Preclinical evaluation of the antithrombotic potential of the most commonly prescribed antiplatelets

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Lay Abstract

Heart attacks and strokes are the leading causes of morbidity and mortality in developed countries. The main function of platelets in the blood is to help stop bleeding; however, they also cause heart attack and stroke. Cholesterol-lowering medications, like atorvastatin, and medications that attenuate platelet activation, like acetylsalicylic acid and clopidogrel, are standard therapies for treating or preventing heart attack and stroke. In our study, we developed a sensitive preclinical method to evaluate the treatment effects of atorvastatin, acetylsalicylic acid and clopidogrel. We found that atorvastatin can also attenuate platelet activation. We also found that a low dose acetylsalicylic acid is better than a higher dose at attenuating platelet activation when used with clopidogrel. Our findings provide valuable information towards guiding treatment strategies for heart attack and stroke. This work could eventually contribute towards reducing morbidity and mortality from these two devastating diseases.

Abstract

Myocardial infarctions and stroke are the leading causes of death in developed countries, and often result from arterial thrombus formation triggered by the rupture of an advanced atherosclerotic plaque. Plasma levels of cholesterol are correlated with risk of cardiovascular disease. In addition, platelets have a predominant role in the formation of arterial thrombi. Thus, lipid-lowering drugs, such as atorvastatin, are used in combination with antiplatelet agents, such as acetylsalicylic acid (ASA) and clopidogrel, to prevent and treat cardiovascular disease.

In the studies described in this thesis, we measured the antiplatelet effects of atorvastatin, ASA and clopidogrel in mice. We discovered that atorvastatin attenuates platelet activation via protease-activated receptor 4 (PAR4, thrombin receptor) signaling in a nitric oxide (NO)-independent manner. We evaluated the antiplatelet effects of all three medications *in vitro*. Furthermore, we measured the antithrombotic potential of these three medications *in vivo* in a murine arterial thrombosis model. We first demonstrated that the sensitivity of the laser injury thrombosis model is improved by measuring platelet activation markers (platelet aggregation, degranulation and phosphatidylserine (PS) exposure). Using this modified laser injury thrombosis model, we found that neither atorvastatin nor ASA reduces thrombus volume, however both delayed *in vivo* platelet activation. Moreover, we showed that in the context of co-administration with clopidogrel (which is the standard-of-care), ASA at 10 mg is more effective at reducing *in vivo* thrombosis than ASA at 40 mg. This supports the

notion that high doses of ASA offset the partial inhibitory effect of $P2Y_{12}$ antagonists by interfering with prostacyclin (PGI_2) formation.

In conclusion, our results support PAR4 signaling as a target for developing new antiplatelets and advocate for ASA dose adjustment in standard-of-care therapeutic strategies that use both ASA and $P2Y_{12}$ antagonists. Our findings will help guide and improve future pharmaceutical intervention strategies for arterial thrombosis patients.

Acknowledgements

At the moment, I am one step away from obtaining a prestigious degree in science from my dream university in Canada. I cannot help but to think back on all of the good memories from the entire journey, which began nearly ten years ago when I landed on this foreign land as a visitor and spans until the present day, where I call Canada home. I understand that the reason I am where I am today is a mixture of luck and a great deal of team effort. I cannot be more thankful to those lovely people who overlooked my limitations in the English language and went out of their way to become mentors and friends. Without all these people, I could never fit into this new home.

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List of Abbreviations

AA – arachidonic acid

AC – adenylyl cyclase

ACS – acute coronary syndromes

ADP – adenosine diphosphate

ARC – arthrogryposis-renal dysfunction-cholestasis

ASA – aspirin / acetylsalicylic acid

ATP – adenosine triphosphate

AUC – area under the curve

BH4 – (6R)-5,6,7,8-tetrahydro-L-biopterin

Ca^{2+} – calcium

CaaX – sequences of CaaX box (**C**: cysteine, **aa**: any aliphatic amino acids, **X**: C-terminal amino acid that determines the substrate specificity)

cAMP – cyclic adenosine monophosphate

cGMP – cyclic guanosine monophosphate

CHS – Chediak-Higashi syndromes

CLEC-2 – C-type lectin-like receptor 2

COX – cyclooxygenase

CRP – C-reactive protein

DAPT – dual-antiplatelet therapy

DMSO – dimethyl sulfoxide

ELISA – enzyme-linked immunosorbent assay

eNOS – endothelial nitric oxide synthase

EPCR – endothelial protein C receptor

FITC – fluorescein isothiocyanate

GC – guanylyl cyclase

GDP – guanosine diphosphate

GI – gastrointestinal

GPCR – G protein-coupled receptor

GPS – grey platelet syndromes

GTP – guanosine triphosphate

GTPases – guanine nucleotide triphosphatases

GYP – GYPGKF, PAR4 agonist peptide (H- Gly-Try-Pro-Gly-Lys-Phe -OH)

HDL – high-density lipoprotein

HMG-CoA reductase – 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase

HPS – Hermansky-Pudlak syndromes

ITAM – immunoreceptor tyrosine-based activation motif

JACC –The American Heart Association and American College of Cardiology

KQAGDV – Lys-Gln-Ala-Gly-Asp-Val sequence

LDL – low-density lipoprotein

MFI – mean fluorescence intensity

Mg^{2+} – magnesium

mRNA – messenger RNA

NO – nitric oxide

OCS – open canalicular system

PAR – protease-activated receptor

PBS – phosphate-buffered saline

PCI – percutaneous coronary intervention

PDE – phosphodiesterases

PE – phycoerythrin

PGE_2 – prostaglandin E_2

PGH_2 – prostaglandin H_2

PGI_2 – prostacyclin

PI3K – phosphatidylinositol 3-kinase

PKC – protein kinase C

PLAs – phospholipases

PLC – phosphorylated phospholipase C

PPAR – peroxisome proliferator-activated receptor

PPP – platelet-poor plasma

PRP – platelet-rich plasma

PS – phosphatidylserine

RGD – Arg-Gly-Asp sequence

ROS – reactive oxygen species

SFLLRN – Ser-Phe-Leu-Leu-Arg-Asn sequence

SNARE – soluble N-ethylmalemide-sensitive factor attachment protein receptor

TCA – trichloroacetic acid

TFPI – tissue factor pathway inhibitor

tPA – tissue plasminogen activator

TXA_2 – thromboxane A_2

uPA – urokinase plasminogen activator

VLDL – very low-density lipoprotein

vWD – von Willebrand disease

vWF – von Willebrand factor

5-HT – 5-hydrotryptamine/serotonin

Chapter 1: Introduction

1.1 Overview of haemostasis and thrombosis

Blood is a bodily fluid, containing cells and a large number of proteins and other molecules, which are pumped by the heart to circulate throughout the body of humans and other animals. Circulating blood distributes essential nutrients and oxygen to cells and removes metabolic waste from these cells. As a result of a wounded cardiovascular system, blood may leak out to the surroundings, disturbing the blood supply to normal cells. In response to these wounds, blood changes from a fluid state to form solid blood clots locally, which prevent further blood loss, restore the normal blood circulation and lead to the repair of the wounded vasculature (Versteeg et al., 2013).

Following acute vessel damage, disturbed endothelial cells that cover the luminal face of the vasculature may not be able to produce adequate anticoagulants; this in turn promotes a procoagulant environment. Most importantly, blood exposure to the subendothelial matrix underneath the endothelium triggers both primary and secondary haemostastic responses, which are facilitated by platelets and coagulation factors, respectively (Versteeg et al., 2013). Collagen in the subendothelium initiates platelet activation through platelet surface adhesion receptors, directing platelets to the compromised vessel sites to form a thin, sealing, layer (Nuyttens et al., 2011). Following this, platelets start aggregating to build a thick platelet plug, which sustains

the initial sealing of the injured vessel (Jackson, 2007). Simultaneously, platelets release their granule contents and release prothrombotic molecules in support of the amplification phase of their own activation (FitzGerald, 1991; Rendu and Brohard-Bohn, 2001). In addition, activated platelets expose phosphatidylserine (PS) from their inner to the outer leaflet of the plasma membrane to allow the assembling of coagulation factors (Zwaal and Schroit, 1997; Roberts et al., 2006). The platelet plasma membrane and cytoskeleton undergo massive rearrangement, thus these activated platelets quickly lose their discoid shape. The morphological changes of activated platelets do not just enhance the density of a platelet plug but also provide an enlarged platform, in the form of a greater plasma membrane surface area, for coagulation reactions to occur.

The coagulation cascade is considered to activate synchronously with platelet activation owing to exposure of subendothelial tissue factor to blood upon vascular damage. Tissue factor binds to circulating factor VII/VIIa to form the extrinsic tenase complex, which activates factor X and factor IX to factor Xa and factor IXa. This process results in a minute amount of thrombin being converted from prothrombin by factor Xa. However, this minute amount of thrombin is sufficient to further amplify the coagulation cascade by catalyzing the conversion of factor XI, factor VIII and factor V to their activated forms. As a result, factor VIIIa and the factor IXa, already produced, form the intrinsic tenase complex, which more efficiently converts factor X to factor Xa. Subsequently, assembling of factor Xa and factor Va on negatively

charged membranes leads to formation of the prothrombinase complex; this results in a large amount of thrombin being generated in a very short period of time. Alternatively, the coagulation cascade is initated through the contact pathway, which begins with factor XII activation. Factor XIIa also activates factor XI to factor XIa, which then activates factor IX and leads to the formation of intrinsic tenase complex. (Butenas and Mann, 2002). Thrombin is the most potent platelet agonist contributing to platelet plug formation (Brass, 2003). In addition, thrombin is the key component in the coagulation cascade because it cleaves soluble fibrinogen to form insoluble fibrin and activates factor XIII to crosslink the fibrin, which in turn strengthen platelet plugs at wounded vessel sites.

To stop bleeding, competent synergistic action among damaged vessels, activated platelets and coagulation factors is required. Many defects in platelets and coagulation factors cause an impaired haemostatic response. Some of these defects are associated with severe bleeding symptoms. For instance, gray platelet syndrome is attributed to platelets missing α-granules, and their cargo, that supports platelet activation; while hemophilia patients lack circulating factor VIII or factor IX. On the other hand, endogenous negative regulation of clot formation is also essential to ensure that haemostatic clots do not continue to grow in excess. Prostacyclin (PGI_2) and nitric oxide (NO) are produced by healthy endothelium and continuously inhibit platelet activation. Antithrombin and tissue factor pathway inhibitor (TFPI) are constitutively present in the circulation and also found in platelet granules (Blair and Flaumenhaft,

2009; Wood et al., 2014). Antithrombin inhibits thrombin, factor Xa, factor IXa, factor XIa and factor XIIa, while TFPI inhibits tissue factor-mediated factor X activation and also factor Va. In the presence of thrombomodulin and endothelial protein C receptors (EPCRs), activated protein C produced by thrombin causes degradation of factor Va and factor VIIIa. In addition, the clot-bound zymogen plasminogen can be converted by tissue plasminogen activator (tPA) or urokinase plasminogen activator (uPA) into its active form, plasmin, which dissolves existing fibrin in blood clots. All together, these regulators will prevent haemostatic clots from forming excessively (Versteeg et al., 2013). Despite the rigorous endogenous regulation of clot formation, irreversible changes to the circulation and vasculature resulting from aging and diseases, such as atherosclerosis and cancers, eventually lead to episodic inadequate regulation of clot formation. The resultant thrombosis is one of the top causes of morbidity and mortality worldwide **(Figure 1.1)** (Mozaffarian et al., 2015).

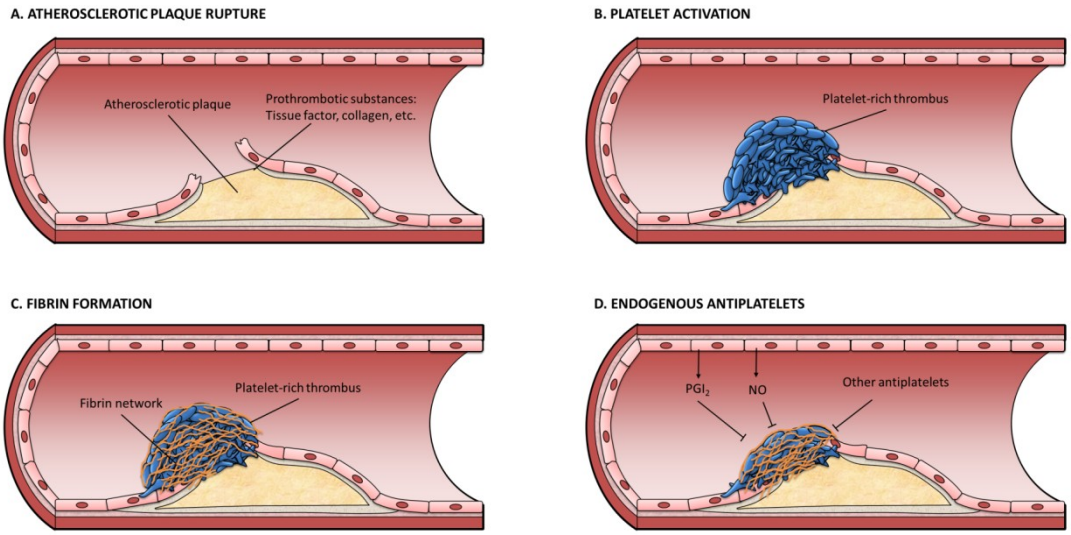
1.2 Atherosclerosis and statins

1.2.1 Lipid-lowering by statins in cardiovascular protection

Atherosclerosis is the major underlying pathological cause of coronary heart disease, stroke and transient ischemic attack. Atherosclerosis is an inevitable chronic disease that gradually alters arterial homeostasis throughout one's life. In the development of atherosclerosis, cholesterol, macrophages, cellular waste and other prothrombotic substances continually accumulate between the endothelium and the

Figure 1.1 Overview of thrombus formation initiated by atherosclerotic plaque rupture.

A. Exposure of prothrombotic substances, such as collagen and tissue factor, upon rupture of an atherosclerotic plaque. **B.** A platelet-rich plug formed on a ruptured atherosclerotic plaque. **C.** Fibrin formation stabilizes a platelet-rich plug. **D.** Endogenous antiplatelets regulate thrombus growth.


A. ATHEROSCLEROTIC PLAQUE RUPTURE
Atherosclerotic plaque
Prothrombotic substances:
Tissue factor, collagen, etc.
B. PLATELET ACTIVATION
Platelet-rich thrombus
C. FIBRIN FORMATION
Fibrin network
Platelet-rich thrombus
D. ENDOGENOUS ANTIPLATELETS
PGI2
NO
Other antiplatelets

tunica media in medium to large arteries. Even though affected vessels can remain asymptomatic for decades, the progression of atherosclerotic plaque will irreversibly narrow down the vessel lumen, which in turn impedes sufficient circulation to the tissue downstream of the affected arteries. More importantly, atherosclerotic plaques can rupture inducing thrombus formation, which may block vessels and cause permanent damage to the downstream tissue (Ross, 1993). The causes of atherosclerosis are not fully understood, however, many risk factors have been identified. These include hypercholesterolemia, smoking and diabetes (Chambless et al., 1997). Atherosclerotic plaques passively trap large amounts of atherogenic lipoproteins, such as low-density lipoprotein (LDL). As such, a logarithmic association between lowering the plasma level of LDL cholesterol and reduction of cardiovascular risks has been established in many studies (Neaton et al., 1992; Verschuren et al., 1995). In the context of slowing down atherosclerotic progression by attenuating lipid deposition, the statin family is the most widely prescribed class of lipid-lowering medications. All statins possess a mevalonate-like pharmacophore, which is structurally similar to the substrate of 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMG-CoA reductase), the rate-limiting enzyme in cholesterol biosynthesis. This characteristic of statins enables their competitive inhibition of HMG-CoA reductase and results in a decrease of cholesterol production and elevated expression of LDL receptors (Blumenthal, 2000; Poli, 2007). A number of large scale clinical trials have proven the lipid-lowering

efficacy of statins, as well as a reduction in cardiovascular morbidity and mortality (Wiviott et al., 2005). The lipid-lowering efficacy of atorvastatin was found to be greater than that of other statins, including fluvastatin, lovastatin, simvastatin and pravastatin (Jones et al., 1998). The exception to this is a new statin family member, rosuvastatin, which appears to be more effective than atorvastatin (McKenney et al., 2003). The therapeutic difference is likely a result of distinct structures of each statin. For instance, statins can be divided into two classes depending on their tissue permeability. Lipophilic statins such as atorvastatin can enter cells via passive diffusion while a hydrophilic statin, such as rosuvastatin, is unlikely to enter most cell types. This feature influences the metabolism of the statin, and in turn affects its efficacy (Germershausen et al., 1989). Presently, atorvastatin remains the most studied and one of the most prescribed statins.

1.2.2 Reduction of inflammation by statins in cardiovascular protection

Atherosclerosis is considered a chronic inflammatory disease owing to the heavy involvement of inflammatory cells, such as monocytes. The presence of atherosclerotic risk factors progressively leads to more interaction between endothelial cells and leukocytes. Endothelial cells at atherosclerotic lesions attract leukocytes to penetrate into the subendothelium and promote atherosclerotic progression through various mechanisms (Libby et al., 2002). Monocytes trapped in the intima, as an example, differentiate into macrophages, which take up accumulating lipoproteins and

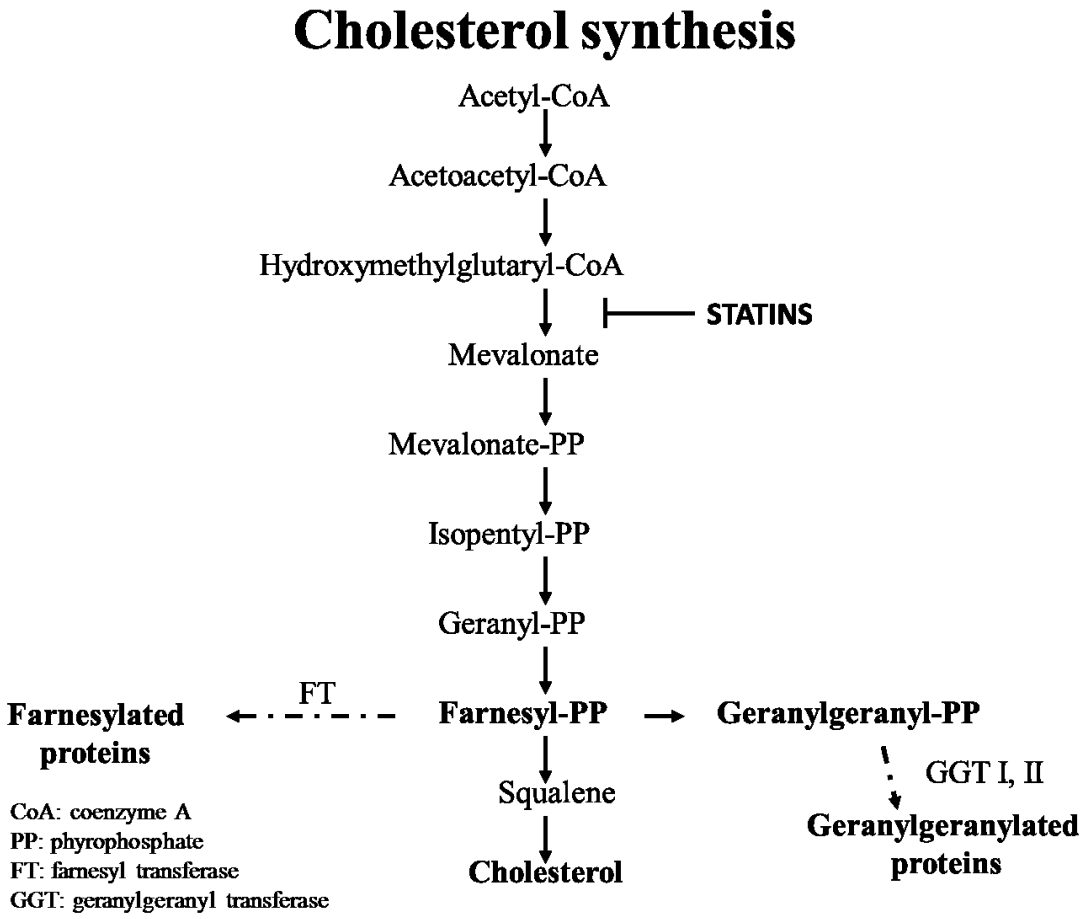
gradually grow into ultra-large foam cells. In addition, macrophages can proliferate and undergo apoptosis to generate cell waste. Apoptotic macrophages contribute to the formation of a necrotic core, which decreases the stability of an atherosclerotic plaque and makes the plaque more prone to rupture (Van Vre et al., 2012; Robbins et al., 2013).

The statin family has anti-inflammatory properties. The PRINCE trial revealed reduced serum C-reactive protein (CRP, an important inflammation marker) levels with statin treatment, independent of lipid-lowering (Albert et al., 2001). Subsequently, decreased circulating proinflammatory cytokine levels, attributable to statin treatment, were reported in patients with metabolic syndrome, hypercholesterolemia and diabetes mellitus (van de Ree et al., 2003; Ascer et al., 2004; Bulcao et al., 2007). As with lipid-lowering, the anti-inflammatory effects of stains are also attributed to their inhibition on HMG-CoA reductase.

Cholesterol biosynthesis is a multi-step process, which requires HMG-CoA reductase activity to firstly convert acetyl-CoA to mevalonate. Owing to HMG-CoA reductase's participation at the early stage of this step-wise synthesis, competitive inhibition of HMG-CoA reductase by statins does not just result in a reduction of cholesterol synthesis, but also affects the biosynthesis of a number of intermediates prior to the generation of cholesterol. These intermediates include farnesyl pyrophosphate and geranylgeranyl pyrophosphate **(Figure 1.2)** (Zhou and Liao, 2009). Importantly, these intermediates are involved in a variety of reactions that are

Figure 1.2 Cholesterol synthesis and statins.

Cholesterol synthesis is a step-wise reaction. Statins inhibit this reaction at the early stage and result in a reduction of farnesyl pyrophosphate and geranylgeranyl pyrophosphate, which participate in protein post-translational modification.


Cholesterol synthesis
Acetyl-CoA
Acetoacetyl-CoA
Hydroxymethylglutaryl-CoA
STATINS
Mevalonate
Mevalonate-PP
Isopentyl-PP
Geranyl-PP
FT
Farnesylated proteins
Farnesyl-PP
Geranylgeranyl-PP
GGT I, II
Geranylgeranylated proteins
Squalene
Cholesterol
CoA: coenzyme A
PP: phyrophosphate
FT: farnesyl transferase
GGT: geranylgeranyl transferase

independent of lipid metabolism. Farnesyl pyrophosphate and geranylgeranyl pyrophosphate can be covalently added to cysteine residues at or near the C-terminus of a number of proteins that contain consensus sequences of CaaX box (**C**: cysteine, **aa**: any aliphatic amino acids, **X**: C-terminal amino acid that determines the substrate specificity) by specific enzymes, farnesyltransferase, geranylgeranyltransferase I and II. These post-translational modifications of proteins are termed prenylation (Zhang and Casey, 1996). Prenylation can determine the cellular location and activity of the proteins. Reduced prenylation by statins impairs signaling pathways of many small guanine nucleotide triphosphatases (GTPases), including Rho, Rac and Ras, resulting in decreased proinflammatory mediators and elevated expression of genes that are associated with protection from atherosclerosis (Zhou and Liao, 2009).

1.2.3 Pleiotropic effects of statins in cardiovascular protection

In addition to the high content of lipoproteins and inflammatory cells, atherosclerotic plaques are also rich in various prothrombotic substances. For instance, the large amounts of collagen and tissue factor in the lesions are directly responsible for robust thrombus growth after the rupture of an atherosclerotic plaque (Libby, 2008). Interestingly, statins were also found to decrease tissue factor expression in atherosclerotic lesions via a Rho-dependent mechanism (Eto et al., 2002). Indeed, many pleiotropic effects of statins independent of lipid-lowering are the result of a reduction in isoprenoid synthesis and downstream events of retarded small GTPase

signaling (Zhou and Liao, 2009). Decreased Rho guanosine triphosphate (GTP)-binding activity in endothelial cells has been found with statin treatment, this in turn results in elevated endothelial nitric oxide synthase (eNOS) expression and activity (Laufs and Liao, 1998). eNOS represents one of the most important anti-atherothrombotic defenses in the vasculature. Constitutive expression of eNOS in the healthy vasculature is responsible for persistent production of NO. As a small gaseous signaling molecule, NO diffuses freely across cell membranes and regulates a number of biological processes. These are potent, despite NO's extremely short half-life of a few seconds. For instance, NO dilates blood vessels by smooth muscle cell relaxation, and NO released into the vascular lumen is a potent platelet inhibitor (Forstermann and Munzel, 2006). Enhanced NO bioavailability is recognized as one of the major cardiovascular benefits of statins.

1.3 Platelets and arterial thrombosis

Platelets are small anucleated cells, which are derived from fragmentation of bone marrow megakaryocytes' cytoplasm (Machlus and Italiano, Jr., 2013). One megakaryocyte can give rise to thousands of platelets and these platelets continue to mature in the circulation to become 2-3 μm in diameter (Paulus, 1975). Everyday approximately 10% of circulating human platelets are newly released from megakaryocytes. Everyday 10% of platelets are removed from the circulation by phagocytosis in the spleen and liver. These two distinct processes enable a constant number of platelets, 150 – 400 thousand per mL in normal human blood (Harker et al.,

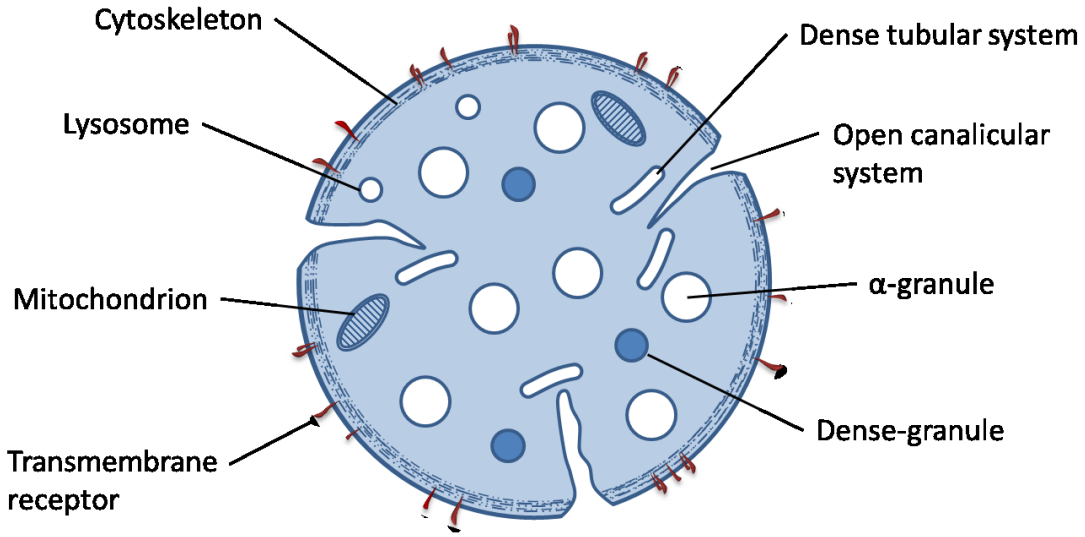
2000). By comparison, murine blood contains a much higher platelet count of 400 – 1600 thousand per mL (Jirouskova et al., 2007).

In addition to their roles in would healing and inflammation, the principal physiological function of platelets is haemorrhage arrest. Circulating platelets flow along the vessel wall (Aarts et al., 1988). This allows platelets to rapidly detect insults to the integrity of the vasculature. Platelets have a unique surface-connected open canalicular system (OCS) and a dense tubular system, near the OCS, beneath their cytoplasmic membrane. On the cell membrane, an abundance of transmembrane receptors can facilitate intricate "inside-out" and "outside-in" signaling in response to changes in their microenvironment. In addition, numerous proteins and small bio-active molecules are stored in platelet granules (Hartwig, 2002). All these features allow platelets to perform their haemostatic function at the site of the damaged vessel **(Figure 1.3)**.

However, excessive platelet activation can be problematic. It is noteworthy that, in opposition to venous systems, platelet-mediated clot formation outweighs coagulation-mediated clot formation in arteries. Completely different shear rates, and distinct inhibitors and stimulators generated in the two types of vessels at least in part determine the fact that venous blood clots are rich in fibrin and arterial clots largely consist of platelets. Moreover, arteries, unlike veins, are highly influenced by atherosclerosis. Indeed, the vast majority of myocardial and cerebral infarctions are associated with thrombus formation on ruptured atherosclerotic plaques. Thus,

Figure 1.3 Simplified cellular structure of a resting platelet.

The platelet surface is rich in transmembrane receptors. Platelets have two unique membrane systems, surface-connected open canalicular system and dense tubular system. Underneath the plasma membrane, the cytoskeleton consist of microtubules, actin and myosin is important for the cell shape. Platelets may possess a few mitochondria and lysosomes. The most abundant granule types inside of a platelet are α-granules and dense-granules.


Cytoskeleton
Lysosome
Mitochondrion
Transmembrane receptor
Dense tubular system
Open canalicular system
α-granule
Dense-granule

anticoagulants are often used to attenuate venous thrombosis even though antiplatelets, such as acetylsalicylic acid (ASA), are also proved to be effective (Brighton et al., 2012). In contrast, although anticoagulants, such as heparin, are useful (Hirsh et al., 2001), drugs that attenuate atherosclerotic progression and antiplatelets together are central in the prevention and treatment of arterial thrombosis.

1.3.1 Platelet adhesion

Adhesive proteins that attract platelets are abundant in the subendothelial matrix of vessels. Among them, collagen is the most important adhesive ligand that directs platelet adhesion upon vessel damage. This is accomplished by interactions of collagen (type I and type III) with three major collagen receptors on the platelet membrane, GPIb-IX-V complex, GPVI and $\alpha_2\beta_1$. The binding of circulating platelets onto an immobilized surface is acknowledged as the first critical step in platelet-mediated hemorrhage arrest and it is also involved in the inflammatory response. Of note, aberrant endothelial cells under the influence of a number of diseases, such as atherosclerosis, can alter their microenvironment to promote platelet adhesion or even thrombosis. When an atherosclerotic plaque ruptures, a large amount of collagen and other trapped adhesive substances activate platelets (Ruggeri and Mendolicchio, 2007).

1.3.1.1 GPIb-IX-V

The average platelet expresses approximately 25,000 GPIb-IX-V complexes. A GPIb-IX-V complex is comprised of four polypeptides, some duplicated: two GPIbα

(143 kDa, 610 amino acids), two GPIbβ (22 kDa, 181 amino acids), two GPIX (20 kDa, 160 amino acids) and one GPV (82 kDa, 544 amino acids). GPV is in the middle of the complex and the other subunits are symmetrically spread in a linear row on each side of GPV. Both GPV and GPIX are non-covalently associated with GPIb. GPIbα and GPIbβ are linked by a disulfide bond (Berndt et al., 2001; Kauskot and Hoylaerts, 2012).

The extracellular portion of GPIb-IX-V complex contains multiple ligand binding sites. The interaction of GPIb-IX-V complex with collagen is indirect. von Willebrand Factor (vWF) is a multimeric glycoprotein synthesized and stored by megakaryocytes/platelets and endothelial cells. Endothelial cells, as the major source of vWF, constitutively release vWF into circulating blood and deposit it onto the subendothelium (Giblin et al., 2008). vWF plays two important roles in haemostasis. In addition to stabilizing factor VIII in the circulation, immobilized vWF acts as a bridge between the GPIb-IX-V complex and collagen (De Meyer et al., 2009). The A1 and A3 domains of vWF mediate the binding to collagen to the subendothelium and the A1 domain binds circulating platelets by tethering to GPIbα subunits of the GPIb-IX-V complex (Arya et al., 2002; Ruggeri, 2002b).

1.3.1.2 GPVI and $\alpha_2\beta_1$

GPVI and $\alpha_2\beta_1$ (GPIa/IIa) are the other two important collagen receptors that mediate platelet activation upon adhesion (Nuyttens et al., 2011). GPVI is a 63 kDa transmembrane glycoprotein exclusively expressed on megakaryocytes and platelets.

The average platelet expresses roughly 3,700 copies of GPVI. Each GPVI molecule is associated with an FcRγ homodimer. The coassembly of GPVI and FcRγ is a prerequisite for normal expression and signaling transduction of GPVI. $\alpha_2\beta_1$ is an integrin originally found on T cells and fibroblasts. Platelets also express a variable amount of $\alpha_2\beta_1$, ranging from 900 to 4,000 copies per platelet. $\alpha_2\beta_1$ is composed of a 150 kDa α_2 chain and a 130 kDa β_1 chain (Clemetson and Clemetson, 2001; Ruggeri and Mendolicchio, 2007; Kauskot and Hoylaerts, 2012).

1.3.1.3 Relative contribution of collagen receptors in platelet adhesion

Deficiencies of components of the GPIb-IX-V complex and of GPVI have been reported in humans. Patients with Bernard-Soulier syndrome have defects in GPIb. These patients often present with thrombocytopenia and a severe bleeding diathesis (Lanza, 2006). Defects in GPVI, in comparison, only result in mild bleeding symptoms in most patients (Dumont et al., 2009; Hermans et al., 2009). Even though complete $\alpha_2\beta_1$ deficiency has not been reported in humans, variable expression of $\alpha_2\beta_1$ caused by several allelic polymorphisms also modestly influences platelet adhesion (Santoro, 1999; Moshfegh et al., 1999).

Mice lacking FcRγ, which results in GPVI deficiency, and mice lacking $\alpha_2\beta_1$, do not exhibit consistent defects in arterial thrombus formation (Ruggeri and Mendolicchio, 2007; Nieswandt et al., 2011). In contrast, GPIbα-deficient mice have a phenotype similar to that of human Bernard-Soulier syndrome and exhibit significantly impaired arterial thrombus formation (Kanaji et al., 2002; Bergmeier et

al., 2006; Konstantinides et al., 2006; Strassel et al., 2007). The GPIb-IX-V complex is believed to primarily mediate reversible platelet adhesion at high shear rates. It is noteworthy that the binding of GPIb-IX-V complex to vWF *ex vivo* not only mediates platelet adhesion but also promotes platelet aggregation at extremely high shear rates (>10,000 sec^{-1}) (Ruggeri et al., 2006; Ruggeri and Mendolicchio, 2007).

1.3.1.4 Other contributors to platelet adhesion

In addition to the three adhesion receptors, GPIV and p65 protein may also interact with immobilized collagen, although their contribution may be limited in terms of supporting platelet adhesion. To a large extent, platelet adhesion is a process that can extend beyond a receptor's direct interactions with collagen owing to the exceedingly promiscuous function of many platelet receptors and the large number of adhesive ligands embedded in the subendothelium. The platelet aggregation receptor $\alpha_{IIb}\beta_3$ recognizes the Arg-Gly-Asp (RGD) sequence of vWF. It also binds to the same motif in fibronectin, which is another abundant adhesive protein in the subendothelium. In addition, fibronectin can be recognized by another platelet receptor, $\alpha_5\beta_1$. As one of the major components in the subendothelium, laminin can trigger platelet binding via GPVI, as well as the laminin receptor, $\alpha_6\beta_1$. It is noteworthy that thrombospondin is another adhesive protein that is not just packed in platelets but also is abundant in atherosclerotic plaques (Ruggeri and Mendolicchio, 2007).

Taken together, the relative role of each known adhesive bond in platelet

adhesion is highly dependent on the local shear rate and the types of ligands that are exposed upon vessel damage. It is also important to understand platelet adhesion when the nonconventional adhesive ligands are presented in the context of other diseases. For instance, tumour podoplanin causes platelets adhesion to the tumour via platelet C-type lectin-like receptor 2 (CLEC-2), which in turn facilitates tumour progression (Suzuki-Inoue et al., 2007). Atherosclerotic plaques have a heightened thrombogenic potential, the aberrant vessel bed may, in turn, greatly alter the relative contribution of specific platelet adhesive bonds compared with the normal haemostatic response.

1.3.2 Platelet aggregation

Following platelet adhesion, immobilized platelets become activated and recruit more circulating platelets to the injury site. In this process, platelets no longer directly interact with the subendothelium but with their own. The self-cohesion of platelets is termed platelet aggregation. The original concept of platelet aggregation is thought to be mainly mediated by the aggregation receptor $\alpha_{IIb}\beta_3$ and its ligands, fibrinogen and fibrin. In addition, platelets can be bridged together via the bonds between $\alpha_{IIb}\beta_3$ and soluble vWF or fibronectin. Recent studies also suggested alternative bonds that contribute to platelet aggregation (Jackson, 2007).

1.3.2.1 $\alpha_{IIb}\beta_3$

$\alpha_{IIb}\beta_3$ (GPIIb/IIIa) is an integrin containing two subunits: α_{IIb} (145 kDa, 1008 amino acids) and β_3 (95 kDa, 762 amino acids). The two subunits are linked by a disulfide bond. $\alpha_{IIb}\beta_3$ is the most abundant receptor in platelets, which contain

approximately 80,000 of them per cell (Kauskot and Hoylaerts, 2012). The majority of $\alpha_{IIb}\beta_3$ is expressed on the plasma membrane of platelets. The receptor is also found in α-granules and the OCS (Nurden et al., 1999). Platelet activation increases the number of $\alpha_{IIb}\beta_3$ molecules on the surface, even before α-granule release, implying that $\alpha_{IIb}\beta_3$ in the OCS can come to the surface (Suzuki et al., 1992; Suzuki et al., 1994). Upon platelet activation, $\alpha_{IIb}\beta_3$ undergoes a conformational change. This leads to the separation of the intracellular and transmembrane portions of the two subunits and ultimately causes the ectodomains to loosen from a highly compact structure to the high affinity state for fibrinogen binding (Coller and Shattil, 2008).

1.3.2.2 Principal ligands of $\alpha_{IIb}\beta_3$

Fibrinogen is a glycoprotein that is synthesized by the liver. It is comprised of three pairs of non-identical chains linked by disulfide bonds. They are the α, β and γ chains with molecular weights of 63.5, 56 and 47 kDa, respectively (McKee et al., 1970). A small portion of fibrinogen is stored inside of α-granules in platelets, though it is predominantly found in plasma (at 200-400 mg/dL in human blood) (Harrison et al., 1989; Tennent et al., 2007). A major role of fibrinogen in haemostasis is to be cleaved by thrombin at the N-termini of the α and β chain to generate fibrin and subsequently form a fibrin network at sites of injured vessels. In addition, both fibrinogen and fibrin are capable of linking adjacent platelets to form a platelet plug during haemostatic arrest. Fibrinogen and fibrin possess two important peptide sequences to enable their interaction with $\alpha_{IIb}\beta_3$. RGD located close to the C-terminus

of α chain and Lys-Gln-Ala-Gly-Asp-Val sequence (KQAGDV) located close to the C-terminus of the γ chain mediate the binding to the ectodomains of the β_3 and α_{IIb} subunit, respectively. It was found that mutated RGD sequence or RGD sequence either blocked by a monoclonal antibody or digested by plasmin had little effect on platelet binding, suggesting that KQAGDV may be a more important binding site than RGD for support of platelet aggregation (Cheresh et al., 1989; Smith et al., 1990; Farrell et al., 1992). Similar to fibrinogen, vWF (10 μg/mL in human blood) and fibronectin (300 - 400 μg/mL in human blood), also possess RGD sequences (Lip and Blann, 1997; To and Midwood, 2011).

1.3.2.3 The role of $\alpha_{IIb}\beta_3$ and its ligands in platelet aggregation

Amongst all the important components in platelet aggregation listed above, congenital deficiencies of $\alpha_{IIb}\beta_3$, fibrinogen and vWF have all been reported in humans. Humans deficient in fibronectin have not been reported. Inherited deficiencies of $\alpha_{IIb}\beta_3$ and fibrinogen, known as Glanzmann thrombasthenia and afibrinogenemia, are extremely rare autosomal recessive disorders and often present with a variable bleeding diathesis (Coller et al., 1987; al Mondhiry and Ehmann, 1994). In contrast, the occurrence of congenital deficiencies of vWF, also known as von Willebrand disease (vWD), is common. In most cases, patients with vWD have an insufficient amount of vWF. These patients remain asymptomatic or have a mild bleeding tendency. However, a rare form of vWD, type 3 vWD, is associated with complete vWF deficiency. Similar to Glanzmann thrombasthenia and afibrinogenemia,

patients inherit type 3 vWD in an autosomal recessive manner and frequently have a very serious bleeding phenotype (Lillicrap, 2013).

Over the past two decades, mice that lack $\alpha_{IIb}\beta_3$, fibrinogen or vWF became available. Even though fibronectin deficiency is embryonically lethal, a conditional knock-out of soluble fibronectin in mouse plasma was viable. Light transmission aggregometry has shown that platelets from $\alpha_{IIb}\beta_3$ and fibrinogen knock-out mice fail to aggregate, suggesting the central roles of the two in platelet aggregation. $\alpha_{IIb}\beta_3$, fibrinogen and vWF-deficient mice all exhibit prolonged bleeding time and spontaneous bleeding events. In addition, all three deficiencies are associated with reduced thrombus formation, suggesting their involvement in both haemostasis and thrombosis. Of importance, mice lacking $\alpha_{IIb}\beta_3$, fibrinogen or vWF display the clinical features of Glanzmann thrombasthenia, afibrinogenemia and vWD, respectively (Denis et al., 1998; Hodivala-Dilke et al., 1999; Ni et al., 2000; Smyth et al., 2001).

1.3.2.4 Other contributors to platelet aggregation

Despite the important roles of the above three ligands in platelet aggregation, no single deficiency leads to the complete absence of thrombus formation. This reflects the compensatory back up of the three ligands in platelet aggregation. Of importance, fibrinogen and vWF deficiency together result in impaired thrombus formation featuring combined phenotypes of the single mutations (Ni et al., 2000). Surprisingly, *in vivo* platelet aggregation can still occur in mice lacking of all three ligands: fibrinogen, vWF and plasma fibronectin (Reheman et al., 2009). Similarly, $\alpha_{IIb}\beta_3$

deficiency results in reduced but not abolished thrombus formation *in vivo* (Smyth et al., 2001). Taken together, the evidence indicates the existence of overlooked or unidentified adhesion molecule partners in platelet aggregation (Brass et al., 2005).

It is noteworthy that vWF multimer and self-assembled fibronectin can potentially provide multiple $\alpha_{IIb}\beta_3$ binding sites (Lip and Blann, 1997; To and Midwood, 2011). vWF multimers have been found to unfold themselves in response to arterial shear rates. The elongated form of a vWF multimer presents an array of binding sites in a linear format, which in turn engage platelets at much greater physical distance. On the other hand, circulating vWF multimers can be degraded by the metalloproteinase, ADAMTS13, which downregulates the activity of these multimers. Most importantly, it has been suggested that unfolded vWF, but not fibrinogen, is the major ligand enabling platelet aggregation against extremely high shear rates. Such platelet aggregation is likely mediated by GPIb-IX-V complex, but not $\alpha_{IIb}\beta_3$ (Siedlecki et al., 1996; Ruggeri et al., 2006; Zheng et al., 2015).

1.3.3 Platelet degranulation

In addition to a few lysosomes and T-granules, the major granule types that platelets possess are α-granules and dense-granules, which are important to the haemostatic function of platelets (Thon et al., 2012; Golebiewska and Poole, 2014). Over 300 active molecules inherited from megakaryocytes, taken from the circulation, or generated by platelets themselves are sorted in these intracellular granules (Rendu

and Brohard-Bohn, 2001). Platelet degranulation, also known as platelet release, is a process driven by a family of proteins called soluble N-ethylmalemide-sensitive factor attachment protein receptors (SNAREs), which lead to the fusion of the platelet cytoplasmic and the granule membrane and exocytosis of the granule contents (Marks, 2012). The regulation of platelet degranulation is poorly understood. However, it is certain that platelets release their granule contents in a focal stimulation-responsive manner. Externalized platelet granule contents are involved in a variety of physiological and pathological events. Platelet degranulation, as a result of platelet activation, also promotes platelet activation, which enables the recruitment of resting platelets by activated platelets during haemostasis (Rendu and Brohard-Bohn, 2001).

1.3.3.1 α-granules

α-granules are the most abundant type of granules in platelets. There are 50-80 per platelet. The fusion of α-granule and cytoplasmic membrane upon platelet activation significantly enlarges the platelet surface area, which supports highly efficient cellular interaction (Blair and Flaumenhaft, 2009). Thus, membrane proteins expressed on the α-granule membrane, including $\alpha_{IIb}\beta_3$ and GPIb-IX-V, become exposed on the platelet surface following the merging of two membrane systems (Suzuki et al., 1992; Berger et al., 1996). In addition, α-granules also carry a number of membrane-associated proteins that are not usually expressed on the surface of circulating platelets. For instance, P-selectin, a cell adhesion protein, is translocated from the membrane of α-granules to the cytoplasmic membrane during α-granule

release (Stenberg et al., 1985).

Numerous adhesive proteins and all three principal aggregation ligands are highly concentrated in platelet α-granules, reflecting the role of externalized α-granule contents in primary haemostasis. Moreover, platelet α-granules are rich in several coagulation factors either endocytosed from plasma, or synthesized and distributed by megakaryocytes, such as factor V, factor XI and factor XIII, suggesting the participation of α-granule releasates in secondary haemostasis. In addition, many soluble proteins originating from platelets, such as β-thromboglobulin and platelet factor-4, are packed in α-granules. Many of these are, too, released to the circulation upon platelet activation and mediate the processes of haemostasis (Rendu and Brohard-Bohn, 2001).

It is noteworthy that platelet α-granules house many proteins that have opposing biological functions. A large number of molecules packed in α-granules are able to suppress platelet activation and coagulation, which can in turn abolish the function of those that promotes platelet activation and coagulation. For instance, antithrombin, TFPI and plasminogen are all found inside of α-granules (Rendu and Brohard-Bohn, 2001; Blair and Flaumenhaft, 2009). This property of α-granules makes it difficult to properly define their haemostatic function. Several lines of evidence have suggested that α-granules may be divided into different populations or α-granules themselves may be divided into small compartments by sorting proteins differently (Italiano, Jr. et al., 2008; Nispen tot et al., 2010). Most importantly, α-granule releasates from

activated platelets are not identical in response to different *in vitro* stimuli (Italiano, Jr. et al., 2008; Battinelli et al., 2011). Taken together, these findings support that platelets can selectively release their α-granule contents according to a change of their microenvironment and that the contribution of α-granules to haemostasis and thrombosis may be finely determined by the local factors that activate platelets.

1.3.3.2 Dense-granules

Individual platelet possesses far fewer, approximately 3-8, dense-granules than α-granules (Blair and Flaumenhaft, 2009). The secretion machineries may also be different between the two types of granules, with a much more rapid release of dense-granule contents occurring, regardless of the stimulus (Jonnalagadda et al., 2012). Moreover, in comparison to these macro-sized proteins packed in α-granules, the molecules housed in dense-granules are smaller. Dense-granules contain a substantial amount of ions such as calcium (Ca^{2+}) and magnesium (Mg^{2+}), and small molecules such as adenosine diphosphate (ADP) and adenosine triphosphate (ATP), serotonin (5-hydroxytryptamine, 5-HT), histamine and polyphosphate (Rendu and Brohard-Bohn, 2001). ADP is one of the major agonists responsible for the amplification phase of platelet activation. ADP does not just enhance intracellular Ca^{2+} level upon platelet activation, but also decreases cyclic adenosine monophosphate (cAMP) formation by adenylyl cyclase (AC) in activated platelets. The latter suppresses platelet inhibition by PGI_2 and therefore further promotes platelet activation (Daniel et al., 1998; Yang et al., 2002).

1.3.3.3 Platelet granules in platelet activation

Congenital deficiencies of platelet α-granules and of dense-granules have been described in humans. Arthrogryposis-Renal dysfunction-Cholestasis (ARC) and Gray Platelet Syndromes (GPS) are characterized by α-granule defects. ARC results from abrogated expression of the trafficking proteins VPS33B or VIPAR (Gissen et al., 2004; Cullinane et al., 2010; Smith et al., 2012). The causative gene for GPS is NBEAL2 (Albers et al., 2011; Gunay-Aygun et al., 2011; Kahr et al., 2011). ARC and GPS mouse models have recently been generated by knocking out the VPS33B or NBEAL2 genes. The two models closely replicate human α-granule deficiencies (Kahr et al., 2013; Bem et al., 2015). Prolonged bleeding times, and partially impaired *in vitro* platelet activation or *in vivo* thrombus formation were observed in mice lacking α-granules and a significant reduction in α-granule cargos. Griscelli, Hermansky-Pudlak (HPS) and Chediak-Higashi Syndromes (CHS) are associated with human dense-granule deficiency. Frequently, patients with dense-granule deficiency display prolonged bleeding times and attenuated secondary aggregation (Huizing et al., 2001; Menasche et al., 2003). Several murine models of Griscelli syndromes, HPS and CHS have also been used to study the function of dense-granules. These includes ashen mice lacking Rab27a, a subclass of GTPases, which exhibit the phenotypes of Griscelli syndromes and HPS (Novak et al., 2002). Mice lacking the LYST gene, also known as beige mice, replicate the lethal defects of human CHS (Ward et al., 2000).

1.3.4 Excitatory agonists and platelet signaling

Platelets possess a large number of important receptors that are highly sensitive and responsive to extracellular stimuli. In addition to collagen and vWF provided by the vessel and ADP externalized from platelet dense-granules, there are various sources of agonists that trigger platelets to activate via platelet surface receptors. Despite a basal level of agonists normally present in blood or carried by circulating cells, platelets remain inactivate by virtue of the consistent suppression by endogenous inhibitors. The accumulation of platelet excitatory agonists above a certain threshold concentration is critically important for platelet activation in high blood flow, which constantly washes these agonists away. The large amounts of agonists that are generated, exposed and trapped at sites of vessel injury can greatly tilt the balance of local pro- and anti-platelet activation properties and enable platelet activation to occur. These include thrombin, ADP and thromboxane A_2 (TXA_2). Following the interaction of excitatory agonists with their respective receptors on platelet surface, the intracellular domains of these receptors mediate robust signal transduction. As a result, platelets aggregate, release their granule contents and alter their shape. All these activities not only promote platelet plug formation but also serve as a positive feedback mechanism that generates more agonists to amplify platelet activation (Bye et al., 2016).

1.3.4.1 Thrombin and its receptors

Thrombin is a “trypsin-like” serine protease with a molecular weight of 37 kDa.

It is believed that the majority of thrombin is generated on the activated platelet surface (Roberts et al., 2006). This concept, however, has recently been challenged by a line of evidence implicating the activated endothelium as the major surface for *in vivo* thrombin generation (Ivanciu et al., 2014). Thrombin has an average half-life of less than a minute *in vivo* and is rapidly inactivated by its inhibitors, such as antithrombin (Ruhl et al., 2012). In addition to the central role of thrombin on fibrin network generation, it has multiple functions in mediating clot formation. As a product of the coagulation cascade, thrombin serves as positive feedback to the reaction by activating an array of coagulation factors, including factor V, factor VIII and factor XI. Contrarily, thrombin negatively mediates its formation by activating protein C to inactivate factor Va and factor VIIIa. Last but not least, thrombin also inhibits fibrinolysis by activating thrombin-activatable fibrinolysis inhibitor and promotes inflammation (Crawley et al., 2007).

The most relevant function of thrombin in this thesis is its potential for activating platelets. Thrombin is the most potent platelet agonist. Human platelets express two types of thrombin receptors. They are protease-activated receptors 1 (PAR1) and 4 (PAR4). The average platelet carries approximately 2,000 copies of PAR1 and the expression number of PAR4 has not yet been confirmed. Both PAR1 and PAR4 mediate thrombin signaling; though PAR1 is considered to predominate because of its activation by lower thrombin concentrations than required for PAR4 activation. Thus, PAR1 is the major thrombin receptor and PAR4 acts as an amplifier in human platelets.

As typical G protein-coupled receptors (GPCRs), each PAR contains seven transmembrane domains, an extracellular N-terminus and an intracellular C-terminus. One of the important features of PARs is that they are receptors carrying their own tethered ligand. Thrombin cleaves a small fragment of PAR1 at the N-terminus to generate a new unmasked N-terminus containing the sequence of Ser-Phe-Leu-Leu-Arg-Asn (SFLLRN). The new terminus serves as a tethered ligand to interact with the second extracellular loop of PAR1 receptor itself, which in turn activates the receptor (Coughlin, 2000). Murine platelets express PAR4 and PAR3 instead of PAR1 and PAR4. Murine PAR4 functions similarly to human PAR1, while PAR3 is an amplifier. The sequence that activates murine PAR4 is Gly-Try-Pro-Gly-Lys-Phe (GYPGKF).

1.3.4.2 ADP and its receptors

ADP is an essential molecule that is involved in cellular energy transfer. ADP consists of a sugar backbone, which is attached to an adenine and two phosphate groups. The addition of a third phosphate group to ADP to generate ATP continually occurs in living subjects, which in turn serves as energy storage. Similar to thrombin, ADP has an extremely short half-life in plasma. This also determines the very local effect of ADP in mediating platelet plug formation. In addition to platelet dense-granules, damaged erythrocytes and endothelial cells are potential resources of ADP and may also contribute to ADP-induced platelet activation (Woulfe et al., 2001).

Platelets possess three types of purinergic receptors, $P2X_1$, $P2Y_1$ and $P2Y_{12}$.

$P2X_1$ is a ligand-gated non-selective cation channel, which mediates low level of platelet activation by allowing extracellular Ca^{2+} to enter platelets in response to ATP. $P2Y_1$ and $P2Y_{12}$ are ADP receptors. There are approximately 150 copies of $P2Y_1$ and 400-1,000 copies of $P2Y_{12}$ found on individual human platelets (Ohlmann et al., 2013). The two are GPCRs, which share a similar molecular structure with PARs. Maximal platelet aggregation by ADP requires the presence of both $P2Y_1$ and $P2Y_{12}$. Of note, the two receptors are associated with different G proteins, which in turn results in diverse activation signaling in response to ADP. $P2Y_1$ signaling causes platelet shape change, a transient rise of cytoplasmic Ca^{2+} and reversible aggregation while $P2Y_{12}$ signaling leads to irreversible platelet aggregation, degranulation and augmentation of platelet activation mediated by other agonists, such as thrombin (Woulfe et al., 2001).

1.3.4.3 TXA_2 and its receptors

Platelets synthesize TXA_2 in response to a variety of agonists, such as thrombin and collagen. The production of TXA_2 starts with arachidonic acid, derived from platelet membrane phospholipids, being freed by phospholipases (PLAs). Arachidonic acid is quickly converted into prostaglandin H_2 (PGH_2) by the membrane-bound enzyme, cyclooxygenase-1 (COX-1) and subsequently converted to TXA_2 by TXA_2 synthase (Samuelsson et al., 1978). Similar to thrombin and ADP, TXA_2 survives for only a short time in plasma, degrading spontaneously with an average half-life of 20 seconds. In contrast to the multiple sources of ADP, platelets are the sole source of TXA_2. Importantly, both ADP and TXA_2 are largely supplied by activated platelets

themselves during clot formation and play critical roles in amplifying platelet activation in an autocrine and a paracrine manner (Bye et al., 2016).

There are roughly 1,500 copies of the TXA_2 receptor, TP, expressed on a platelet (Nurden et al., 2003). Similar to PARs and P2Ys, TP is also a GPCR. Two isoforms of TP, TP_α and TP_β, have both been reported in human platelets. TP_α is preferentially identified as the predominant one mediating platelet activation in response to TXA_2 stimulation, while the expression and function of TP_β in platelets remains controversial (Dorn, 1989). TP signaling leads to increased cytoplasmic level of Ca^{2+} in platelets, platelet shape change and aggregation (Paul et al., 1999).

1.3.4.4 Other agonists and receptors in platelet activation

Thrombin, ADP and TXA_2 are identified as the major soluble platelet agonists responsible for platelet activation. In addition to these, there are a number of weak platelet agonists that have also been identified. These include serotonin and epinephrine. Serotonin does not independently induce platelet aggregation and degranulation. It, however, potentiates platelet activation in response to thrombin and ADP after binding to its GPCR, 5-HT_{2A} receptor (Li et al., 1997). Epinephrine, a circulating hormone, may be capable of independently mediating platelet aggregation, though its primary role is, too, to potentiate platelet activation by other excitatory agonists after binding to its GPCR, α_2-adrenergic receptor (Shattil et al., 1989). The recent discovery of the expression of CLEC-2 on platelet surface has introduced an important platelet agonist, podoplanin (Suzuki-Inoue et al., 2007). Other excitatory

agonists and their receptors may still remain unknown **(Figure 1.4)**.

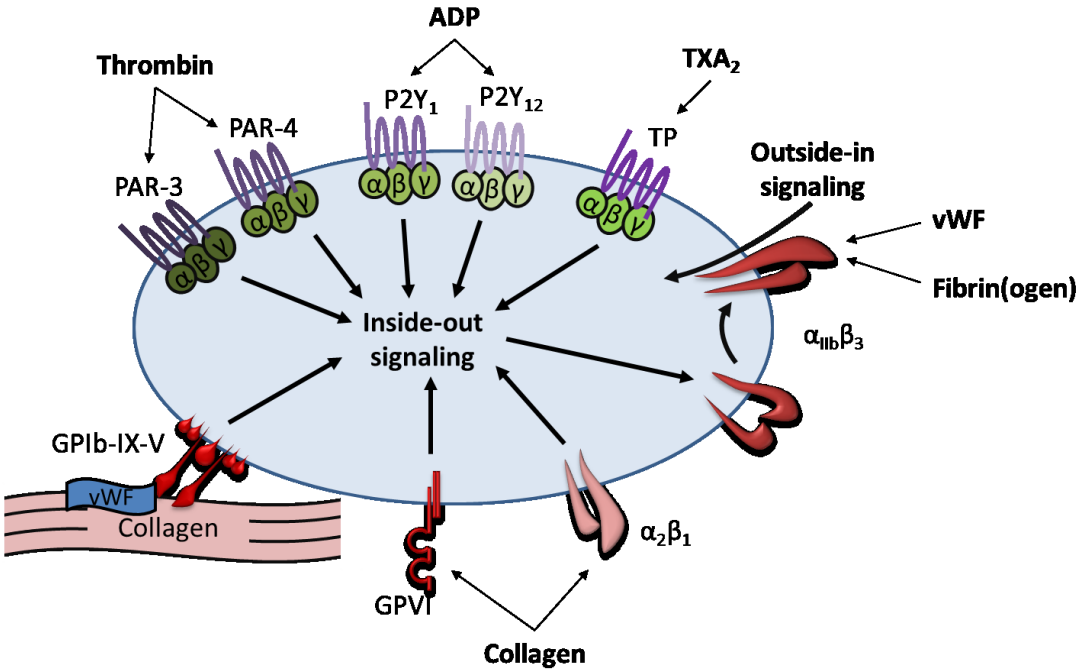
1.3.4.5 G proteins

Platelet activation signaling through GPCRs in response to many agonists is mediated by G protein complexes, which interact with the intracellular regions of cytoplasmic membrane-bound GPCRs. G protein complexes are comprised of three heterotrimeric subunits, α, β, and γ, and each of them has several isoforms. Twenty three α, 7 β, and 12 γ isoforms have been identified and many of them have been found in platelets. G protein complexes possess different cellular function, which is determined by the subunit isoforms. Currently, G protein complexes are classified based on the α-subunit isoforms (Oldham and Hamm, 2008).

An individual G protein complex contains one of each α, β, and γ subunits and is associated with a molecule of guanosine diphosphate (GDP) at the resting state. Following ligand binding to a GPCR, the GPCR undergoes a conformational change and the signal is subsequently transmitted to the relevant G protein complex and leads to the exchange of GTP for GDP in the G protein complex. This results in the separation of α-subunit from the βγ-dimer. Dissociated Gα-GTP and βγ-dimer can independently activate effector proteins in signal transduction. In the end, GTPase activity of α-subunit enables the hydrolyzation of the attached GTP to GDP and allows re-association of α-subunit and βγ-dimer to return to the resting state of a G protein complex (Smrcka, 2008; Oldham and Hamm, 2008).

Figure 1.4 Major mouse platelet receptors and their ligands.

Platelet adhesion receptors, GPIb-IX-V complex, GPVI and $\alpha_2\beta_1$ immobilize platelets by interacting with subendothelial collagen. Platelet aggregation receptor $\alpha_{IIb}\beta_3$ facilitates the interaction between platelets via fibrin(ogen) and vWF. Platelets respond to thrombin stimulation through receptors PAR4 and PAR3. Platelets respond to ADP stimulation through receptors $P2Y_{12}$ and $P2Y_1$. Platelet respond to TXA_2 stimulation through receptor TP. Signaling through these receptors lead to platelet activation and promote platelet aggregation.


ADP
Thrombin
TXA2
P2Y1
P2Y12
PAR-4
PAR-3
TP
Outside-in signaling
vWF
Fibrin(ogen)
Inside-out signaling
αIIbβ3
GPIb-IX-V
vWF
Collagen
α2β1
GPVI
Collagen

1.3.4.6 Major cellular signaling in platelet activation and endogenous platelet inhibition

Platelet receptors execute two major functions in platelet activation, 1) they receive extracellular signals and 2) initiate a cellular response. Robust signaling pathways comprised of numerous molecules, such as G protein complexes, occur within activated platelets and ultimately converge into common signaling events, including platelet shape change, aggregation and degranulation (Offermanns, 2006).

Briefly, following vessel injury, adhesion receptors first are engaged. The cytoplasmic tails of both GPIb-IX-V complex and GPVI bind to the tyrosine kinase Src family. The two receptors have also been reported to be associated with the Fc receptor, which provides a motif, the immunoreceptor tyrosine-based activation motif (ITAM), which is phosphorylated by Src upon activation. This results in enhanced intracellular Ca^{2+} levels. In addition, it will lead to platelet aggregation and degranulation mediated by protein kinase C (PKC) (Bye et al., 2016). The platelet adhesion receptor, $\alpha_2\beta_1$, and the aggregation receptor, $\alpha_{IIb}\beta_3$, are integrins, which share similar signaling transduction mechanisms. With $\alpha_{IIb}\beta_3$ as an example, activation signal received by platelet adhesion receptors and many GPCRs result in $\alpha_{IIb}\beta_3$ activation. This is termed as “inside-out” signaling. The process is at least in part mediated by a small GTPase, Rap1b. Rap1b activation promotes the interaction between the head domain of a cytoskeletal protein, talin, and the intracellular tail of β_3 subunit. The associated talin mechanically forces $\alpha_{IIb}\beta_3$ to undergo a conformational change. On the other hand, ligand binding to $\alpha_{IIb}\beta_3$ initiates “outside-in” signaling,

which also causes activation of the integrin (Chrzanowska-Wodnicka et al., 2005; Nieswandt et al., 2007; Petrich et al., 2007). The association of the G protein complex, G_{13}, and the cytoplasmic domain of β_3 is an essential step in $\alpha_{IIb}\beta_3$ "outside-in" signaling (Gong et al., 2010). Src kinase activation, as a result of G_{13} activation, leads to a series of cellular events. These include promoting the interaction of the β_3 tail with intracellular molecules, activating phospholipase C (PLC) γ 2 and a small GTPase, RhoA.

In addition to the activation signals through platelet adhesion, aggregation receptors and their ligands, platelet excitatory agonists and their GPCRs are also indispensable in platelet signaling. The G protein complexes involved in platelet activation by major platelet agonists have been revealed. PARs and TP are found to associate with G_{13} and Gq, while $P2Y_1$ and $P2Y_{12}$ mediate signal transduction via Gq and Gi, respectively. Gq activation results in phosphorylation of PLCβ3 and increased intracellular Ca^{2+} levels, which in turn lead to platelet aggregation and degranulation. RhoA activation by activated G_{13} does not just mediate $\alpha_{IIb}\beta_3$ activation but also leads to platelet shape change and degranulation. Activated Gi triggers Rap1b-mediated platelet aggregation and degranulation. The crosstalk of abundant signaling pathways within platelets adds to the complexity of platelet activation. For instance, platelet aggregation and degranulation mediated by phosphatidylinositol 3-kinase (PI3K) is a common pathway that can be activated by both receptor associated proteins, Src and G protein complex (Offermanns, 2006; Bye et al., 2016).

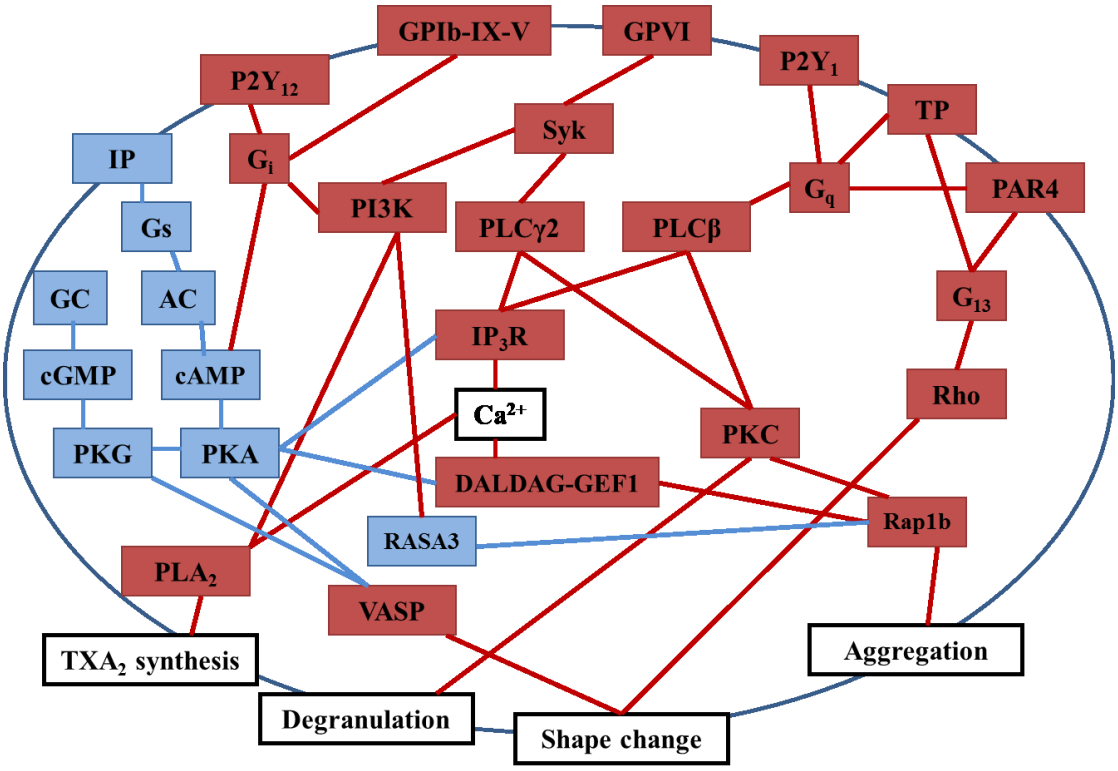
It is noteworthy that G protein complexes do not just participate in activation signaling transduction but are also involved in the negative regulation of platelet activation. Endothelial cells constantly prevent platelets from activating by producing PGI_2 and NO. Both raise platelet cellular level of cyclic nucleotides, cyclic guanosine monophosphate (cGMP) and cAMP, by activating guanylyl cyclase (GC) and AC, respectively. In contrast to the direct action of NO on GC activation, PGI_2 activates AC through a Gs protein-coupled receptor, the IP receptor. In addition, adenosine is released from damaged cells or from converted ADP in the circulation. Adenosine also elevates platelet cAMP through its Gs protein-coupled receptor, adenosine receptor. Together, cGMP and cAMP act as inhibitory second messengers to persistently suppress broad aspects of platelet activation **(Figure 1.5)** (Offermanns, 2006).

1.4 Antiplatelets used to treat arterial thrombosis

Platelets have been the principle target of medical interventions to reduce the thrombotic burden of acute coronary syndrome (ACS) and ischemic stroke. These pathologies are characterized by decreased blood supply from atherosclerosis in myocardial and cerebral arteries, respectively. ACS and ischemic stroke are predominately triggered by plaque disruption and are the leading cause of death worldwide (Mozaffarian et al., 2015). As previously mentioned, lipid-lowering medications, specifically statins, can reduce the likelihood of such arterial thrombosis by slowing down atherosclerosis progression. In addition, percutaneous coronary intervention (PCI) is also a commonly used non-surgical procedure to restore blood

Figure 1.5 Complexity of mouse platelet signaling networks.

Platelet signaling *in vivo* is highly complex and involves many signaling pathways activated by various agonists and simultaneously regulated by inhibitors. Blue boxes and lines represent inhibitory regulators and signaling that suppress platelet activation. Red boxes and lines represent platelet activation mediated by activation receptors and intracellular molecules. Blank boxes represent the common activation events: elevated intracellular Ca^{2+} levels, platelet aggregation, degranulation, shape change and TXA_2 synthesis. (modified figure obtained from (Bye et al., 2016))


GPIb-IX-V
GPVI
P2Y12
P2Y1
TP
Syk
IP
Gi
Gq
PAR4
PI3K
PLCγ2
PLCβ
Gs
GC
AC
G13
IP3R
cGMP
cAMP
Rho
Ca2+
PKC
PKG
PKA
DALDAG-GEF1
Rap1b
RASA3
PLA2
VASP
TXA2 synthesis
Aggregation
Degranulation
Shape change

flow to atherosclerotic vessel segments by opening up the affected arteries with a balloon or stent. Antiplatelets attenuate stent thrombosis and thrombus formation upon plaque rupture to prevent arterial blockage, which can result in ischemia to the heart and brain (Mehta et al., 2001; Jneid et al., 2003; Meadows and Bhatt, 2007).

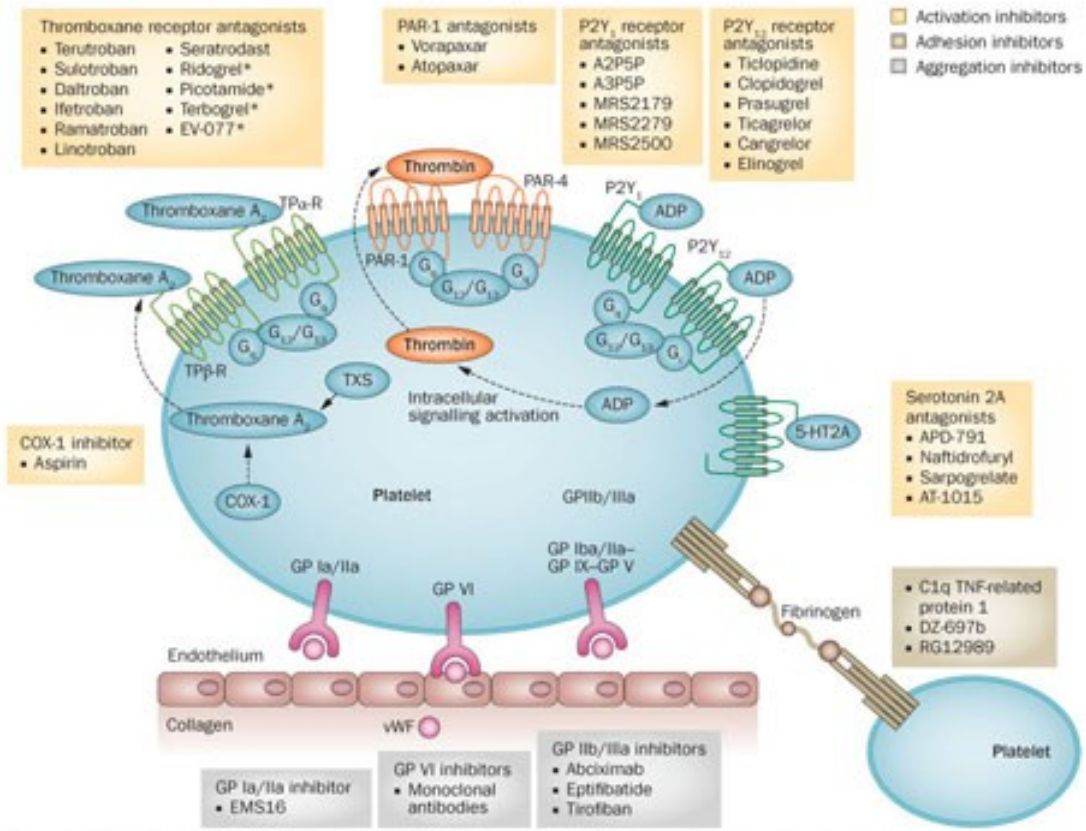
Given that platelets play a pivotal role in haemostasis, a good antiplatelet therapy must not just maximally abrogate unwanted platelet plug formation and growth but also minimally interrupt the haemostatic function of platelets. Despite many antiplatelet therapies introduced to target different aspects of platelet activation in the past two decades, only a handful of these fit into the narrow "therapeutic window" of being effective without causing excess bleeding. Current antiplatelet therapies greatly reduce arterial thrombosis related morbidity and mortality, however, recurrent arterial thrombosis continues in some treated patients. This incomplete inhibition of clinical thrombosis by current antiplatelet therapies drives the demand for better strategies, either the adjustment of current antiplatelets, or the development of new antiplatelets (Capodanno et al., 2013; Franchi and Angiolillo, 2015).

1.4.1 Currently available antiplatelets

At present, several classes of antiplatelet therapies have been approved for clinical use **(Figure 1.6)**. The combinations of the COX-1 inhibitor, ASA, and one of the $P2Y_{12}$ antagonists is called dual-antiplatelet therapy (DAPT), and has long been the standard-of-care; it is recommended in current guidelines for prevention and

Figure 1.6 Platelet activation pathways targeted by current and novel antiplatelets.

Current antiplatelets are: COX-1 inhibitor aspirin (ASA) and ADP receptor $P2Y_{12}$ inhibitors, including clopidogrel, ticagrelor and prasugrel. Novel antiplatelets under development include: new $P2Y_{12}$ inhibitors, cangrelor and elinogrel, agents that inhibit thrombin receptor PAR1, TXA_2 receptor TP, adhesion receptors and ADP receptor $P2Y_1$, etc. (Franchi and Angiolillo, 2015).


Thromboxane receptor antagonists
• Terutroban
• Sulotroban
• Daltroban
• Ifetroban
• Ramatroban
• Linotroban
• Seratrodast
• Ridogrel*
• Picotamide*
• Terbogrel*
• EV-077*
PAR-1 antagonists
• Vorapaxar
• Atopaxar
P2Y1 receptor antagonists
• A2P5P
• A3P5P
• MRS2179
• MRS2279
• MRS2500
P2Y12 receptor antagonists
• Ticlopidine
• Clopidogrel
• Prasugrel
• Ticagrelor
• Cangrelor
• Elinogrel
Activation inhibitors
Adhesion inhibitors
Aggregation inhibitors
Thrombin
PAR-4
PAR-1
P2Y1
ADP
P2Y12
ADP
Thromboxane A2
TPα-R
Thromboxane A2
TPβ-R
TXS
Thromboxane A2
Thrombin
Intracellular signalling activation
ADP
5-HT2A
Serotonin 2A antagonists
• APD-791
• Naftidrofuryl
• Sarpogrelate
• AT-1015
COX-1 inhibitor
• Aspirin
COX-1
Platelet
GPIIb/IIIa
GP Ia/IIa
GP VI
GP Iba/IIa–GP IX–GP V
Fibrinogen
• C1q TNF-related protein 1
• DZ-697b
• RG12989
Endothelium
Collagen
vWF
Platelet
GP Ia/IIa inhibitor
• EMS16
GP VI inhibitors
• Monoclonal antibodies
GP IIb/IIIa inhibitors
• Abciximab
• Eptifibatide
• Tirofiban

treatment in patients with ACS and post PCI (Levine et al., 2016). In addition, phosphodiesterase (PDE) inhibitors, dipyridamole and cilostazol, enhance the platelet inhibitory effect of cAMP and cGMP by preventing their degradation (Gresele et al., 2011). The PAR1 antagonist, vorapaxar, was recently approved, and it has been tested in a combination with standard-of-care. The addition of vorapaxar had an overall benefit over standard therapy in reducing thrombotic events, but at the expense of increasing the likelihood of bleeding. Therefore, vorapaxar is not recommended for all patients (Tricoci et al., 2012).

The principle mechanism of ASA's antiplatelet effect is to irreversibly suppress TXA_2 biosynthesis. ASA inhibits the interaction between arachidonic acid and COX-1 by acetylating a specific serine moiety in COX-1's hydrophobic pocket, which ultimately results in diminished COX-1 function (Roth and Majerus, 1975; Loll et al., 1995). Three approved $P2Y_{12}$ antagonists, clopidogrel, prasugrel and ticagrelor, are currently in clinical use. Clopidogrel and prasugrel are prodrugs, which are converted into active metabolites *in vivo* following oral administration. The active metabolites covalently and irreversibly bind to $P2Y_{12}$ receptors to abolish $P2Y_{12}$ signaling through the life span of these platelets. Ticagrelor is a high affinity ADP analogue, which competitively interacts with $P2Y_{12}$ receptors, resulting in reversible inhibition of $P2Y_{12}$ signaling (Cattaneo, 2010).

1.4.2 The antithrombotic efficacy of ASA and $P2Y_{12}$ antagonists is proved in clinical trials

ASA is the longest used antithrombotic agent and has been tested in a large number of clinical trials. Despite dosing disparity, it has been well established that ASA administration is effective in the primary prevention of myocardial infarction and in the secondary prevention of both myocardial and cerebral infarction (Baigent et al., 2009). $P2Y_{12}$ antagonists are rather new, compared to ASA. A comparison of clinical thrombotic events was made between different $P2Y_{12}$ antagonists (Cattaneo, 2010). Importantly, $P2Y_{12}$ inhibition was proven to be more potent than ASA in reducing the incidence of arterial thrombosis. Specifically, in the CAPRIE trial, clopidogrel was found to more effectively decrease the risks of myocardial infarction, ischaemic stroke and vascular death compared to ASA (CAPRIE Steering Committee, 1996). The goal of administrating ASA and a $P2Y_{12}$ antagonist together (DAPT) is to concomitantly inhibit TXA_2- and ADP-mediated platelet activation in order to achieve greater antithrombotic potential. The superiority of DAPT over ASA alone in arterial thrombosis was demonstrated in the CURE trial and the CHARISMA trial (Yusuf et al., 2001; Bhatt et al., 2006). Up to now, MATCH is the only clinical trial that assessed the therapeutic efficacy of DAPT compared to clopidogrel alone. Because the MATCH trial revealed a non-significant difference of risk reduction at the expense of increased bleeding with DAPT administration, the therapeutic value of DAPT versus a $P2Y_{12}$ antagonist alone remains inconclusive (Diener et al., 2004).

1.4.3 Novel antiplatelets under development

Within the same catalog of inhibiting TXA_2- and ADP-induced platelet activation, several new inhibitors have been developed. To avoid the side-effects of ASA, and TP receptor activation-mediated by other eicosanoids, targeting TP receptors or both TP receptor and COX-1 were suggested as potential targets for better clinical outcomes. The majority of the newly developed drugs of this kind, however, have shown to be inferior to ASA (van der Wieken et al., 1995; Bousser et al., 2011). Only a dual TP/COX-1 inhibitor has reached phase II trial. Similar to ticagrelor, cangrelor is an ATP analogue that inhibits $P2Y_{12}$ receptors by directly and reversibly interacting with the receptor. Cangrelor has completed three major phase III trials and has shown efficacy to reduce arterial thrombotic events. Different from all the approved $P2Y_{12}$ antagonists, cangrelor has a rapid offset of action. Although cangrelor has not yet been approved for clinical use, it is an attractive bridging strategy for patients who require $P2Y_{12}$ inhibition and undergo surgery (Capodanno et al., 2013; Franchi and Angiolillo, 2015).

In addition to the continuing studies of novel inhibitors on these conventional targets, a number of unconventional platelet signaling pathways have been proposed and many of their inhibitors are at the early stages of investigations. These include drugs that target adhesion signaling pathways by decreasing the bioavailability of collagen or directly blocking the adhesion receptors, GPVI and the GPIb-IX-V complex; 5-HT receptor antagonists; reagents that alter signaling proteins within

platelets, such as NO donors and PI3K inhibitors **(Figure 1.6)** (Capodanno et al., 2013; Franchi and Angiolillo, 2015).

1.4.4 Future perspective of antiplatelets

Theoretically, therapies that weaken platelet activation by downregulating the generation of platelet excitatory agonists, the function of receptors, or relevant signaling molecules, can potentially be used to suppress human arterial thrombosis. However, many drugs developed in the past failed to prove their therapeutic value. In addition to targeting platelet-specific molecules in order to minimize drug effects beyond haemostasis and thrombosis, identifying the platelet activation pathways that are disproportionally involved in thrombosis is important to avoid the adverse bleeding effects associated with the new inhibitors. Despite the imperfection of the combination of ASA and a $P2Y_{12}$ antagonist, the regimen has not yet been challenged over a decade and it remains as the standardized therapy in arterial thrombosis. Of note, the dosage and treatment duration chosen in early stages of clinical studies, at least in part, influence the net benefit and determine the fate of new antiplatelets. Furthermore, continuing investigation of the optimal dosage and treatment duration can go on even after these drugs have been approved in clinical use. The subtle correction of these parameters may also greatly improve drug efficacy in treating arterial thrombosis in future.

Chapter 2: Objectives

Atherosclerosis and platelets play pivotal roles in arterial thrombosis, which is one of the leading causes of morbidity and mortality worldwide. Atorvastatin is a commonly prescribed medication to suppress atherosclerotic progression by lowering LDL cholesterol. Recent studies have revealed pleiotropic benefits associated with statins. These include promoting platelet inhibition in an eNOS-dependent fashion. Dual-antiplatelet therapy (DAPT), which consists of ASA and a $P2Y_{12}$ antagonist, usually clopidogrel, is the most commonly prescribed approach to attenuate platelet activation by TXA_2 and ADP. Although atorvastatin, ASA and clopidogrel have proven to be effective in reducing arterial thrombosis in many clinical trials, preclinical evidence of their antiplatelet effects are lacking. In fact, the antiplatelet effect of ASA has not yet been shown using any animal thrombosis models. There is a dearth of both preclinical and clinical evidence to support the optimal combination of ASA and clopidogrel to treat arterial thrombosis. In addition, because patients at risk of cardiovascular disease often have dysfunctional eNOS, it is important to know if atorvastatin also inhibits platelet activation in an eNOS-independent fashion. Thus, the specific objectives of the thesis are:

1. To improve an existing mouse thrombosis model to make it more sensitive to detect platelet inhibition in preclinical studies.
2. To investigate NO-independent platelet inhibition by atorvastatin.
3. To guide optimal dosing of ASA and clopidogrel to improve DAPT.

Chapter 3: High-speed, volumetric, confocal imaging identifies discrete platelet activation events during thrombus formation.

Author list: Ran Ni, Xufang Bai, Bruno Esposito, Ji Zhou, Margaret L. Rand, Jeffrey I. Weitz, Peter L. Gross

Foreword

This manuscript is in preparation. The project was designed by Dr. Peter L. Gross and experiments contained in the project were performed by me. The blinded evaluation of the location of platelet activation markers was performed by Dr. Ji Zhou. We thank former laboratory technicians Bruno Esposito and Dr. Xufang Bai for training with mouse surgeries and the proper use of intravital microscopy, and for obtaining pilot data. Dr. Margaret L. Rand and Dr. Jeffrey I. Weitz have contributed to the experimental design and analysis of experimental data. Chapter 3 is a direct representation of the ongoing manuscript.

3.1 Abstract

Objective: Most heart attacks and strokes are caused by platelet-rich thrombi superimposed on disrupted atherosclerotic plaques. Because of the central role of platelets in these disorders, platelet inhibitors, such as acetylsalicylic acid (ASA/aspirin), are a mainstay of their prevention and treatment. Despite the importance of platelets, our knowledge about the dynamics of platelet activation comes mainly from *in vitro* studies of platelet aggregation in response to single agonists or from *in vivo* monitoring of platelet accumulation/thrombus volume after vessel wall injury. Our objective was to monitor the spatial and temporal aspects of platelet activation that occur after laser injury to cremaster arterioles in mice using high-speed two-color confocal microscopy.

Methods and Results: Platelets were labelled with an antibody against GPIbβ and platelet activation was monitored using fluorescently-labelled antibodies against platelet integrin CD41 that increases in number upon platelet activation, and P-selectin, a marker of α-granule release, and Diannexin, a marker of phosphatidylserine (PS) exposure on the platelet surface. The ratio of fluorescence of the activation label to the platelet label at each pixel space within the thrombus was calculated. CD41 upregulation occurs more rapidly than α-granule release. PS exposure is the slowest event. These activation events occur in distinct locations within the thrombus: whereas CD41 upregulation occurs diffusely, P-selectin and PS exposure are localized to the vessel wall at the site of injury. ASA had no effect on platelet accumulation/thrombus

volume or P-selectin expression, but delayed CD41 upregulation.

Conclusions: This system appears capable of monitoring platelet activation events *in vivo*.

3.2 Introduction

Heart attacks and strokes are the leading cause of morbidity and mortality worldwide. Most are triggered by arterial thrombi that form at sites where the structural or functional integrity of the endothelium has been disrupted (Ruggeri, 2002a). Arterial thrombi are predominantly composed of platelet aggregates. Consequently, current drug strategies for prevention and treatment of arterial thrombosis focus on agents that inhibit platelet activation or aggregation.

Platelet activation in response to various single agonists has been well characterized *in vitro*. In more recent work, the response of platelets to pairs of agonists has been evaluated. Such studies have identified subpopulations of activated platelets, including sustained calcium (Ca^{2+})-induced platelets (SCIP), which express procoagulant PS and are targets for transglutaminases (Kulkarni and Jackson, 2004), and collagen- and thrombin-activated (COAT) platelets, which express serotonin-binding sites on their surface (Szasz and Dale, 2002). The relevance of these and other subpopulations of activated platelets is uncertain because the nature and concentration of platelet agonists that are generated in response to vessel injury *in vivo* are unknown.

Mouse thrombosis models have largely focused on assessment of platelet

accumulation/thrombus volume in response to various injuries (Ni et al., 2000; Smyth et al., 2001; Ni et al., 2001; Falati et al., 2002; Ni et al., 2003; Massberg et al., 2003; Schlachterman et al., 2005; Dubois et al., 2006; Erhardt et al., 2006; Dubois et al., 2007; Vandendries et al., 2007; Kalia et al., 2008; Konopatskaya et al., 2009; Hechler et al., 2010). Morphological examination of thrombi reveals that most of the platelets at the periphery of thrombi are discoid and not round like the platelets closer to the vessel wall (Kulkarni et al., 2000). Evaluation of Ca^{2+} concentrations in single platelets within thrombi demonstrates considerable heterogeneity (van Gestel et al., 2002). Using PS expression as a marker of platelet activation, a distinct sequence of platelet activation occurs over time (Hayashi et al., 2008). Thus, fibrinogen-binding and PS-expressing platelets are observed in adjacent but distinct locations, while P-selectin expression, which is indicative of α-granule release, colocalizes with PS expression (Munnix et al., 2007).

Capitalizing on recent advances in high-speed two-color confocal microscopy, we evaluated the dynamics of platelet activation in developing thrombi *in vivo*. With one fluorophore imaging a marker of platelet activation and the other non-overlapping fluorophore imaging an activation-independent platelet marker, pair-wise volumetric confocal slices were analyzed. This methodology permits separation of markers of integrin upregulation, α-granule release and PS surface expression in time and space. Using this technology, we demonstrate time dependence to α_{IIb} integrin upregulation and P-selectin and PS expression within thrombi and we provide evidence that ASA

has distinct inhibitory effects on platelet activation within thrombi.

3.3 Materials and Methods

3.3.1 Materials

Wild-type C57BL/6J male mice were purchased from Jackson Laboratories USA. All chemicals were purchased from Sigma Aldrich USA unless otherwise stated. Antibodies used in the detection of platelet activation and platelet accumulation *in vitro* and *in vivo* were from Emfret Analytics Germany, with the exception that the anti-P-selectin antibody (RB40.34) was purchased from Becton Dickinson (BD) Biosciences USA. Diannexin is a kind gift from A. Allison at Alavita Pharmaceuticals, Mountain View, CA. Antibody F_{ab} fragments were generated using a F_{ab} preparation kit from Pierce USA. Fluorophore conjugation was performed using protein labeling kits from Pierce USA and Invitrogen USA. Anesthesia solutions ketamine, xylazine, atropine and Nembutal were purchased from Ayerst, Bayer, Ormond Veterinary and Abbott laboratories (Canada), respectively. General surgical supplies were from Fine Scientific Tools (Canada) and BD Biosciences (USA). Pure ASA powder was purchased from Sigma USA. Twenty gauge feeding needles were from Popper and Sons Inc. USA. 60 X water immersion objective was purchased from Olympus Japan. Photodiode velocimeter (a Microvessel Velocity OD-RT Doppler) was obtained from CircuSoft Instrumentation Germany. Piezo-electric focuser was obtained from Physiks International Germany. Nitrogen dye laser was from Micropoint System USA. The

rest components for intravital microscopy including Slidebook software (5.0) were purchased from Intelligent Imaging Innovations USA. Anticoagulant heparin was from Leo Pharma Inc. Canada. Platelet inhibitors apyrase and prostacyclin (PGI_2) used in platelet isolation were purchased from Sigma and Calbiochem USA, respectively. Thrombin used in flow cytometry was from Roche Diagnostics USA. PAR4 agonist peptide (H-Gly-Try-Pro-Gly-Lys-Phe-OH, GYP) was from Bachem Switzerland and collagen was from Nycomed Pharma Germany. DiO was purchased from Invitrogen USA. Disposable hemocytometers were from Incyto Korea. The apparatus and reagents used in immunoblot were from BioRad USA and the determination was done using a Typhoon 9410 from GE Healthcare/Amersham Biosciences USA.

3.3.2 Methods

3.3.2.1 Mouse handling

Age-matched male wild-type mice were used between 8 to 12 weeks old and weighed at least 25 grams. Where stated, ASA (1.25 mg/kg body weight) or gelatin vehicle was administered by gavage using a 20-gauge feeding needle 60 minutes prior to initiating thrombus formation. All experiments were performed according to Canadian Council of Animal Care Guidelines and all animal utilization protocols were approved by the Animal Research Ethics Board at McMaster University.

3.3.2.2 Cremaster muscle preparation

Mice were pre-anesthetized with an intraperitoneal injection of 125 mg/kg

ketamine, 12.5 mg/kg of xylazine and 0.25 mg/kg of atropine. Anesthesia was maintained with Nembutal, which was administered via a cannulus inserted in the jugular vein. The airway was stabilized by insertion of a tracheal tube and body temperature was maintained at 37^{o}C using a thermo-controlled rodent blanket. The scrotum was incised and the testicle and surrounding cremaster muscle were then exteriorized onto an intravital microscopy tray. The cremaster muscle was superfused with thermo-controlled (37^{o}C) and aerated (95% N_2, 5% CO_2) bicarbonate-buffered saline for the duration of the experiment.

3.3.2.3 Infusion of detecting antibodies and proteins

Mice were infused via the jugular cannulus with X488 (0.1 µg/g body weight), a monoclonal antibody directed against GPIbβ that is tagged with Dylight-488. Where indicated, mice were infused with Dylight-647-tagged F_{ab} fragments (1.3 µg/g body weight) derived from rat anti-mouse CD41 (MWReg30), a monoclonal antibody directed against the platelet-specific integrin α_{IIb} that recognizes both the resting and activated forms of the molecule, Dylight-647-tagged antibody against P-selectin (2 µg/g body weight, RB40.34), or Dylight-647-tagged Diannexin (0.025 µg/g body weight), a recombinant homodimer of annexin A5 (Kuypers et al., 2007). Fluorophore conjugation and F_{ab} preparation were performed according to directions provided by the supplier.

3.3.2.4 Intravital videomicroscopy

The microcirculation of the cremaster muscle was visualized using intravital

videomicroscopy as previously described (Falati et al., 2002; Celi et al., 2003; Gross et al., 2005; Bai et al., 2009), but with the following modifications. Micro-vessel data were obtained using an Olympus BX series microscope with a 60 X 0.9 NA water immersion objective. To ensure that the shear rate in evaluated arterioles was at least 1500 s^{-1} prior to injury, the centerline erythrocyte velocity was measured in real-time using a photo-diode velocimeter. Digital images were captured with a 640 x 480 format CCD camera set at 2 x 2 binning. Confocal images were obtained using a Yokagowa CSU22 spinning-disk, set at 4400 rpm, illuminated by diode lasers with acoustic optical tuned frequency (AOTF) switching. Three-dimensional images were obtained with a piezo-electric focuser. The camera and illumination were controlled, and the images analyzed, using Slidebook. In all experiments, 160 pair-wise image slices, 0.5 μm apart, were captured in sequence for each time point over 20 seconds as follows: Fl488 (channel with excitation: 488 nm, emission: 520 nm peak with 20 nm bandwidth), Fl647 (channel with excitation: 647 nm, emission: 670 nm with 25 nm bandwidth), next slice; Fl488, Fl647, next slice; etc.

3.3.2.5 Laser-induced injury

Vessel wall injury was induced using a nitrogen dye laser focused through the microscope objective and aimed at the vessel wall (Falati et al., 2002; Celi et al., 2003). Over the course of 3 hours, multiple injuries were induced upstream from the previous one so as to minimize the possibility that earlier formed thrombi would affect subsequent results. There were no characteristic trends in thrombus size or

composition in sequential thrombi generated in a single mouse.

3.3.2.6 Image analysis by pixel ratio

Data were discarded if the Fl488 fluorescence of thrombi, an index of platelet accumulation/thrombus volume, did not increase above baseline levels. For every slice and at every pixel where the Fl488 intensity was above threshold (determined as previously described) (Vandendries et al., 2007), the intensity of each activation label, Fl647 **(Figure 3.2 A and C)**, was divided by the Fl488 intensity (X488) at that pixel to obtain a ratio **(Figure 3.2 B and D)**. The means of these ratios over the entire thrombus volume were calculated at each time-point and normalized to the maximal mean ratio at any time for that thrombus, and the time to half-maximal ratio (for each activation marker) was determined.

3.3.2.7 Analysis of location of platelet activation

An evaluator blinded to the activation label categorized the location of each activation marker within the three-dimensions of the thrombus over time. Categories were: i) eccentric (located at the periphery of the thrombus), ii) diffuse, or iii) indeterminate. Chi-squared analysis was used to compare the distribution of the categories among the three activation labels.

3.3.2.8 Flow cytometry analysis of activation of mouse platelets

Blood (1 mL) was collected via carotid cannulation into TBS/Heparin (9:1 vol/vol, Tris-buffered saline: 20 mM Tris-HCl, 137 mM NaCl, pH 7.3, containing 20 U/mL heparin. Platelet-rich plasma was obtained by centrifugation at 500 x g for 5 minutes. Platelets were pelleted at 1,500 x g for 5 minutes, resuspended and then

washed twice in Tyrode's buffer (134 mM NaCl, 0.34 mM $Na2HPO_4$, 2.9 mM KCl, 12 mM $NaHCO_3$, 20 mM HEPES, pH 7.0, 5 mM glucose, 0.35% bovine serum albumin) in the presence of 20 mU/mL apyrase and 0.5 nM PGI_2. Platelets were resuspended in 500 µL Tyrode's buffer supplemented with 40 mU/mL apyrase and were used after 30 minutes incubation at 37ºC as described on the Emfret Analytics website (www.emfret.com). Platelets were activated with: 0.1 U/mL thrombin, 12 mM H-Gly-Tyr-Pro-Gly-Lys-Phe-OH (GYP; a protease activated receptor-4 (PAR4) agonist peptide), 250 µg/mL collagen, or 250 µg/mL collagen plus 0.1 U/mL thrombin. Activation was monitored by flow cytometry using the fluorescently tagged-antibodies or proteins described above; gating excluded microparticles. Values were expressed as mean fluorescence intensity.

3.3.2.9 Preparation of labeled platelets for transfusion

Platelet-rich plasma (PRP) was obtained as described above and platelets were isolated in the void volume of a Sepharose 2B column into PIPES buffer, pH 7.4. After 20 minutes incubation with 5 µg DiO, platelets were pelleted at 1,500 x g, resuspended in PIPES buffer and counted in a hemocytometer using a fluorescent microscope. A total of $1x10^5$ labeled platelets were infused into each recipient mouse via a jugular cannulus. After inducing laser injury as described above, thrombi were observed in wide-field at 1x1 binning in bright field and using Fl488 at one frame every 300 milliseconds. Sequential frames were observed off-line in bright-field to determine the times that individual platelets arrived and left the thrombus.

3.3.2.10 Statistical Analysis

Group data were expressed as mean ± SEM. Significance of differences was determined using Student's t-tests or two-way ANOVA. Significance of differences among classifications of activation marker location was determined by chi-squared analysis. A *P value < 0.05 was considered statistically significant.

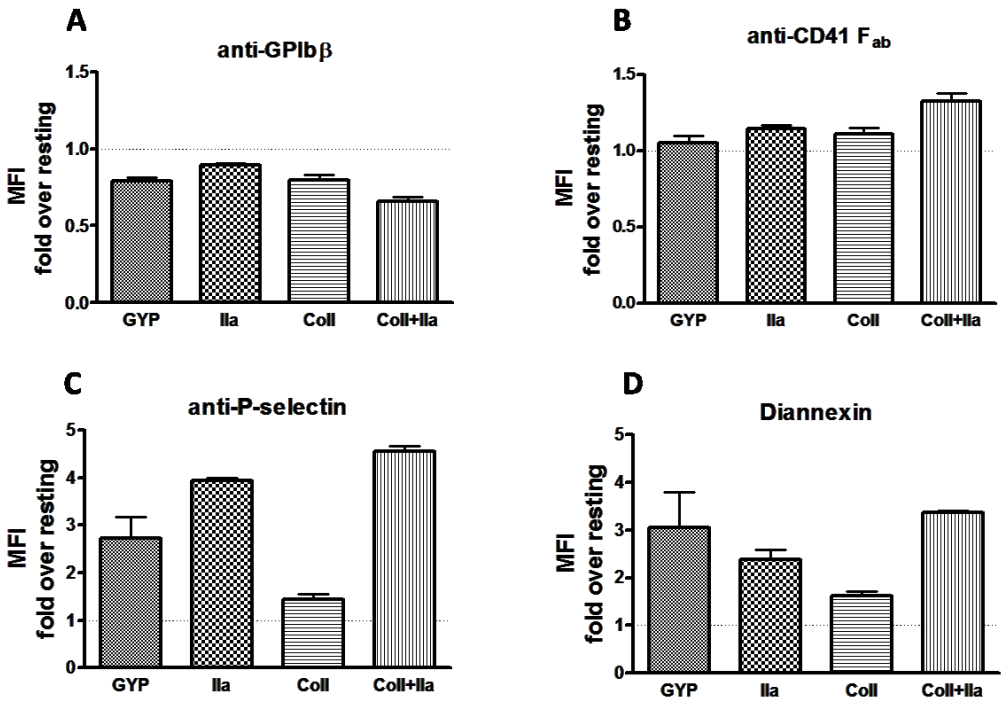
3.4 Results

3.4.1 Platelet activation by flow cytometry

Activation with GYP, thrombin, collagen or collagen plus thrombin reduced the level of surface-expressed GPIbβ on platelets as determined by the mean fluorescence intensity (MFI) of X488 binding **(Figure 3.1)**. Activation with GYP or thrombin did not alter the amount of GPIbβ detected by immunoblot analysis of platelet lysates (data not shown). After activation with collagen and centrifugation at 1,500 x g a reduced amount of GPIbβ was detected by immunoblot analysis of platelet lysates, but collagen-activated platelets centrifuged at 13,000 x g had a similar amount of GPIbβ detected by immunoblot analysis as resting platelets. These findings suggest that GPIbβ is localized to microparticles that are released from platelets after activation with collagen. Our findings are not consistent with the concept that GPIbβ is cleaved from mouse platelets after activation. Because X488 binding to platelets and platelet microparticles does not change after activation, we chose it as a reliable indicator of platelet accumulation/thrombus volume. After platelet activation with GYP, thrombin, collagen or collagen plus thrombin *in vitro*, the MFI of Dylight-647-tagged CD41-F_{ab}

Figure 3.1 Agonist-induced change in platelet accumulation and activation markers by flow cytometry.

Washed mouse platelets were treated with PAR4 agonist peptide (GYP), thrombin (IIa), collagen (coll) or collagen plus thrombin (coll+IIa). Mean ± SEM of mean fluorescence intensity (MFI) over resting platelets of the indicated markers are shown. **A. Anti-GPIbβ**, platelet accumulation (thrombus volume) marker X488. **B. Anti-CD41 F_{ab},** platelet aggregation marker MWReg30. **C. Anti-P-selectin,** platelet degranulation marker RB40.34. **D. Diannexin,** PS exposure marker, a dimer of annexin V.


A
anti-GPIbβ
MFI
fold over resting
1.5
1.0
0.5
0.0
GYP
IIa
CoII
CoII+IIa
B
anti-CD41 Fab
MFI
fold over resting
1.5
1.0
0.5
0.0
GYP
IIa
CoII
CoII+IIa
C
anti-P-selectin
MFI
fold over resting
5
4
3
2
1
0
GYP
IIa
CoII
CoII+IIa
D
Diannexin
MFI
fold over resting
5
4
3
2
1
0
GYP
IIa
CoII
CoII+IIa

fragments, anti-P-selectin and Diannexin increased. To test whether the low concentrations of markers used impaired thrombus formation *in vivo*, platelet accumulation/thrombus volume (as measured by X488) in the presence of the different markers was compared with that in their absence. There were no decreases in maximal or integrated platelet accumulation/thrombus volume (as measured by X488) in the presence of the platelet activation markers (data not shown). Because more anti-CD41 F_{ab}, anti-P-selectin and Diannexin bound to activated platelets than to resting platelets, they were chosen as useful markers of the biological properties of activation that they represent, namely: integrin upregulation (CD41), α-granule release (P-selectin) and PS expression on the outer leaflet of the platelet membrane (Diannexin binding). Thus, we validated these tools as markers of platelet accumulation/thrombus volume and different parameters of platelet activation.

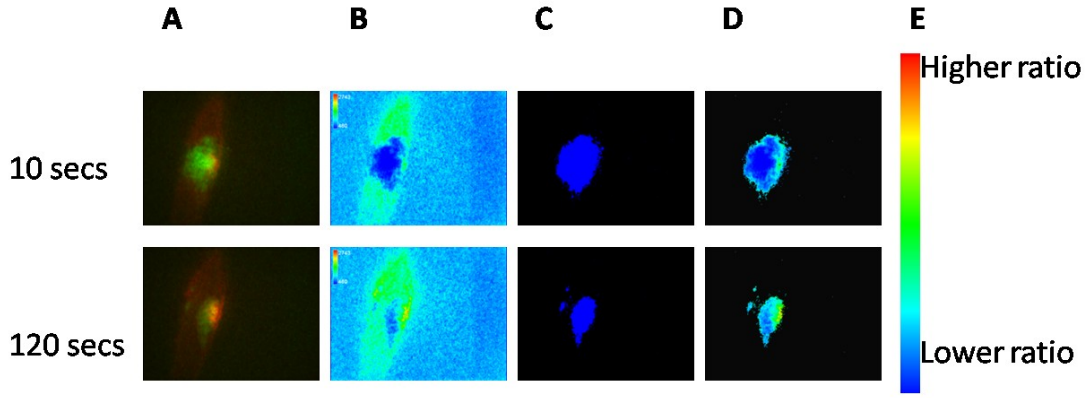
3.4.2 Biological processes of platelet activation are time-resolved in vivo

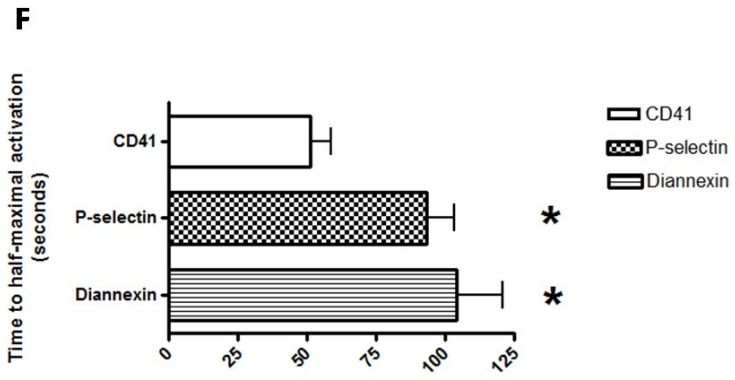
We determined the time to half-maximal activation ratio of the different markers of platelet activation **(Figure 3.2)**. The increase in CD41 relative to GPIbβ occurs most rapidly with a time to half-maximal of 51 ± 7 seconds (N=26); a time that coincided with the time to maximal platelet accumulation (as measured by X488), mean of 50 ± 7 seconds. The increase in P-selectin relative to GPIbβ occurs with a time to half-maximal of 93 ± 10 sec (N=18), while the increase in PS relative to GPIbβ occurs with a time to half-maximal of 124 ± 8 sec (N=49). The differences in

Figure 3.2 Analysis of two-color confocal images to obtain an activation ratio.

Shown are representations of a single mid-volume *z* -slice images 10 and 120 seconds after laser injury to illustrate how activation ratio is calculated. **A. A single mid-volume confocal plane.** Ungated 12-bit images of fluorescence from Dylight-488-tagged anti-GPIbβ binding (green) and from Dylight-647-tagged anti-P-selectin binding (red) are shown. **B. Ratio image of P-selectin to GPIbβ in a single plane.** The ratio of fluorescence intensity in the 647 channel to that in the 488 channel is calculated at every pixel. Depicted is a temperature look-up table (red is high, blue is low) of a single plane. **C. Fluorescence intensity of Anti-GPIbβ above threshold.** A mask was created by segmenting pixels where the anti-GPIbβ fluorescence intensity was above background (the fluorescence intensity of anti-GPIbβ in non-injured vessel). **D. Ratio image of P-selectin to GPIbβ in the thrombus.** Only pixels where the fluorescence of anti-GPIbβ is above threshold (see **C**) are included in the analysis. Depicted is a temperature look-up table (red is high, blue is low) of a single plane. **E. A temperature look-up table. F. Time to half-maximal activation ratio of the indicated platelet activation markers.** Dylight-488-tagged anti-GPIbβ was paired with Dylight-647-tagged anti-CD41 F_{ab}, or Dylight-647-tagged anti-P-selectin, or Dylight-647-tagged Diannexin. A mean activation ratio (calculated as illustrated above) was obtained for each volume (consisting of 120 z-slices) of a thrombus at each time point. The time for the mean activation ratio to reach half-maximal was calculated for each thrombus. Mean ± SEM

are shown. *$P < 0.05$ compared with anti-CD41 F_{ab}.


A
B
C
D
E
10 secs
120 secs
Higher ratio
Lower ratio


F
Time to half-maximal activation (seconds)
CD41
P-selectin
Diannexin
0
25
50
75
100
125
*
*
CD41
P-selectin
Diannexin

time to half-maximal activation ratios amongst the markers were statistically significant **(Figure 3.2 F)**. Thus, these markers of platelet activation are time resolved *in vivo* and do not all occur at the time of maximum increase in thrombus volume.

3.4.3 Biological processes of platelet activation are at distinct locations within a thrombus in vivo

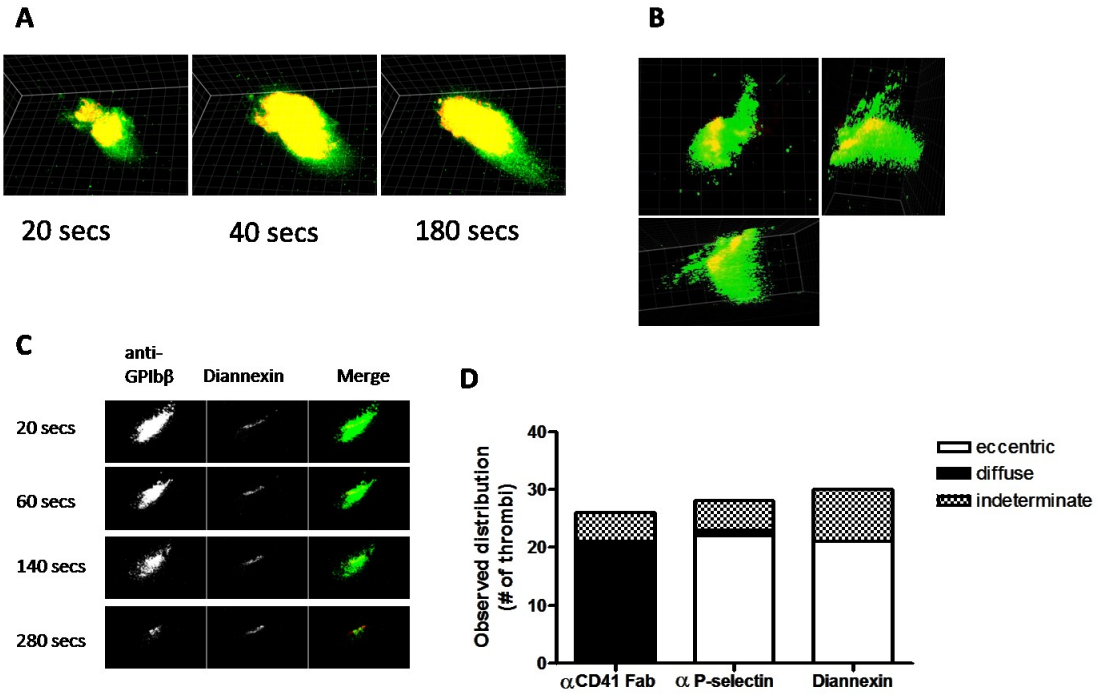
An investigator blinded to the activation marker assessed the four-dimensional images (three-dimensional images over time) to identify the location of the high activation ratio signal during the initial 5 minutes of thrombus formation. The locations of the high ratio signal were classified as either eccentric in the thrombus (at the vessel wall), diffuse within the thrombus or indeterminate. The majority of high-ratio signals from anti-CD41 F_{ab} were classified as diffuse. In contrast, the majority of high-ratio signals from anti-P-selectin and Diannexin were classified as eccentric and were localized to the vessel wall **(Figure 3.3)**. Thus, the markers of platelet activation are found in distinct locations within the laser-induced arteriolar thrombus. Markers that reflect stronger activation (α-granule release and PS exposure) are found mainly at the vessel wall where the injury was induced.

3.4.4 Time to half-maximal activation ratio of CD41, but not P-selectin, is delayed by ASA.

ASA is the most widely used antiplatelet drug; however, its antithrombotic effects in animal models are modest.(Doutremepuich et al., 2010) We were unable to

Figure 3.3 Distinct location of increase in platelet activation markers.

A. Representative reconstructed three-dimensional images showing anti-CD41 F_{ab} binding in a thrombus over time. Green indicates anti-GPIbβ. Red indicates anti-CD41 F_{ab}. Yellow indicates colocalization of anti-GPIbβ and anti-CD41F_{ab}. Reconstruction used a maximal image projection method. **B. Representative reconstructed three-dimensional image showing anti-P-selectin binding in a thrombus.** Green indicates anti-GPIbβ. Red indicates anti-P-selectin. Yellow indicates colocalization of anti-GPIbβ and anti-P-selectin at 200 seconds after laser injury. A view from the above, the right and the front is shown. Reconstruction used a maximal image projection method. **C. Multiple time points showing Diannexin binding in a thrombus.** A single plane at 20, 60, 140 and 280 seconds after laser injury is shown. In the merge column green indicates anti-GPIbβ, red indicates Diannexin, and yellow indicates colocalization of anti-GPIbβ and Diannexin. **D. Classification of location of platelet activation marker increase.** An observer blinded to the platelet activation markers used in time-lapse three-dimensional images (as in **C**) and classified the location of the marker (anti-CD41F_{ab}, anti-P-selectin and Diannexin binding) increase as eccentric, diffuse, or indeterminate. The observed distributions of anti-P-selectin and Diannexin were both significantly different to the distribution of anti-CD41 F_{ab} ($P < 0.001$, Chi-squared test, 18 to 26 thrombi per activation marker group).


A
20 secs
40 secs
180 secs
B
C
anti-GPIbβ
Diannexin
Merge
20 secs
60 secs
140 secs
280 secs
D
Observed distribution (# of thrombi)
40
30
20
10
0
αCD41 Fab
α P-selectin
Diannexin
eccentric
diffuse
indeterminate

demonstrate an inhibitory effect of ASA treatment on maximal or integrated platelet accumulation/thrombus volume (GPIbβ, measured by X488) in laser-injured cremaster arterioles **(Figure 3.4, A and B)**. However, when we compared platelet activation in thrombi formed in ASA-treated mice with those formed in control mice given vehicle only **(Figure 3.4 C)**, we found that ASA-treatment significantly (P=0.009) prolonged the time to half-maximal activation ratio of CD41 from 50 ± 5 seconds (N=18) to 88 ± 12 seconds (N=16), The time to half-maximal activation ratio of P-selectin **(Figure 3.4 D)** or Diannexin (data not shown) was unaltered by ASA treatment. Thus, our method can discern subtle changes in platelet activation events in the presence of a clinically effective antiplatelet drug.

3.4.5 Most platelets spend only seconds in a thrombus

Because we observed delayed upregulation of CD41 on platelets in the thrombus by ASA, we hypothesized that the platelet-platelet interactions prior to CD41 upregulation would be weak that few adherent platelets would remain firmly attached to the thrombus. To explore this possibility, a small number of DiO-labeled platelets were infused into mice prior to laser injury. Typically, 5 or 6 platelets interacted with each thrombus over the 4 minutes of observation. In 44 thrombi in 6 mice, a total of 265 individual platelets were monitored: the median length of time a platelet remained adherent to the thrombus was 15.3 seconds (range 0.6 to 240 seconds); the distribution of time platelets remained attached to the thrombus is shown in **Figure 3.5**. Most of

Figure 3.4 Platelet accumulation and activation in thrombi in: effect of ASA treatment.

ASA (1.25 mg/kg body weight) or gelatin vehicle where administered by gavage 60 minutes before induction of thrombus formation. **A. The distribution of the integrated platelet (anti-GPIbβ binding) fluorescence for each thrombus at maximal size.** Data for individual thrombi are shown for 27 thrombi in vehicle-treated mice and 36 thrombi in ASA-treated mice. Mean ± SEM are indicated by the line. **B. The distribution of the integrated platelet fluorescence (anti-GPIbβ binding) over 5 minutes for each thrombus.** Data for individual thrombi, as in **A** are shown. **C. Time to half-maximal activation ratio of CD41F$_{ab}$.** Mean ± SEM of 18 thrombi in vehicle-treated mice and 16 thrombi in ASA-treated mice are shown. *$P < 0.05$ compared with vehicle. **D. Time to half-maximal activation ratio of anti-P-selectin.** Mean ± SEM of 18 thrombi in vehicle-treated mice and 25 thrombi in ASA-treated mice are shown.

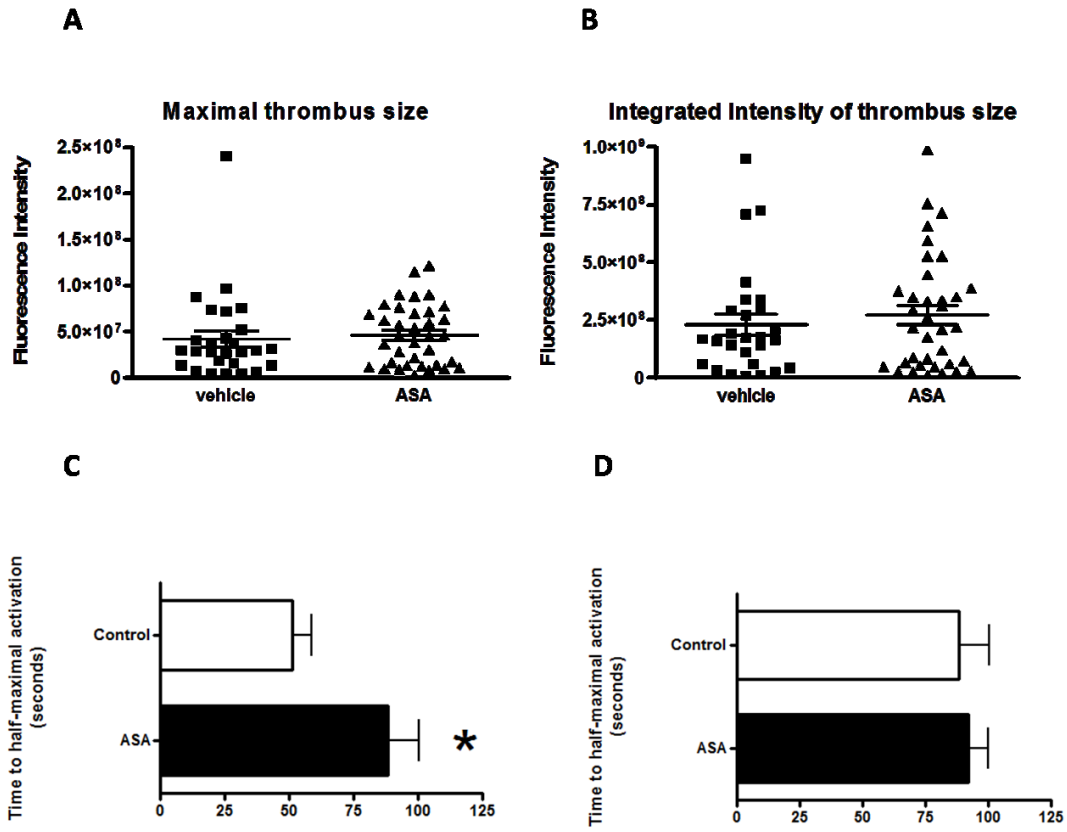
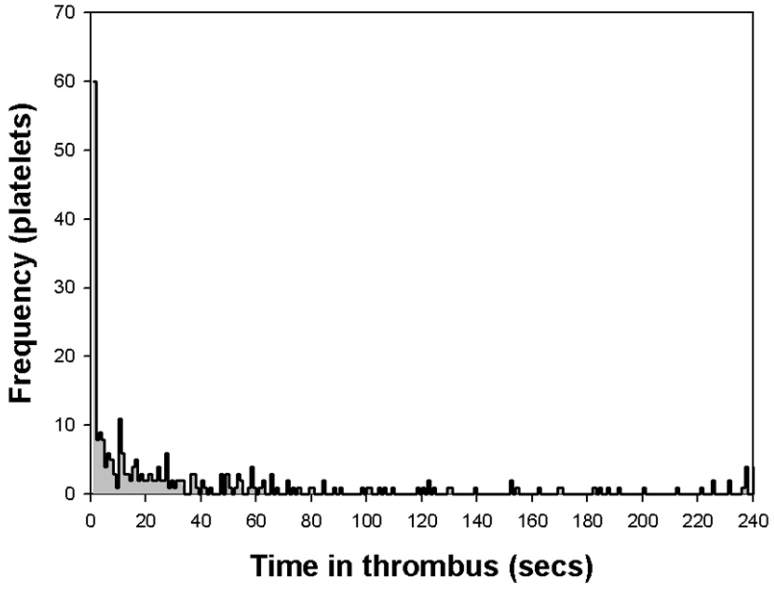

A
Maximal thrombus size
Fluorescence Intensity
2.5×10⁸
2.0×10⁸
1.5×10⁸
1.0×10⁸
5.0×10⁷
0
vehicle
ASA
B
Integrated Intensity of thrombus size
Fluorescence Intensity
1.0×10⁹
7.5×10⁸
5.0×10⁸
2.5×10⁸
0
vehicle
ASA
C
Time to half-maximal activation (seconds)
Control
ASA
*
0
25
50
75
100
125
D
Time to half-maximal activation (seconds)
Control
ASA
0
25
50
75
100
125

Figure 3.5 Distribution of the amount of time platelets spend in a thrombus.

DiO-labeled platelets ($1x10^5$) were infused into mice prior to thrombus formation. Using fluorescence microscopy the thrombi were imaged every 300 milliseconds. The amount of time each individual DiO-labeled platelet was adherent to a thrombus was recorded. Typically, 5 or 6 DiO-labeled platelets interacted with the thrombus during the 240 seconds of recording. Shown is a binned histogram of 265 DiO-labeled platelets in 44 thrombi in 6 mice. The median length of time a transfused platelet was adherent to the thrombus was 15.3 seconds (range from 0.6 to 240 seconds); the mode value was 0.6 seconds.


Frequency (platelets)
70
60
50
40
30
20
10
0
0
20
40
60
80
100
120
140
160
180
200
220
240
Time in thrombus (secs)

the thrombus-associated platelets observed early after laser injury remained adherent for only a brief period of time, suggesting that this cell-adhesion interaction is weak and is thus unlikely to involve integrin upregulation.

3.5 Discussion

We have developed a novel technique to evaluate platelet activation in a thrombus *in vivo*. This method monitors different aspects of platelet activation over time and at specific locations within the thrombus. Platelets rapidly accumulate after laser injury to cremaster arterioles, as determined by X488 binding to GPIbβ. Initially, most of the platelets in the thrombus are not activated, although they are adherent one to another. This is followed by upregulation of CD41 expression, which reaches half-maximal within 50 seconds and occurs diffusely throughout the thrombus. In contrast, P-selectin expression, a marker of α-granule release, occurs more slowly and Diannexin binding, representing PS expression, takes even longer to reach a maximum within the thrombus. By the time these activation events reach their peak, the platelet volume (determined by GPIbβ) within the thrombus is already decreasing. Consistent with this concept is our finding that most of the platelets remain adherent to the thrombus for less than 30 seconds. In addition, the increase in P-selectin and PS expression is mostly localized to the vessel wall and likely at the site of the laser injury. Thus, we can now separate various parameters of platelet activation *in vivo* in time and space.

A major limitation of the laser injury cremaster arteriole model is the large

variation (over 2 log) in the extent of platelet accumulation in thrombi (Dubois et al., 2006) (and Figure 4), which likely reflects subtle differences in the extent of injury produced by the laser (Hechler et al., 2010). Despite this variation, however, mice deficient in PAR-4, talin1, kindlin-3, LAT, ESAM or protein kinase Cα exhibit reduced platelet accumulation at sites of injury (Chou et al., 2004; Dubois et al., 2006; Nieswandt et al., 2007; Vandendries et al., 2007; Kalia et al., 2008; Moser et al., 2008; Konopatskaya et al., 2009; Stalker et al., 2009). However, mice with more subtle platelet defects or those given ASA do not show consistent reductions in platelet accumulation/thrombus volume. The advantage of high-speed two-color confocal microscopy is that it permits assessment of platelet activation as well as platelet accumulation/thrombus volume. This advantage is best illustrated by the findings with ASA, which has no effect on platelet accumulation/thrombus volume or P-selectin expression, but delays the upregulation of CD41 expression. These findings are consistent with the effects of ASA on *in vitro* platelet activation in the presence of physiological levels of Ca^{2+} (Rand et al., 1996) and suggest that assessment of the effect of platelet inhibitors on platelet activation may provide information that would be missed if only platelet accumulation/thrombus volume were measured. It is important to note that potent inhibitors of coagulation, such as high-dose hirudin, block platelet accumulation (as measured using GPIbβ) in thrombi to such an extent that CD41 upregulation and P-selectin expression cannot be measured (data not shown).

Our system is unable to identify the change in conformation of CD41/CD61 ($\alpha_{IIb}\beta_3$ integrin) upon platelet activation. In mice, this conformational change results in increased binding of phycoerythrin (PE)-tagged JON/A antibody; untagged antibody fails to recognize the activated conformation of the integrin. Unfortunately, PE is not suitable for *in vivo* imaging. Also, our system is not fast enough to simultaneously visualize three activation markers. Therefore, we are unable to determine whether PS-expressing platelets and those exhibiting integrin-α_{IIb} upregulation represent distinct subsets, as has been reported based on observations made at a single time-point after injury to the carotid artery (Munnix et al., 2007). We did not observe a decrease in CD41 relative to GPIbβ over the initial 5 minutes of observation; a decrease in activated CD41/CD61 relative to annexin binding was observed *in vitro* 10 minutes after activation (Munnix et al., 2007).

We found that Diannexin (a recombinant homodimer of annexin A5) bound to platelets in the eccentric portion of the thrombus proximal to the vessel wall and the site of laser injury; a finding consistent with a report by Hayashi et al. (Hayashi et al., 2008) who evaluated annexin binding to laser-injured, low shear (approx. 100 second^{-1}) mesenteric venules. However, whilst we found that Diannexin labeling was maximal within 40 seconds of thrombus formation, Hayashi et al. reported continued annexin binding to platelets in thrombi up to 300 seconds after injury. It is noteworthy that Hayashi et al. and others (Munnix et al., 2007) used annexin as a label *in vivo* at a final concentration in blood in the 100 (Hayashi et al., 2008) to 1000 nM (Munnix et

al., 2007) range. In contrast, we used Diannexin at a final concentration of 5 nM, a concentration that, in our system, did not inhibit platelet accumulation in thrombi. Consistent with the findings with high concentrations of annexin, Diannexin was inhibitory in preliminary studies in our system when administered at concentrations over 100 nM (data not shown).

Like others (Grosse et al., 2007; Hechler et al., 2010), we assume that GPIbβ is a valid marker of platelet accumulation/thrombus volume that does not decrease after platelet activation. Although this is controversial, by immunoblot analysis of platelet lysates, we failed to detect a decrease in GPIbβ after thrombin activation. After collagen activation, GPIbβ decreases in the intact platelet fraction, but not in the fraction that is sedimented after centrifugation at 13,000 x g, which presumably includes platelet microparticles. It is unknown whether platelet microparticles accumulate within thrombi *in vivo*. We are making the assumption that platelet microparticles and activated platelets accumulate in an identical fashion; thus, GPIbβ can be used as a marker of intact platelet or platelet microparticle accumulation. If this assumption is incorrect and GPIbβ in a thrombus decreases with platelet activation, our activation ratio parameter would be amplified. Because GPIbβ is used as a marker for platelets in conjunction with all of the markers of activation, however, our conclusions would be unaltered unless platelet microparticles preferentially contain GPIbβ relative to CD41, P-selectin and PS, all of which are found in microparticles.

Our findings of distinct locations of activation cannot be explained by steric

hindrance with some of the markers. F_{ab} fragments of the antibody against CD41 are small, but are found diffusely within the thrombus. Although Diannexin and P-selectin antibody differ in size, they bind to similar locations at the periphery, mostly deep within the thrombus. The observation that PS and P-selectin expressing platelets localize to the periphery of the thrombus confirms the findings of Munnix et al., which was made at a single time-point after injury to the carotid artery using annexin as a label (see above) (Munnix et al., 2007).

Our observation that the arterial thrombus is largely composed of platelets that are not fully activated is consistent with the results of studies that used DIC microscopy to evaluate platelet morphology in thrombi (Kulkarni et al., 2000; Jackson et al., 2003). Initially, most of the platelets in a thrombus are not activated as evidenced by their short dwell time and the lack of upregulation of CD41. These platelets are likely adhering via low affinity interactions with von Willebrand factor (vWF) and/or fibrinogen; morphologically they are discoid (Kulkarni et al., 2000; Jackson et al., 2003; Nesbitt and Jackson, 2006). A fraction of arriving platelets become activated, but even these activated platelets leave the thrombus (individually or in aggregates) as evidenced by the decreasing absolute amount of anti-P-selectin and Diannexin binding to the thrombus over time (data not shown). The relatively slow activation of platelets within thrombi may explain the clinical success of antiplatelet therapy. ASA is the foundation of such treatment, produces a 20% to 25% reduction in the risk of cardiovascular death, myocardial infarction and stroke in

patients with atherosclerotic disease (Becker et al., 2008). Despite its clear benefit in humans, however, ASA has only modest effects in animal models of thrombosis. If routine intravital videomicroscopy were used for screening, drugs like ASA would be dismissed because they fail to attenuate platelet accumulation/thrombus volume. In contrast, with high-speed two-color confocal microscopy, we can detect the specific effects of relatively weak antiplatelet agents on platelet activation. Therefore, our system may not only be useful for monitoring the dynamics of platelet activation, but also may help to better define the action of existing antiplatelet agents *in vivo* and screen new antiplatelets.

Chapter 4: Atorvastatin delays platelet activation *in vivo* even in the absence of endothelial NO synthase.

Author list: Ran Ni, Tal Peleg, Peter L. Gross

Foreword

This manuscript has been published in Arteriosclerosis, Thrombosis, and Vascular Biology (PMID: 22995523, Vol: 32, No.: 11, pp2609-15, November 2012). All experiments contained in the article were performed by me. Dr. Tal Peleg helped with the measurement of platelet activation in eNOS-deficient mice using flow cytometry. The manuscript was written by me and Dr. Peter L. Gross. According to the policies of the Journal of the American Heart Association, no formal permission is required to reproduce this article for non-commercial purposes. Chapter 4 is a direct representation of the above referenced article.

4.1 Abstract

Objective: Statins decrease mortality in patients with vascular disorders, and evidence for the pleiotropic effects of statins is accumulating. Statins enhance endothelial NO synthase (eNOS) expression, thereby attenuating platelet activation and thrombus formation. Our goal was to determine whether statins have eNOS-independent effects on platelet activation.

Methods and Results: Wild-type and eNOS-deficient mice were given a 14-day course of oral atorvastatin, and platelet activation was evaluated *in vitro* and *in vivo*. Whereas in wild-type mice atorvastatin inhibited platelet activation *in vitro* in response to numerous agonists, in eNOS-deficient mice, atorvastatin inhibited only thrombin-induced and protease-activated receptor 4 (PAR4) agonist peptide-induced platelet activation. Consistent with an eNOS-independent effect, atorvastatin inhibited platelet activation *in vivo* in both wild-type and eNOS-deficient mice.

Conclusion: Atorvastatin inhibits platelet activation via eNOS-dependent and eNOS-independent mechanisms with the latter restricted to PAR4-induced activation downstream of the receptor.

4.2 Introduction

Statins, inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A reductase, effectively reduce the burden of atherothrombotic disease (Sever et al., 2004). Recent evidence suggests that this may be partly attributed to processes that are independent of lipid reduction (Ray, 2003; Calabro and Yeh, 2005). Statins seem to attenuate

platelet activation in hypercholesterolemic patients (Puccetti et al., 2002; Puccetti et al., 2003) and in animal models (Alfon et al., 1999; Gaddam et al., 2002; Thompson et al., 2002; Schirmer et al., 2012). Normocholesterolemic mice that received statins were protected in stroke and myocardial infarction models; they also exhibited decreased endothelial Rho GTP-binding activity, increased endothelial and platelet endothelial nitric oxide synthase (eNOS) mRNA expression, and lower circulating levels of platelet activation markers, including β-thromboglobulin and platelet factor-4 (Laufs et al., 1998; Laufs et al., 2000; Laufs et al., 2002; Gertz et al., 2003).

Nitric oxide (NO) inhibits platelet activation (Radomski et al., 1987; Kroll and Sullivan, 1988; Freedman et al., 1997; Freedman et al., 1999; Freedman and Loscalzo, 2003) through a variety of mechanisms (see review by Loscalzo (Loscalzo, 2001)). NO activates guanylate cyclase (GC) in platelets, leading to an increase in cyclic guanosine monophosphate (cGMP) (Pigazzi et al., 1999). This can alter many signaling proteins in platelets and is considered to be the major mediator of NO signal transduction. Rosuvastatin treatment of rats reduced phosphorylation of platelet vasodilator-stimulated phosphoprotein, an indicator of NO activity (Schafer et al., 2005). Platelets from rats treated with cerivastatin exhibited reduced platelet aggregation and greater NO release, and these rats had delayed times to occlusion in a carotid artery injury model of thrombosis – all of these effects are abolished with L-arginine methylester, an NO inhibitor (Yokoyama et al., 2005). *In vitro*, simvastatin inhibits collagen- and arachidonic acid-induced aggregation of rabbit platelets and

enhances NO and cGMP production – effects that are attenuated with NO scavengers or inhibitors (Chou et al., 2008). Together, these findings provide evidence that statins inhibit platelets in an NO-dependent fashion. However, it remains unclear whether statins also have NO-independent effects on platelets. To explore this possibility, we compared the effects of a 14-day course of oral atorvastatin on *in vitro* and *in vivo* platelet function in eNOS-deficient mice with those in wild-type mice.

4.3 Materials and Methods

4.3.1 Materials

Wild-type C57BL/6J and eNOS-deficient mice were purchased from Jackson Laboratories USA. Atorvastatin tablets were obtained from Hamilton Health Sciences Pharmacy, Canada. Atorvastatin tablets were ground into a powder, which was then mixed with regular mouse diet ingredients (AIN-76A) at a concentration of 0.03 g/kg before compression into pellets. The mouse diet was prepared by Dyets Inc. USA. All chemicals were purchased from Sigma Aldrich USA unless otherwise stated. Antibodies used in the detection of platelet activation and platelet accumulation *in vitro* and *in vivo* were from Emfret Analytics Germany, with the exception that the anti-P-selectin antibody (RB40.34) was purchased from Becton Dickinson (BD), goat anti-PAR4 antibodies and an irrelevant anti-goat antibody were purchased from Santa Cruz USA. Antibody F_{ab} fragments were generated using a F_{ab} preparation kit from Pierce USA. Fluorophore conjugation was performed using protein labeling kits from

Pierce USA and Invitrogen USA. Anesthesia solutions ketamine, xylazine, atropine and Nembutal were purchased from Ayerst, Bayer, Ormond Veterinary and Abbott laboratories (Canada), respectively. General surgical supplies were from Fine Scientific Tools (Canada) and BD Biosciences (USA). 60 X water immersion objective was purchased from Olympus Japan. Photodiode velocimeter (a Microvessel Velocity OD-RT Doppler) was obtained from CircuSoft Instrumentation Germany. Piezo-electric focuser was obtained from Physiks International Germany. Nitrogen dye laser was from Micropoint System USA. The rest components for intravital microscopy including Slidebook software (5.0) were purchased from Intelligent Imaging Innovations USA. Anticoagulants heparin and sodium citrate were purchased from Leo Pharma Inc. Canada and BD Biosciences USA, respectively. A direct cGMP ELISA kit was purchased from Enzo Life Sciences USA. Bovine thrombin used in Multiplate Analyzer (Diapharma) was from Sigma USA and platelet aggregation was measured using Multiplate mini test cells (Diapharma). Platelet inhibitors apyrase and prostacyclin (PGI_2) used in platelet isolation were purchased from Sigma and Calbiochem USA, respectively. Disposable hemocytometers were from Incyto Korea. Bovine thrombin used in flow cytometry was from Roche Diagnostics USA. PAR4 agonist peptide (H-Gly-Try-Pro-Gly-Lys-Phe-OH, GYP) was from Bachem Switzerland, collagen was from Nycomed Pharma Germany and ADP was purchased from Sigma USA.

4.3.2 Methods

4.3.2.1 Oral administration of atorvastatin

Age-matched male wild-type mice were used between 8 to 12 weeks old and weighed at least 25 grams. Both wild-type C57BL/6J and eNOS-deficient mice were divided into two groups. The control group received the same mouse diet without the active drug, while the other group received the diet containing atorvastatin at a concentration of 0.03 g/kg of diet. All mice consumed roughly 5 g of diet per day, such that the experimental group received around 7.5 mg/kg of atorvastatin per day. Studies were done according to Canadian Council of Animal Care Guidelines, and all animal use protocols were approved by the Animal Research Ethics Board at McMaster University.

4.3.2.2 Isolation of washed mouse platelets

Blood (1 mL) obtained by carotid cannulation was collected into 0.1 mL of Tris-buffered saline (20 mM Tris-HCl, 137 mM NaCl, pH 7.3) containing 20 U/mL heparin (9:1 vol/vol). Platelet-rich plasma (PRP) was obtained by centrifugation at 500 x g for 5 minutes. After a second centrifugation at 1,500 x g for 5 minutes, the platelet pellet was washed twice in Tyrode's buffer (134 mM NaCl, 0.34 mM Na2HPO$_4$, 2.9 mM KCl, 12 mM NaHCO$_3$, 20 mM HEPES, pH 7.0, 5 mM glucose, 0.35% (w/v) bovine serum albumin) containing 20 mU/mL apyrase and 0.5 nM PGI$_2$ and then resuspended in 500 μL Tyrode's buffer supplemented with 40 mU/mL apyrase. Experiments with washed platelets were performed after they were incubated

for 30 minutes at 37℃; this method is described in detail on the Emfret Analytics Web site (www.emfret.com).

4.3.2.3 Determination of platelet cGMP levels

After lysis of 1 X 10^9 washed platelets with 0.1 M HCl, the cellular debris was pelleted by centrifugation at 600 x g. After acetylation using acetic anhydride and triethylamine, cGMP in the supernatant was assayed using a competitive ELISA. To reduce the potential of between-day variation, samples were analyzed in pairs (wild-type and eNOS-deficient mice, or control and atorvastatin-treated mice), and results were expressed relative to the values for wild-type mice on a control diet.

4.3.2.4 Flow cytometry assessment of platelet activation

Platelet activation was determined by conformational changes of platelet aggregation receptor, $\alpha_{IIb}\beta_3$, and P-selectin expression on platelet surface. The two aspects of platelet activation were detected using the combination of a PE-conjugated antibody, JON/A, directly against activated form of $\alpha_{IIb}\beta_3$ and a FITC-conjugated antibody, Wug.E9, directly against P-selectin. Washed platelets were adjusted to 1 x 10^6 cells per reaction in calcium-free Tyrode's buffer and 0.1 M $CaCl_2$ was then added to all samples prior to the addition of platelet excitatory agonists. Washed mouse platelets were activated with ADP (0.12 to 122 μM/mL), with thrombin (2.4 to 12.1 mU/mL), with PAR4 agonist peptide (GYP, 1.67 to 5.28 mM), or with collagen (33.3 to 216 μg/mL). The reaction was terminated at 15 minutes by 400 μL PBS containing 1% formaldehyde. Platelet activation was monitored by flow cytometry and values

were expressed as mean fluorescence intensity (MFI).

4.3.2.5 Multiplate determination of platelet activation

Blood samples were taken via carotid cannulation into 3.2% sodium citrate (9:1 vol/vol), and 175 uL of citrated whole blood was mixed with an equal volume of normal saline containing 3 mM $CaCl_2$ in the cup portion of a pediatric cuvette (mini multiplate test cell). After 3-minute incubation at 37 ℃, aggregation was initiated by an addition of 162 U thrombin or 7 μg/mL collagen with consistent agitation at 800 rpm. Electrical impedance caused by platelet adhesion and aggregation was monitored at approximately 0.5 second intervals for 6 minutes using a Multiplate Analyzer. Values were expressed as area under the curve (AUC). In some experiments, 4 mM acetylsalicylic acid (ASA, aspirin) was incubated with blood for 10 minutes at room temperature before recalcification.

4.3.2.6 PAR4 expression on mouse platelets

PAR4 expression on washed mouse platelets was quantified by flow cytometry using 53 μg/mL of a polyclonal goat antibody directed against the N terminus of mouse PAR4, or irrelevant goat IgG, and detected with 53 μg/mL of PE-conjugated anti-goat IgG, a secondary antibody directed against the primary antibody; a Dylight-488-tagged monoclonal antibody directed against platelet glycoprotein GPIbβ, was used to gate the platelet population. Values were expressed as geometric mean fluorescence intensity.

4.3.2.7 Intravital analysis of platelet accumulation and activation after laser-induced injury to cremaster muscle arterioles

In anesthetized mice, the cremaster muscle was prepared as previously described (Rand et al., 2012). Using the jugular vein cannulus, mice were given an infusion of 0.1 μg/g of Dylight-488-tagged anti-GPIbβ antibody X488. Where indicated, an infusion of 1.3 μg/g of Dylight-647-tagged F_{ab} fragments of rat anti-mouse CD41 (MWReg30), a monoclonal antibody directed against α_{IIb} that recognizes both the resting and activated form of this platelet-specific integrin, or 2 μg/g of a Dylight-647-tagged antibody against P-selectin (RB40.34) was also administered (Falati et al., 2002). Fluorophore conjugation and F_{ab} fragment preparation were performed according to the directions from the supplier. After laser injury to cremaster arterioles, platelet accumulation and activation were measured and analyzed as previously described (Rand et al., 2012), except that platelet activation was assessed on the basis of both CD41 upregulation and P-selectin surface expression (see detailed methods in Chapter 3).

4.3.2.8 Statistical analyses

Data were analyzed using Prism 5.0. Agonist-induced platelet activation detected by flow cytometry (using JON/A-PE and anti-P-selectin-FITC) was fitted to a sigmoidal dose-response curve with 4 parameters and fixing the parameter bottom at zero. The significance of difference between fitted curves was examined using an extra sum of squares *F* test (Prism). Group data were expressed as means±SEM. Significance of differences was determined using Student's *t*-tests. cGMP levels were

normalized relative to control values, and significance of the means relative to 1.0 was determined using *t*-tests. In all analyses, **P*<0.05 was considered statistically significant.

4.4 Results

4.4.1 Atorvastatin treatment increases platelet cGMP levels

cGMP levels were measured using a competitive ELISA. Compared with controls, atorvastatin treatment increased platelet cGMP levels by 1.5-fold (95% CI, 1.3- to 1.7-fold) in wild-type mice. In contrast, cGMP levels in eNOS-deficient mice were 33.6% lower than those in wild-type mice (95% CI, 18.5-48.8%) and were not changed with atorvastatin treatment (95% CI, 0.86- to 1.28-fold) **(Figure 4.1)**. Thus, atorvastatin increases cGMP levels in platelets from wild-type mice, but not in platelets from eNOS-deficient mice, consistent with the known capacity of statins to upregulate eNOS activity.

4.4.2 Atorvastatin treatment inhibits platelet activation in vitro even in platelets from eNOS-deficient mice

In platelets from wild-type mice, atorvastatin treatment inhibited platelet activation induced by ADP, collagen, GYP and thrombin as measured by $\alpha_{IIb}\beta_3$ activation (JON/A-PE binding), and by GYP and thrombin as measured by P-selectin surface expression (anti-P-selectin-FITC binding) **(Figure 4.2)**. ADP did not increase P-selection surface expression (data not shown), and collagen-induced P-selectin

Figure 4.1 Effect of atorvastatin treatment on platelet cGMP levels in wild-type and eNOS-deficient mice.

Platelets from wild-type and eNOS-deficient mice were obtained and analyzed in pairs (N=3); platelets were obtained from eNOS-deficient (N=5) and wild-type mice (N=4) treated with control diet or atorvastatin diet at 0.03 g/kg for 14 days and analyzed in pairs. cGMP was extracted from washed platelets and measured by using a competitive ELISA. **A.** cGMP levels in eNOS-deficient mice by atorvastatin. **B.** cGMP levels in wild-type mice by atorvastatin and cGMP levels in eNOS-deficient mice. Data show mean ± SEM of the amount of the cGMP relative to that in the wild-type mice without atorvastatin treatment. *$P < 0.05$ compared with the control. (one-sample *t*-test).

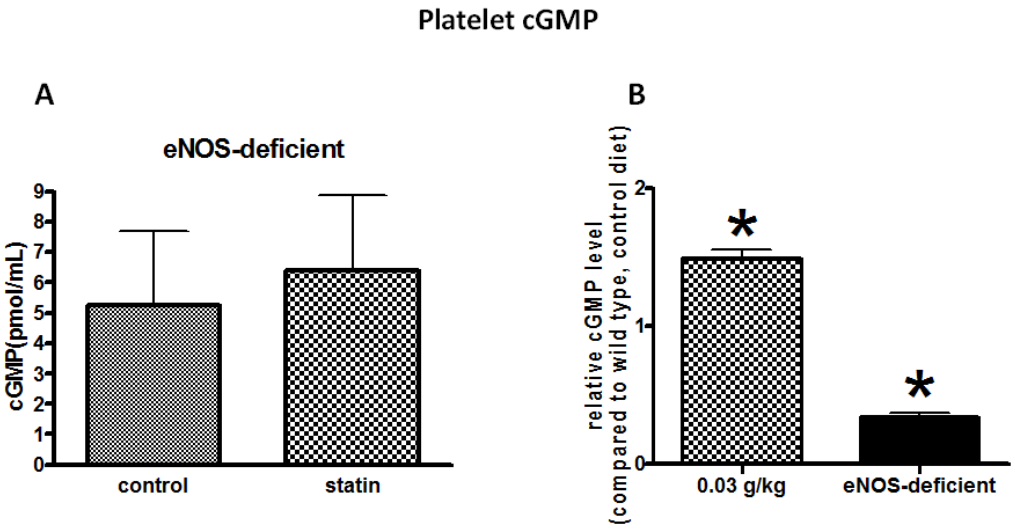
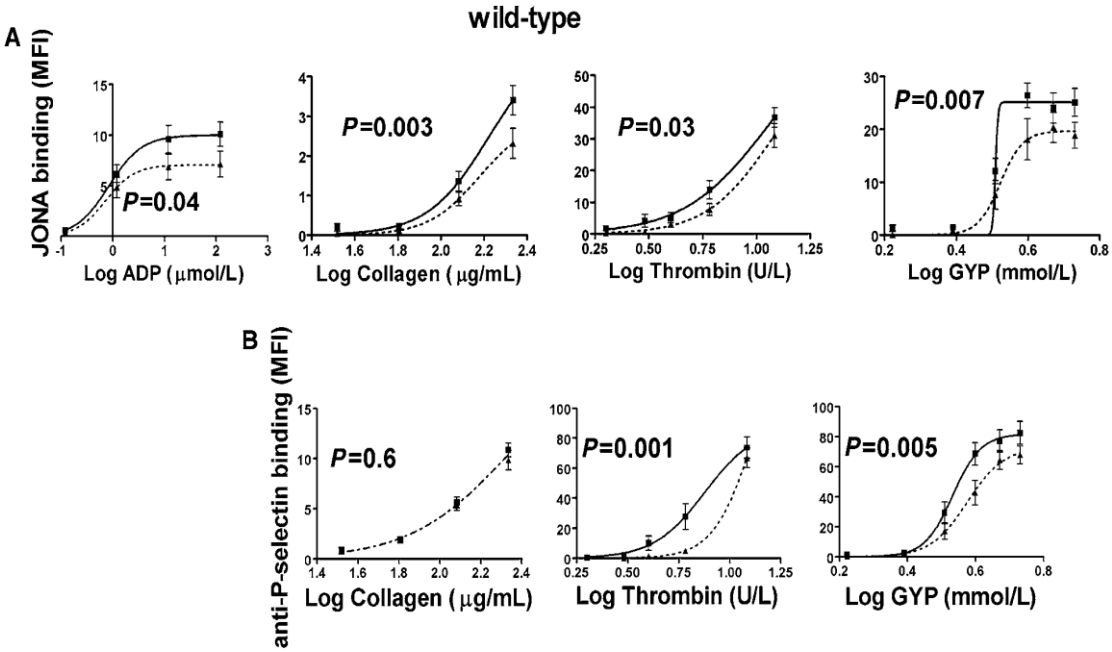

Platelet cGMP
A
eNOS-deficient
cGMP(pmol/mL)
0
1
2
3
4
5
6
7
8
9
control
statin
B
relative cGMP level
(compared to wild type, control diet)
0
1
2
*
*
0.03 g/kg
eNOS-deficient

Figure 4.2 Effect of atorvastatin diet on the flow cytometry markers of platelet activation in wild-type mice.

Washed platelets were obtained from mice fed with atorvastatin diet at 0.03 g/kg (triangles, dashed lines) or control diet (squares, solid lines) for 14 days. Data show mean ± SEM, N=14 mice per group. The *x* axis depicts the log of the agonist concentration. Best-fit sigmoidal dose-response curves were generated using Prism, assuming a bottom of 0. *P* values are from an extra sum of squares *F* test of the null hypothesis that the 3 parameters (slope, EC50, and top) are same between the groups. If the 3 parameters are the same ($P>0.05$), then the shared fit is drawn as a dash-dot line. **A.** JON/A-PE binding after activation by ADP, GYP, collagen, or thrombin. **B.** Anti-P-selectin-FITC binding in the same samples. No anti-P-selectin-FITC binding occurred after ADP activation. MFI indicates mean fluorescence intensity.


wild-type
A
JONA binding (MFI)
P=0.04
Log ADP (μmol/L)
P=0.003
Log Collagen (μg/mL)
P=0.03
Log Thrombin (U/L)
P=0.007
Log GYP (mmol/L)
B
anti-P-selectin binding (MFI)
P=0.6
Log Collagen (μg/mL)
P=0.001
Log Thrombin (U/L)
P=0.005
Log GYP (mmol/L)

surface expression was not inhibited by atorvastatin. In eNOS-deficient mice, atorvastatin treatment significantly inhibited platelet activation by collagen, GYP and thrombin as measured by JON/A-PE binding, and by GYP and thrombin as measured by anti-P-selectin-FITC binding **(Figure 4.3)**. In these mice, atorvastatin did not significantly alter platelet activation by ADP as measured by JON/A-PE binding or by collagen as measured by anti-P-selection-FITC binding **(Figure 4.3)**. Thus, although atorvastatin inhibits platelet activation in response to a wide range of agonists in wild-type mice, it only inhibits platelet activation in response to GYP and thrombin in eNOS-deficient mice. Likewise, in the Multiplate Analyzer, atorvastatin significantly reduced (P=0.04) AUC of aggregation induced by thrombin in whole blood from eNOS-deficient mice, but had no significant effect on collagen-induced aggregation **(Figure 4.4 A)**. To interrogate the effects of atorvastatin on the thromboxane (TXA_2) pathway, citrated whole blood from eNOS-deficient mice were treated with ASA *in vitro* for 10 minutes prior to Multiplate analysis. ASA pretreatment decreased aggregation induced by collagen in atorvastatin-treated eNOS-deficient mice to the same extent as it did in control eNOS-deficient mice **(Figure 4.4 B)**. Taken together, these results suggest that in eNOS-deficient mice, atorvastatin treatment inhibits GYP- and thrombin-induced platelet activation. In contrast, in these mice, atorvastatin does not influence collagen-induced platelet activation nor does it seem to alter the TXA_2 pathway.

Figure 4.3 Effect of atorvastatin diet on the flow cytometry markers of platelet activation in eNOS-deficient mice.

Washed platelets were obtained from eNOS-deficient mice fed with atorvastatin diet at 0.03 g/kg (triangles, dashed lines) or control diet (squares, solid lines) for 14 days. Data show mean ± SEM, N=14 mice per group. The *x* axis depicts the log of the agonist concentration. Best-fit sigmoidal dose-response curves were generated using Prism assuming a bottom of 0. *P* values are an extra sum of squares *F* test of the null hypothesis that the 3 parameters (slope, EC50, and top) are same between the groups. If the 3 parameters are the same ($P>0.05$), then the shared fit is drawn as a dash-dot line. **A.** JON/A-PE binding after activation by ADP, GYP, collagen or thrombin. **B.** Anti-P-selectin-FITC binding in the same samples. MFI indicates mean fluorescence intensity.

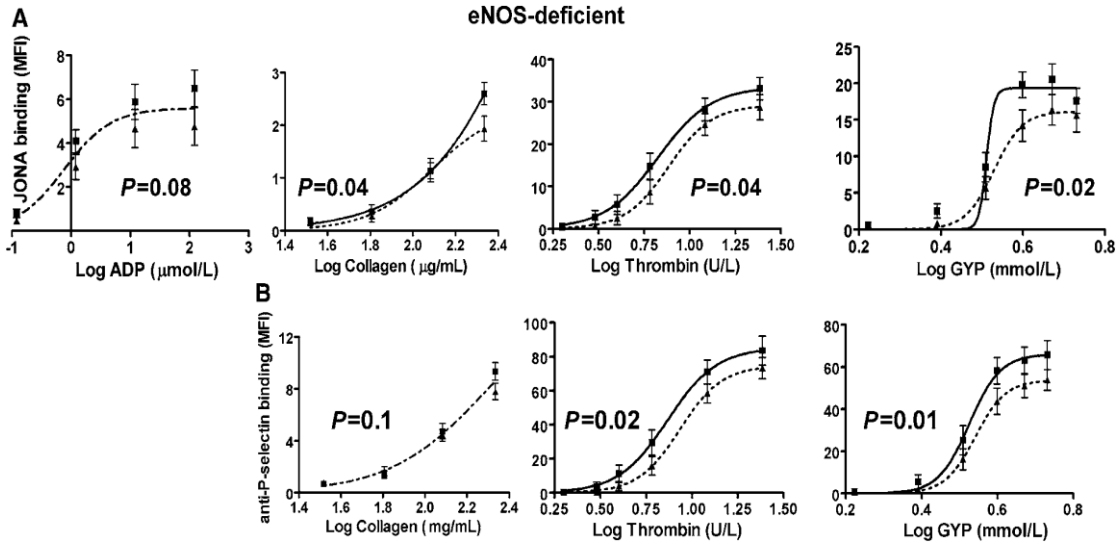
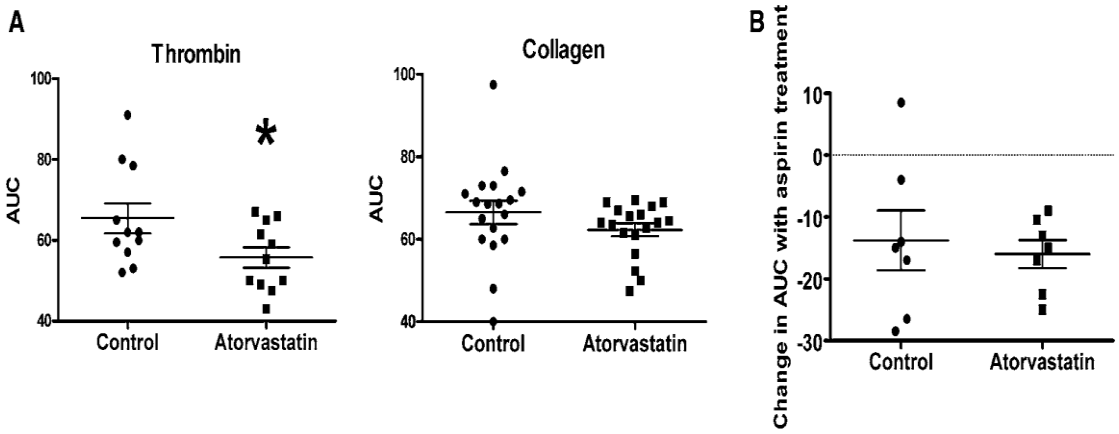

A
eNOS-deficient
JONA binding (MFI)
P=0.08
Log ADP (μmol/L)
P=0.04
Log Collagen (μg/mL)
P=0.04
Log Thrombin (U/L)
P=0.02
Log GYP (mmol/L)
B
anti-P-selectin binding (MFI)
P=0.1
Log Collagen (mg/mL)
P=0.02
Log Thrombin (U/L)
P=0.01
Log GYP (mmol/L)

Figure 4.4 Effect of atorvastatin diet on platelet aggregation in eNOS-deficient mice measured by Multiplate Analyzer.

Whole blood (in citrate) from control mice or mice treated with atorvastatin at 0.03 g/kg was diluted and recalcified before agonist addition. **A.** Area under the curve (AUC) of aggregation after thrombin (162 U, N=11) or collagen (7 μg/mL, N=18). *P<0.05. **B.** Blood was treated with acetylsalicylic acid (aspirin, ASA) *in vitro* before aggregation by collagen (7 μg/mL, N=7). Shown are individual values of the change in AUC with ASA treatment and mean ± SEM.


A
Thrombin
AUC
100
80
60
40
*
Control
Atorvastatin
Collagen
AUC
100
80
60
40
Control
Atorvastatin
B
Change in AUC with aspirin treatment
10
0
-10
-20
-30
Control
Atorvastatin

4.4.3 PAR4 expression on platelets is not altered by atorvastatin treatment

To exclude the possibility that the impaired response to GYP and thrombin in platelets from atorvastatin-treated mice reflects reduced expression of PAR4 on their platelets, PAR4 expression on platelets from wild-type mice was quantified using flow cytometry with antibodies directly against PAR4 extracellular domain. Although the fluorescence intensity of PAR4 antibody binding to the resting platelets was low, it was above background and was similar in atorvastatin-treated mice and in the untreated controls **(Figure 4.5)**.

4.4.4 Atorvastatin treatment has no effect on platelet accumulation in thrombi in vivo

Platelet accumulation in laser-injured cremaster arterioles was assessed using established methods. In both wild-type and eNOS-deficient mice, atorvastatin has no effect on the integrated intensity of GPIbβ, maximal intensity, or time to maximal intensity (data not shown).

4.4.5 Time to half-maximal activation ratio of CD41 and P-selectin of platelets in thrombi is delayed by atorvastatin treatment of wild-type mice in vivo

Because atorvastatin had no effect on platelet accumulation/thrombus volume after laser-induced injury in wild-type mice, we examined the effect of atorvastatin on platelets activation in thrombi formed in wild-type mice **(Figure 4.6)**. Compared with controls, atorvastatin treatment significantly ($P<0.0001$) prolonged the time to

Figure 4.5 Effect of atorvastatin diet on PAR4 surface expression in mouse platelets.

Mean ± SEM of the geometric mean fluorescence of PAR4 antibody binding to washed resting platelets from 10 mice on control diet (clear bar) and 10 atorvastatin-treated mice (checked bar) are shown.

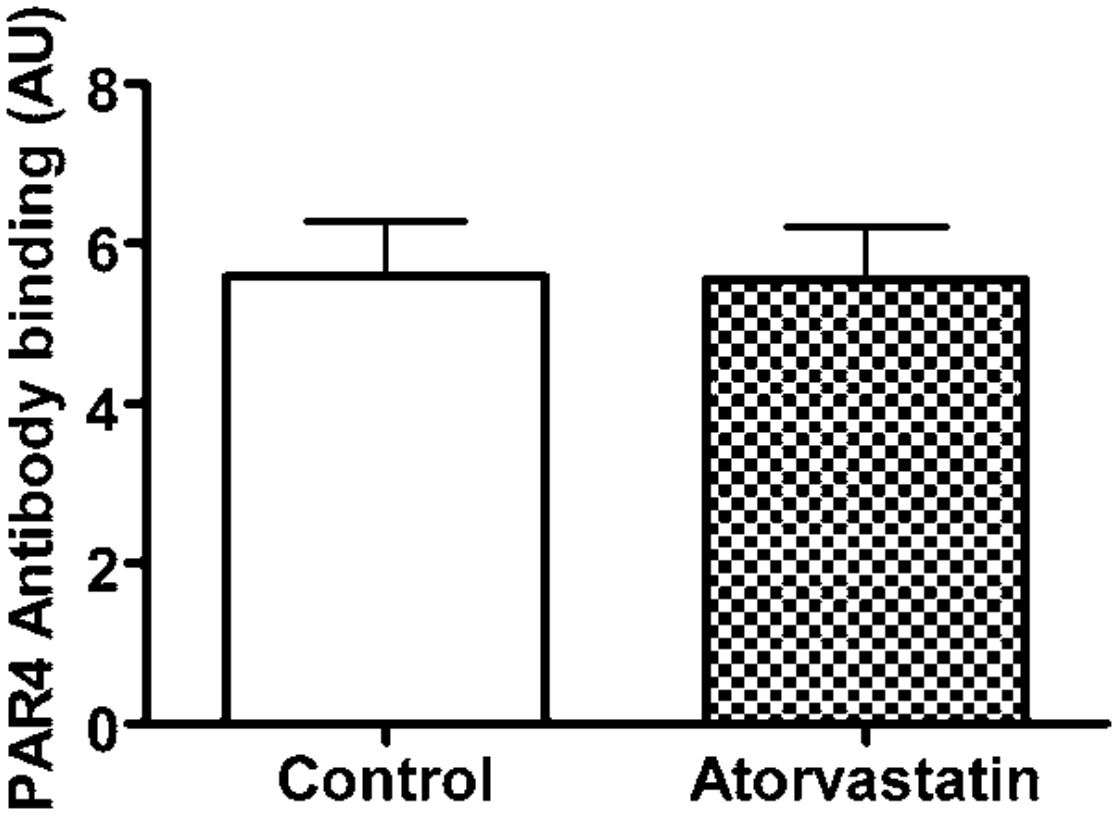
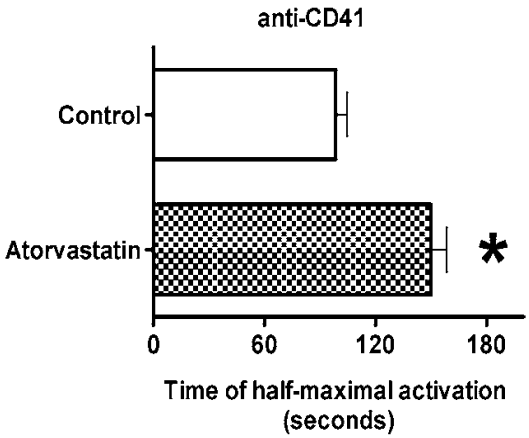
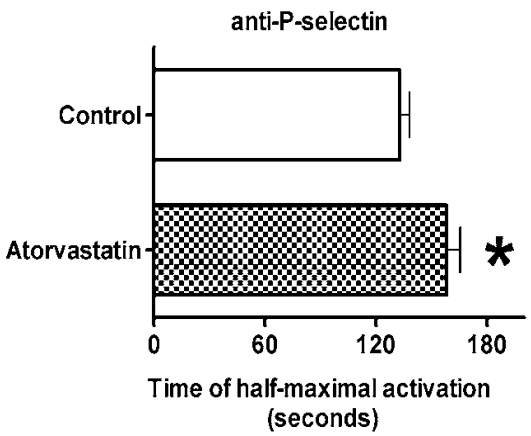

PAR4 Antibody binding (AU)
8
6
4
2
0
Control
Atorvastatin

Figure 4.6 Effect of atorvastatin diet on platelet activation in thrombi in wild-type mice.

Time to half-maximal activation ratio of anti-CD41 (left). Mean ± SEM of 33 thrombi in control diet mice (clear bar) and 46 thrombi in atorvastatin-treated mice (checked bar) are shown. Time to half-maximal activation ratio of anti-P-selectin (right). Mean ± SEM of 28 thrombi in control diet mice and 32 thrombi in atorvastatin-treated mice are shown. *P<0.05 compared with control diet.


anti-CD41
Control
Atorvastatin
*
0
60
120
180
Time of half-maximal activation
(seconds)


anti-P-selectin
Control
Atorvastatin
*
0
60
120
180
Time of half-maximal activation
(seconds)

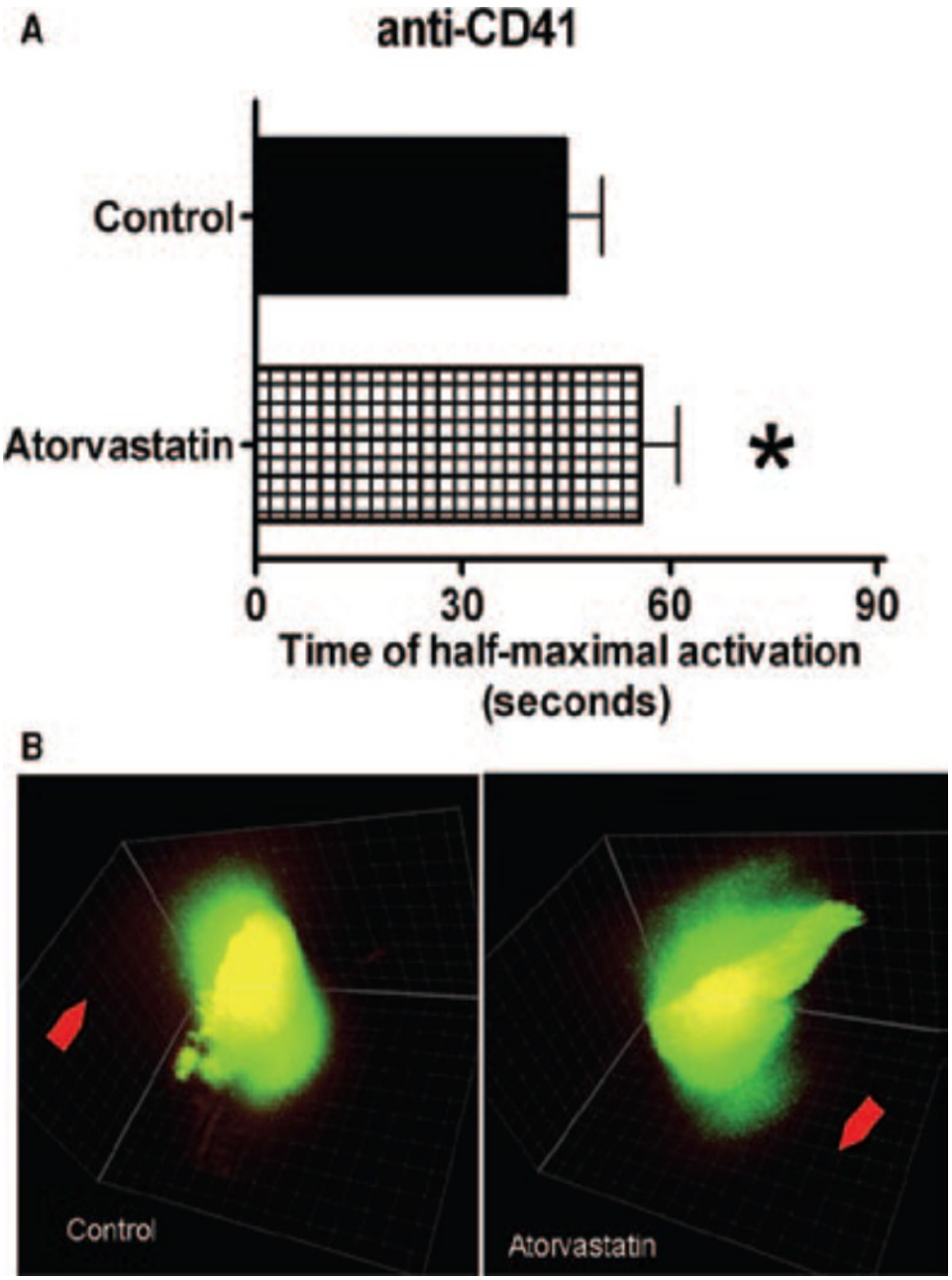
half-maximal activation ratio of CD41 1.5-fold from 98±6 seconds (N=33) to 150±9 seconds (N=46) **(Figure 4.6)**. The time to half-maximal activation ratio of P-selectin was also significantly prolonged (P<0.0044) by atorvastatin treatment by 1.2-fold from 132±5 seconds (N=62) to 158±7 seconds (N=44) **(Figure 4.6)**. Thus, although atorvastatin treatment does not alter platelet accumulation in the thrombi that form at sites of laser injury, it delays platelet activation events within these thrombi.

4.4.6 Time to half-maximal activation ratio of CD41 upregulation of platelets in thrombi is delayed by atorvastatin treatment of eNOS-deficient mice in vivo

We examined the effects of atorvastatin on platelet activation, as measured by CD41 upregulation, in thrombi formed in eNOS-deficient mice **(Figure 4.7 A)**. Similar to its effects in wild-type mice, atorvastatin significantly prolonged (P=0.044) the time to half-maximal activation ratio of CD41 upregulation by 1.3-fold from 44±5 seconds (N=46) to 57±5 seconds (N=50). To confirm these findings, 3-dimensional rendered images of the thrombi were evaluated, and thrombi of similar sizes in atorvastatin-treated mice were compared with those in controls. In all thrombi, CD41 upregulation was evident throughout the thrombus, and visual comparisons revealed less anti-CD41 antibody binding in similar-sized thrombi in atorvastatin-treated mice than in controls **(Figure 4.7 B)**. Thus, although atorvastatin treatment inhibited only GYP- and thrombin-induced platelet activation in eNOS-deficient mice, it still modulates platelet activation events in thrombi in these mice.

Figure 4.7 Effect of atorvastatin diet on CD41 upregulation within thrombi in eNOS-deficient mice.

A. Time to half-maximal activation ratio of 46 thrombi in control diet (solid bar), and 50 thrombi in atorvastatin-treated (checked bar) mice. Mean ± SEM are shown; **P*<0.05 compared with control diet. **B.** Representative of 3-dimensional rendering of thrombi in control and atorvastatin-treated eNOS-deficient mice. Green indicates anti-GPIbβ binding; yellow indicates the colocalization of anti-CD41 binding and anti-GPIbβ binding. Each square is 10 μm. These images were collected between 60 and 80 seconds after thrombus formation. The red arrow indicates the direction of blood flow.


A
anti-CD41
Control
Atorvastatin
*
0
30
60
90
Time of half-maximal activation
(seconds)
B
Control
Atorvastatin

4.5 Discussion

There is mounting evidence that statins inhibit platelet activation in an eNOS-dependent fashion. This is not surprising given that NO is a potent inhibitor of platelet activation. However, it is unknown whether statins have eNOS-independent effects on platelets. To address this possibility, we compared the effects of atorvastatin on platelet activation *in vitro* and *in vivo* in mice deficient in eNOS with those in wild-type mice. Atorvastatin treatment of eNOS-deficient mice inhibited platelet activation induced by thrombin and GYP, inhibited aggregation induced by thrombin, and delayed platelet activation in thrombi, as measured by CD41 upregulation.

This report adds to the literature supporting the inhibition of platelet activation by statins, specifically a chronic oral dose of atorvastatin. The mice consumed approximately 7.5 mg/kg body weight of atorvastatin per day, but because of poor oral absorption of atorvastatin in mice, reported plasma levels in mice are lower than that would be expected with the same dose in humans (Shum et al., 1996; Black et al., 1998; Gertz et al., 2003; Yokoyama et al., 2005). A higher dose of atorvastatin inhibited ADP- and PAR-induced platelet activation in hyperlipidemic patients with coronary artery disease, whereas a lower dose of atorvastatin plus ezetimibe did not, even though both treatment regimens reduced low-density lipoprotein cholesterol to a similar extent (Piorkowski et al., 2007), implying that statin effects on platelets might be dose dependent and not related to lipoprotein level. Recent studies have also shown that intravenously administered statins have acute effects on platelet function *in vitro*

and *in vivo* (Ali et al., 2009; Obi et al., 2009).

The effect of statins on eNOS is well studied. NO, synthesized from L-arginine by NO synthase, activates intracellular soluble GC and results in the formation of cGMP, an important modulator in many physiological and pathological conditions (Moncada et al., 1991). It is important that in platelets, the NO/cGMP system has been shown to provide an inhibitory pathway-regulating platelet adhesion and aggregation (Radomski et al., 1987; Freedman et al., 1997; Freedman et al., 1999; Pigazzi et al., 1999; Emerson et al., 1999; Loscalzo, 2001; Freedman and Loscalzo, 2003). In addition, statins increase NO bioavailability in vascular cells and platelets by enhancing eNOS expression, stabilizing eNOS mRNA, and possibly decreasing superoxide formation (Tannous et al., 1999; Kalinowski et al., 2002; Zhou et al., 2004), suggesting that induced NO formation may be the major mechanism of the platelet inhibitory activity of statins. At least two studies have sought an eNOS-independent vascular protective effect of statins. The same dosing regimen of atorvastatin that used to lower the plasma levels of β-thromboglobulin and platelet factor-4 (markers of platelet α-granule release) in wild-type mice but had no effect on these parameters in eNOS-deficient mice (Laufs et al., 2000). Likewise, an intraperitoneal dose of simvastatin reduced myocardial infarct size after a myocardial ischemia reperfusion protocol in wild-type mice but not in eNOS-deficient mice (Yamakuchi et al., 2005). Thus, our demonstration that atorvastatin inhibits platelet activation in eNOS-deficient mice is important, because we evaluated platelet function

directly to reveal effects that were not seen with indirect measurements of platelet activation.

Because the comparison of the effects of atorvastatin treatment on platelet activation *in vitro* was evaluated in pairs, we are unable to make firm conclusions about the effect of eNOS-deficiency per se (compared with wild-type mice) on these parameters of platelet activation. The Loscalzo group has reported that platelets from eNOS-deficient mice were hyper-active (Freedman et al., 1999). Consistent with this concept, we show that cGMP levels were lower in eNOS-deficient mice than in wild-type mice. Moreover, platelet activation *in vivo*, as measured by CD41 upregulation occurred more rapidly in eNOS-deficient mice that were given a control diet than in their wild-type mice counterparts. Using the same comparison, atorvastatin prolonged the time to half-maximal CD41 upregulation by 1.5-fold in wild-type mice and 1.3-fold in eNOS-deficient mice, implying that around half of the atorvastatin effect on this parameter is eNOS independent.

That atorvastatin inhibits platelet activation in the absence of eNOS broadens the clinical importance of statins as platelet inhibitors. In patients with cardiovascular risk factors, eNOS can be uncoupled (Forstermann and Munzel, 2006). Uncoupling refers to the state where eNOS products combine with products of nicotinamide adenine dinucleotide phosphate-oxidases to form peroxynitrite, which oxidizes (6R)-5,6,7,8-tetrahydro-L-biopterin (BH4), an essential eNOS cofactor. In the absence of BH4, eNOS products superoxide instead of NO, which contributes to vascular

stress. Our findings predict that the antiplatelet effect of statins might persist in patients with metabolic syndrome even though eNOS is uncoupled and NO production is impaired.

Our observation that atorvastatin inhibits thrombin- and GYP-induced activation in platelets from eNOS-deficient mice is novel. The effects of statins on PAR-signaling have been previously reported in humans (Serebruany et al., 2006). In patients with metabolic syndrome, statins have been reported to reduce the expression of PAR1 (the dominant PAR on human platelets). In our study, statin treatment had no effect on PAR4 expression. Although this may reflect differences in the effect of statin treatment on human and mouse platelets, the inhibited platelet activation that we observed in eNOS-deficient mice seems to reflect events downstream to the PAR4 receptor because thrombin- and GYP-induced platelet activation was inhibited. This concept is supported by studies with human platelets that demonstrate inhibition of PAR1 activation after 2 hours of incubation with a statin. In these studies, addition of prenyl substrates restored PAR1 activation, suggesting that impaired prenylation downstream to PAR1 may be responsible for the statin effect (Fenton et al., 2002; Fenton et al., 2005). Alternatively, the eNOS-independent effects of statins might involve peroxisome proliferator-activated receptor-γ (PPARγ) because simvastatin has been reported to inhibit platelet protein kinase C (PKC) α via interactions with PPARγ (Ali et al., 2009). In this report, the PPARγ-dependent effect of simvastatin was observed when platelets were activated by arachidonic acid or ADP. In our studies,

atorvastatin inhibited only ADP-induced platelet activation in wild-type mice, raising the possibility that if this inhibitory pathway depends on PPARγ, it may also require the presence of eNOS.

Although atorvastatin did not inhibit platelet accumulation/thrombus volume, it inhibited the upregulation of markers of platelet activation in thrombi formed in both eNOS-deficient and wild-type mice. This finding provides further validation for this high-speed, 2-color microscopy method (Chapter 3) for evaluating the effects of inhibitors on the markers of platelet activation *in vivo*. The observation that atorvastatin delayed CD41 upregulation in eNOS-deficient mice, despite the fact that it only inhibited thrombin- and GYP-induced platelet activation *in vitro*, is consistent with previous observations that platelet activation in the laser-induced arteriolar thrombosis model is thrombin dependent (Vandendries et al., 2007; Kretz et al., 2010).

In the conclusion, we have extended our understanding of how statins inhibit platelet activation. Independent of the effects on eNOS, atorvastatin inhibits thrombin- and GYP-induced platelet activation without altering the expression of PAR4.

Chapter 5: The dose effect of acetylsalicylic acid in combination with clopidogrel on arterial thrombus formation in mice.

Author list: Ran Ni, Nima Vaezzadeh, Ji Zhou, Marco Cattaneo, Peter L. Gross

Foreword

This manuscript is in preparation. The experiments were designed by Dr. Peter L. Gross and me. All experiments contained in the manuscript were performed by me. Dr. Nima Vaezzadeh helped with experimental sample preparation and Dr. Ji Zhou trained me in mouse handling. Dr. Marco Cattaneo has contributed to experimental design. The manuscript was drafted by me under the guidance of Dr. Peter L. Gross. Chapter 5 is a direct representation of the manuscript under preparation.

5.1 Abstract

A combination of acetylsalicylic acid (ASA) and a $P2Y_{12}$ antagonist (dual-antiplatelet therapy, DAPT) is the preferred choice to treat arterial thrombosis. This regimen combines the inhibition of two separate platelet activation pathways, mediated by thromboxane A_2 (TXA_2) and by adenosine diphosphate (ADP). However, ASA and $P2Y_{12}$ antagonists can also converge to influence the level of cyclic adenosine monophosphate (cAMP) in platelets, with ASA inhibiting endothelial prostacyclin (PGI_2) formation and thus impairing platelet cAMP formation, and $P2Y_{12}$ antagonists conversely potentiating cAMP formation. Given that cAMP is a potent platelet inhibitor, administration of $P2Y_{12}$ antagonists with ASA, at high enough doses, may at least to a certain extent, offset the positive effects of $P2Y_{12}$ antagonists on cAMP generation, resulting in reduced platelet inhibition. Therefore, we hypothesize that, with an ASA dose that does not inhibit endothelial formation of PGI_2 but inhibits platelet formation of TXA_2, DAPT may provide greater protection to cardiovascular patients. In our study, wild-type mice were dosed orally with ASA, clopidogrel, or a combination of both. We found that ASA dosing at 40 mg resulted in a maximal inhibition of PGI_2 production; 10 mg of ASA did not inhibit PGI_2 production. ASA inhibited arachidonic acid-induced platelet activation *in vitro* in a dose-dependent manner. Significant inhibition of low arachidonic acid-induced platelet aggregation was shown by ASA at 10 mg. Importantly, we demonstrated a greater inhibition of murine arterial thrombus formation by DAPT containing 10 mg ASA compared to

DAPT containing 40 mg ASA. Our findings argue for relatively low doses of ASA in the context of using DAPT to attenuate arterial thrombosis. We suggest that prescribing ASA at a dose that preserves PGI_2 formation will be likely to increase the therapeutic value of DAPT in treating human arterial thrombosis.

5.2 Introduction

Arterial thrombosis is the leading cause of morbidity and mortality in developed countries (Lozano et al., 2012; Raskob et al., 2014). Platelets play a pivotal part in arterial thrombosis and therefore are the main target for therapies to prevent and treat arterial thrombosis. Upon activation, platelets release their granule contents, such as ADP, and synthesize TXA_2. Together, ADP and TXA_2 amplify platelet activation and promote thrombus growth via their receptors $P2Y_1$/$P2Y_{12}$ and TP, respectively (Li et al., 2010). $P2Y_{12}$ antagonists and the COX-1 inhibitor, ASA, respectively reduce ADP- and TXA_2-induced platelet activation, thereby suppressing arterial thrombosis. $P2Y_{12}$ antagonists and ASA have both shown high efficacy in preventing and treating major cardiac events (Awtry and Loscalzo, 2000; Cattaneo, 2010). Presently, DAPT, combining ASA and a $P2Y_{12}$ antagonist, is the standard-of-care in patients with acute coronary syndromes (ACS) and after percutaneous coronary intervention (PCI) (Levine et al., 2016).

ASA is the longest used antithrombotic and the principal mechanism for its antiplatelet effect is the irreversible suppression of TXA_2 biosynthesis. In the process of TXA_2 signaling, activated platelets release membrane phospholipid-derived

arachidonic acid, which is quickly converted into prostaglandin H_2 (PGH_2) by the platelet membrane-bound enzyme, cyclooxygenase-1 (COX-1) and subsequently converted to TXA_2 by TXA_2 synthase (Samuelsson et al., 1978). TXA_2 activates platelets via TP, thereby leading to increased cytoplasmic Ca^{2+} levels and shape change in platelets (Paul et al., 1999). ASA inhibits the interaction between arachidonic acid and COX-1 by acetylating a specific serine moiety in COX-1's hydrophobic pocket, which ultimately results in diminished TXA_2-mediated platelet activation (Roth and Majerus, 1975; Loll et al., 1995). The antithrombotic properties of ASA have long been confirmed in human subjects. Meta-analysis in six primary prevention trials has shown that long-term ASA allocation yielded a significant reduction of non-fatal myocardial infarction compared with controls. Furthermore, meta-analysis in 16 secondary prevention trials has shown that ASA is of substantial net benefit in reducing both myocardial infarction and stroke versus controls (Baigent et al., 2009).

Of note, COX-1 is ubiquitously expressed in almost all tissues, which gives ASA pleiotropic effects. The profile of prostaglandins generated by COX-1 from different cell types is highly dependent on the expression and activity of a series of specific isomerases and synthases that are coupled with COX-1 (Dubois et al., 1998; Smyth et al., 2009). COX-1 in gastrointestinal (GI) mucosa is highly coupled with prostaglandin E_2 (PGE_2) synthase to produce PGE_2. Because PGE_2 plays an important role in GI integrity, administration of ASA results in decreased arterial thrombosis

with a well-known side-effect of GI toxicity, including ulcers and bleeding (Roderick et al., 1993). Importantly, COX-1 in endothelial cells is coupled with prostaglandin I_2 (PGI_2) synthase and produces PGI_2. PGI_2 activates AC in platelets to generate the second messenger, cAMP, a potent inhibitor of platelet activation. Endothelial cells are the predominant donor of PGI_2 in the circulation to continuingly suppress platelet activation (Mitchell et al., 2008). Thus, ASA, at high enough doses, may at least to a certain extent, decrease PGI_2-mediated platelet suppression by inhibiting endothelial cell COX-1.

Platelets have two ADP receptors, $P2Y_1$ and $P2Y_{12}$. Only targeting $P2Y_{12}$ has established clinical relevance in human cardiovascular diseases (Gachet, 2008). $P2Y_{12}$ signaling leads to enhanced cytoplasmic Ca^{2+} levels, platelet shape change and aggregation. In addition, $P2Y_{12}$ signaling is also important to potentiate platelet aggregation and degranulation by thrombin (Cattaneo, 2015). The $P2Y_{12}$ antagonists that are currently approved for treatment of arterial thrombosis include clopidogrel, prasugrel and ticagrelor (Cattaneo, 2010). Prasugrel and ticagrelor are newer $P2Y_{12}$ antagonists, their superiority over clopidogrel in reducing arterial thrombotic events have been proven in the TRITON-TIMI 38 and PLATO trials, respectively (Wiviott et al., 2007; Wallentin et al., 2009). Among these $P2Y_{12}$ antagonists, clopidogrel is the most commonly prescribed. Clopidogrel works as a prodrug and it is converted into its active metabolites *in vivo*. The active metabolites covalently bind to $P2Y_{12}$ by forming a disulfide bond with cysteine residues of $P2Y_{12}$. This interaction, in turn, irreversibly

inhibits the receptor. Of note, it is often overlooked that $P2Y_{12}$ activation also leads to AC inhibition, which in turn attenuates platelet cAMP formation and reduces platelet inhibition (Cattaneo, 2010). Thus, $P2Y_{12}$ antagonists promote platelet inhibition by cAMP via abrogating AC inhibition. However, the clinical relevance of this particular antiplatelet effect of $P2Y_{12}$ antagonists has not yet been addressed directly.

Despite DAPT, some patients continue to experience thrombotic events. There is a consensus on the dosage of $P2Y_{12}$ antagonists in use. As an example, maintenance clopidogrel is administrated at 75 mg, once a day. However, there is disparity in the dose of ASA used. Recent analyses in the clinical trial, PLATO, have revealed geographic difference between patients receiving DAPT. It was shown that North American patients had slightly less benefit on DAPT than the rest of the world. One hypothesized cause for this observation is the higher doses of concomitant ASA given to patients in North America in the trial (Mahaffey et al., 2011). Cattaneo's group has previously reported that PGI_2 inhibits thrombin-induced washed platelet aggregation from healthy human subjects in the presence of a $P2Y_{12}$ antagonist, cangrelor. However, PGI_2 does not affect platelet aggregation when $P2Y_{12}$ signaling remains intact (Cattaneo and Lecchi, 2007). This line of evidence indicates that $P2Y_{12}$ inhibition potentiates PGI_2-mediated platelet inhibition. Taken together, a possible explanation for the adverse effect with DAPT containing high doses of ASA observed in PLATO is that decreased PGI_2 levels by high doses of ASA may result in less pronounced effect on AC activation promoted by $P2Y_{12}$ antagonists. To support this

hypothesis and underscore the importance of preserving PGI_2 levels in optimizing the antiplatelet effect of DAPT, in this study, we evaluated the effects of ASA dosing on PGI_2 formation and the overall antiplatelet effect of DAPT using a murine arterial thrombosis model and thus provide a potential strategy to improve current patient care.

5.3 Materials and Methods

5.3.1 Materials

Wild-type C57BL/6J male mice were purchased from Jackson Laboratories USA. All chemicals were purchased from Sigma Aldrich USA unless otherwise stated. Antibodies used in the detection of platelet activation and platelet accumulation *in vivo* were from Emfret Analytics Germany, with the exception that the anti-P-selectin antibody (RB40.34) was purchased from Becton Dickinson (BD) Biosciences USA. Antibody F_{ab} fragments were generated using a F_{ab} preparation kit from Pierce USA. Fluorophore conjugation was performed using protein labeling kits from Pierce USA and Invitrogen USA. Anesthesia solutions ketamine, xylazine, atropine and Nembutal were purchased from Ayerst, Bayer, Ormond Veterinary and Abbott laboratories (Canada), respectively. General surgical supplies were from Fine Scientific Tools (Canada) and BD Biosciences (USA). Pure ASA powder was purchased from Sigma USA. Clopidogrel tablets (Plavix) were obtained from Hamilton Health Sciences Pharmacy, Canada. Twenty gauge feeding needles were from Popper and Sons Inc.

USA. 60 X water immersion objective was purchased from Olympus Japan. Photodiode velocimeter (a Microvessel Velocity OD-RT Doppler) was obtained from CircuSoft Instrumentation Germany. Piezo-electric focuser was obtained from Physiks International Germany. Nitrogen dye laser was from Micropoint System USA. The rest components for intravital microscopy including Slidebook software (5.0) were purchased from Intelligent Imaging Innovations USA. Anticoagulants heparin and sodium citrate were purchased from Leo Pharma Inc. Canada and BD Biosciences USA, respectively. Platelet inhibitors apyrase and prostacyclin (PGI_2) used in platelet isolation were purchased from Sigma and Calbiochem USA, respectively. ADP and bovine thrombin were from Sigma USA. Arachidonic acid was from Diapharma. Platelet aggregation was measured using Multiplate mini test cells (pediatric cuvettes, Diapharma). Disposable hemocytometers were from Incyto Korea. cAMP and 6-keto-PGF1α ELISA kits were from Cayman Chemical.

5.3.2 Methods

5.3.2.1 Oral administration of ASA and clopidogrel

Pure ASA crystals and clopidogrel 75 mg tablets were dissolved in distilled water and diluted apple jelly, respectively, using a sonicator bath. ASA and clopidogrel in solution were freshly made daily and given to mice within an hour of preparation. Age-matched male wild-type mice were used between 8 to 12 weeks old and weighed at least 25 grams. All mice received a gavage feeding, using 20-gauge feeding needles, at 24 and 4 hours before experimentation, containing a volume of 100 μL of vehicle or

antiplatelet reagents. Mice treated with ASA were given the drug once, 4 hours prior to experiments. Mice treated with clopidogrel received the drug twice, 24 hours and 4 hours prior to experiments. Thus in some experiments, clopidogrel was co-administered with ASA. Blood samples were collected 24 hours following the first gavage and *in vivo* experiments were carried out between 24-25 hours after the first gavage. Studies were done according to Canadian Council of Animal Care Guidelines, and all animal use protocols were approved by the Animal Research Ethics Board at McMaster University.

5.3.2.2 Multiplate determination of platelet inhibition by ASA

Mice received ASA in a range from 0 to 5 mg/kg (equivalent to human dosing of 0, 10, 20, 40, 80, 160, 240 and 320 mg ASA). Whole blood was collected via carotid artery cannulation into 3.2% sodium citrate at a ratio of 9:1 (vol:vol). Whole blood (175 μL) was mixed with an equal volume of normal saline containing 3 mM $CaCl_2$ in the cup portion of a pediatric Multiplate test cell. Following a three-minute incubation at 37 ℃, platelet aggregation was initiated by adding 25 μM (low), 37.5 μM (medium) or 50 μM (high) arachidonic acid. Electrical impedance caused by platelet adhesion and aggregation was recorded at approximately 0.5 second intervals for 6 minutes using a Multiplate Analyzer. Values were expressed as area under the curve (AUC).

5.3.2.3 Multiplate determination of platelet inhibition by clopidogrel and PGI_2

To determine the antiplatelet effect of clopidogrel, platelet aggregation was initiated in citrated blood from mice who were given clopidogrel in a range from 0 to

5 mg/kg (equivalent to human dosing of 0, 75, 150, 225 and 300 mg clopidogrel) by adding 10 μM ADP to the Multiplate test cell. In some experiments, platelet aggregation was initiated with bovine thrombin (0.8 U/mL) with or without addition of PGI_2 (15 μM). Platelet aggregation was analyzed using the Multiplate Analyzer.

5.3.2.4 Determination of ASA's effect on 6-keto-PGF1α level in plasma

Platelet-poor plasma (PPP) was obtained from mice treated with ASA in a range from 0 to 5 mg/kg by centrifuging the citrated blood twice at 3,000 x g for 5 minutes at room temperature. All the samples were stored at -80 ℃ in the presence of indomethacin (10 μg/mL). Plasma 6-keto-PGF1α was determined using a competitive ELISA.

5.3.2.5 Determination of clopidogrel's effect on platelet cAMP level

Platelet-rich plasma (PRP) was obtained from heparinized whole blood of mice received vehicle or 5 mg/kg clopidogrel by centrifugation at 500 x g for 5 minutes. Platelets were washed with Tyrode's buffer (134 mM NaCl, 0.34 mM $Na2HPO_4$, 2.9 mM KCl, 12 mM $NaHCO_3$, 20 mM HEPES, pH 7.0, 5 mM glucose, 0.35% (w/v) bovine serum albumin) containing 20 mU/mL apyrase by centrifugation PRP at 1,500 for 5 minutes and resuspending the pellet twice. The platelets were then resuspended in 500 μL Tyrode's buffer containing 40 mU/mL apyrase and incubated at 37 ℃ for 30 minutes. Platelet counts were adjusted to 1 X 10^8 per mL before use.

Platelet suspensions (5 x 10^7 platelets in 500 μL of Tyrode's buffer) were treated with 0.8 U/mL thrombin, or 0.8 U/mL thrombin and 15 μM PGI_2 at room temperature

in presence of 1 mM $CaCl_2$ for 2 minutes. Following the incubation, 2.5% trichloroacetic acid (TCA) was immediately added and samples were snap-frozen in liquid nitrogen. Samples were later thawed and shaken at 4 ℃ for 45 minutes. Protein precipitates were excluded from the samples by centrifugation at 4 ℃ for 30 minutes. TCA in the supernatant was extracted three times by mixing with 5 volumes of water-saturated ether. The residual ether was then removed by heating the samples at 70 ℃ for 10 minutes. All samples were stored at -80 ℃ before assayed using a competitive ELISA.

5.3.2.6 Intravital analysis of platelet accumulation and activation after laser-induced injury to cremaster muscle arterioles

In anesthetized mice, the cremaster muscle was prepared as previously described (Rand et al., 2012). Using the jugular vein cannulus, mice were given an infusion of 0.1 μg/g of Dylight-488-tagged anti-GPIbβ antibody X488. Where indicated, an infusion of 1.3 μg/g of Dylight-647-tagged F_{ab} fragments of rat anti-mouse CD41 (MWReg30), a monoclonal antibody directed against α_{IIb} that recognizes both the resting and activated form of this platelet-specific integrin, or 2 μg/g of a Dylight-647-tagged antibody against P-selectin (RB40.34) was also administered. Fluorophore conjugation and F_{ab} fragment preparation were performed according to the directions from the supplier. After laser injury to cremaster arterioles, platelet accumulation and activation were measured and analyzed as previously described, except that platelet activation was assessed on the basis of both CD41 upregulation and P-selectin surface expression (see detailed methods in Chapter 3).

5.3.2.7 Statistical Analyses

Data were expressed as means±SEM. Student's *t*-tests, one-way ANOVA and two-way ANOVA were used to compare means. A *P value under 0.05 was considered statistically significant.

5.4 Results

5.4.1 ASA at 40 mg, but not 10 mg, attenuates circulating PGI_2 level in mice

Owing to the extremely short half-life of PGI_2, we determined the effects of ASA on PGI_2 formation by measuring its degradation product, 6-keto-PGF1α, in plasma from mice treated with 0 to 320 mg ASA. We found that ASA at 10 mg did not affect circulating PGI_2 levels in mice. However, ASA at 20 mg interfered with PGI_2 levels. Most importantly, ASA at or above 40 mg provided the maximal inhibition on PGI_2 formation in mice, showing nearly 50% reduction of circulating 6-keto-PGF1α level four hours after ASA administration **(Figure 5.1)**.

5.4.2 ASA dosing inhibits arachidonic acid- and collagen-induced platelet aggregation in vitro

We found that mice treated with ASA had inhibition of arachidonic acid-induced platelet aggregation in a dose-dependent manner. Platelet aggregation by 25 μM arachidonic acid was significantly suppressed by ASA as low as 10 mg and was nearly fully inhibited by ASA at 40 mg **(Figure 5.2 A)**. Platelet aggregation by 37.5 μM arachidonic acid was inhibited by ASA dosed between 10 and 320 mg **(Figure 5.2 B)**.

Figure 5.1 Dose effect of ASA on circulating 6-keto-PGF1α levels.

Citrated plasma from wild-type mice was obtained 4 hours following the oral administration of vehicle or ASA at doses of 10, 20, 40, 80, 160, 240 or 320 mg. PGI_2 degradation product, 6-keto-PGF1α, in plasma was determined using a competitive ELISA. A best-fit sigmoidal dose-response curve was generated using Prism. Mean ± SEM are shown, (N = 9 or 10 mice per dose group), $P<0.0001$.

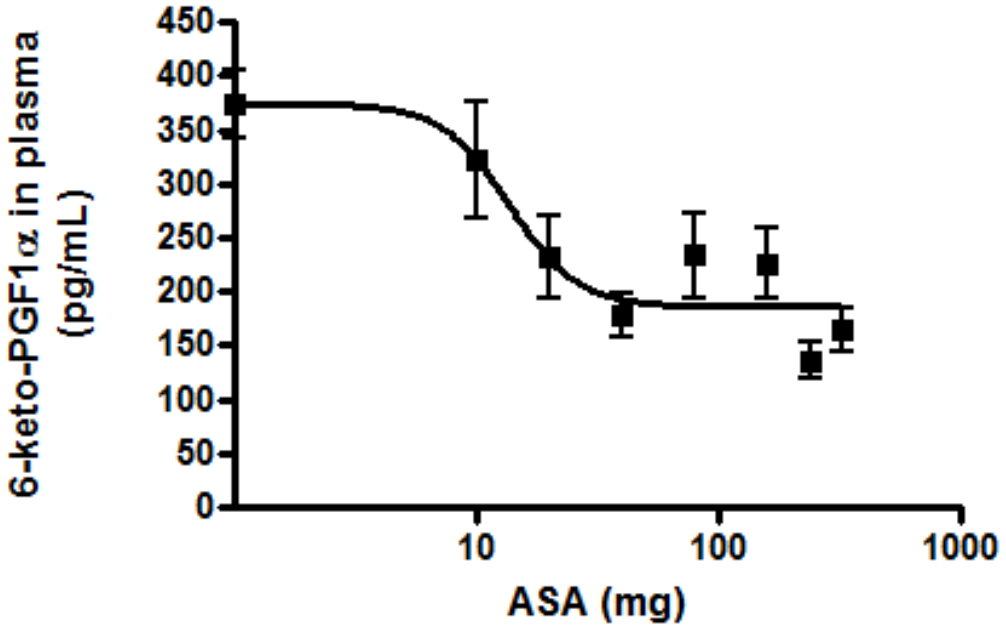
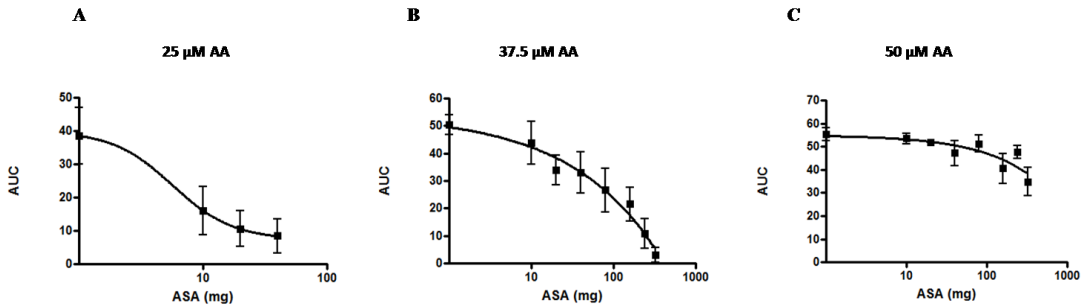

6-keto-PGF1α in plasma (pg/mL)
450
400
350
300
250
200
150
100
50
0
10
100
1000
ASA (mg)

Figure 5.2 Dose effect of ASA on platelet aggregation by arachidonic acid (AA).

Citrated whole blood from wild-type mice was obtained between 4 to 5 hours following the oral administration of vehicle or ASA at doses of 10, 20, 40, 80, 160, 240 or 320 mg. Blood was recalcified with 1.5 mM $CaCl_2$ and whole blood aggregation was recorded for 6 minutes after AA addition using a Multiplate Analyzer. Best-fit sigmoidal dose-response curves were generated using Prism. Mean ± SEM are shown. **A.** Area under the curve (AUC) of platelet aggregation by 25 μM AA (N = 7 mice per dose group), $P<0.05$. **B.** AUC of platelet aggregation by 37.5 μM AA (N = 10 mice per dose group), $P<0.0001$. **C.** AUC of platelet aggregation by 50 μM AA (N = 10 mice per dose group), $P<0.05$.


A
25 µM AA
AUC
ASA (mg)
B
37.5 µM AA
AUC
ASA (mg)
C
50 µM AA
AUC
ASA (mg)

However the inhibitory effect of ASA on platelet aggregation, by 37.5 μM arachidonic acid compared to that by 25 μM arachidonic acid, was noticeably milder **(Figure 5.2, A and B)**. Partial inhibition was observed with ASA at 10 or 40 mg and full inhibition was achieved with ASA at 320 mg **(Figure 5.2 B)**. In addition, platelet aggregation by 50 μM arachidonic acid was only marginally impaired by very high doses of ASA that we tested **(Figure 5.2 C)**.

Because platelet activation by collagen cannot occur completely without TXA_2 formation, we also evaluated collagen-induced platelet activation in response to oral administration of ASA. We found that, similar to 50 μM arachidonic acid-induced platelet aggregation, platelet activation by collagen was only moderately affected by even high doses of ASA that we tested **(Figure 5.3)**.

5.4.3 Clopidogrel at 5 mg/kg inhibits ADP-induced platelet activation

In order to investigate the PGI_2 inhibitory effect independent of $P2Y_{12}$ signaling, we need to determine the minimal dose of clopidogrel that would maximally inhibit $P2Y_{12}$-induced platelet activation. To do this, we measured platelet aggregation by 10 μM ADP in response to clopidogrel treatments at 0, 1.25, 2.5, 3.75 and 5 mg/kg. We found that clopidogrel inhibits ADP-induced platelet aggregation in a dose-dependent manner. Clopidogrel at 1.25, 2.5 and 3.75 mg/kg results in partial inhibition of ADP-induced platelet aggregation. Clopidogrel at 5 mg/kg or higher (data not shown) abolished platelet aggregation by ADP **(Figure 5.4)**.

Figure 5.3 Dose effect of ASA on platelet aggregation by collagen.

Citrated whole blood from wild-type mice was obtained between 4 to 5 hours following the oral administration of vehicle or ASA at doses of 10, 20, 40, 80, 160, 240 or 320 mg. Blood was recalcified with 1.5 mM $CaCl_2$ and whole blood aggregation was recorded for 6 minutes after addition of 1.6 μg/mL collagen using a Multiplate Analyzer, expressed as area-under-the-curve (AUC). A best-fit sigmoidal dose-response curve was generated using Prism. Mean ± SEM are shown, (N = 10 mice per dose group), $P<0.05$.

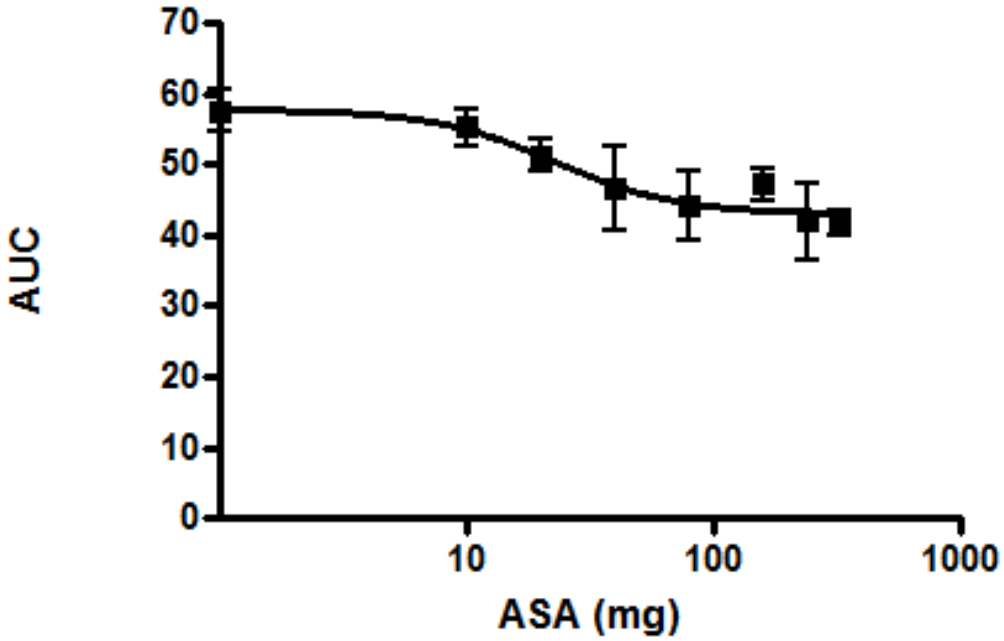
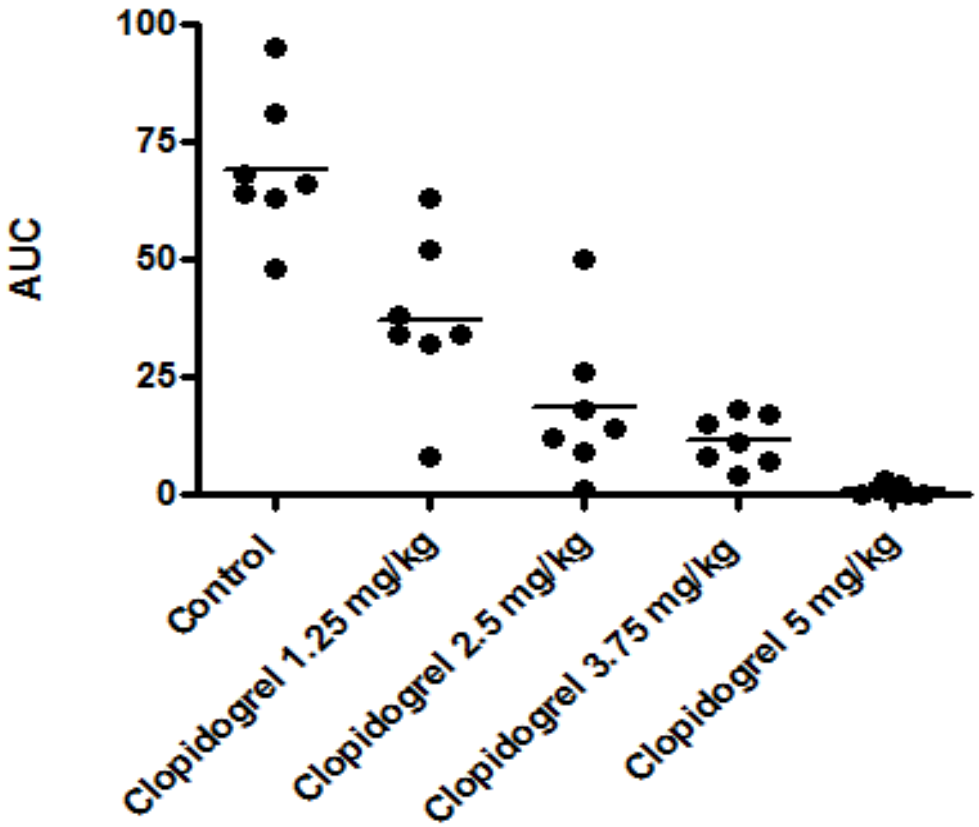

70
60
50
40
30
20
10
0
AUC
10
100
1000
ASA (mg)

Figure 5.4 Dose effect of clopidogrel on platelet aggregation by ADP.

Citrated whole blood from wild-type mice was obtained between 4 to 5 hours following the oral administration of vehicle or clopidogrel at doses of 1.25, 2.5, 3.75 or 5 mg/kg body weight. Blood was recalcified with 1.5 mM $CaCl_2$ and whole blood aggregation was recorded for 6 minutes after 10 μM ADP initiation using a Multiplate Analyzer. Mean ± SEM are shown, (N = 7 mice per dose group), $P<0.0001$.


AUC
100
75
50
25
0
Control
Clopidogrel 1.25 mg/kg
Clopidogrel 2.5 mg/kg
Clopidogrel 3.75 mg/kg
Clopidogrel 5 mg/kg

5.4.4 DAPT containing 10 mg ASA has a stronger inhibitory effect on murine arterial thrombus formation in vivo compared to that containing 40 mg ASA

Because we found that ASA at 40 mg but not at 10 mg impaired PGI_2 formation, and both doses of ASA at least partly attenuated platelet aggregation *in vitro*, we evaluated the inhibitory effects of DAPT containing clopidogrel with either 10 mg or 40 mg ASA on murine thrombus formation using the laser injury thrombosis model. We found that arterial thrombus volume (measured by GPIbβ) with DAPT containing 10 mg ASA was smaller compared to those with DAPT containing 40 mg ASA **(Figure 5.5)**. There was no difference on platelet activation markers (measured by CD41 upregulation and P-selectin surface expression) by DAPT containing 10 mg and 40 mg ASA (data not shown).

5.4.5 Clopidogrel potentiates the antiplatelet effect of PGI_2 in vitro

Even though clopidogrel at 5 mg/kg can almost completely inhibit ADP-induced platelet aggregation, we found that clopidogrel at this dose did not suppress thrombin-induced (0.8 U/mL) platelet aggregation. We also found that 15 μM PGI_2 did not suppress 0.8 U/mL thrombin-induced platelet aggregation in control mice. However, 0.8 U/mL thrombin-induced platelet aggregation from mice who received 5 mg/kg clopidogrel was significantly inhibited in the presence of 15 μM PGI_2 **(Figure 5.6)**. Thus, when PGI_2 is present, clopidogrel can inhibit potent thrombin-induced platelet activation, confirming the work of Cattaneo's group.

Figure 5.5 Effect of DAPT containing 10 or 40 mg ASA on murine arterial thrombus formation.

Wild-type mice were orally given 5 mg/kg clopidogrel combined with either 10 or 40 mg ASA. Laser injury-induced thrombi in cremaster muscle arterioles were evaluated between the 4^{th} to 5^{th} hour following the last gavage treatment. **A.** Fl488 fluorescence intensity of each thrombus at the peak is shown. **B.** Fl488 fluorescence intensity of total thrombus volume throughout the 220 seconds of recording of each thrombus is shown. Mean ± SEM are shown, (45 thrombi in the group of DAPT with 10 mg ASA and 44 thrombi in the group of DAPT with 40 mg ASA, 8 mice in each treatment group, were examined), $*P<0.05$.

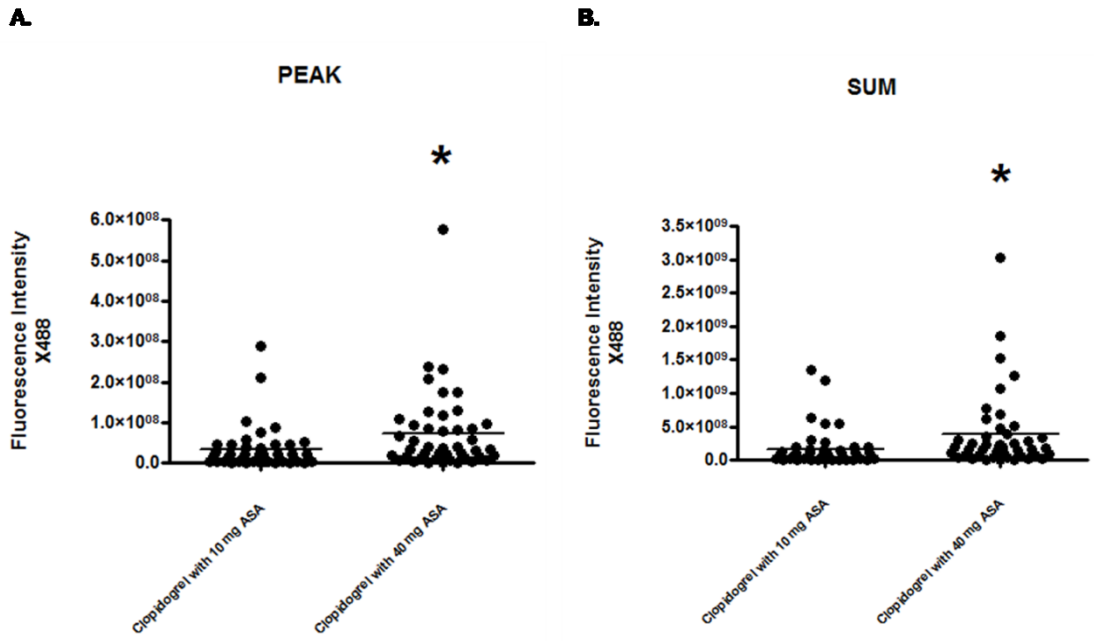
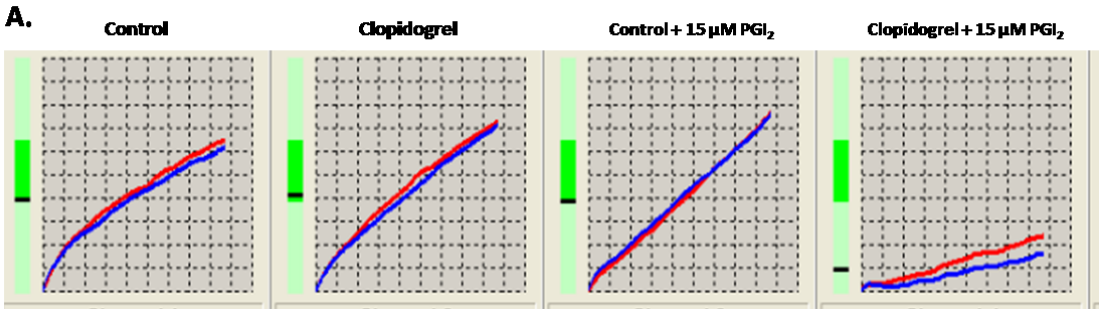
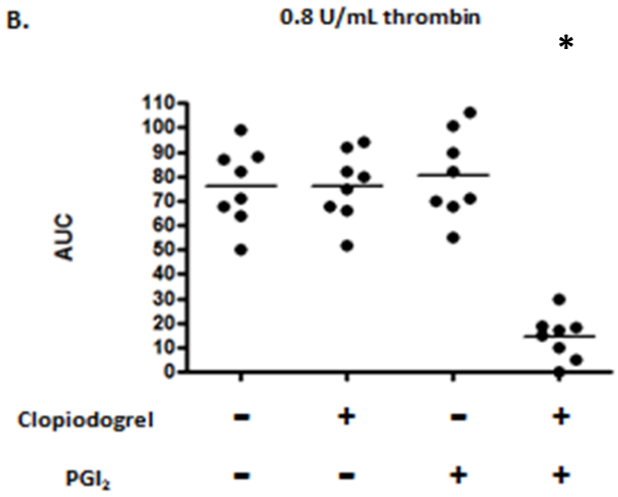

A.
PEAK
Fluorescence Intensity X488
6.0×10^08
5.0×10^08
4.0×10^08
3.0×10^08
2.0×10^08
1.0×10^08
0.0
*
Clopidogrel with 10 mg ASA
Clopidogrel with 40 mg ASA
B.
SUM
Fluorescence Intensity X488
3.5×10^09
3.0×10^09
2.5×10^09
2.0×10^09
1.5×10^09
1.0×10^09
5.0×10^08
0.0
*
Clopidogrel with 10 mg ASA
Clopidogrel with 40 mg ASA

Figure 5.6 Effect of clopidogrel and PGI_2 on platelet aggregation by thrombin.

Citrated whole blood from wild-type mice was obtained between 4 to 5 hours following the oral administration of vehicle or clopidogrel at 5 mg/kg body weight. Blood was recalcified with 1.5 mM $CaCl_2$ and whole blood aggregation was recorded for 6 minutes after adding 0.8 U/mL thrombin or 0.8 U/mL thrombin with 15 μM PGI_2 using a Multiplate Analyzer. **A.** Representative of whole blood aggregation initiated by thrombin. Blue and red lines represent two independent measurements of platelet aggregation simultaneously by Multiplate Analyzer. **B.** Quantification of whole blood aggregation initiated by thrombin. Mean ± SEM are shown, (N = 8), **P*<0.0001.



Whole blood aggregation by 0.8 U/mL Thrombin (AUC)



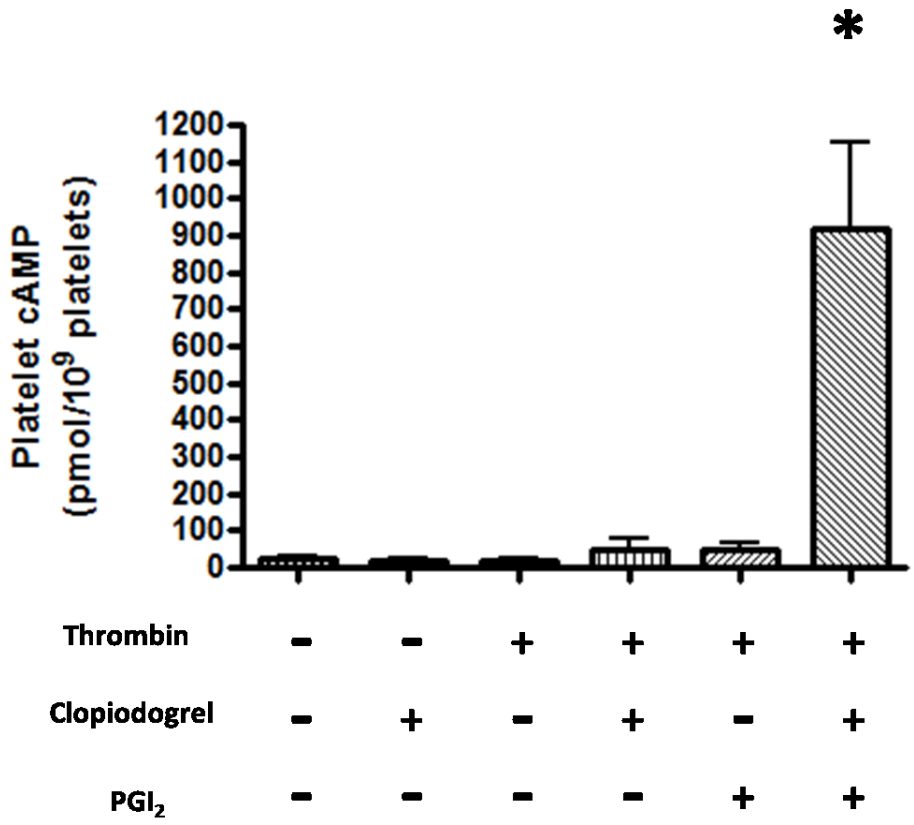
To further confirm the mechanism of the synergistic inhibitory effect of PGI_2 and clopidogrel, we also measured the inhibitory second messenger platelet cAMP. We found that resting platelets from control and clopidogrel-treated mice had similar levels of cAMP. cAMP levels in thrombin-activated platelets from both control and clopidogrel-treated mice did not differ from those in resting platelets in the absence of PGI_2. We found that thrombin activation did not increase cAMP levels in platelets from control mice in the presence of PGI_2. In contrast, in the presence of PGI_2, cAMP levels in thrombin-activated platelets from clopidogrel-treated mice rose about 18 times from the basal level **(Figure 5.7)**. This confirms the mechanism of the synergistic inhibitory effect of PGI_2 and clopidogrel, as platelet cAMP is greatly increased.

5.5 Discussion

Antiplatelet drugs, ASA and clopidogrel inhibit TXA_2- and ADP-induced platelet activation, respectively (Awtry and Loscalzo, 2000; Cattaneo, 2010). The two antiplatelets also can influence the levels of the inhibitory platelet second messenger cAMP in opposite directions. ASA can reduce platelet cAMP formation by decreasing endothelial production of PGI_2, while clopidogrel promotes platelet cAMP formation by abrogating the inhibitory effect of $P2Y_{12}$ signaling on AC activation. Although the combination of ASA and clopidogrel (DAPT) decreases clinical arterial thrombotic events (CAPRIE Steering Committee, 1996; Baigent et al., 2009), the overall antithrombotic effect of DAPT by the interplay of ASA and clopidogrel on cAMP has

Figure 5.7 Effect of clopidogrel and PGI_2 on platelet cAMP levels.

Whole blood (in heparin) was obtained from wild-type mice treated with either vehicle or clopidogrel at 5 mg/kg body weight and platelets were then isolated. cAMP levels in resting platelets, in 0.8 U/mL thrombin-activated platelets, and in 0.8 U/mL thrombin-activated platelets in the presence of 15 μM PGI_2, were determined using a competitive ELISA. Mean ± SEM are shown, (N = 4), **P*<0.0001.


*
Platelet cAMP
(pmol/10^9 platelets)
1200
1100
1000
900
800
700
600
500
400
300
200
100
0
Thrombin - - + + + +
Clopiodogrel - + - + - +
PGI2 - - - - + +

not been studied directly. To prove the importance of preserving PGI_2 in optimizing the antiplatelet effect of DAPT, we evaluated the effect of ASA dosing on arterial thrombus formation by DAPT in mice. We found that DAPT containing a dose of ASA that is not inhibitory to PGI_2 formation resulted in smaller murine arterial thrombi compared to DAPT containing a dose of ASA that is inhibitory to PGI_2 formation.

TXA_2 and PGI_2 generated by platelet and endothelial COX-1, respectively, are both inhibited by ASA (Awtry and Loscalzo, 2000). In our study, we revealed very different inhibition patterns on murine TXA_2 and PGI_2 formation by ASA treatment. ASA at 10 mg did not affect PGI_2 formation by endothelial COX-1. However, ASA at 40 mg or above maximally reduced PGI_2 formation, indicating saturated endothelial COX-1 inhibition by ASA as low as 40 mg. In contrast, we found that ASA inhibits TXA_2 formation at doses as low as 10 mg. ASA provides a dose-dependent reduction of TXA_2 formation as evidenced by decreased arachidonic acid-induced platelet aggregation. There is controversy over the effects of ASA on TXA_2 and PGI_2 formation - some believe that ASA is unlikely to inhibit TXA_2 formation without interfering with PGI_2 formation, while others argue that ASA only inhibits PGI_2 formation when very high doses are administered (Burch et al., 1978; Preston et al., 1981; Patrignani et al., 1982; Weksler et al., 1983; FitzGerald et al., 1983; Patrono et al., 1985; Kyrle et al., 1987), our findings support the studies suggesting the feasibility of using ASA, at low enough doses, to inhibit TXA_2 formation without interfering

with PGI_2 formation.

Importantly, we found that DAPT of clopidogrel co-administered with 10 mg ASA has stronger inhibitory effects on *in vivo* arterial thrombus formation in mice compared to DAPT containing 40 mg ASA. Our finding is in an agreement with the observation from a number of clinical trials, which suggest that there is not a linear correlation between antithrombotic effects and increasing doses of ASA (Peters et al., 2003; Jolly et al., 2009; Yu et al., 2012). In addition, this finding is consistent with the PLATO trial, which argued for the use of lower ASA doses in the context of DAPT involving a $P2Y_{12}$ antagonist (Mahaffey et al., 2011). It is noteworthy that, in another dual anti-platelet regimen "Aggrenox", a very low dose of ASA, at 25 mg b.i.d., is recommended to be concomitantly administrated with dipyridamole, which inhibits platelets by preventing cAMP from degradation (Diener et al., 1996). Moreover, ASA has been found to be effective at 30 mg once a day to reduce arterial thrombotic events (The Dutch TIA Trial Study Group, 1991). Such low doses of ASA have not been tested or recommended in the current standard-of-care. The American Heart Association and American College of Cardiology (JACC) guidelines had long been recommending an ASA dose range of 75-325 mg, which was generally higher than the European Society of Cardiology guideline's recommended dose of below 100 mg (Silber et al., 2005; King, III et al., 2008). In 2016, JACC lowered the recommended ASA dose in DAPT to 81 mg (Levine et al., 2016). However, this may not be the end of the journey of ASA dose adjustments in DAPT. Our study in mice suggests that a

dose of ASA that preserves an adequate amount of PGI_2 produced by endothelial COX-1 is pivotal in optimizing the overall antiplatelet effect of DAPT. At present, the maximal dose of ASA that does not inhibit PGI_2 formation but still, at least partially, inhibits TXA_2 formation in humans remains unclear.

It is generally believed that the clinical benefits of $P2Y_{12}$ antagonists are attributed to their inhibition of ADP-mediated platelet activation. Moreover, platelet activation by ADP potentiates platelet response to thrombin, therefore, the use of $P2Y_{12}$ antagonists, to certain extent, is also effective inhibiting thrombin-induced platelet activation (Cattaneo, 2015). In our study, we have shown that neither clopidogrel at a dose that maximally inhibits ADP-induced platelet activation, nor a high concentration of PGI_2, inhibit platelet aggregation in whole blood induced by a high concentration of thrombin. However, the combination of clopidogrel and PGI_2 are synergistic to reduce platelet aggregation in whole blood induced by the same high concentration of thrombin. Mechanistically, we also showed that thrombin-activated platelets from mice who received clopidogrel have a dramatic increase of cellular cAMP levels in the presence of exogenous PGI_2. Our results confirm the previous findings from Cattaneo's group of the synergistic effects of cangrelor and PGI_2 on inhibiting human platelet aggregation and elevating human platelet cAMP levels by thrombin (Cattaneo and Lecchi, 2007). Taken together, the results highlight the overlooked platelet inhibitory effect of $P2Y_{12}$ inhibition by potentiating PGI_2 signaling and imply the essential role of PGI_2 in optimizing the antithrombotic effect of DAPT.

The promotion of $P2Y_{12}$ antagonists on PGI_2-induced elevations of platelet cAMP may also explain why, in the same means of inhibiting ADP-induced platelet activation, $P2Y_{12}$ serves as a better antithrombotic target than $P2Y_1$ in clinical settings. Signaling through $P2Y_1$ does not alter cAMP levels in platelets.

Taken together, our findings support the speculation that reduced PGI_2 formation from co-administration of ASA may blunt the overall efficacy of $P2Y_{12}$ inhibition. It was proven that clopidogrel is more effective than ASA in reducing arterial thrombosis in the CAPRIE trial (CAPRIE Steering Committee, 1996). The superiority of DAPT over ASA alone in arterial thrombosis has also been established in the CURE trial (Yusuf et al., 2001). However, clinical evidence to manifest the efficacy of DAPT versus clopidogrel alone is lacking (Diener et al., 2004). Even though our study has demonstrated that a dose of ASA that preserves PGI_2 formation is essential in determining the overall antithrombotic effect of DAPT, we did not prove that DAPT containing such a dose of ASA is better than clopidogrel alone. The evaluation of the effect of DAPT and clopidogrel on murine arterial thrombus formation will be informative to validate the necessity to combine ASA and clopidogrel in treating human arterial thrombosis.

In conclusion, we demonstrated that DAPT containing a dose of ASA that does not interfere with PGI_2 formation is superior to DAPT with a dose of ASA that is inhibitory to PGI_2 formation in reducing murine arterial thrombus formation. Because the two doses of ASA both inhibit TXA_2-mediated platelet activation, we suggest that

proper dosing of ASA, to preserve PGI_2 formation by endothelial cells, is an important prerequisite for the optimal effect of DAPT in reducing arterial thrombosis.

Chapter 6: General Discussion

Atherosclerosis is a chronic disease that irreversibly alters healthy vasculature throughout one's life, which makes the elderly more prone to arterial thrombotic diseases (Ross, 1993). Human postmortems of infarcted tissues have shown that a large volume of arterial thrombi are platelets, thus platelets have long been the major target of pharmaceutical interventions to treat arterial thrombosis (Fuster et al., 1988; Meadows and Bhatt, 2007). Despite a variety of platelet activation pathways thought to be important in the pathological development of arterial thrombosis, the majority of new inhibitors targeting these pathways failed to be approved for use in humans owing to their toxicity, therapeutic ineffectiveness or disproportional enhancement of bleeding risk. In addition, a small portion of arterial thrombotic patients even with the best current pharmaceutical interventions continue to experience thrombotic or bleeding events. The imperfection of the current antiplatelet therapy drives the demand for novel platelet inhibitors (Capodanno et al., 2013; Franchi and Angiolillo, 2015).

The studies described in this thesis were undertaken to evaluate the efficacy of platelet inhibition with existing pharmaceutical reagents using the mouse laser injury arterial thrombosis model. Amongst all three medications that are given to arterial thrombotic patients, statins, $P2Y_{12}$ antagonists and ASA, statins are prescribed for the main purpose of limiting the progression of atherosclerotic plaques, which was originally thought to be independent of platelet inhibition. Later, statins were shown to

suppress platelet activation predominately by enhancing the bioavailability of NO, a potent platelet inhibitor and vasodilator (Zhou and Liao, 2009). Because the pleiotropic benefits and low bleeding incidence associated with statin use, exploring activation signaling pathways in platelets that are inhibited by statins could extend their use beyond the purpose of lipid-lowering and may inform us about safe targets for the development of future antiplatelets.

$P2Y_{12}$ antagonists and ASA function as platelet inhibitors to decrease the risk of thrombosis. A combination of a $P2Y_{12}$ antagonist and ASA is the standard-of-care in patients with ACS and those that undergo PCI. This dual-antiplatelet therapy (DAPT) combines the inhibition of ADP- and TXA_2-induced platelet activation and is thus far the most widely used pharmaceutical intervention in arterial thrombosis (Levine et al., 2016). Presently, dispute over proper dosing of ASA in DAPT continues, in an attempt to balance the likelihood of causing bleeding, and to avoid reducing PGI_2 production by endothelial cells. Moreover, $P2Y_{12}$ activation has been found to interfere with PGI_2 signaling in platelets, which in turn complicates the antithrombotic effect of ASA dosing in the overall efficacy of the standard-of-care regimen of ASA and a $P2Y_{12}$ antagonist.

The goals of our studies were to 1) improve an existing mouse thrombosis model to make it more sensitive to detect platelet inhibition in preclinical studies, to 2) investigate NO-independent platelet inhibition by atorvastatin, and to 3) guide optimal dosing of ASA and clopidogrel to improve DAPT. We have shown that our laser injury

mouse model can detect the heterogeneous nature of arterial thrombi and it is a useful tool to evaluate subtle platelet inhibition. By using this mouse model, we have found that atorvastatin attenuates *in vivo* platelet activation via thrombin signaling in addition to the well-known effect of enhancing NO production. Moreover, we have found that PGI_2 increases cAMP in platelets and plays an important role in determining the overall antithrombotic potential of DAPT containing a $P2Y_{12}$ antagonist and ASA in mice. We suggest that an ASA dose that does not interfere with PGI_2 production by endothelial cells in DAPT may provide greater protection to arterial thrombotic patients. This chapter contains an integrated discussion of these three projects that use the laser injury mouse thrombosis model to guide better pharmaceutical interventions in humans.

6.1 What does our mouse model add to *in vivo* thrombosis research

Before animal models were introduced to thrombosis research, our understanding of platelets' role in haemostasis and thrombosis largely relied on the information gained from platelet activation *in vitro* in response to, usually single, excitatory agonists (Yardumian et al., 1986). However, platelets are expected to function differently *in vivo* during haemostatic or thrombotic plug formation for a number of reasons. Briefly, activated endothelium that plays an important role in modulating platelet activation is not usually included in the *in vitro* studies. Studies *in vitro* usually evaluate one or two platelet agonists. Yet, many molecular participants in platelet activation have not been identified. Moreover, the momentary concentrations

of these excitatory agonists and inhibitors being generated and detained at the site of platelet plugs remain unknown. Shear rate is a variable, which is difficult to replicate under *in vitro* conditions. Shear rate is synergistically determined by rheological blood flow and the vasculature under physical and pathological conditions. Shear rate does not just affect the concentrations of platelet agonists and inhibitors but is an independent factor in mediating platelet plug formation. These influential components that are never all included in *in vitro* platelet activation studies can be better controlled by using *in vivo* animal models. Animal models allow us to investigate the overall contribution of distinct platelet activation pathways in haemostatic and thrombotic plug formation (Jackson, 2007; Sachs and Nieswandt, 2007). Currently, proof of target efficacy in preclinical testing is required prior to an antiplatelet is approved to be tested in humans.

Many animal species are used in the research of human haemostasis and thrombosis. Mice are the most commonly used because they are inexpensive and breed fast. Importantly, the majority of platelet agonists, receptors, and intracellular molecules that mediate platelet activation in humans have also been found to be similar in mice. In addition, advanced transgenic technology in mice greatly facilitates efficient screening and evaluation of causative genes and potential inhibitors in thrombosis (Westrick and Ginsburg, 2009). There are several ways to trigger murine thrombus formation *in vivo*. These include intravenous injection of platelet agonists and mechanical injuries by ligation or micropuncture (Sachs and Nieswandt, 2007).

The most commonly used ones are chemical, photochemical and laser pulse. In the chemical injury model, ferric chloride ($FeCl_3$) is applied for a short time over vessels, which in turn causes biological receptor/ligand interaction-independent aggregates to occur on the intact endothelium (Ciciliano et al., 2015). In photochemical models, in the presence of a photosensitizing dye, such as Rose Bengal, vessels are irradiated by high-intensity light for limited time. The process produces reactive oxygen species (ROS) to cause focal damage to the endothelium (Watson et al., 1985; Inamo et al., 1996). In the laser injury model, a short pulse of focused high-energy laser directly causes heat damage to a very limited region, aimed at the endothelium, which in turn causes a platelet plug to grow but does not involve severe trauma to the surrounding endothelial cells (Falati et al., 2004). These thrombosis models are useful tools to unravel the complexity of real-time thrombus formation; however, all three have advantages and limitations. For instance, the $FeCl_3$ model often generates large thrombi and thrombotic potential is determined by the parameter of time to form an occlusive thrombus. These features make the model less ideal for studying real-time ultrastructure or cellular components of the thrombi in the presence of shear stress beyond occlusion. In contrast, the photochemical and laser injury models produce relatively small and non-occlusive thrombi, which are feasible for fluorescence microscopic imaging. However, thrombus size in these two models is highly influenced by the chosen vessels and the duration, intensity or localization of the light energy.

We took the advantage of advanced microscope imaging technology that enables high-speed three-dimensional monitoring of multiple fluorescent compounds. In our study presented in Chapter 3, two fluorescent probes were simultaneously administered intravenously to experimental mice to allow for evaluation of platelet activation. This adds the parameter of platelet activation to the conventional parameter of platelet plug size/thrombus volume, in the determination of prothrombotic potential using this model. The distinct markers of different aspects of platelet activation allowed us to successfully detect platelet subpopulations temporally and spatially within the thrombus during real-time platelet plug formation. This greatly enhanced our knowledge of *in vivo* arterial thrombus formation. Our study adds to the previous findings supporting the heterogeneous nature of platelet-rich plugs. It has been shown that platelets located at the outside region of platelet-rich plugs remain in discord shape and are loosely packed, which in turn results to the high permeability of this region in contrast to the densely packed central region. $P2Y_{12}$ antagonist, cangrelor, reduced plug volume from the loosely packed region and thrombin inhibitor, hirudin, reduced plug volume from the densely packed region (Stalker et al., 2013). We found that distinct platelet activation events within a thrombus occur differently in time and space. The restricted location of Diannexin binding we found in a thrombus explains why fibrin formation is not fully colocalized with a forming platelet-rich plug in the laser injury thrombosis model. In addition, fibrin formation and Diannexin binding are detected at the injury site, which is the central region of a platelet-rich plug. Together,

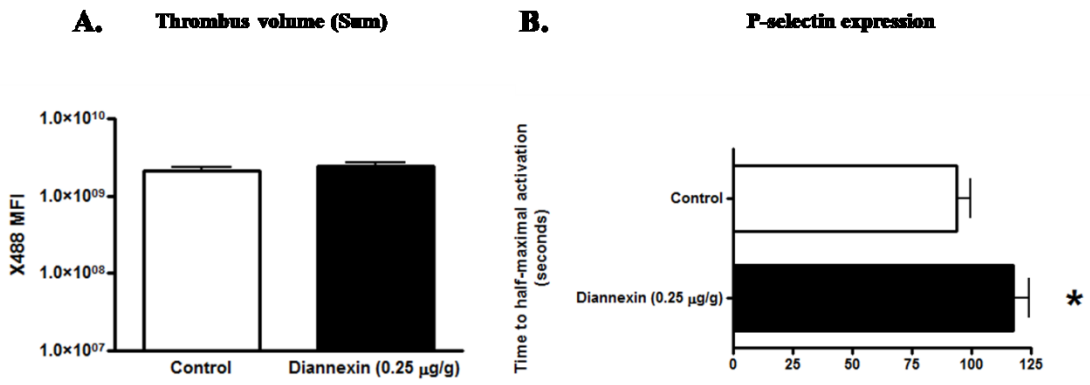
the evidence supports the restricted regional effect of hirudin inhibition. Most importantly, we highlighted the importance of involving the heterogeneous nature of platelet-rich plugs in the evaluation of antiplatelets' effects. Even though the weak platelet inhibitor, ASA, does not alter plug volume as observed with cangrelor and hirudin inhibition, ASA suppresses *in vivo* platelet activation.

The establishment of a new parameter - time to half-maximal platelet activation (to plug size), to a large extent, enhanced the sensitivity of this model in detecting subtle inhibitory effects. One of the major concerns of the laser injury model has been the poor reproducibility of the size of the thrombus formed by laser injury (Falati et al., 2004). The variation of thrombus size within the same testing group can largely shadow the difference of thrombus size between testing groups. Given that *in vivo* platelet activation appears to progress at a rate independent of thrombus size, the overall consistency of the time to half-maximal platelet activation is much greater compared to that of thrombus volume. As a result, this novel parameter requires less experimental numbers to reflect treatment effects; in statistical terms it has a greater relative efficiency. The usefulness of this method was further verified in our demonstration of platelet inhibition *in vivo* by atorvastatin in Chapter 4.

Even though we have found that ASA (Chapter 3), atorvastatin (Chapter 4) and Diannexin at a low dose **(Figure 6.1)** (Rand et al., 2012) inhibited *in vivo* platelet activation events without altering thrombus volume, it is currently not clear how impaired platelet activation (within the same magnitude of thrombus volume)

Figure 6.1 Effect of low dose Diannexin on platelet accumulation and activation in thrombi *in vivo*.

A. Thrombus volume/platelet accumulation by low dose Diannexin was measured by X488 (Dylight-488-tagged antibody against GPIbβ). **B.** Time to half-maximal ratio of P-selectin expression by low dose Diannexin. Mean ± SEM are shown, (47 thrombi from control group and 45 thrombi from Diannexin-treated group), **P*<0.01. (Rand et al., 2012)

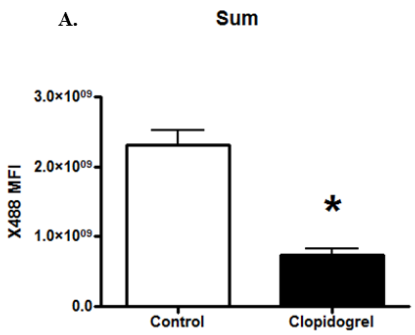

A.
Thrombus volume (Sum)
X488 MFI
1.0×10^10
1.0×10^09
1.0×10^08
1.0×10^07
Control
Diannexin (0.25 μg/g)
B.
P-selectin expression
Time to half-maximal activation (seconds)
Control
Diannexin (0.25 μg/g)
*
0
25
50
75
100
125

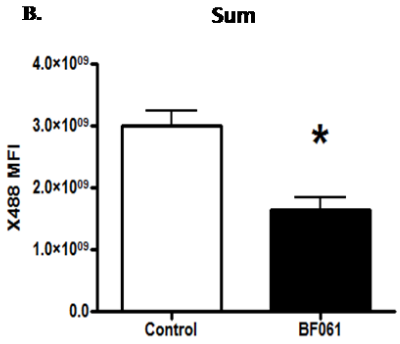
contributes to thrombus suppression. One explanation is that disorganized thrombus architecture as a result of this type of inhibition may make thrombi vulnerable to endogenous platelet inhibitors, other antithrombotic agents and flow. We demonstrated that a large number of the platelets participating in a thrombus detach themselves from the existing thrombus within seconds. The loss of thrombus volume by leaving platelets can be substituted quickly with new platelets supplied by the circulation. Our result indicates that the turnover of individual platelets over time may be the primary mechanism of volume loss from a thrombus. The upregulation of CD41 surface expression may strengthen the reversible platelet aggregation against platelets to detach from a thrombus. In contrast, decreased CD41 surface upregulation by ASA inhibition may weaken the reversible platelet aggregation and allow platelets to break off from a thrombus. Even though in our study, thrombus volume was not changed by ASA treatment, we do not exclude the possibility that ASA changes thrombus volume at a later stage of thrombus progression, beyond 4 minutes of recording time. The importance of inhibiting platelet activation *in vivo* by ASA, atorvastatin and low dose Diannexin require further elucidation.

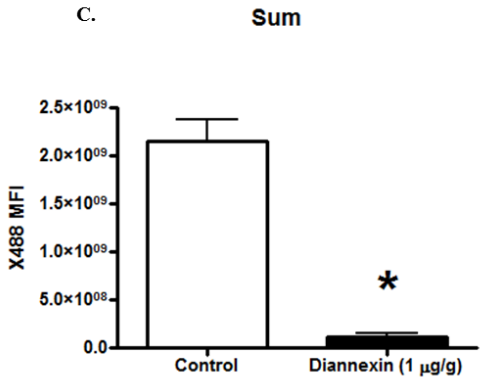
It is important to note that the modified model does not challenge the validity of using conventional laser injury thrombosis model to detect strong platelet inhibition. For instance, we have shown that $P2Y_{12}$ antagonists, Diannexin at a high dose and NBEAL2 deficiency reduce thrombus volume using the conventional laser injury model **(Figure 6.2)** (Hu et al., 2011; Rand et al., 2012; Kahr et al., 2013). In addition,

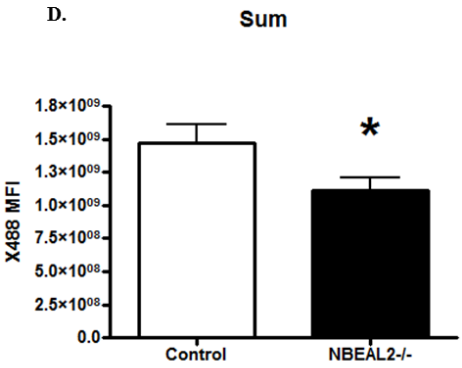
Figure 6.2 Effect of $P2Y_{12}$ antagonists, high dose Diannexin and NBEAL2 deficiency on platelet accumulation/thrombus volume *in vivo*.

A. Thrombus volume/platelet accumulation by clopidogrel at 30 mg/kg, (88 thrombi from control group and 73 thrombi from clopidogrel-treated group), **P*<0.0001, (unpublished data). **B.** by novel $P2Y_{12}$ antagonist BF061 at 25 mg/kg, (66 thrombi from control group and 63 thrombi from BF061-treated group), **P*<0.001. **C.** by high dose Diannexin, (72 thrombi from control group and 22 thrombi from Diannexin-treated group), **P*<0.05. **D.** in NBEAL2-deficient mice was measured by X488 (Dylight-488-tagged antibody against GPIbβ) (170 thrombi from control group and 155 thrombi from NBEAL2-/- group), **P*<0.05. (Hu et al., 2011; Rand et al., 2012; Kahr et al., 2013)


A.
Sum
X488 MFI
3.0×10^09
2.0×10^09
1.0×10^09
0.0
*
Control
Clopidogrel


B.
Sum
X488 MFI
4.0×10^09
3.0×10^09
2.0×10^09
1.0×10^09
0.0
*
Control
BF061


C.
Sum
X488 MFI
2.5×10^09
2.0×10^09
1.5×10^09
1.0×10^09
5.0×10^08
0.0
*
Control
Diannexin (1 μg/g)


D.
Sum
X488 MFI
1.8×10^09
1.5×10^09
1.3×10^09
1.0×10^09
7.5×10^08
5.0×10^08
2.5×10^08
0.0
*
Control
NBEAL2-/-

we have also demonstrated that time to half-maximal activation was not altered by either $P2Y_{12}$ antagonists, Diannexin at a high dose or NBEAL2 deficiency (data not shown). Thus we suggest that if a condition results in potent inhibition of platelet activation, this will result in a smaller thrombus volume within minutes. The platelets that form this smaller thrombus appear to be as activated as platelets in an untreated thrombus.

Despite the large body of work using mouse models to inform us of mechanisms and targets for therapeutics in arterial thrombosis, proper interpretation ought to weigh in the relevance of these results to human diseases and proper human validation of the effect of potential platelet inhibitors should occur. After all, some molecules of the two species that mediate platelet activation are not identical (Westrick and Ginsburg, 2009). Of note, notwithstanding mouse platelets are smaller in size; they express higher copies of many important platelet receptors compared to human platelets **(Table 6.1)** (Zeiler et al., 2014). Last but not least, given that shear rates vary amongst species, in different vessel beds, in the same individual, or even in exactly the same vessel bed before and after pathological conditions, shear-dependent platelet activation *in vivo* in mouse models may not closely reflect that in humans (Mailhac et al., 1994; Siegel et al., 1994; Bluestein et al., 1997).

Together, mouse models remain a powerful tool in thrombosis research and continue to evolve to eliminate their drawbacks. We believe that dual-fluorescent labels improve the conventional laser injury model to be a better tool for future

Table 6.1 Copies of major human and mouse receptors on platelets.

Comparison of expression levels of platelet adhesion, aggregation, and major signaling receptors in humans and mice.

Receptors	Human	Murine
GPIb-IX-V	25,000	35,600
GPVI	3,700	19,700
$\alpha_2\beta_1$	900 – 4,000	16,000
$\alpha_{IIb}\beta_3$	80,000	120,000
PAR1/4	2,000	5,200
$P2Y_1$	150	1,000
$P2Y_{12}$	400 – 1,000	2,700
TP	1,500	1,900

thrombosis research. However, the valuable evidence gained from animal studies will only support the feasibility of antiplatelets in human testing. Human testing is an inevitable step to validate new antiplatelets. This subject will be discussed in greater details in the following sections.

6.2 What is the mechanism by which statin treatment alters PAR signaling

Our finding of eNOS-independent platelet inhibition by atorvastatin in mice adds to the previous literature supporting statin's inhibitory effect on thrombin signaling in humans. In human subjects, statin use is found to decrease PAR1 expression on platelets (Serebruany et al., 2006). In contrast, we demonstrated that atorvastatin treatment of mice resulted in platelets with no alteration of PAR4 expression but with decreased PAR4 signaling. Our findings suggest that an impaired intracellular signaling response downstream of thrombin-induced PAR activation might have been overlooked as another aspect of platelet inhibition by statins. Attenuating thrombin signaling via intracellular molecules, as statins do, without directly blocking PAR1, may in part explain the much milder platelet inhibitory effect of statins compared to PAR1 inhibitor, vorapaxar.

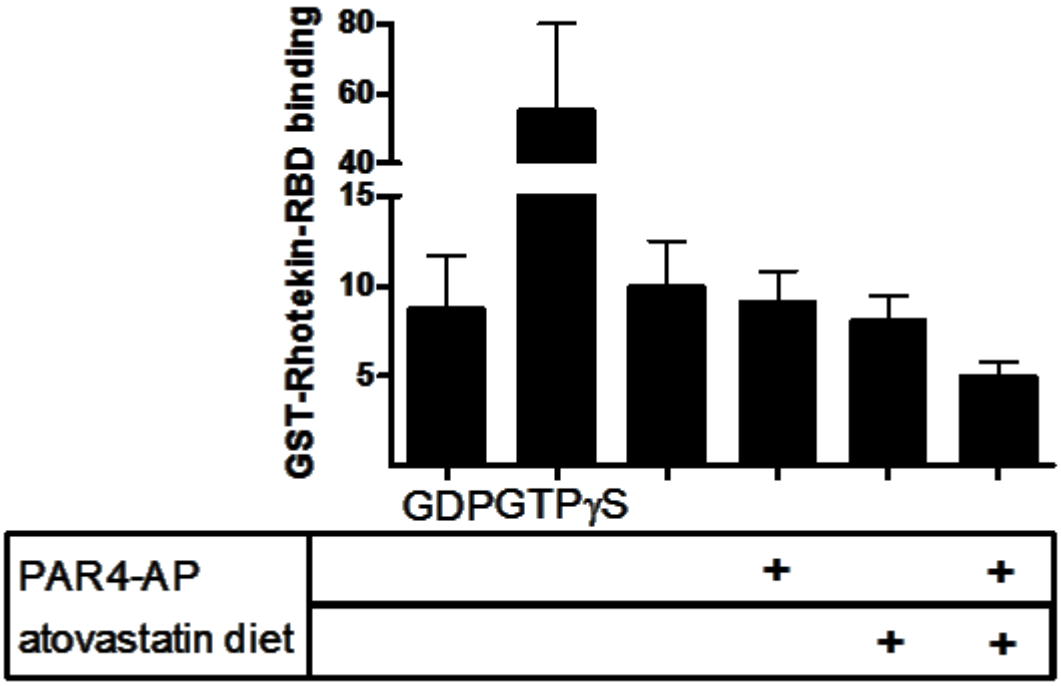
Amongst the cellular signaling molecules that are involved in thrombin-mediated platelet activation, two of them possess the consensus sequence of CaaX that can be modified by prenylation, which is likely affected by statin use. The first one is the G protein complex, which interacts with PAR4 (Zhang and Casey, 1996). The other one is Rho GTPase, which acts as an effector molecule to mediate signal transduction

downstream of activated G proteins (Zhou and Liao, 2009). In fact, as mentioned earlier, eNOS-dependent platelet inhibition by statins is a result of depressed Rho GTPase activity in endothelial cells (Laufs and Liao, 1998). Rho activation plays the central role in cytoskeleton rearrangement during platelet activation, and determine the size of a resting platelet (Pleines et al., 2012; Bye et al., 2016). In our study, we did not notice a size difference of resting platelets by statin treatment (data not shown), however, our preliminary result showed that GYP (PAR4 agonist peptide)-mediated platelet Rho activation was decreased in statin-treated mice **(Figure 6.3)**. Taken together, there may be multiple aspects of platelet inhibition as a result of attenuated Rho activities by statins.

In order to efficiently mediate signal transduction from membrane-bound GPCRs to many membrane-bound effector molecules, G protein complexes ought to be in the close proximity to plasma membrane. However, none of the three G protein subunits contains a transmembrane-spanning domain to facilitate membrane interactions. It was found that palmitoylation of α-subunit and prenylation of γ-subunit are the major post-translational modifications that anchor G protein complexes to plasma membrane (Higgins and Casey, 1996). Palmitoylation and prenylation are both suppressed by statins. Impaired prenylation by statins is attributed to a reduction of the two distinct isoprenoids in the process of cholesterol synthesis, while palmitoylation may be directly regulated by cellular cholesterol levels (Parsons et al., 2006; Chini and Parenti, 2009). In our study, atorvastatin at 0.2 g/kg does not influence murine plasma

Figure 6.3 Effect of atorvastatin on GYP (PAR4 agonist peptide)-induced Rho activation (Rho-GTP) in platelets from eNOS-deficient mice.

Activated Rho protein (Rho-GTP) in resting and GYP-activated platelets from eNOS-deficient mice fed with control diet or atorvastatin diet at 0.03 g/kg for 14 days was collected using a Rho pull-down assay and quantified by western blot (Preliminary data, N=2).


GST-Rhotekin-RBD binding
80
60
40
15
10
5
GDPGTPγS
PAR4-AP
atovastatin diet
+
+
+
+

cholesterol levels **(Table 6.2)**, thus, we rule out the effects of altered α-subunit palmitoylation in the inhibition of PAR4 intracellular signaling by statin treatment.

Cellular signaling downstream of ADP receptor $P2Y_1$ and TXA_2 receptor TP seem to share G protein complexes, Gq and G_{13}, named for the specific G protein α-subunit, with thrombin receptors (Offermanns, 2006). It is cryptic that the activation of either $P2Y_1$ or TP is not altered by atorvastatin treatment in an eNOS-independent manner in our study. Given that there is growing body of evidence suggesting GPCR selectivity for particular βγ-dimers and the imperative function of βγ-dimers in G protein-mediated signaling transduction (Smrcka, 2008), it is reasonable to hypothesize that Gq and G_{13} under different GPCRs in platelets may be further classified by the subtypes of engaged βγ-dimers. Moreover, we hypothesize that prenylation of distinct βγ-dimer in the G protein complexes associated with PAR4 is inhibited by statin treatment and this is responsible for the restrictive inhibitory effects found in PAR4 signaling by atorvastatin in our study.

In comparison to α-subunit, βγ-dimers in signaling have long been overlooked. α-subunit activation has been well studied and the subtypes were found to be specific for a GPCR in a tissue context (Oldham and Hamm, 2008). To date, less is known to the interaction between βγ-dimers and their effector proteins. Also, how the selectivity of different βγ-dimers and their prenylation state influence distinct cellular signaling are puzzles that remain to be solved. Recent studies have identified numerous effector molecules of βγ-dimers and this has led to the discoveries of a number of

Table 6.2 Effect of atorvastatin diet on lipid profile.

Plasma levels of very low-density lipoprotein (VLDL), LDL and high-density lipoprotein (HDL) from mice fed with atorvastatin diet at 0.2 g/kg for 14 days (N=7).

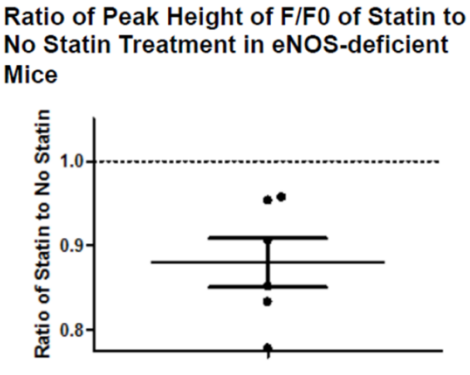
Lipid Profile	Control (mM)	Atorvastatin-treated (mM)
VLDL	0.10 ±0.04	0.08 ±0.03
LDL	0.11±0.05	0.10 ±0.04
HDL	0.68 ±0.12	0.58 ±0.15
Total	0.89 ±0.16	0.76 ±0.20

downstream cellular events. These include PLCβ isoforms and downstream event of intracellular event of Ca^{2+} mobilization (Park et al., 1993). In support of these, we evaluated intracellular Ca^{2+} levels in PAR4 peptide-activated platelets from control and atorvastatin-treated mice **(Figure 6.4)**. We found decreased Ca^{2+} mobilization in atorvastatin-treated platelets, implying the possibility of alteration on βγ-dimer property by atorvastatin in PAR4 signaling. Importantly, prenylation may not just determine the potency of βγ-dimers to activate effector molecules but may, at least in part, determine the affinity of a G protein complex to interact with its GPCR (Wedegaertner et al., 1995). Given that conventional Gq α-subunit signaling is more potent compared to βγ-dimers in activating PLCβ, prenylation of distinct βγ-dimer, alternatively, may indirectly attenuate PLCβ activation through Gq α-subunit by attenuating the coupling of a G protein complex with PAR4 (Park et al., 1993). This investigation can potentially provide insights to the ongoing debate on the chronological sequence of G protein coupling and post-translational modifications of α- and βγ-subunits. The work may also potentially extend the use of prenylation inhibitors, to suppress activities of oncogenic Ras protein in cancer research, to Rho and G proteins in thrombosis research (Berndt et al., 2011). Importantly, to identify the isoforms of βγ-dimer that are involved in PAR4 signaling may lead to new platelet intracellular targets for future pharmaceutical interventions.

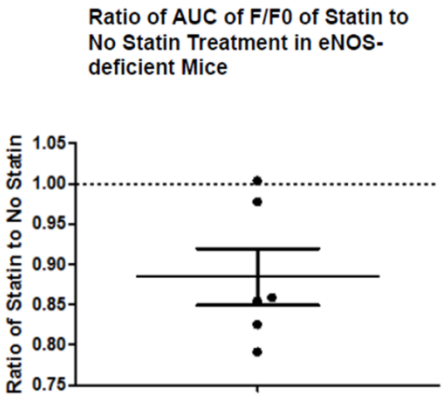
Figure 6.4 Effect of atorvastatin on GYP (PAR4 agonist peptide)-induced intracellular Ca^{2+} mobilization in platelets from eNOS-deficient mice.

Washed platelets from eNOS-deficient mice fed with control diet or atorvastatin diet at 0.03 g/kg for 14 days were activated by GYP in the presence of 1 mM Ca^{2+}. **A.** Data show peak values and **B.** AUC values of intracellular Ca^{2+} mobilization in activated platelets from eNOS-deficient mice fed with atorvastatin relative to those from eNOS-deficient mice fed with control diet (dash lines). Mean±SEM are shown, (N = 6), $P<0.05$.

A.


Ratio of Peak Height of F/F0 of Statin to No Statin Treatment in eNOS-deficient Mice
Ratio of Statin to No Statin
1.0
0.9
0.8

B.


Ratio of AUC of F/F0 of Statin to No Statin Treatment in eNOS-deficient Mice
Ratio of Statin to No Statin
1.05
1.00
0.95
0.90
0.85
0.80
0.75

6.3 The effectiveness of the most commonly prescribed antiplatelet regimens that have multiple targets using mouse models

Platelet activation is a complex of cellular processes. This starts with abundant heterogeneous platelet stimuli and ligands available at clot forming sites and is followed by extensive crosstalk and sharing of small signaling molecules between numerous platelet receptors. Signal transduction ultimately leads to the common platelet activation events of platelet aggregation, degranulation and shape change, etc. (Bye et al., 2016). Because most platelet signaling pathways are involved in both pathological and physiological clot formation, modern antiplatelets favor attenuating platelet activation by multiple platelet inhibitors that target different platelet signaling pathways over inhibition of a single pathway provided by a sole, more potent, platelet inhibitor. In our study presented in Chapter 5, we have highlighted the importance of the platelet inhibitory pathway, PGI_2-IP-AC-cAMP, in the determination of overall effectiveness of DAPT that contains clopidogrel and ASA using a murine arterial thrombosis model. This study highlights the importance of animal studies to test theories of drug combinations, even if the therapeutic value of the individual drugs is certain.

It is of note that arterial thrombotic patients are frequently prescribed a statin to slow down atherosclerosis progression. Others' and our demonstration in Chapter 4 of the fact that atorvastatin is able to attenuate platelet activation independent of lipid-lowering, qualifies atorvastatin as a platelet inhibitor. Taken together, the regimen combining atorvastatin, $P2Y_{12}$ antagonist and ASA inhibits thrombin-, ADP-

and TXA_2-induced platelet activation. In spite of the extensive platelet inhibition provided by the regimen, the overall potency of platelet inhibition by all three platelet inhibitors together in a combination remains unclear owing to their individual and reciprocal roles in regulating platelet inhibitory machineries. This could be evaluated in the future.

In previous studies, it has been strongly suggested that the major inhibitory effect of statins on platelet activation is to induce eNOS and thus NO production (Laufs and Liao, 1998). We confirmed that atorvastatin can broadly inhibit platelet activation *in vitro* induced by multiple individual agonists and platelet activation within forming thrombi *in vivo* in the presence of functional eNOS. Our finding supports previous studies that elevated platelet cGMP, a second messenger, is a result of increased NO availability, which serves as important means of persistent endogenous platelet inhibition beyond its role in vessel dilation (Forstermann and Munzel, 2006). $P2Y_{12}$ antagonists also enhance platelet inhibitory effects by another chief vessel tone regulator PGI_2. This is through promoting platelet cAMP generation by abrogating AC inhibition from $P2Y_{12}$ activation (Yang et al., 2002). In contrast to $P2Y_{12}$ antagonists, ASA may decrease platelet cAMP by downregulating PGI_2 production (Smyth et al., 2009). At present, how important elevated cAMP levels are to the net clinical benefits of $P2Y_{12}$ inhibition is underappreciated. The counteracting effect of PGI_2 downregulation by ASA in the net clinical benefits of COX-1 inhibition is not yet well proven. Thus the additive effect of ASA with $P2Y_{12}$ antagonist is speculative and not

proven. In our study, we were able to confirm the effectiveness of DAPT combining clopidogrel and ASA, at a low dose, in reducing arterial thrombus formation in mice. Importantly, we highlighted the importance of $P2Y_{12}$ inhibition on cAMP generation and called for caution on proper ASA dosing in DAPT in order to maintain PGI_2 production, which enables cAMP promotion by $P2Y_{12}$ inhibition.

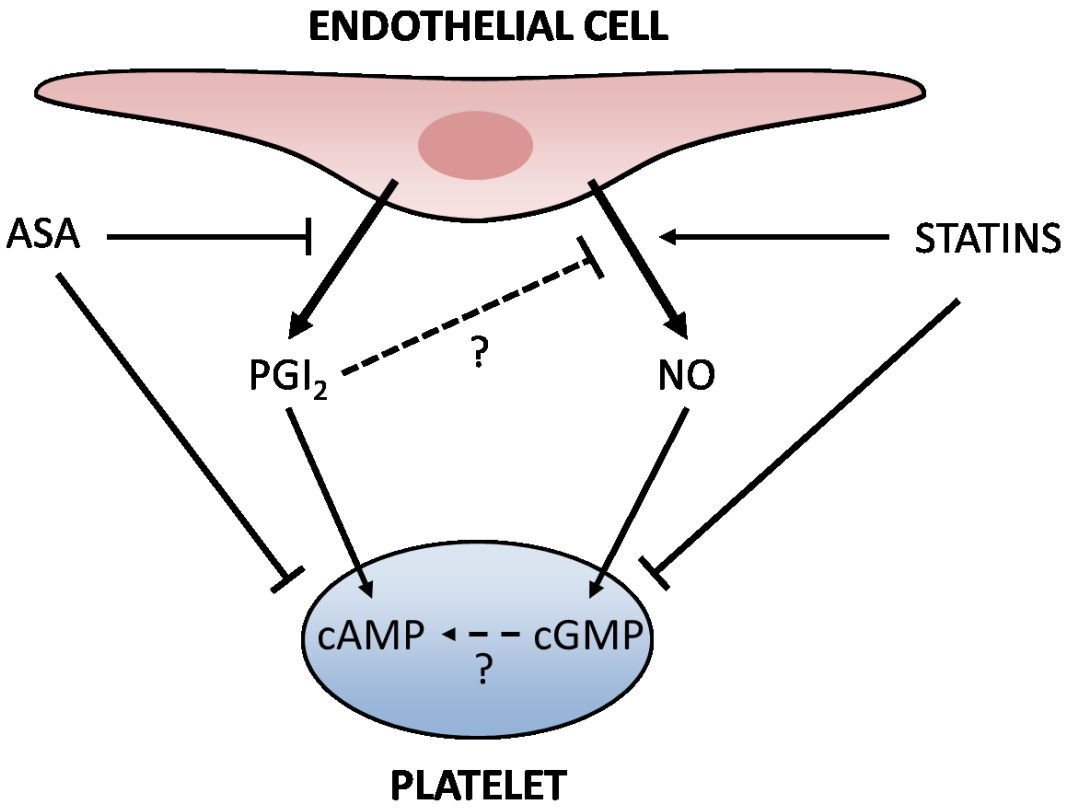
In addition to attenuating PGI_2-mediated platelet inhibition, $P2Y_{12}$ activation has recently been found to desensitize platelet inhibition by NO. The study also revealed that $P2Y_{12}$ inhibition dramatically enhanced the inhibitory effect of NO on platelets similar to what is observed between the interplay of $P2Y_{12}$ blockade and PGI_2. The difference is that $P2Y_{12}$ inhibition does not elevate cGMP production or prevent cGMP degradation but alters the effectors downstream of cGMP (Kirkby et al., 2013). Although the synergy between $P2Y_{12}$ antagonists and NO remains to be further explored, the phenomenon supports concomitant administration of $P2Y_{12}$ antagonists and statins in regard to platelet inhibition.

Taken together, the combination of a $P2Y_{12}$ antagonist, a statin and a dose of ASA that allows normal PGI_2 production, presumably, will result to an enhancement of endogenous platelet inhibition by both PGI_2 and NO, in addition to attenuated ADP, thrombin and TXA_2 signaling. However, the potential interplay of PGI_2 and NO also weighs in to this assumption and might suggest otherwise. A study of endothelial cells, the dominant donor of endogenous PGI_2 and NO, has shown that attenuated PGI_2 levels lead to an increase of NO production in a Ca^{2+}-dependent manner (Bolz and

Pohl, 1997). Furthermore, studies on downstream products of PGI_2 and NO in platelets have shown that cellular cGMP can positively regulate cAMP levels by preventing it from being degraded (Maurice and Haslam, 1990; Dickinson et al., 1997). These two lines of evidence raise two questions: 1) does it matter when a dose of ASA that interferes with PGI_2 production is prescribed with $P2Y_{12}$ antagonists and statins, given that such inhibition may augment NO inhibition instead? And, 2) are elevated platelet cGMP levels by statins sufficient to recover platelet cAMP levels when PGI_2 production is completely abrogated by ASA **(Figure 6.5)**? In future studies, to answer these questions, endothelial production of PGI_2 and NO under basal conditions and under the influence of $P2Y_{12}$ antagonists, statins, or/and ASA needs to be investigated. Secondly, cAMP and cGMP levels in platelets under these treatment conditions will also unmask the reciprocal effects amongst these medications. In addition, the comparison of PGI_2 and NO potency on platelet inhibition needs to be determined. It is presently held that the inhibitory effect of PGI_2 is more potent compared to that of NO. A study, nevertheless, has argued for the opposing observation when $P2Y_{12}$ antagonists were used (Ozuyaman et al., 2005; Kirkby et al., 2013). The extremely short half-lives of PGI_2 and NO will make the accurate determination on the effects of these medications challenging to study *in vitro*. Ultimately, evaluation of *in vivo* thrombus formation using our murine thrombosis model will allow the continuing evaluation of the participation of endothelial cells, under control and in all treatment conditions. This will in turn more precisely reveal

Figure 6.5 A model of the interplay between ASA and statins on platelet inhibition.

ASA and statins can directly inhibit platelet activation in PGI_2- and NO-independent manners. ASA decreases PGI_2 formation by endothelial cells, while statins promote NO formation by endothelial cells. The formation of PGI_2 and NO leads to elevated levels of platelet cAMP and cGMP, respectively, which in turn inhibit platelet activation. PGI_2 may be inhibitory to NO formation by endothelial cells. cGMP may elevate cAMP levels in platelets.


ENDOTHELIAL CELL
ASA
STATINS
PGI2
?
NO
cAMP
cGMP
?
PLATELET

the combined effects of these three platelet inhibitors on arterial thrombosis.

6.4 To translate our findings in mice to human subjects

In our studies, we have shown significant downregulation of PGI_2 by ASA at a relatively low dose of 40 mg in mice, but not at a dose of 10 mg, and at least some antiplatelet effects of ASA at 10 mg (human equivalent). Moreover, we have demonstrated that administration of a $P2Y_{12}$ antagonist with 10 mg ASA had a stronger inhibitory effect on mouse thrombus formation compared to that of DAPT with 40 mg ASA (Chapter 5). Together, we propose a novel approach to ASA dosing in DAPT to achieve the greatest effectiveness in reducing arterial thrombosis in mice. The ultimate goal of this study is to be able to interpret what we have found in mice to human patients who receive DAPT. As a hypothesis from our findings in mice, our suggested optimal dose of ASA in DAPT was one that did not inhibit COX-1 in endothelial cells, but inhibited TXA_2-mediated platelet activation even if only partially.

In the past, with the goal of maximally inhibiting TXA_2-mediated platelet activation, a great number of studies aimed at searching for the most favorable dose of ASA in treating human thrombosis. The recommended dosing has been amended a number of times towards to lower ones owing to frequent occurrences of GI bleeding associated with higher doses of ASA (Roderick et al., 1993). Presently, the most commonly prescribed ASA dose is 81 mg, which is referred in North America as the low dose (Levine et al., 2016). We do not challenge the fact that ASA at 81 mg acts as

an effective platelet inhibitor when it is administered alone. Higher doses of ASA most likely provide greater inhibition to TXA_2-mediated platelet activation than ASA at low doses (Tohgi et al., 1992). ASA is a weak platelet inhibitor, which does not affect platelet response to ADP and thrombin (Rinder et al., 1993; Undas et al., 2007). However, in the course of thrombus formation, the participation of various stimuli including ADP is inevitable because of amplification. Even if a given dose of ASA does not interfere with endothelial COX-1 function to produce adequate PGI_2, AC inhibition resulted from $P2Y_{12}$ activation would, nevertheless, invalidate the inhibitory effect of PGI_2 on platelets. In this case, the availability of PGI_2 would not drive the demand to lower the ASA dose.

With the same goal of treating arterial thrombosis by attenuating TXA_2-mediated platelet activation, the ASA dose determination that we suggest in the context of a regimen containing both ASA and a $P2Y_{12}$ antagonist is disparate from the previous ASA dose determination. The finding of synergistic inhibitory effect of $P2Y_{12}$ inhibition and PGI_2 on a high concentration of thrombin-mediated platelet activation *in vitro* is profound. This line of evidence implies that a $P2Y_{12}$ antagonist does not just inhibit ADP-mediated platelet activation *in vivo*, but also functions as a potent inhibitor to thrombin-mediated platelet activation *in vivo* owing to the presence of endogenous PGI_2. Thus, the proper dosing of ASA in DAPT should be determined by the preservation of PGI_2 formation, and not adverse effects or the maximal suppression of TXA_2 formation. Such a distinct approach in dose determination

should result in clinical trials evaluating DAPT with conventional ASA dosing compared to DAPT with ASA dosing based on our findings.

Based on existing evidence from clinical trials, we hypothesize that ASA at a dose lower than 81 mg in DAPT will add additional protection to arterial thrombotic patients. Firstly, administration of ASA alone at doses much lower than 81 mg has been used and effectively inhibited arterial thrombosis in European patients (The Dutch TIA Trial Study Group, 1991). Secondly, ASA at 25 mg twice a day is recommended in co-administration with dipyridamole (another dual-antiplatelet regimen termed Aggrenox) (Diener et al., 1996). Last but not least, in spite of a number of clinical trials on DAPT have shown that the antithrombotic effect of ASA at high doses is not more potent compared to low doses, the PLATO trial has revealed the superiority of ASA at low doses (approximate 100 mg) versus high doses (>300 mg) (Mahaffey et al., 2011). Given that the potent *in vitro* synergistic effects of $P2Y_{12}$ inhibition and PGI_2 on platelet activation by thrombin were found in both mice and human subjects, it is surprising that there is a dearth of clinical evidence to validate ASA dose lowering in the promotion of such a synergistic effect. In another words, evidence indicates that the current ASA dose of 81 mg is inhibitory to endothelial COX-1, which in turn disables the generation of an adequate amount of PGI_2 similar to what higher dose of ASA would do. In fact, there is no evidence that ASA, at doses studied in clinical trials thus far, adds any additional antithrombotic effect to a $P2Y_{12}$ antagonist.

Presently, the amount of PGI_2 required for *in vivo* synergistic effects with $P2Y_{12}$ inhibition in humans is unclear. Once again, what is the highest dose of ASA that is not inhibitory to the generation of PGI_2 and whether this dose is still effective on TXA_2-mediated platelet activation in humans remain questions to be answered. To directly lower ASA dose from 81 mg in DAPT without knowing the precise dose target would potentially be risky to arterial thrombotic patients, even within a study. Therefore, the production of PGI_2 in response to ASA at various doses that are below 81 mg in healthy individuals ought to be firstly determined. Similar to the study we carried on in mice, plasma levels of 6-keto-$PGF_{1\alpha}$ can be used as a surrogate of PGI_2 production. Furthermore, whether these doses of ASA are antithrombotic also need to be confirmed. *In vitro* platelet activation by AA in response to ASA treatment can closely reflect ASA's effectiveness, as we demonstrated in mice.

The limitations of our proposed future study include that ASA's effect is influenced by gender and disease (Dalen, 2006). In addition, we cannot rule out the possibility that individuals simply respond to ASA differently. These two pitfalls can be overcome through promoting "personalized medicine" and find the optimal ASA dose via titration for each patient. The ultimate goal of our studies will be to prove the feasibility of ASA dose lowering in DAPT in a clinical trial. This would eventually involve atherothrombotic patients who may react to ASA treatment differently from healthy individuals and require for further ASA dose adjustment. It is of note that the interplay of PGI_2 and NO found in endothelial cells and platelets, as what we have

discussed earlier, may alter the outcome from what we expect with ASA dose lowering in DAPT. Recall that statins are NO enhancers and are commonly prescribed to patients who receive DAPT. In this case, PGI_2 and NO production by endothelial cells in response to ASA or/and statins need to be evaluated in human subjects. cAMP and cGMP in platelets in response to ASA, statins or/and $P2Y_{12}$ antagonists will also need to be evaluated. Moreover, the determination of PGI_2 and NO potency in platelet inhibition will serve as a prerequisite prior to a clinical trial.

6.5 The implications of our studies in future antiplatelet regimens

The potentiation of endogenous platelet inhibitors by $P2Y_{12}$ inhibition, in addition to attenuated ADP-mediated platelet activation, found in our study and others implies the central position of targeting $P2Y_{12}$ signaling in modern antiplatelets. This does not just support the continued search for better $P2Y_{12}$ antagonists, but may also lead to the next generation of better DAPTs or triple drug combinations with a $P2Y_{12}$ antagonist in the prophylaxis and treatment of arterial thrombosis.

It is of note that endothelial dysfunction is a hallmark of cardiovascular diseases and depressed NO produced by endothelial cells is a key feature of endothelial dysfunction (Davignon and Ganz, 2004). Even though the recognition of the major cardiovascular benefits of statin use independent of lipid-lowering was to promote NO generation, combining a $P2Y_{12}$ antagonist and NO together was found to be potent, but NO itself was found inadequate to inhibit thrombin-mediated platelet aggregation *in vitro* by others (Kirkby et al., 2013). This line of evidence suggests that NO

elevation by statin treatment alone may have little effect on platelets in the absence of $P2Y_{12}$ antagonists. It also highlighted the importance of our finding on eNOS-independent platelet inhibition by statins in the presence of active $P2Y_{12}$ signaling (Chapter 4). In future studies, the synergistic effect of $P2Y_{12}$ antagonists and NO *in vitro* found in human platelets needs to be further verified *in vivo* using animal thrombosis models. Given that broad platelet inhibitory effects by the two endogenous platelet inhibitors are well known. The fact that dramatically magnified PGI_2 and NO signaling in platelets is amplified by $P2Y_{12}$ inhibition builds a strong argument for a combination of drugs that promote PGI_2 or/and NO signaling with $P2Y_{12}$ antagonists to achieve greater antithrombotic potential.

PGI_2 and NO donor drugs, such as iloprost and nitroglycerin, have been used to ease ischemia symptoms by promoting vessel dilation (Baysal et al., 2006; Yurtseven et al., 2006; Rex et al., 2008). Their inhibitory effects on platelet activation have also been suggested, the clinical relevance of such effects, however, is less clear. The evidence we list in this thesis imply that these drugs may not be great platelet inhibitors by themselves. Moreover, we suggest that they may have potent antiplatelet effects when they are administrated to patients who receive a $P2Y_{12}$ antagonist. PGI_2 and NO donor drugs are often short-acting agents. Therefore, they may not be ideal for outpatients. However, under medical monitoring, combining PGI_2 or/and NO donors with a fast-acting $P2Y_{12}$ antagonist, for instance cangrelor, may serve an effective treatment to rapidly reverse ACS in inpatients. It is of note that nitroglycerin

is presently one of the immediate treatments given to patients when ACS is suspected in the hospital. In the context of promoting the generation of platelet cyclic nucleotides, the novel therapeutic class of soluble GC stimulators, such as riociguat, in use to treat pulmonary arterial hypertension, may also be very beneficial to arterial thrombotic patients (Ghofrani et al., 2013).

An alternative approach to magnify the antiplatelet effects of PGI_2 and NO by $P2Y_{12}$ antagonists is to prevent inactivation of their second messengers, cAMP and cGMP, in platelets. Phosphodiesterases (PDEs) are a group of enzymes that catalyze the hydrolysis of cAMP and cGMP, which reduces cellular levels of these two second messenger molecules. Over sixty isoforms of PDE have been identified and their distribution appears to be tissue specific. Platelets express three PDE isoforms, PDE2, PDE3 and PDE5. PDE2 and PDE3 degrade both cAMP and cGMP while PDE5 has cGMP as the sole substrate. Existing evidence suggests that some nonselective PDE inhibitors have platelet inhibitory effects (Gresele et al., 2011). However, their ability to reduce ischaemic cardiovascular events is not certain. Given that PDEs are ubiquitously expressed, selective inhibitors that target platelet PDE isoforms are likely more effective and less problematic. At present, a PDE3 inhibitor, cilostazol, and a PDE3/PDE5 inhibitor, dipyridamole, have been tested in large scale clinical trials and have proven to be useful in thrombotic patients. The effectiveness of cilostazol was found when it was compared to ASA or placebo (Uchiyama et al., 2009; Shinohara et al., 2010). The superiority and non-inferiority were found in the comparisons of

dipyridamole administrated with ASA to ASA alone or clopidogrel alone (Diener et al., 1996; Sacco et al., 2008). To date, there is little clinical evidence regarding the antithrombotic effect of concomitantly administrating PDE inhibitors and $P2Y_{12}$ antagonists. The antithrombotic potential has been tested when cilostazol is added on top of DAPT (triple antiplatelet regimen) in two trials. However, the superiority of the triple antiplatelet regimen over conventional DAPT was only shown in one of the studies (Han et al., 2009; Suh et al., 2011). It is noteworthy that in both studies, loading and maintaining doses of ASA were set at 300 mg and 100 mg, respectively. Because our hypothesis of much lower dose of ASA is required for normal levels of PGI_2 production (Chapter 5), we suggest that reduced PGI_2 by ASA at ≥ 100 mg in these trials may have made the retention of cAMP by cilostazol less pronounced. In order to unmask the synergism of $P2Y_{12}$ inhibition and elevation of these cyclic nucleotides by cilostazol or dipyridamole, similar comparisons should be carried out without ASA administration or using a PGI_2-sparing dose of ASA in future clinical studies.

In addition to combining antiplatelets that have different platelet inhibitory properties, it is also appealing to develop new antiplatelets that carry multiple platelet inhibitory properties. With dipyridamole as an example, its initial use was for vessel dilation and a number of its antiplatelet actions were then established. Aside from inhibiting the degradation of cAMP and cGMP, it can block plasma adenosine uptake by erythrocytes and in turn enhances adenosine-mediated platelet inhibition (Gresele

et al., 1983; Gresele et al., 1986). It was also found to elevate PGI_2 production by scavenging free radicals, which cause inactivation of endothelial COX-1. In support of the concept of finding more potent antiplatelets, we have earlier described a novel $P2Y_{12}$ antagonist, BF061, which has anti-PDE activity. We also confirmed that the antithrombotic effect of BF061 is not inferior to clopidogrel in mice (Hu et al., 2011). Taken together, we suggest that antiplatelet regimens featuring the promotion of endogenous platelet inhibitors in addition to $P2Y_{12}$ inhibition will offer greater antithrombotic potential.

6.6 Conclusions and Significance

In this thesis, we first described an updated laser injury mouse thrombosis model. This enabled the detection of three distinct aspects of platelet activation (platelet aggregation, degranulation and PS surface expression) *in vivo*. Importantly, we introduced a new parameter – the time to half-maximal activation to determine antithrombotic effects. Our updated model can provide sensitive and efficient detection of platelet inhibition *in vivo*. We later investigated the antithrombotic potential of three widely used platelet inhibitors, atorvastatin, clopidogrel and ASA, using this laser injury model. We successfully evaluated the antithrombotic effects of all three drugs individually. We highlighted the usefulness of our updated model with the fact that we were able to detect subtle differences of platelet activation within comparably formed thrombi by atorvastatin and ASA. Our demonstration of eNOS-independent platelet inhibition by atorvastatin implies the potential of targeting

the PAR signaling pathway in developing future pharmaceutical interventions in arterial thrombosis. In addition, we evaluated the overall efficacy of DAPT containing clopidogrel and ASA in the reduction of murine arterial thrombus formation. We suggested that ASA at a PGI_2-sparing dose may be necessary for the optimal therapeutic efficacy of this regimen in arterial thrombotic patients. Others' and our demonstration of the synergy between endogenous platelet inhibitors and $P2Y_{12}$ inhibition indicated that the discovery of $P2Y_{12}$ antagonists is the cornerstone of modern antiplatelet medicine. Our finding does not just imply the significance of interplay between drugs in the determination of their overall antithrombotic potential, but also elicits more potent drug combinations in the future to provide greater protection to arterial thrombotic patients.

Without exception, the future antiplatelet regimens proposed in the thesis and beyond will require substantial evidence from animal models to support their usefulness in reducing arterial thrombotic burden to move forward to human testing. Moreover, as part of the evaluation, preservation of normal haemostatic function by these regimens in animals will also need to be proved. Most importantly, proper dosing of a medication in patients can only be guided by preclinical studies and these always require further investigation with human subjects. Ample evidence in addition to our study on DAPT shows the influence of dosing on the effectiveness of a medication. With the statin family as an example, endothelial toxicity was found to associate with high doses of statins presumably owing to excessive inhibition of

protein prenylation (Laufs et al., 1998). In addition, we found that atorvastatin at 0.2 g/kg (a dose that is 6-fold higher than the effective dose used in Chapter 4) did not inhibit murine platelet activation *in vitro* (data not shown). Taken together, the multistep testing in drug development warrants patients the best protection with minimal side-effects. In summary, the studies in the thesis provide insight to the future of antiplatelet medicine. In addition, our findings also support the necessity of animal studies in assisting the discoveries and development of future antiplatelet regimens.

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