothelial cells *in vitro* and in reducing histamine-induced general edema in experimental animals *in vivo* (Rippe and Grega, 1978). The effect is exerted quickly and occurs both in endothelial cells under basal conditions and after exposure to vasoactive agents. The efficacy of cAMP is independent whether it is increased by activation of adenylyl cyclase or by the inhibition of cAMP-degrading phosphodiestareses. The suggested underlying signaling mechanism involves Rac-1 small GTPase. It was shown recently that cAMP can protect the endothelial barrier by preventing the inhibition of Rac-1 in microvascular endothelial cell cultures *in vitro* as well as in intact vessels . It is hypothesized that Rac-1 controls the endothelial barrier function by mechanisms involving VE-cadherin-mediated cell-to-cell adhesion (J. Waschke, 2004). β2-adrenergic agonists, serotonin and prostaglandins, such as PGI_2 and PGE_2 increase cAMP levels in endothelial cells and were shown to improve endothelial barrier function *in vitro* and *in vivo* (for the effects of prostaglandins on endothelial barrier function please see below).

Glucocorticoids, together with the mineralocorticoid aldosterone, belong to the group of corticosteroids and play an essential role in the clinical treatment of brain diseases with impaired blood brain barrier, e.g. brain tumors or brain edema. Corticosteroid therapy was first introduced in the early 1960s (Kaal and Vecht, 2004). Corticosteroids rapidly reduce edema formation, and capillary permeability is observed to be reduced within a few hours after one single dose of a corticosteroid; however, the underlying signaling mechanism is not elucidated in depth. Recently, corticosteroids were shown to attenuate the expression of the edema-inducing factor VEGF, which is involved in the regulation of endothelial dedifferentiation (Kaal & Vecht, 2004). Dexamethasone, the clinically most widely used corticosteroid, is at least 30 times more potent than cortisol (hydrocortisone), the endogenously active glucocorticoid in the bloodstream. Corticosteroids can act by both suppressing inflammation and directly on the endothelial junctions by regulating the structure, the phosphorylation and content of the protein components of the tight junctions (Felinski and Antonetti, 2005).

3.3. Prostaglandin I_2 (PGI_2) and endotelial barrier function

PGI_2 is overtly known to inhibit platelet aggregation and to induce vasodilatation by relaxing vascular smooth muscle cells. Its receptor, IP, is found mainly in megakariocytes and smooth muscle cells, but is also expressed in endothelial cells . IP is preferentially coupled to Gs subunits, and PGI_2 binding activates adenylyl cyclase to increase cAMP levels. The subsequent activation of PKA impedes ERK signaling, and thus interferes with signaling by TXA2 . In endothelial cells, the activation of PKA by PGI_2 is also involved in Rac activation and an IP selective agonist was found to enhance endothelial barrier formation by Rac mediated signaling . In addition to binding IP, PGI_2 is a natural ligand of the nuclear receptor peroxisome proliferator–activated receptor, PPAR β/δ, and also binds PPAR γ with similar affinity . Endothelial progenitor cells, (EPCs) also express IP receptors, and PGI_2 was shown to play pro-angiogenic and vasodilator effects via activation of PPAR γ . Cellular functions, like adhesion, migration and proliferation on fibronectin were significantly attenuated in IP-deficient EPCs compared with wild-type EPCs, which suggests the involvement of cell surface IP receptor . In human lung fibroblasts, PGI_2 analogs induced VEGF production . PGI_2 analogs induced angiogensis in an *in vivo* murine

corneal model of angiogenesis, where the effect was mediated by PPAR γ activation and VEGF production .

3.4. Prostaglandin E_2 and endothelial function

PGE_2 exerts its cellular effects by binding to four different E-prostanoid receptors (EP1 to EP4) which belong to the family of seven transmembrane G protein-coupled receptors, and each of these receptors has a distinct pharmacological signature based on its pharmacophore and intracellular signal transduction. EP2 and EP4 expression has been found in large vessel endothelial cells in culture , and EP1, EP3 and EP4 can be detected in brain microvascular ECs *in vitro* and *in vivo* . EP1 receptor binding triggers an increase in free intracellular Ca^{2+} and PKC activity, probably arising from its coupling to Gq and the subsequent activation of PLC. However, this idea has been seriously challenged, since the weak increase in inositol triphosphate (IP3) mediated by EP1 cannot account for the strong increase in intracellular Ca^{2+} concentrations observed . EP2 and EP4 are both functionally coupled to Gs, which activates adenylyl cyclase to generate cAMP. Despite this similarity, these receptors display remarkable structural and functional differences in terms of ligand binding affinity and internalization and desensitization rates. The generation of cAMP leads to the activation of PKA, which is central to the downstream events triggered by both receptors. In some cell types PKA phosphorylates and activates the transcription factor cAMP response element binding protein (CREB) , and PKA activity also impairs ERK 1, 2 signalling, which seems to be responsible for the decrease in matrix metalloprotease (MMP)-1 release in synoviocytes . In endothelial cells, PKA activity is implicated in the activation of the small GTPase Rac by PGE_2 . PKA can also phosphorylate and inhibit glycogen synthase kinase (GSK) 3α, thereby promoting transcription of COX-2. Interestingly, PGE_2 stimulation of EP4-expressing HEK cells can negatively regulate the activity of PKA through the activation of PI3K signaling . EP4 also specifically activates the PI3K/Akt pathway, which leads to phosphorylation and inhibition of GSK3a in HEK cells that were stably transfected with human EP4 receptors . In some cell types the activated PI3K augments ERK activity, which mediates expression of the transcription factor early growth response protein (EGR) 1 and subsequent transcription of genes such as mPGES-1 . Mouse EP4 receptor plays a critical role in PGE_2-

dependent *in vitro* migration/tubulogenesis which is mediated by activation of the ERK pathway . EP3 receptors are more complex, in that several EP3 isoforms can be generated by mRNA splicing—four bovine isoforms, three mouse isoforms, and eight human variants have been reported so far. The diversity of EP3 receptors has interesting functional implications, as human EP3 isoforms show discrepancies in constitutive activities . Human EP3 mainly couples to Gi proteins, and its activation generally inhibits cAMP synthesis. A result of this is an increase in ERK activity, which has been reported in some cell types (Burkey and Regan, 1995). In contrast, the EP3C isoform has been shown to activate Gs proteins in Chinese hamster ovary cells . Similarly, as a consequence of its coupling to G13, EP3 activates the Rho pathway in PC12 and MDCK cells . PGE_2 can regulate the JNK and p38 MAP kinase pathways in a number of cell types, such as synovial fibroblasts (where it induces COX-2 mRNA stabilization), and endothelial cells . Transactivation of tyrosine kinase receptors by PGE_2 provides a further level of complexity to the signaling events triggered by this prostanoid. PGE_2 transactivates the EGF receptor via activation of c-Src, which either activates EGFR directly or indirectly, by inducing a matrix metalloproteinase activity that releases membrane-bound TGFa . Recently a distinct transactivation mechanism has been identified, in which PGE_2 modulates PPARy nuclear receptor activity through the PI3K/Akt pathway .

The possible co-expression of several EP variants raises the question of how a particular cell can process the mixed and conflicting set of signals generated by simultaneous binding of PGE_2 to different EP receptors. This interesting issue has not been extensively investigated, but the final biological outcome will probably depend on many factors. Among these, ligand affinity for the receptor is likely to be important: in the case of PGE_2 its affinity is tenfold higher for EP3 and EP4 than for EP1; and fivefold higher for EP4 than for EP2 . Other potential factors are crosstalk among the different signal transduction pathways triggered by the stimulus, and the internalization and desensitization rate of each variant. EP4 and EP2 are a clear example of this last factor: EP4 is more rapidly internalized and desensitized, resulting in comparatively lower cAMP production .

4. THE ROLE OF EOSINOPHILS IN ASTHMA

It is generally accepted that the numbers of activated eosinophils in the asthmatic lung broadly correlate with diseases severity (Corrigan and Kay, 1992). Recently it was shown that asthmatic patients that received treatment based on eosinophil counts in sputum had significantly fewer severe asthma exacerbations than patients treated according to standard management therapy Eosinophils are considered as pro-inflammatory cells with a substantial potential for tissue destruction. The biological activities exerted by the eosinophils are related to the products released from their granules. Mucosal damage in chronic asthma is associated with cytotoxic and pro-inflammatory mediators release from activated eosinophils. These products include reactive oxygen species and cytotoxic granule and vesicular and cytotoxic granule and vesicular proteins: major basic protein (MBP), eosinophil cationic protein (ECP), eosinophil peroxidise (EPO), eosinophil-derived neurotoxin (EDN), as well as phospholipid-derived mediators, LTB_4 and cysLTs. In addition to their toxins and lipid-derived mediators, eosinophils themselves may also produce and release pro-inflammatory cytokines and growth factors, as evidenced by their gene expression for IL-4, IL-5, IL-6, IL-8, IL-13, TNF-α and GM-CSF .

Eosinophils are capable of generating eicisanoid derivatives of arachidonis acid by means of cyclooxygenase and the 5- and 15-lipoxygenase pathways. Moreover, due to the expression of LTC4 synthase, eosinophils are a major source of 5-lipoxygenase-derived cysLTs, which are potent paracrine mediators of bronchial obstruction and inflammation pertinent to asthma (Drazen and Austen, 1987). The local generation of eicosanoids at distinct sites within eosinophils may be important for the roles of these eicosanoids, both as paracrine mediators pertinent to inflammation and as intracrine signal-transducing mediators that help regulate cellular responses of eosinophils (Bandeira-Melo and Weller, 2005).

The local generation of eicosanoids at distinct sites within eosinophils may be important for the roles of these eicosanoids, both as paracrine mediators pertinent to inflammation and as intracrine signal-transducing mediators that help regulate cellular responses of eosinophils (Bandeira-Melo and Weller, 2005). The accumulation and activation of eosinophils in the lungs are governed by the up-regulation of adhesion molecules on lung endothelial cells and the production of various cytokines and chemotactic molecules by mast cells, T cells, epithelial cells and endothelial cells

themselves. Cytokines released from Th2-type cells, particularly IL-3, IL-5 and GM-SCF, are thought to regulate eosinophil priming, activation and survival

4.1. Eosinophil recruitment in allergic inflammation

During inflammation, leukocytes typically accumulate in response to certain mediators produced within the tissue itself. The passage of leukocytes through the vascular lumen into tissues occurs in several phases of the adhesion cascade, including in several phases, including rolling, activation, firm adhesion, transendothelial migration, and subendothelial migration. Although infiltration of eosinophil leukocytes is one of the most important aspects of allergic inflammatory reactions, eosinophils also participate in non-allergic inflammation. Eosinophil accumulation is regulated not only by endothelial adhesion molecules, but also by interactions between eosinophil adhesion molecules and extracellular matrix elements. This interaction probably occurs within capillary vessels that have small diameters. Adhesion molecules are grouped into three subfamilies: selectins, integrins, and the immunoglobulin supergene family.

Figure 2.

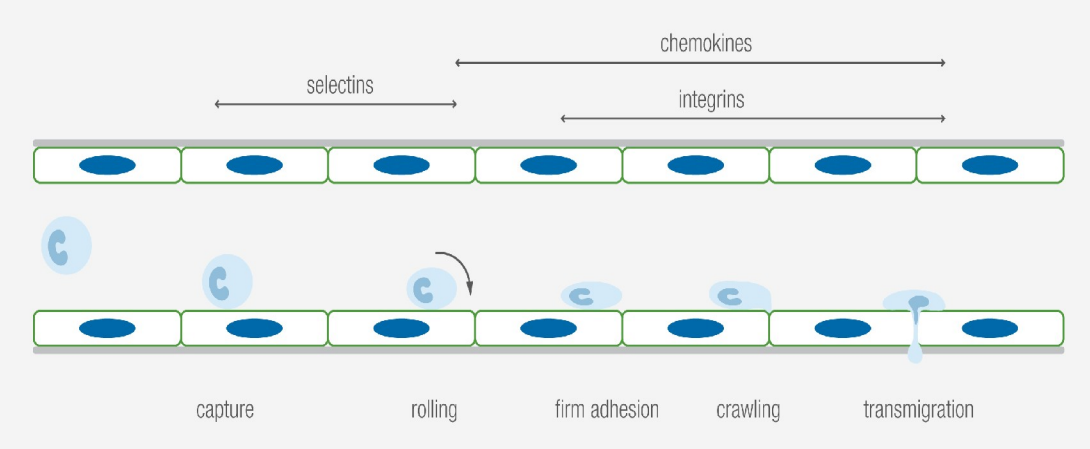

Figure 2. Adhesion cascade. *Eosinophils circulating in the blood can become captured by selectin molecules to the endothelium by inflammatory stimuli. The selectin-mediated weak adhesion allows the eosinophils to roll on the surface of endothelial cells to the site of inflammation. The preferred site is identified by chemotaxis towards the specific chemokines released by inflammatory cells or activated endothelial cells. At the preferred spot the eosinophils are arrested by firm adhesion through the activation of integrins and endothelial cell adhesion molecules; subsequently activated eosinophils crawl to the rearranged endothelial junctions and transmigrate into the tissue.*

4.1.1. Endothelial – eosinophil interactions

Eosinophil rolling and adhesion. The first step in the extravasation of eosinophils is their tethering to the vascular endothelium. Adhesion molecules are up-regulated by, e.g. IL-1, IL-4, TNF-α and IFN-γ and the same cytokines may also increase the avidity of adhesion molecules both on eosinophils and endothelial cells . Rolling, the initial contact of circulating eosinophils with the blood vessel wall prior to extravasation is mediated by selectins. Selectins are Ca^{2+}-dependent type-I transmembrane glycoproteins. E- and P-selectins are specific for endothelial cells, but P-selectin is also expressed on platelets, while L-selectin is specific for leukocytes. Each of these molecules has a lectin-like domain at the NH2-terminus, which is crucial for the binding properties of the protein. Counterligands for the selectins are sialylated fucosylated mucin-like ligands, and molecules such as the tetrasaccharide sialyl Lewis x, which contains α-2,3-linked terminal sialic acid residues, or α-1,3-linked fucose that can bind to all three selectins . An *in vivo* inflammatory model has shown that eosinophil rolling is dependent upon prior tethering by endothelial selectins . Although eosinophils can use all of the selectins to form attachments under shear conditions, P-selectin is much better than E-selectin at mediating eosinophil recruitment *in vivo* (Sriramarao and Broide, 1996) and in vitro (Reinhardt and Kubes, 1998). P-selectin (CD62P) is an integral membrane protein stored in Weibel–Palade bodies of endothelial cells and alpha granules of platelets and the cell surface expression is up-regulated when endothelial cells are stimulated with IL-4, IL-13 or histamin . P-selectin glycoprotein ligand 1 (PSGL-1), the primary ligand for P-selectin, is constitutively expressed on the

surface of circulating leukocytes. As many as ten-fold more eosinophils than neutrophils exhibit adhesion to airway endothelium (nasal polyps), suggesting that there is an eosinophil-specific adhesion pathway, which is explained with the higher aviditiy of eosinophils in binding to low concentrations of P-selectin under flow conditions .
The revesible adhesion between the selectins and their ligands makes the eosinophils move slowly along the vascular endothelium. This helps the eosinophils to sense the activation signals (chemoattractants) oming from tissue resident cells and/or the endothelium, and to adhere firmly by interaction of integrins and their ligands

Eosinophil specific chemoattractants. Selective eosinophil chemotaxis under the influence of potent eosinophil chemoattractants is a crucial factor for the characteristic increase of the number of eosinophils in the asthmatic lung. Until the last decade, the most effective eosinophil chemoattractants, which had been identified, such as platelet-activating factor (PAF) , complement 5a (C5a) and leukotriene B_4 (Czarnetzki and Rosenbach, 1986) were also active on neutrophils. In 1992, the chemokine RANTES (regulation upon activation, normal T-cell expressed and secreted) was shown to be an effective eosinophil chemoattractant, which was not active on neutrophils . Several other chemokines, in particular those of the CC-chemokine family which are acting through the chemokine receptor CCR3, have also been described as potent eosinophil chemoattractants. They include the eotaxin family comprising eotaxin (CCL11) (Rothenberg, 1999), eotaxin-2 (CCL24) and eotaxin-3 (CCL26) , and the monocyte chemoattractant proteins MCP-3 and MCP-4 . Increasing interest is also being paid to the chemotactic properties of cytokines and growth factors. IL-3, GM-CSF (Resnick and Weller, 1993), IL-5 (Hom and Estridge, 1994), IL-8 and IL-13 have been recognized as activators of eosinophil function, including migration. Furthermore, the lipid-derived mediator prostaglandin (PG) D_2 plays a key role in asthma as it potently stimulates eosinophil chemotaxis .

Integrin mediated eosinophil rolling. Additionally to selectins, integrins can also contribute to the rolling of eosinophils, via the α4 β1 integrin (very late antigen-4, VLA-4, CD29/CD49d), and the α4 β7 integrin which are specific eosinophil integrins. VCAM-1 is thought to be the primary ligand for α4-integrin-dependent leukocyte recruitment, as the α4 integrin binds to purified VCAM-1 protein or to VCAM-1-transfected cell lines under static or laminar flow conditions *in vitro* . The principal ligand of α4 β7 integrin is MAdCAM-1, a molecule that has been shown to support eosinophil rolling and firm adhesion under conditions of flow . VCAM-1 is more likely than MAdCAM-1 to play a significant role in eosinophil recruitment to the lung, because VCAM-1 is expressed in lung, whereas MAdCAM-1 is predominantly expressed in the gastrointestinal tract . Eosinophils express additional integrins, like αMβ2 (Mac-1, CD11b/CD18) and the αLβ2 (lymphocyte function associated antigen, LFA-1, CD11aCD18). β2 integrins also support rolling. ICAM-1, intracellular adhesion molecule-1, is the main ligand of β2 integrins, but αMβ2 can also recognize and bind to VCAM-1.

Eosinophil arrest. Integrins are receptors which need to be activated, to greatly increase their ligand-binding capability, i.e. avidity. Increased integrin-affinity corresponds to conformational changes of the adhesion molecule subunits that leads to increased ligand-binding energy and decreased rate of ligand dissociation. Valency describes the receptor density per area of plasma membrane involved in cell adhesion, which is dependent of lateral mobility or, in other words, the clustering of integrin complexes and the cell surface expression levels of integrins. Modulation in the affinity of an integrin for its ligand is the crucial step in the chemokine induced arrest . Chemokine-stimulated signaling induces conformational changes only in a fraction of integrins at the site of adhesion to endothelial cells; however, the average avidity of the entire integrin population in a cell is increased. Chemoattractant receptors are generally GPCRs; the intarcellular signaling cascade started from GPCRs to integrin activation occurs in three different stages, which comprise phospholipase C (PLC) signaling, activation of small GTPases, and induction of transitional integrin conformational changes via the association with actin-binding proteins, like talin-1 . In addition to mediating adhesion, integrins regulate various cellular functions, including cell motility, proliferation, respiratory burst, degranulation and apoptosis (Shattil, 2005).

Eosinophil transendothelial migration

Crawling. Transmigration through the vascular walls is the final step of leukocyte emigration into the inflamed tissue and can occur with minimal disruption in the integrity of the vessel wall. Before crossing the wall of capillaries, leukocytes crawl inside the blood vessel along the endothelium in a Mac-1-ICAM-1 dependent manner , seeking preferred sites of transmigration. Leukocyte migration across the endothelial cell barrier can occur rather fast, within 2-5 minutes, but crossing the endothelial cell basement membrane can take considerably longer (5-15 minutes). Transendothelial cell migration can be triggered by luminal chemoattractants and different levels of shear stress . The interaction of leukocyte integrins with their endothelial cell-specific ligands (ICAM-1, VCAM-1) may also stimulate endothelial cells in a manner that they further induce leukocyte transmigration. Adherent leukocytes can promote docking structure or transmigratory cup formation on the apical site of endothelial cells in the form of cellular projection that are rich in ICAM-1 and VCAM-1 and cytoskeletal components (vinculin, α-actinin) . These projections can further induce and support paracellular or transcelullar migration pathway.

Paracellular migration pathway. Ligation of endothelial cell adhesion molecules can lead to reduced interendothelial contacts and facilitate the migration of leukocytes via the endothelial junctions. The event is connected to and dependent on increased levels of endothelial free intracellular Ca^{2+}, a cellular response that can further promote junction opening, via activation of myosin light-chain kinase and subsequent endothelial contraction . In the inflamed tissue, endothelial junctions are subject to rearrangement of junctional molecules; the ones supporting leukocyte transmigration (ICAM-1, VCAM-1, platelet-endothelial cell adehesion molecule, PECAM-1, endothelial cell-selective adhesion molecule, ESAM and junctional adhesion molecule, JAM-A, B) are increased (Muller, 2003; Vestweber, 2002), while those that might act as obstacles in leukocyte transmigration (VE-Cadherin) are distributed away from the junctions . Thus, numerous molecular interactions must occur in sequence for paracellular transmigration to be successful.

Although eosinophils can spontaneously migrate through endothelial cell monolayers , the migration of eosinophils from the blood into tissues has been shown to be dependent on β2 integrins . Transmigration of adherent leukocytes, including eosinophils, across cultured endothelial cells is dependent upon the interaction of LFA-1 and Mac-1 integrins with their endothelial counterreceptors, ICAM-1 and ICAM-2 . C5a-mediated eosinophil transmigration across HUVEC monolayers is primarily dependent on LFA-1 integrin . Eosinophil-priming cytokines, such as IL-3, IL-5, and GM-CSF, promote chemoattractant-dependent migration of eosinophils through endothelial cell monolayers (Bochner and Schleimer, 2001). Eosinophils require shear stress to reach maximal levels of transmigration. However, shear stress alone is not capable of promoting eosinophil transmigration (Cuvelier and Patel, 2001).

Transcellular migration pathway. In the central nervous system and in some inflammatory scenarios leukocyte transmigration can occur via the transcellular route . Although this pathway allows the leukocyte to pass the endothelium faster than via the paracellular route (<1 minute), only a minority (~5-20%) of leukocytes migrate transcellularly. Vesiculo-vacuolar organelles (VVOs) are small continous membrane-associated passageways that can act as a gateway for leukocytes through the body of endothelial cells (Dvorak and Feng, 2001). Leukocyte migration starts with the extension of membrane protrusions into endothelial cells . Ligated ICAM-1 becomes translocated from the apical side of endothelial cells to caveolae and F-actin reach regions, and then by caveolin-1 transport to the basal membrane . The process leads to formation of intraendothelial channels through which the leukocytes can migrate. The same molecules that mediate paracellular leukocyte migration can be involved in the transcellular pathway.

4.1.2. Eosinophil degranulation

Upon reaching the inflamed tissue, eosinophils become activated by interactions with cytokines, lipid mediators, immune globulins, complement fragments and integrin ligands, and start to degranulate. They release toxic mediators (e.g. granule proteins, lipid mediators, oxygen metabolites, proteases, and cytokines) and cause tissue damage,

including the epithelium, smooth muscle cells and neurons, worsening the symptoms of allergic diseases.

5. PHARMACOTHERAPY OF ASTHMA

The pathogenic basis of atopy and related disorders is still only partially understood and no casual therapy, except for specific immunotherapy in selected cases, is available to-date. Symptomatic treatment of asthma includes β2-adrenoceptor agonists which are the most effective bronchodilators and were developed from catecholamines from the adrenal medulla, whereas coricosteroids (Norn and Clementsen, 1988), from the adrenal cortwx, are by far the most effective controllers of the underlying inflammatory processes in the airways. In detail, corticosteroids can inhibit the biosynthesis of cytokines and prostaglandins, the expression of their receptors and of adhesion molecules. Moreover, corticosteroids facilitate the biosynthesis of ani-inflammatory mediators, such as lipocortin and IL-10 . Thus, the gold standard of asthma therapy is a combination of on-demand delivery of β_2-agoinst with regular (i.e. basal) inhaled corticosteroid medication. Alternatives for asthma medication include inhaled cromoglycate (Edwards and Norris, 1994) to ameliorate inflammation, and inhaled muscarinic receptor antagonists or oral thephylline (Weinberger and Hendeles, 1996), abronchodilators. Antagonists of cysLT receptors combine bronchodilation with an anti-inflammatory effect, but are not efficacious in many patients.Overall, these symptomatic regimens are effective and most patients are sufficiently managed. However, a portion of asthmatic patients is resistant to any pharmacological treatment, including corticosteroids. In addition, therapy is often accompanied by serious side effects when applied at high doses (e.g. corticosteroids). Therefore, there is growing demand for additional treatments of allergic disease, particularly for those who need to avoid corticosteroids (e.g. children), and those who are insufficiently treated with standard therapeutic regimens. In the case of bronchial asthma, these patients rely mainly on bronchodilator therapy and are at risk of suffering both from side-effects of these drugs and uncontrolled pulmonary inflammation leading to airway remodeling .

In addition to allergic diseases , eosinophils are currently considered a major therapeutic target in several other diseases, such as eosinophilic esophagitis (Straumann

and Simon, 2005), colitis ulcerosa (Wedemeyer and Vosskuhl, 2008), or renal disease falsh: type 2 Diabetes . Controlling eosinophil trafficking and endothelial barrier function might open novel therapeutic avenues for these diseases as well.

6. AIMS OF THE THESIS

As described above, eosinophils are important effector cells in allergic inflammation and other eosinophilic diseases leading to airway hyperreactivity and tissue damage. Therefore, considerable effort has been invested in the development of drugs that can effectively control the trafficking and activation of eosinophils in order to ameliorate the inflammatory response. Unfortunately, currently available regimens such as anti-IL-5 antibodies or LTC_4 receptor antagonists have proven little effective in clinical practice. The disappointing performance of these drugs might be explained by their selectivity, as they do not alter the responsiveness of eosinophils to other potent eosinophil activators, such as the CCR3 ligands, PGD_2 or PAF. A promising alternative approach could be the use of drugs that directly initiate inhibitory pathways in eosinophils, thereby rendering them unresponsive to any stimulus.

Available literature suggests that PGE_2 is capable of impeding eosinophil trafficking, but the usefulness of PGE_2 as a therapeutic agent is certainly limited because of its known clinical side effects such as acute bronchoconstriction, retrosternal soreness, transient cough anf flu-like symptoms . These adverse effects pf PGE_2 are thought to arise from EP1 or EP3 receptor stimulation, whereas selective IP or EP4 agonists might have more favourable pharmacological profiles.

Therefore, this thesis was based on the hypothesis that stimulation of EP4 and/or IP receptors can effectively abrogate the trafficking of eosinophils and thereby control eosinophilic inflammation, tissue damage and organ impairment. The following specific questions were addressed in detail:

- Are EP4 and IP receptors expressed by eosinophils?

- Does the selective stimulation of EP4 and IP receptors attenuate eosinophil chemotaxis *in vitro*?
- How are major intracellular pathways (e.g. cAMP, PKA, PKC) coupled to the inhibitory effects of EP4 and/or IP agonists?
- Are EP4 and IP receptors modulators of the cellular interaction of eosinophils with the endothelium as a crucial step in eosinophil extravasation?
- Does the selective stimulation of EP4 and IP receptors modulate the barrier function of endothelial cells against invading eosinophils?

The answers to these questions will show whether selective prostaglandin IP or EP4 receptor agonists might be of potential therapeutic value in allergic disease by controlling eosinophilic inflammation.

II. MATERIALS AND METHODS

REAGENTS

Laboratory reagents were purchased mainly from Sigma-Aldrich (Vienna, Austria), unless specified. Human Eotaxin/CCL11 and TNF-α were from Peprotech (London, United Kingdom). PGI_2, PGE_2, PGD_2, EP2 receptor agonist butaprost, the EP3 receptor agonist sulprostone and the polyclonal anti-EP4and anti-IP antibodies were obtained from Cayman (Ann Arbor, Mich). The EP4 agonist ONO AE1-329 and the EP4 antagonist ONO AE3-208 were gifts from ONO Pharmaceutical (Osaka, Japan). Anti-rabbit and anti-mouse IgG secondary antibody conjugated with Alexa fluor-488 was from Invitrogen. Monoclonal antibody 24 (activation sensitive CD11/18 Ab) was from Hycult Biotechnology (Uden, The Netherlands). Monoclonal anti-VE-Cadherin antibody was obtained from Santa Cruz Biotechnology. Phalloidin–Texas Red was from Molecular Probes (Eugene, Ore). Anti-VCAM-1/CD106-FITC, anti-ICAM-1/CD54-PE, anti-CD11b-FITC, anti-CD16-PE and anti-E-selectin/CD62E-PE were from BD Pharmingen (Vienna, Austria). Vectashield/DAPI was purchased from Vector Laboratories (Burlingame, Calif). Ultra V Blocking solution was purchased from Lab Vision Corp. (Fermont, CA, USA), Antibody diluent was purchased from Dako (Carpinteria, CA, USA). The adenylyl cyclase inhibitor SQ22536 was supplied by Biomol (Hamburg, Germany). CellFix and FACS-Flow were from Becton Dickinson (Vienna, Austria). Assay buffer was made from Dulbecco modified PBS (with 0.9 mmol/L Ca^{2+} and 0.5 mmol/L Mg^{2+}; Invitrogen, Vienna, Austria), 0.1% BSA, 10 mmol/L HEPES, and 10 mmol/L glucose, pH 7.4. Fixative solution was prepared by adding 9 mL of distilled water and 30 mL of FACS-Flow to 1 mL of CellFix. Drugs were dissolved in ethanol, distilled water or dimethyl sulfoxide and further diluted in assay buffer to produce a final concentration of the solvents of less than 0.1%.

METHODS

CELL MODELS

Culture of endothelial cells

Human lung microvascular endothelial cells (HMVEC-L) cryopreserved as tertiary cultures were purchased from Lonza (Verviers, Belgium) and were maintained in EGM-2 MV Bullet kit medium (Lonza) with 5% FCS. All culture surfaces were pre-coated with 1% gelatin for 1 hour at 37°C to promote endothelial cell attachment and growth. The medium was changed every 2 days and cells were passaged when they reached 90% confluency (5-6 days); the cultures were used between 5 and 10 passages (Weinberger and Hendeles, 1996) .

Preparation of human peripheral blood eosinophils and neutrophils

Blood was sampled from healthy nonatopic volunteers, who were not taking any medication, after informed consent was provided according to a protocol approved by the Ethics Committee of the Medical University of Graz. Polymorphonuclear leukocytes (PMNL, containing eosinophils and neutrophils) were prepared by means of dextran sedimentation of erythrocytes followed by centrifugation on Histopaque gradients as previously described . In some cases PMNL were further purified to gain eosinophils by using negative magnetic selection with an antibody cocktail (CD2, CD14, CD16, CD19, CD56, and glycophorin A) and colloidal magnetic particles from StemCell Technologies (Vancouver, Canada). All separation steps were performed at room temperature. The resulting purity and viability of eosinophil and neutrophil preparations was typically greater than 95%.

ASSAYS

Migration assays

Chemotaxis assay

Chemotaxis of purified eosinophils or neutrophils (10^5 per well) was determined by using a 48-well micro-Boyden chamber with a 5-µm pore-size PVP-free polycarbonate filter (NeuroProbe, Inc, Gaithersburg, Md) at 37°C in a humidified CO_2 incubator for 1 h. Cells that had migrated into the bottom wells of the chamber were counted with a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA), and eosinophils or neutrophils were distinguished from contaminating cells by means of forward-scatter/side-scatter gating and autofluorescence, as previously described

Leukocyte transendothelial migration assay

Endothelial cells were grown to confluence in 5-µm filters in 6.5-mm Transwell inserts (Corning, NY) to study neutrophil or eosinophil transmigration across endothelial monolayers. Confluence of the monolayers was confirmed by measuring transendothelial electrical resistance with an Endohm device (WPI, Sarasota, Fla).19. The monolayers grown in the inserts were treated differentially; further on they were placed into 24-well plates containing medium or eotaxin (3 nM) or IL-8 (3 nM) in the bottom wells and $2x10^5$ neutrophils or eosinophils were added into the top wells.

The plates were incubated at 37°C in a humidified incubator for 2 or 4 h, and the non-migrated and transmigrated neutrophils or eosinophils were enumerated by means of flow cytometry .

Adhesion assays

Eosinophil adhesion to fibronectin

Adhesion of purified eosinophils to fibronectin was determined by incubating the eosinophils ($6x10^4$ per well) for 30 min at 37°C in flat-bottom 96-well plates that had been pre-coated with 5 μg/mL fibronectin for 2 hours . After incubation, non-adherent cells were collected first, and adherent eosinophils were harvested with an EDTA (3 mmol/L) buffer. Both non-adherent and adherent cells were enumerated by means of flow cytometry. Eosinophil adhesion was calculated as the percentage of adherent eosinophil on the basis of the total cells added.

Eosinophil adhesion to endothelial cells

Adhesion of eosinophils or neutrophils to endothelial monolayers, which had been grown to confluence in 48-well plates, was investigated by allowing purified eosinophils or neutrophils ($6x10^4$/well) to become adherent for 30 min at 37°C (Yamamoto and Nagata, 1999). Thereafter, non-adherent cells were first removed, then adherent eosinophils or neutrophils together with the endothelial cells were removed by trypsinizing. The cells were enumerated from the two fractions by means of flow cytometry. Adhesion was calculated as the percentage of adherent neutrophils on the basis of the total cells added.

Immunofluorescence staining of eosinophil adhesion

For the staining of F-actin in eosinophils, chamber slides were pre-coated with 5 μg/mL fibronectin for 2 h, and purified eosinophils ($3x10^5$/chamber) were then allowed to adhere for 30 min at 37°C. After removal of nonadherent cells, adherent eosinophils were fixed with 3.7% formaldehyde, permeabilized with 0.1% Triton X-100, blocked with 1% BSA, stained with phalloidin–Texas Red (5 U/ mL) for 20 minutes at room temperature in the dark, and mounted with Vectashield/DAPI . Immunofluorescent

microscopic analysis was performed with an Olympus UPlanApo–60x/1.20 lens using an Olympus IX70 fluorescence microscope, an Olympus DP50-CU digital camera and Olympus Cell^P software (Olympus, Lake Success, NY).

Upregulation, activation and clustering of eosinophil adhesion molecules

Polymorphonuclear leukocyte preparations were incubated with agonists for 15 minutes at 37°C and then stained with anti-CD11b (fluorescein isothiocyanate, 1:30) and anti-CD16 (phycoerythrin, 1:50) antibodies. CD11b expression on $CD16^{-neg}$ eosinophils was quantified by means of flow cytometry and expressed as the fold increase over baseline expression .

Eotaxin-induced activation of β2-integrins was determined by means of indirect immunofluorescence flow cytometry with mAb 24, which recognizes only the activated conformation of CD11b/CD18 . Purified eosinophils were incubated with vehicle or eotaxin (3 nM) for 15 min at 37°C and in the presence of mAb 24 (1:50) or isotype control followed by staining with Alexa fluor 488–labeled secondary antibody (1:500) for 30 min at 4°C. The stained samples were either directly measured with flow cytometry or were cytospinned to microscope slides to visually analyse the clustering of activated CD11b/CD18 integrins. Microscope slides were observed with an Olympus UPlanApo–60x/1.20 lens using an Olympus IX70 fluorescence microscope, an Olympus DP50-CU digital camera and Olympus Cell^P software (Olympus, Lake Success, NY).

Eosinophil apoptosis assay

Eosinophil viability was determined with the means of the Annexin V-FITC apoptosis detection kit, where apoptotic cells were quantified by flow cytometry. Purified eosinophils were placed into 96-well plates, the cells were harvested at 0h-1h-3h time points and stained with FITC-labeled annexin V and propidium iodide for x min in the dark and then analysed by flow cytometry. Annexin V^{-pos} cells were counted as

apoptotic cells (FL-1 fluorescence channel) while propidium iodide^{-pos} cells were regarded as necrotic (FL-2) .

Flow cytometric detection of IP and EP4 receptors

IP and EP4 receptor expression on purified human eosinophils, neutophils or endothelial cells was determined by using indirect immunofluorescence flow cytometry with polyclonal IP and EP4 antibodies directed against the C-terminus of the receptors. Therefore, cells were first permeabilized with Fix&Perm (ADG, Kaumberg, Austria) for 15 min fixation and 15 min permeabilization of the cells at room temperature. The nonspecific binding sites were blocked with 1% BSA for 30 min at 4°C. Samples were then incubated with the following antibodies, each for 30 min at 4°C: 20 µg/mL polyclonal rabbit IP antibody or IP antibody, the binding sites of which had been blocked with 5-fold amount of the corresponding immunogenic sequence of the IP receptor (blocking peptide) as control. The same concentrations were used in case of EP4 receptor staining, however as control, an isotype specific rabbit polyclonal antibody was used. Finally, 4 µg/mL goat anti-rabbit IgG secondary antibody, conjugated with Alexa fluor-488 was added and cells were fixed and analyzed with flow cytometry .

Western blotting of IP receptors

For Western blot detection of IP receptor purified eosinophils were lysed in a buffer containing 50 mM Tris-HCl, 25 mM KCl, 5 mM MgCl2, and 0.2% Nonidet P-40 supplemented with protease inhibitors, as previously described . Protein samples (40 µg) were resolved on 10% Tris-HCl polyacrylamide gels for 1h at 150V and subsequently transferred to a nitrocellulose membrane. Membranes were probed with rabbit polyclonal IP antibodies (20 µg/mL), followed by horseradish peroxidase–conjugated anti-rabbit secondary antibodies (4 µg/mL) and Amersham ECL Plus (GE Healthcare, Vienna, Austria) detection reagents.

Transendothelial electrical resistance measurement

Confluence of the monolayers was confirmed by measuring transendothelial electrical resistance (TEER) with an Endohm device (WPI, Sarasota, Fla) . Endothelial cells were grown to confluence in 5-µm filters in 6.5-mm Transwell inserts; confluent monolayers were treated differentially and changes in monolayer resistance were recorded. Further on, they were subjected to leukocyte transendothelial migration assays (as described above). After eosinophil or neutrophil transendothelial migration the TEER was recorded again.

Electrical wound healing assay

To study the regenerative properties of endothelial monolayers, the cells were grown on small gold electrodes and monitored by using electric cell-substrate impedance sensing (ECIS, Applied Biophysics, Troy, New York). After the endothelial cells formed a confluent layer i.e. the resistance reached a maximal plateau phase, the cells were subjected to high current (wounding protocol: 5 sec, 900 µA, 48 kHz), resulting in severe electroporation and subsequent cell death as indicated by a dramatic drop in the resistance. After the electrical wounding, the electrode's resistance was again monitored to screen the repopulation and ultimately healing of the wound .

Immunofluorescence stainings of endothelial cells

VE-Cadherin staining

Differentially treated endothelial monolayers grown in Permanox chamber slides were fixed (3.7% formaldehyde in PBS) for 10 min, followed by permeabilization with 0.1% Triton X-100 in PBS for 15 minutes at room temperature. The nonspecific binding sites were blocked with Ultra V Blocking solution for 30 min. Incubation with the mouse monoclonal VE-cadherin antibody (1 µg/mL) or isotype-matched control

antibody was performed in the antibody diluent solution for 1 h at room temperature, followed by 30 minutes staining with Alexa fluor 488–conjugated secondary antibody (4 μg/mL) . After being mounted with Vectashield/DAPI medium, the samples were analyzed on an Olympus IX70 fluorescence microscope and an Olympus UPlanApo–60x/1.20 lens using an Olympus DP50-CU digital camera and Olympus Cell^P software (Olympus, Lake Success, NY).

IP receptor staining

For microscopic immunofluorescence staining, endothelial cells were seeded on chamber slides, then the cells were fixed and blocked for 30 min with Ultra V Blocking solution. Incubation with the primary rabbit anti-IP antibody lasted for 30 min at room temperature; AF-488-labeled goat anti-rabbit secondary antibody was incubated with the cells for further 30 min in dark at 4˚C. After being mounted with Vectashield/DAPI medium, the samples were analyzed with an Olympus UPlanApo–60x/1.20 lens using an Olympus IX70 fluorescence microscope, an Olympus DP50-CU digital camera and Olympus Cell^P software (Olympus, Lake Success, NY).

Endothelial adhesion molecule expression

Endothelial cells were grown into monolayer in 48-well plates. After differential treatments the cells were detached by using a detachment buffer (25 mM HEPES, 10 mM EDTA in PBS). The endothelial cells were then stained with directly labelled VCAM-1/ICAM-1/E-selectin antibodies for 30 min at 4°C in dark . The expression of endothelial adhesion molecules was quantified by means of flow cytometry and expressed as percentage of the maximal staining intensity (TNFα 10pM).

Enzyme Immunoassay

PGE_2 and the stable PGI_2 metabolite 6-keto-$PGF_{1\alpha}$ released by cultured endothelial cells was measured from the conditioned media by using an in house radioimmunoassay and and an enzyme immunoassay (Cayman), respectively. The detection limit was 5 pg/mL .

Statistical analyses

All data are shown as means ± SEMs for *n* observations. Comparisons of groups were performed by using 1-way ANOVA or 2-way ANOVA for repeated measurements, and P values of less than 0.05 were considered statistically significant.

III. RESULTS

Part I - Endogenous prostaglandin I_2 limits the eosinophil – endothelial interaction*

* published data :

Viktoria Konya, Eva Maria Sturm, Petra Schratl, Eckhardt Beubler, Gunther Marsche, Rufina Schuligoi, Irmgard Th. Lippe, Bernhard A. Peskar, Akos Heinemann. **Endothelium-derived prostaglandin I_2 controls the migration of eosinophils**.

J Allergy Clin Immunol 125, 5: 1105-1113 (May 2010)

Background

Enhanced eosinophil recruitment from the blood into the tissue is a characteristic feature of allergic diseases, such as bronchial asthma. Prostaglandin (PG) I_2 is the major prostanoid released by endothelial cells, but its role in allergic inflammation has not been elucidated in depth. The effects of PGI_2 are mediated by a single G protein–coupled receptor, IP, which is coupled to Gs proteins and stimulates adenylyl cyclase. Mice deficient for PGI_2 receptors (IPs) show exaggerated eosinophilic inflammation in response to allergen in lung and skin and enhanced airway remodeling . Recent studies also demonstrated that PGI_2 attenuates the migration of murine TH2 cells and inhibits cytokine production and T-cell stimulatory function of murine bone marrow–derived dendritic cells , and treatment of endothelial cells with PGI_2 reduces subsequent adhesion of neutrophils .

Aims

The aim of the first part of my work was to test the hypothesis that PGI_2 is involved in eosinophil - endothelial interaction. Therefore functional assays were carried out on human eosinophils and on eosinophils in interaction with human pulmonary microvascular endothelial cells.

Pharmacological inhibitors, selective agonists and antagonists were used to specify the observed effects.

Aim 1: To asses the role of PGI_2 in eosinophil functions

Aim 2: To evaluate the role of PGI_2 in eosinophil – endothelial interaction

Aim 1: To asses the role of PGI_2 in eosinophil functions

PGI_2 inhibits eosinophil chemotaxis

The inhibitory effect of Prostaglandin I_2 on human eosinophil functions was first investigated in an *in vitro* chemotaxis assay model. Chemotaxis, the directed migration of cells towards a chemoattractant is a basic function of leukocytes. Purified human eosinophil leukocytes were pre-incubated with vehicle or serial dilutions of PGI_2 for 5 min at room temperature and allowed to migrate for 60 min in a micro-Boyden chemotaxis chamber toward eotaxin or complement factor 5a (C5a; 1 nM each) placed in the bottom wells.

Figure 3A shows that PGI_2 markedly inhibited the migration of human eosinophils toward eotaxin and C5a achieving 50% inhibition at concentrations of 3 to 10 nM. On average, migration of 10% of input cells was stimulated by 1 nM eotaxin. As control cells, human neutrophil leukocytes were used; neutrophil chemotaxis was induced by IL-8 or C5a placed in the bottom wells. C5a behaves as chemoattractants both for neutrophils and eosinophils. In comparison with eosinophils, the same concentrations of PGI_2 did not affect the migration of neutrophils toward IL-8 (1 nM) or C5a (1 nM; ***Figure 3B***). Pharmacological inhibitors were used to test the specific involvement of IP receptors and the anticipated underlying signaling pathway (activation of adenylyl cyclase and intracellular cAMP increase). Pretreatment of eosinophils for 15 min with an IP antagonist, CAY10441 (300 nM), or an inhibitor of adenylyl cyclase, SQ22536 (10 mM), prevented specifically the PGI_2 inhibition of eosinophil chemotaxis (***Figure 3C***) in comparison to the vehicle-pretreated eosinophils.

Figure 3.

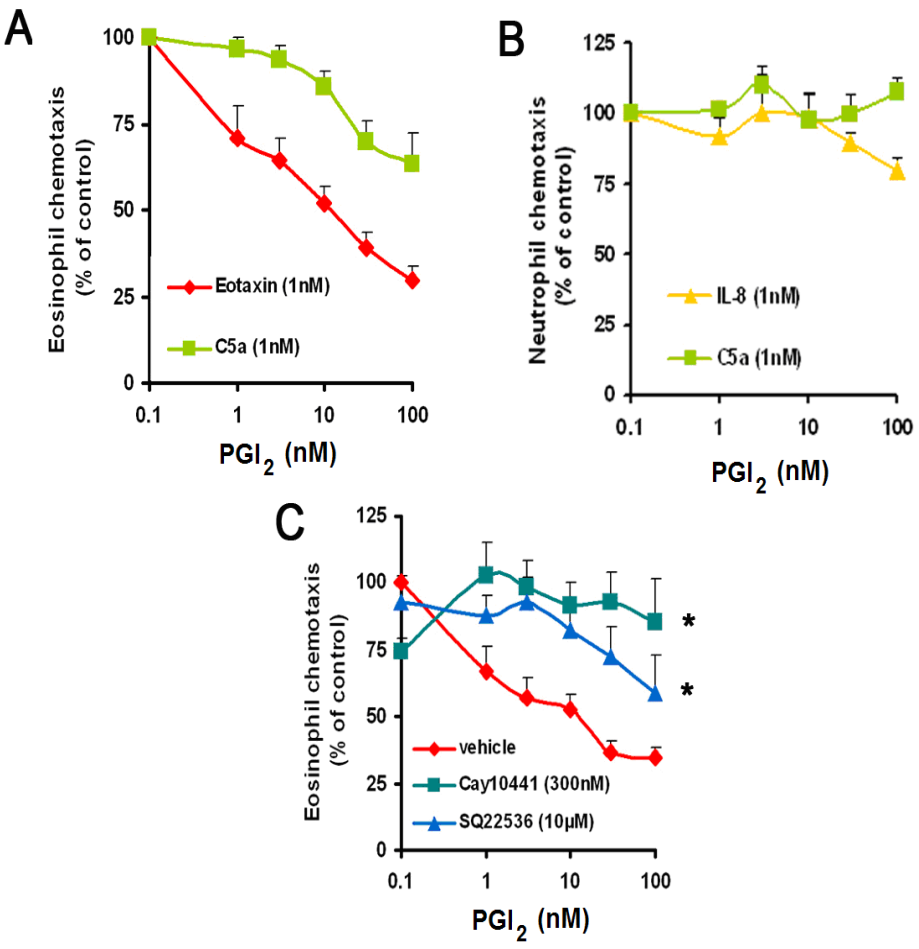

Figure 3. PGI_2 selectively inhibits the chemotaxis of eosinophils via activation of IP receptor and adenylyl cyclase pathway. *Human purified eosinophils (**A**) and neutrophils (**B**) were incubated with increasing concentrations of PGI_2 and were then allowed to migrate towards eotaxin, C5a or IL-8 (1 nM each) for 1 h in a micro-Boyden chamber. In **C**, eosinophils were pretreated with vehicle, an IP receptor antagonist, CAY10441 (300 nM) or an inhibitor of adenylyl cyclase, SQ22536 (10µM). Cells were then incubated with increasing concentrations of PGI_2 which was followed by migration towards eotaxin (1 nM) for 1 h. Cells in the bottom wells were enumerated by flow cytometry and responses were expressed as percent of the control response in the absence of PGI_2. n=4-8; * P<0.05 versus vehicle. (These experiments were performed by Eva Sturm.)*

IP, the PGI_2 receptor is expressed on human eosinophils

Prompted by the observation of the inhibitory PGI_2 effects on eosinophils, I investigated whether eosinophils expressed the cognate PGI_2 receptor, IP. In fact, flow cytometric and Western blot data revealed the presence of IP receptors on eosinophils. Specificity of the polyclonal rabbit IP antibody was demonstrated by preabsorption of the specific binding sites of the antibody with the immunogenic blocking peptide of the IP receptor, which markedly reduced the binding of the antibody to the cells. ***Figure 4A*** shows the histograms of unstained eosinophils or neutrophils, and cells stained with 2nd antibody only. Specific staining of IP receptors is demonstrated in the red-colored histogram, while the pink histogram shows reduced staining with the preabsorped IP antibody. In contrast, neutrophils did not express IP receptors (***Figure 4A***, right panel). The presence of IP receptors on eosinophils was also confirmed by means of Western blotting with the same anti-IP antibody, yielding a band with the predicted molecular size of 67 kDa (***Figure 4B***). Platelets were used (PL) as positive control.

Figure 4.

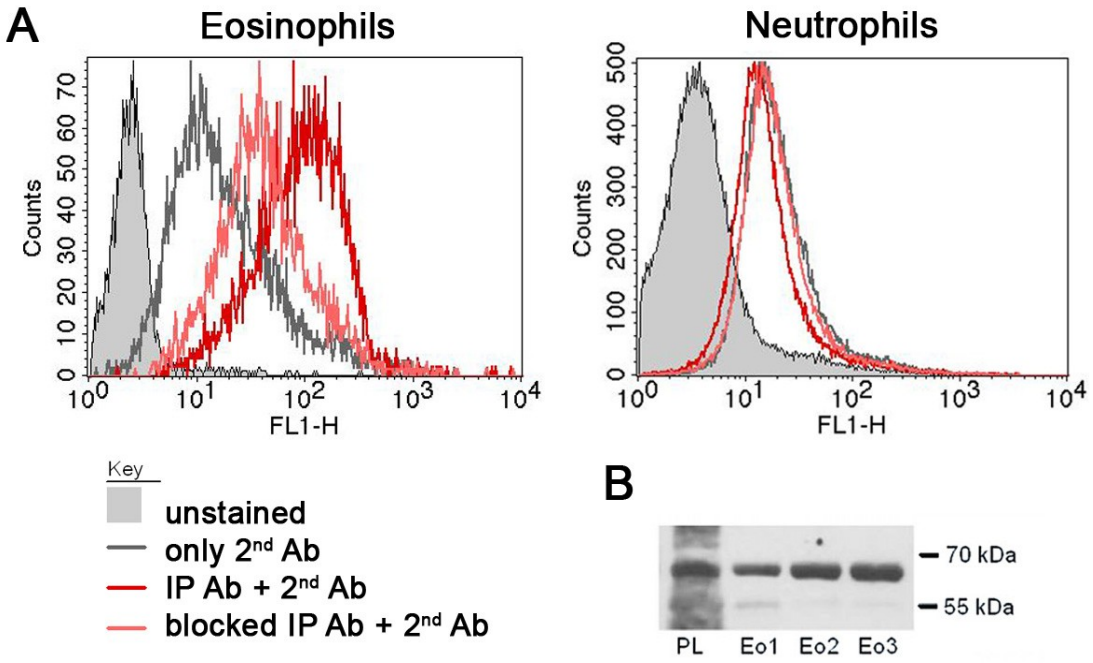

Figure 4. Expression of IP receptors on purified eosinophils was detected by means of flow cytometry and Western blotting. *In **A**, a flow cytometric staining is shown on eosinophils and neutrophils; as controls, unstained and only 2nd antibody-stained*

samples were used. The specificity of the IP antibody was proved by pre-absorbing the IP receptor antibody with its specific blocking peptide sequence (blocked IP Ab). ***B****, shows a representative Western Blot of the IP receptor expression. Eo1-3, Eosinophils from 3 donors; PL, platelets were used as positive control. n = 4-8. *P <0 .05 versus vehicle.*

PGI_2 inhibits eosinophil adhesion to fibronectin

Since adhesion is a crucial step in leukocyte extravasation and also modulates effector functions such as respiratory burst and degranulation, I investigated the effect of PGI_2 on eosinophil adhesion. First, eosinophil adhesion assay was assessed in fibronectin (5 μg/ml) pre-coated multiwell plates, where the adhesion of eosinophils was induced by increasing concentrations of eotaxin (0.3-3 nM). The effect of PGI_2 on the eosinophil adhesion to fibronectin was determined by pre-incubating purified eosinophils with vehicle or PGI_2 (100 nM) for 5 min at room temperature. Eosinophils were then placed onto fibronectin-coated 96-well plates containing increasing concentrations of eotaxin (0.3-3 nM) and allowed to become adherent while incubating for 30 min at 37˚C in a humidified incubator. ***Figure 5A*** demonstrates that PGI_2 reduced the eotaxin-induced adhesion of eosinophils to fibronectin by 40% to 60%, as determined by flow cytometric enumeration of adherent cells.

Next, eosinophil adhesion stimulated by eotaxin was visualized by staining the actin filaments with the highly photostable dye phalloidin-Texas Red. Eosinophils were allowed to adhere to the 'chamber slide' bottom surface which was pre-coated with fibronectin (5 μg/ml). Eosinophils were pretreated with vehicle or PGI_2 (100 nM) for 5 min at room temperature. After the pre-treatment, eosinophils were stimulated with 3nM eotaxin to become adherent on the fibronectin coated slides for 30 min at 37˚C in a humidified incubator. In the next step, non-adhered eosinophils were aspirated, the adherent cells were fixed, permeabilized, blocked and finally stained with phalloidin-Texas Red. The slides were mounted, and analysed by fluorescence microscopy. F-actin staining in adherent eosinophils revealed distinct morphologic alterations. ***Figure 5B*** shows that unstimulated eosinophils were round shaped and small sized, but upon activation with eotaxin (3 nM) they acquired a polarized, elongated shape and formed discrete protrusions containing dense F-actin network, consistent with podosomes.

Pretreatment of eosinophils with 100 nM PGI_2 did not prevent the eotaxin-induced polarization of the cells but abrogated their ability to form podosome-like structures (***Figure 5B***). Quantitatively, eotaxin-stimulated eosinophils expressed an average of 19.9 ± 1.9 of these structures (300 eosinophils were analyzed from 3 different blood donors), whereas PGI_2 reduced their number to 6.6 ± 1.7 ($P < .01$). These data suggested that PGI_2 reduces the adhesion of eotaxin-activated eosinophils to fibronectin-coated plastic surfaces, and furthermore prevents the morphologic changes, i.e. the formation of adhesive structures in eosinophils that are required for effective adhesion, crawling and migration.

Figure 5.

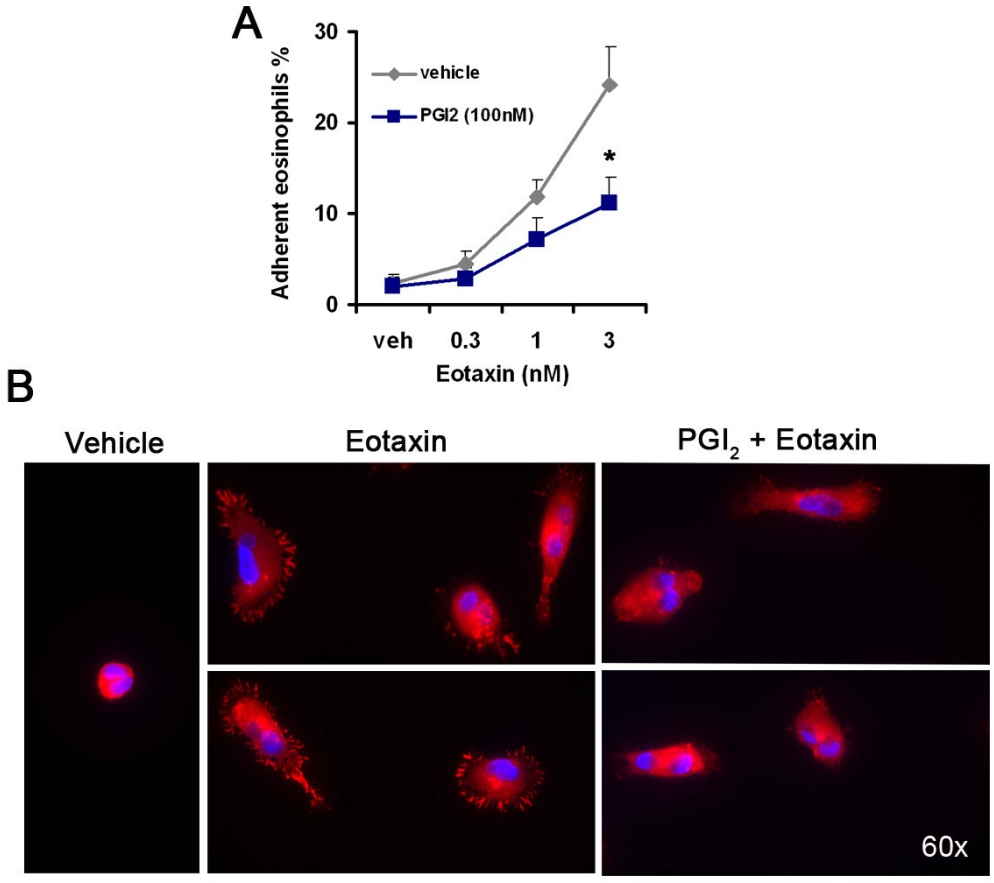

Figure 5. PGI_2 inhibits eosinophil adhesion to fibronectin and reduces eosinophil podosome formation.

*In **A**, eosinophil preparations were pre-incubated with PGI_2 (100 nM) or its vehicle for 5 min and were then allowed to adhere to fibronectin-coated 96 well plates in the absence or presence of increasing concentrations of eotaxin (0.3-3 nM) for 30 min. The portion of adherent eosinophils was determined via enumeration of adhered and non-*

*adhered eosinophils by flow cytometry. In **B**, eosinophil adhesion to fibronectin-coated chamber slides and the adhesive structure formation were visualized by phalloidin-Texas Red and the nuclear dye DAPI (blue) staining. Eosinophils were pre-incubated with PGI_2 (100 nM) or its vehicle for 5 min, eosinophil adhesion was induced by eotaxin (3 nM) for 15 min, non-adhered eosinophils were discarded. n=6-8; *P<0.05 PGI_2 versus vehicle.*

PGI_2 does not affect the viability of eosinophils

As it was shown above, PGI_2 inhibits various functions of eosinophils; in order to prove that the inhibitory effect is not due to influencing the cell viability, i.e. inducing apoptosis of eosinophils, we used propidium iodide/Annexin V-FITC staining, where apoptotic cells are recognised by Annexin V binding to phosphatydyl serine on the outer leaflet of the cell membrane, while staining with propidium iodide discriminates necrotic cells. Eosinophils were incubated with vehicle, 30 nM or 100 nM of PGI_2 or iloprost, a stable PGI_2 analogue for 0h, 1h and 3h. Samples were then stained with propidium iodide/Annexin V-FITC and analyzed by flow cytometry. In these assays, neither PGI_2 nor iloprost affected the viability of the cells as indicated by unaltered numbers of apoptotic cells (***Figure 6A and B***). Similarly, the portion of necrotic cells (generally below 5% after 3h) was not altered by IP receptor stimulation (not shown). These data showed that the IP receptor mediated inhibition of eosinophil chemotaxis, adhesion and podosome formation is not due to affecting the viability of eosinophils.

Figure 6.

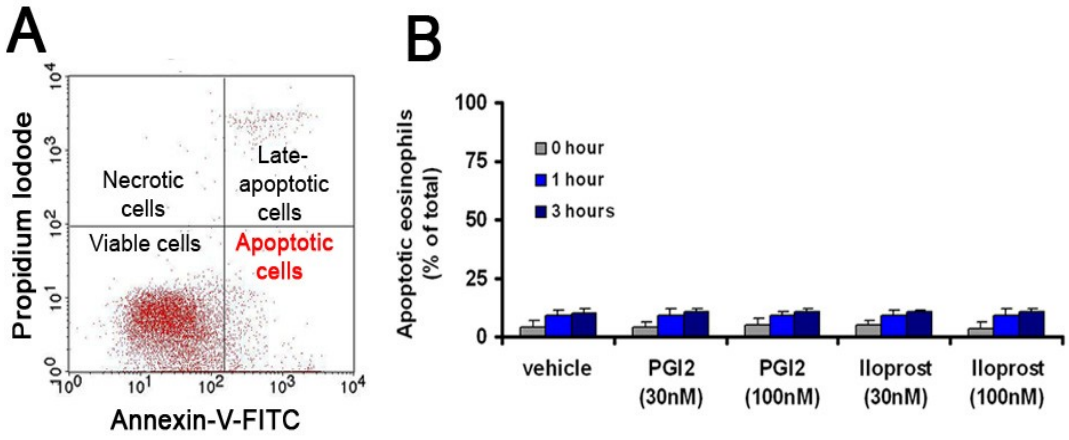

Figure 6. PGI_2 does not affect the viability of eosinophils. *Purified eosinophils were incubated with vehicle or PGI_2 (30-100 nM) or a stable PGI_2 analogue, iloprost (30-100 nM) in non-tissue culture treated 96 well plates for 0-3 h. The harvested cells were double stained with Annexin V conjugated with FITC and propidium iodide dyes and the samples were analyzed by flow cytometry. In **A**, a flow cytometric dot plot is shown, where acquired eosinophils are divided by a quadrant based on their emitted fluorescence in FL-1 (Annexin V-FITC) and in FL-2 (propidium iodide). Annexin-V^{-pos} / propidium $iodide^{-neg}$ eosinophils were identified as apoptotic cells. In **B**, apoptotic eosinophils are displayed as the percentage of total cells acquired after 0h, 1h and 3h of vehicle, PGI_2 or iloprost treatment. n = 4.*

<u>PGI_2 inhibits the up-regulation and activation of CD11b/CD18 integrin on eosinophils</u>

Since increased podosome formation and adhesiveness of eosinophils after allergen challenge is strongly related to the adhesion molecule CD11b/CD18 , we investigated whether PGI_2 was also able to block the rapid up-regulation of CD11b integrin subunits on the cell surface of eosinophils, a process that is important in mediating firm adhesion and activation of leukocytes. The flow cytometric determination of changes in eosinophil CD11b surface expression was performed using polymorphonuclear leukocyte (PMNL) preparations. Therefore, cell suspensions were stained with anti-CD11b-FITC in combination with anti-CD16-PE and incubated with serial dilutions of eotaxin (0.01-10 nM) for 15 min at 37 °C to stimulate CD11b

expression. CD11b expression on $CD16^{-neg}$ eosinophils was quantified and presented as percent of maximal CD11b expression. Before eotaxin stimulation, isolated PMNL were first pretreated with vehicle, PGI_2 (100 nM) or forskolin (20 µM), an adenylyl cylcase activator, for 5 min at room temperature. As illustrated in ***Figure 7A*** the eotaxin concentration-response curves were shifted to the right by treatment with PGI_2 (100 nM), displaying a concentration-dependent attenuation of CD11b up-regulation on the surface of eosinophil. The effect of PGI_2 was mimicked by the adenylyl cyclase activator forskolin (20 µM), which likewise blunted the eotaxin-stimulated CD11b up-regulation.

Figure 7

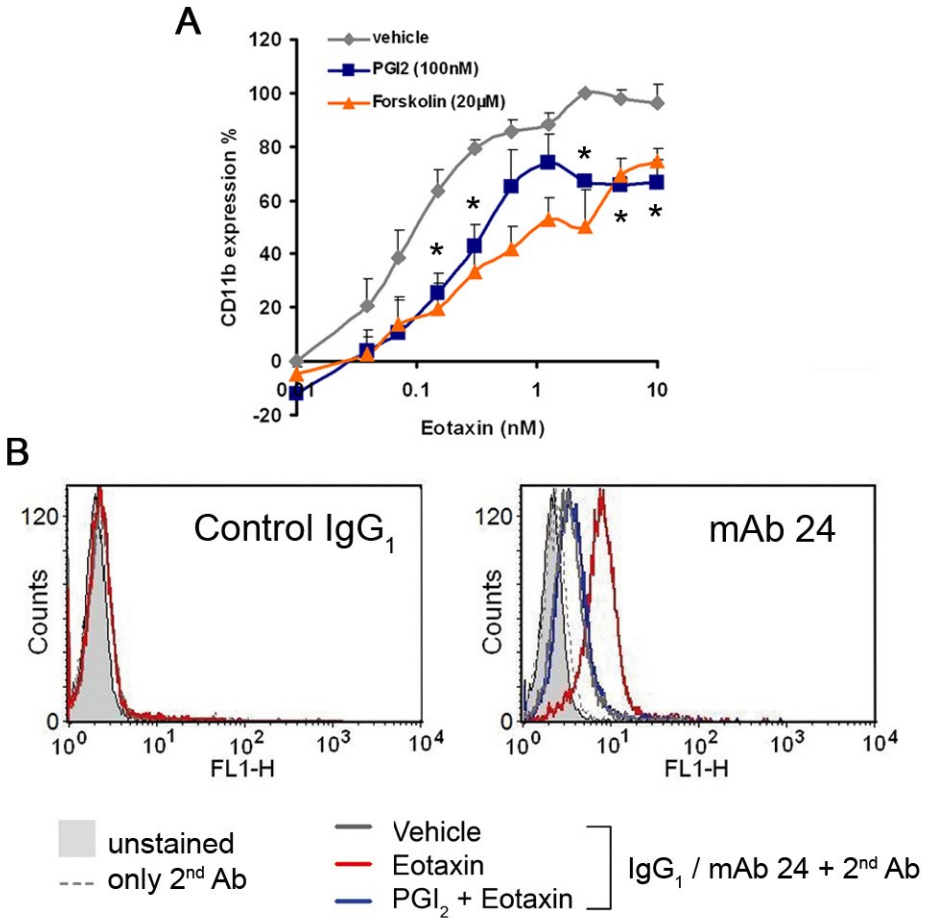

Figure 7. PGI_2 attenuates the cell surface up-regulation of the CD11b integrin subunit and prevents the activation of the CD11b/CD18 adhesion molecule complex.
*In **A**, purified eosinophils were pre-incubated with PGI_2 (100 nM), forskolin (20 µM) or its vehicle for 5 min and were then stimulated with increasing concentrations of eotaxin (0.01-10nM) for 15 min. The cell surface up-regulation of CD11b on eosinophils was*

*determined by flow cytometry and was expressed as percent of the maximal control response. In **B**, flow cytometric stainings are shown for isotype control antibody or mAb 24, an antibody specific for activated CD11b/CD18 integrin complex on eosinophils. Cells were incubated in the absence or presence of eotaxin (3 nM), PGI_2 (100 nM), or both. As controls, unstained and only 2nd antibody stained eosinophils were used. n = 4-8. *P <0.05, PGI_2 versus vehicle.*

In addition to upregulating the surface expression of CD11b, chemoattractants are capable of inducing the activation of CD11b/CD18 complexes which relies on conformational change of the integrin complex from a low ligand binding avidity status to a high ligand binding avidity status . The conformational change can be detected by specific antibodies that bind in a conformation-sensitive manner, like mAb 24 which recognizes only the activated CD11b/CD18 complexes. Here, purified eosinophils were pre-treated with either vehicle or PGI_2 (100 nM) for 5 min at room temperature. Activation of CD11b/CD18 was induced by stimulating eosinophils with eotaxin (3 nM) for 15 min at 37 ˚C, which was followed by incubating stimulated cells with mAb 24 or an isotype control antibody, and cells were stained with the Alexa fluor 488-labeled 2nd antibody. Samples were analyzed by flow cytometry and showed that eotaxin enhanced the binding of mAb 24 to eosinophils, while binding of the isotype-control antibody was not changed. The eotaxin-induced binding of mAb 24 was completely prevented by PGI_2 (***Figure 7B***). These data demonstrated that PGI_2 blocks the mobilization of intracellular pools of CD11b integrin subunit and the activation of CD11b/18 complexes, expressed on the cell surface that can further explain the effect of PGI_2 causing reduced adhesion of eosinophils.

Summary – Aim 1

The experiments presented in this first part of the thesis suggest that PGI_2 is an important negative determinant of eosinophil functions. I have shown by flow cytometry and western blot that IP, the sole receptor for PGI_2 is expressed on eosinophils; furthermore, activation of IP receptor leads to attenuation of agonist-induced eosinophil chemotaxis. The IP specific downstream signaling pathway was

identified as key mediator of the inhibitory effect of PGI_2, as selective antagonism of IP receptors and inhibition of adenylyl cyclase enzyme could rescue the inhibitory effect of PGI_2. Concomitantly, PGI_2 decreased the eosinophil adhesion to fibronectin. Morphological studies of eosinophil adhesion revealed that PGI_2 treatment does not only reduce the number of adhering eosinophils, but prevents the morphological changes needed for effective locomotion, i.e. the formation of eosinophil adhesive structures and podosomes. Furthermore, PGI_2 abrogated the mobilization of intracellular pools of CD11b integrin subunits and the activation of CD11b/CD18 complexes expressed on the cell surface, a finding that can further explain how PGI_2 reduces the adhesion of eosinophils. Importantly, the inhibitory effects of PGI_2 are carried out without affecting the viability of eosinophils.

Aim 2: To evaluate the role of PGI_2 in eosinophil – endothelial interaction

Human lung microvascular endothelial cells release PGI_2

PGI_2 is the major prostanoid released in abundance by endothelial cells, but its role in allergic inflammation has not been investigated in details. In fact, human lung microvascular endothelial cells produced significant amounts of PGI_2, as estimated by its stable metabolite 6-keto-PGF_{1a} in the supernatants (11.7 ± 2.8 pg/ml in 30 min, n = 4) but not PGE_2 (not shown). Treatment of endothelial cells with 10 μM of the COX inhibitor diclofenac, a concentration that corresponds to plasma levels in patients (Giagoudakis and Markantonis, 2005), attenuated the PGI_2 biosynthesis by 90% (1.3 ± 1.5 pg/ml, n = 4; ***Figure 9B***).

Endothelial-derived PGI_2 attenuates eosinophil adhesion to endothelial monolayers

The role of endogenous PGI_2 production in eosinophil adhesion and transmigration was studied. Endothelial monolayers were grown in multiwell plates and PGI_2 release was inhibited with diclofenac (10 µM) treatment for 30 min. Purified eosinophils were then added to the confluent endothelial monolayers in the presence or absence of diclofenac, and in the absence or presence of various concentrations of eotaxin (0.3-3 nM) or PGD_2 (10-100 nM). The portion of adherent eosinophils relative to the total numbers of input eosinophils was determined after 30 min incubation at 37°C. Eotaxin and PGD_2 concentration-dependently enhanced eosinophil adhesion to endothelial cells (***Figure 8A and B***). Diclofenac not only enhanced basal adhesion of eosinophils to endothelial cells, but also enhanced the eotaxin- and PGD_2-stimulated adhesion. To further substantiate the role for endothelial PGI_2, a more specific approach was taken, by pretreating eosinophils with the IP receptor antagonist CAY10441 (300 nM) for 10 min. ***Figure 8A*** demonstrates that, like diclofenac treatment, blocking IP receptors markedly enhanced the adhesiveness of eosinophils to the endothelial monolayers, both under basal conditions and after stimulation of eosinophils with eotaxin or PGD_2. The effect of diclofenac on eosinophil adhesion to endothelial cells appeared to be a dual one: first, baseline adhesion increased, and second, the responsiveness of eosinophils to stimulation was enhanced.

Figure 8.

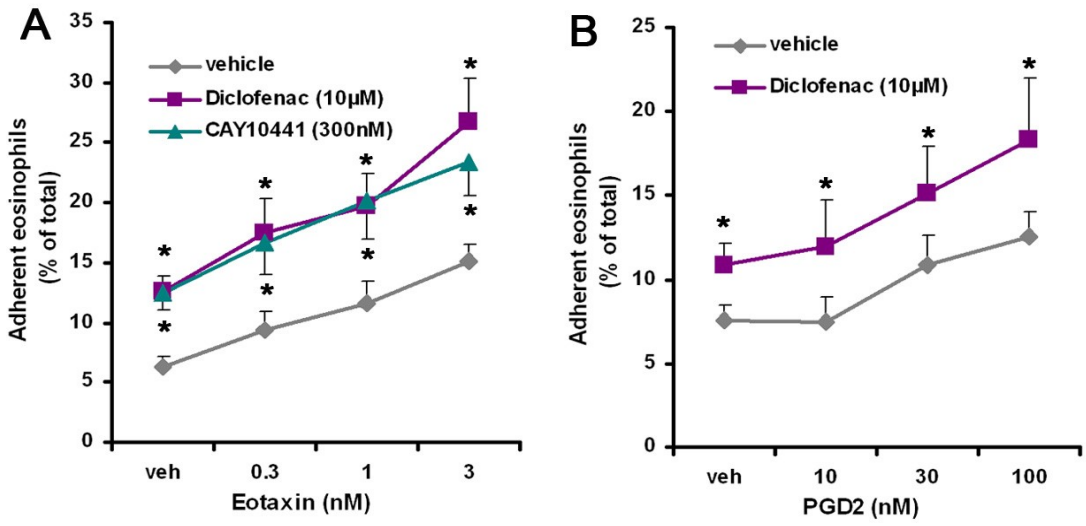

Figure 8. Endothelial derived PGI_2 attenuates the adhesion of eosinophils to human pulmonary microvascular endothelial cells. *Confluent endothelial monolayers were treated with vehicle or a cyclooxygenase inhibitor, diclofenac (10 μM) for 30 min in 48 well-plates. Alternatively, eosinophils were pretreated with vehicle or an IP receptor antagonist, CAY10441 (300 nM) for 10 min, prior to addition to the endothelial cell monolayers. Eosinophils were allowed to adhere in the presence or absence of increasing concentrations of eotaxin (0.3-3 nM) (**A**), or PGD_2 (10-30 nM) (**B**). The portion of adherent eosinophils relative to input eosinophils was determined 30 min later by flow cytometric enumeration of the cells. n=6-8; *P<0.05 diclofenac versus vehicle and CAY10441 versus vehicle.*

Endothelial-derived PGI_2 attenuates eosinophil transendothelial migration

In further experiments we assessed the effect of endogenous PGI_2 on eosinophils undergoing transendothelial migration. Diclofenac (10 μM) or its solvent were supplemented to the cell medium for 2 and 5 h before purified eosinophils were added to the top wells of the Transwell inserts. Eosinophils were allowed to migrate toward vehicle or eotaxin (1 nM) in the bottom wells for 4 h. Eotaxin enhanced the portion of eosinophils that had migrated through the endothelial monolayer into the bottom wells by 3- to 4-fold compared with vehicle, and pretreatment of the monolayers with diclofenac further enhanced the chemotactic response to eotaxin (***Figure 9A***). Interestingly, the duration of COX inhibition in endothelial cells was correlated with the enhancement of eosinophil transendothelial migration, with 5 h of diclofenac treatment being more effective than 2 h of treatment. Under these conditions, approximately 6% of input eosinophils could be recovered from the bottom wells.

Figure 9.

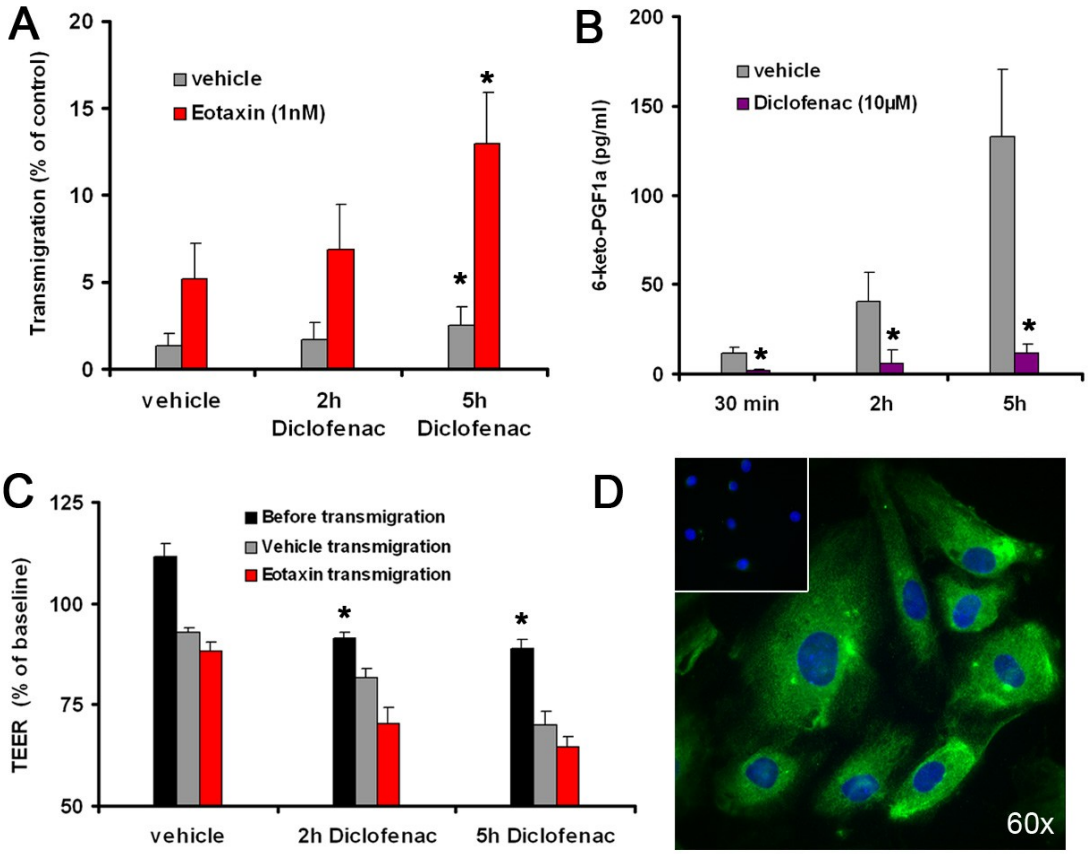

Figure 9. Inhibition of PGI_2 release impairs the barrier function of endothelial monolayers and leads to enhanced eosinophil transendothelial migration. *Endothelial monolayers were grown until confluence in Transwell plates and were treated with vehicle or the cyclooxygenase inhibitor, diclofenac (10 μM) for 2 or 5 h. In **A**, purified eosinophils were added to the top wells and were allowed to transmigrate into the bottom wells containing vehicle or eotaxin (1 nM). 4 h later, transmigrated eosinophils were enumerated from the bottom wells by flow cytometry and data were expressed as percent of cells that migrated towards eotaxin (1 nM) across blank filters. In **B**, PGI_2 release from endothelial cells that had been incubated with vehicle or diclofenac (10 μM) for 30 min, 2 and 5 h, was estimated by measuring the stable metabolite 6-keto-$PGF_{1\alpha}$ by enzyme immunoassay. In **C**, transendothelial electrical resistance (TEER) of the endothelial monolayers grown in Transwell plates was measured before and after eosinophil transmigration and was expressed as percent of baseline, i.e. before diclofenac or vehicle was added. n=3-6; *P<0.05 diclofenac versus vehicle of diclofenac. In **D**, immunofluorescence staining of endothelial IP receptor expression is*

shown. The insert depicts the staining with isotype control antibody and the nuclei stained with DAPI. The staining shown is representative of 3 independent experiments.

Endothelial-derived PGI_2 sustains the integrity of endothelial junctions

Loss of endogenous PGI_2 leads to decrease of transendothelial electrical resistance

The endothelial cells were grown to confluent monolayers in Transwell inserts. Confluence of the monolayers was confirmed by measuring transendothelial electrical resistance daily until the assay was performed. Mean electrical resistance of confluent endothelial monolayers increased to values of 75 to 106 Ω and remained constant thereafter. Diclofenac (10 µM) or its solvent were supplemented to the cell medium. Treatment of endothelial cells with the COX inhibitor diclofenac (10 µM) for 2 and 5 h reduced the PGI_2 biosynthesis by 90% (***Figure 9B***). Unexpectedly, transendothelial electrical resistance of the endothelial monolayers was reduced after diclofenac treatment (***Figure 9C***), suggesting that endogenous PGI_2 release is an important autocrine factor in supporting endothelial barrier function. Moreover, transendothelial electrical resistance of the endothelial monolayers was further decreased after eosinophil transmigration (***Figure 9C***). These observations suggested that the loss of endogenous PGI_2 not only augments eosinophil transmigration but also compromises the barrier function of the endothelial monolayer.

IP receptor is expressed on pulmonary microvascular endothelial cells

PGI_2 seemed to act in an autocrine fashion on endothelial cells. Therefore, I assessed the expression of IP receptors in the human lung microvascular endothelial cells using a polyclonal IP receptor antibody and corresponding isotype-matched control antibody. In fact, indirect immunofluorescence staining confirmed the presence of IP receptors on endothelial cells (***Figure 9C***).

Visualizing endothelial junctions - immunofluorescence staining of VE-cadherin

In order to investigate in more detail whether inhibition of COX altered the barrier function of the endothelial layer, cells were grown on glass cover slips and treated with diclofenac for 2 and 5 h. Fluorescence staining of VE-cadherin, a major component of the endothelial barrier by forming adherens junctions, and of F-actin was carried out. The vehicle-treated endothelial monolayers presented with tight, closed cell

junctions and subtle F-actin staining mainly distributed to the subcortical actin rim (***Figure 10***). Diclofenac treatment disrupted the endothelial cell junctions, and after 5 h of diclofenac treatment, even pore formation between endothelial cells was detected. As a result of blocked PGI_2 biosynthesis, more intense actin polymerization was observed, with formation of F-actin stress fibers after 2 h and redistribution of dense actin filaments to the subcortical region after 5 h. Co-incubation of endothelial cells with diclofenac plus the stable PGI_2 analogue iloprost (300 nM) prevented the loss of tight adherent junctions and gap formation, and also delayed the reorganization of the actin cytoskeleton (***Figure 10***).

Figure 10.

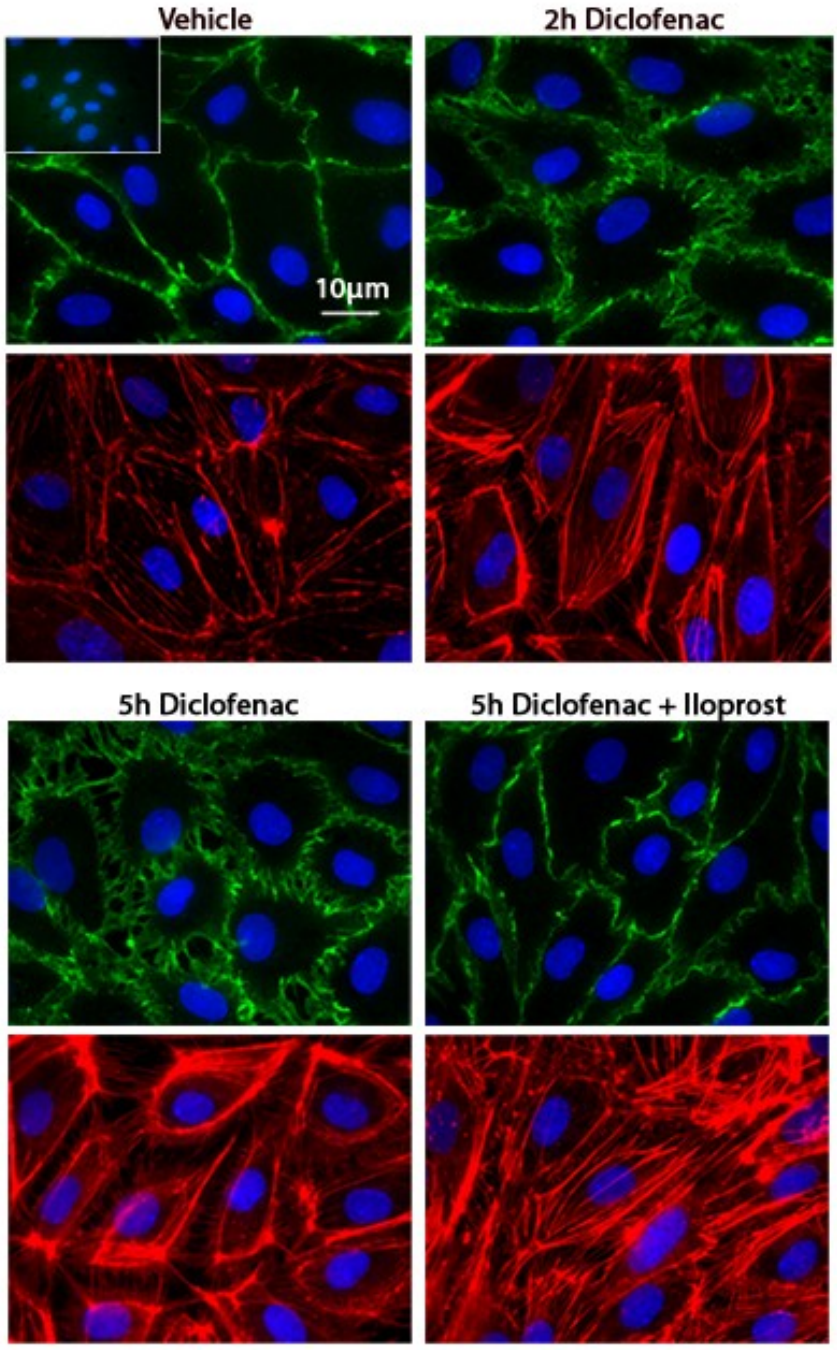

Figure 10. Immunofluorescence staining of VE-cadherin and F-actin shows that inhibition of PGI_2 release leads to morphological changes of endothelial monolayers. *Confluent endothelial monolayers grown on chamber slides were incubated with vehicle or the cyclooxygenase inhibitor diclofenac (10 µM) or diclofenac plus the IP receptor agonist, iloprost (100 nM) for 2 or 5 h and were then stained with anti-VE-cadherin antibody (green) or phalloidin-Texas Red conjugate (red). The stainings shown are representative of 3 independent experiments. The insert shows staining with an isotype-control antibody.*

Summary – Aim 2

In this part I have shown that human pulmonary microvascular endothelial cells release PGI_2 (as it was demonstrated by measuring its stable metabolite, 6-keto-PGF_{1a} in the conditioned supernatant). The endothelial release of PGI_2 was reduced by diclofenac, a non-selective COX-inhibitor. In interaction assays with diclofenac-pretreated endothelial cells, adhesion and transendothelial migration of eosinophils were enhanced significantly, suggesting that the endogenously produced PGI_2 is an important factor in limiting interactions of endothelium with the inflammatory effector cells, e.g. eosinophils. Furthermore, PGI_2 in an autocrine manner seems to be involved in promoting the integrity of endothelial junctional network, as it was observed that the loss of endogenous PGI_2 led to decrease of transendothelial electrical resistance of the endothelial monolayer. Additionally, I demonstrated the expression of PGI_2 receptors, IP, by immunofluorescence microscopy. In order to further investigate the permeability changes of endothelial monolayers caused by the loss of endogenous PGI_2, the junctional alterations were visualized by staining of VE-cadherin, the major component of adherens junctions. Collectively, these data suggest a dual role for endogenous PGI_2 in the interaction of endothelial cells and eosinophils. Firstly, endothelial PGI_2 attenuates the adhesion and transendothelial migration of eosinophils in a paracrine fashion; secondly, PGI_2 shields the endothelial junctional connections in an autocrine manner.

Part II : The role of PGE_2 – EP4 signaling in endothelial barrier function and transendothelial trafficking of eosinophils

Background

PGE_2 is the predominant cyclooxygenase (COX) product of epithelial cells, airway macrophages and smooth muscle cells, and is regarded as a potent inflammatory mediator due to its effects on vasodilation, vascular permeability and nociception; however, the role of PGE_2 in allergic inflammation is less clear. COX-1 and COX-2 deficient mice exhibited significantly increased lung inflammation and airway hyperresponsiveness in OVA induced asthma model, which is correlated with abrogation of PGE_2 biosynthesis . Furthermore, PGE_2 besides preventing allergen-induced bronchoconstriction , it is observed to inhibit allergen-induced airway inflammation, including decreased airway eosinophilia in asthma patients and Th2 cytokine production in the OVA-induced murine model . Endothelial cells express all four EP receptors (EP1-4) .

Aims:

In the second part of my work I focused on one PGE_2 receptor, the EP4 receptor. I hypothesized that the selective activation of EP4 receptor might promote the barrier function of endothelium. Therefore different resistance measurements were carried out, endothelial adhesion molecule expression assays were performed, and trafficking of inflammatory eosinophils was studied in order to understand the role of EP4 activation in this cellular interaction under allergic inflammatory conditions.

Aim 1: To assess the role of PGE_2-EP4 signaling in the regulation of endothelial barrier function

Aim 2: To obtain evidence for the importance of PGE_2-EP4 signaling in the trafficking of eosinophils

Aim 1: To assess the role of PGE_2-EP4 signaling in the regulation of endothelial barrier function

Prostaglandin E_2 and and EP4 receptor agonist prevent thrombin-induced endothelial barrier disruption

In order to investigate how PGE_2 and the specific EP4 agonist (ONO-AE1-329) affect the barrier function of the endothelial monolayer, cells were grown on chamber slides until they formed tight monolayers. After pretreatment with vehicle, PGE_2 or EP4 agonist (30 nM) for 15 min at 37 °C, endothelial cells were incubated with 0.5 U/mL thrombin or vehicle for 20 min at 37°C. Fluorescence staining of VE-cadherin, the major component of endothelial adherens junctions was carried out. The vehicle-treated endothelial monolayers showed tight, closed junctions and relaxed cells (***Figure 11***). Thrombin treatment disrupted the cell junctions, and small pores appeared between the retracted endothelial cells. PGE_2 and EP4 agonist (30 nM each) prevented the thrombin-induced barrier disruption and cell retraction. In the absence of thrombin stimulation of the endothelial monolayer (control treatment), PGE_2, EP4 agonist or EP4 antagonist pretreatments did not alter markedly the junctional network. However, a selective EP4 antagonist (ONO-AE3-208 at 300 nM) prevented the protective effect of PGE_2 on the endothelial monolayer, thus enabling thrombin-induced interendothelial gap formation (***Figure 11***). These data suggested that PGE_2 is protective in maintaining the integrity of enodothelial cell layer when it is exposed to a barrier disruptive agent like thrombin. Moreover, PGE_2 exerts its effect via activating EP4 receptor which was shown by using a selective EP4 receptor agonist. Importantly, an EP4-selective agonist fully mimicked the protective effect of PGE_2 on the endothelial monolayers.

Figure 11.

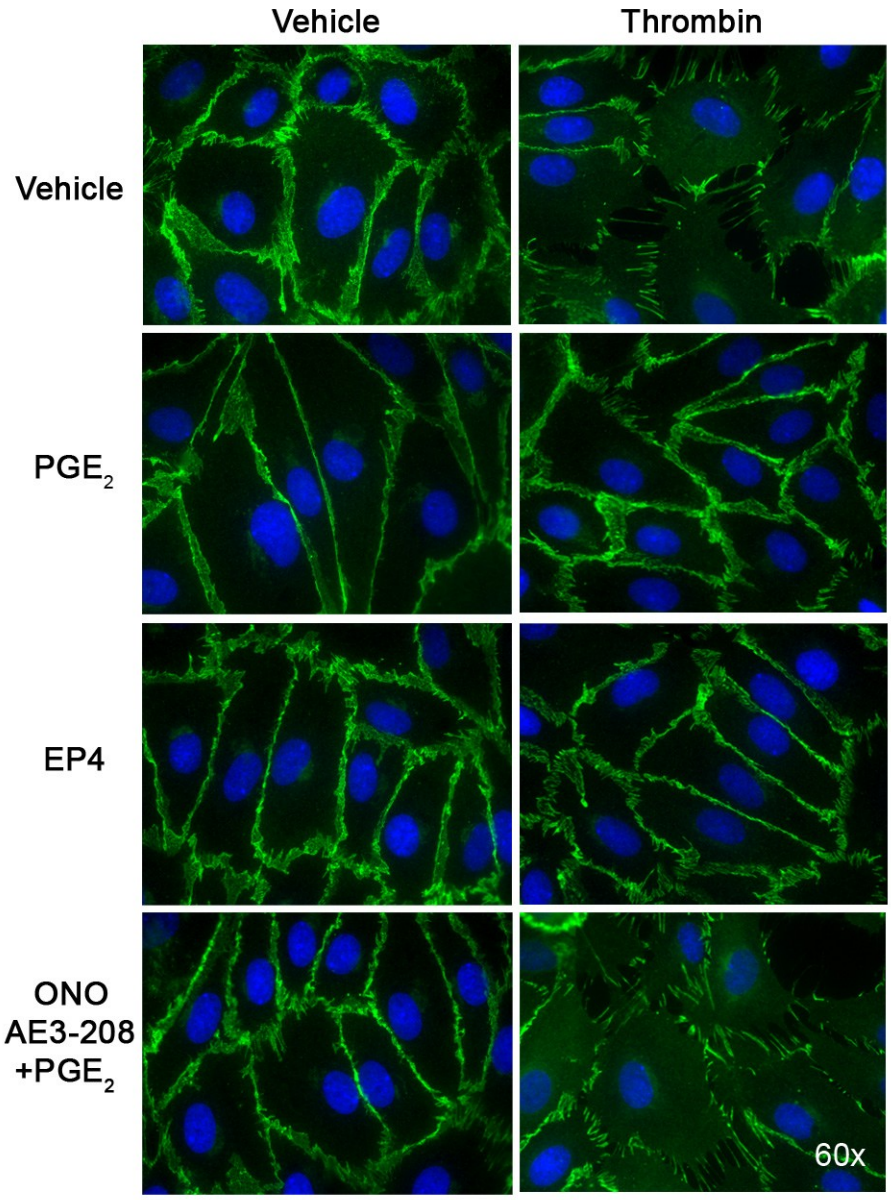

Figure 11. ***Immunofluorescence staining of VE-Cadherin reveals that EP4 receptor activation protects against thrombin-induced disintegration of the endothelial layer.*** *Confluent endothelial monolayers grown on chamber slides were preincubated with vehicle, PGE_2 or EP4 agonist (30 nM each) for 15 min or ONO-AE3-208 (EP4 selective antagonist, 300 nM, 20 min prior to PGE_2). The monolayers were exposed to thrombin (0.5 U/ml) or vehicle for 20 min and were then stained with anti-VE-cadherin antibody (green); the nuclei are stained with DAPI (blue). The micrographs are representative of 3 independent experiments.*

Prostaglandin E_2 and a selective EP4 receptor agonist promote wound healing of endothelial monolayers

To study the effect of PGE_2 and the role of EP4 receptors in the regenerative potential of endothelial layers, the cells were grown on small gold electrodes and monitored by using electric cell-substrate impedance sensing (ECIS, Applied Biophysics, Troy, New York). After the endothelial cells formed a confluent layer and the resistance reached a maximal plateau phase (6000 Ω at 48 kHz), the monolayers were electrically wounded (standard protocol: 900 μA, 5 sec, 48 kHz). Repopulation of the electrodes, i.e. healing of the wound was recorded online. Pretreatment of the monolayers with PGE_2 and EP4 agonist (30 nM each) for 15 min before the wounding, promoted markedly the regeneration of the cell layers (***Figure 12***). Already 4 h after the wounding it became evident that the PGE_2 or EP4 agonist-treated endothelial cells showed faster recovery, i.e. the increase of resistance was higher than in vehicle-treated cells. Between 8 and 10 h after wounding, the PGE_2 and EP4 agonist-pretreated endothelial cells showed statistically significant differences versus the vehicle treatment. Complete recovery of the monolayers was observed 11 h after the wounding in the vehicle-treated endothelial cells, but already after 8 h in PGE_2 or EP4 agonist-treated endothelial cells. Vascular endothelial growth factor (VEGF), a known barrier promoting agent was used as a positive control which resulted in regeneration of the endothelial layer in a very similar manner, like PGE_2 or the EP4 agonist.

Figure 12.

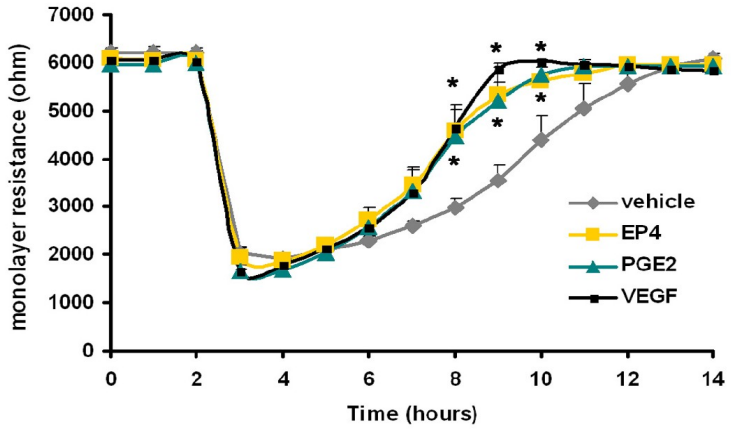

Figure 12. Wound healing of endothelial monolayers is promoted by EP4 agonist and PGE_2 treatment. *Endothelial cells were grown on small gold electrodes and monitored for changes in resistance by using electric cell-substrate impedance sensing (ECIS). After recording the baseline resistance, the monolayers were pretreated with EP4 agonist (30 nM) and PGE_2 (30 nM) for 15 min and they were subjected to high field pulse according to a standard wounding protocol. The resistance changes were followed until total recovery of the monolayers. Vascular endothelial growth factor (VEGF) was used as positive control. n=4-6; *P<0.05 EP4, PGE_2 and VEGF versus vehicle.*

The specific involvement of EP4 receptors in the endothelial barrier regulation in response to PGE_2 was assessed by using a selective EP4 antagonist (ONO-AE3-208). Blockade of EP4 receptors for 20 min before administration of PGE_2 reversed the barrier-promoting effect of PGE_2 and resulted in slower wound healing, similar to untreated cells (***Figure 13A***). In contrast, the selective EP2 receptor agonist, butaprost, and the selective EP3 receptor agonist, sulprostone failed to promote the healing of the wound (***Figure 13B***). These data show that PGE_2 via activating EP4 receptors does not only help to maintain the tightly connected network of endothelial cells but even promotes the regeneration of injured monolayers.

Figure 13.

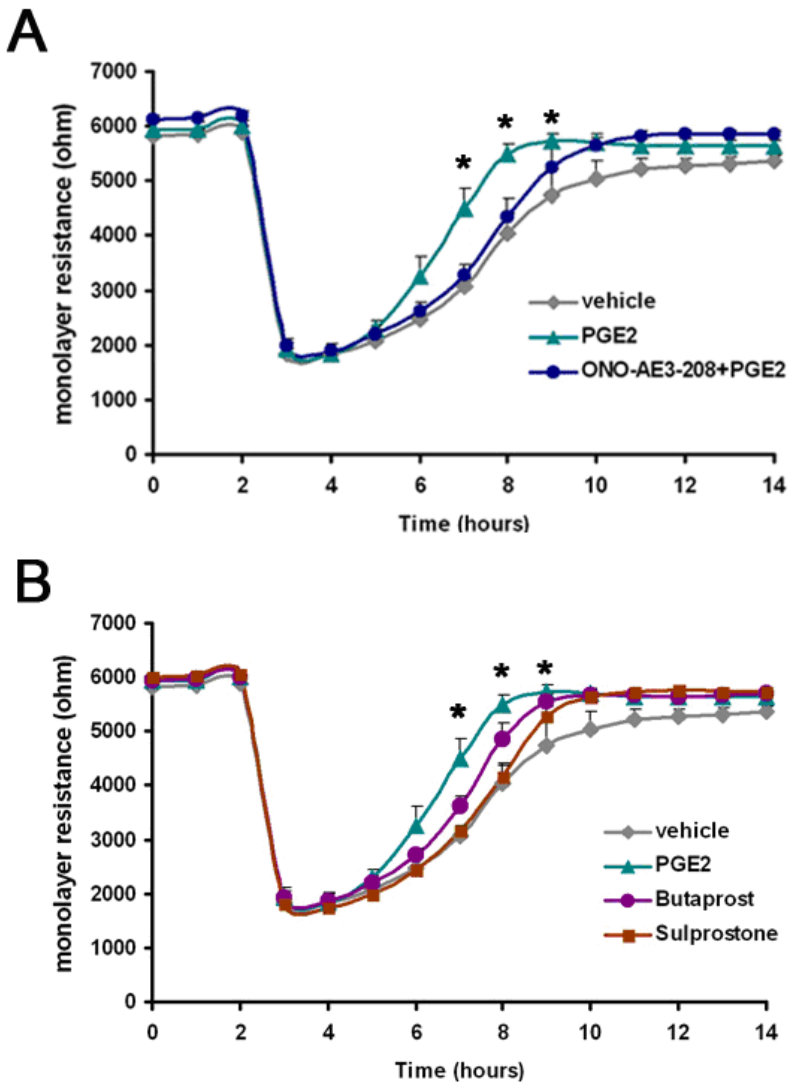

Figure 13. Selective activation of EP4 receptors mediates the PGE_2-induced enhancement of wound healing of endothelial monolayers. *In **A,** endothelial cells grown on small gold electrodes were pretreated with the EP4-selective antagonist, ONO-AE3-208 (300 nM) for 20 min, and then treated with PGE_2 (30 nM) for 15 min prior to electrical wounding of the monolayers. In **B,** endothelial cells were pretreated with either PGE_2 (30 nM) or butaprost (EP2 receptor-selective agonist, 30 nM) or sulprostone (EP3-selective agonist, 30 nM) for 15 min prior to electrical wounding. n=4-6; *P<0.05 PGE_2 versus vehicle. *P>0.05 butaprost or sulprostone versus vehicle.*

Stimulation of EP4 receptors blocks the transendothelial migration of eosinophils

To study the barrier function of endothelial cells against transmigrating eosinophils, I performed transendothelial migration assays where endothelial cells were grown into confluent monolayers in Transwell inserts with 5 μm pore size.

In order to trigger eosinophil transendothelial migration, endothelial permeability was induced by the addition of 0.5 U/mL thrombin for 20 min. Eosinophils were allowed to transmigrate for 4 h towards vehicle or 3 nM eotaxin. The basal transmigration towards vehicle was approximately 6% of the cells added to the top inserts (***Figure 14***). The presence of 3 nM eotaxin in the bottom wells triggered 13% eosinophil transmigration, and EP4 agonist pretreatment of the endothelial layer diminished the migratory response to 7%. After endothelial monolayers had been disintegrated with 0.5 U/mL thrombin, 3 nM eotaxin caused the migration of 35% of eosinophils across the endothelial layer. The thrombin-enhanced eosinophil transmigration was reduced to 11.26% by EP4 agonist pretreatment of the endothelial layer, to similar values observed in the absence of thrombin. Thrombin evolves its barrier-disrupting effect in a reversible manner, but it also leads to endothelial release of different cytokines; therefore, thrombin was added just 20 min prior to the addition of eosinophils, to open up the endothelial junctions for the eosinophil transmigration.

Figure 14.

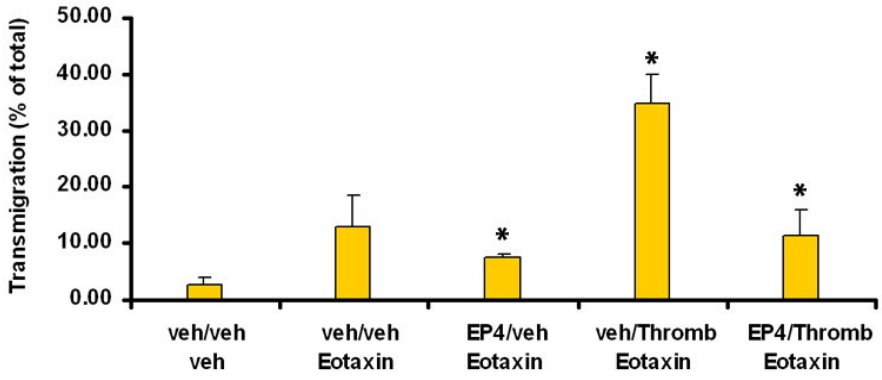

Figure 14. EP4 receptor activation attenuates the thrombin induced eosinophil transendothelial migration. *Endothelial monolayers grown in Transwell plates were pretreated with the EP4-selective agonist ONO AE1-329 (30 nM) or vehicle for 15 min prior to exposing the monolayers to thrombin (0.5 U/ml) for 15 min. The monolayers were then subjected to eotaxin (3 nM) triggered eosinophil transmigration. Four h*

*later, the non-migrated cells from the top wells and the transmigrated cells from the bottom wells were collected and enumerated by flow cytometry. n=6; *P<0.05 EP4 agonist/vehicle versus vehicle/vehicle and EP4 agonist/thrombin versus vehicle/thrombin.*

EP4 agonist prevents the thrombin-induced endothelial hyperpermeability

Confluence of the monolayers before conducting eosinophil transmigration assays, was confirmed indirectly by measuring transendothelial electrical resistance (TEER) using an Endohm device. Endothelial layers were used for experiments when the electrical resistance had increased to 75-105 Ω, as described . TEER changes were recorded prior to endothelial treatment, following the treatment and after the transmigration assay. As measured before transmigration, thrombin reduced the TEER of the monolayer by 70% with respect to the basal resistance (***Figure 15***). The EP4 receptor agonist slightly, but significantly increased the electrical resistance of the monolayers by 11% in the absence of thrombin and prevented the thrombin-induced drop in resistance by approximately 50%. After eosinophil transmigration, the TEER of the monolayers was further decreased.

Figure 15.

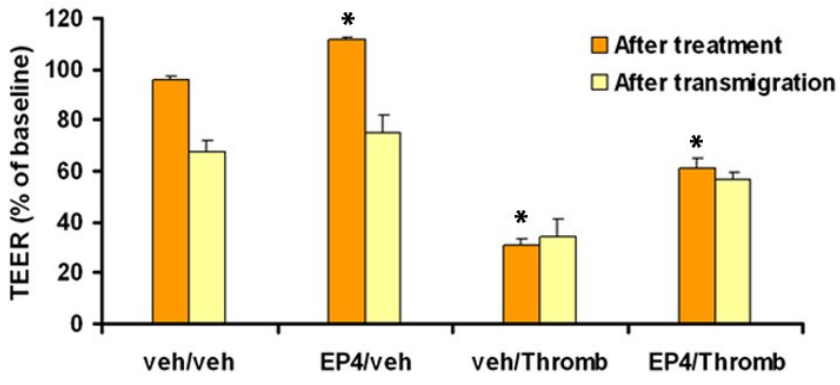

Figure 15. Activation of EP4 receptors shields the endothelial monolayer resistance upon thrombin induced endothelial hyperpermeability. *Transendothelial electrical resistance (TEER) of the endothelial monolayers grown in Transwell plates was measured before and after EP4 agonist (ONO AE1-329, 30 nM) or vehicle pretreatment*

*for 15 min and after eosinophil transmigration triggered by eotaxin (3 nM) and was expressed as percent of baseline, i.e. before treatment. n=6; *P<0.05 EP4 agonist/vehicle versus vehicle/vehicle and EP4 agonist/thrombin versus vehicle/thrombin.*

PGE_2 and the EP4 agonist attenuate the TNFα-induced upregulation of endothelial adhesion molecule expression

Under inflammatory conditions the expression of endothelial adhesion molecules, like E-selectin, VCAM-1 and ICAM-1 is upregulated. The impact of PGE_2 and stimulation of EP4 receptors on the TNFα-induced upregulation of the expression of VCAM-1, ICAM-1 and E-selectin was investigated. Endothelial cells, grown in 48-well plates, were activated with 1 pM and 10 pM TNFα for 4 h at 37˚C. Upregulation of the adhesion molecules was determined by using fluorochrome-labeled antibodies. Endothelial cells were pretreated with 30nM of PGE_2, the EP4 agonist ONO AE1-329 or vehicle for 15 min at 37˚C before TNFα was added. TNFα markedly upregulated all three adhesion molecules on the surface of endothelial cells. ICAM-1 and VCAM-1 upregulation could not be significantly reduced by PGE_2 or the EP4 agonist (***Figure 16A and B***). In contrast, PGE_2 and the EP4 agonist significantly reduced the TNFα-induced upregulation of E-selectin expression on the endothelial cells by approximately 50% (Fig 14, B). In order to investigate the signaling mechanism behind the inhibitory effect of EP4 receptor activation, the adenylyl cyclase inhibitor SQ22536 (10 μM) and the protein kinase C (PKC) inhibitor chelerythrine (1μM) were employed. Inhibition of adenylyl cyclase could not prevent the inhibitory effect of the EP4 receptor agonist or PGE_2 on the TNFα-induced endothelial E-selectin expression. In contrast, chelerythrine almost completely reversed the effect of EP4 receptor agonist (96.33% of control) and PGE_2 (88.76% of control) at attenuateing the E-selectin expression. These data suggested that PKC, but not the adenylyl cyclase/cAMP pathway, is involved in the signaling of the EP4 mediated attenuation of E-selectin expression.

Figure 16.

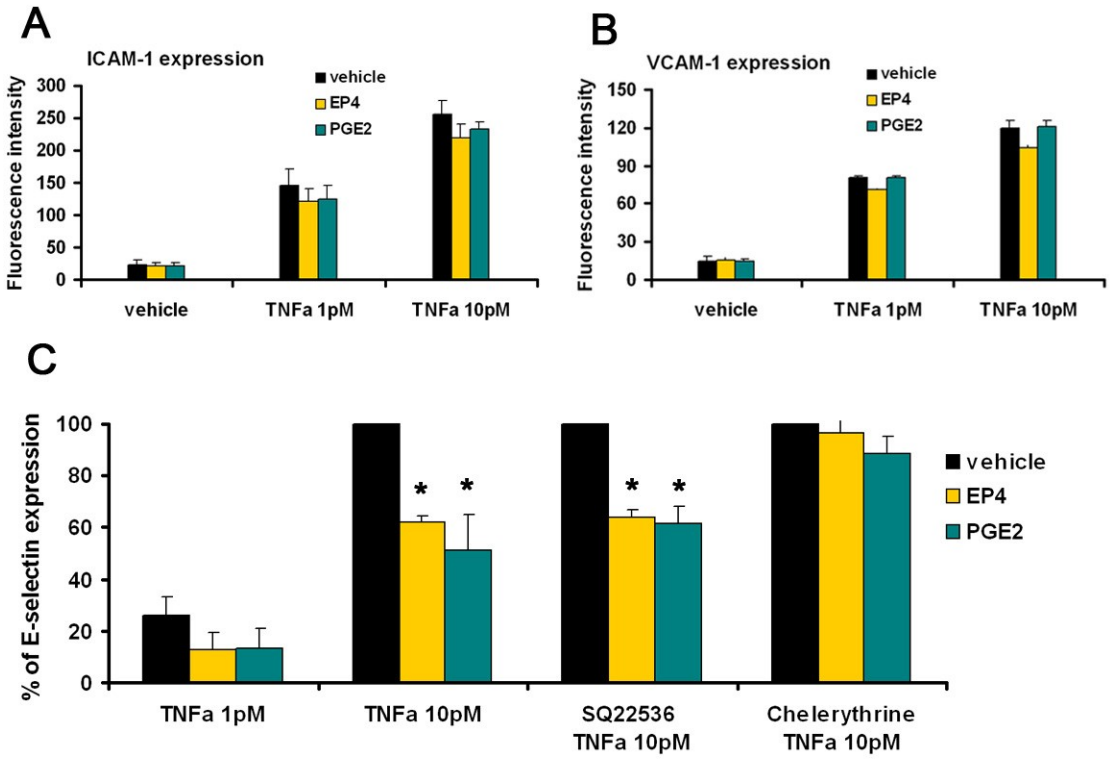

Figure 16. PGE_2 or stimulation of EP4 receptors reduced the TNFα-induced E-selectin expression in endothelial cells. *Prior to inducing endothelial cell adhesion molecule expression with TNFα (1 - 10 pM), endothelial cells grown in 48-well plates were pretreated with vehicle, the EP4 agonist ONO AE1-329 or PGE_2 (30 nM each) for 15 min. Adhesion molecule expression was detected by using fluorophore-labeled antibodies against ICAM-1, VCAM-1 and E-selectin. In **A,** ICAM-1, in **B,** VCAM-1 expression changes are displayed as raw data (fluorescence intensity). In **C,** E-selectin expression changes are shown as % of E-selectin expression, where 100% is the maximal E-selectin expression observed upon 10 pM TNFa stimulation of vehicle pretreated endothelial cells. SQ22536 (adenylyl cyclase inhibitor, 10 μM) or chelerythrine (PKC inhibitor, 1 μM) were added to the endothelial cells 15 min before further treatments were given. n=6; *P<0.05 EP4 agonist versus vehicle and PGE_2 versus vehicle.*

EP4 receptor expression on endothelial cells and eosinophils

The presence of EP4 receptors on endothelial cells and eosinophils was investigated by indirect immunofluorescence flow cytometry. Histograms show human lung microvascular endothelial cell (HMVEC-L) and eosinophil EP4 receptor staining (***Figure 17***). Isotype-matched irrelevant antibodies or the second antibody alone were used as negative controls.

Figure 17.

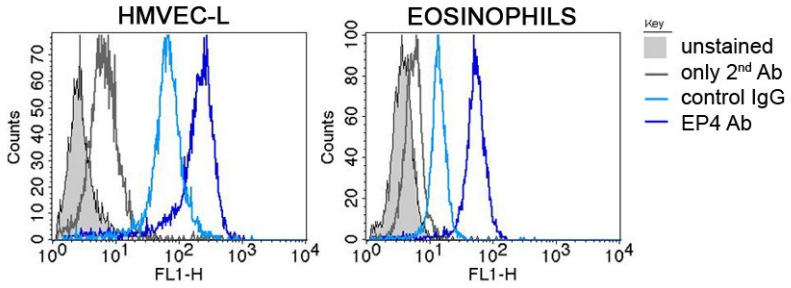

Figure 17. EP4 receptor is expressed on endothelial cells and eosinophils. *Flow cytometric histograms are shown for* human lung microvascular endothelial cell (HMVEC-L) *and eosinophils. As controls, unstained cells, only 2nd antibody or isotype control IgG_1 plus the 2nd antibody were used. The stainings shown are representative of three different experiments.*

Summary – Aim 1

These data showed that stimulation of EP4 receptors promotes the barrier function of endothelial monolayers. Selective activation of EP4 receptors can prevent the hypermeability induced by barrier disrupting agents, such as thrombin, as shown by visualizing the junctional network of endothelial monolayers and performing TEER measurements on endothelial cells grown in Transwell plates. The recovery of electrically wounded endothelial layers was also stimulated by PGE_2 and EP4 agonist pretreatment of the endothelial cells. Selective EP4 receptor blockade and other PGE_2

analogs specific for distinct PGE_2 receptors (EP2, EP3) were applied for proving the specificity of the involvement of EP4 receptors. Furthermore, EP4 receptor activation attenuated eosinophil trafficking towards eotaxin through the endothelium, as an indirect measure of the endothelial barrier properties. The endothelial adhesion molecule, E-selectin, when induced by TNFα was also negatively modulated by PGE_2 and an EP4 agonist, and the data showed that PKC, but not the adenylyl cyclase/cAMP pathway, is involved in the signaling of the EP4-mediated attenuation of E-selectin expression. Finally, I showed the EP4 receptor expression on endothelial cells by indirect immune flow cytometric staining. Importantly, eosinophils were also found to express EP4 receptors.

Aim 2: To obtain evidence for the importance of PGE_2-EP4 signaling in the trafficking of eosinophils

<u>EP4 agonist blocked the upregulation and activation of CD11b/CD18 integrins</u>

One of the most important determinants of eosinophil-endothelial interaction is the group of integrins that mediate eosinophil firm adhesion to endothelial cells. Activation of CD11b/CD18 integrin was assessed by the specific binding of the activation-sensitive antibody mAb 24. Purified eosinophils were pre-treated with vehicle or the EP4 agonist (30 nM), then incubated with 3nM eotaxin or 30 nM PGD_2 together with mAb 24, or an IgG_1 control antibody for 15 min at 37°C. After the primary antibodies, samples were incubated with the secondary antibody. Flow cytometric measurements showed that the eotaxin or PGD_2-induced activation of CD11b/CD18 integrin complexes was prevented by the EP4 receptor agonist (***Figure 18A***).

Next, I examined whether the EP4 agonist and PGE_2 were able to inhibit the quick up-regulation of CD11b integrin subunits on the cell surface of eosinophils. Changes of CD11b surface expression on eosinophils was determined by flow cytometry using polymorphonuclear leukocyte (PMNL) preparations. PMNL were stained with anti-CD11b-FITC in combination with anti-CD16-PE and stimulated with

increasing concentrations of eotaxin (0.01-10 nM) for 15 min at 37 °C to increase CD11b expression. CD11b expression on $CD16^{-neg}$ eosinophils was quantified and presented as percent of maximal CD11b expression. Before eotaxin stimulation, PMNL preparations were first pretreated with vehicle, PGE_2 (30 nM) or an EP4 agonist (30 nM) for 5 min at room temperature. As shown in ***Figure 18B*** the eotaxin-induced CD11b upregulation was reduced by treatment with PGE_2 and EP4 agonist (30 nM each), showing a concentration-dependent inhibition of CD11b up-regulation eosinophil cell surface.

Figure 18.

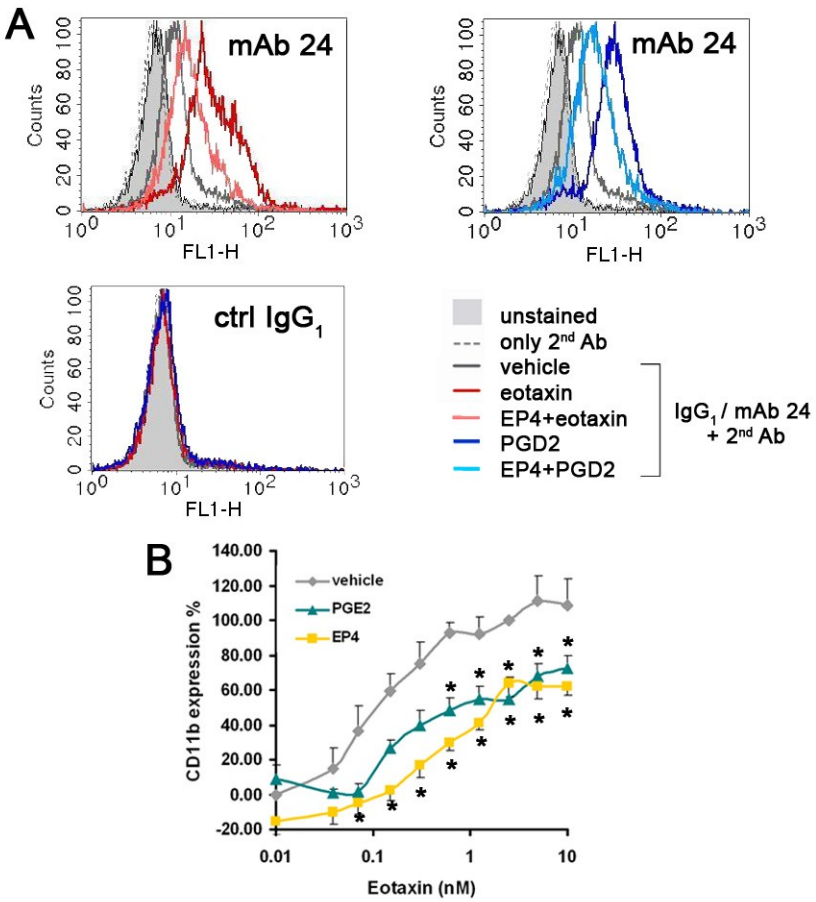

Figure 18. EP4 agonist attenuates the cell surface up-regulation of the CD11b integrin subunit and prevents the activation of CD11b/CD18 adhesion molecule complex. *In* ***A,*** *purified eosinophils were pre-incubated with the EP4 agonist ONO AE1 329 (30 nM) or its vehicle for 10 min and were then stimulated with vehicle, eotaxin (3 nM) or* PGD_2 *(30 nM) for 15 min in the presence of the mAb 24 or the control* IgG_1 *antibody. Flow cytometric staining is depicted with isotype control antibody or mAb 24, an antibody, specific for activated CD11b/CD18 integrin complex*

*on eosinophils. As controls, unstained and only 2nd antibody-stained eosinophils are shown. n = 6. In **B**, purified eosinophils were pre-incubated with EP4 agonist (30 nM), PGE_2 (30 nM) or its vehicle for 5 min and were then stimulated with increasing concentrations of eotaxin (0.01-10nM) for 15 min. The cell surface up-regulation of CD11b on eosinophils was determined by flow cytometry and was expressed as percent of the maximal control response. n = 4-8. *P <0.05, EP4 agonist or PGE_2 versus vehicle.*

<u>PGE_2 and EP4 agonist inhibit the clustering of activated CD11b/CD18 integrins</u>

Following integrin activation, the clustering of activated integrins on leukocytes has been suggested to play an important role in successful and efficient adhesion to endothelial cells. Clustered integrins determine preferred sites of adhesion and enable the eosinophils at these sites to strongly bind to endothelial adhesion molecules. Following the staining procedure for detecting the activated CD11b/18 integrins (see above), stimulated and stained cells were cytospinned to microscope slides. Immunofluorescent images of cytospinned eosinophils revealed that stimulation of eosinophils with eotaxin led to the clustering of CD11b/CD18 which can be seen as distinct dots on the eosinophil cell surface (***Figure 19***). However, PGE_2 and EP4 agonist pretreatment of eosinophils prevented the eotaxin-triggered clustering of CD11b/CD18: staining in the presence of PGE_2 or the EP4 agonist was homogenous on eosinophils, similar to unstimulated cells.

Figure 19.

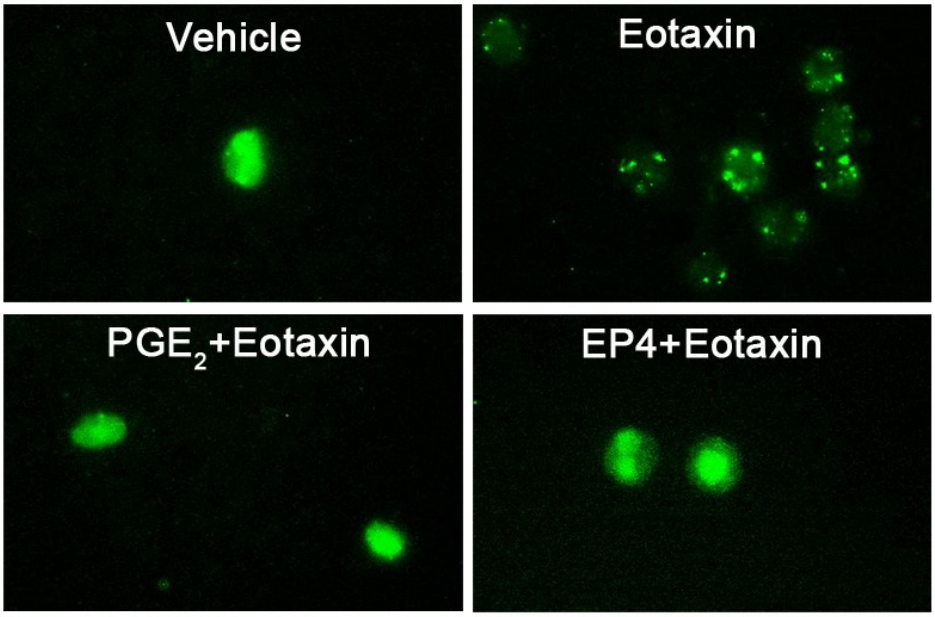

Figure 19. EP4 agonist and PGE_2 inhibit the cell surface clustering of activated CD11b/CD18 integrins. *Purified eosinophils were pre-incubated with the EP4 agonist ONO AE1-329 or PGE_2 (30 nM each) or with vehicle for 10 min and were then incubated with vehicle, eotaxin (3 nM) for 15 min together with the mAb 24 or the control IgG_1 antibody. Immunofluorescent images are magnifications of the original images captured with 60x oil immersion objectives. The stainings shown are representative of 3 independent experiments.*

Podosome formation of adherent eosinophils is attenuated by PGE_2 and EP4 an agonist

Eosinophil adhesion and adhesive structure formation was demonstrated with Phalloidin-Texas Red staining of F-actin in eosinophils that adhered to fibronectin-coated chamber slides. Purified eosinophils were pretreated with vehicle, PGE_2 or EP4 agonist (30 nM each) for 10 min at room temperature. After the pre-treatment, eosinophils were stimulated with 3nM eotaxin to become adherent on the fibronectin coated slides for 30 min at 37˚C in a humidified incubator. As the next step, non-adherent eosinophils were aspirated, the adhered cells were fixed, permeabilized, blocked and finally stained with phalloidin-Texas Red. The slides were mounted and

analyzed with fluorescence microscopy. F-actin staining in adherent eosinophils revealed distinct morphologic alterations (***Figure 20***). The micrographs of vehicle-treated eosinophils showed round shaped small cells. This morphology was remarkably altered upon activation of the eosinophils; eotaxin induced the polarization and elongation of the cell shape, and caused the formation of adhesive structures, reminiscent of podosomes, which are dynamic adhesive structures considered to be important for directed cell motility. Podosome formation was quantified from the micrographs and compared in all treatment groups (300 cells / treatment). Eotaxin induced extensive podosome formation, while PGE_2 and the EP4 agonist markedly reduced the podosome numbers of adherent eosinophils (***Figure 20B***).

Figure 20.

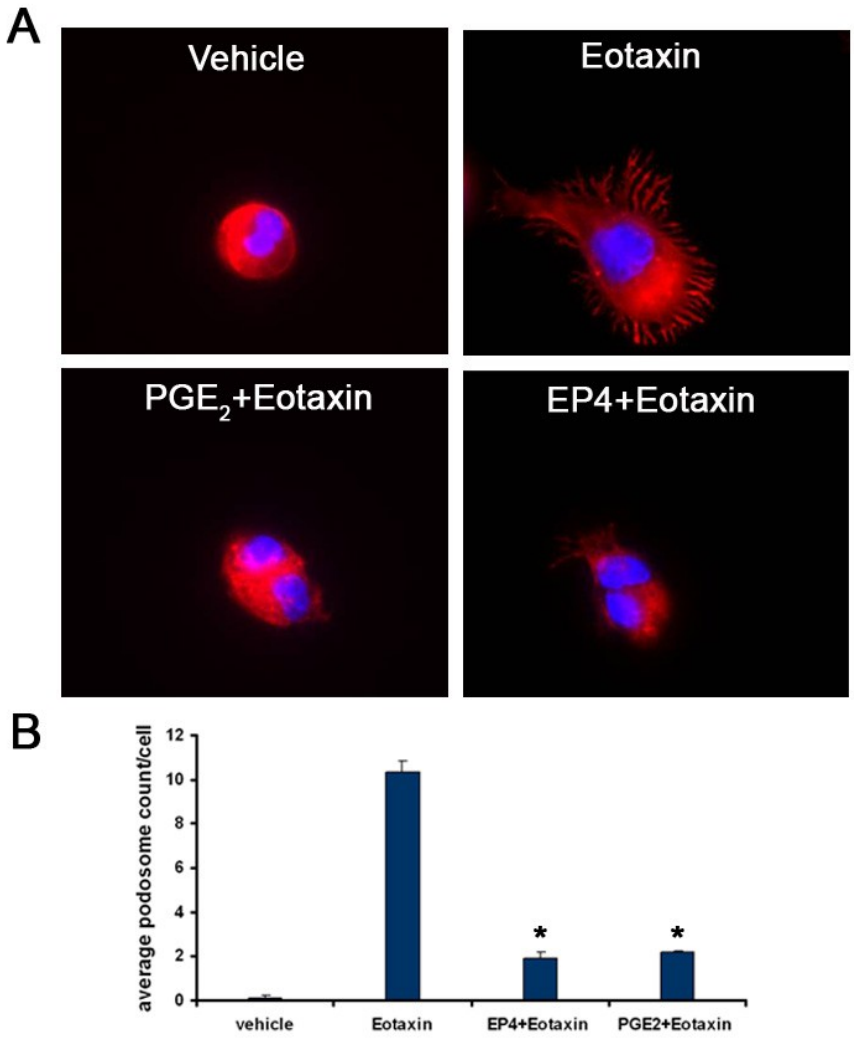

Figure 20. Podosome formation in activated eosinophils is prevented by PGE_2 and EP4 agonist treatment. *In **A,** adhesion of eosinophils to fibronectin- coated chamber slides and the adhesive structure formation were visualized by phalloidin-Texas Red staining of the F-actin. Eosinophils were pre-incubated with vehicle, the EP4 agonist ONO AE1-329 or PGE_2 (30 nM each) for 10 min and were then incubated with vehicle or eotaxin (3 nM) for 30 min to induce eosinophil adhesion. Non-adhered eosinophils were then discarded. Representative images are magnifications of the original micrographs recorded with 60x oil immersion objective. In **B,** quantification of podosome formation/cell is shown. n=300; *P<0.05 PGE_2/eotaxin versus vehicle/eotaxin and EP4/eotaxin versus vehicle/eotaxin.*

EP4 receptor activation on eosinophils abrogates their chemotactic responsiveness

Finally we tested the hypothesis that negative modulation of eosinophil expression/activation of adhesion molecules by EP4 receptors translates to reduced chemotaxis in response to chemoattractants. For that purpose we pretreated purified human eosinophils with the EP4 receptor antagonists GW627368X (1 μM) or ONO AE3-208 (100 nM) or their vehicle for 15 minutes at 37°C, and then mixed them with various concentrations of PGE_2 (3-100 nM). Migration towards eotaxin (1 nM) was determined thereafter. PGE_2 led to a decrease of eosinophil migration in a concentration-dependent manner; at the highest concentration of PGE_2 (100 nM) migration was reduced by more than 70%. The inhibitory effect of PGE_2 was markedly attenuated by the EP4 receptor antagonists GW627368X (***Figure 21***) or ONO AE3-208 (n=4, data not shown). In agreement with these findings we also observed that the EP4-selective agonist ONO AE1-329 (3-100 nM) mimicked the effect of PGE_2 at inhibiting eosinophil migration towards eotaxin (1 nM, ***Figure 21***) and PGD_2 (30 nM, data not shown) with the same efficacy and potency as PGE_2.

Figure 21.

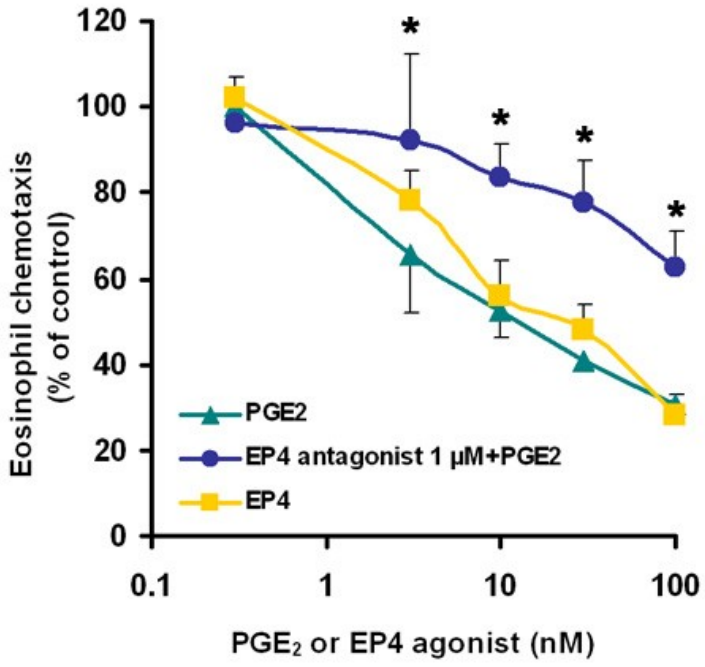

Figure 21. EP4 receptors mediate the inhibitory effect of PGE_2 on eosinophil migration. *Purified eosinophils were pretreated with the EP4 receptor antagonist*

*GW627368X (1 μM) or vehicle for 15 min at 37 °C, mixed with various concentrations of PGE_2 or the EP4 agonist ONO AE1-329 and then loaded into the top wells of a microBoyden chamber. Cells were allowed to migrate towards eotaxin (1 nM) in the bottom wells for 1 h. Responses were expressed as percent of the control response, i.e. to eotaxin only. n = 5-8. *P < 0.05 vs vehicle. (This experiment was performed by Petra Luschnig.)*

Summary – Aim 2

In the final part of my thesis, I focused on the involvement of EP4 receptor in the regulation of eosinophil functions and endothelial-eosinophil interaction. This set of data suggests that PGE_2 and selective stimulation of EP4 receptors can prevent the activation of CD11b/CD18 integrins as induced by eotaxin or PGD_2. Furthermore, I observed that PGE_2 and an EP4 agonist can affect the cell surface clustering of activated CD11b/18 integrins in chemoattractant-stimulated eosinophils. Correspondingly, eosinophil adhesion and podosome formation were also significantly reduced by PGE_2 and EP4 treatment of eosinophils. Finally, both PGE_2 and an EP4 agonist attenuated the chemotaxis of eosinophils. These data demonstrate that EP4 receptors might modulate the recruitment of eosinophils to tissue by a dual action, first by activating eosinophil EP4 receptors and thereby attenuating eosinophil function; and second, by binding to endothelial EP4 receptors, strengthening endothelial barrier function and preventing the activation of endothelial cells by inflammatory mediators.

IV. DISCUSSION

Asthma is one of the most common respiratory disorders in the world, still there is no single comprehensive therapy available for adequate management of the asthmatic patient's condition. Asthma is a disease characterized by bronchial hyperresponsiveness and ongoing airway inflammation and airway remodeling. The prevalence of asthma has been steadily increasing in the past 2 decades. Airway inflammation and associated bronchial hyperresponsiveness can be initiated acutely by exposure to allergens to which the person is sensitized or viral infection, and result in at least reversible airway obstruction. However, asthma can be a persistent disease as well with chronic airway inflammation and airway remodeling. The symptoms of asthma are highly variable; therefore, defining asthma with the expression of multiple phenotypes is very complex. At the cellular level, in particular, eosinophils, mast cells, macrophages and T helper 2 cells appear to be the most pivotal effectors of the disease. An essential feauture of asthma exacerbation is the infiltration of eosinophils from the blood circulation to the site of inflammation. Eosinophils are key cells in chronic inflammation, promoting the release of proinflammatory mediators and cytokines that result in damage and remodeling of the airways and the surrounding tissue.

During the present study, I have shown that PGI_2 and PGE_2 are effective modulators of eosinophil – endothelial interactions. It was observed that receptors for PGI_2 and PGE_2 are expressed both on eosinophils and endothelial cells and that these prostanoids directly regulate the biological functions of eosinophils and endothelial cells. Therefore, the negative regulatory role of PGI_2 and PGE_2 appeared to be two-sided. On the one hand, PGI_2 and PGE_2 attenuated specific functions of eosinophils, such as chemotaxis, adhesion to substratum, or adhesion to and transmigration across endothelial monolayers. On the other hand, these prostaglandins turned out to promote the barrier function of endothelial cells by strengthening the endothelial junctional network, thereby protecting the monolayer from permeability increase upon exposure to permeability increasing agonists or extravasating eosinophils. The mechanisms how PGI_2 and PGE_2 exert their biological effects are characteristically different. From the the cellular sources that produce them, through their receptor specificities to the activated signaling pathway, PGI_2 and PGE_2 show distinct patterns. Still, the phenotypic outcome

in the context of eosinophil-endothelial interaction under allergic inflammatory conditions might be very similar.

In the first part of the thesis, I described the regulatory role of PGI_2 in eosinophil locomotion. As it is well recognized, the major source of PGI_2 is the endothelium. Since under normal, physiological conditions PGI_2 is abundantly released by the endothelium, the first experiments were performed to elucidate the effects of exogenously applied PGI_2 on human eosinophils. In fact, we were able to show that human peripheral blood eosinophils express the PGI_2 receptor IP and that exogenously added PGI_2 markedly inhibits the migration of eosinophils through polycarbonate filters toward the chemoattractants eotaxin and C5a. The inhibitory potency of PGI_2 was remarkable, with a concentration achieving 50% of it maximal inhibitory effect (IC50) of 1-3 nM and a maximal inhibitory effect of more than 70%. The involvement of IP receptors in this inhibition of eosinophil migration was confirmed by a selective IP antagonist, CAY10441, which completely prevented the inhibitory effect of PGI_2 on eosinophil migration. The inhibitory effect of PGI_2 appeared to be selective for eosinophils because neutrophil migration toward IL-8 was not attenuated by PGI_2. The current data have also shown that the inhibitory effect of PGI_2 is largely mediated by cAMP increase because the adenylyl cyclase inhibitor SQ22536 largely reversed the PGI_2-induced attenuation of eosinophil chemotaxis. This observation was in line with the known signaling partner of IP receptors being Gαs proteins coupled to adenylyl cyclase.

In contrast, PGE_2 is the predominant cyclooxygenase (COX) product of airway macrophages, epithelial cells and smooth muscle cells. The effect of PGE_2 can be mediated via four distinct receptors (EP1-4); accordingly, the cellular response is a fine-tuned balance of receptor expression, avidity of the receptors to PGE_2 and the interactions of these receptors with respect to intracellular signaling pathways. It was recently observed by our group that LPS-induced PGE_2 release of monocytes attenuated the locomotion of eosinophils, and that this effect was mimicked by the selective EP2 and EP4 agonists, butaprost and ONO AE1-329, respectively . The inhibitory efficacy and potency of the PGE_2 and the EP4 agonist was similar to that of PGI_2. Although activation of both EP2 and EP4 receptors has been previously shown to stimulate cAMP production, the inhibitory effect of PGE_2 was not reversed by the adenylyl cyclase inhibitor SQ22536 or the protein kinase (PK) A inhibitor H-89. In contrast, inhibitors of phosphatidylinositol 3-kinase (PI-3K) and PKC largely prevented the inhibitory effect

of PGE_2 . Similarly, the adenylyl cyclase inhibitor had no effect on the negative modulation by the EP4 agonist of eosinophil chemotaxis towards eotaxin (data not shown), which was in sharp contrast to the PGI_2-induced abrogation of eosinophil migration. Collectively, although the functional consequences of IP and EP4 receptor activation are similar, IP-mediated eosinophil inhibition is signaled for by the adenylyl cyclase/cAMP pathway, while EP4-induced eosinophil inhibition is independent from cAMP but essentially involves PI-3K and PKC. Similarly, I found that PGE_2 or the EP4 agonist attenuated the TNFα-induced E-selectin expression in endothelial cells and this effect was left unaltered by the adenylyl cyclase inhibitor, but was abrogated by the PKC inhibitor. Further discrepancies between IP and EP4 signaling in eosinophils were revealed by assays of flow cytometric shape change, respiratory burst and intracellular Ca^{2+} mobilization. While EP4 receptor stimulation potently curbed all of these responses, PGI_2 had no effect (data not shown).

Since these data implicated PKC as a negative regulator of eosinophil migration we investigated the effect of the PKC activator PMA on eosinophil migration. As expected, PMA very potently mimicked the inhibitory effect of the EP4 receptor agonist on eosinophil migration (data not shown). The inhibitory effect of PMA, however, could not be prevented by the PI-3K inhibitor. Therefore, our data indicate that both PI-3K and PKC are involved in the attenuation of eosinophil migration upon EP4 receptor activation and confirm previous data that PI-3K is acting up-stream of PKC (Hirai and Chida, 2003). Stimulation of EP4 has been shown to cause phosphorylation of extracellular signal-regulated kinases (ERKs) through a PI3K-dependent mechanism . Additionally, the existence of an alternative EP2/EP4 signaling pathway, linked to PKC activation has been postulated (Reno and Cannas, 2005). In agreement with our observation it has also been suggested that modulation of PI3K mediates the inhibitory effect of PGE_2 in neutrophils .

In addition to directed migration, a further important function of eosinophils is adhesion. Adhesion is mediated by integrin heterodimers which are composed of two subunits; α and β chain. The extracellular domains have specific binding sites for endothelial adhesion molecules, like VCAMs and ICAMs. However, integrins can recognize and attach to determined sequences in proteins of the extracellular matrix, such fibronectin via Arg-Gly-Asp (RGD) repeats. The capacity of leukocytes to adhere

is not only crucial for locomotion but also an important determinant of further effector functions, such as respiratory burst, mediator release, degranulation or apoptosis .
Therefore, I investigated the effect of PGI_2 on eosinophil adhesion to fibronectin. I observed that the adhesion of eosinophils was concentration-dependently stimulated by eotaxin and that exogenously added PGI_2 inhibited this response. Morphologic studies suggested that PGI_2 did not affect the eotaxin-induced flattening and polarization of eosinophils. Similarly, eotaxin-induced actin-dependent rearrangement of the cytoskeleton, as investigated with the flow cytometric shape-change assay, was not altered by PGI_2 (data not shown). In contrast, F-actin staining of adherent eosinophils revealed that the eotaxin-stimulated formation of F-actin–dense protrusions, most likely podosomes, was specifically inhibited by PGI_2. Podosomes are transient assemblies of cytoskeletal and membrane proteins that are believed to be important for cell movement and invading of eosinophils, via lysing VCAM-1 and matrix proteins (Linder and Aepfelbacher, 2003) . Interestingly, airway eosinophils purified from antigen-challenged subjects demonstrated enhanced podosome formation and upregulation of the adhesion molecule Mac-1 (CD11b/CD18) and were more adhesive and migratory than unchallenged eosinophils .

In a PGI_2-similar manner, PGE_2 and selective activation of EP4 receptor by the EP4 selective agonist ONO-AE1-329 reduced the adhesion of eosinophils to fibronectin. Eosinophil podosome formation was significantly reduced by PGE_2 or the EP4 selective agonist, as it was quantified from immunofluorescence images. Although podosome formation has been investigated in some detail, not much is known about the signaling mechanism that leads to podosome disassembly. Small GTPases, members of the Rho family, such as RhoA, Rac1 and Cdc42 are key regulators of adhesion dynamics that control the formation and polymerization of filamentous actin as well as microtubules, and modulate contractility . Activation of RhoA is often accompanied by the loss of Rac1 activity, while active forms of these two Rho GTPases can produce opposing effects on the cytoskeleton . Podosome assembly is essentially important for the highly migratory and invasive cells, like dendritic cells. Concerning PGE_2-mediated signaling, it was shown that PGE_2 is able to induce activation of RhoA which is followed by a decrease of both Rac1 and Cdc42 activity in mature dendritic cells . In dendritic cells, the implication of Rac1 and Cdc42 in podosome formation was demonstrated previously .

In eosinophil attachment to the extracellular matrix or to endothelial cells, the roles of CD11b/CD18 (Mac-1) and CD49d/CD29 (VLA-4) are the most prominent ones. Integrin activation is carried out in three very effective and rapid ways, firstly by cell surface upregulation of integrin subunits, secondly, by conformation change of the heterodimer into a status with higher avidity for its ligand, and finally by lateral shifting of heterodimers in the cell membrane in order to form preferred sites for adhesion to the endothelial cells. In my studies, I focused mainly on the modulation of CD11b/CD18 integrin. I found that the rapid eotaxin-induced upregulation of the adhesion molecule subunit CD11b was attenuated by PGI_2, and this effect was mimicked by the adenylyl cyclase activator forskolin. Moreover, activation of the CD11b/CD18 complex was likewise prevented by PGI_2 as detected by the active conformation-specific antibody mAb24.

PGE_2 and the EP4 receptor agonist were likewise observed to augment the eotaxin-induced cell surface upregulation of CD11b integrin subunit and activation of EP4 receptor prevented the eotaxin or PGD_2-induced CD11b/CD18 integrin activation. Furthermore, visual examination provided evidence that clustering of activated CD11b/CD18 integrins on eotaxin-activated eosinophils was inhibited by PGE_2 or the EP4-selective agonist. Similarly, PGE_2 significantly attenuated the upregulation and activation of β_1 integrins in human peripheral blood monocytes and macrophages, where the highest inhibition was found by selective activation of EP4 receptor . Apart from adhesion, CD11b/CD18 is an important modulator of leukocyte responses, including migration , phagocytosis , mediator release , and apoptosis . Therefore it is conceivable that the PGI_2 and PGE_2-induced reduction of CD11b expression and activation might have profound effects on eosinophil accumulation and function at sites of inflammation. There is evidence for modulated mediator release in mouse neutrophils, where the selective activation of EP4 receptors inhibited the LPS-induced TNFα release

In the interaction assays, it appeared that the effects of PGI_2 and PGE_2 are brought about by a dual action, (i) by directly targeting eosinophils through IP and EP4 receptors, respectively; and (ii) by strengthening endothelial barrier function. To investigate the role of endogenous PGI_2, we used human pulmonary microvascular endothelial cells as a physiologic source of PGI_2 and studied the interaction of eosinophils and the endothelium. In fact, inhibition of PGI_2 biosynthesis in endothelial cells by using a COX inhibitor, which almost completely abrogated PGI_2 biosynthesis, or an IP antagonist markedly enhanced eosinophil adhesion to the endothelium on the one hand, and

augmented the transendothelial migration of eosinophils on the other hand. These observations demonstrated that endothelium-derived PGI_2 was able to control the migratory capacity of eosinophils. PGI_2 did not attenuate eosinophil migration by reducing eosinophil viability because a 3-hour incubation of eosinophils with PGI_2 or the IP agonist iloprost (30-100 nM) did not alter the binding of Annexin V as a measure of apoptosis, or the uptake of propidium iodide, which is indicative of necrosis. Because endothelial cells also express IP receptors (Turner and Kinsella, 2009) and an IP agonist was found to enhance endothelial barrier formation , I tested the hypothesis that inhibition of endogenous PGI_2 biosynthesis might enhance eosinophil transmigration also by compromising the barrier function of the endothelium. Incubation of endothelial monolayers with the COX inhibitor in fact reduced the transendothelial electrical resistance of the monolayers. This effect corresponded with disruption of endothelial adherent junctions after COX inhibition, as revealed by immunostaining of VE-cadherin, which was accompanied by actin rearrangement and formation of stress fibers. These morphologic alterations could be largely prevented by treatment of the monolayers with the IP agonist iloprost, which unequivocally showed that the lack of PGI_2 leads to enhanced permeability of the endothelial monolayer.

PGE_2, on the contrary to PGI_2, is not released by endothelial cells under normal conditions; however under inflammatory conditions COX-2 is induced which results in equal PGE_2 and PGI_2 release from HUVEC and the pulmonary microvascular endothelium (data not shown). Endothelial cells are known to express all four EP receptors, but my experiments suggested that selective stimulation of EP4 receptors was the most efficacious in promoting the barrier function of human microvascular endothelial layers. In fact, EP4 receptor stimulation accounted almost completely for the barrier-promoting effect of PGE_2. Increase in endothelial permeability was induced by thrombin stimulation of endothelial monolayers, which developed within 15 minutes, as it was visualized by immunofluorescent staining of VE-cadherin. Stimulation of EP4 receptors on endothelial cells shielded the tightly connected endothelial cells from permeability increase upon thrombin stimulation which was detected by measuring the transendothelial electrical resistance (TEER) changes of the monolayer. Immunofluorescent staining of the endothelial adherens junctions revealed that PGE_2 via specifically activating EP4 receptors prevented the disintegration of the endothelial adherens junctions. The specific involvement of EP4 receptor was further proved by using an EP4 selective antagonist, ONO-AE3-208, which completely prevented the

barrier-protective effect of PGE_2 on thrombin-stimulated endothelial monolayers. When endothelial cells preincubated in the above mentioned manner were subjected to eosinophil transendothelial migration, EP4 receptor activation prevented the eosinophil invasion across the thrombin-activated monolayer. These observations suggested that PGE_2 via activating EP4 receptors on endothelial cells protects against agonist-induced eosinophil transendothelial migration. In the process of eosinophil adhesion to, and transmigration across, the endothelial monolayer, endothelial adhesion molecules are essential for molecular interactions with eosinophil integrins. The expression of ICAM-1 and VCAM-1 induced by TNFα was unaffected by PGE_2 or EP4 agonist treatment of the endothelial cells. However, TNFα-induced E-selectin expression was reduced by PGE_2 and the EP4 agonist and these data showed that protein kinase C, but not the adenylyl cyclase/cAMP pathway, is involved in the signaling of the EP4 mediated attenuation of E-selectin expression. These results can contribute to and further explain the reduced eosinophil trafficking across the EP4 receptor agonist-treated endothelial monolayer.

For further investigation of the barrier-promoting effect of the PGE_2-EP4 signaling, wound healing assays were performed. The recovery of electrically wounded endothelial monolayers was accelerated by PGE_2 and EP4 agonist pretreatment of the endothelial cells. A selective EP4 antagonist (ONO AE3-208), and EP2 and EP3 receptor agonists (butaprost and sulprostone, respectively) were applied for further substantiation of the specificity of EP4 receptor involvement. Supporting these data, it was shown very recently that upregulation of COX-2 by H_2O_2 is crucial in the repair of mechanically wounded HUVEC monolayers, where the COX-2 downstream products, PGE_2 and PGI_2 were implicated in the observed effect . Elsewhere, selective activation of EP4 receptor played a critical role in PGE_2-dependent *in vitro* tubulogenesis of mouse embryonal endothelial cells and the specific effect of EP4 receptor activation was coupled to the activation of ERK signaling pathway .

In this study I could demonstrate that endothelium-derived PGI_2 is an important modulator of eosinophil-endothelial interaction and might have a bearing on eosinophil accumulation at sites of allergic reaction. Our data might hence explain previous findings that deletion of IP receptors in mice augments the eosinophilic infiltrate in allergic responses of the lung and skin and enhances airway remodeling . Moreover, eosinophil influx has been shown to be exaggerated in COX-1 or COX-2 knockout mice

and also in mice treated with selective COX-1 or COX-2 inhibitors , which might be consistent with the loss of the inhibitory action of PGI_2 on transendothelial migration of eosinophils. Endothelial dysfunction has been described in patients with bronchial asthma , and plasma levels of 6-keto-$PGF_{1\alpha}$, the stable metabolite of PGI_2, are significantly lower during symptomatic periods in asthmatic patients than in healthy subjects (Wen, 1990). Interestingly, the plasma levels of 6-keto-$PGF_{1\alpha}$ after allergen challenge were inversely correlated with the frequency of asthmatic late-phase responses , which are usually accompanied by increased eosinophil infiltration of the bronchi. Therefore, clinical data suggest that attenuated release of PGI_2 caused by endothelial dysfunction or, alternatively, treatment with COX inhibitors might favor allergic late-phase responses. In fact, COX inhibitors precipitate asthma attacks in certain asthmatic patients.

The prevalence of aspirin intolerance in the normal population is estimated as being 5-6% and up to 10% among asthmatic patients . It can display a wide range of clinical pictures, such as acute asthma attacks, urticaria, angioedema, chronic rhinitis, and anaphylactic shock . The combination of rhinitis, nasal polyposis and aspirin-induced asthma is referred to as Samter's triad. Within 3 hours of ingestion of aspirin or other NSAIDs, individuals with aspirin-intolerant asthma develop bronchoconstriction, often accompanied by rhinorrhea, conjunctival irritation, and scarlet flush. In severe cases, a single therapeutic dose of aspirin/NSAID can provoke violent bronchospasm, loss of consciousness, and respiratory arrest . Although several hypotheses have been developed over time to explain how inhibition of prostaglandin biosynthesis can trigger an allergy-like cascade of symptoms in these predisposed patients, the pathogenesis of aspirin intolerance has not yet been unequivocally elucidated: modifications of eicosanoid metabolism leading to overproduction of cysteinyl leukotrienes, associations with distinct HLA alleles, and single nucleotide polymorphisms in 5-lipoxygenase or FcεRIα promoter genes have been shown .

The initial event in aspirin-intolerant asthma appears to be the interruption of the synthesis of PGE_2, since exogenous PGE_2 prevents aspirin-precipitated bronchoconstriction . PGE_2 has profound regulatory effects on other inflammatory systems. It reduces leukotriene synthesis by inhibiting 5-lipoxygenase, inhibits cholinergic transmission, and prevents mediator release from mast cells (Stevenson and Szczeklik, 2006). Interestingly, single nucleotide polymorphisms (SNPs) in the promoter region of the gene encoding the EP2 receptor were associated with aspirin-

intolerant asthma . Therefore, even if PGE_2 is synthesized in normal amounts, the inhibitory effects of PGE_2 may be impaired due to reduced expression of EP2 receptors.

Chronic, persistent inflammation is the hallmark of patients with aspirin-induced asthma (Nasser, Pfister, 1996). Eosinophils are consistently found in blood, nasal and bronchial secretions as well as in bronchial biopsy specimens of patients afflicted with aspirin-induced asthma . The airway expression of IL-5 is also markedly increased in patients with aspirin intolerance . IL-5 is a key regulator of eosinophil lineage and is involved in eosinophilopoeisis, eosinophil recruitment, activation, and survival. Bronchial biopsy studies also have revealed that eosinophils are the predominant cells containing LTC_4 synthase. Therefore, increased numbers of eosinophils and the presence of an increased amount of LTC_4 synthase activity may be responsible for the pathophysiology of aspirin-induced asthma. The loss of the anti-inflammatory effect of PGI_2 or PGE_2 after intake of COX inhibitors might be counterbalanced in patients with aspirin-tolerant asthma by means of concomitant abrogation of proinflammatory prostaglandins, such as PGD_2 or PGH_2, which are potent chemoattractants for eosinophils .

Conclusions

The results of my work have shown that endothelial release of PGI_2 might be an important defense mechanism against inappropriate eosinophil infiltration and might limit allergic responses by means of a dual action: (i) inhibition of eosinophil responsiveness to chemoattractants in terms of adhesion and migration and (ii) strengthening of the barrier function of the endothelium against infiltrating leukocytes. The latter effect became evident after inhibition of PGI_2 biosynthesis with a COX inhibitor, which disrupted the texture of endothelial monolayers and markedly augmented eosinophil transmigration through the endothelium. These observations suggested that the endothelium plays a key role through PGI_2 as a gatekeeper by limiting the extravasation of eosinophils from the circulation into the tissue and that endothelial dysfunction, where PGI_2 biosynthesis is compromised, might contribute to eosinophilic inflammation in patients with allergic disease.

Similarly, PGE_2 released at sites of allergic inflammation by macrophages, epithelial cells or smooth muscle cells might be important for counter-balancing the plethora of proinflammatory mediators that are released during the allergic response.

Like PGI_2, activation of EP4 receptors can down-modulate allergic inflammation with respect to (i) activation of endothelial cells and (ii) locomotion of eosinophils. On the endothelial side, PGI_2 and PGE_2 strengthen the junctional network, prevent endothelial cell retraction, down-regulate the cytokine-induced expression of adhesion molecules and promote endothelial regeneration. On the side of the eosinophil, these prostanoids can attenuate the chemotactic responsiveness, the adhesion and chemokine-induced up-regulation and activation of adhesion molecules. Most importantly, this study was the first to demonstrate eosinophil expression of the corresponding prostaglandin receptors, IP and EP4.

While the cellular effectors that mediate the inhibitory effects of PGI_2 and PGE_2 are similar, the intracellular signaling pathways show clear disparities: while PGI_2 activation of IP receptors stimulates adenylyl cyclase and the inhibitory effect of PGI_2 integrally depends on cAMP, activation of EP4 receptors inhibits endothelial and eosinophil functions independently from cAMP but relies on PI3K and PKC. Collectively, these data suggest that selective IP or EP4 receptor agonists might be useful therapeutic options for otherwise inadequately controlled inflammation in patients with asthma and allergic diseases. Moreover, patients suffering from other diseases that likewise implicate inadequate eosinophilic infiltration and tissue damage, such as eosinophilic esophagitis, colitis ulcerosa, hypereosinophilic syndrome or renal disease might also benefit from such prostaglandin-based therapies.

The present data could predict that generally, PGI_2 and PGE_2 can be amongst the crucial factors mediating the major function of endothelium, forming the vessel wall barrier between the circulating blood and the surrounding tissue. In addition to the special scenario of asthma bronchiale and eosinophil tissue recruitment, PGI_2 and PGE_2 might prevent the extravasation of other inflammatory cells, such as neutrophils, monocytes or T lymphocytes that are involved in chronic obstructive pulmonary disease, neurologic dysfunction, atherosclerosis and leukaemia, respectively.

REFERENCES

Aceves, S.S. and Broide, D.H. (2008) Airway fibrosis and angiogenesis due to eosinophil trafficking in chronic asthma. *Curr Mol Med* **8**, 350-8.

Ausprunk, D.H. and Folkman, J. (1977) Migration and proliferation of endothelial cells in preformed and newly formed blood vessels during tumor angiogenesis. *Microvasc Res* **14**, 53-65.

Ayalon, O., Sabanai, H., Lampugnani, M.G., Dejana, E. and Geiger, B. (1994) Spatial and temporal relationships between cadherins and PECAM-1 in cell-cell junctions of human endothelial cells. *J Cell Biol* **126**, 247-58.

Babu, K.S. and Salvi, S.S. (2000) Aspirin and asthma. *Chest* **118**, 1470-1476.

Badewa, A.P., Hudson, C.E. and Heiman, A.S. (2002) Regulatory effects of eotaxin, eotaxin-2, and eotaxin-3 on eosinophil degranulation and superoxide anion generation. *Exp Biol Med (Maywood)* **227**, 645-51.

Baelder, R., Fuchs, B., Bautsch, W., Zwirner, J., Kohl, J., Hoymann, H.G., Glaab, T., Erpenbeck, V., Krug, N. and Braun, A. (2005) Pharmacological targeting of anaphylatoxin receptors during the effector phase of allergic asthma suppresses airway hyperresponsiveness and airway inflammation. *J Immunol* **174**, 783-9.

Bandeira-Melo, C. and Weller, P.F. (2005) Mechanisms of eosinophil cytokine release. *Mem Inst Oswaldo Cruz* **100 Suppl 1**, 73-81.

Barnes, P.J. (1998) Anti-inflammatory actions of glucocorticoids: molecular mechanisms. *Clin Sci (Lond)* **94**, 557-72.

Barreiro, O., Yanez-Mo, M., Serrador, J.M., Montoya, M.C., Vicente-Manzanares, M., Tejedor, R., Furthmayr, H. and Sanchez-Madrid, F. (2002) Dynamic interaction of VCAM-1 and ICAM-1 with moesin and ezrin in a novel endothelial docking structure for adherent leukocytes. *J Cell Biol* **157**, 1233-45.

Barthel, S.R., Jarjour, N.N., Mosher, D.F. and Johansson, M.W. (2006) Dissection of the hyperadhesive phenotype of airway eosinophils in asthma. *Am J Respir Cell Mol Biol* **35**, 378-86.

Bates, D.O. (1998) The chronic effect of vascular endothelial growth factor on individually perfused frog mesenteric microvessels. *J Physiol* **513 (Pt 1)**, 225-33.

Bazzoni, G. and Dejana, E. (2004) Endothelial cell-to-cell junctions: molecular organization and role in vascular homeostasis. *Physiol Rev* **84**, 869-901.

Becker, P.M., Verin, A.D., Booth, M.A., Liu, F., Birukova, A. and Garcia, J.G. (2001) Differential regulation of diverse physiological responses to VEGF in pulmonary endothelial cells. *Am J Physiol Lung Cell Mol Physiol* **281**, L1500-11.

Berlin, C., Bargatze, R.F., Campbell, J.J., von Andrian, U.H., Szabo, M.C., Hasslen, S.R., Nelson, R.D., Berg, E.L., Erlandsen, S.L. and Butcher, E.C. (1995) alpha 4 integrins mediate lymphocyte attachment and rolling under physiologic flow. *Cell* **80**, 413-22.

Berman, R.S., Frew, J.D. and Martin, W. (1993) Endotoxin-induced arterial endothelial barrier dysfunction assessed by an in vitro model. *Br J Pharmacol* **110**, 1282-4.

Betz, M. and Fox, B.S. (1991) Prostaglandin E2 inhibits production of Th1 lymphokines but not of Th2 lymphokines. *J Immunol* **146**, 108-13.

Bhattacharya, M., Peri, K., Ribeiro-da-Silva, A., Almazan, G., Shichi, H., Hou, X., Varma, D.R. and Chemtob, S. (1999) Localization of functional prostaglandin E2 receptors EP3 and EP4 in the nuclear envelope. *J Biol Chem* **274**, 15719-24.

Birukova, A.A., Zagranichnaya, T., Fu, P., Alekseeva, E., Chen, W., Jacobson, J.R. and Birukov, K.G. (2007) Prostaglandins PGE(2) and PGI(2) promote endothelial barrier enhancement via PKA- and Epac1/Rap1-dependent Rac activation. *Exp Cell Res* **313**, 2504-20.

Blum, M.S., Toninelli, E., Anderson, J.M., Balda, M.S., Zhou, J., O'Donnell, L., Pardi, R. and Bender, J.R. (1997) Cytoskeletal rearrangement mediates human microvascular endothelial tight junction modulation by cytokines. *Am J Physiol* **273**, H286-94.

Bochner, B.S. and Schleimer, R.P. (2001) Mast cells, basophils, and eosinophils: distinct but overlapping pathways for recruitment. *Immunol Rev* **179**, 5-15.

Broide, D.H. (2001) Molecular and cellular mechanisms of allergic disease. *J Allergy Clin Immunol* **108**, S65-71.

Buchanan, F.G., Wang, D., Bargiacchi, F. and DuBois, R.N. (2003) Prostaglandin E2 regulates cell migration via the intracellular activation of the epidermal growth factor receptor. *J Biol Chem* **278**, 35451-7.

Burelout, C., Thibault, N., Levasseur, S., Simard, S., Naccache, P.H. and Bourgoin, S.G. (2004) Prostaglandin E2 inhibits the phospholipase D pathway stimulated by formyl-methionyl-leucyl-phenylalanine in human neutrophils. Involvement of EP2 receptors and phosphatidylinositol 3-kinase gamma. *Mol Pharmacol* **66**, 293-301.

Burka, J.F. and Garland, L.G. (1977) A possible modulatory role for prostacyclin (PGI2) INIgGa-induced release of slow-reacting substance of anaphylaxis in rats. *Br J Pharmacol* **61**, 697-9.

Burkey, T.H. and Regan, J.W. (1995) Activation of mitogen-activated protein kinase by the human prostaglandin EP3A receptor. *Biochem Biophys Res Commun* **211**, 152-8.

Burns, S., Thrasher, A.J., Blundell, M.P., Machesky, L. and Jones, G.E. (2001) Configuration of human dendritic cell cytoskeleton by Rho GTPases, the WAS protein, and differentiation. *Blood* **98**, 1142-9.

Camacho, M., Lopez-Belmonte, J. and Vila, L. (1998) Rate of vasoconstrictor prostanoids released by endothelial cells depends on cyclooxygenase-2 expression and prostaglandin I synthase activity. *Circ Res* **83**, 353-65.

Carlos, T.M. and Harlan, J.M. (1994) Leukocyte-endothelial adhesion molecules. *Blood* **84**, 2068-101.

Carmeliet, P., Lampugnani, M.G., Moons, L., Breviario, F., Compernolle, V., Bono, F., Balconi, G., Spagnuolo, R., Oosthuyse, B., Dewerchin, M., Zanetti, A., Angellilo, A., Mattot, V., Nuyens, D., Lutgens, E., Clotman, F., de Ruiter, M.C., Gittenberger-de Groot, A., Poelmann, R., Lupu, F., Herbert, J.M., Collen, D. and Dejana, E. (1999) Targeted deficiency or cytosolic truncation of the VE-cadherin gene in mice impairs VEGF-mediated endothelial survival and angiogenesis. *Cell* **98**, 147-57.

Carson, M.R., Shasby, S.S. and Shasby, D.M. (1989) Histamine and inositol phosphate accumulation in endothelium: cAMP and a G protein. *Am J Physiol* **257**, L259-64.

Chan, J.R., Hyduk, S.J. and Cybulsky, M.I. (2001) Chemoattractants induce a rapid and transient upregulation of monocyte alpha4 integrin affinity for vascular cell adhesion molecule 1 which mediates arrest: an early step in the process of emigration. *J Exp Med* **193**, 1149-58.

Cinamon, G., Shinder, V., Shamri, R. and Alon, R. (2004) Chemoattractant signals and beta 2 integrin occupancy at apical endothelial contacts combine with shear stress signals to promote transendothelial neutrophil migration. *J Immunol* **173**, 7282-91.

Cohen, J. (2002) The immunopathogenesis of sepsis. *Nature* **420** , 885-91.

Corrigan, C.J. and Kay, A.B. (1992) T cells and eosinophils in the pathogenesis of asthma. *Immunol Today* **13**, 501-7.

Coughlin, S.R. (2000) Thrombin signalling and protease-activated receptors. *Nature* **407**, 258-64.

Couturier, C., Haeffner-Cavaillon, N., Weiss, L., Fischer, E. and Kazatchkine, M.D. (1990) Induction of cell-associated interleukin 1 through stimulation of the adhesion-promoting proteins LFA-1 (CD11a/CD18) and CR3 (CD11b/CD18) of human monocytes. *Eur J Immunol* **20**, 999-1005.

Cowburn, A.S., Sladek, K., Soja, J., Adamek, L., Nizankowska, E., Szczeklik, A., Lam, B.K., Penrose, J.F., Austen, F.K., Holgate, S.T. and Sampson, A.P. (1998) Overexpression of leukotriene C4 synthase in bronchial biopsies from patients with aspirin-intolerant asthma. *J Clin Invest* **101**, 834-46.

Cuvelier, S.L. and Patel, K.D. (2001) Shear-dependent eosinophil transmigration on interleukin 4-stimulated endothelial cells: a role for endothelium-associated eotaxin-3. *J Exp Med* **194**, 1699-709.

Czarnetzki, B.M. and Rosenbach, T. (1986) Chemotaxis of human neutrophils and eosinophils towards leukotriene B4 and its 20-w-oxidation products in vitro. *Prostaglandins* **31**, 851-8.

Dahlen, S.E., Hansson, G., Hedqvist, P., Bjorck, T., Granstrom, E. and Dahlen, B. (1983) Allergen challenge of lung tissue from asthmatics elicits bronchial contraction that correlates with the release of leukotrienes C4, D4, and E4. *Proc Natl Acad Sci U S A* **80**, 1712-6.

Dallaire, M.J., Ferland, C., Page, N., Lavigne, S., Davoine, F. and Laviolette, M. (2003) Endothelial cells modulate eosinophil surface markers and mediator release. *Eur Respir J* **21**, 918-24.

Das, A.M., Williams, T.J., Lobb, R. and Nourshargh, S. (1995) Lung eosinophilia is dependent on IL-5 and the adhesion molecules CD18 and VLA-4, in a guinea-pig model. *Immunology* **84**, 41-6.

De la Roche, M.A., Smith, J.L., Betapudi, V., Egelhoff, T.T. and Cote, G.P. (2002) Signaling pathways regulating Dictyostelium myosin II. *J Muscle Res Cell Motil* **23**, 703-18.

Delamere, F., Holland, E., Patel, S., Bennett, J., Pavord, I. and Knox, A. (1994) Production of PGE2 by bovine cultured airway smooth muscle cells and its inhibition by cyclo-oxygenase inhibitors. *Br J Pharmacol* **111**, 983-8.

Desai, S., April, H., Nwaneshiudu, C. and Ashby, B. (2000) Comparison of agonist-induced internalization of the human EP2 and EP4 prostaglandin receptors: role of the carboxyl terminus in EP4 receptor sequestration. *Mol Pharmacol* **58**, 1279-86.

DiScipio, R.G., Daffern, P.J., Jagels, M.A., Broide, D.H. and Sriramarao, P. (1999) A comparison of C3a and C5a-mediated stable adhesion of rolling eosinophils in postcapillary venules and transendothelial migration in vitro and in vivo. *J Immunol* **162**, 1127-36.

Dormond, O., Bezzi, M., Mariotti, A. and Ruegg, C. (2002) Prostaglandin E2 promotes integrin alpha Vbeta 3-dependent endothelial cell adhesion, rac-activation, and spreading through cAMP/PKA-dependent signaling. *J Biol Chem* **277**, 45838-46.

Drazen, J.M. and Austen, K.F. (1987) Leukotrienes and airway responses. *Am Rev Respir Dis* **136**, 985-98.

Dudek, S.M., Camp, S.M., Chiang, E.T., Singleton, P.A., Usatyuk, P.V., Zhao, Y., Natarajan, V. and Garcia, J.G. (2007) Pulmonary endothelial cell barrier enhancement by FTY720 does not require the S1P1 receptor. *Cell Signal* **19**, 1754-64.

Dvorak, A.M. and Feng, D. (2001) The vesiculo-vacuolar organelle (VVO). A new endothelial cell permeability organelle. *J Histochem Cytochem* **49**, 419-32.

Dvorak, H.F. (2002) Vascular permeability factor/vascular endothelial growth factor: a critical cytokine in tumor angiogenesis and a potential target for diagnosis and therapy. *J Clin Oncol* **20**, 4368-80.

Ebisawa, M., Bochner, B.S., Georas, S.N. and Schleimer, R.P. (1992) Eosinophil transendothelial migration induced by cytokines. I. Role of endothelial and eosinophil adhesion molecules in IL-1 beta-induced transendothelial migration. *J Immunol* **149**, 4021-8.

Edwards, A.M. and Norris, A.A. (1994) Cromoglycate and asthma. *Lancet* **343**, 426

Eligini, S., Arenaz, I., Barbieri, S.S., Faleri, M.L., Crisci, M., Tremoli, E. and Colli, S. (2009) Cyclooxygenase-2 mediates hydrogen peroxide-induced wound repair in human endothelial cells. *Free Radic Biol Med* **46**, 1428-36.

Essler, M., Staddon, J.M., Weber, P.C. and Aepfelbacher, M. (2000) Cyclic AMP blocks bacterial lipopolysaccharide-induced myosin light chain phosphorylation in endothelial cells through inhibition of Rho/Rho kinase signaling. *J Immunol* **164**, 6543-9.

Faour, W.H., He, Y., He, Q.W., de Ladurantaye, M., Quintero, M., Mancini, A. and Di Battista, J.A. (2001) Prostaglandin E(2) regulates the level and stability of cyclooxygenase-2 mRNA through activation of p38 mitogen-activated protein kinase in interleukin-1 beta-treated human synovial fibroblasts. *J Biol Chem* **276**, 31720-31.

Felinski, E.A. and Antonetti, D.A. (2005) Glucocorticoid regulation of endothelial cell tight junction gene expression: novel treatments for diabetic retinopathy. *Curr Eye Res* **30**, 949-57.

Feng, D., Nagy, J.A., Pyne, K., Dvorak, H.F. and Dvorak, A.M. (1998) Neutrophils emigrate from venules by a transendothelial cell pathway in response to FMLP. *J Exp Med* **187**, 903-15.

Ferro, T., Neumann, P., Gertzberg, N., Clements, R. and Johnson, A. (2000) Protein kinase C-alpha mediates endothelial barrier dysfunction induced by TNF-alpha. *Am J Physiol Lung Cell Mol Physiol* **278**, L1107-17.

Foley, S. and Hamid, Q. (2006) Severe asthma. *J Allergy Clin Immunol* **117**, 714-7.

Forman, B.M., Chen, J. and Evans, R.M. (1997) Hypolipidemic drugs, polyunsaturated fatty acids, and eicosanoids are ligands for peroxisome proliferator-activated receptors alpha and delta. *Proc Natl Acad Sci U S A* **94**, 4312-7.

Forssmann, U., Uguccioni, M., Loetscher, P., Dahinden, C.A., Langen, H., Thelen, M. and Baggiolini, M. (1997) Eotaxin-2, a novel CC chemokine that is selective for the chemokine receptor CCR3, and acts like eotaxin on human eosinophil and basophil leukocytes. *J Exp Med* **185**, 2171-6.

Foster, P.S., Rosenberg, H.F., Asquith, K.L. and Kumar, R.K. (2008) Targeting eosinophils in asthma. *Curr Mol Med* **8**, 585-90.

Fujino, H., Salvi, S. and Regan, J.W. (2005) Differential regulation of phosphorylation of the cAMP response element-binding protein after activation of EP2 and EP4 prostanoid receptors by prostaglandin E2. *Mol Pharmacol* **68** , 251-9.

Fujino, H., West, K.A. and Regan, J.W. (2002) Phosphorylation of glycogen synthase kinase-3 and stimulation of T-cell factor signaling following activation of EP2 and EP4 prostanoid receptors by prostaglandin E2. *J Biol Chem* **277**, 2614-9.

Fujino, H., Xu, W. and Regan, J.W. (2003) Prostaglandin E2 induced functional expression of early growth response factor-1 by EP4, but not EP2, prostanoid receptors via the phosphatidylinositol 3-kinase and extracellular signal-regulated kinases. *J Biol Chem* **278**, 12151-6.

Fukuda, T., Numao, T., Akutsu, I. and Makino, S. (1992) Platelet-activating factor (PAF)-induced chemotaxis and PAF binding to human eosinophils. *J Lipid Mediat* **5**, 145-9.

Fukui, M., Tanaka, M., Hamaguchi, M., Senmaru, T., Sakabe, K., Shiraishi, E., Harusato, I., Yamazaki, M., Hasegawa, G. and Nakamura, N. (2009) Eosinophil count is positively correlated with albumin excretion rate in men with type 2 diabetes. *Clin J Am Soc Nephrol* **4**, 1761-5.

Funk, C.D. (2001) Prostaglandins and leukotrienes: advances in eicosanoid biology. *Science* **294**, 1871-5.

Furuse, M., Sasaki, H. and Tsukita, S. (1999) Manner of interaction of heterogeneous claudin species within and between tight junction strands. *J Cell Biol* **147**, 891-903.

Garcia, J.G., Davis, H.W. and Patterson, C.E. (1995) Regulation of endothelial cell gap formation and barrier dysfunction: role of myosin light chain phosphorylation. *J Cell Physiol* **163**, 510-22.

Garcia-Zepeda, E.A., Combadiere, C., Rothenberg, M.E., Sarafi, M.N., Lavigne, F., Hamid, Q., Murphy, P.M. and Luster, A.D. (1996) Human monocyte chemoattractant protein (MCP)-4 is a novel CC chemokine with activities on monocytes, eosinophils, and basophils induced in allergic and nonallergic inflammation that signals through the CC chemokine receptors (CCR)-2 and -3. *J Immunol* **157**, 5613-26.

Gauvreau, G.M., Watson, R.M. and O'Byrne, P.M. (1999) Protective effects of inhaled PGE2 on allergen-induced airway responses and airway inflammation. *Am J Respir Crit Care Med* **159**, 31-6.

Gavett, S.H., Madison, S.L., Chulada, P.C., Scarborough, P.E., Qu, W., Boyle, J.E., Tiano, H.F., Lee, C.A., Langenbach, R., Roggli, V.L. and Zeldin, D.C. (1999) Allergic lung responses are increased in prostaglandin H synthase-deficient mice. *J Clin Invest* **104**, 721-32.

Giagoudakis, G. and Markantonis, S.L. (2005) Relationships between the concentrations of prostaglandins and the nonsteroidal antiinflammatory drugs indomethacin, diclofenac, and ibuprofen. *Pharmacotherapy* **25**, 18-25.

Gonzalez-Mariscal, L., Betanzos, A. and Avila-Flores, A. (2000) MAGUK proteins: structure and role in the tight junction. *Semin Cell Dev Biol* **11**, 315-24.

Graham, I.L., Gresham, H.D. and Brown, E.J. (1989) An immobile subset of plasma membrane CD11b/CD18 (Mac-1) is involved in phagocytosis of targets recognized by multiple receptors. *J Immunol* **142**, 2352-8.

Groeneveld, A.B. (2002) Vascular pharmacology of acute lung injury and acute respiratory distress syndrome. *Vascul Pharmacol* **39**, 247-56.

Hartert, T.V., Dworski, R.T., Mellen, B.G., Oates, J.A., Murray, J.J. and Sheller, J.R. (2000) Prostaglandin E(2) decreases allergen-stimulated release of prostaglandin D(2) in airways of subjects with asthma. *Am J Respir Crit Care Med* **162**, 637-40.

Hartnell, A., Heinemann, A., Conroy, D.M., Wait, R., Sturm, G.J., Caversaccio, M., Jose, P.J. and Williams, T.J. (2004) Identification of selective basophil chemoattractants in human nasal polyps as insulin-like growth factor-1 and insulin-like growth factor-2. *J Immunol* **173**, 6448-57.

Hasegawa, S., Ichiyama, T., Kohno, F., Korenaga, Y., Ohsaki, A., Hirano, R., Haneda, Y., Fukano, R. and Furukawa, S. (2010) Prostaglandin E2 suppresses beta1-integrin expression via E-prostanoid receptor in human monocytes/macrophages. *Cell Immunol* **263**, 161-5.

Hatae, N., Sugimoto, Y. and Ichikawa, A. (2002) Prostaglandin receptors: advances in the study of EP3 receptor signaling. *J Biochem* **131**, 781-4.

He, T., Lu, T., d'Uscio, L.V., Lam, C.F., Lee, H.C. and Katusic, Z.S. (2008) Angiogenic function of prostacyclin biosynthesis in human endothelial progenitor cells. *Circ Res* **103**, 80-8.

Heinemann, A., Schuligoi, R., Sabroe, I., Hartnell, A. and Peskar, B.A. (2003) Delta 12-prostaglandin J2,

a plasma metabolite of prostaglandin D2, causes eosinophil mobilization from the bone marrow and primes eosinophils for chemotaxis. *J Immunol* **170**, 4752-8.

Heinemann, A., Sturm, G.J., Ofner, M., Sturm, E.M., Weller, C., Peskar, B.A. and Hartnell, A. (2005) Stem cell factor stimulates the chemotaxis, integrin upregulation, and survival of human basophils. *J Allergy Clin Immunol* **116**, 820-6.

Hill, S.J. (1990) Distribution, properties, and functional characteristics of three classes of histamine receptor. *Pharmacol Rev* **42**, 45-83.

Hirai, T. and Chida, K. (2003) Protein kinase Czeta (PKCzeta): activation mechanisms and cellular functions. *J Biochem* **133**, 1-7.

Hla, T., Bishop-Bailey, D., Liu, C.H., Schaefers, H.J. and Trifan, O.C. (1999) Cyclooxygenase-1 and -2 isoenzymes. *Int J Biochem Cell Biol* **31**, 551-7.

Hocking, D.C., Phillips, P.G., Ferro, T.J. and Johnson, A. (1990) Mechanisms of pulmonary edema induced by tumor necrosis factor-alpha. *Circ Res* **67**, 68-77.

Holinstat, M., Mehta, D., Kozasa, T., Minshall, R.D. and Malik, A.B. (2003) Protein kinase Calpha-induced p115RhoGEF phosphorylation signals endothelial cytoskeletal rearrangement. *J Biol Chem* **278**, 28793-8.

Hom, J.T. and Estridge, T. (1994) Antigen-induced recruitment of eosinophils: importance of CD4+ T cells, IL5, and mast cells. *Clin Immunol Immunopathol* **73**, 305-11.

Horie, S., Okubo, Y., Hossain, M., Sato, E., Nomura, H., Koyama, S., Suzuki, J., Isobe, M. and Sekiguchi, M. (1997) Interleukin-13 but not interleukin-4 prolongs eosinophil survival and induces eosinophil chemotaxis. *Intern Med* **36**, 179-85.

Huang, A.J., Manning, J.E., Bandak, T.M., Ratau, M.C., Hanser, K.R. and Silverstein, S.C. (1993) Endothelial cell cytosolic free calcium regulates neutrophil migration across monolayers of endothelial cells. *J Cell Biol* **120**, 1371-80.

Imhof, B.A. and Aurrand-Lions, M. (2004) Adhesion mechanisms regulating the migration of monocytes. *Nat Rev Immunol* **4**, 432-44.

Ingraham, L.M., Coates, T.D., Allen, J.M., Higgins, C.P., Baehner, R.L. and Boxer, L.A. (1982) Metabolic, membrane, and functional responses of human polymorphonuclear leukocytes to platelet-activating factor. *Blood* **59**, 1259-66.

Jaffar, Z., Ferrini, M.E., Buford, M.C., Fitzgerald, G.A. and Roberts, K. (2007) Prostaglandin I2-IP signaling blocks allergic pulmonary inflammation by preventing recruitment of CD4+ Th2 cells into the airways in a mouse model of asthma. *J Immunol* **179**, 6193-203.

Jaffar, Z., Wan, K.S. and Roberts, K. (2002) A key role for prostaglandin I2 in limiting lung mucosal Th2, but not Th1, responses to inhaled allergen. *J Immunol* **169**, 5997-6004.

Jenneck, C., Juergens, U., Buecheler, M. and Novak, N. (2007) Pathogenesis, diagnosis, and treatment of aspirin intolerance. *Ann Allergy Asthma Immunol* **99**, 13-21.

Jinnai, N., Sakagami, T., Sekigawa, T., Kakihara, M., Nakajima, T., Yoshida, K., Goto, S., Hasegawa, T., Koshino, T., Hasegawa, Y., Inoue, H., Suzuki, N., Sano, Y. and Inoue, I. (2004) Polymorphisms in the prostaglandin E2 receptor subtype 2 gene confer susceptibility to aspirin-intolerant asthma: a candidate gene approach. *Hum Mol Genet* **13**, 3203-17.

Johansson, M.W., Lye, M.H., Barthel, S.R., Duffy, A.K., Annis, D.S. and Mosher, D.F. (2004) Eosinophils adhere to vascular cell adhesion molecule-1 via podosomes. *Am J Respir Cell Mol Biol* **31**, 413-22.

John, M., Lim, S., Seybold, J., Jose, P., Robichaud, A., O'Connor, B., Barnes, P.J. and Chung, K.F.

(1998) Inhaled corticosteroids increase interleukin-10 but reduce macrophage inflammatory protein-1alpha, granulocyte-macrophage colony-stimulating factor, and interferon-gamma release from alveolar macrophages in asthma. *Am J Respir Crit Care Med* **157**, 256-62.

Jones, D.A., Benjamin, C.W. and Linseman, D.A. (1995) Activation of thromboxane and prostacyclin receptors elicits opposing effects on vascular smooth muscle cell growth and mitogen-activated protein kinase signaling cascades. *Mol Pharmacol* **48**, 890-6.

Kaal, E.C. and Vecht, C.J. (2004) The management of brain edema in brain tumors. *Curr Opin Oncol* **16**, 593-600.

Kamata, T., Tieu, K.K., Tarui, T., Puzon-McLaughlin, W., Hogg, N. and Takada, Y. (2002) The role of the CPNKEKEC sequence in the beta(2) subunit I domain in regulation of integrin alpha(L)beta(2) (LFA-1). *J Immunol* **168**, 2296-301.

Kamio, K., Sato, T., Liu, X., Sugiura, H., Togo, S., Kobayashi, T., Kawasaki, S., Wang, X., Mao, L., Ahn, Y., Holz, O., Magnussen, H. and Rennard, S.I. (2008) Prostacyclin analogs stimulate VEGF production from human lung fibroblasts in culture. *Am J Physiol Lung Cell Mol Physiol* **294**, L1226-32.

Kanwar, S., Bullard, D.C., Hickey, M.J., Smith, C.W., Beaudet, A.L., Wolitzky, B.A. and Kubes, P. (1997) The association between alpha4-integrin, P-selectin, and E-selectin in an allergic model of inflammation. *J Exp Med* **185**, 1077-87.

Katamura, K., Shintaku, N., Yamauchi, Y., Fukui, T., Ohshima, Y., Mayumi, M. and Furusho, K. (1995) Prostaglandin E2 at priming of naive CD4+ T cells inhibits acquisition of ability to produce IFN-gamma and IL-2, but not IL-4 and IL-5. *J Immunol* **155**, 4604-12.

Kawabe, J., Yuhki, K., Okada, M., Kanno, T., Yamauchi, A., Tashiro, N., Sasaki, T., Okumura, S., Nakagawa, N., Aburakawa, Y., Takehara, N., Fujino, T., Hasebe, N., Narumiya, S. and Ushikubi, F. (2010) Prostaglandin I2 promotes recruitment of endothelial progenitor cells and limits vascular remodeling. *Arterioscler Thromb Vasc Biol* **30**, 464-70.

Keese, C.R., Wegener, J., Walker, S.R. and Giaever, I. (2004) Electrical wound-healing assay for cells in vitro. *Proc Natl Acad Sci U S A* **101**, 1554-9.

Kim, S.H., Ye, Y.M., Lee, S.K. and Park, H.S. (2006) Genetic mechanism of aspirin-induced urticaria/angioedema. *Curr Opin Allergy Clin Immunol* **6**, 266-270.

Knudsen, K.A., Soler, A.P., Johnson, K.R. and Wheelock, M.J. (1995) Interaction of alpha-actinin with the cadherin/catenin cell-cell adhesion complex via alpha-catenin. *J Cell Biol* **130**, 67-77.

Komhoff, M., Grone, H.J., Klein, T., Seyberth, H.W. and Nusing, R.M. (1997) Localization of cyclooxygenase-1 and -2 in adult and fetal human kidney: implication for renal function. *Am J Physiol* **272**, F460-8.

Kroegel, C., Warner, J.A., Virchow, J.C. Jr and Matthys, H. (1994) Pulmonary immune cells in health and disease: the eosinophil leucocyte (Part II). *Eur Respir J* **7**, 743-60.

Kuypers, F.A., Lewis, R.A., Hua, M., Schott, M.A., Discher, D., Ernst, J.D. and Lubin, B.H. (1996) Detection of altered membrane phospholipid asymmetry in subpopulations of human red blood cells using fluorescently labeled annexin V. *Blood* **87**, 1179-87.

Lindemann, S., Gierer, C. and Darius, H. (2003) Prostacyclin inhibits adhesion of polymorphonuclear leukocytes to human vascular endothelial cells due to adhesion molecule independent regulatory mechanisms. *Basic Res Cardiol* **98**, 8-15.

Linder, S. and Aepfelbacher, M. (2003) Podosomes: adhesion hot-spots of invasive cells. *Trends Cell Biol* **13**, 376-85.

Lintomen, L., Franchi, G., Nowill, A., Condino-Neto, A., de Nucci, G., Zanesco, A. and Antunes, E.

(2008) Human eosinophil adhesion and degranulation stimulated with eotaxin and RANTES in vitro: lack of interaction with nitric oxide. *BMC Pulm Med* **8**, 13

Liu, F., Schaphorst, K.L., Verin, A.D., Jacobs, K., Birukova, A., Day, R.M., Bogatcheva, N., Bottaro, D.P. and Garcia, J.G. (2002) Hepatocyte growth factor enhances endothelial cell barrier function and cortical cytoskeletal rearrangement: potential role of glycogen synthase kinase-3beta. *FASEB J* **16** , 950-62.

Liu, Y., Wada, R., Yamashita, T., Mi, Y., Deng, C.X., Hobson, J.P., Rosenfeldt, H.M., Nava, V.E., Chae, S.S., Lee, M.J., Liu, C.H., Hla, T., Spiegel, S. and Proia, R.L. (2000) Edg-1, the G protein-coupled receptor for sphingosine-1-phosphate, is essential for vascular maturation. *J Clin Invest* **106**, 951-61.

Lopez, A.F., Sanderson, C.J., Gamble, J.R., Campbell, H.D., Young, I.G. and Vadas, M.A. (1988) Recombinant human interleukin 5 is a selective activator of human eosinophil function. *J Exp Med* **167**, 219-24.

Lorenzon, P., Vecile, E., Nardon, E., Ferrero, E., Harlan, J.M., Tedesco, F. and Dobrina, A. (1998) Endothelial cell E- and P-selectin and vascular cell adhesion molecule-1 function as signaling receptors. *J Cell Biol* **142**, 1381-91.

Lum, H. and Malik, A.B. (1996) Mechanisms of increased endothelial permeability. *Can J Physiol Pharmacol* **74**, 787-800.

Mathe, A.A. and Hedqvist, P. (1975) Effect of prostaglandins F2 alpha and E2 on airway conductance in healthy subjects and asthmatic patients. *Am Rev Respir Dis* **111**, 313-20.

Matsumoto, K., Sterbinsky, S.A., Bickel, C.A., Zhou, D.F., Kovach, N.L. and Bochner, B.S. (1997) Regulation of alpha 4 integrin-mediated adhesion of human eosinophils to fibronectin and vascular cell adhesion molecule-1. *J Allergy Clin Immunol* **99**, 648-56.

Matsumoto, T. and Claesson-Welsh, L. (2001) VEGF receptor signal transduction. *Sci STKE* **2001**, re21

McVerry, B.J., Peng, X., Hassoun, P.M., Sammani, S., Simon, B.A. and Garcia, J.G. (2004) Sphingosine 1-phosphate reduces vascular leak in murine and canine models of acute lung injury. *Am J Respir Crit Care Med* **170**, 987-93.

Mehta, D., Tiruppathi, C., Sandoval, R., Minshall, R.D., Holinstat, M. and Malik, A.B. (2002) Modulatory role of focal adhesion kinase in regulating human pulmonary arterial endothelial barrier function. *J Physiol* **539**, 779-89.

Melillo, E., Woolley, K.L., Manning, P.J., Watson, R.M. and O'Byrne, P.M. (1994) Effect of inhaled PGE2 on exercise-induced bronchoconstriction in asthmatic subjects. *Am J Respir Crit Care Med* **149**, 1138-41.

Millan, J., Hewlett, L., Glyn, M., Toomre, D., Clark, P. and Ridley, A.J. (2006) Lymphocyte transcellular migration occurs through recruitment of endothelial ICAM-1 to caveola- and F-actin-rich domains. *Nat Cell Biol* **8**, 113-23.

Miyazaki, Y., Satoh, T., Nishioka, K. and Yokozeki, H. (2006) STAT-6-mediated control of P-selectin by substance P and interleukin-4 in human dermal endothelial cells. *Am J Pathol* **169**, 697-707.

Monneret, G., Gravel, S., Diamond, M., Rokach, J. and Powell, W.S. (2001) Prostaglandin D2 is a potent chemoattractant for human eosinophils that acts via a novel DP receptor. *Blood* **98**, 1942-8.

Moy, A.B., Bodmer, J.E., Blackwell, K., Shasby, S. and Shasby, D.M. (1998) cAMP protects endothelial barrier function independent of inhibiting MLC20-dependent tension development. *Am J Physiol* **274**, L1024-9.

Moy, A.B., Van Engelenhoven, J., Bodmer, J., Kamath, J., Keese, C., Giaever, I., Shasby, S. and Shasby, D.M. (1996) Histamine and thrombin modulate endothelial focal adhesion through centripetal

and centrifugal forces. *J Clin Invest* **97**, 1020-7.

Muller, W.A. (2003) Leukocyte-endothelial-cell interactions in leukocyte transmigration and the inflammatory response. *Trends Immunol* **24**, 327-34.

Nagao, K., Tanaka, H., Komai, M., Masuda, T., Narumiya, S. and Nagai, H. (2003) Role of prostaglandin I2 in airway remodeling induced by repeated allergen challenge in mice. *Am J Respir Cell Mol Biol* **29**, 314-20.

Nakata, J., Kondo, M., Tamaoki, J., Takemiya, T., Nohara, M., Yamagata, K. and Nagai, A. (2005) Augmentation of allergic inflammation in the airways of cyclooxygenase-2-deficient mice. *Respirology* **10**, 149-56.

Narumiya, S., Sugimoto, Y. and Ushikubi, F. (1999) Prostanoid receptors: structures, properties, and functions. *Physiol Rev* **79**, 1193-226.

Nathan, C., Srimal, S., Farber, C., Sanchez, E., Kabbash, L., Asch, A., Gailit, J. and Wright, S.D. (1989) Cytokine-induced respiratory burst of human neutrophils: dependence on extracellular matrix proteins and CD11/CD18 integrins. *J Cell Biol* **109**, 1341-9.

Negishi, M., Namba, T., Sugimoto, Y., Irie, A., Katada, T., Narumiya, S. and Ichikawa, A. (1993) Opposite coupling of prostaglandin E receptor EP3C with Gs and G(o). Stimulation of Gs and inhibition of G(o). *J Biol Chem* **268**, 26067-70.

Norn, S. and Clementsen, P. (1988) Bronchial asthma: pathophysiological mechanisms and corticosteroids. *Allergy* **43**, 401-5.

Noso, N., Proost, P., Van Damme, J. and Schroder, J.M. (1994) Human monocyte chemotactic proteins-2 and 3 (MCP-2 and MCP-3) attract human eosinophils and desensitize the chemotactic responses towards RANTES. *Biochem Biophys Res Commun* **200**, 1470-6.

Obase, Y., Matsuse, H., Shimoda, T., Haahtela, T. and Kohno, S. (2005) Pathogenesis and management of aspirin-intolerant asthma. *Treat Respir Med* **4**, 325-336.

Pai, R., Soreghan, B., Szabo, I.L., Pavelka, M., Baatar, D. and Tarnawski, A.S. (2002) Prostaglandin E2 transactivates EGF receptor: a novel mechanism for promoting colon cancer growth and gastrointestinal hypertrophy. *Nat Med* **8**, 289-93.

Pai, R., Szabo, I.L., Soreghan, B.A., Atay, S., Kawanaka, H. and Tarnawski, A.S. (2001) PGE(2) stimulates VEGF expression in endothelial cells via ERK2/JNK1 signaling pathways. *Biochem Biophys Res Commun* **286**, 923-8.

Palmeri, D., van Zante, A., Huang, C.C., Hemmerich, S. and Rosen, S.D. (2000) Vascular endothelial junction-associated molecule, a novel member of the immunoglobulin superfamily, is localized to intercellular boundaries of endothelial cells. *J Biol Chem* **275**, 19139-45.

Parent, J.L., Labrecque, P., Orsini, M.J. and Benovic, J.L. (1999) Internalization of the TXA2 receptor alpha and beta isoforms. Role of the differentially spliced cooh terminus in agonist-promoted receptor internalization. *J Biol Chem* **274**, 8941-8.

Patel, K.D., Cuvelier, S.L. and Wiehler, S. (2002) Selectins: critical mediators of leukocyte recruitment. *Semin Immunol* **14**, 73-81.

Pavord, I.D., Wong, C.S., Williams, J. and Tattersfield, A.E. (1993) Effect of inhaled prostaglandin E2 on allergen-induced asthma. *Am Rev Respir Dis* **148**, 87-90.

Peebles, R.S. Jr, Dworski, R., Collins, R.D., Jarzecka, K., Mitchell, D.B., Graham, B.S. and Sheller, J.R. (2000) Cyclooxygenase inhibition increases interleukin 5 and interleukin 13 production and airway hyperresponsiveness in allergic mice. *Am J Respir Crit Care Med* **162**, 676-81.

Peebles, R.S. Jr, Hashimoto, K., Morrow, J.D., Dworski, R., Collins, R.D., Hashimoto, Y., Christman,

J.W., Kang, K.H., Jarzecka, K., Furlong, J., Mitchell, D.B., Talati, M., Graham, B.S. and Sheller, J.R. (2002) Selective cyclooxygenase-1 and -2 inhibitors each increase allergic inflammation and airway hyperresponsiveness in mice. *Am J Respir Crit Care Med* **165**, 1154-60.

Penn, M.S. and Chisolm, G.M. (1991) Relation between lipopolysaccharide-induced endothelial cell injury and entry of macromolecules into the rat aorta in vivo. *Circ Res* **68**, 1259-69.

Phillipson, M., Heit, B., Colarusso, P., Liu, L., Ballantyne, C.M. and Kubes, P. (2006) Intraluminal crawling of neutrophils to emigration sites: a molecularly distinct process from adhesion in the recruitment cascade. *J Exp Med* **203**, 2569-75.

Piali, L., Hammel, P., Uherek, C., Bachmann, F., Gisler, R.H., Dunon, D. and Imhof, B.A. (1995) CD31/PECAM-1 is a ligand for alpha v beta 3 integrin involved in adhesion of leukocytes to endothelium. *J Cell Biol* **130**, 451-60.

Pillinger, M.H., Rosenthal, P.B., Tolani, S.N., Apsel, B., Dinsell, V., Greenberg, J., Chan, E.S., Gomez, P.F. and Abramson, S.B. (2003) Cyclooxygenase-2-derived E prostaglandins down-regulate matrix metalloproteinase-1 expression in fibroblast-like synoviocytes via inhibition of extracellular signal-regulated kinase activation. *J Immunol* **171**, 6080-9.

Plager, D.A., Loegering, D.A., Weiler, D.A., Checkel, J.L., Wagner, J.M., Clarke, N.J., Naylor, S., Page, S.M., Thomas, L.L., Akerblom, I., Cocks, B., Stuart, S. and Gleich, G.J. (1999) A novel and highly divergent homolog of human eosinophil granule major basic protein. *J Biol Chem* **274**, 14464-73.

Pola, R., Gaetani, E., Flex, A., Aprahamian, T.R., Bosch-Marce, M., Losordo, D.W., Smith, R.C. and Pola, P. (2004) Comparative analysis of the in vivo angiogenic properties of stable prostacyclin analogs: a possible role for peroxisome proliferator-activated receptors. *J Mol Cell Cardiol* **36**, 363-70.

Predescu, D. and Palade, G.E. (1993) Plasmalemmal vesicles represent the large pore system of continuous microvascular endothelium. *Am J Physiol* **265**, H725-33.

Price, L.S., Leng, J., Schwartz, M.A. and Bokoch, G.M. (1998) Activation of Rac and Cdc42 by integrins mediates cell spreading. *Mol Biol Cell* **9**, 1863-71.

Rainger, G.E., Buckley, C., Simmons, D.L. and Nash, G.B. (1997) Cross-talk between cell adhesion molecules regulates the migration velocity of neutrophils. *Curr Biol* **7**, 316-25.

Rao, R., Redha, R., Macias-Perez, I., Su, Y., Hao, C., Zent, R., Breyer, M.D. and Pozzi, A. (2007) Prostaglandin E2-EP4 receptor promotes endothelial cell migration via ERK activation and angiogenesis in vivo. *J Biol Chem* **282**, 16959-68.

Reid, D.M., Perry, V.H., Andersson, P.B. and Gordon, S. (1993) Mitosis and apoptosis of microglia in vivo induced by an anti-CR3 antibody which crosses the blood-brain barrier. *Neuroscience* **56**, 529-33.

Reinhardt, P.H. and Kubes, P. (1998) Differential leukocyte recruitment from whole blood via endothelial adhesion molecules under shear conditions. *Blood* **92**, 4691-9.

Reno, F. and Cannas, M. (2005) Effect of prostaglandin E2 on PMA-induced macrophage differentiation. *Prostaglandins Other Lipid Mediat* **75**, 13-24.

Resnick, M.B. and Weller, P.F. (1993) Mechanisms of eosinophil recruitment. *Am J Respir Cell Mol Biol* **8**, 349-55.

Richter, J., Ng-Sikorski, J., Olsson, I. and Andersson, T. (1990) Tumor necrosis factor-induced degranulation in adherent human neutrophils is dependent on CD11b/CD18-integrin-triggered oscillations of cytosolic free Ca2+. *Proc Natl Acad Sci U S A* **87**, 9472-6.

Rippe, B. and Grega, G.J. (1978) Effects of isoprenaline and cooling on histamine induced changes of

capillary permeability in the rat hindquarter vascular bed. *Acta Physiol Scand* **103**, 252-62.

Rot, A., Krieger, M., Brunner, T., Bischoff, S.C., Schall, T.J. and Dahinden, C.A. (1992) RANTES and macrophage inflammatory protein 1 alpha induce the migration and activation of normal human eosinophil granulocytes. *J Exp Med* **176**, 1489-95.

Rothenberg, M.E. (1999) Eotaxin. An essential mediator of eosinophil trafficking into mucosal tissues. *Am J Respir Cell Mol Biol* **21**, 291-5.

Rothenberg, M.E., Owen, W.F. Jr, Silberstein, D.S., Woods, J., Soberman, R.J., Austen, K.F. and Stevens, R.L. (1988) Human eosinophils have prolonged survival, enhanced functional properties, and become hypodense when exposed to human interleukin 3. *J Clin Invest* **81**, 1986-92.

Schratl, P., Sturm, E.M., Royer, J.F., Sturm, G.J., Lippe, I.T., Peskar, B.A. and Heinemann, A. (2006) Hierarchy of eosinophil chemoattractants: role of p38 mitogen-activated protein kinase. *Eur J Immunol* **36**, 2401-9.

Schuligoi, R., Sedej, M., Waldhoer, M., Vukoja, A., Sturm, E.M., Lippe, I.T., Peskar, B.A. and Heinemann, A. (2009) Prostaglandin H2 induces the migration of human eosinophils through the chemoattractant receptor homologous molecule of Th2 cells, CRTH2. *J Leukoc Biol* **85**, 136-45.

Schumann, R.R., Leong, S.R., Flaggs, G.W., Gray, P.W., Wright, S.D., Mathison, J.C., Tobias, P.S. and Ulevitch, R.J. (1990) Structure and function of lipopolysaccharide binding protein. *Science* **249**, 1429-31.

Sedgwick, J.B., Menon, I., Gern, J.E. and Busse, W.W. (2002) Effects of inflammatory cytokines on the permeability of human lung microvascular endothelial cell monolayers and differential eosinophil transmigration. *J Allergy Clin Immunol* **110**, 752-6.

Sehmi, R., Cromwell, O., Wardlaw, A.J., Moqbel, R. and Kay, A.B. (1993) Interleukin-8 is a chemo-attractant for eosinophils purified from subjects with a blood eosinophilia but not from normal healthy subjects. *Clin Exp Allergy* **23**, 1027-36.

Shasby, D.M., Ries, D.R., Shasby, S.S. and Winter, M.C. (2002) Histamine stimulates phosphorylation of adherens junction proteins and alters their link to vimentin. *Am J Physiol Lung Cell Mol Physiol* **282**, L1330-8.

Shattil, S.J. (2005) Integrins and Src: dynamic duo of adhesion signaling. *Trends Cell Biol* **15**, 399-403.

Shaw, S.K., Bamba, P.S., Perkins, B.N. and Luscinskas, F.W. (2001) Real-time imaging of vascular endothelial-cadherin during leukocyte transmigration across endothelium. *J Immunol* **167**, 2323-30.

Shephard, E.G., Malan, L., Macfarlane, C.M., Mouton, W. and Joubert, J.R. (1985) Lung function and plasma levels of thromboxane B2, 6-ketoprostaglandin F1 alpha and beta-thromboglobulin in antigen-induced asthma before and after indomethacin pretreatment. *Br J Clin Pharmacol* **19**, 459-70.

Smith, W.L. (1992) Prostanoid biosynthesis and mechanisms of action. *Am J Physiol* **263**, F181-91.

Smith, W.L. and Dewitt, D.L. (1996) Prostaglandin endoperoxide H synthases-1 and -2. *Adv Immunol* **62**, 167-215.

Smyth, E.M., Austin, S.C., Reilly, M.P. and FitzGerald, G.A. (2000) Internalization and sequestration of the human prostacyclin receptor. *J Biol Chem* **275**, 32037-45.

Sousa, A.R., Lams, B.E., Pfister, R., Christie, P.E., Schmitz, M. and Lee, T.H. (1997) Expression of interleukin-5 and granulocyte-macrophage colony-stimulating factor in aspirin-sensitive and non-aspirin-sensitive asthmatic airways. *Am J Respir Crit Care Med* **156**, 1384-1389.

Sriramarao, P. and Broide, D.H. (1996) Differential regulation of eosinophil adhesion under conditions of flow in vivo. *Ann N Y Acad Sci* **796**, 218-25.

Sriramarao, P., DiScipio, R.G., Cobb, R.R., Cybulsky, M., Stachnick, G., Castaneda, D., Elices, M. and Broide, D.H. (2000) VCAM-1 is more effective than MAdCAM-1 in supporting eosinophil rolling under conditions of shear flow. *Blood* **95**, 592-601.

Stevenson, D.D. and Szczeklik, A. (2006) Clinical and pathologic perspectives on aspirin sensitivity and asthma. *J Allergy Clin Immunol* **118**, 773-786.

Straumann, A. and Simon, H.U. (2005) Eosinophilic esophagitis: escalating epidemiology? *J Allergy Clin Immunol* **115**, 418-9.

Sturm, E.M., Schratl, P., Schuligoi, R., Konya, V., Sturm, G.J., Lippe, I.T., Peskar, B.A. and Heinemann, A. (2008) Prostaglandin E2 inhibits eosinophil trafficking through E-prostanoid 2 receptors. *J Immunol* **181**, 7273-83.

Symon, F.A., Lawrence, M.B., Williamson, M.L., Walsh, G.M., Watson, S.R. and Wardlaw, A.J. (1996) Functional and structural characterization of the eosinophil P-selectin ligand. *J Immunol* **157**, 1711-9.

Szczeklik, A., Nizankowska, E., Sanak, M. and Swierczynska, M. (2001) Aspirin-induced rhinitis and asthma. *Curr Opin Allergy Clin Immunol* **1**, 27-33.

Szczeklik, A., Sladek, K., Dworski, R., Nizankowska, E., Soja, J., Sheller, J. and Oates, J. (1996) Bronchial aspirin challenge causes specific eicosanoid response in aspirin-sensitive asthmatics. *Am J Respir Crit Care Med* **154**, 1608-1614.

Taha, R., Olivenstein, R., Utsumi, T., Ernst, P., Barnes, P.J., Rodger, I.W. and Giaid, A. (2000) Prostaglandin H synthase 2 expression in airway cells from patients with asthma and chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* **161**, 636-40.

Takahashi, Y., Tokuoka, S., Masuda, T., Hirano, Y., Nagao, M., Tanaka, H., Inagaki, N., Narumiya, S. and Nagai, H. (2002) Augmentation of allergic inflammation in prostanoid IP receptor deficient mice. *Br J Pharmacol* **137**, 315-22.

Takayama, K., Garcia-Cardena, G., Sukhova, G.K., Comander, J., Gimbrone, M.A. Jr and Libby, P. (2002) Prostaglandin E2 suppresses chemokine production in human macrophages through the EP4 receptor. *J Biol Chem* **277**, 44147-54.

Thompson, R.D., Wakelin, M.W., Larbi, K.Y., Dewar, A., Asimakopoulos, G., Horton, M.A., Nakada, M.T. and Nourshargh, S. (2000) Divergent effects of platelet-endothelial cell adhesion molecule-1 and beta 3 integrin blockade on leukocyte transmigration in vivo. *J Immunol* **165**, 426-34.

True, A.L., Rahman, A. and Malik, A.B. (2000) Activation of NF-kappaB induced by H(2)O(2) and TNF-alpha and its effects on ICAM-1 expression in endothelial cells. *Am J Physiol Lung Cell Mol Physiol* **279**, L302-11.

Turner, E.C. and Kinsella, B.T. (2009) Transcriptional regulation of the human prostacyclin receptor gene is dependent on Sp1, PU.1 and Oct-1 in megakaryocytes and endothelial cells. *J Mol Biol* **386**, 579-97.

Ulcar, R., Peskar, B.A., Schuligoi, R., Heinemann, A., Kessler, H.H., Santner, B.I. and Amann, R. (2004) Cyclooxygenase inhibition in human monocytes increases endotoxin-induced TNF alpha without affecting cyclooxygenase-2 expression. *Eur J Pharmacol* **501**, 9-17.

van Helden, S.F., Oud, M.M., Joosten, B., Peterse, N., Figdor, C.G. and van Leeuwen, F.N. (2008) PGE2-mediated podosome loss in dendritic cells is dependent on actomyosin contraction downstream of the RhoA-Rho-kinase axis. *J Cell Sci* **121**, 1096-106.

van Nieuw Amerongen, G.P. and van Hinsbergh, V.W. (2002) Targets for pharmacological intervention of endothelial hyperpermeability and barrier function. *Vascul Pharmacol* **39**, 257-72.

Van Rijen, H., van Kempen, M.J., Analbers, L.J., Rook, M.B., van Ginneken, A.C., Gros, D. and Jongsma, H.J. (1997) Gap junctions in human umbilical cord endothelial cells contain multiple connexins. *Am J Physiol* **272**, C117-30.

Vasioukhin, V., Bauer, C., Yin, M. and Fuchs, E. (2000) Directed actin polymerization is the driving force for epithelial cell-cell adhesion. *Cell* **100**, 209-19.

Verzijl, D., Peters, S.L. and Alewijnse, A.E. (2010) Sphingosine-1-phosphate receptors: zooming in on ligand-induced intracellular trafficking and its functional implications. *Mol Cells* **29**, 99-104.

Vestweber, D. (2002) Regulation of endothelial cell contacts during leukocyte extravasation. *Curr Opin Cell Biol* **14**, 587-93.

Vittet, D., Buchou, T., Schweitzer, A., Dejana, E. and Huber, P. (1997) Targeted null-mutation in the vascular endothelial-cadherin gene impairs the organization of vascular-like structures in embryoid bodies. *Proc Natl Acad Sci U S A* **94**, 6273-8.

Walsh, M.T., Foley, J.F. and Kinsella, B.T. (2000) The alpha, but not the beta, isoform of the human thromboxane A2 receptor is a target for prostacyclin-mediated desensitization. *J Biol Chem* **275**, 20412-23.

Walters, E.H., Bevan, C., Parrish, R.W., Davies, B.H. and Smith, A.P. (1982) Time-dependent effect of prostaglandin E2 inhalation on airway responses to bronchoconstrictor agents in normal subjects. *Thorax* **37**, 438-42.

Wang, C.B., Wong, C.K., Ip, W.K., Li, M.L., Tian, Y.P. and Lam, C.W. (2005) Induction of IL-6 in co-culture of bronchial epithelial cells and eosinophils is regulated by p38 MAPK and NF-kappaB. *Allergy* **60**, 1378-85.

Wang, D., Wang, H., Shi, Q., Katkuri, S., Walhi, W., Desvergne, B., Das, S.K., Dey, S.K. and DuBois, R.N. (2004) Prostaglandin E(2) promotes colorectal adenoma growth via transactivation of the nuclear peroxisome proliferator-activated receptor delta. *Cancer Cell* **6**, 285-95.

Waschke, J., Drenckhahn, D., Adamson, R.H., Barth, H. and Curry, F.E. (2004) cAMP protects endothelial barrier functions by preventing Rac-1 inhibition. *Am J Physiol Heart Circ Physiol* **287**, H2427-33.

Watabe, A., Sugimoto, Y., Honda, A., Irie, A., Namba, T., Negishi, M., Ito, S., Narumiya, S. and Ichikawa, A. (1993) Cloning and expression of cDNA for a mouse EP1 subtype of prostaglandin E receptor. *J Biol Chem* **268**, 20175-8.

Wedemeyer, J. and Vosskuhl, K. (2008) Role of gastrointestinal eosinophils in inflammatory bowel disease and intestinal tumours. *Best Pract Res Clin Gastroenterol* **22**, 537-49.

Wegener, J., Keese, C.R. and Giaever, I. (2002) Recovery of adherent cells after in situ electroporation monitored electrically. *Biotechniques* **33** , 348, 350, 352 passim.

Wegener, K.L., Partridge, A.W., Han, J., Pickford, A.R., Liddington, R.C., Ginsberg, M.H. and Campbell, I.D. (2007) Structural basis of integrin activation by talin. *Cell* **128**, 171-82.

Weinberger, M. and Hendeles, L. (1996) Theophylline in asthma. *N Engl J Med* **334**, 1380-8.

Wen, Z.G. (1990) [Changes in plasma thromboxane B2 and 6-keto-prostaglandin F1 alpha in bronchial asthma]. *Zhonghua Jie He He Hu Xi Za Zhi* **13**, 224-6, 255.

Wraight, J.M., Smith, A.D., Cowan, J.O., Flannery, E.M., Herbison, G.P. and Taylor, D.R. (2004) Adverse effects of short-acting beta-agonists: potential impact when anti-inflammatory therapy is inadequate. *Respirology* **9**, 215-21.

Yamamoto, H. and Nagata, M. (1999) Regulatory mechanisms of eosinophil adhesion to and transmigration across endothelial cells by alpha4 and beta2 integrins. *Int Arch Allergy Immunol* **120 Suppl 1**, 24-6.

Yamamoto, H., Nagata, M. and Sakamoto, Y. (2005) CC chemokines and transmigration of eosinophils in the presence of vascular cell adhesion molecule 1. *Ann Allergy Asthma Immunol* **94**, 292-300.

Yamane, H., Sugimoto, Y., Tanaka, S. and Ichikawa, A. (2000) Prostaglandin E(2) receptors, EP2 and EP4, differentially modulate TNF-alpha and IL-6 production induced by lipopolysaccharide in mouse peritoneal neutrophils. *Biochem Biophys Res Commun* **278**, 224-8.

Yildiz, P., Oflaz, H., Cine, N., Genchallac, H., Erginel-Unaltuna, N., Yildiz, A. and Yilmaz, V. (2004) Endothelial dysfunction in patients with asthma: the role of polymorphisms of ACE and endothelial NOS genes. *J Asthma* **41**, 159-66.

Zhou, W., Hashimoto, K., Goleniewska, K., O'Neal, J.F., Ji, S., Blackwell, T.S., Fitzgerald, G.A., Egan, K.M., Geraci, M.W. and Peebles, R.S. Jr (2007) Prostaglandin I2 analogs inhibit proinflammatory cytokine production and T cell stimulatory function of dendritic cells. *J Immunol* **178**, 702-10.

ABBREVIATIONS

Abbreviation	*Definition*
AA	arachidonic acid
AJ	adherent junction
BAL-fluid	bronchoalveolar lavage
Ca^{2+}	calcium ion
cAMP	cyclic adenosine monophosphate
C5a	complement 5a
COX-1, 2	cyclooxygenase-1, 2
CREB	cAMP response element-binding
CTD	C-terminal domain
ECP	eosinophil cationic protein
edg-1	endothelial differentiation gene-1, S1PR1
EDN	eosinophil-derived neurotoxin
EGF	epidermal growth factor
EP	E-type prostanoid receptor
ERK	extracellular signal regulated kinase
EPC	endothelial progenitor cell
EPO	eosinophil peroxidise
E-selectin	endothelial-selectin
DP	D-type prostanoid receptor
F-actin	filamentous actin
FP	F-type prostanoid receptor
GJ	gap junction
GM-CSF	granulocyte-monocyte-colony stimulating factor
GRK	G protein-coupled receptor kinase
GSK	glycogen synthase kinase
H1-4	histamine receptor 1-4
HGF	hepatocyte growth factor
HMVEC-L	human microvascular endothelial cells from lung
ICAM-1	intercellular cell adhesion molecule-1
IgE	Immunoglobulin E

IL	Interleukin
IP	I-type prostanoid receptor
IP3	inositol triphosphate
JMD	juxtamembrane domain
JAMs	junctional adhesion molecules
LBP	LPS binding protein
LFA-1	lymphocyte function associated antigen-1
LPS	lipopolysaccharide
LTs	leukotrienes
MAC-1	macrophage-1 antigen, CD11b/CD18
MAPK	mitogen-activated protein kinase
MBP	major basic protein
MCP	monocyte chemoattractant protein
MMP	matrix metalloprotease
MLCK	myosin light chain kianse
mPGES	microsomal PGE2 synthase
NF-κB	nuclear factor-kappa B
PAF	platelet-activating factor
PAR-1	protease activated receptor-1
PECAM-1	platelet-endothelial cell adhesion molecule-1
PGI_2	prostaglandin I_2, prostacyclin
PGE_2	prostaglandin E_2
PGD_2	prostaglandin D_2
PI3K	phosphoinositide 3-kinase
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
PMA	phorbol myristyl acetate
PPARγ	peroxisome proliferator-activated receptor gamma
Rac-1	Ras-related C3 botulinum toxin substrate 1
RANTES	regulated upon activation, normal T-cell expressed, and secreted
S1P	sphingosine-1-phosphate
S1PR1-5	sphingosin-1-phosphate receptor 1-5

Th2	T-helper cell type 2
TJ	tight junction
TLR4	toll-like receptor-4
TRPC1	transient receptor potential cation channel 1
TNF-a	tumor necrosis factor alpha
TXs	thromboxanes
VASP	vasodilator-stimulated phosphoprotein
VCAM-1	vascular cell adhesion molecule-1
VE-Cadherin	vascular endothelial cadherin
VEGF	vascular endothelial growth factor
VEGFR2	vascular endothelial growth factor receptor 2
VLA-4	very late antigen-4
VVO	vesiculo-vacuolar organelle
ZO-1	zonula occludens-1

CURRICULUM VITAE

Personal

Birth : 7 December 1982, in Szombathely, Hungary

Affiliation : Institute of Experimental and Clinical Pharmacology
Medical University Graz
Universitätsplatz 4
A-8010 Graz, Austria
Tel.: 43 (316) 380 4511
Fax.: 43 (316) 380 9645
E-mail: viktoria.konya@medunigraz.at
http://www.medunigraz.at/pharmakologie/staff.html

Education

2007 - 2010: PhD study at the Medical University of Graz, Austria

2007: Master thesis: "*Analysis of the immune suppression mechanisms of human bone marrow mesenchymal stem cells; an apoptosis study in T cells"* Dr. Eva Monostori, Lymphocyte Transduction Laboratory, Hungarian Academy of Science, Szeged

2005-2006: Internship for 8 months in the Toxicological Laboratory, Prof. Dr. Robert Kiss, ULB-Universite Libre de Bruxelles, Brussels, Belgium: *"Characterization of the role of galectin-1 in defense mechanisms of human glioma cells against apoptosis and autophagy"*

2003 - 2007 : Diploma of English scientific translator

2001 - 2007 : MSc study in Biology at the University of Szeged, Hungary

Awards

2004-2007: Scholarship of the Republic of Hungary, Ministry of Culture and Education, Budapest, Hungary

2005-2006: TEMPUS-ERASMUS Scholarship, VUB-Vrije Universiteit Brussel, Belgium

2005-2007: Scholarship of the town Szeged, Annual Regional Student Science Competition -1st prize

2007: Annual National Student Competition - distinguished award

Publications

Original articles:

Konya V, Sturm EM, Schratl P, Beubler E, Marsche G, Schuligoi R, Lippe IT, Peskar BA, Heinemann A. Endothelium-derived prostaglandin I_2 controls the migration of eosinophils. ***J Allergy Clin Immunol.*** 2010; 125, 5: 1105-1113.

Schuligoi R, Sturm E, Luschnig P, Konya V, Philipose S, Sedej M, Waldhoer M, Peskar BA, Heinemann A. CRTH2 and D-Type prostanoid receptor antagonists as novel therapeutic agents for inflammatory diseases. ***Pharmacology,*** 2010; 85, 6: 372-382.

Sturm EM, Schratl P, Schuligoi R, Konya V, Sturm GJ, Lippe IT, Peskar BA, Heinemann A. Prostaglandin E2 inhibits eosinophil trafficking through E-prostanoid 2 receptors. ***J Immunol***. 2008;181:7273-83.

Konya V, Meszaros PG, Viskolcz B and Csizmadia IG. Conformational analysis of oxidized vitamin-C. ***J Mol Struct (THEOCHEM)***. 2003; 666-667:397-400.

Posters and oral presentations:

Konya V, Sturm EM, Graziani A and Akos Heinemann. Endothelial prostaglandin I_2 restrains eosinophil trafficking. *Presented at the Annual Meeting of the Austrian Pharmacological Society, Graz, Austria, November 2009*

Konya V, Sturm EM, Graziani A and Akos Heinemann. The role of prostacyclin in endothelial-eosinophil interaction. *Presented at the Annual Meeting of European Respiratory Society, Vienna, Austria, September 2009*

Konya V, Sturm EM, Balint Z, Krenn K, Heinemann A. Prostacyclin in endothelial-eosinophil interaction. *Presented at the Allergy & Asthma Symposium: Bridging Innate and Adaptive Immunity; Bruges, Belgium. Mai 2009*

Konya V, Sturm EM, Balint Z, Krenn K, Heinemann A. The role of prostacyclin in the interaction of endothelial cells and eosinophil granulocytes. *Presented at the Annual Meeting of the Austrian Pharmacological Society, Innsbruck, Austria, November 2008*

Konya V, Sturm EM, Balint Z, Krenn K, Heinemann A. The role of prostacyclin in endothelium-eosinophil interactions. *Presented at the Annual Doctoral Day, Graz, Austria, October 2008*

Acknowledgements

I started my PhD studies in 2007 at the Institute of Experimental and Clinical Pharmacology. Over the last three years, I gained deeper knowledge and experience in the field of inflammation research. Therefore, I thank the whole staff of our institute for providing a friendly and inspiring atmosphere.

First of all, I want to express my gratitude to my supervisor, Prof. Akos Heinemann for his continuous guidance throughout these years. His broad knowledge and experience, his creative attitude were essential for completing this work. I want to thank to the other two members of my Thesis Committee, Prof. Rufina Schuligoi and Prof. Wolfgang Graier for their supportive suggestions.

I want to express my thanks to Martina Ofner, Birgit Brodacz, Gerald Parzmair and Ilse Lanz for their excellent technical assistance.

I am grateful to my colleagues, Petra Luschnig, Eva Sturm and Miriam Sedej for their collaboration. Special thanks to my friend and colleague, Sonia Philipose for very inspiring scientific or semi-scientific discussions.

I wish to thank my family for the emotional support which I always got when I needed it.

Finally, I am indebted to our PhD Program "Molecular Medicine" and the Medical University of Graz for financially supporting my work; personally, I am very grateful to Mag. Karin Osibow who was on non-stop duty at organizing our student life over the last three years. Special thanks to our PhD Dean, Prof. Andrea Olschewski for steering the PhD Program.

Declaration

I hereby declare that this thesis is my own original work and that I have fully acknowledged by name all of those individuals and organisations that have contributed to the research for this thesis. Due acknowledgement has been made in the text to all other material used. Throughout this thesis and in all related publications I followed the guidelines of "Good Scientific Practice".

Date *Signature*