Master Thesis

The role of prostaglandin receptors on platelet aggregation

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Table of Contents

Abstract

Platelets are small anucleate blood cells that circulate in the blood stream and get activated upon tissue injury. During activation platelets lose their distinct shape and gain their functional ability to support hemostasis. Interactions between platelets and the injured vessel wall depends on receptor mediated platelet adhesion, activation and aggregation. The importance of normal platelet count and proper platelet function is indicated in platelet associated bleeding disorders. Although thrombus formation is essential in supporting haemostasis after injury, the pro-thrombotic potential of inappropriate activated platelets increases the risk of thrombotic cardiovascular events like myocardial infarction and stroke. Thus, development of antiplatelet drugs that interfere with platelet activation is an important issue.

Prostanoids have a different impact on platelets and therefore differently affect haemostasis. Thromboxan A_2 (TXA_2) via activation of the TP receptor, promotes platelet aggregation, while prostaglandin (PG)D_2, via activation of the DP receptor and PGI_2 via the IP receptor, inhibit platelet aggregation. For PGE_2 a biphasic, concentration dependent effect has been reported. While activation of the EP4 receptor inhibits, the EP3 receptor aggravate agonist – induced aggregation. Platelet prostaglandin receptors are proposed to be useful targets in antithrombotic therapy, since blocking certain receptors, was shown to reduce platelet activation without interfering with the essential function of platelets in haemostasis.

In the first part of my thesis I investigated the effect of the lipid lowering drug Tredaptive® - a combination of niacin and the DP antagonist laropiprant - on platelet function. The addition of laropiprant to niacin reduces the side effect of “flush”, which is mediated mainly by PGD_2. I could show that in addition to its antagonistic effect on the DP receptor, laropiprant has antithrombotic properties which are mediated via the TP receptor, since collagen induced platelet aggregation was inhibited by higher concentrations of laropiprant. I also show that niacin inhibits *in vitro* thrombogenesis under flow – an effect that was, in contrast to the niacin caused „flush“ response, not due to further prostaglandin release. The fact that the known niacin receptor GPR109A is not present on platelets together with the long niacin incubation time that is needed to see an effect, further suggests an indirect effect on platelets that

might depend on shear stress. The obtained *in vitro* data suggest that Tredaptive®, beside its potential to treat dyslipedemias, might have an additional positive impact on thrombotic cardiovascular disease by decreasing the functional potential of platelets. However, further studies are required to identify the underlying mechanisms of action of niacin.

In the second part of my thesis I investigated the effect of the EP3 agonist sulprostone and the EP3 antagonist L-798106 on platelet function. Since an EP3 antagonist was reported to be useful in platelet therapy and an EP3 antagonist is currently in clinical trials, I wanted to extend the knowledge of this potential therapeutic target. I show that sulprostone increased collagen and fibrinogen induced platelet adhesion under flow – an effect that was totally reversed by pretreatment with the EP3 antagonist L-798 106. In contrast, sulprostone failed to affect whole blood *in vitro* thrombus formation under flow, although the pro-aggregatory effect of sulprostone in whole blood has already been shown. However, these results demonstrate that there are a lot of unsolved questions with regard to the EP3 receptor functionality, and a deeper understanding is needed.

In summary the results obtained deepen the understanding of a therapeutically used drug and add knowledge about the function of the EP3 receptor on platelets.

Zusammenfassung

Thrombozyten sind kleine kernlose Blutzellen, die im Blutkreislauf zirkulieren und bei Gewebeverletzung aktiviert werden. Während der Aktivierung verändern Thrombozyten ihre Form und erwerben die funktionelle Fähigkeit die Hämostase aufrecht zu erhalten. Die Wechselwirkungen zwischen Thrombozyten und der verletzten Gefäßwand sind abhängig von den rezeptorvermittelten Thrombozyten - Adhäsion, Aktivierung und Aggregation. Die Bedeutung dieser Zellen zeigt sich in Störungen der Blutgerinnung bei veränderter Thrombozytenanzahl und/oder Thrombozytenfunktion. Obwohl die Thrombusbildung nach einer Verletzung eine wesentliche Rolle beim Aufrechterhalten der Hämostase spielt, erhöht sich aber durch unerwünschte Thrombozytenaktivierung das Risiko für Thrombose assoziierte Krankheiten wie Herzinfarkt und Schlaganfall. Dies macht die Entwicklung von Thrombozytenaggregationshemmern, die in die Thrombozytenaktivierung eingreifen, zu einem relevanten Forschungsthema.

Prostanoide haben einen unterschiedlichen Effekt auf Thrombozyten und greifen somit unterschiedlich in die Hämostase ein. Während Thromboxan A_2 (TXA_2) die Thrombozytenaggregation fördert indem es den TP Rezeptor aktiviert, führen Prostaglandin (PG) D_2 über die Aktivierung des DP Rezeptors und PGI_2 über die Aktivierung des IP Rezeptors zu einer Hemmung der Thrombozytenaggregation. PGE_2 ist bekannt für seinen biphasischen, konzentrationsabhängigen Effekt. Während die Aktivierung des EP4 Rezeptors eine Hemmung der Thrombozytenaggregation bewirkt, steigert die Aktivierung des EP3 Rezeptors die Thrombozytenaggregation. Die Prostaglandinrezeptoren auf Thrombozyten stellen wichtige Angriffspunkte in der antithrombotischen Therapie dar, da die Blockierung bestimmter Rezeptoren die Thrombozytenaktivierung vermindert, ohne ihre essentielle Funktion in der Hämostasis zu beeinträchtigen.

Im ersten Teil meiner Masterarbeit habe ich das lipidsenkende Arzneimittel Tredaptive®, das aus Niacin und dem DP-Antagonisten Laropiprant besteht, auf seine Wirkung auf die Thrombozytenaggregation untersucht. Niacin wird in Kombination mit Laropiprant verabreicht, um die hauptsächlich durch PGD_2

verursachte Nebenwirkung "Flush" zu reduzieren. Es konnte gezeigt werden, dass Laropiprant zusätzlich zu seiner Wirkung auf den DP Rezeptor auch TP Rezeptor vermittelte antithrombotische Eigenschaften besitzt, da eine hohe Konzentration von Laropiprant die Kollagen induzierte Thrombozytenaggregation inhibierte. Zusätzlich konnte ich zeigen, dass Niacin die *in vitro* Thrombogenese unter Flussbedingung hemmt, ein Effekt, der im Gegensatz zum "Flush" nicht auf Prostaglandinfreisetzung zurückzuführen ist.

Ich konnte zeigen, dass der Niacin Rezeptor GPR109A nicht auf Thrombozyten vorhanden ist und der Effekt von Niacin des Weiteren von einer langen Inkubationszeit abhängig ist. Dies lässt eine indirekte Wirkung auf die Thrombozyten schließen. Diese *in vitro* gewonnenen Daten lassen vermuten, dass Tredaptive®, neben seinem Effekt in der Behandlung von Dyslipidämien, durch die Verminderung der Thrombozytenreaktivität einen zusätzlichen positiven Einfluss auf thrombotische kardiovaskuläre Erkrankungen haben könnte. Es sind jedoch weitere Studien erforderlich, um die zugrunde liegenden Mechanismen der Niacinwirkung zu identifizieren.

Im zweiten Teil meiner Arbeit habe ich die Wirkung des EP3 Agonisten Sulproston und des EP3 Antagonisten L-798106 auf die Thrombozytenfunktion untersucht. Da ein EP3 Antagonist derzeit in einer klinischen Studie in Bezug auf seine anti-aggregatorische Wirkung untersucht wird, wollte ich die funktionellen Effekte des EP3 Rezeptors an Thrombozyten genauer erforschen. Ich konnte zeigen, dass Sulproston zu einer erhöhten Kollagen- und Fibrinogen-induzierten Thrombozytenadhäsion führt und, dass dieser Effekt durch Vorbehandlung mit dem EP3-Antagonisten L-798106 reversibel ist. Im Gegensatz zu bereits publizierten Daten, die zeigten, dass Sulproston im Vollblut zu einer Steigerung der Aggregation führt, konnte ich keinen Effekt auf die Thrombusbildung unter Flussbedingungen nachweisen. Diese Ergebnisse zeigen, dass es noch eine Vielzahl ungelöster Fragen im Bezug auf die Funktion des EP3 Rezeptors gibt und ein tieferes Verständnis erforderlich ist.

Zusammenfassend vertiefen die Ergebnisse das Wissen über ein therapeutisch eingesetztes Arzneimittel und erweitern das Verstehen über die Funktion des EP3 Rezeptors auf Thrombozyten.

1. Introduktion

1.1 Platelets

Platelets or thrombocytes are small, anucleate cells circulating in the blood stream. They got their name due to their distinct plate shape, one main characteristic of unstimulated platelets. Beside red blood cells, they are the most abundant blood cells with about 150-350 000 platelets/ ml blood. The average lifespan of platelets is only 5-10 days. Platelets first enter the blood stream when they are released from large bone marrow cells known as Megakaryocytes. Platelet production is under hormonal control of thrombopoitin, which provides a constant, normal platelet count that is necessary to maintain platelet function. Their functional involvement ranges from acting in microbial infections, thrombosis, wound healing and inflammation. Nevertheless, the primary function of platelets is to support haemostasis by interacting with the injured vessel wall (Broos et al., 2011). These interactions are mediated by platelet surface receptors that are the key players in platelet adhesion, activation and aggregation as described below.

1.1.2 Prevention of platelet function

Under normal physiological circumstances platelets do not adhere to the vessel wall. The functional endothelium acts as physical barrier that inhibit interactions with the high thrombotic components of the extracellular matrix (ECM). In addition, cells of the endothelium release substances that inhibit binding of circulating platelets and therefore maintain blood fluidity. Among these negative regulators, prostacyclin, nitric oxide and the ADPase Cd39 play the key role in preventing unwanted platelet activation (Brass, 2003; Broos et al., 2011) . Prostacyclin is a member of the prostanoid family, and is also known as prostaglandin I_2 (PGI_2). Platelets express a corresponding $G_{\alpha s}$-protein coupled receptor for this prostaglandin, referred as PGI_2 receptor IP. Stimulation of IP immediately leads to cAMP production via activation of

the adenylate cyclase, counteracting an increase of intracellular Ca^{2+} levels that are essential for platelet activation (Weiss and Turitto, 1979; Edwards et al., 1987).

The ADPase Cd39 is involved in thromboregulation by hydrolyzing ADP, a soluble agonist for platelet activation. The reduction of small ADP amounts counteracts aggregation response and therefore prevents thrombus formation (Marcus et al., 1997; Gayle et al., 1998).

A further part of the thromboregulatory system is nitric oxide (NO). Nitric oxide synthases (NOS) catalyze the production of NO from L-arginine (Alderton et al., 2001). NO diffuses through the platelet surface membrane (Dangel et al., 2010), and activates a NO sensitive guanylyl cyclase that mediates cGMP production finally leading to inhibition of Ca^{2+} rises (Radomski et al., 1990).

The above described systems are essential to oppose inappropriate platelet activation leading to unwanted platelet function that could cause risk for blood vessel occlusion (Brass, 2003)

1.1.3 Platelet adhesion

After a vessel wall injury, components of the ECM are exposed to the blood stream where they present a prothrombotic surface to platelets. Platelets marginating along the endothelium react on the altered surface as they bind to adhesive ECM proteins. The most important interactions occur with collagen, von Willebrand factor (VWF), laminin, fibronectin and thrombospondin. These interactions are mediated by specific glycoprotein receptors (GP) expressed on platelets surface (Broos et al., 2011). Among these adhesive surface proteins, collagen plays the key role in the initial phase of platelet adhesion and platelet-collagen interactions finally result in platelet intracellular signaling. The initiation of platelet adhesion depends on the local shear stress. At high shear stress conditions VWF-platelet interactions are required to recruit fast flowing platelets, while at low shear stress ($<1000\ s^{-1}$) collagen, fibronectin and laminin are the primary interaction partners of platelets (Broos et al., 2011). Binding to endothelial VWF is mediated by glycoprotein Ibα (GPIbα) and

triggers platelet tethering. This is crucial for platelet adhesion to collagen, that finally allows firm platelet adhesion (Coburn et al., 2011).

1.1.3.1 Receptors involved in platelet adhesion

Collagen receptors GPVI and α2β1

Collagen functions as key component for both, platelet adhesion and platelet aggregation. Platelet-collagen interactions are mainly mediated by the integrin α2β1 and glycoprotein VI (GPVI) (Farndale et al., 2003). Their exact roles are still a matter of debate, but recent research indicates that the first contacts at site of injury are predominantly mediated by GPVI (Nieswandt and Watson, 2003). These interactions lead subsequently to intracellular signaling that triggers changes in the integrin α2β1(Nieswandt et al., 2001). The integrin is therefore shifting to a high affinity binding state for collagen (Van de Walle et al., 2005), that promotes signaling pathways and firm adhesion by increasing the affinity for GPVI–collagen interactions (Atkinson et al., 2003)

GPIbα – receptor for VWF

Upon vascular damage, circulating VWF interacts with collagen mainly via its A3 but also with its A1 domain (Lankhof et al., 1996). The immobilized VWF now has the ability to interact with GPIbα, expressed on circulating, non activated platelets. GPIbα is a high copy number protein that can be found in different platelet glycoprotein complexes (Andrews et al., 2003). To avoid interactions between platelets and VWF under normal vascular circumstances, VWFs A1 domain is only active when (a) VWF is immobilized or (b) under high shear rates. Receptor binding is then accomplished by certain VWF domains that expose the prior shielded A1 domain (Martin et al., 2007). Platelet interactions with VWF are insufficient for stable adhesion, but provide a good starting position for further interactions by keeping platelets close to the endothelium (Broos et al., 2011). The evidence that these interactions are essential for platelet function is shown in bleeding disorders, like Willebrand disease and

Bernard– Soulier syndrome that results from a functional lack in VWF or GPIbα (De Meyer et al., 2009; Salles et al., 2008).

β1 and β3 integrins

The β1 and β3 integrins are especially important in the last phase of platelet adhesion and their binding ability depends on the activation of platelets. Upon platelet activation conformational receptor changes allow high affinity binding of β1 and β3 integrins to their ECM ligands. This illustrates that adhesion and activation of platelets are overlapping processes (Broos et al., 2011).

1.1.4 Platelet activation and aggregation

Platelet activation is a multistep process initiated by agonist-receptor binding that results in platelet shape change (increases the area of surface contact) and granule release via an exocytotic process. During activation, platelets gain the ability to bind fibrinogen, an essential prerequisite for platelet aggregation (Broos et al., 2011).

In general, platelet thrombus formation following a vessel wall injury can be divided in different stages: initiation, extension and stabilization.

1.1.4.1 Stages of thrombus formation

The initial phase is described by capturing of circulating platelets (see platelet adhesion) which are therefore activated. This is followed by a shape change that involves spreading of the adhered platelets and results in formation of a platelet monolayer at site of injury (Brass, 2003).

During shape change, platelets secret their storage granule that are classified as dense and α-granule (Flaumenhaft, 2003). The locally increased concentration of effector molecules is necessary for further plug formation. Further platelet accumulation on the initial collagen bound platelet monolayer indicates the second

phase of plug formation termed as plug extension. For extension of the platelet plug, thromboxan A_2 (TXA_2), thrombin and ADP play the key role. These substances activate further platelets by binding on their G-protein coupled surface receptor (see platelet signaling pathways) and the thereby triggered activation let them adhere to the already formed platelet monolayer (Michelson, 2004; Brass, 2003).

While ADP is secreted from dense granule of activated platelets (Fogelson and Wang, 1996), the prostanoid TXA_2 derives from the arachidonic acid cascade (see prostanoid formation). TXA_2 mediates its activatory potential via the platelet TP receptor (Knezevic et al., 1993) and ADP acts as activating agent via the G- protein coupled receptors $P2Y_1$ and $P2Y_{12}$ (Murugappa and Kunapuli, 2006).

The thrombin-platelet interactions are not elucidated in detail, but PAR1 and PAR4 (protease activated receptor 1 and 4) seem to be the key players in thrombin induced human platelet activation (Coughlin, 1999). Thrombin acts by cleaving a single peptide bond of the extracellular receptor domain that results in the exposure of a new N-terminus referred as tethered ligand. The tethered ligand self-activates the receptor through specific binding and intracellular signalling cascades promote platelet activation (Vu et al., 1991). The ultimate step of platelet activation is platelet-platelet interaction (see integrin signaling) (Brass, 2003).

The last phase in plug formation includes all mechanism involved in plug stabilization. Platelet-platelet interactions are stable and long-lasting what allows contact dependent signalling cascades (see integrin signaling). Upon activation, platelets express the CD40 ligand (CD40L) which allows them direct interaction with the active conformation of GPIIb/IIIa, leading to an additional positive impact on plug establishment (Inwald et al., 2003).

1.1.5 Platelet Signaling pathways

In general , agonist induced platelet activation can be divided in 3 different phases: (1) the early signaling involving interactions between agonists and their distinct platelet surface receptors (2) intermediate signaling and (3) signaling through integrin activation. However, platelet activation occurs as a dynamic process involving crosstalk between the activation pathways and multiple amplification mechanism, allowing tight regulation of platelet function (Li et al., 2010)

1.1.5.1 Early signalling

Signaling through G-protein coupled receptors (GPCRs)

GPCRs belong to a large receptor superfamily consisting of seven transmembrane domains that are coupled to an inner membrane located G-protein. G-proteins are trimeric proteins consisting of an α, β and γ subunit. When soluble agonists like ADP and thromboxan bind to their respective GPCRs, the transmitted signals lead to activation of the G-protein. This occurs through conversion of the α-subunit from an inactive (GDP bound) to an active (GTP bound) form. The activated α-subunit as well as the βγ dimer interact with downstream targets that finally promote implementing of the receptor signal in platelet function (Offermanns, 2006).

Depending on α-subunit similarities, G-proteins are divided in subfamilies, whereas plateteles express G_i, G_q, G_s and $G_{12/13}$. Beside G_s, all G-proteins present in platelets are involved in proaggregatory signaling pathways (Offermanns, 2006).

ADP binds to platelets through $P2Y_1$ and $P2Y_{12}$ coupled to G_q and G_i respectively (Ohlmann et al., 1995). Thrombin acts on PAR receptors, and the human PAR1 and PAR4 receptors are both G_q and $G_{12/13}$ coupled (Kahn et al., 1998; Nakanishi-Matsui et al., 2000) . The TP receptor mediates TXA_2 signals via G_q and $G_{12/13}$ (Knezevic et al., 1993b). G_s activation is a result of IP receptor activation through binding of PGI_2, released from the endothelium under circumstances of vascular integrity (Weiss and Turitto, 1979).

Signaling through the tyrosine cascade

Collagen induces tyrosine signaling via binding of the GPVI receptor. The collagen receptor GPVI is associated with two Syr tyrosine kinases (Fyn and Lyn) that include a SRC Homology 3 (SH3) domain which mediates receptor binding. Upon collagen binding, Fyn and Lyn are phosphorylated by ITAM, leading to recruitment of a further tyrosine kinase (Syk). This results in a complex cascade that finally leads to phoshorylation of the SH2 domain of phospholipase Cγ2 (PLCγ2) (Watson et al., 2010). PLCγ2 hydrolyzes phosphatidylinositol-4,5-bisphosphate, resulting in certain second messenger molecules that raises the cytosolic Ca^{2+} levels (Pasquet et al., 1999). The increase in Ca^{2+} levels allow platelet shape change, prostanoid synthesis via the arachidonic acid cycle and platelet granule release - all steps that are required for the ultimate step of platelet aggregation (Varga-Szabo et al., 2009; Bergmeier and Stefanini, 2009).

1.1.5.2 Intermediate platelet signalling

Although initial stimulation of platelet activation occurs differently, most of the cascade involve PLC activation (e.g. G_q and ITAM pathways) (Varga-Szabo et al., 2009) . This phosporylation step is required for elevation of intracellular Ca^{2+} release acting as a key player for initiation of signaling events like activation of protein kinase C (PKC). There are a lot of PKC isoforms known and controversial roles regarding platelet function have been reported. For PKCα its role in platelet granule release is well established (Konopatskaya et al., 2009). Granule release is required for the recruitment of flowing platelets, activation amplification and finally stabilisation of the thrombus (Reed et al., 2000; Ren et al., 2008).

Upon α-granule release, the adhesion molecule P-selectin (CD62P) is exposed to the outer platelet membrane (Stenberg et al., 1985). P-selectin is also expressed on cells of the endothelium (Ramos et al., 1999). P-selectin is reported for its stabilising role of GPIIb/IIIa-fibrinogen interactions, leading to firm aggregates (Merten and Thiagarajan, 2000). P-selectin is an important research tool since its expression does not change over time (Ruf and Patscheke, 1995).

The role for P-selectin as a receptor for monocytes and neutrophils is known for decades (Larsen et al., 1989) and plays a key role for the initial phase in leukocyte adhesion, by mediating the rolling of monocytes along the activated endothelium (Ramos et al., 1999). Since inflammation associated diseases are characterized by leucocyte migration, P-selectin interactions with platelets and neutrophils can recruit platelets as active participants to atherosclerotic lesions. (Burger and Wagner, 2003).

1.1.5.3 Integrin signaling

Integrins consist of 2 subunits (α and β) that form non covalently bound heterodimers. Platelets express a lot of different integrin complexes on their surface, allowing them to interact with collagen (α2β1), fibronectin (α5β1), laminin (α6β1), vitronectin (αvβ1), and fibrinogen (αIIbβ3) (Li et al., 2010). The integrin αIIbβ3 (GPIIb/IIIa) is the most common platelet receptor and can be found on circulating platelets in a non active, aggregation preventing form.

Integrin signaling always occurs bidirectional, that means that the proper function of integrins depends on two different signaling cascades- referred as „inside-out“ and „outside-in“ signaling. Platelet receptors involved in early platelet activation induce intracellular signal cascades (Shattil et al., 1985). This triggers binding of talin (major protein of the cytoskeleton) and kindlin to the cytoplasmic integrin tail of GPIIb/IIIa (β3 subunit). Consequently, the extracellular part of the receptor undergoes conformational changes resulting in a high affinity ligand binding state (Tadokoro et al., 2003). It was shown that the talin head domain (THD) is sufficient for this ”inside-out” signaling (Calderwood et al., 1999). The conformational changes in GPIIb/IIIa from a bent to an extended activated receptor, allows fibrinogen binding and ultimately results in platelet aggregation (Coller and Shattil, 2008; Vinogradova et al., 2002). Thereby the platelet thrombus is extended.

GPIIb/IIIa is further able to stabilize the platelet thrombus by ”outside-in“ signaling. Upon ligand-receptor interactions signals are transmitted from the outside to the inside of platelets (Leisner et al., 1999). Beside platelet plug stabilisation, this cascade can further result in platelet spreading (by promoting cytosceletal reorganisation), platelet granule secretion and also clot retraction (Shattil and Newman, 2004).

1.1.6 Antiplatelet drugs

Antiplatelet drugs can be divided in 3 different groups: (1) COX-inhibitors like aspirin, (2) thienopyridins like clopidogrel and (3) GPIIb/IIIa receptor antagonists like abciximab. A short overview of their mechanism of function is shown in **Figure 1**.

1.1.6.1 Aspirin

Aspirin or acetylsalicylic acid is a nonsteroidal anti-inflammatory drug (NSAIDs). The fever-reducing and at higher concentrations anti-inflammatory properties of these drugs have already been well described (Singh and Triadafilopoulos, 1999). Aspirin has a unique antithrombotic effect by preventing platelet function irreversible (Funk et al., 1991). The mechanism of action of aspirin was elucidated in the 1970s, where it was shown that aspirin prevents prostaglandin release by inhibition of cyclooxygenase (COX) (Vane, 1978) and nowadays it is further known that COX inhibition is due to acetylation of serin 529. (Funk et al., 1991). The main product of COX in platelets is TXA_2 , known for promoting platelet activation through binding the platelet TP receptor (Knezevic et al., 1993). So, by interfering with TXA_2 production platelets lose their potential to aggregate for their whole lifespan. Based on the short lifespan of platelets, irreversible COX inhibition prevents platelet function up to 7 days, whereas during these days TXA_2 production increases continuously by synthesis of new, functional platelets (Patrono et al., 2004). The balance of TXA_2 and the platelet inhibitor PGI_2 is required to support proper platelet function.

Treatment with low dose of acetylsalicylic acid can reduce the risk of cardiovascular events since most of the myocardial infarctions are a direct result from thrombotic arterial occlusion (Willard et al., 1992). Aspirin further serves as antiplatelet agent in acute diseases that could lead to thrombosis (Schrör, 1995). Aspirin has been reported to cause gastrointestinal haemorrhage (Patrono et al., 2004) but a low aspirin dose given in antiplatelet therapy has little systemic effects due to its high first pass effect.

1.1.6.2 Clopidogrel

Clopidogrel is a thienopyridine and therapeutically used for its effect to selectively inhibit platelet aggregation induced by ADP. In contrast to aspirin, prostaglandin synthesis is unaffected (Quinn and Fitzgerald, 1999). The functional properties of clopidogel are mediated by a reduction of ADP binding sites, and the receptor is thereby irreversibly modified. Clopidogrel is a prodrug and the active metabolite, formed in the liver, acts on platelet $P2Y_{12}$ receptor. Clopidogrel fails to affect platelets *in vitro* (Savi et al., 2000). Cytochrom P450 is responsible for the metabolic conversion of clopidogrel, since a global cytochrom C450 inhibitor decreased the functional potential of the platelet inhibitor drug (Savi et al., 1994).

Clopidogrel and ticlopidine, which was prior on the market, have the same mechanism of action and via their function as antiplatelet drugs they prevent stroke and myocardial infarction with the same potential as aspirin. The disadvantages of ticlopidine are clearly shown with regard to the common side effects. So, it was not surprising that clopidogrel - described as well tolerated in long term clinical trials - rapidly replaced ticlopidine as platelet ADP receptor inhibitor (Quinn and Fitzgerald, 1999).

Recently prasugel, which has the same mode of action as ticlopidin and clopidogrel was approved in the United States and Europe for the treatment of patients with acute coronary syndromes (Gurbel and Tantry, 2008). Compared to clopidogrel and ticlopidine, the metabolic conversion is considerably faster, therefore prasugel has an enhanced and more rapid effect on platelets (Mousa et al., 2010). A combined therapy of aspirin and thienopyridins has been proposed useful, since increased antithrombotic efficiency has been reported by simultaneous application of both substances (Herbert et al., 1998)

1.1.6.3 GPIIb/IIIa receptor antagonists

Platelet activation is a complex process and occurs through binding of different platelet agonist leading to several signaling pathways. The varibility of platelet activation indicates that finding of a proper platelet drug is not that easy: ADP receptor-- and COX – inhibitors for instance fail to prevent thrombin induced platelet aggregation, since they can only block certain activation pathways. The integrin receptor GPIIb/IIIa was suggested as a promising antiplatelet drug, since activation of this receptor is the final outcome of all activation associated signaling pathways. (Coller, 1997). GPIIb/IIIa receptor antagonists were first developed in the early 1980s. Inhibitors include antibodies and short peptids or nonpeptids containing a specific receptor recognition sequence. The antiplatelet agent abciximab is the Fab-fragment of a monoclonal antibody specific for GPIIb/IIIa and acts by cross-reaction with the receptor (Topol et al., 1999). About 40 000 molecules are interacting with a single platelet, and a significant inhibition occurs when half of the receptors are blocked. A nearly complete inhibition is reached when 80 % of platelet receptors are blocked. A extremely prolonged bleeding time was only detected by high abciximab concentrations (> 90 %) (Coller et al., 1983). The role of abciximab in preventing vascular occlusion is well established and it is indicated for use in individuals undergoing percutanous coronary intervention (Dery et al., 2004).

Beside its role to inhibit platelet function via receptor blocking, it was suggested that the anti-aggregatory potential of abciximab is partially due to a decrease in thrombin formation. (Reverter et al., 1996). Abciximab is further known to bind the vitronectin receptor at higher doses and for its potential to bind an active leukocyte receptor. Until now, it is not known if these interactions are involved in the functional potential of abciximab (Coller et al., 1983; Coller et al, 1997).

Natural GPIIb/IIIa ligands have a short recognition sequence containing -Arg-Gly-Asp- (RGD) that allows them interactions with several integrins. RGD served as initial-point for the development of tirofiban (Topol et al., 1999), which was shown to be more active in platelet inhibition than abciximab. However, this could only be reported at late time points after drug administration (Smith and Gandhi, 2001).

Eptifibatide is the third GPIIb/IIIa blocker and contains the aminoacids -Lys-Gly-Asp- and is a structural analogue of the C-terminus γ-chain of fibrinogen that is responsible for the receptor binding of the ligand (Topol et al., 1999; Phillips and Scarborough, 1997). All 3 blockers interact with GPIIb/IIIa independently of its activation state. Although their different nature, their ability to prevent platelet aggregation underlies the same mechanism of action and all of these inhibitors are indicated for percutanous coronoary intervention (Topol et al., 1999).

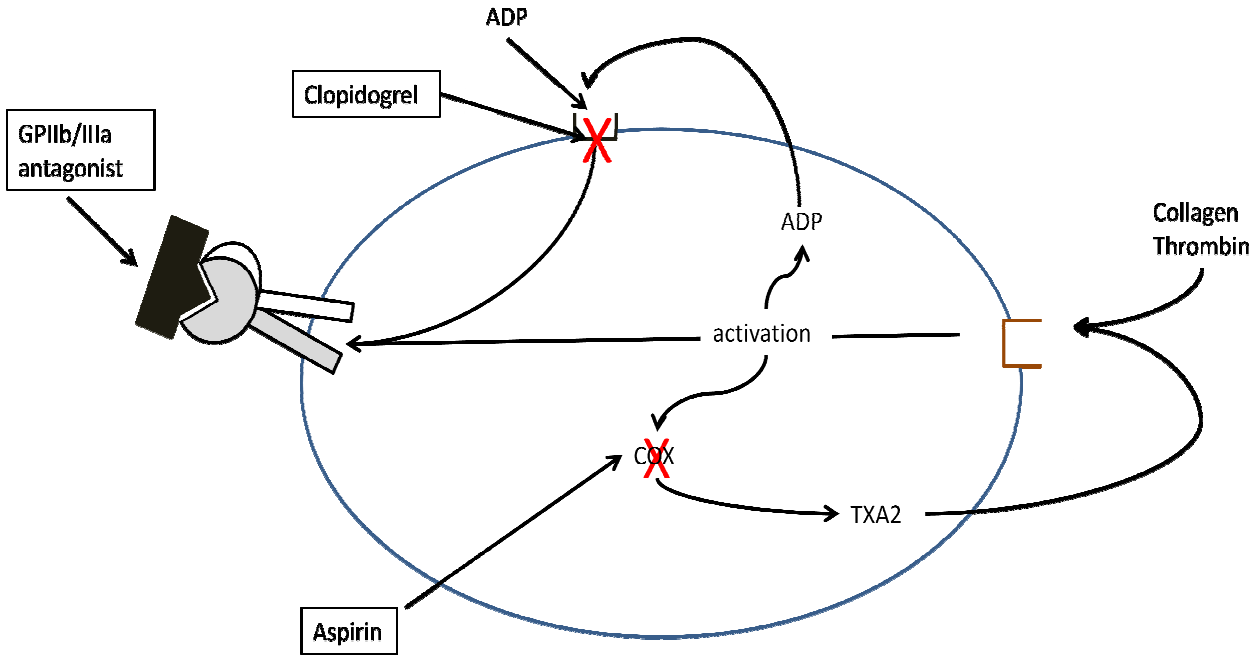

Figure 1: Targets of antiplatelet drugs

Asprin inhibits the formation of the TXA_2 by irreversible inhibition of COX, the enzyme for prostanoid synthesis. TXA2 is an potent platelet aggregation stimulus. Clopidogrel acts on the platelet ADP receptors and prevents GPIIb/IIIa activation, and therefore platelet aggregation. GPIIb/IIIa antagonists inhibit platelet aggregation by blocking interactions with fibrinogen and VWF that are essential for aggregate formation.

Excursion: platelet-bacteria interactions

Recently, the idea that bacteria as infectious agents play a role in cardiovascular events gained researches attention. Upon entry of bacteria in the blood stream, they interact with platelets, suggesting an important role of platelets in infection (Kerrigan and Cox, 2009). Interactions between platelets and the microbe can occur either directly or indirectly, whereas the latter means that a plasma protein mediates the contact via a plasma bridge. The second possibility would be the secretion of platelet - stimulating agents by the bacteria (like lipopolysaccharides (LPS)). In addition, cytokines whose release result from pathogen induced inflammation, can interact with platelets and therefore cause their activation (Fitzgerald et al., 2006) Platelet-bacteria interactions vary between different species and strains, but they have in common that all interactions underlie an all-or-nothing principle. This means that, in contrast to typical platelet agonists, a certain concentration of bacteria is needed to cause an effect, but this effect would not be further increased by elevated levels of bacteria (Kerrigan and Cox, 2010).

Platelet response to bacterial invaders can cause different pathological phenotypes. One main consequence following bacterial platelet stimulation is thrombus formation. Further, a systemic activation of platelets can also cause thrombocytopenia, which is associated with bleeding problems (Fitzgerald et al., 2006) and often occurs due to a response of shiga toxin (Amirlak and Amirlak, 2006). Granule secretion is induced by platelet-bacteria interactions and when the locally cytokine concentration is increased. Cytokines are inflammatory mediators, involved in pathogenesis like atherosclerosis (Coppinger et al., 2004). Since bacterial activated platelets or their products participate in processes with a negative impact on the organism, it was suggested that blocking these interactions would have a beneficial effect regarding inflammation and subsequent diseases. Therefore blocking of the platelet Fcγ-receptor was proposed (Kerrigan and Cox, 2010). For streptococci it has already been shown that they failed to induce platelet aggregation in the absence of the Fcγ-receptor (Ford et al., 1997). Beside reduction of the pathogenic potential of platelets, one further advantage would be that the main function of platelets in hemostasis is not disturbed, since common platelets agonists act on different receptors (Kerrigan and Cox, 2010).

1.2 Prostanoids

Inflammation occurs as a response to infection and injury. One typical sign of inflammation is the highly increased biosynthesis of lipid-derived autacoids referred as prostanoids (Hata and Breyer, 2004).

Thereby, arachidonic acid (AA) is released from membrane phospholipids in a phospolipase-A_2 catalyzed reaction. AA is oxidized by the enzyme COX resulting in the formation of the prostanoid precursor form PGH_2 (Smith et al., 2000). Particularly the COX isoform COX-2 is involved in prostanoid production following an inflammatory stimulus, while COX-1 maintains the basal synthesis (FitzGerald and Patrono, 2001). The newly formed unstable PGH_2 is further metabolized by tissue specific isomerases that decide solely which prostanoids are synthesized. Prostanoids include 4 bioactive in vivo formed prostaglandins (PGs), termed PGE_2, PGI_2, PGD_2, PGF_2 and TXA_2. Prostanoid production depends on the cell type, whereas most cells produce one or two dominant products. Prostaglandins mediate their different effects through binding on GPCRs. There are 9 known receptors on which binding of prostanoids can occur: PGE binds to 4 subtypes of E prostanoid receptors (EP1-4), PGD_2 activates the DP and the chemoattractant receptor-homologous molecule expressed on TH2 (CRTH2) receptor, PGF_2 activates the FP-, PGI_2 the IP- and TXA_2 the TP- receptor. Activated receptors mediate their effects via their intracellular receptor coupled intracellular signaling pathway (Smyth et al., 2008).

Prostaglandins can act in an autocrine and juxtacrine manner and are thereby potent modulators of different functional body systems (CNS, respiratory, endocrine, gastrointestinal, cardiovascular and immune system). Prostaglandins are involved in fever, cancer metastasis and cardiovascular disease and haemostasis. Therefore, inhibition of their biosynthesis is clinically relevant. NSAIDs inhibit COX and therefore the complete prostaglandin production. In platelets prostaglandins mediate pro- as well as antiaggregatory effects, therefore, specific receptor antagonists that only affect certain pathways may be more appropriate and advantageous for patients that need anti-platelet therapy. (Hata and Breyer, 2004a).

1.2.1 Prostaglandins in platelet function

As already mentioned above TXA_2 via activation of the TP receptor is pivotal for platelet aggregation and adhesion (Knezevic et al., 1993a).

In contrast PGI_2, that is synthesized in endothelial cells, via acting on the IP receptor is a potent inhibitor of platelet aggregation and an essential part of the tight regulation system that opposes unwanted platelet function (Brass, 2003) as already described above. Like other platelet inhibitors, the PGI_2 mediated effect results from an increase in cAMP and cGMP leading to phosphorylation of VASP (vasodilator stimulated phosphoprotein). VASP is a actin filament associated protein and mediates changes of the platelet cytoskeleton as a result of cAMP or cGMP dependent phosphorylation (Reinhard et al., 1992). The increase of cAMP is mainly due to IP receptor coupled G_s-protein and leads to vasodilation and anti-aggregatory platelet effects (Sasaki et al., 1994).

The unstable nature of PGI_2 makes this prostaglandin inappropriate for experimental use, therefore a variety of stable analogues have been developed. Amongst those, iloprost is most widely used in research (Boie et al., 1994). Furthermore, iloprost is also clinically relevant for the treatment of patients with primary pulmonary hypertension (McLaughlin et al., 1998)

PGD_2 is also well known to inhibit platelet aggregation via the DP receptor (Giles et al., 1989a). The inhibitory potential of this receptor is also due to elevation of intracellular cAMP levels. (Hirata et al., 1994a). In addition, prevention of Ca^{2+} mobilisation and the inhibition of platelet aggregation have been reported (Ito et al., 1989). PGD_2 is produced by eosinophils, macrophages, Th2 cells and endothelial cells (Camacho et al., 1998) and is also released in high concentrations by mast cells during anaphylaxis (Peskar, 1978). Beside DP, PGD_2 can also interact with CRTH2. This receptor is expressed on TH2 cells, eosinophils and basophils and is known for its role in chemotaxis (Schuligoi et al., 2010). In contrast to DP, CRTH2 is not involved in platelet inhibition (Royer et al., 2008).

PGE_2 is the most common prostaglandin produced in the human body and acts on 4 different GPCRs: EP 1- 4 (Sugimoto and Narumiya, 2007). PGE_2 shows a biphasic function on platelets: while PGE_2 inhibits platelet aggregation at higher

concentrations, low concentrations show an inhibitory effect (Vezza et al., 1993; Weiss et al., 1976). Platelet mRNA indicates the presence of EP1, EP3 and EP4, while the EP2 seems not to be present on the platelet surface (Paul et al., 1998). EP2 and EP4 receptor activation is associated with an increase of intracellular cAMP as a result of G_s activation that finally leads to inhibition of platelet aggregation (Sugimoto and Narumiya, 2007). In contrast, activation of the EP3 receptor, enhances intracellular Ca^{2+} levels and an increases platelet aggregation (Matthews and Jones, 1993a).

Beside the appearance of EP subtypes, it was further revealed that there exist different splicing variants of the EP3 receptor that derive from alternative splicing of the C-terminus (Sugimoto and Narumiya, 2007). These EP3 subtypes are further increasing the variability of PGE_2 mediated actions, since EP subtypes and splice variants differ in their intracellular signaling. Different splice variants of the EP3 have already been reported in human and in mice (Hata and Breyer, 2004b). The investigation of the different EP3 splice variants in mice lead to further understanding for the diversity of PGE_2 triggered cellular responses, since it could be shown that also splice variants coupled to the same intracellular signaling cascade, provided different functions as a result of different subcellular targeting of the receptor. The C-terminal tails seem to be crucial for this activity (Hasegawa et al., 2000). In addition, constitutive activity of the splice variants can already differ (Hata and Breyer, 2004b). Initial reports described EP3 as coupled to the Gi-protein and therefore leading to an inhibition of intracellular cAMP (Sugimoto et al., 1992)

PGE_2 has a biphasic effect on platelets: it increases agonist- induced platelet aggregation at low concentrations via the EP3 receptor and it decreases platelet aggregation at higher concentrations via the EP4 receptor, as previously shown in our group (Philipose et. al, 2011). The EP4 receptor mediate its effect through the coupled Gs protein, leading to cAMP production (Breyer et al., 2001). Recently, a decrease of Ca^{2+} mobilization, inhibition of GPIIb/IIIa heterodimerization and a reduction of P-selectin expression was reported upon EP4 receptor activation. In addition, EP4 receptor activation lead to a decrease in platelet adhesion and thrombus formation, thus an EP4 receptor agonist might be useful as antithrombotic agent (Philipose et. al, 2010).

Compared to the other EP receptors, the platelet EP1 receptor is not that well descibed. EP1 receptor activation leads to an increase of intracellular Ca^{2+} levels, but whether this occurs through G-protein involvement is not sure (Narumiya et al., 1999).

1.3 Niacin and Laropiprant

Niacin, also known as nicotinic acid, is an essential human nutrient that became of research interest because of its lipid modifying effects. The niacin function on lipid metabolism is unique, since niacin treatment has a positive impact on all lipid abnormalities. This means that niacin shows potential to decrease, triglycerides, cholesterol, low density lipoprotein (LDL) levels while increasing high density lipoprotein (HDL). This effects of niacin are advantageous compared to statins with regard to mixed hyperdislipidemia (Bodor and Offermanns, 2008a).

Although niacin is highly effective for dyslipidemia treatment, common side effects interfere with the beneficial potential. When niacin acts on its receptor GPR109A prostaglandin production is stimulated in mast cells, karatinocytes and monocytes (Kamanna and Kashyap, 2008; Hanson et al., 2010). Especially PGD_2 acting on the DP receptor, has been reported to be responsible for the niacin caused side effects like facial vasodilation and cutaneous warmth which are referred as “flush” response (Papaliodis et al., 2008). Although, flushing is transient following niacin intake, about 5 % of the patients discontinue niacin treatment due to these complications (Brinton et al., 2011). As a consequence, a combination of laropiprant (a DP receptor antagonist) with niacin is currently marketed for treatment of dyslipidemias in Europe under the trade name Tredaptive® (Sanyal et al., 2010). Safety concerns may have played in role in the US, since the drug was rejected by the U.S. Federal Drug Administration in 2008. Although Tredaptive® has been shown to be effective and well tolerated by patients suffering from dyslipedemia (McKenney et al., 2010; Bays et al., 2010), it has not been revealed how this combination affects thrombotic cardiovascular events. Since prostaglandins are key regulators of platelet function, laropiprant through blocking the antiaggegatory function of the DP receptor (Feinstein et al., 1983a) could cause thrombotic cardiovascular risks .

2. Material and Methods

2.1 Material

2.1.1 Reagents

3,3′-dihexyloxacarbocyanine iodide	Sigma (Vienna, Austria)
ADP	Probe & Go (Osburg, Germany), Sigma
Antibody diluent	DAKO (Vancouver, Canada)
BD Cell Fix	BD Biosciences (San Jose, CA, USA)
BD FACS Flow	BD Biosciences
Bovine Serum Albumin	Sigma
$C_6H_5Na_3O_7 * 2H_2O$	Merck (Darmstadt, Germany)
$CaCl_2$	Merck
Collagen	Probe & Go
Cytochalasin B	Sigma
D(+)-Glucose monohydrate	Merck
Dextran	Sigma
DMSO	Carl Roth GMBH (Karlsruhe, Germany)
Dulbecco´s PBS 1x liquid - $CaCl_2$, $MgCl_2$	Invitrogen (Lofer, Austria)
Dulbecco´s PBS 1x liquid +$CaCl_2$, $MgCl_2$	Invitrogen
EDTA	Carl Roth GMBH
Fibrinogen	Sigma
Fix&Perm: reagent A	ADG (Kaumberg, Austria)

HEPES	Sigma
Histopaque	Sigma
Iloprost	Cayman (Ann Arbor, MI, USA)
KCl	Merck
L-798106	Tocris (Bristol, United Kingdom)
Laropiprant	Cayman
$MgCl_2$	Carl Roth GMBH
Na_2HPO_4 * 2 H_2O	Merck
NaCl	Carl Roth GMBH
$NaHCO_3$	Merck
Niacin	Sigma
Sodium citrate	Sigma
Toluidine blue	Sigma
Ultra-V blocking solution	Thermo Scientific (Waltham, MA, USA)

2.1.2 Antibodies

anti-hHM74A/GPR109A antibody-FITC	R&D Systems (Minneapolis, MN, USA)
$CD16^+$ antibody-PE	BD Biosciences
CD41 antibody-FITC	Invitrogen
CD61 antibody-PE	BD Biosciences
CD62P antibody-FITC	BD Biosciences
IgG Isotype control antibody-FITC	Sigma
IgG Isotype control antibody-PE	BD Biosciences
IgG Isotype control IgG_{2B}-FITC	R&D Systems
PAC1 antibody-FITC	BD Biosciences

2.1.3 Buffers and Solutions

Fix solution
30 ml FACS flow
10 ml distilled water
1 ml CellFix

Wash buffer for preparation of washed platelets (platelet aggregometry)
140 mM NaCl
10 mM $NaHCO_3$
2.5 mM KCl
0.9 mM Na_2HPO_4 * 2 H_20
2.1 mM $MgCl_2$
22 mM $C_6H_5Na_3O_7$
0.055 mM D(+)-Glucose monohydrate
0.35% BSA
pH 6.5

Tyrode Buffer
10 mM HEPES
134 mM NaCl
1 mM $CaCl_2$
12 mM $NaHCO_3$
2.9 mM KCl
0.34 mM Na_2HPO_4 * 2 H_2O
1mM $MgCl_2$
0.055 mM D(+)-Glucose monohydrate
pH 7.4

Isolation buffer
50 ml Dulbecco´s PBS with $CaCl_2$(0.9 mM) and $MgCl_2$(0.5 mM)
0.1% BSA
10 mM HEPES
10 mM Glucose
pH 7.4

Kimura`s stain
11 ml 0.05% Toluidine blue
5 ml phosphate buffer (pH 6.4)
0.8 ml 0.03% light green
0.5 ml saturated saponin solution

2.1.4 Equipment and Software

Vena8fluoro+ biochips	THP Medical Products (Vienna, Austria)
ABX Micros 60	Horiba Medical (Tulln, Austria)
Apact 4004	LABiTec (Ahrensburg, Germany)
CellixVenaFlux software	Cellix Ltd (Dublin, Ireland)
DucoCell software	Cellix Ltd
FACS Calibur flow cytometer	Becton-Dickinson (Mountain View, USA)
Hamamatsu ORCA-03G digital camera	Hamamatsu (Herrsching, Germany)
Mirus nanopump	Cellix Ltd
Olympus IX70 fluorescence microscope	Olympus (Vienna, Austria)
Zeiss Axiovert 40 CFL microscope	Zeiss (Vienna, Austria)

2.2 Methods

The following study was approved by the Ethics Committee of the Medical University of Graz. Whole blood taken from healthy individuals was collected in the presence of the anticoagulant sodium citrate (3.8 %). Immediately after blood withdrawal aliquots were used to perform whole blood experiments. To obtain platelet rich plasma (PRP) fresh whole blood was centrifuged at 400 x g for 20 min without break. The supernatant was collected and either used as PRP or to produce washed platelets. Therefore, washing steps, as described below, had to be performed in order to isolate platelets from non cellular plasma compounds.

2.2.3 Platelet aggregation

Platelet aggregation was performed using different stimuli to induce aggregation in PRP or washed platelets. Therefore, the 4-channel platelet aggregometer APACT4004 (LABiTec,Ahrensburg, Germany) was used for aggregation measurements using the principle of light transmission aggregometry (LTA) first developed by Born (**Figure 2**).

Prior aggregation was induced in PRP, a small aliquot was taken and centrifugated (700 x g for 15 min without break) to obtain platelet poor plasma (PPP). The aggregometer was calibrated by arbitrarily setting PRP to 0 % aggregation and PPP to 100% aggregation. If not mentioned elsewhere, PRP was incubated with the antagonist for 10 min followed by agonist treatment for further 5 min and subsequently addition of $CaCl_2$ (1 mM). Incubation occurred at 37° C and aggregation was started 2 min after $CaCl_2$ addition with ADP (1.25-10 µM) or collagen (1.25-10 µg/ml) as proaggregatory stimuli. To record a potential change on aggregate formation, platelets were activated with ADP or collagen concentrations that induced submaximal aggregation. Aggregation was recorded for at least 4 minutes with constant stirring (1000 rpm) at 37° C. The recorded changes in light transmission resulted from platelet response to the stimuli, where a direct correlation of increased light transmission and platelet aggregation existed. PRP experiments were performed not longer than 3.5 hours after blood withdrawal.

Light transmission aggregometry experiments were partially performed with washed platelets acquired in further centrifugation steps. For washed platelet preparation fresh PRP was mixed with EDTA 2% in a 1:20 ratio to the plasma volume. This was followed by centrifugation at 1000 x g for 15 min and resuspension of the pellet with wash buffer (140 mM NaCl,10 mM $NaHCO_3$, 2.5 mM KCl, 0.9 mM Na_2HPO_4 * 2 H_20, 2.1 mM $MgCl_2$, 22 mM $C_6H_5Na_3O_7$, 0.055 mM D(+)-Glucose monohydrate and 0.35% bovine serum albumin (BSA); pH 6.5). The resuspended pellet was washed twice with 10 ml wash buffer by centrifugation at 1000 x g for 15 min. This time, the obtained pellet was resuspended in Tyrode buffer (10 mM HEPES, 134 mM NaCl, 1

mM $CaCl_2$, 12 mM $NaHCO_3$, 2.9 mM KCl, 0.34 mM Na_2HPO_4 * 2 H_2O, 1mM $MgCl_2$, 0.055 mM D(+)-Glucose monohydrate; pH 7.4) and the suspension was subsequently filled up to the initially used PRP volume. Light transmission measurement for washed platelets was performed as mentioned above for PRP, with the difference that Tyrode buffer was considered to be 100% of light transmission/platelet aggregation.

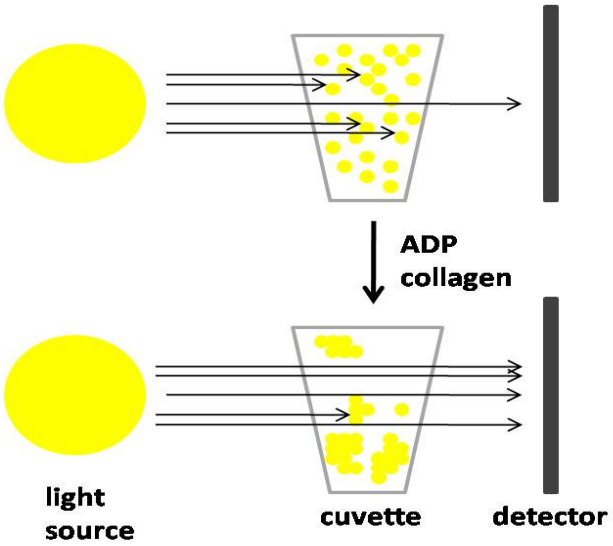

Figure 2: Principle of light transmission aggregometry
After addition of certain stimuli the prior unactivated and therefore constant distributed platelets gain the ability to form aggregates. The more aggregate formation occurs the more light can pass, so the increase in light detection directly correlates with the increase in platelet aggregation.

2.2.2 Flow cytometric detection of GPR109A

GPR109A receptor staining experiments were performed with washed platelets and polymorph nuclear leukocytes (PMNLs), since evidence for niacin receptor expression was previously shown for neutrophils (Kostylina et al., 2007)

2.2.2.1 Preparation of washed platelets for GPR109A receptor staining

Washed platelets were prepared as described above, despite that after the resuspension an additional centrifugation step at lower force (400 x g) for 7 min was performed. Thereafter the supernatant was carefully removed and platelets resuspended in 2 ml of wash buffer. To obtain a certain platelet concentration (1000/ µl) the ABX Micros 60 (Horiba Medical, Austria, Tulln) was used for cell number determination.

2.2.2.2 GPR109A staining of washed platelets

Once washed platelets were diluted to the required concentration, 100 µl of the suspension was transferred to FACS tubes and fixed for 15 min at room temperature using 50 µl of reagent A from the Fix&Perm Kit (ADG, Kaumberg, Austria). After incubation, samples were washed with 250 µl of wash buffer and centrifuged at 400 x g for 7 min. This washing step was repeated after blocking the cells with the Ultra-V blocking solution (Thermo Scientific,Waltham, MA, USA) for 30 min at 4°C. To remove the blocking solution the same washing step was repeated once more, and samples were then incubated with the anti-hHM74A/GPR109A FITC conjugated antibody (2.5 µg/ ml) and the control antibody IgG_{2B}-FITC. The antibodies were prepared in antibody diluent, (Dako, Vancouver, Canada) and 50 µl of the diluted antibody were added to one sample. Experiments were made in triplicates, whereas 3 untreated samples served as an additional control. After incubation with the antibody mixture for 30 min at 4 °C and a subsequent washing step, samples were resuspendend in fix solution (2% BD Cellfix, 20% distilled water and 60% cold FACS flow) and either directly measured with a FACS Calibur flow cytometer (Becton-Dickinson) or stored at 4° C until measurement.

2.2.2.3 Neutrophil isolation and counterstaining

Neutrophils were isolated from the daily taken fresh whole blood. After centrifugation of the blood like described above, the supernatant was removed to prepare washed platelets while all other blood cells remained in the 50 ml falcon tube. Then leukocytes and erythrocytes were separated using 6 ml of 6 % dextran. The falcon was filled up with 0.9 % saline and the mixture was incubated at room temperature for 30 min.

For density centrifugation the upper phase of the dextran sedimentation including leukocytes was carefully placed onto 15 ml of Histopaque solution. After centrifugation (400 x g for 20 min without break) the upper phase, the peripheral blood mononuclear cells (PBMCs) and the Histopaque were removed. The remaining pellet was resuspended in 10 ml isolation buffer (50 ml Dulbecco´s PBS containing $CaCl_2$ (0.9 mM) and $MgCl_2$ (0.5 mM), 0.1% BSA, 10 mM HEPES, 10 mM Glucose, pH 7.4) and centrifuged at 400 x g for 7 min. The supernatant was discarded and the pellet again resuspended, this time in 10 ml 0.2% saline. This step needed for erythrocyte lysis and was followed immediately by addition of 10ml 1.6% saline to equilibrate to the physiological conditions. After addition of 10 ml isolation buffer and a centrifugation step (400 x g for 7 min) the pellet was resuspeded in 10 ml isolation buffer. Cell count was determined using a Neubauer chamber. For that purpose, the cell suspension was diluted 1:10 with Kimura stain.

5 million isolated PMNLs were then incubated with an anti-$CD16^+$-PE antibody in a 1:50 dilution with PBS without $CaCl_2$ and $MgCl_2$ in a total volume of 500 µl. After incubation with the antibody for 5 min, the suspension was filled up to 5 ml with PBS containing $CaCl_2$ (0.9 mM) and $MgCl_2$ (0.5 mM) and centrifuged at 400 x g for 7 min. Cells were then resuspended in 5 ml PBS to obtain a concentration of 1000 cells /ml. 100 µl of this suspension was transferred to FACS tubes. The following sample treatment including fixation and antibody staining was performed as mentioned above for washed platelets.

2.2.2.4 Flow cytometric analysis of GPR109A receptor staining

Platelet staining was shown in FL-1 channel (used for measuring of FITC conjugated antibodies) against side scatter (SSC). For PMNLs a different setting was used: PMNLs were first selected in a forward scatter (FSC) against side scatter (SSC) dotplot and then the ability of neutrophils to bind to the CD16+-PE antibody was further utilized to distinguish neutrophils from eosinophils. Therefore, FL-1/ FL-2 channel was used (**Figure 3**).

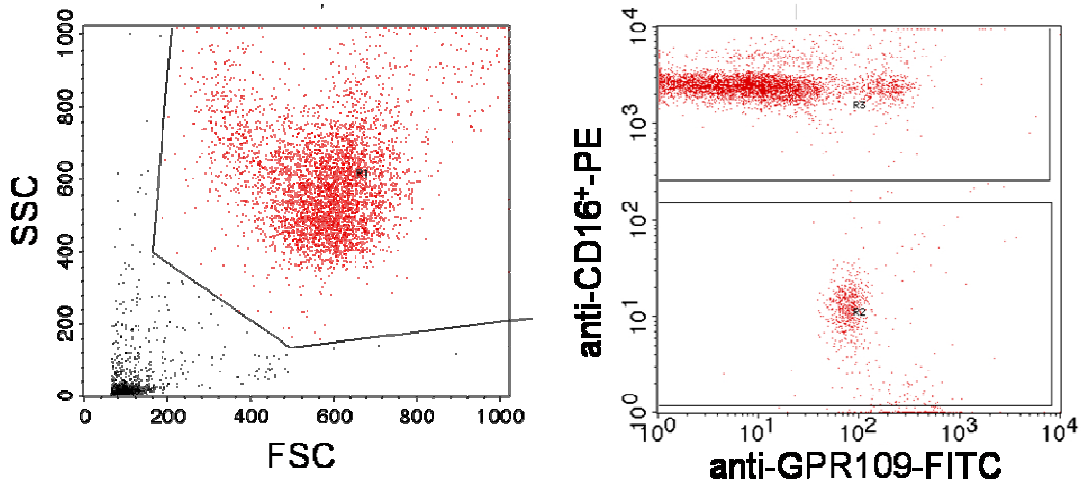

Figure 3: Flow cytometric staining of neutrophils and eosinophils

FSC against SSC dotplot (left) allowed selection of PMNLs which were further distinguished in a second dotplot (right) based on $CD16^+$-PE positive neutrophils seen in FL-2.

2.2.4 In vitro thrombogenisis

Thrombus formation *in vivo* occurs when platelets get activated after a vessel wall injury. Platelets adhere to collagen, a component of the ECM that is exposed to the blood stream. This results in formation of firm thrombi.

With the Cellix system *in vitro* assays can be performed mimicking these *in vivo* conditions. The Vena8Flouro+ Biochip (Cellix Ltd., Dublin, Ireland) used here is a chip with 8 microcappillaries which can be coated with different adhesion molecules.

This allows investigation of specific interactions between adhesion molecules and certain cells that can be perfused over the channels. Therefore a part of the VenaFlux™ Platform (**Figure 4**), the Mirus™ 2.0 Nanopump produces shear stress levels equivalent to physiological conditions in blood vessels.

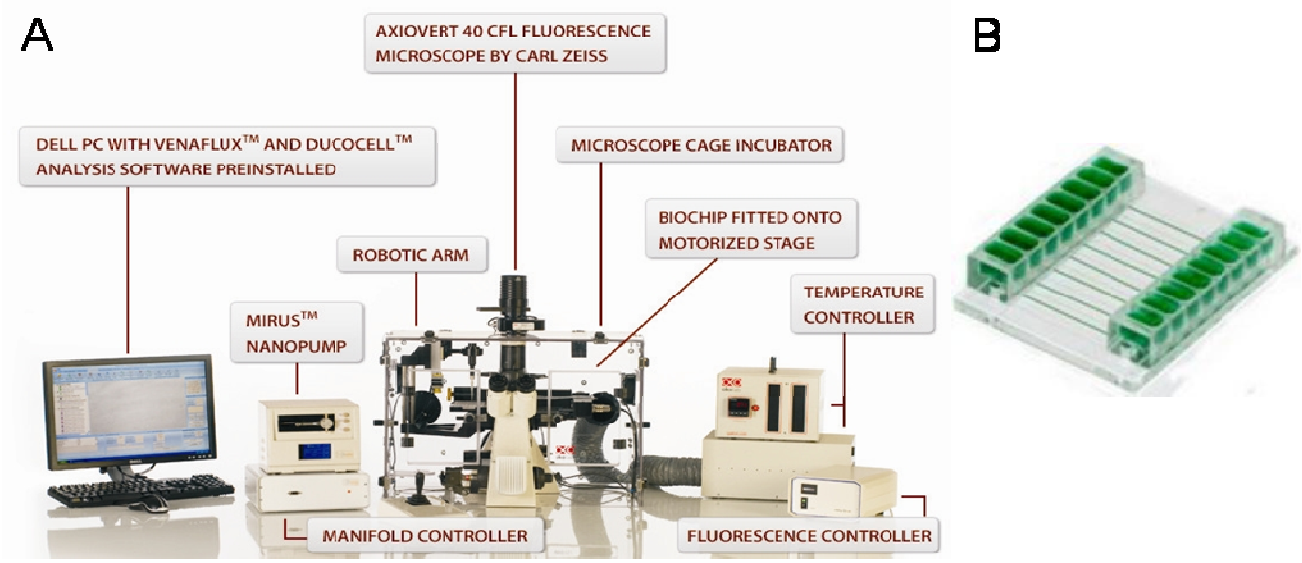

Figure 4: VenaFluxTM Platform and Vena8Flouro+ Biochip
(A) Overview of the semi-automated VenaFluxTM Platform. The MirusTM 2.0 Nanopump produces the required shear stress to perfuse cells over the channels allowing live imaging using the Zeiss Axiovert 40 CFL microscope connected with the VenaFlux software. (B) The eight microcapillaries of the Vena8Flouro+ Biochip are used for studying interactions between adhesion molecules and certain cells mimicking in vivo conditions

For *in vitro* thrombogenisis experiments, the 8 channels of the Vena8Flouro+ Biochip were coated with 14 µl of collagen (125 µg/ ml) respectively, at 4℃ overnight. The next day, the channels were blocked with BSA (10 µg/ ml) for 20 min to avoid unspecific platelet binding. Washing steps with distilled water were performed prior and after the blocking with BSA to remove the unbound collagen/BSA. In both washing steps every channel was washed twice. After the blood was withdrawn, 5 ml of fresh whole blood was incubated with 3, 3′-dihexyloxacarbocyanine iodide (1 µM) in the dark for at least 10 min. The used green-fluorescent dye is selective for the mitochondria of viable cells. If not mentioned elsewhere, whole blood was incubated with the antagonist 10 min prior addition of the agonist which was incubated for another 5 min. After agonist/antagonist incubation blood was mixed with $CaCl_2$ at a final concentration of 1 mM. After 2 min blood was perfused over the collagen coated

channel at a physiological shear rate of 30 dynes cm^{-2}. The formation of thrombi occurred in a time depended manner and was recorded by a Zeiss Axiovert 40 CFL microscope, using Hamamatsu ORCA-03G digital camera and Cellix VenaFlux software. Images were taken 3 min after the perfusion start and later analysed with the DucoCell analysis software (Cellix Ldt). This program allows the analysis of thrombi covered areas.

2.2.5 Platelet adhesion

Platelet adhesion experiments were performed with the adhesion molecules collagen and fibrinogen. Platelets were prepared as washed platelets, like mentioned above for platelet aggregometry.

2.2.5.1 Platelet adhesion to collagen

The coating and washing steps of the applied Vena8Flouro+ Biochips were performed like described above. After the fresh prepared washed platelets (as described for platelet aggregation experiments) were incubated with antagonists or vehicle for 10 min, agonists were added and incubated for further 5 min. After agonist/antagonist incubation washed platelets were mixed with $CaCl_2$ at a final concentration of 1 mM. 2 min after $CaCl_2$ addition washed platelets were perfused over the collagen coated channel using the Mirus^{TM} 2.0 Nanopump at a constant low shear stress of 2.5 dynes cm^{-2}. Platelet adhesion to collagen occurred in a time depended manner and was recorded by light microscopy (Zeiss Axiovert 40 CFL microscope), using Hamamatsu ORCA-03G digital camera and Cellix VenaFlux software. Images were taken between 3-9 min after the perfusion start and later analysed with the DucoCell analysis software (Cellix). The area covered by aggregates was analysed. All data are represented as mean+SEM of different experiments and are normalized to the respective vehicle.

2.2.5.1 Platelet adhesion to fibrinogen

Vena8Flouro+ Biochips were coated with fibrinogen (100µg/ ml) at 4° C overnight. The next day, the channels were blocked with BSA (10 µg/ ml) for 20 min to avoid unspecific platelet binding. Washing steps with PBS containing $CaCl_2$ (0.9 mM) and $MgCl_2$ (0.5 mM) were performed prior and after the blocking with BSA to remove the unbound fibringen/BSA. In both washing steps every channel was washed twice. After the fresh prepared washed platelets were incubated with antagonists for 10 min, agonists were added and incubated for further 5 min. After agonist/antagonist incubation washed platelets were mixed with $CaCl_2$ at a final concentration of 1 mM. 2 min after $CaCl_2$ addition samples were mixed with ADP (10 µM) and then directly perfused over the fibrinogen coated channels. Pre-activation of platelets with ADP is crucial since heterodimerization of GPIIb/IIIa, which is the receptor for fibrinogen, is only present on activated platelets. Further steps were performed like described for platelet adhesion to collagen.

2.2.6 Flow Cytometric Immunofluorescence Staining of P-selectin and GPIIb/IIIa

When platelets become activated, they express certain receptors specific for the activated platelet state. This method is based on the staining of these receptors with fluorescence conjungated antibodies. Prior the staining platelets were activated by addition of certain stimuli after samples were incubated with agonists.The effect of agonists on platelet receptor activation/heterodimerization which results in the end in changes in platelet aggregation and thrombus formation was revealed using different staining protocols.

2.2.6.1 Flow Cytometric Immunofluorescence Staining of P-selectin in platelet rich plasma

Fresh PRP was incubated with niacin (3 mM) and vehicle for 30 min. Then plasma was mixed with PBS containing $CaCl_2$ (0.9 mM) and $MgCl_2$ (0.5 mM), thereafter 10 µl of plasma were added in a FACS tube containing 190 µl PBS. This was followed by the addition of ADP (3 µM) after cytochalasin B at a final concentration of (5 µg/ ml) was added to the samples. The latter substance causes degranulation of platelet α-granulas needed for the exposure of P-selectin to the surface. When collagen (4 µg) was used as a stimulus, addition of cytochalasin B is unnecessary, since collagen activates platelets via a different mechanism which includes platelet granula release. Directly after stimulus addition, platelet receptors were stained with anti-CD62P-FITC conjugated antibody, which recognizes P-selectin (BD, Vienna, Austria) and anti-IgG-FITC conjugated antibody (Sigma) served as isotype control. After incubation with the antibodies for 15 min at 37°C the reaction was stopped using ice cold PBS without $CaCl_2$ and $MgCl_2$. This was followed by a centrifugation step at 400 x g for 7 min. After this washing step the supernatant was removed and cells were fixed with fix solution (1 ml Cellfix, 10 ml distilled water and 30 ml cold FACS flow). Sample measurement was performed with the FACS Calibur flow cytometer (Becton-Dickinson). Platelets were selected first in a forward scatter against side scatter dotplot and P-selectin upregulation was then shown in a further dotplot which distinguished fluorescence positive from fluorescence negative cells by using the FL-1 channel.

2.2.6.2 Flow Cytometric Immunofluorescence Staining of P-selectin and GPIIb/IIIa in platelet rich plasma II

The difference between this staining procedure and the one already described above is mainly due to the agonist incubation performed here in fresh whole blood. In addition to niacin the IP agonist iloprost, known for its downregulating effect on P-selectin expression and inhibiting effect on GPIIb/IIIa heterodimerization was used as control. Consequently, whole blood was incubated with niacin (3 mM) and iloprost at 30 nM, a concentration which showed a high reduction in P-selectin expression and

GPIIb/IIIa heterodimerization in previous experimens performed by our group (Philipose et al., 2010). Then PRP was prepared by centrifugation of pretreated whole blood at 400 x g for 20 min with low break. PRP was carefully taken off and slightly mixed prior 10 µl of PRP were added to FACS tubes containing 190 µl of PBS containing $CaCl_2$ (0.9 mM) and $MgCl_2$ (0.5 mM). The samples were activated using ADP (3 µM) or collagen (4 µg/ ml) and stained using anti-CD62P-FITC conjugated antibody, PAC-1-FITC (BD, Vienna, Austria) antibody and anti-IgG-FITC conjugated antibody as control antibody. P-selectin staining was exactly performed as mentioned above.

GPIIb/IIIa heterodimerization was detected using PAC-1-FITC antibody which reconformation dependent determinant of the GPIIb/IIIa complex recognizes a 50 µl of the antibody were usedin a 1:10 dilution with PBS with $CaCl_2$ (0.9 mM) and $MgCl_2$ (0.5 mM) after addition of the activation stimulus. After incubation for 30 min at room temperature the reaction was stopped using ice cold PBS without $CaCl_2$ and $MgCl_2$. Fixation and sample measurement for GPIIb/IIIa and P-selectin was performed as described above.

2.2.6.3 Flow Cytometric Immunofluorescence Staining of P-selectin and GPIIb/IIIa in whole blood

Fresh whole blood was incubated with niacin (3 mM), iloprost (30 nM) and respective vehicles for 30 min. In certain experiments whole blood was incubated with iloprostor vehicle for only 7 min and then directly used for further staining procedure. After incubation, 10 µl of blood was added to FACS tubes containing 190 µl of PBS containing $CaCl_2$ (0.9 mM) and $MgCl_2$ (0.5 mM).

Platelet activation and staining was performed like described above, with the major difference that additionally 50 µl of anti-CD61-PE antibody (BD, Vienna, Austria) in a 1:10 dilution was added to each sample prior to PAC1 and anti-CD62P antibody. CD61 is known as glycoprotein IIIa (GPIIIa) and is present on all platelets irrespective of their activation state. The staining of all present platelets was necessary to distinguish platelets from other blood cells. Sample incubation and fixation was performed as mentioned above. Samples were measured with the FACS Calibur flow

cytometer using a protocol for sample measurement that first allows selection of platelets from other blood cells with a forward scatter (FSC) against side scatter (SSC) dotplot and a further dotplot that allows the selection of PE-positive platelets (Figure 5). These positive cells are further verified with respect to binding of FITC conjugated antibodies demonstrating P-selectin expression/GPIIb/IIIa heterodimerization.

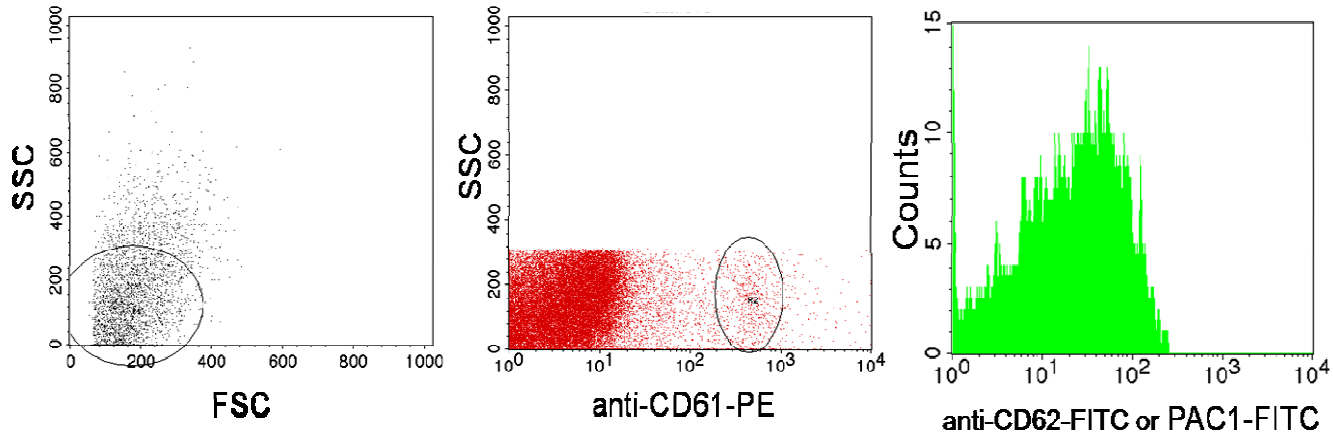

Figure 5: Flow cytometric staining of activated platelets in whole blood
Cells selected in a forward against side scatter dotplot were screened for PE-positive platelets. To further distinguish between activated and unactivated platelets only platelets bound to both fluorescence conjugated antibodies are shown in the FL-1 histogram.

2.2.7 Data presentation and Statistical analysis

All data are represented as mean+SEM of different experiments and are normalized to the respective vehicle. Numbers (n) of experiments performed are shown in the respective Figure legends. Statistical data analysis was performed using Sigma Plot (Systat Software Inc; San Jose, CA, USA). For comparisons one-way ANOVA for repeated measurements or one-way Anova for repeated measurements on ranks were calculated and statistical differences evaluated with Dennett's or Bonferroni's post test based on ranks. Values $P<0.05$ were considered statistically significant.

3. Results

3.1 Part I: Tredaptive® studies

Background

Recently, our group was interested in revealing the effect of niacin and laropiprant with regard to platelet function. Both substances are active compounds of a new developed drug called Tredaptive®, which is considered as well tolerated and effective in the treatment of lipid disorders (McKenney et al., 2010). Niacin acts as lipid lowering agent but the most common side effect is flushing, which is mediated by the relase of PGD_2 (Papaliodis et al., 2008). Laropiprant is an antagonist of the PGD_2 receptor DP and prevents this side effect (Bodor and Offermanns, 2008b). Since 2008, Tredaptive® is licensed by the the European Medicines Agency for treatment of dislipidemias, while in the US this drug was rejected by the Food and Drug Administration the same year. Although this was not commented, safety concerns may have played a role (Sanyal et al., 2010).

Since the function of Tredaptive® was not examined on thrombotic cardivascular events, our group wanted to elucidate how platelet function is affected under niacin and laropiprant exposure. Prostaglandins play a key role in haemostasis by activation of inhibition of platelets (see introduction). The PGD_2 receptor DP is expressed on platelets and evidence for its antiaggregatory function has already been shown (Feinstein et al., 1983b); (Giles et al., 1989b); Keery and Lumley, 1988). Therefore, our group was interested to investigate the pharmacological properties of laropiprant in detail, with respect to possible thrombotic risks.

Recently, we could show that laropiprant counteracted the DP receptor mediated effects in platelet aggregation, VASP phosporylation, P-selectin expression and GPIIb/IIIa heterodimerization. In addition to the results obtained in human plasma, the inhibitory effect of PGD_2 on *in vitro* thrombogenesis was reversed when whole blood was pretreated with the DP antagonist laropiprant at a concentration of 1 µM.

To summerize these results, the role of laropiprant as a DP antagonist on platelets has been clearly demonstrated.

Additionally, we found out in a further set of experiments that niacin and a higher concentration of laropiprant (10 µM) on its own inhibited *in vitro* thrombogenesis, outweighing a potential thrombotic risk via blocking of the DP receptor. This inhibitory effect of laropiprant on thrombus formation might be due to its antagonistic effect on the thromboxane receptor TP and as we could show, on its antagonistic effect on the PGE_2 receptor EP3. Thus, we reasoned that Tredaptive®, beside its lipid lowering potential, could have an additional beneficial effect on thrombotic events.

Aims of the project

Since it was shown that niacin and laropiprant can reduce thrombus formation *in vitro* I wanted to investigate the effect of of niacin and laropiprant on platelet activation by using a number of different approaches.

While it was recently shown that laropiprant acts via the blocking of DP, TP and EP3 receptors (Philipose et. al, 2012), the underlying mechanism behind the niacin effect was still unclear. Therefore, my aims were

a) To investigate whether niacin directly interacts with prostaglandin receptors expressed on human platelets

b) to show whether the niacin receptor GPR109A is involved in the inhibitory effect of niacin on *in vitro* thrombogenesis

With regard to laropiprant I wanted to investigate its effect on collagen-induced platelet aggregation on its own. Therefore, my aim was

c) To examine whether laropiprant inhibits collagen- induced platelet aggregation on its own

3.1.1 Niacin and laropiprant inhibit in vitro thrombogenisis*

Since previously obtained data served as the starting point for this research, I decided to show these results first to explain the following experimental approach.

Based on its role as a DP, EP3 and TP receptor antagonist, we were interested how laropiprant would affect *in vitro* thrombogenesis. Therefore, whole blood was incubated with different concentrations of the antagonist for 10 min and then perfused over a collagen coated channel.

Since niacin was recently reported to inhibit platelet aggregation *in vitro* (Serebruany et al., 2010), we were further interested if niacin had the ability to affect thrombus formation *in vitro*. First I performed time course experiments. Whole blood was incubated with niacin (3 mM) for 10, 15 and 30 min. We found that treatment of blood with niacin for 30 min gave the maximal effect (**Figure 6A**). Next we did a concentration response curve by incubating whole blood for 30 min with different concentration of niacain and found that 3 mM was the most effective concentration (**Figure 6B**). Niacin, as well as a high concentration of laropiprant (10 µM) caused pronounced inhibition of thrombus formation *in vitro* (**Figure 6C and D**). The inhibitory effect observed after niacin treatment was not influenced when whole blood was preincubated with acetylsalicylic acid (1 mM) or laropiprant (1 µM and 10 µM).

This suggests that, in contrast to the niacin caused flush response, the involvement of PGD_2 on this effect can be excluded. Furthermore, this result lets us assume that niacin mediates its inhibitory effect on platelets independently from prostaglandin formation.

* Data was obtained in the Project laboratory 16.08.2012-1.10.2012 together with Sonia Philipose

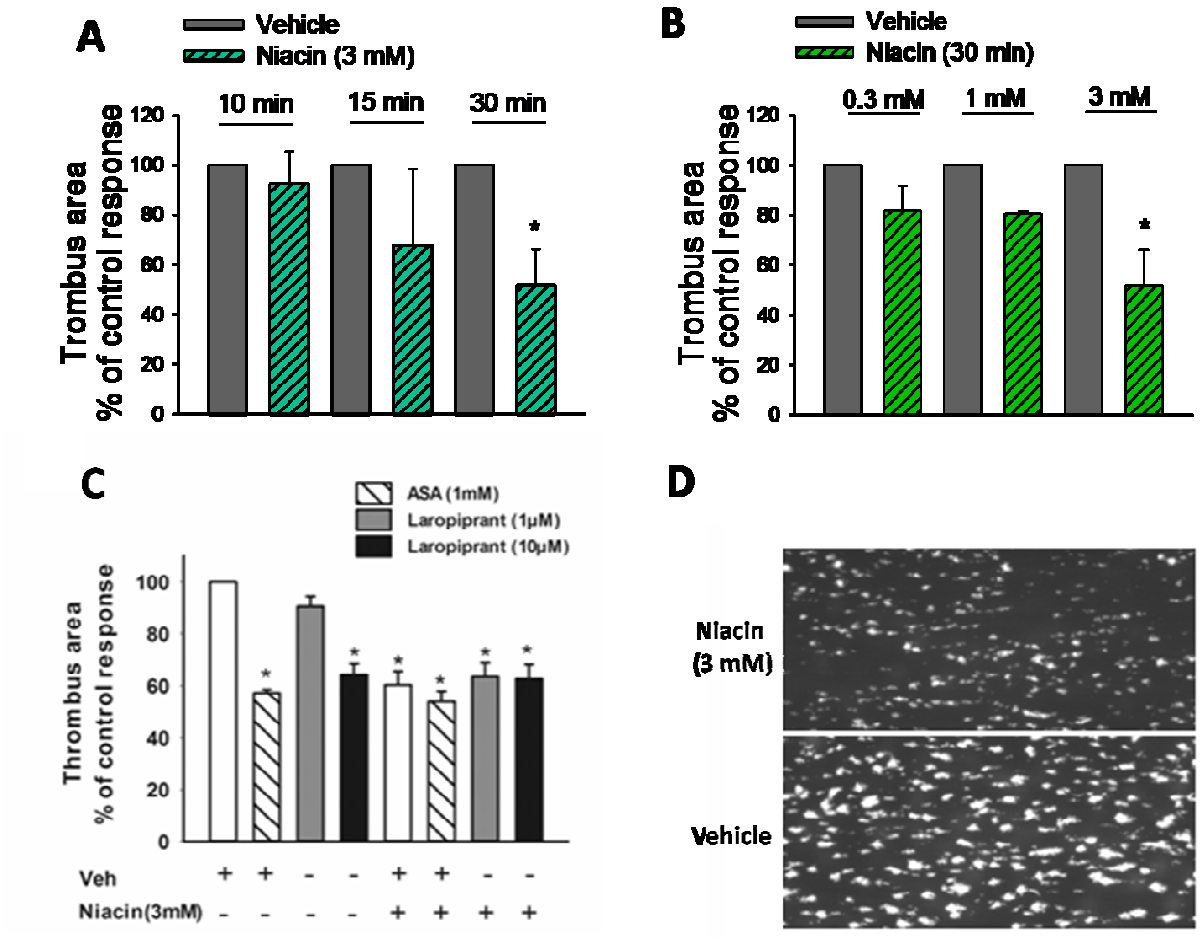

Figure 6 : Niacin and laropiprant (10 µM) inhibit thrombus formation *in vitro*

Fresh whole blood was perfused with constant shear stress (30 dyne cm^{-2}) over collagen coated channels of Vena8+ biochips. Whole blood was prior incubated with a fluorescence dye (3, 3'-dihexyloxacarbocyanine iodide*)* that allows recording of thrombus formation by fluorescence microscopy. Images were taken 3 min after the perfusion start and 4 representative images were analyzed. (A, B) Niacin incubation time (A) and niacin concentration (B) was optimized. Therefore, whole blood was incubated with a constant concentration of niacin (3 mM) for 10, 15 and 30 min. In a second setting niacin at different concentrations (0.3, 1 and 3 mM) was exposed to whole blood for 30 min. Niacin at the highest revealed dose had the ability to inhibit thrombus formation in vitro when blood was pretreated for 30 min. (C) Both, laropiprant and niacin affected thrombus formation. The effect of laropiprant was dose dependent: whereas 1 µM of the substance had no effect on thrombus formation, a significant reduction could be seen when the concentration was increased to 10 µM. Niacin (3 mM) showed significant reduction which was not influenced by pretreatment with acetylsalicylic acid (1 mM) or laropiprant. (D) Typical images obtained 3 min after perfusion start. Blood pretreated with niacin (3 mM) showed inhibition of thrombus covered area compared to vehicle treated whole blood. Images were analyzed with the DucoCell analysis program. Results are shown as mean+SEM of n= 3-6 different experiments and are normalized to the respective vehicle. Statistical significance is determined by *P<0.05.

3.1. 2 Laropiprant (1 µM), in contrast to niacin (3 mM), inhibits platelet aggregation

Since laropiprant was known to interact with platelet prostaglandin receptor EP3, DP and TP and is capable to reduce thrombus formation *in vitro*, we wanted to elucidate the effect of laropiprant on collagen-induced platelet aggregation. Therefore, PRP was used for incubation of laropiprant. Platelet aggregation was induced with a collagen concentration (1.5 – 10 µM) that caused 60-80 % aggregation. Laropiprant significantly reduced platelet aggregation already at a concentration of 1 µM, and this effect was increased dose-dependent (**Figure 7**). Further, we wanted to test the impact of niacin on platelet aggregation. In contrast to laropiprant, niacin (3mM) incubated in PRP for 30 min was not able to affect platelet aggregation stimulated with collagen (data not shown).

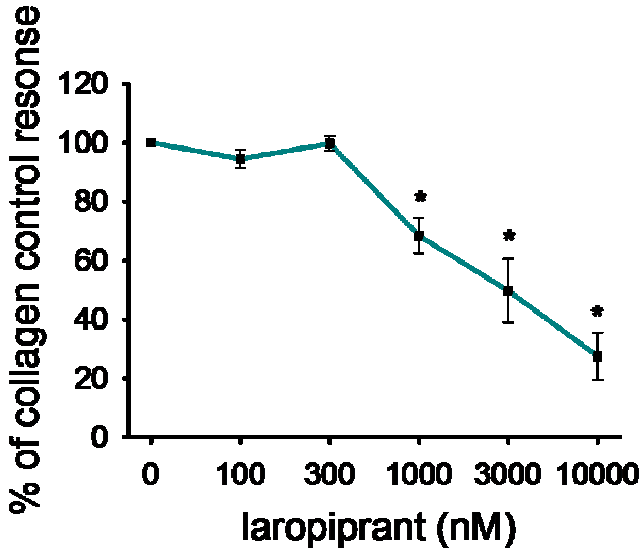

Figure 7: Laropiprant inhibits platelet aggregation in a dose dependent manner
Pretreatment of PRP samples with laropiprant affected collagen stimulated platelet aggregation as recorded with light transmission aggregometry. Aggregation was unaffected with concentrations below 1 µM but then significantly decreased. Results are shown as mean±SEM of n= 4 different experiments and are normalized to the respective vehicle. Statistical significance is determined by *P<0.05.

3.1.3 Niacin (3 mM) has no effect on P-selectin upregulation in PRP

The fact that niacin had no effect on platelet aggregation, using PRP, led us to the assumption that there is no direct interaction between niacin and platelet surface receptors. To determine whether this is the case, the impact of niacin was further tested on P-selectin expression. The adhesion molecule P-selectin is released from α-granulas during platelet activation and functions as a key player on platelet aggregation. PRP was incubated with niacin (3 mM) for 30 min and stimulated with ADP (3 µM) and collagen (6 µg). Prior activation with ADP, samples were treated with cytochalasin B (5 µg/ml) to permit translocation of P-selectin to the surface of platelets. Receptor staining was performed subsequently by addition of a CD62P fluorescence conjungated antibody which was incubated for 15 min at 37 ℃. Niacin preincubation did not influence P-selectin expression neither when ADP (**Figure 8A**) nor when collagen (**Figure 8B**) was used as stimulus. This is consistent with the previous observation that niacin showed no effect on platelet aggregation in PRP, supporting the hypothesis that niacin mediates its effect through an indirect mechanism.

3.1. 4 Neutrophils, but not platelets and eosinophils express the niacin receptor GPR109A

Results obtained during aggregation and P-selectin upregulation experiments, supported the hypothesis, that the niacin caused inhibition on *in vitro* thrombus formation is not directly mediated. It has been established in different studies that niacin mediate its actions via the G-protein coupled receptor GPR109A (Kamanna and Kashyap, 2007) and it has been shown that this receptor is expressed on human neutrophils (Kostylina et al., 2007). To definitely exclude that niacin acts on platelets through a direct mechanism I wanted to investigate if the niacin receptor GPR109A is present on platelet surface. Therefore, I incubated washed platelets with an FITC conjugated anti-GPR109A antibody. Neutrophils served as positive control, therefore PMNLs were isolated and subsequently distinguished by counterstaining of neutrophils with an anti- CD16+ antibody. The GPR109A receptor was not

detectable on platelets as determined by flow cytometry, since the fluorescence values of GPR109A antibody and the respective isotype control were not different (**Figure 9A and C**). Receptor staining of neutrophils and eosinophils confirmed previous observations (Kostylina et al., 2007): while receptor expression was found on neutrophils (**Figure 9B and C**), no evidence for the presence of this receptor could be detected on eosinophils (**Figure 9C**).

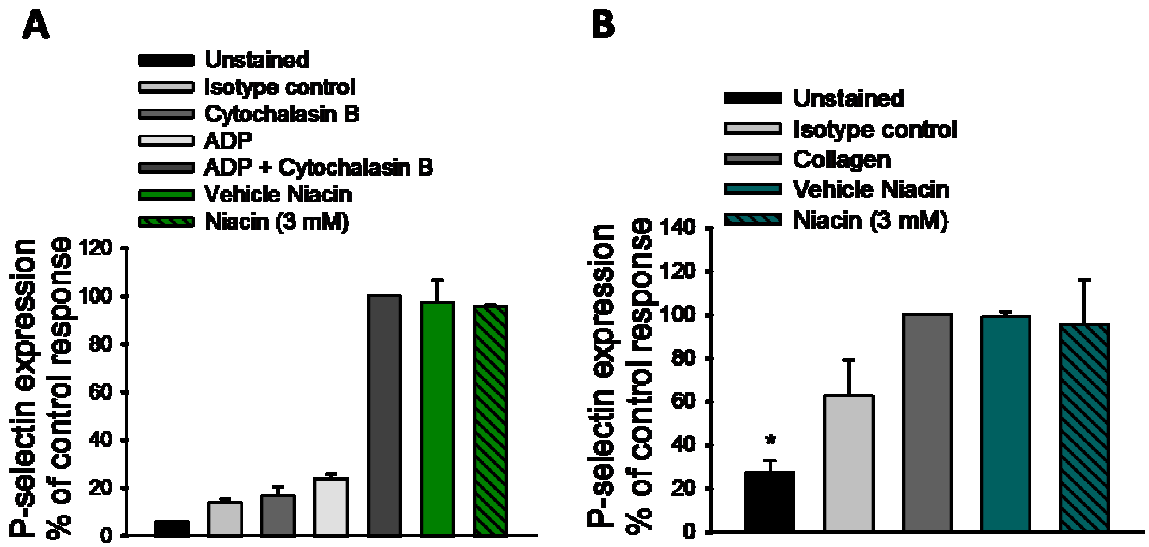

Figure 8: P-selectin upregulation in platelet rich plasma is not affected by niacin (3mM)
ADP and collagen induced surface expression of P-selectin was detected by flow cytometry via the fluorescence conjugated antibody CD62P. ADP stimulation was performed in the presence of Cytochalasin B (5 µg/ml) (A) ADP stimulated P-selectin upregulation was not affected when niacin was exposed to PRP, this was conformable with (B) collagen dependent activation of platelets, that neither led to inhibition of P-selectin upregulation. Results are shown as mean+SEM of n= 4 different experiments and are normalized to the respective vehicle. Statistical significance is determined by *P<0.05.

3.1. 5 Niacin (3 mM) incubated in whole blood, has no effect on P-selectin expression in PRP

Since the presence of the niacin receptor (GPR109A) on platelets could be excluded and neither platelet aggregation nor P-selectin expression was affected in PRP in the presence of niacin (3 mM) I wanted to know if niacin is able to inhibit P-selectin upregulation via an indirect mechanism. Therefore, whole blood was mixed with niacin (3 mM) for 30 min, thereafter blood was centrifuged to obtain PRP which was activated with ADP in the presence of cytochalasin B (5 µg/ ml) or collagen and

stained with CD62P-FITC as mentioned above. The IP receptor agonist iloprost (30 nM) served as a methodical control, since it is well known that IP receptor activation prevents the upregulation of P-selectin in PRP (Philipose et al., 2010).

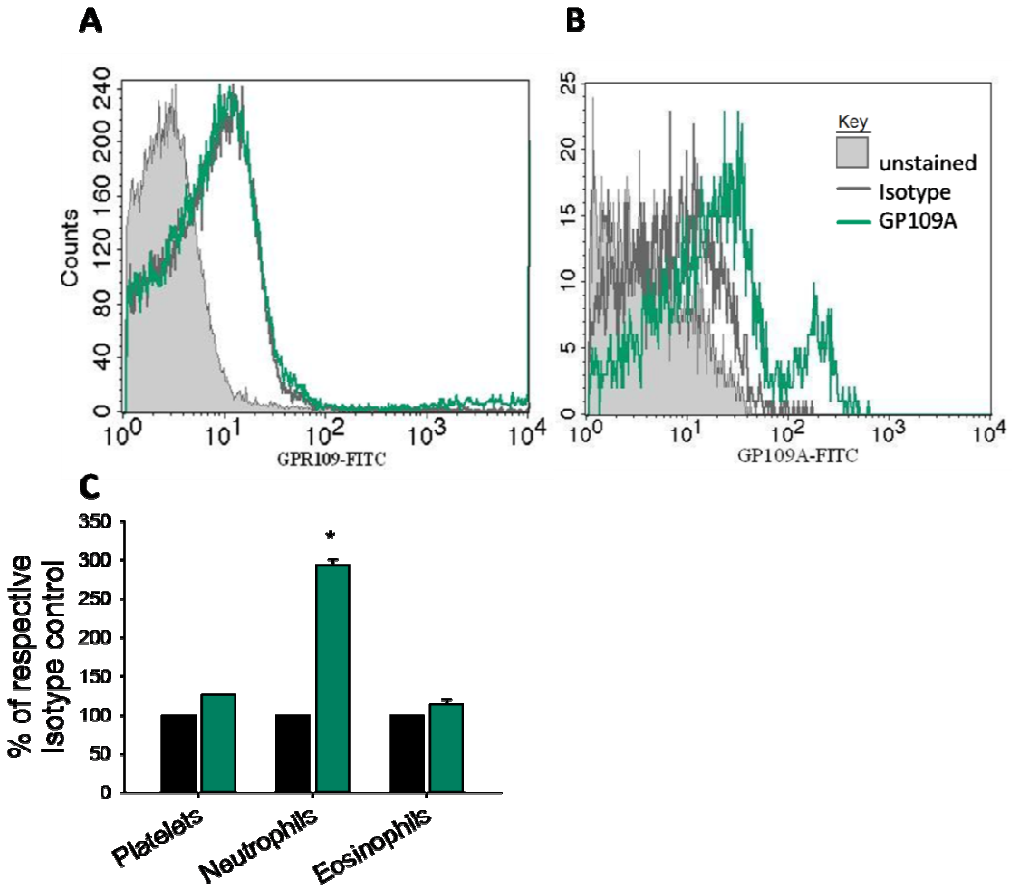

Figure 9: Neutrophils express GPR109A whereas the niacin receptor is not present on platelets or eosinophils

Niacin receptor was detected with a fluorescence conjugated antibody against GPR109A. Platelets and PMNLs were isolated from whole blood and after incubation with the antibody, receptor expression was determined using flow cytometry. . (A) GPR109A was not present on human platelets. No increase in fluorescence units could be detected by comparing GPR109A antibody treated samples with the respective isotype control. (B) GPR109A was expressed on human neutrophils. Unstained (light grey), isotype control (dark grey) and GPR109A (green) staining of one representative experiment are shown in this overlay. The increase of fluorescence intensity observed with the FITC labelled GPR109A antibody indicates the presence of this receptor on neutrophils (C) Fluorescence units obtained in flow cytometric staining of platelets, neutrophils and eosinophils are normalized to their respective isotype control. There is 3-fold fluorescence increase detected in neutrophil receptor staining whereas fluorescence detection is almost unaffected in platelets and eosinophils. Normalized results are shown as mean+SEM of n= 4 different experiments. Statistical significance is determined by *P<0.05.

P-selectin upregulation was not affected by niacin using ADP (**Figure 10A**) or collagen (**Figure 10B**) as platelet stimuli. Interestingly, iloprost (30 nM) acted as inhibitor of P-selectin expression only in ADP activated samples.

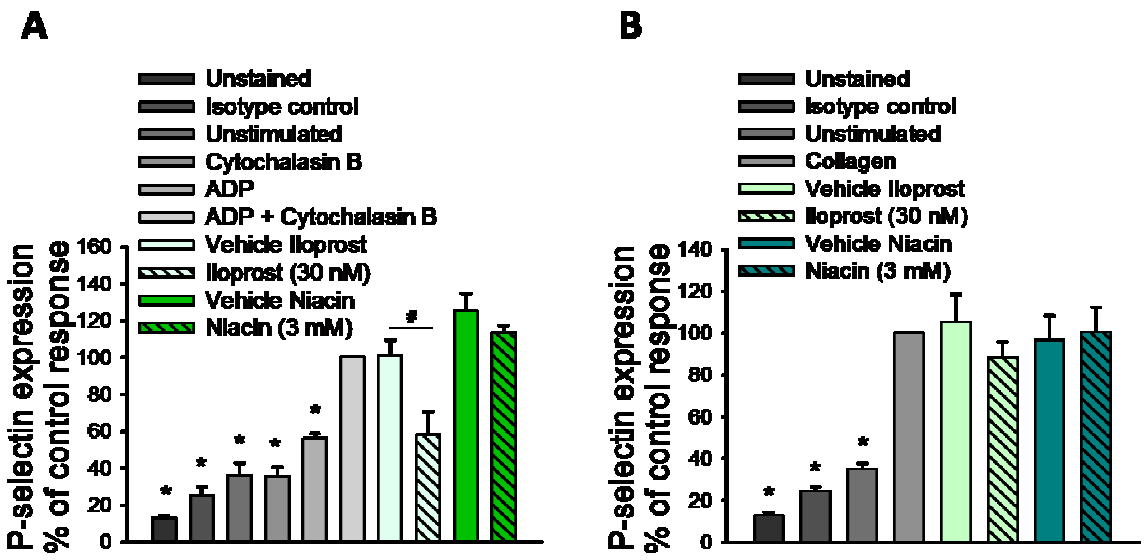

Figure 10: P-selectin upregulation in platelet rich plasma is not affected by niacin (3 mM) preincubated in whole blood

ADP and collagen induced surface expression of P-selectin in the presence of cytochalasin B (5 µg/ml) was detected by flow cytometry via the fluorescence conjugated antibody CD62P. PRP used for the staining was prepared by centrifugation of whole blood that was prior incubated with niacin and iloprost. (A) ADP stimulated P-selectin upregulation was not affected under niacin exposure, wheras iloprost (30 nM) showed significant inhibition compared to vehicle. (B) Niacin (3 mM) could not change P-selectin expression caused by collagen, but interestingly also iloprost (30 nM) failed to inhibit P-selectin upregulation. Results are shown as mean+SEM of n= 3-4 different experiments and are normalized to stimulated samples. Statistical significance is determined by *P<0.05.

The fact that iloprost treated samples showed only partially ability to reduce P-selectin expression, let me consider that the staining procedure was suboptimal and needed further optimizing. Therefore, I used niacin (3 mM) and iloprost (30 nM) pretreated whole blood for a direct staining procedure. Staining occured with CD62P-FITC (as mentioned above) in the presence of CD61-PE. This different labeled antibodies were required to select platelets (CD61 positive) from whole blood by binding on their surface in an activation independent way. As already described,

cytochalasin B (5 µg/ml) was added to ADP activated samples to induce granule release. Again, P-selectin staining was performed using ADP (3 µM) and collagen (6 µg). P-selectin upregulation was measured by flow cytometry. Niacin pretreated samples showed again unaltered fluorescence intensity compared to the vehicle (**Figure 11A and B**). While niacin could not affect P-selectin upregulation, iloprost (30 nM) showed a significant reduction in ADP and collagen induced P-selectin expression, as expected.

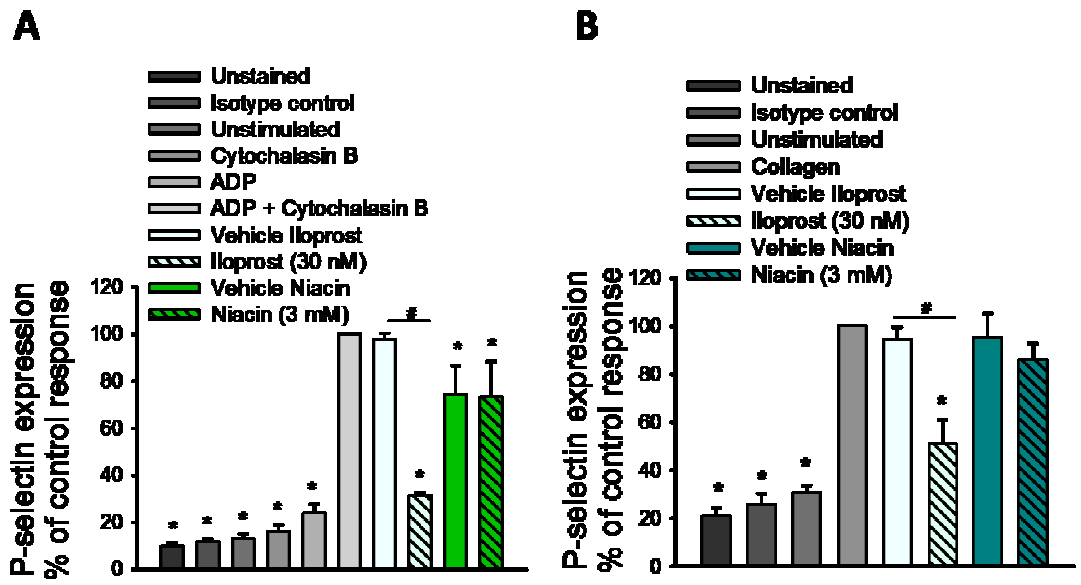

Figure 11: P-selectin upregulation in whole blood is not affected by niacin (3 mM)
ADP in the presence of Cytochalasin B (5 µg/ml) and collagen induced P-selectin expression was detected by flow cytometry via the fluorescence conjugated antibody CD62P-FITC. Simultaneously to CD62P, the PE-conjugated antibody CD61 was used to prior select all platelets from whole blood before measuring P-selectin upregulation in a different fluorescence channel. (A) ADP stimulated P-selectin upregulation was not affected under niacin exposure, wheras iloprost (30 nM) showed significant inhibition compared to vehicle. (B) Niacin (3 mM) could not change P-selectin expression caused by collagen, but iloprost (30 nM) treatment resulted in significant inhibition of receptor expression. Results are shown as mean+SEM of n= 3-4 different experiments and are normalized to stimulated samples. Statistical significance is determined by *P<0.05.

3.1.6 Flow cytometric detection of GPIIb/IIIa was not affected by niacin (3 mM)

In addition to revealing the effect of niacin on P-selectin expression I wanted to examine a potential effect of niacin on GPIIb/IIIa heterodimerization. This receptor conformation occurs during platelet activation and is essential to bind fibrinogen. The conformation dependent antibody PAC1-FITC was used to detect the GPIIb/IIIa heterodimerization on the platelet surface. Since I could show that the niacin receptor was not directly expressed on the surface of platelets, all GPIIb/IIIa staining experiments were performed using whole blood for niacin (3 mM) incubation. Similar, as described above for P-selectin staining, the ability of niacin to affect GPIIb/IIIa heterodimerization was revealed using two different staining approaches.

First, niacin (3 mM) was incubated for 30 min in whole blood which was subsequently centrifuged to obtain PRP. Then, platelets were activated with ADP (3 µM) and cytochalasin B (5 µg/ml) or collagen (6 µg) and incubated with the PAC1-FITC antibody for 30 min at room temperature in the dark. Niacin (3 mM) had no effect on GPIIb/IIIa receptor heterodimerziation, while iloprost clearly inhibited the heterodimerization (**Figure 12A**). Also in collagen activated platelets, niacin did not affect GPIIb/IIIa heterodimerzation (**Figure 12B**). Interestingly, also iloprost showed no inhibitory effect in the collagen stimulated series of experiments, suggesting that the experimental conditions were suboptimal.

Since it was described in literature (Shattil et al., 1987a) that these whole blood stainings should be performed within 10 min after the blood withdrawal, I wanted to examine if the long incubation of blood (30 min) was responsible for the lack of effect of iloprost. Therefore, iloprost (30 nM) was mixed with fresh whole blood and stained after a shortened incubation time of 7 min. Indeed, using this different approach, iloprost (30 nM) was able to inhibit GPIIb/IIIa heterodimerization (**Figure 12C**), supporting the concept that incubation time is a relevant factor for whole blood staining experiments. However, since we showed that for the effect of niacin 30 min of incubation time is needed, this protocol could not be applied for testing this substance.

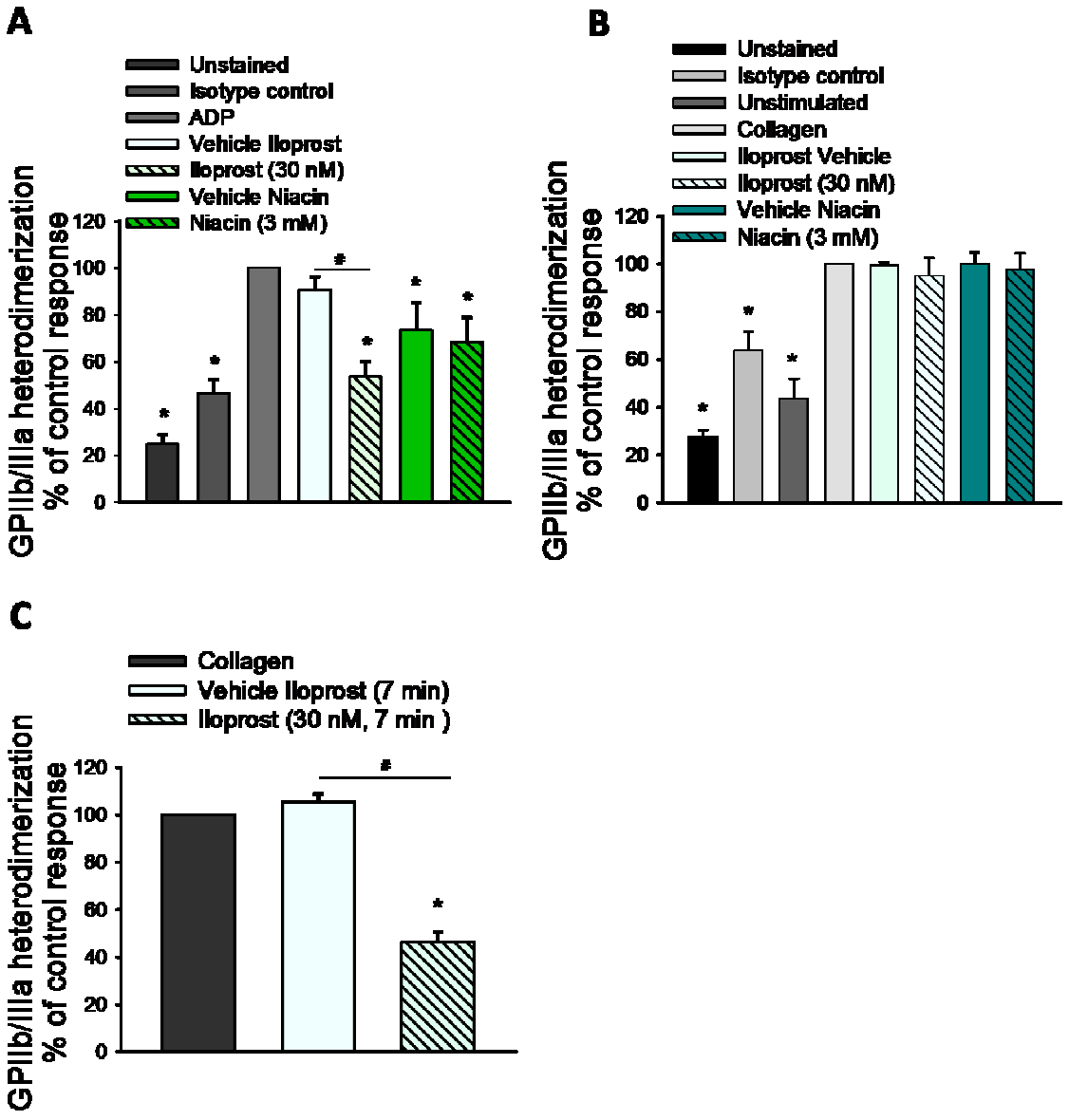

Figure 12: GPIIb/IIIa heterodimerzation in PRP is not affected by niacin (3mM) preincubated in whole blood

ADP and collagen induced heterodimerization was detected by flow cytometry via the fluorescence conjugated antibody PAC1-FITC. PRP used for the staining was prepared by centrifugation of whole blood that was prior incubated with niacin and iloprost. (A) ADP stimulated GPIIBb/IIIa heterodimerization was not affected under niacin exposure, wheras iloprost (30 nM) showed significant inhibition compared to vehicle. (B) Niacin (3 mM) could not change GPIIb/IIIa heterodimerization caused by collagen, but interestingly iloprost (30 nM) failed to affect GPIIb/IIIa heterodimerization. (C) Iloprost (30 nM) could cause significant reduction of GPIIb/IIIa heterodimerzation when incubation time was shortened to 7 min.Results are shown as mean+SEM of n= 3 different experiments and are normalized to stimulated samples. Statistical significance is determined by *P<0.05.

In a further set of experiments, whole blood was directly stained after being pretreated with niacin (3 mM) and iloprost (30 nM) for 30 min. ADP (3 µM) and cytochalasin B (5 µg/ml) was used to initiate GPIIb/IIIa receptor formation which was detected by PAC1-FITC antibody in the presence of CD61-PE. CD61 antibody binding occurred in an activation independent manner that allowed prior selection of platelets from whole blood before GPIIb/IIIa heterodimerization was measured. Similar with prior obtained results, niacin (3 mM) did not affect heterodimerization of the fibrinogen receptor (**Figure 13A**). Also iloprost had no effect (**Figure 13A**). I also investigated whether a shorter incubation period improved the experimental approach. Again, as described for the PRP above, the reduction of the incubation time resulted in the expected results for iloprost, showing an inhibition of GPIIb/IIIa expression (**Figure 13B**). In this regard it has been further established that in all GPIIb/IIIa staining experiments the necessarily long incubation time of niacin limits this experimental approach. Whole blood measurements caused problems of the FACS calibur and therefore experiments could not be continued.

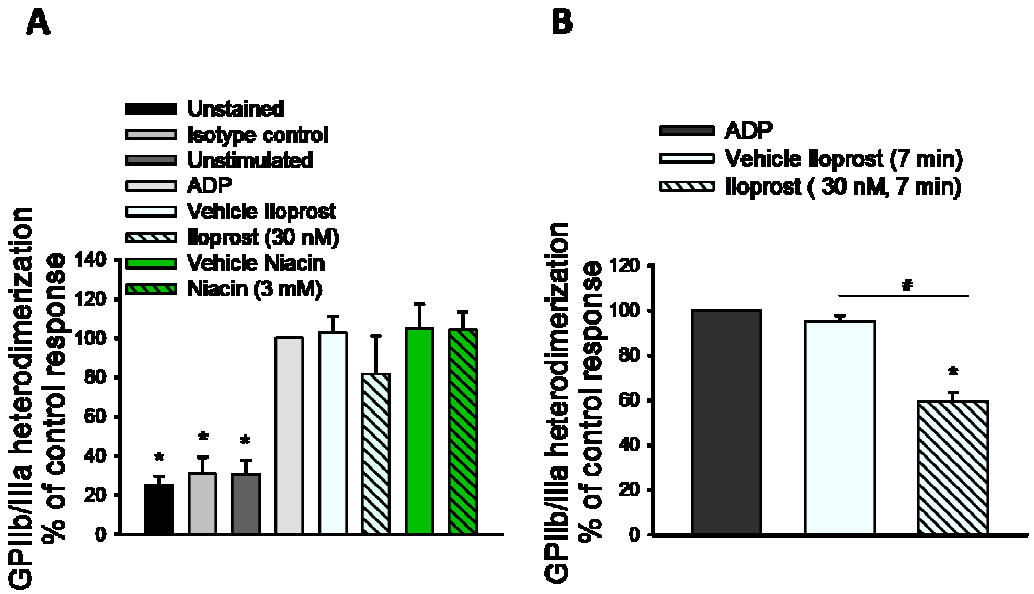

Figure 13: Platelet GPIIb/IIIa heterodimerzation in whole blood is not affected by niacin (3mM)

ADP (3 µM) induced heterodimerization was detected by flow cytometry via the fluorescence conjugated antibody PAC1-FITC in the presence of CD61. Prior whole blood was incubated with niacin (3 mM) and iloprost (30 nM). (A) ADP stimulated GPIIBb/IIIa heterodimerization was not affected neither under niacin exposure nor when whole blood was incubated with iloprost. (B) Whole blood which was directly stained after 7 min incubation with iloprost showed significant inhibition of GPIIb/IIIa receptor heterodimerization.Results are shown as mean+SEM of n= 3 different experiments and are normalized to stimulated samples. Statistical significance is determined by *P<0.05.

3.2 Part II: Platelet EP3 receptor studies with sulprostone and L-798106

Background and Aim

Beside its potential to bind the platelet DP and TP receptor, laropiprant also showed affinity for the EP3 receptor as previously shown in our group (Philipose et al., 2012). Laropiprant inhibited platelet aggregation by blocking the EP3 receptor, and it counteracted its well-described proaggregatory function (Schober et al., 2010; Fabre et al., 2001; Petrucci et al., 2011) .

Based on the involvement of the EP3 receptor on the laropiprant mediated effect on platelet function, I wanted to expand the current knowledge of this receptor with regard to platelet function. Therefore, I used the selective EP3 agonist sulprostone and the EP3 antagonist L-798106. Although, the role of EP3 as a proaggregatory receptor on platelets has already been well described, it was not shown how platelet adhesion and thrombus formation under flow conditions *in vitro* are affected by activation of the EP3 receptor. Therefore, my aim was to elucidate the role of the platelet EP3 receptor with regard to these platelet functions.

3.2.1 L-798106 reverses the sulprostone caused increase on platelet aggregation in washed platelets, but not in PRP

Initially, I was interested in the potential of the EP3 antagonist L-798106 to reverse the sulprostone-caused increase on platelet aggregation.

Since it is already well established that sulprostone increases stimulus-evoked platelet aggregation via the EP3 receptor (Matthews and Jones, 1993a; Heptinstall et al., 2008b; Dovlatova et al., 2008) I wanted to reverse this effect by using the EP3 antagonist L-79816 in PRP. Therefore, a concentration response curve with the antagonist was performed. Prior testing the antagonist on its EP3 receptor blocking effect, the potentiation of platelet aggregation was revealed by performing a concentration response curve of sulprostone. Collagen concentrations (1.5-5 µM) that induced 30-50 % platelet aggregation were used as proaggregatory stimuli. In line with previous data, sulprostone was able to increase aggregate formation concentration dependently (**Figure 14A**). For platelet aggregation studies with L-798106, samples were pre-incubated with the EP3 antagonist, followed by sulprostone treatment (100 nM). The increase in aggregate formation could not be reversed by the EP3 antagonist, even with the highest concentration used (3000 nM), (**Figure 14B**).

Based on these results I wanted to know if L-798106 could counteract the increase on aggregation by sulprostone when ADP (1.5-5 µM) was used for platelet activation. Experiments were performed the same way as described above. Sulprostone already at 10 nM enhanced ADP- induced platelet aggregation 2-fold (**Figure 14C**). PRP was incubated with increasing concentrations of L-798106 (100-3000 nM), but as already shown for collagen activated platelets, the EP3 antagonist could not counteract the enhancement of aggregation by sulprostone (10 nM and 30 nM) on ADP activated platelets (**Figure 14D**)

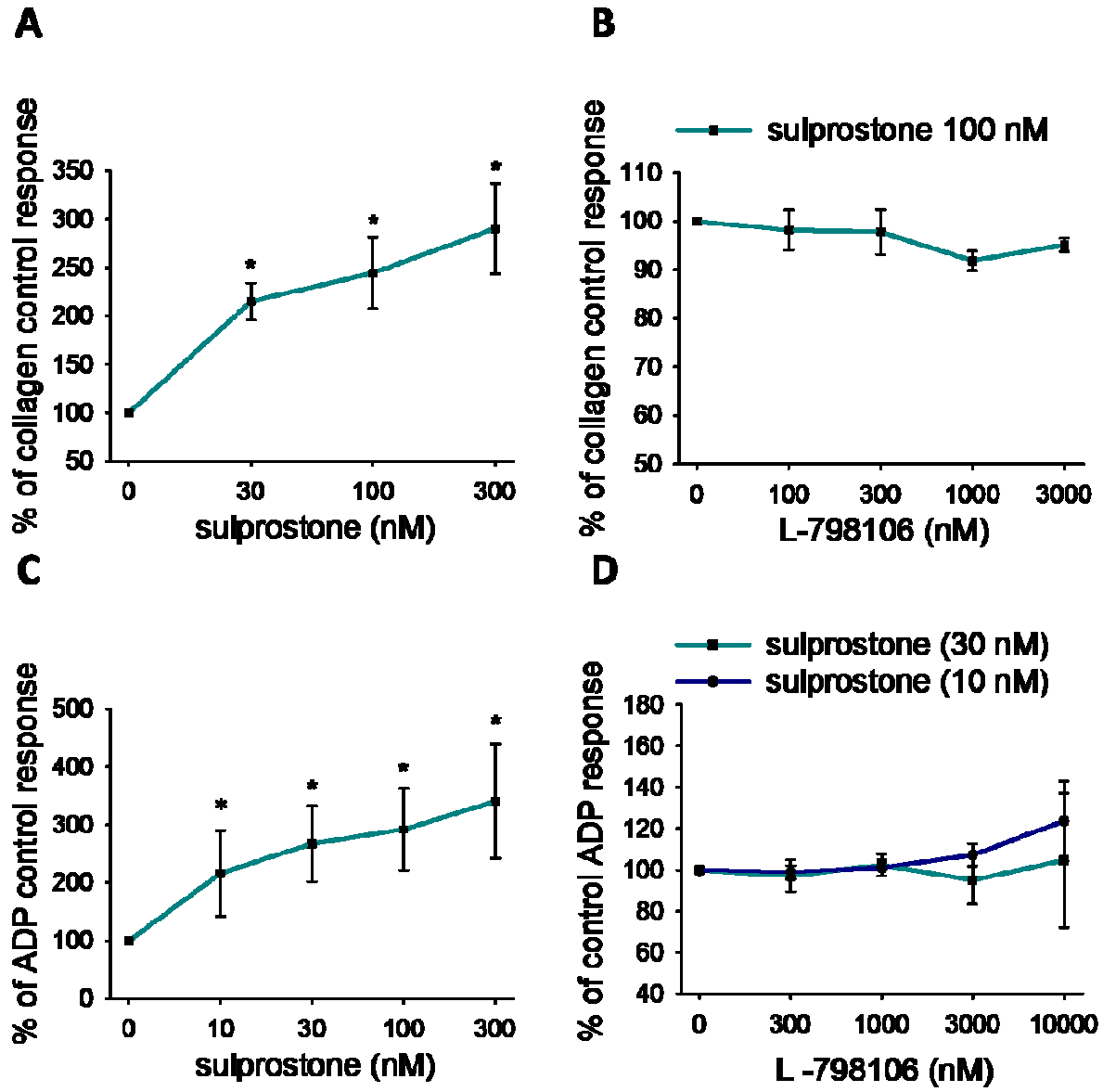

Figure 14: The sulprostone caused increase on platelet aggregation in PRP is unaffected by the EP3 antagonist L-798106

PRP was incubated with the EP3 agonist sulprostone for 5 min. When L-798106 was used, the EP3 antagonist was mixed with PRP 10 min before the agonist was added. Pretreated PRP was then stimulated with collagen or ADP and aggregate formation was measured with a 4-channel aggregometer at 37 °C during constant stirring (1000 rpm). (A) Pretreatment of PRP samples with sulprostone (30, 100, 300 nM) affected collagen (1.5-5 µM) stimulated platelets by increasing platelet aggregation. (B) The sulprostone (100 nM) caused increase on collagen induced platelet aggregation was not affected by the EP3 antagonist L-798109 (100-3000 nM) (C) As seen in collagen induced platelet aggregation, sulprostone (10, 30, 100, 300 nM) significantly increased platelet aggregation when stimulated with ADP (1.5-5 µM). (D) The sulprostone caused increase on ADP induced platelet aggregation was not affected by the EP3 antagonist L-798109. Results are shown as mean±SEM of n= 3-6 different experiments and are normalized to the respective vehicle. Statistical significance is determined by *P<0.05

To test, whether missing functionality of L-798106 on sulprostone increased platelet aggregation was due to binding of the antagonist to plasma proteins, a further set of experiments was performed using washed platelets instead of PRP. Therefore, washed platelets were freshly prepared and incubated with L-798106 and sulprostone as described above. Aggregation was stimulated with collagen (1.5-5 µM), and washed platelets pretreated with sulprostone (10, 30 and 100 nM) showed a 2 to 3-fold increase on platelet aggregate formation(**Figure 15A**) which was in line with prior obtained results using PRP. In contrast to previous results obtained in PRP, the EP3 antagonist L-798106 was able to counteract the sulprostone effect in washed platelets at a concentration of 1000 nM (**Figure 15B**). Higher concentrations (3 µM) of L-798106 caused a decrease in platelet aggregation on its own.

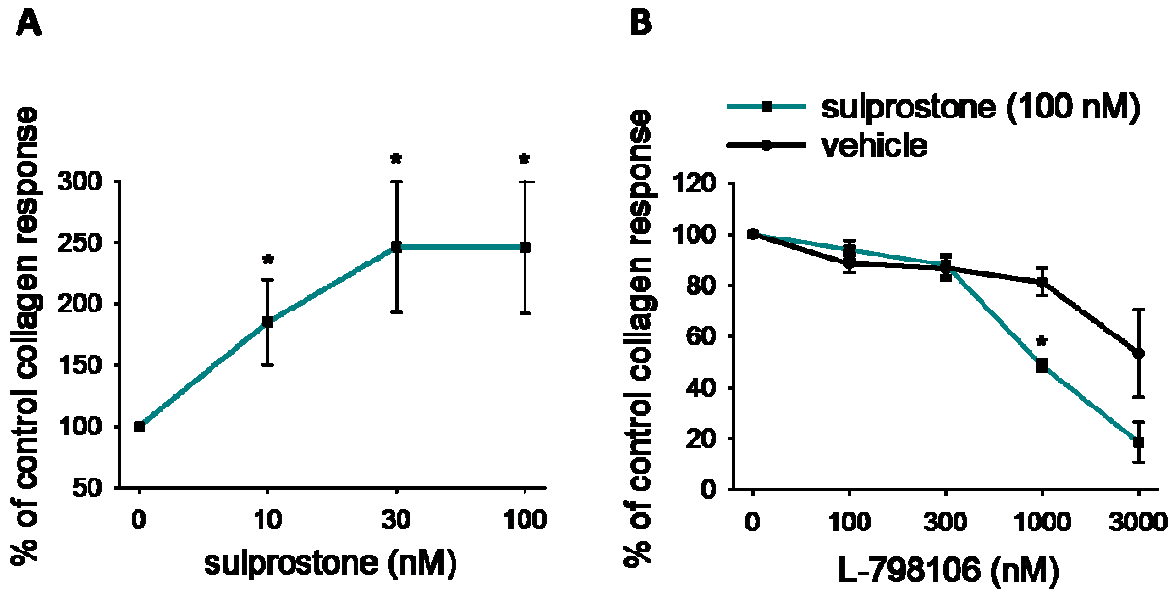

Figure 15: The sulprostone-caused increase of platelet aggregation in washed platelets is reversed by the EP3 antagonist L-798106

Freshly prepared washed platelets were incubated with the EP3 agonist sulprostone for 5 min. When L-798106 was used, the EP3 antagonist was mixed with PRP 10 min prior agonist addition. Pretreated washed platelets were then stimulated with collagen (1.5-5 µM) and aggregate formation was measured with a 4-channel aggregometer at 37 °C during constant stirring (1000 rpm). (A) Pretreatment of washed platelet samples with sulprostone (10, 30 and 100 nM) caused a significant increase in collagen stimulated platelet aggregation. (B) The sulprostone (100 nM) caused increase on collagen induced platelet aggregation was reversed with 1 µM of the EP3 antagonist L-798106. Results are shown as mean+SEM of n= 4-5 different experiments and are normalized to the respective vehicle. Statistical significance is determined by *P<0.05

3.2.2 L-798106 reverses the sulprostone caused increase on platelet adhesion to fibrinogen and collagen.

Since the EP3 antagonist L-798106 finally prevented sulprostone binding to the EP3 receptor, I wanted to know whether this effect can be seen on platelet adhesion under flow condition. Therefore, I used collagen (125 µg/ml) or fibrinogen (100 µg/ml) coated Vena8+fluoro biochips. Again, washed platelets were prepared and pretreated with L-798106 (1000 nM) and sulprostone (100 nM) as described for the aggregation experiments. In case of adhesion to fibrinogen, platelets were mixed with ADP (10 µM) 2 min prior perfusion over the fibrinogen coated channels. Images were taken during the first 9 min after start of the perfusion. The adhesion of ADP activated platelets to fibrinogen was increased when washed platelets were incubated with sulprostone (100 nM) and this effect could be reversed by pretreatment of the samples with L-798106 (1000 nM) (**Figure 16A**).

In the case of collagen, images were also taken during the first 9 min after start of the perfusion. When washed platelets were perfused over collagen coated channels, adhesion was increased with sulprostone (100 nM) and L-798106 (1000 nM) again counteracted the sulprostone induced increase on platelet adhesion, and L-798106 (1000 nM) alone had no effect on collagen induced adhesion (**Figure 16B**).

3.2.3 Sulprostone did not affect *in vitro* thrombogenesis

Since there are no data in the literature with regard to the effect of sulprostone on *in-vitro* thrombogenesis, I wanted to reveal this effect on thrombus formation *in vitro* under flow condition. Therefore whole blood was incubated with the fluorescence dye (3, 3'-dihexyloxacarbocyanine iodide*)* and different concentrations of sulprostone and then perfused over collagen coated channels (125 µg/ ml) of Vena8+ fluoro biochips. In contrast to adhesion experiments, sulprostone (10 – 100 nM) had no effect on thrombus formation (**Figure 17**).

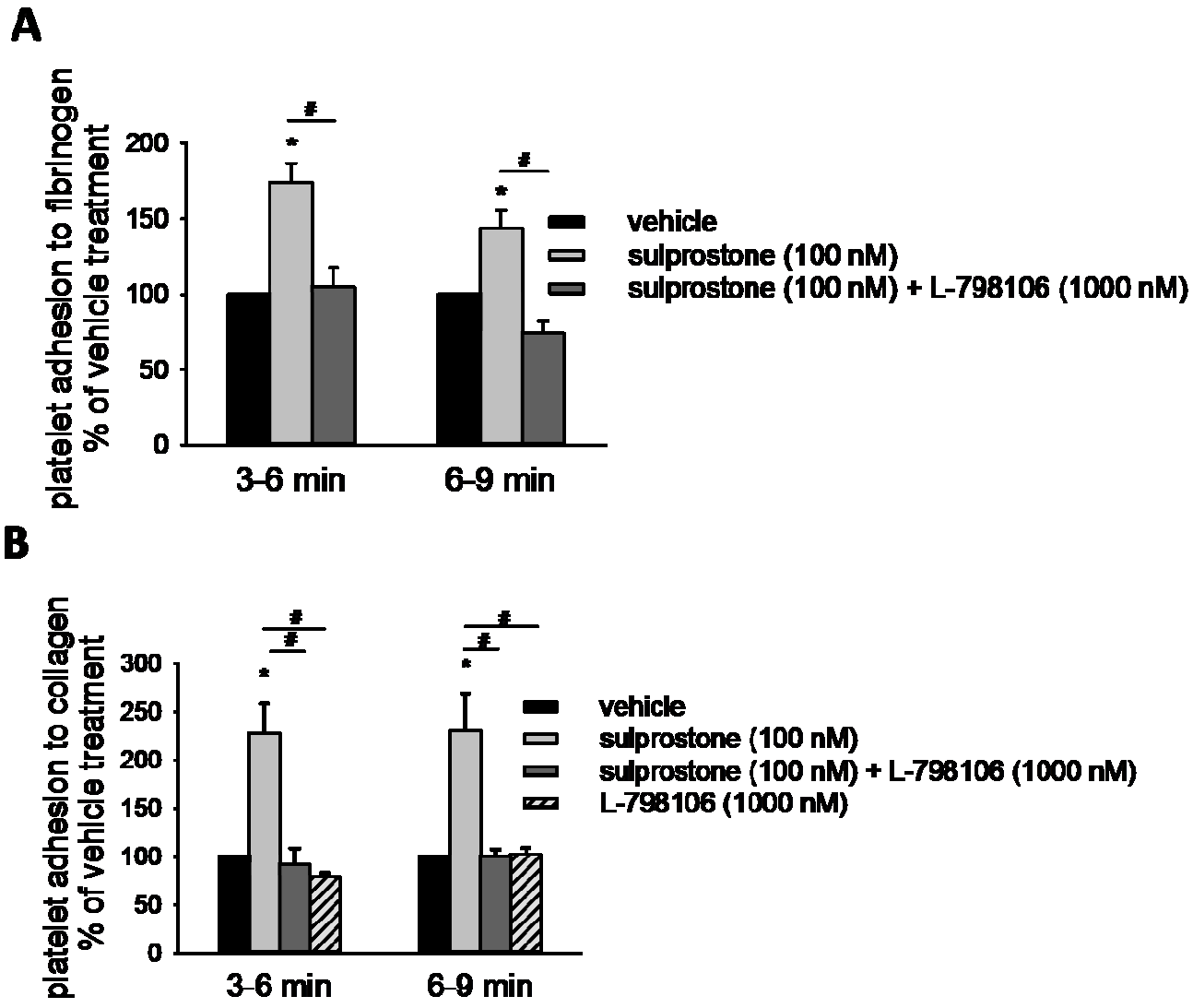

Figure 16: The sulprostone (100 nm) caused increase on platelet adhesion to collagen and fibrinogen, is reversed by the EP3 antagonist L-798106 (1000 nM)

Freshly prepared washed platelets were perfused with constant shear stress (0.5 dyne cm^{-2}) over fibrinogen or collagen coated channels of Vena8+ biochips. Washed platelets were prior incubated with L-798106 (1000 nM) and sulprostone (100 nM). Platelets were preactivated with ADP (10 μM) when perfusion occurred over fibrinogen coated channels. Adhesion was recorded by light microscopy. Images were taken during the first 9 min after the perfusion start and 6 representative images were analyzed between 3-6 min and between 6-9 min. Results are shown as mean+SEM of n= 5-6 different experiments and are normalized to the respective vehicle. Statistical significance is determined by *P<0.05

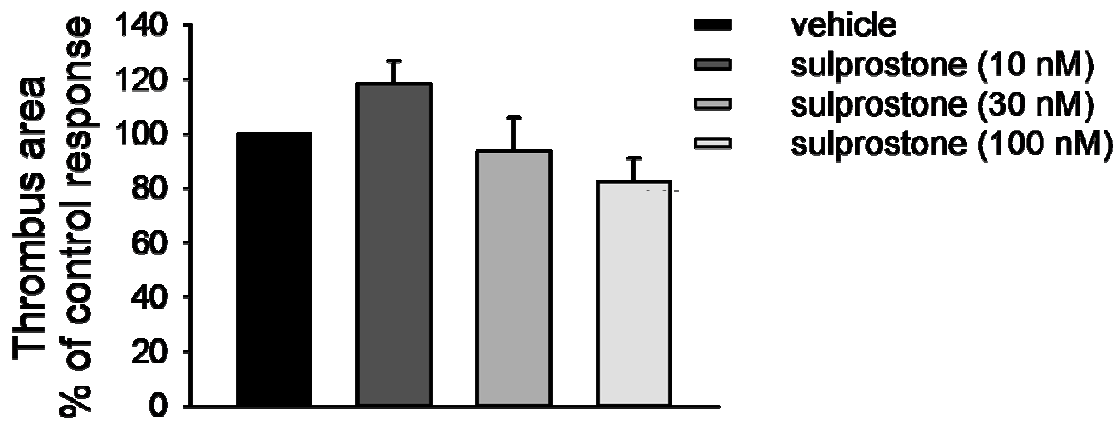

Figure 17: Sulprostone (10, 30 and 100 nM) did not affect in vitro thrombus formation

Fresh whole blood was perfused with constant shear stress (30 dyne cm^{-2}) over collagen coated channels of Vena8+ biochips. Whole blood was prior incubated with a fluorescence dye (3, 3'-dihexyloxacarbocyanine iodide*)* that allows recording of thrombus formation by fluorescence microscopy. Sulprostone at different concentrations (10, 30 and 100) was mixed with whole blood for 5 min. Images were taken 3 min after the start of the perfusion and 4 representative images were analyzed. Images were analyzed with the DucoCell analysis program. Results are shown as mean+SEM of n= 4 different experiments and are normalized to the respective vehicle.

4. Discussion

Part I

In the first part of my thesis I was involved in an ongoing study that revealed the effect of niacin and laropiprant with regard to platelet function. These two substances are currently marketed under the name Tredaptive®, a lipid lowering drug reported to be effective and well tolerated (Bays et al., 2010; McKenney, 2004), although the impact of this combination regarding thrombotic cardiovascular risk was not investigated in detail until now. While niacin acts on the lipid metabolism, laropiprant, a DP receptor antagonist, is active in preventing the niacin caused side effect of "flush" that is caused mainly due to PGD_2 release (Papaliodis et al., 2008). PGD_2 acts on platelets by reducing their potential to aggregate and laropiprant by blocking the PGD_2 target receptor DP could therefore interfere with the antiaggregatory potential of platelets. A resulting elevation in platelet activity could cause a higher thrombotic risk for dyslipedemia patients treated with Tredaptive®.

PGD_2 is known to increase intracellular cAMP production and Ca^{2+} mobilization via the DP receptor, leading to a decreased platelet potential (Feinstein et al., 1983a). Recent experiments of our group could further confirm the inhibitory potential of PGD_2 and the antagonistic effect of laropiprant on the DP receptor. It could be shown that PGD_2 has inhibitory potential in collagen as well as in ADP induced platelet aggregation and that this effect solely occurred due to DP activation (Philipose et. al, 2012).

For niacin a weak, but significant antiaggregatory effect in collagen induced platelet aggregation was reported and it was suggested that this was mediated by an inhibitory effect of niacin due to further prostaglandin release (Serebruany et al., 2010).

Based on these previously obtained results we were interested how (a) niacin and laropiprant on its own and (b) in combination would affect *in vitro* collagen- induced thrombogenesis under flow condition. Since it was not elucidated how niacin affects

platelet functionality, *in vitro* thrombogenesis was performed using different conditions with the result that niacin at a therapeutical relevant dose (3 mM) could inhibit collagen-induced thrombus formation under flow condition, however a perincubation period of 30 min in whole blood for was needed as shown in Figure 6.

These results let us hypothesize that niacin may not directly interact with platelets, since short incubation times are in general sufficient for receptor activation following an agonist stimulus. To investigate whether the effect of niacin was due to prostaglandin release, we pretreated blood (1) with acetylsalicylic acid to block COX and therefore, prostaglandin synthesis or (2) laropiprant to see whether PGD_2 is involved in this effect. Interestingly, none of the pretreatments affected the niacin caused inhibition of thrombogenesis. Thus, in contrast to the „flush" effect, prostaglandins seem not to be involved in the niacin mediated effect on thrombus formation.

Interestingly, laropiprant at higher concentrations (10 µM) also prevented collagen-induced thrombus formation under flow. Collagen is known to causes TXA_2 formation (Watts et al., 1991) and therefore the inhibition may result from counteracting the pro-trombotic function of the TP receptor. The affinity of laropiprant to the TP receptor has already been reported (Lai et al., 2008). In addition our group further substantiated TP antagonistic effects by showing that laropiprant counteracted the TP receptor agonist U46619 induced effects on aggregation, on Ca^{2+} mobilization, P-selectin expression and GPIIb/IIIa heterodimerization (Philipose et al., 2012)

These results, together with the data obtained in aggregation experiments (Figure 7) where I could show the concentration-dependent decrease of collagen induced platelet aggregation by laropiprant , provide further evidence for the TP receptor mediated reduction on collagen induced *in vitro* thrombus formation under flow. Thus, confirming a further role of laropiprant as anti-aggregatory agent, however, at higher concentrations as therapeutically used. However, it was shown that laropiprant, also at higher concentrations is well tolerated (Lai et al., 2008).

In contrast to laropiprant, niacin preincubation did not result in reduced platelet aggregation, which was not consistent with already published data (Serebruany et al., 2010). Based on this finding, I wanted to proof whether a direct effect of niacin on platelets could be seen on P-selectin expression. P-selectin is essential in platelet-

leukocyte-endothelium interactions (Larsen et al., 1989) and is an established reseach tool to reveal platelet activation (Ruf and Patscheke, 1995). In line with data obtained in platelet aggregation, P-selectin expression was unaffected as depicted in Figure 8 upon niacin treatment of PRP, thus strengthening the hypothesis of an indirect mechanism of niacin on platelets.

GPR109A is the known target receptor for niacin and present on keratinocytes, monocytes/macrophages (Hanson et al., 2010b) and neutrophils (Kostylina et al., 2007). In contrast to neutrophils, platelets do not express the GPR109A receptor as determined by flow cytometry as shown in Figure 9-further confirming an indirect effect of niacin on platelet function.

The role of niacin as an inhibitor of platelet function was clearly shown in the *in vitro* thrombogenisis experiments, but could not be detected in the subsequent aggregation and P-selectin experiments when niacin was incubated in PRP. However, these methods would have been solely able to detect an effect of niacin that results from direct niacin-platelet interactions.

To determine whether niacin has an impact on platelet activation via an indirect mechanism on P-selectin expression, niacin was incubated in whole blood, since niacin may cause the release of substances from other blood cells which could influence platelet activation. Thereafter blood was either (1) centrifuged to obtain PRP or (2) directly stained for P-selectin expression. The advantage of the whole bloodstaining might be that the further washing and centrifugation steps that could additionally lead to unspecific platelet activation (Shattil et al., 1987b), are avoided. However, in both approaches niacin could neither affect ADP nor collagen induced P-selectin expression (depicted in Figure 10A and B). The IP receptor agonist iloprost (30 nM) was used as a control, due to its already well defined inhibitory effect on platelet activation/ P-selectin expression (Philipose et. al, 2010). While the ADP induced effects were inhibited by iloprost in PRP as well as in whole blood in this stetting, the effects of collagen were only inhibited when whole blood was directly stained and not when PRP was stained. This leads to the conclusion that this experimental setup does not work properly. I assume that this might be is a result of the long incubation time that had to be chosen for the incubation with niacin. In general, all obtained results suggest that the inhibitory potential of iloprost decreases when samples are not stained within a few minutes as compared to prior performed

P-selectin experiments upon iloprost treatment (Philipose et. al, 2010). However, the results obtained from the whole blood staining experiments suggest, that niacin does not affect ADP- or collagen- induced P-selectin expression.

GPIIb/IIIa is the major platelet integrin involved in platelet aggregation and only active upon heterodimerisation. The receptor converts to a competent fibrinogen receptor upon activation and can therefore be detected by the conformation-dependent antibody PAC-1 (Shattil et al., 1987b). Niacin failed to affect GPIIb/IIIa heterodimerization as depicted in Figure 12A and B. While iloprost could inhibit ADP induced GPIIb/IIIa activation, collagen induced GPIIb/IIIa heterodimerization was not affected when iloprost was incubated for 30 min, but iloprost was effective after a short incubation periode (7 min), directly followed by antibody staining. This results suggest that time is an important factor for these sort of staining experiments. This assumption is in line with an already reported hypothesis that platelet stability is one major variable for platelet activation dependent antibody-staining (Shattil et al., 1987b). A further finding was that spontaneous platelet activation depends very much on the factor time, since spontaneous platelet activation did not occur when samples were stained within 10 min after blood withdrawal.

For both P-selectin expression and GPIIb/IIIa heterodimerisation the prolongend incubation time required for niacin could interfere with its inhibitory potential on platelet activation as shown in Figure 12C where the methodical controls (iloprost) were not effective. However, in the other experimental settings the iloprost control showed a significant inhibition of P-selectin and GPIIb/IIIa heterodimerization and niacin failed to do so. These controversial results confirmed that the time factor is important in these staining experiments. The failure of niacin to affect P-selectin expression and GPIIb/IIIa heterodimerization may be explained that (a) this method is not sensible enough to detect niacin caused effect on platelets or (b) niacin has no ability to affect platelet activation in this setting. Further research has to be done to solve these questions.

To summerize the results of the Tredaptive® study, laropiprant (10 µM) was able to inhibit the collagen- induced thrombus formation *in vitro* under flow. This suggests an additional anti-aggregatory role of laropiprant on platelet function that seems to be

due to TP receptor activation. It was also shown in our group, that laropirant blocks the pro-aggregatory PGE_2 receptor EP3, (Philipose et. al, 2012) thus leading to a further positive impact on platelet function, since an EP3 receptor is suggested to be a useful target in antiplatelet therapy, and the EP3 antagonist DG-041 is currently in clinical trials (Heptinstall et al., 2008a). The ability of laropiprant to block the TP and EP3 receptor outweighs its pro-thrombotic effect by blocking the antiaggregatory DP receptor.

Niacin, showed inhibitory potential on *in vitro* thrombogenesis but failed to effect platelet aggregation and flow cytometric staining for the activation markers P-selectin and GPIIb/IIIa also in whole blood staining. We could show that the niacin effect observed in collagen-induced thrombus formation under flow was (a) not due to further prostaglandin release and (b) that the known niacin receptor GPR109 was not directly involved since this receptor is not present on platelets surface. The fact that niacin recquired an incubation time of 30 min further strengthens the hypothesis that niacin effects platelets via in indirect way. Our experiments further indicate that the niacin caused inhibitory effect might depend on flow conditions.

The final conclusion of the Tredaptive® study is that niacin as well as tredaptive may have positive impacts regarding platelet function, supporting an additional beneficial effect, besides its function as lipid lowering drug. Whereas the mechanism of action of laropiprant *in vivo*, have been investigated in detail (Philipose et al., 2012), a deeper knowledge of underlying mechanisms of the niacin caused effect remains to be investigated

Part II

In this part of my thesis I was interested to enhance knowledge with regard to the platelet EP3 receptor, that was reported to be a potential target for antiplatelet therapy (Heptinstall et al., 2008a). Since involvement of this receptor could be shown in the anti-thrombotic effect of laropiprant (as described in the first part of the thesis, Philiopose et al., 2012) and an EP3 antagonist DG-041 is currently in phase 2 clinical trials (Heptinstall et al., 2008a) I was interested to further elucidate the role of this receptor with regard to platelet function.

The natural EP3 agonist PGE_2 was inappropriate to reveal specific functions of the EP3 receptor due to its additional binding affinity for all other EP receptors. Thus, I used the selective EP3 agonist sulprostone.

In platelet aggregation experiments I could confirm the already described role of sulprostone as enhancing collagen and ADP induced platelet aggregation (Schober et al., 2010; Fabre et al., 2001; Petrucci et al., 2011) in PRP. To reveal whether this effect solely depends on EP3 receptor activation, I used the EP3 antagonist L-798106. Interestingly, the sulprostone induced increase of platelet aggregation was not affected either in collagen (Figure 14b) nor in ADP (Figure 14D) stimulated platelets when PRP samples were preincubated with L-798 106 – even at very high concentrations (10 µM).

We speculated that the failure of L-798106 to counteract the sulprostone-induced increase in agonist-induce platelet aggregation might be due to binding of this substance to plasma proteins that would prevent platelet receptor interactions with L-798106. This assumption was proofed by using washed platelets instead of PRP. L-7981106 inhibited the sulprostone caused increase in collagen induced platelet aggregation as depicted in Figure 15B. This confirms that the effect of sulprostone on platelet aggregation is mediated by the EP3 receptor.

The effect of sulprostone on platelet adhesion under flow has not been investigated so far, thus I wanted to elucidate whether platelet adhesion is affected the same way as platelet aggregation. Platelet adhesion induced by collagen was significantly increased when washed platelets were pretreated with sulprostone, an effect that

could be totally reversed by L-798106 Figure 16A The platelet adhesion to fibrinogen was also increasedby sulprostone and the EP3 antagonist L-798106 counteracted again the sulprostone induced amplification in platelet adhesion, without showing an effect on its own, as depicted in Figure 16B.

In contrast to data obtained in platelet adhesion under flow, sulprostone did not affect whole blood *in vitro* thrombogenesis under flow. There are different studies about the effect of sulprostone on platelet aggregation in whole blood that all found that sulprostone promote platelet aggregation (Matthews and Jones, 1993b; Vezza et al., 1993; Heptinstall et al., 2008a). Since different groups provided consistent data with regard to the ability of sulprostone to affect platelet aggregation it can be speculated that the missing effect of sulprostone on thrombus formation under flow *in vitro* might be a result of the shear stress. Small variations of the shear stress (using different flow conditions) did not alter thrombus formation (data not shown) and too high/to less shear stress permitted platelet collagen interactions.

Until now, 10 different mRNA splice variants have been identified for the human EP3 receptor, that are shown to couple to different G-proteins and therefore can lead to several signaling cascades (Israel and Regan, 2009).On platelets at least three isoforms are present (Paul et al., 1998) One can speculate that under flow conditions other receptor isoforms are predominant, which then become the target of sulprostone under shear stress, thus leading to an altered impact of sulprostone on thrombus formation.

Summerized, the role of sulprostone as a selective EP3 agonist could be established in assays for platelet aggregation. I showed for the first time, that this EP3 agonist enhances collagen and fibrinogen induced platelet adhesion under flow. Although, the pro-aggregatory effect of sulprostone in whole blood has already been shown, sulprostone failed to affect collagen- induced thrombus formation of whole blood under flow. It can be speculated that this might be due to activation of different EP3 splice variants under flow conditions, however, this remains to be investigated. In summary, this results demonstate that there are a lot of unsolved questions with regard to the effects of EP3 receptor activation in platelets,

Parts of my results contributed to a manuscript that is accepted for publication.

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Abbreviations

AA	arachidonic acid
ADP	adenosine diphosphate
ASA	acetyl salicylic acid
ATP	adenosine triphosphate
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
COX	cyclooxygenase
ECM	extracellular matrix
GP	glycoprotein receptors
GTP	guanosine triphosphate
HDL	high density lipoprotein
LDL	low density lipoprotein
LPS	lipopolysaccharides
LTA	light transmission aggregometry
NO	nitric oxide
PG	prostalandin
PKC	phosphokinase C
PLC	phospholipase C
PMNLs	polymorph nuclear leukocytes
PPP	platelet poor plasma
PRP	platelet rich plasma
Syk	spleen tyrosine kinase
Th2	T-helper cell type 2
VWF	von Willebrand factor