Platelet GPIb and downstream activation by *S. sanguis*

by

Ahmed Y. Abdulrehman

A Thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba

in partial fulfilment of the requirements of the degree of

MASTER OF SCIENCE

Faculty of Dentistry

Department of Oral Biology

University of Manitoba

Winnipeg

TABLE OF CONTENTS

SUMMARY

There is increasing evidence suggesting the contribution of bacterial infections in atherothrombotic conditions. Studies have demonstrated that bacteria residing within the oral cavity activate platelets once they enter circulation. *S. sanguis* 2017-78 is capable of stimulating platelet aggregation in a thromboxane-dependent manner. In the present study, the signaling events associated with *S. sanguis* have been studied further. *S. sanguis* 2017-78 caused the phosphorylation of p38 MAP kinase and subsequently $cPLA_2$. The p38 MAP kinase inhibitor, SB203580 inhibited *S. sanguis* 2017-78-induced platelet aggregation as well as the phosphorylation of both p38 MAP kinase and $cPLA_2$. These data are consistent with $cPLA_2$ as a physiological target of p38.

A second component of the study examined the effects of aspirin, a known inhibitor of cyclooxygenase, on these signalling pathways. As previously shown, aspirin inhibited *S. sanguis* 2017-78-induced platelet aggregation, however aspirin also inhibited the phosphorylation of p38. Platelets treated with aspirin in the presence of SB203580, however displayed an enhanced phosphorylation suggesting that aspirin also inhibits p38 phosphorylation, resulting in an increase in p38 phosphorylation upstream of thromboxane A_2 in the pathway.

A third component of the study looked at the effects of blocking GPIb/IX/V, a platelet receptor that stimulates the activation of PLCγ2, on *S. sanguis* 2017-78-induced platelet activation. AN51, the GPIb/IX/V inhibitor blocked *S. sanguis* 2017-78-induced PLCγ2 phosphorylation, consistent with previous findings. Additionally, in aspirin treated platelets, blockade of GPIb enhanced the phosphorlyation of p38 MAP kinase. These findings suggest

that blockade of GPIb/IX/V inhibits a negative regulatory pathway, likely the previously reported PECAM-1 pathway, in platelet activation leading to enhanced platelet activity.

Taken together these results suggest a highly complex, multi-receptor, multi-signalling mechanism underlying platelet activation in response to *S. sanguis* 2017-78.

List of Figures

List of Tables

List of Abbreviations

AA	arachidonic acid
Ab	antibody
ADP	adenosine diphosphate
ASA	aspirin
ATP	adenosine triphosphate
BSA	bovine serum albumin
$[Ca^{2+}]$	cytosolic free calcium
COX	cyclooxygenase
$cPLA_2$	cytosolic phospholipase A_2
DAG	diacylglycerol
DTS	dense tubular system
ERK	extracellular regulated kinase
GP	glycoprotein
GDP	guanosine diphosphate
GTP	guanosine triphosphate
Ig	immunoglobulin

IP_3	inositol 1,4,5-triphosphate
LAMP	lysosomal associated membrane protein
LDL	low density lipoprotein
MAPK	mitogen-activated protein kinase
MI	myocardial infarction
OCS	open canalicular system
PG	prostaglandin
PLC	phospholipase C
PKC	protein kinase C
PKG	protein kinase G
PMSF	phenylmethylsulfonyl fluoride
PRP	platelet rich plasma
S. sanguis	*Streptococcus sanguis*
TxA_2	thromboxane A_2
TXS	thromboxane synthase
vWF	von Willebrand factor

1. INTRODUCTION

The first Surgeon General's Report on Oral Health in the United States of America in 2000 recognized the link between good oral hygiene and good systemic health (Satcher, 2000). The report highlighted the link between periodontal disease and multiple systemic health conditions including cardiovascular disease, diabetes and certain lung diseases. The recognition of such a causal relationship may change the priority of the general public by encouraging a preventative approach to good oral health status. In addition, individuals in charge of public health sectors will be able to include comprehensive oral health treatment protocols when managing individuals with systemic health disorders.

2. LITERATURE REVIEW

2.1. Periodontal Disease

Periodontitis is a bacterially-induced inflammatory disease (Wilson and Kornamn, 2003). Less severe forms of periodontal disease affect 75% of adults in the United States while severe forms are present in 20 to 30% of adults. Bacteria, although required, are not sufficient *per se* for the onset of periodontitis but rather, factors such as individual susceptibility and oral hygiene are integral and important to the establishment of the diseases (Socransky and Haffajee, 1992). The severity of the disease correlates with a variety of other factors including oral hygiene, smoking, general systemic health, genetics disposition and several prescription medications. Symptoms include gingival bleeding, halitosis and the loosening of teeth resulting in tooth loss (Coventry *et al.,* 2000).

2.1.1 Pathogenesis of periodontal disease

The accumulation of virulent oral pathogens such as *Porphyromonas gingivalis*, *Bacteroides forsythus* and *Actinobacillus actinomycetemcomitans*, and their respective by-products (eg amines, fatty acids, sulphur compounds) irritate the gums and promote an inflammatory response (Wilson and Kornman, 2003). The gums detach from teeth thereby forming "pockets" that are further colonized by bacteria. This results in the entry of bacterial components including lipopolysaccharide (LPS) which promotes the recruitment of neutrophils, macrophages and lymphocytes which, in the prolonged presence of bacteria, enhance the inflammatory response, have an adverse effect on the periodontium including inducing connective tissue destruction, the breakdown of bone and tooth loss (Hausmann *et al.,* 1970).

2.2. Association between periodontal disease and cardiovascular disease

There is increasing interest in the relationship between oral health status and systemic health. Poor oral health has been correlated with the onset of diabetes, low birth weight babies, obesity and, of particular interest, the development of various forms of cardiovascular disease (Hollister and Weintraub, 1993; Tonetii and Claffey, 2005). As recently reviewed there is a positive correlation between poor dental health, notably periodontitis, and the development of cardiovascular disease (Persson and Persson 2008). Vettore and colleagues reported that individuals with periodontal disease had 19% increased chance of developing cardiovascular disease, and this risk rose to 44% in individuals 65 years of age or older (Vettore *et al.*, 2003).

The underlying mechanism is unclear, however low-level bacteraemias, such as those associated with periodontitis, initiate host responses altering coagulability, endothelial and vessel wall integrity and platelet function, and subsequently result in atherosclerotic and thrombotic events (Silver *et al.*, 1977; Iwai, 2009). Several cytokines associated with periodontitis, such as IL-1β and TNFα, and a variety of growth factors are known to be highly atherogenic (Mealey, 1991).

2.3. Platelets

Platelets are small anucleate cells, derived from precursor megakaryocyte in the bone marrow, which exist in circulation for 7-10 days and are removed by the spleen. At rest, platelets have a biconcave disc appearance with an equatorial diameter of 2-3µm (Figure 2.1).

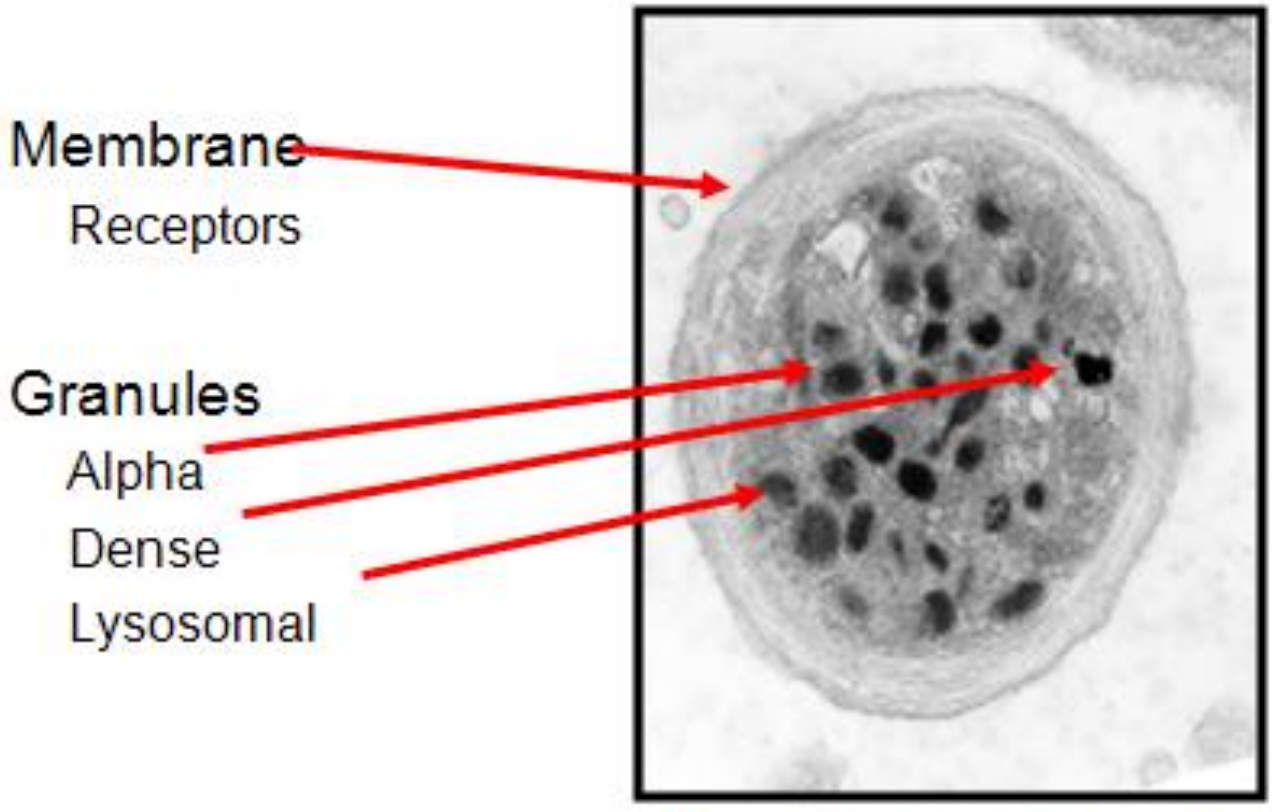


Figure 2.1. Electron microscope image of a platelet. The clearly defined platelet membrane is visible with the intracellular granules clearly outlined within the platelet.

Source:cytoplasm.http://www.umanitoba.ca/institutes/manitoba_institute_cell_biology/MICB/israels_sarahtm

2.3.1. Platelet membrane

The exterior of the platelet includes the glycocalyx above the typical plasma membrane bilayer. The membrane consists of an asymmetric distribution of phospholipids with phosphatidylcholine present equally on both sides. At rest sphingomyelin is found in greater proportions in the outer layer and phosphatidylserine primarily on the inner leaflet (Shattil and Cooper, 1978). The plasma membrane has a series of invaginations, the open canalicular system (OCS), which increases the surface area of the membrane and contributes to the expansion and spreading of the platelet upon activation.

Inherent in the membrane are receptors which are integral to platelet function. These include receptors for adhesive proteins and receptors for soluble platelet agonists.

2.3.2 Platelet cytoskeleton

Immediately distal to the plasma membrane is a microtubule coil which maintains the discoid shape of the resting platelet (Michelson, 2007). Platelets have up to 800 000 actin molecules that combine to create 2000-5000 actin polymers. Upon activation, actin interacts with myosin to mediate the change in shape outlined below (Fox, 2001). A number of signalling proteins are also incorporated into the cytoskeleton following platelet activation, including Rap 1b and members of the MAP kinase family (Fischer *et al.*, 1994; Nadal *et al.*, 1997).

2.3.3 Dense tubular system (DTS)

The DTS is an internal structure that has a similar function to the smooth endoplasmic reticulum in other cells. It accumulates Ca^{2+} by an ATPase and is the major Ca^{2+}-storing organelle in platelets (Cutler *et al.,* 1978). Receptors for inositol 1,4,5-triphosphate (IP_3) are located on the DTS membrane which when engaged mobilize Ca^{2+} (Miyakawa *et al.,* 1999). Additionally the DTS is the site of thromboxane synthetase activity, leading to the generation of thromboxane A_2 (TxA_2) (Gerrard *et al.,* 1976).

2.3.4 Platelet granules

Platelets have three type of secretory granules; alpha granules, dense granules and lysosomal granules.

2.3.4.1 Alpha granules

Alpha granules are the most numerous organelles in platelets with an average of 80 per platelet. These granules range from 200 to 500 nm in diameter. Alpha granules contain, and secrete, a large number of adhesion molecules, chemokines, immunological proteins, coagulation factors and protease inhibitors (Cramer *et al.*, 1994). Biosynthesis of these proteins occurs at the megakaryocyte stage (Grinstein and Furuya, 1983).

The composition of the alpha granule membrane is similar to that of the plasma membrane with the result that the surface expression of several proteins with functional significance is up regulated following exocytosis. Of particular importance are the adhesive receptors αIIbβ3 (Gogstad *et al.,* 1981), GPIb/V/IX (Berger *et al.,* 1996), P-selectin (Koedam *et*

al., 1992), GMP-140 (Stenberg *et al.,* 1985) and PECAM-1 (Cramer *et al.,* 1994). In addition to these integral proteins, alpha granules membranes contains a Mg^{2+} dependant ATPase proton pump which maintains a low intra-granule pH crucial for the process of exocytosis (Grinstein and Furuya, 1983).

2.3.4.2 Lysosomal granules

Lysosomal granules contain hydrolytic enzymes and proteins. Proportionately platelets possess more lysosomal enzymes than other cells (McNicol and Gerrard, 1997). Unlike alpha and dense granules, these granules do not secrete all of their contents, but rather secrete only 30-60% of their hydrolases, selectively releasing different proportions (Holmsen, 1994).

The platelet lysosomal granule membrane contains a Mg^{2+} ATPase proton pump which maintains the acidic intragranular environment (Polasek, 1989). In addition these membranes contain LAMP 1, LAMP2 and CD63 which, following platelet activation, are expressed on the surface (Michelson, 2007). The precise role of these proteins has not yet been determined, however, they may promote the adhesive capability of platelets (Febbraio and Silverstein, 1990).

2.3.4.3 Dense granules

Platelets contain 3-8 dense granules per platelet, are 250-300 nm in diameter (Holmsen, 1985; McNicol and Israels, 1999) and contain a variety of ions, nucleotides and amines.

A variety of proteins are inherent in the dense granule membrane, including a H^{+} ion ATPase responsible for serotonin uptake (Fishkes and Rudnick 1982), P-selectin, CD63 and

LAMP-2 (Michelson, 2007). Many of these proteins are expressed on the platelet surface following exocytosis (Israels and Gerrard 1996).

α-granules	Dense granules	Lysosomal granules
vWF	Serotonin	Cathepsin D
von Willebrand antigen II	ATP	Cathepsin E
Thrombospondin	ADP	Carboxypeptidae A
C1 inhibitor	Calcium	Carboxypeptidase B
Endothelial cell growth factor		Acid phosphatase
Albumin		Arylsulphtase
Fibrinogen		Proline
Fibronectin		Acid phosphatise
Factor V		β-D-glururonidase
IgG, IgA, IgM		β-D-galactosidase
Endothelial cell growth factor		β-D-fucosidase
Plasminogen		β-D-glucosidase
Platelet derived collagenase inhibitor		β-N-acetyl-D-hexosaminidase
Tissue factor pathway inhibitor		α-D-glucosidase
Histidien rich glycoprotein		α-L-fucosidase
Vitronectin		α-D-mannosidase
Osteonectin		α-L-arabinofuranosidase
Protein S		
Interleukin-1 β		

Table 2.1. Platelet granule contents. (Sources: Harrison and Cramer, 1993; McNicol and Israels, 1999; van Oost, 1986).

2.4 Platelets in haemostasis

The primary function of platelets is haemostasis. The dynamics of blood flow dictate that platelets are found at the periphery of blood vessels, thereby facilitating a fast response to vessel damage. Damage to blood vessels exposes platelets to the subendothelial matrix which initiates a cascade of events leading platelet clot formation and the termination of bleeding.

Under high shear conditions, the initial adhesion occurs by the interaction of von Willebrand factor (vWF) with the platelet GPIb/IX/V. Under low shear conditions however, exposed collagen will also bind directly to the platelet via the α2β1 integrin. Subsequent to both adhesive events, collagen binds to GPVI and CD36 and stimulates platelets (Moog *et al.*, 2001). Once stimulated, platelets change from a round discoid shape with extended pseudopods, exocytosis and TxA_2 generation occurs, there is the expression of a procoaggulant surface and a variety of adhesive receptors are exposed.

2.4.1 Platelet agonists

2.4.1.1 Collagen

Collagen is the most thrombogenic component located in the subendothelium (Baumgartner and Haudenschild, 1972). Upon vascular damage, collagen is exposed to the blood where it causes platelet activation (Poole and Watson, 1995; Morton *et al.*, 1989; Cowan, 1981). Platelets contain several collagen receptors, integrin α2β1, which promotes adhesion, GPVI, which contributes to the activation of platelets (Morton *et al.*, 1989; Santoro *et al.*, 1991), and CD36 also promotes adhesion (Tandon *et al.*, 1989). Platelet activation by high concentrations of collagen is mediated through GPVI/FcRγ-chain complex leading to the

formation of the LAT signalosome - a complex of signalling enzymes such as tyrosine kinases, phosphatidylinositol-3-kinase (PI-3-K) and PLCγ2 (see section 5.2) (Rittenhouse and Allen, 1982; Watson *et al.*, 1985).

2.4.1.2 von Willebrand factor (vWF)

von Willebrand factor (vWF) is a multimeric glycoprotein released from endothelial cells and platelets following vessel injury. As outlined above, vWF mediates the attachment between platelets and vessel walls. The absence of, or mutations in vWF, the heterogenic condition known as von Willebrand Disease results in bleeding disorders (Kao *et al.*, 1979).

The vWF receptor is GPIb/IX/V which is expressed on the platelet surface at numbers near 25 000 per platelet. It is composed of four transmembrane regions; GPIbα, which is linked to GPIbβ by a disulfide bond as well as the covalently attached GPIX and GPV. GPIb forms a 1:1 complex with GPIX. Once formed, this complex is capable of mediating ligand binding and signalling functions (Canobbio *et al.*, 2004; Cunningham *et al.*, 1996).

AN51 is a monoclonal antibody that competitively prevents the binding of vWF to GPIb/IX/V (Ruan *et al.*, 2008). However AN51, does not inhibit platelet aggregation in response to ADP, collagen type I or type III, thrombin or AA (Ruan *et al.*, 1987).

GPIb/IX/V elicits a signalling pathway although the precise mechanism(s) involved is unclear (Rivera *et al.* 2009; Gibbins 2004). However protein tyrosine kinases, such as Src, Fyn, Lyn and SYK along with PLCγ2, have each been implicated (Gibbins 2004).

2.4.1.3 Thrombin

The serine protease thrombin is the final product of the coagulation cascade where it acts to convert fibrinogen into fibrin (Turgeon and Houenou, 1997); it is also the most potent platelet stimuli (Fenton *et al.,* 1979). Platelets express three thrombin receptors, GPIb, PAR1 and PAR4 (Brass, 2003). PAR1 and PAR4 are prototypical protease activated receptors where thrombin cleaves the amino terminal portion of the receptor to generate a novel terminal which autostimulates the receptor (Brass, 2003). PAR1 and PAR4 are both G-protein coupled-receptors which lead to the activation of phospholipase Cβ (see section 5.1) (Brass, 2003). Under certain conditions GPIb may enhance the signaling through the PAR1 receptor (Han *et al.,* 2003).

2.4.1.4 ADP/ATP

ADP is released from dense granules upon platelet activation where it acts to propagate platelet aggregation in a positive feedback mechanism. ADP-induced platelet activation is mediated by two G protein-coupled receptors $P2Y_1$ and $P2Y_{12}$; the former is linked to activation of PLCβ and the latter to the inhibition of adenylate cyclase (Kunapuli *et al.,* 2003; Jantzen *et al.,* 2001). The importance of ADP to platelet function is demonstrated by the clinical use of the $P2Y_{12}$ antagonist clopidogrel (Plavix®).

ATP is also released from platelets $P2X_1$ the ligand-gated Ca^{2+} channels leading to an increase in cytosolic free Ca^{2+} levels ($[Ca^{2+}]_I$) (Rolf *et al.,* 2001; Toth-Zsamboki *et al.,* 2003). This effect is critical for agonist-induced shape change (section 4.2.1) and it also enhances other platelet responses (Oury *et al.,* 2002; Toth-Zsamboki *et al.,* 2003).

2.4.1.5 Thromboxane A_2

TxA_2 is synthesized and released from platelets and acts as an agonist which amplifies the response of platelets at the site of injury. On the platelet surface TxA_2 binds to the two different receptor subtypes; the Gq-coupled receptor, TPα and the Gi-coupled TPβ receptor associated with PLCβ activation (see section 5.1) (Narumiya *et al.*, 1993). Aspirin is an inhibitor of COX1 activity in platelets which inhibits TxA_2 synthesis and this accounts for the prophylactic antithrombotic action of low dose aspirin preparations (Marnett *et al.*, 1999).

2.4.1.6 Immunoglobulin G (IgG)

IgG is an antibody that contains four peptide chains, two of which are classified as heavy chains and two of which are classified as light chains. IgG also has two antigen binding sites. Once bound by its Fc region to the platelet Fc receptor, FcγRIIA dimerizes resulting in the events leading to platelet activation.

Platelets have a low affinity IgG receptor FcγRIIA (Anderson *et al.*, 1995). Studies carried out by Pampolina and McNicol (2005) have shown that in the presence of IgG, *S. sanguis* 2017-78 induced the tyrosine phosphorylation of FcγRIIA 30s after stimulation which also resulted in the phosphorylation of PLCγ2, LAT and Syk.

Other studies looking at the indirect phosphorylation of the IgG receptor have documented that upon the binding of vWF to GPIb/IX/V, FcγRIIA also undergoes tyrosine phosphorylation (Canobbio *et al.*, 2001). Currently, the explanation for this may rely on the close physical proximity between these two receptors as a current measurement through

fluorescent energy transfer studies indicates that they are less than 10nm apart (Sullam *et al.,* 1998; Sun *et al.,* 1999).

2.4.2 Platelet responses

2.4.2.1 Platelet shape change

Upon activation by most agonists, platelets undergo a change in shape from a discoid to a spherical appearance, with the extension of filopodia (Paul *et al.,* 1999). The mechanism involves the agonist-induced increase $[Ca^{2+}]_I$, activation of a Ca^{2+}-dependent myosin light chain kinase, phosphorylation of myosin and its consequent interaction with actin. This modification in cytoskeletal proteins is responsible for the conversion from its resting disc shape into spheres with extended filopodia (Daniel *et al.,* 1984). Antonikov and colleagues demonstrated that shape change is not always associated with an aggregation response, however is capable of occurring in the absence of aggregation (Antonikov *et al* 1981).

2.4.2.2 Exocytosis

Platelet activation leads to cytoskeletal rearrangement, centralization of granules and subsequent exocytosis of granular contents (Gordon, 1976). There are two potential mechanisms underlying exocytosis; firstly a model where individual granules fuse to the plasma membrane prior to secretion, or alternatively, a model where granules fuse together and then to the membrane (Reed *et al.,* 2000). Regardless, the exocytotic process leads to the release of granular contents and the expression of granular membrane proteins, such as P-selectin and αIIbβ3 on the surface of activated platelets (Reed *et al.,* 2000).

2.4.2.3 TxA_2 synthesis

Arachidonic acid (AA) is released from membrane phospholipids by phospholipase A_2 (PLA_2) and is converted, by cyclooxygenase 1 (COX1), to the prostaglandin endoperoxides PGG_2 and PGH_2, which in turn is converted by thromboxane synthetase to TxA_2. TxA_2 and PGH_2 are platelet agonists however both have extremely short half lives (Armstrong, 1996).

2.4.2.4 Procoagulant surface expression

Once platelets become activated, the external leaflet of their membrane has an increased expression of the negatively charged phospholipid phosphatidylserine. This phosphatidylserine enriched membrane provides a surface for coagulation to proceed, leading to thrombin generation (Sims *et al.,* 2001). Concurrent with this is the shedding of phosphatidylserine-enriched microparticles, which similarly contributes a procoaggulant surface. The consequence of this is the formation of a hemostatic plug (McNicol *et al.,* 1993)

2.4.2.5 Aggregation

Platelet activation is associated with the activation of the platelet fibrinogen receptor, αIIbβ3. In the resting platelet αIIbβ3 is present on the plasma membrane in a latent form. Once activated αIIbβ3 undergoes a conformational change allowing it to bind circulating fibrinogen. Fibrinogen is a large divalent protein released from endothelial cells and platelets which binds to platelets, thus facilitating the formation of a platelet aggregate (Barkalow *et al.,* 2003; Ragab *et al.,* 2003; Cooney *et al.,* 2001). Platelet signalling events can be divided into 1) inside out signalling; signals from agonist bound receptors that result in the activation of the fibrinogen

receptor, or 2) outside in signalling; signals resulting from the binding of fibrinogen to the platelet.

2.5. Platelet intracellular signalling

Platelet activation initiated by individual agonists acting on their respective receptors is mediated by a wide range of intracellular signalling cascades. However the final outcome is the formation of a stable, effective platelet plug.

2.5.1. Phospholipase C Beta (PLCβ)

Platelet PAR1, PAR4, TPα and $P2Y_1$ are each G protein coupled receptors. In each case the G protein involved is Gq which is associated with PLCβ. There are multiple forms of PLCβ of which PLCβ2 is the most predominant isoform in platelets (Lee *et al.,* 1996). Engagement of these receptors results in the dissociation of the α subunit of Gq which activates PLCβ which in turn hydrolyses the relatively minor membrane phospholipid component phosphatidylinositol 4,5-bisphosphate (PIP_2) to generate two second messengers, IP_3 and DAG (see sections 5.3 and 5.4) (Lian *et al.*, 2005)

2.5.2. Phospholipase C Gamma 2 (PLCγ2)

Engagement of either the GPVI/FcRγ-chain complex by collagen or FcγRIIA by IgG leads to the Syk-mediated phosphotyrosine phosphorylation of Src homology-2(SH2) domains on the adaptor protein LAT. Several signalling molecules recognize, and bind to, the phosphorylated SH2 domain, notably phosphatidylinositol-3-kinase and PLCγ2. Once bound to

LAT, PLCγ2 is subjected to phosphotyrosine phosphorylation which activates the enzyme (Barkalow *et al.,* 2003; Ragab *et al.,* 2003; Cooney *et al.,* 2001). As outlined above for PLCβ, PLCγ2 converts PIP_2 to IP_3 and DAG (Abrams 2005, Fitzgerald *et al.,* 2006)

2.5.3. IP_3 and calcium

IP_3, generated by the actions of PLCβ or PLCγ2, binds to specific receptors on the DTS to release Ca^{2+} from these stores thereby elevating $[Ca^{2+}]_I$. This elevated $[Ca^{2+}]_I$ in turn activates several enzymes, notably myosin light chain kinase, which subsequently mediate multiple platelet responses, such as shape change, exocytosis and adhesive receptor expression (Ardlie *et al.,* 1986; Roberts and Bose, 2002; Hathaway and Adelstein, 1979).

2.5.4. DAG and Protein Kinase C

Platelets have been reported to contain 14 serine/threonine kinases, of which ten are activated in response to thrombin (Ferrell and Martin, 1988), although most have not been identified. Of those identified, most attention has focused on protein kinase C (PKC) and the mitogen-activated protein kinases (MAPKs; see section 2.5.5).

Two major sub-families of PKC are present in platelets, the conventional (cPKC) forms, which are both DAG- and Ca^{2+}-dependent, and the novel forms (nPKC) which are DAG-dependent but Ca^{2+}-independent. Both forms are activated distal to DAG generation and in turn lead to the phosphorylation of multiple proteins, including pleckstrin and protein kinase D (Kroll *et al.,* 1991). Activation of protein kinase C synergises with Ca^{2+} -mediated phosphorylation events leading to the final platelet response (Newton 1995; Nishizuka 1992)

Previous research has shown the activation of the platelet fibrinogen receptor through PKC pathways (Shattil *et al.,* 1998; van Willegen and Akkerman, 1991; Hers *et al.,* 1998; van Willegen and Akkerman, 1992, Watson *et al.,* 1988; Quinton *et al.,* 2002). Additional evidence supporting this proposal is through data showing that the inhibition of PKC through the use of staurosporine resulted in the absence of αIIbβ3 activation (Shattil *et al.,* 1992; Trybulec *et al.,* 1993)

2.5.5. MAP kinases

In common with other cells, platelet stimulation results in the activation of MAP kinases (Kramer *et al.,* 1995). As outlined in Figure 2.2, MAP kinases exist as cascades that relay extracellular signals to intracellular targets (Pearson *et al.,* 2001). The binding of a ligand to its appropriate receptor leads to the autophosphorylation of tyrosine residues on the receptor. This is followed by the recruitment of adaptor proteins, such as Grb, via SH2 domains to the phosphotyrosine residue of the receptor. Grb is closely associated with Sos, a GTP/GDP exchange protein which increases the rate of dissociation of GDP from the small G protein Ras. This allows GTP to bind to, and activate, Ras. Ras activates Raf (MAPKKK), serine/threonine kinases, which phosphorylate and activate MEK (MAPKK), threonine/tyrosine kinases, which in turn phosphorylate and activate MAPK, serine/threonine kinases (Cobb, 1999).

Four groups of MAP kinases have been studied and described in detail. These include the extracellular signal-regulated kinases (ERKs), c-jun N-terminal or stress-activated protein kinases, ERK5/big MAP kinase 1 (BMK1), and the p38 group of protein kinases (Ono and Han, 1999). Although ERK1 and ERK2 have been implicated in calcium entry (Rosado and Sage,

2001), their role in platelet activation is controversial (McNicol and Jackson 2003; Jackson and McNicol, 2010).

The p38 MAPK family is divided into 4 isoforms, α, β, γ, and δ, where the α and β families share 75% homology with the γ and δ families sharing less homology (Brown *et al.,* 2008). In platelets p38 is activated in response to a variety of agonists including thrombin, TxA_2, ADP and vWF (Begonja *et al.,* 2007; Canobbio *et al.,* 2004). Activation of p38, primarily the α isoform, phosphorylates cytosolic phospholipase A_2 ($cPLA_2$) in response to vWF, high concentrations of collagen and thrombin, resulting in AA release (Kramer *et al.,* 1996). The role of p38 in platelets stimulated by other agonists such as low concentrations of collagen and TxA_2 is unknown (Saklatvala *et al.,* 1996).

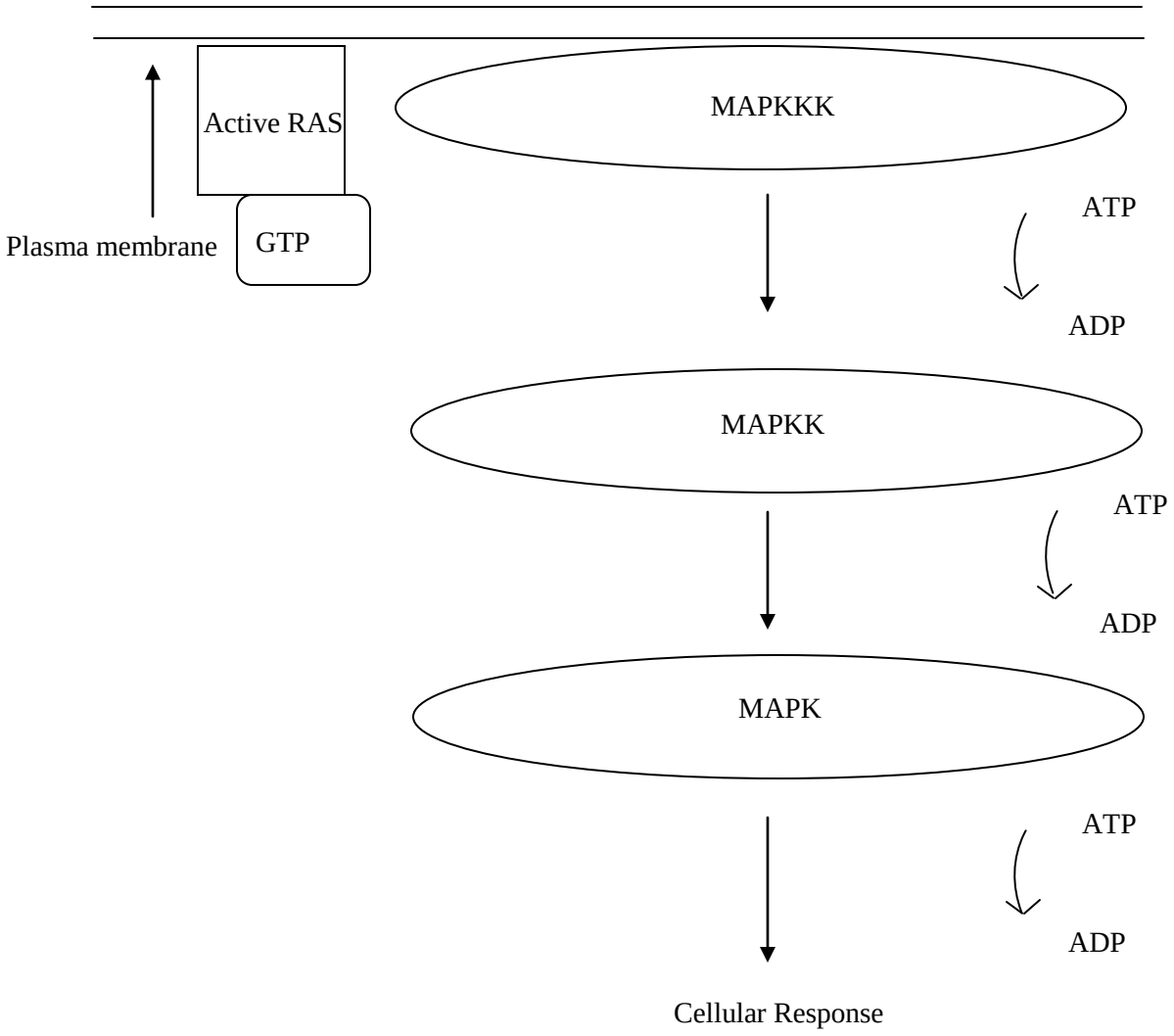


Figure 2.2. MAP kinase intracellular response

The MAP kinase module has 3 components and initiates through the MAPKKK (Raf). Ras recruits Raf to the plasma membrane and stimulates its activation. Through the expenditure of ATP, Raf activates the MAPKK which subsequently activates the final MAPK.

2.5.6. Cytosolic phospholipase A_2 ($cPLA_2$)

Cytosolic phospholipase A_2 ($cPLA_2$) is a Ca^{2+}-dependent 85 kDa enzyme which liberates AA by the hydrolysis of sn-2-acyl bonds of membrane phospholipids (Guy *et al.*, 2009). It possesses a high degree of substrate variability with AA liberated from multiple phospholipids including phosphatidylethanolamine, phosphatidylserine and phosphatidylinositol. Lin and colleagues demonstrated that $cPLA_2$ requires phosphorylation for activity by substituting Ser^{505} with Ala which removed the AA liberating capacity of the enzyme (Lin *et al.*, 1993). In addition this mutant was not phosphorylated by MAPK, suggesting a role for a MAPK in $cPLA_2$ activity (Lin *et al.*, 1993).

Subsequently, as outlined above, Kramer and colleagues reported that in platelets, $cPLA_2$ is a substrate for p38. The p38 inhibitor, SB203580 blocked both $cPLA_2$ phosphorylation and platelet aggregation (Kramer *et al.*, 1996). Additionally, experimental data has shown that SB203580 inhibited the conversion of exogenous AA to TxA_2 (Borsch Haubold *et al.*, 1998). Canobbio and colleagues showed that AA release stimulated by vWF was strongly reduced by inhibition of p38MAPK by SB203580 (Canobbio et *al.*, 2004). A second AA liberating enzyme, diglyceride lipase, is present in platelets (Bell *et al.*, 1979). Although diglyceride lipase is activated by thrombin (Bell *et al.*, 1979), its regulatory mechanisms and relative contribution to AA release, and thus TxA_2 generation, are unknown.

2.5.7. GPIb/IX/V signalling crosstalk

GPIb/IX/V is involved in "signalling crosstalk" with other platelet receptors notably FcγRIIA, the platelet IgG receptor. Studies have suggested a close physical proximity of the two receptors GPIbα and FcγRIIA (Sun *et al.*, 1999; Sullam *et al.*, 1998). Moreover, FcγRIIA and

GPIbα co-immunoprecipitate, suggesting a co-localization on the platelet membrane (Sullam *et al.,* 1998). In fact, these studies have identified that there is a physical proximity of less than 10 nm between FcγRIIA and GPIbα.

GPIb/IX/V contributes to the activation of the platelet fibrinogen receptor, αIIbβ3. According to general consensus, the transitory interaction that takes place between vWF and GPIb/IX/V enables GPIb/IX/V to send signals towards the cell interior resulting in the activation of the fibrinogen receptor (Jackson *et al.,* 2003; Ruggeri *et al.,* 1999)

2.5.8. Platelet endothelial cell adhesion molecule (PECAM)

PECAM-1 is expressed on the surface of circulating platelets and is a 130 kDa member of the immunoglobulin gene family. PECAM-1 functions as an inhibitory receptor that inhibits the actions of activating receptors that have cytoplasmic immunoreceptor tyrosine based activation motifs (ITAMs). The cytoplasmic component of PECAM-1 has 2 immunoreceptor tyrosine based inhibitory motif (ITIMS). When phosphorylated on tyrosine residues, these ITIMS signal for the recruitment of and activate the protein tyrosine phosphatases SHP2 and SHP1 (Newman and Newman, 2003).

2.6. Platelets and Disease

2.6.1. Platelets and thrombosis

Pathologically, it is recognized that platelets play a significant role in acute arterial thrombosis. This is characterized by the inappropriate activation, or over activation of platelets in the circulation. In the former situation platelets become activated with no vascular injury leading to vessel occlusion and in the latter a haemostatic plug is formed but a portion breaks off and

occludes a smaller vessel downstream. Regardless, the result is vascular occlusion, or thrombosis, which leads to clinical conditions such as myocardial infarction and transient ischemic events (Jackson and Clagett, 2001).

Thrombosis is the pathological extension of the normal haemostatic process. The process involves adhesion, exocytosis, TxA_2 generation and aggregation as outlined above. Clinically individuals at risk of thrombotic events are managed by either aspirin or clopidogrel each of which reduce the platelet responsiveness (Myers, 2005).

2.6.2. Platelets and Atherosclerosis

Atherosclerosis is a highly complex progressive cardiovascular disease characterized by an accumulation of lipids in arterial blood vessels (Lusis, 2000). High levels of low density lipoprotein (LDL) diffuse between endothelial cells into the sub-endothelial matrix where oxidation occurs leading to the formation, and trapping, of minimally oxidized LDL. This process activates the overlying endothelial cells which compromises their integrity, produces cytokines and expresses leukocyte adhesive proteins. The leukocytes adhere to, and migrate through, the endothelial layer into the subendothelial matrix where, under the influence of the cytokines they mature into macrophages which engulf LDL to form foam cells. Concurrently macrophage-release cytokines attract smooth muscle cells and eventually, under the influence of cytokines and growth factors, a mature atherosclerotic fibrous plaque is formed with an enlarged calcified necrotic core (Lusis, 2000).

Tissue factor, which is found within the core of the atherosclerotic lesion, initiates the coagulation cascade resulting in thrombin generation which in turn activates platelets and promotes a thrombotic response (Lusis, 2000).

However it is now becoming increasingly evident that platelets play an active role in the development of the atherosclerotic lesion. Multiple studies have shown that platelets, through receptors such as P-selectin and CD40L, bind to activated endothelial cells (Henn *et al.,* 1998; Slupsky *et al.,* 1998; Lindmark *et al.,* 2000). This then stimulates the release of chemokines, cytokines and growth from alpha granules, notably of RANTES, which is a major contributing factor to the vascular inflammation associated with atherosclerosis (Libby and Simon, 2001).

2.6.3. Platelets and other inflammatory conditions

In addition to atherosclerosis, platelets have been implicated in the development of several other inflammatory conditions. These include inflammatory bowel disease, rheumatoid arthritis, psoriasis, migraine and inflammatory pulmonary disease (Andoh *et al.,* 2006; Sarchielli and Gallai, 2001; Wang *et al.,* 2007). Although the evidence for the role of platelets in some of these conditions is relatively weak, it does emphasis the multifunctional nature of platelets in disease processes.

2.7. Oral Bacteria and platelets

The oral cavity contains over 300 bacteria species many of which can elicit platelet aggregation *in vitro* (Herzberg *et al.,* 1983). Traditionally the interaction of orally-derived bacteria with platelets has been associated with the onset and progression of bacterial endocarditis, however there is increasing evidence for a role for both gram positive and gram negative organisms in acute thrombotic disorders (van Gorp *et al.,* 1999)

2.7.1. Platelets and Endocarditis

Endocaridits is an infection of heart valves and the endocardium (Sandre and Shafran, 1996) which can lead to complications such as paravalvular abscess formation, congestive heart failure and embolic stroke (Sexton and Spelman, 2003).

Streptococci, staphylococci and enterococci are the most commonly isolated organisms from endocarditic lesions (Johnson, 1993) and these organisms often gain entry to the circulation as the result of oral, gastrointestinal and genitourinary surgical procedures (Steckelberg and Wilson, 1993). In addition to these procedures, most of the cases of endocarditis are caused by the chronic inflammation, resulting from periodontal disease (Lockhart, 2000).

Streptococci have surface adhesins that enable them to bind with platelets. For example, *S. sanguis* produces a surface polysaccharide that promotes the interaction with fibrin-platelet clots *in vitro* (Scheld *et al.,* 1978). The inactivation of fibronectin binding activity in *S. sanguis* reduces the ability of *S. sanguis* to promote the progression of endocarditis in rats (Lowrance *et al* 1990; Curley *et al* 1995).

Subsequent to colonization by these bacteria, platelets and fibrin form deposits over the colonies thereby preventing these bacteria from becoming targets of the immune response. In addition an aggregating strain of *S. sanguis* produced larger lesions in rabbits than a non-aggregating strain of *S. sanguis* (Herzberg *et al* 1992).

2.7.2. Oral bacteria induced platelet aggregation

Platelets can be activated by several different bacterial species implying a thrombogenic ability of these organisms, however only the proposed mechanisms of platelet activation for *S. mutans*, *P. gingivalis* and *S. aureus* will be discussed.

S. mutans, a gram positive member of the viridans group of streptococci, is often isolated from endocarditis lesions. Certain strains of this species are able to stimulate activation and aggregation in rabbit platelets. *S. mutans*-induced aggregation is dependent on plasma protein including IgG (Chia *et al.*, 2004). Additionally, *S. mutans* adhesion to, and aggregation of, platelets is mediated by cell wall polysaccharides (Chia *et al.*, 2004).

P. gingivalis is a gram negative periopathogen certain strains of which interact with platelets. *P. gingivalis* expresses fimbriae that are believed to bind to currently undefined platelet receptors or directly to fibrinogen (Sharma *et al.*, 2000). It has further been speculated that these fimbriae may also trap *P. gingivalis* membrane vesicles and concentrate them near the platelet (Herzberg *et al.*, 1994; Sharma *et al.*, 2000). Certain strains of *P. gingivalis* can induce platelet aggregation releasing R-gingipains, cysteine proteases that activate platelet thrombin receptors PAR1 and PAR4 (Lourbakos *et al.*, 2001; Pham *et al.*, 2002).

S. aureus is a gram positive bacterium often implicated in endovascular infection and infective endocarditis (Moreillon *et al.*, 2002). Platelet-activating strains of *S. aureus* express surface proteins which interact with extracellular matrix molecules, such as fibrinogen and vWF, as well as platelet receptors. *S. aureus*-induced platelet activation is similar to that of *S. sanguis* induced platelet activation, however it has been shown to be independent of platelet secretions

since studies by O'Brien *et al* (2002) have shown that the ADP hydrolase apyrase had no effect on *S. aureus* induced aggregation.

O'Brien *et al* (2002) have identified surface proteins on *S. aureus* that contribute to platelet activation. Specifically, protein A expressed on the *S. aureus* surface binds to the Fc component of IgG (O'Brien *et al.,* 2002). In contrast, vWF does not induce aggregation independently (Hartlieb *et al.,* 2000), but rather amplifies the aggregation response in the presence of additional bacterial surface proteins (O'Brien *et al.,* 2002).

2.7.3. *S. sanguis*-induced platelet activation

S. sanguis is a gram positive facultative coccus residing in the oral cavity, commonly found within dental plaque (Douglas *et al.,* 1993).

Herzberg and colleagues initially isolated 78 *S. sanguis* strains from patients with endocarditis and reported that 76 of these strains caused platelet aggregation (Herzberg *et al.,* 1983). Subsequently a rhamnose-rich glycoprotein, termed Platelet-Aggregation-Associated Protein (PAAP), with a platelet-interacting peptide sequence of Pro-Gly-Glu-Gln-Gly-Pro-Lys (Erickson and Herzberg 1993) has been implicated in platelet aggregation. This sequence is similar to a collagen octapeptide region, Lys-Pro-Gly-Glu-Pro-Gly-Pro-Lys that is required for platelet aggregation (Legrand *et al.,* 1980; Gong *et al.,* 1995). PAAP, which interacts with platelet membrane proteins of 175 kDa and 230 kDa (Gong *et al*., 1995) initiates the binding of *S. sanguis* to platelets as well as inducing platelet aggregation (Gong *et al.,* 1995).

Not all strains of *S. sanguis* induce platelet aggregation. Kerrigan and colleagues (Kerrigan *et al.,* 2002) have suggested that *S. sanguis* strains can be divided into three types

based on their relative capacity to activate platelets *in vitro*. Under this classification Type I strains induce platelet aggregation with a short delay, Type II strains have a longer delay and Type III strains do not induce platelet aggregation (Kerrigan *et al.,* 2002).

The platelet receptors for *S. sanguis* have also been partially identified. Type I *S. sanguis* strains directly interact with the N terminal of GPIb/IX/V (Kerrigan *et al.,* 2002). Kerrigan and colleagues reported that the anti-GPIb antibody AN51 inhibited platelet aggregation by all strains of *S. sanguis* (Kerrigan *et al.,* 2002). Therefore GPIb/IX/V has been implicated as the possible receptor mediating activation. vWF promotes platelet adhesion via interactions with collagen and GPIb/IX/V on the platelet surface (McNicol *et al.,* 2007). Research carried out by McNicol *et al* (2007) observed that the platelets from a type 3 von Willebrand disease (vWD) patient did not respond to *S. sanguis* 2017-78. Additionally, they re-suspended the platelets from a normal individual in the vWD patient plasma and observed that these platelets did not respond to *S. sanguis* 2017-78. Their results demonstrate that vWF is involved in 2017-78 induced aggregation.

S. sanguis also requires the interaction of plasma IgG with the platelet FcγRIIa in order to elicit a full aggregation response (Pampolina and McNicol, 2005). The removal of IgG from plasma, as well as the prevention of IgG binding to FcγRIIa, inhibits platelet aggregation in response to some strains of *S. sanguis* (Ford *et al.,* 1997).

S. sanguis-induced platelet activation is a "true activation response" as it includes shape change, granule secretion and TxA_2 release (Ford *et al* 1993). A donor dependent lag phase precedes *S. sanguis*-induced platelet aggregation which is dependent on Ca^{2+}, TxA_2 and ADP (Sullam *et al.,* 1987; Ford *et al,* 1993).

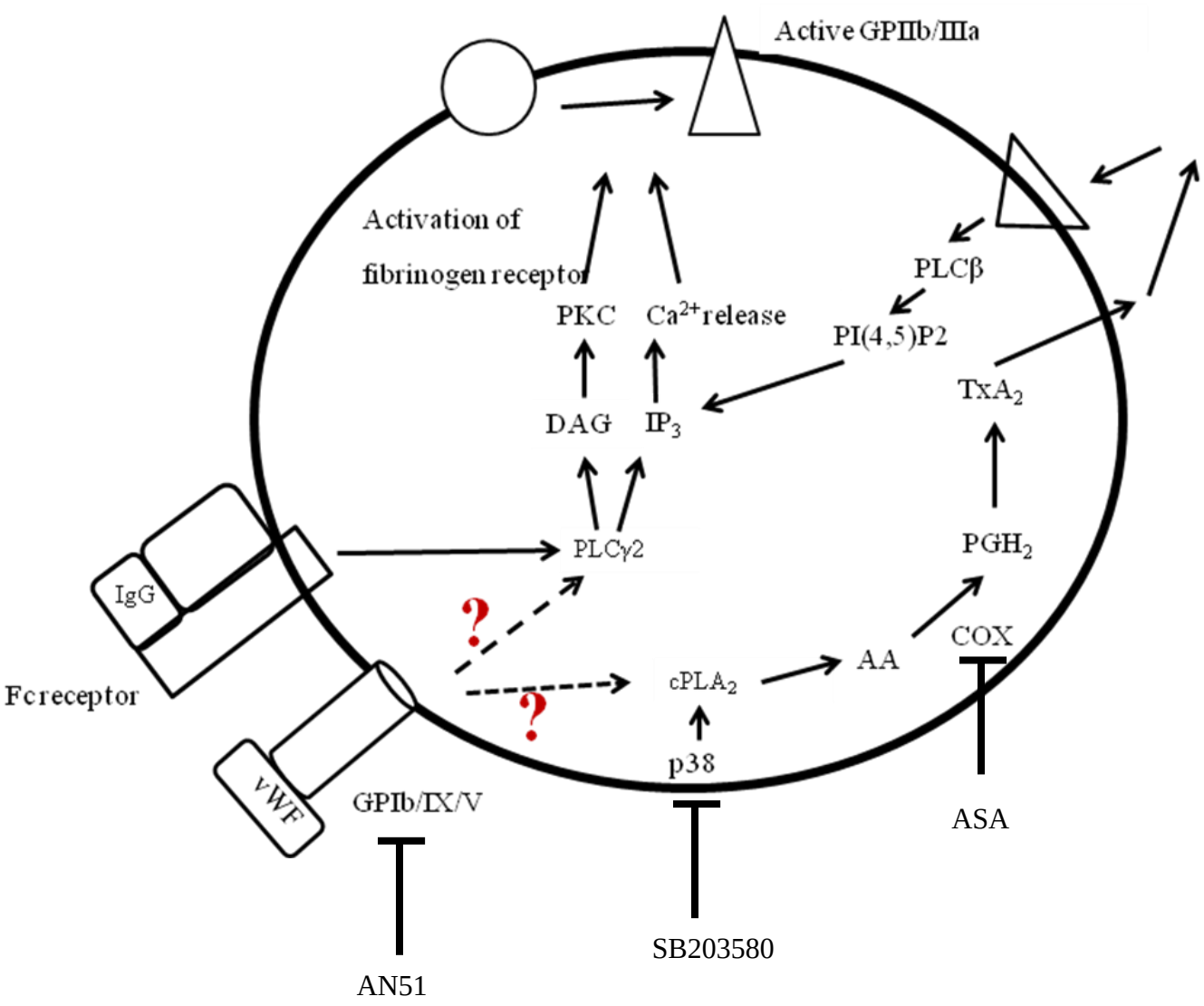


Figure 2.3. Platelet signalling events and *S. sanguis*

Engagement of the Fc receptor, leads to PLCγ2 phosphorylation and subsequent activation pathways. PLCγ2 activation results in the release of inositol tri-phosphate (IP_3) and diacylglycerol (DAG). IP_3 binds to its receptor leading to calcium release and increased intracellular calcium concentrations. DAG activates protein kinase (PKC) which activates downstream events. Cytosolic phospholipase A_2 ($cPLA_2$) cleaves arachidonic acid (AA) from membrane phospholipids. AA is then converted to prostaglandin H_2 (PGH_2), by cyclooxygenase (COX), which is subsequently converted to to thromboxane A_2 (TxA_2). TxA_2 is released from the platelet and acts to promote the formation of a thrombus. Von Willebrand Factor (vWF) binds to GPIb/IX/V, however the downstream pathway requires further elucidation. Inhibitors that interfere with components of this pathway are AN51 (monoclonal anti GPIb antibody), SB203580 (selective p38 kinase inhibitor) and ASA (COX inhibitor).

3. Objectives

The objectives of this research are to further characterize the interactions that occur between *S. sanguis* strain 2017-78 and platelet GPIb/IX/V and additionally, to determine the events that occur after engagement of this receptor. *S. sanguis* strain 2017-78 has been shown previously to bind to, and activate, human platelets (Herzberg *et al.*, 1983). Additionally, this interaction has been characterized as taking place at the N-terminal of GPIb (Kerrigan *et al.*, 2002). This interaction has been shown to stimulate the activation of the p38 MAP kinase and the subsequent activation of $cPLA_2$ (Canobbio *et al.*, 2004). Therefore, the primary hypothesis to be tested is that p38 activation is a crucial component of platelet aggregation and lies upstream of $cPLA_2$. A second project will address the contribution of GPIb towards *S. sanguis* induced platelet aggregation and subsequently, will further clarify the downstream events of GPIb engagement.

4. MATERIALS AND METHODS

4.1. Materials

S. sanguis strain 2017-78 was isolated from an individual with sub-acute bacterial endocarditis (Herzberg *et al.*, 1983) and was a gift provided by Dr. Mark Herzberg (University of Minnesota, Minneapolis, Minnesota). Sheep's blood agar blood plates were purchased from the microbiological laboratories at the Health Sciences Center (Winnipeg, MB). Bovine serum albumin (BSA), Igepal CA-630, sodium orthovanadate, Tween 20, acetyl salicylic acid (ASA), staurosporine, RGDS, wortmanin were purchased from Sigma Aldrich Canada Ltd. (Oakville, On) and SB203580 from Calbiochem (La Jolla, CA). Methanol, hydrochloric acid and sodium citrate were purchased from Fisher Scientific Ltd. (Whitby, ON). ECL western blotting detection agents and Restore Western Blot Stripping Buffer were purchased from Thermo Scientific (Rockford, IL), and protease inhibitor tablets were purchased from Roche Diagnostics Canada (Laval, PQ). Nitrocellulose membranes, TRIS, glycine, 30% acrylamide-bis, temed, sodium dodecyl sulfate (SDS), prestained SDS Page standards and all electrophoresis and immunoblotting supplies were purchased from BioRad Laboratories Ltd. (Missisauga, ON). Protein A and G beads were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Vacutainer needles and syringes were purchased from Becton Dickinson Vacutainer systems (Franklin Lakes, NJ).

4.2. Antibodies

Anti-human platelet glycoprotein Ib antibody (Ab), AN51 was purchased from the DAKO group (Dako, Denmark). The anti-phospho-p38 MAP Kinase (Thr180/Tyr182), anti- p38 MAP kinase and anti-mouse IgG horseradish peroxidase (HRP)-linked Abs were all purchased

from Cell Signalling Technologies (Boston, MA). PLCγ2 and Abs to $cPLA_2$ and $pcPLA_2$ were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-phosphotyrosine Ab 4G10 was purchased from Millipore (Temecula, CA). The anti-rabbit HRP-Linked Ab was purchased from New England Biolabs Inc (Boston, MA).

4.3. Preparation of bacterial suspensions

S. sanguis 2017-78 was grown on blood agar plates (18 hours in candle jars at 37^0C). Bacteria was then harvested and washed twice with 500µL of 1X HEPES-tyrodes buffer. Bacterial cells were sonicated (10 seconds on ice). The absorbance (660 nM) was measured and the bacterial count was measured using a standard curve of absorbance versus bacteria/mL.

4.4. Preparation of Platelet Rich Plasma

These studies were approved by the Research Ethics Board of the University of Manitoba. Blood was drawn, following informed consent, by venipuncture from healthy adults who denied talking medications known to interfere with platelet function within the previous 2 weeks. Blood was drawn into a syringe containing 0.1x volume of 3.8% sodium citrate. The blood was centrifuged at 37 000g for 20 minutes at room temperature to obtain platelet rich plasma (PRP). After centrifugation, PRP was transferred to a vial and equilibrated in the water bath for 30 minutes at 37°C prior to use.

4.5. Platelet Aggregation

Platelets (2.5×10^8 platelets/mL) were stirred (7000 rpm at 37^0C) in a Payton dual channel aggregometer and aggregation monitored continuously as an increase in light

transmission. For studies involving inhibitors, platelets were pre-incubated with the inhibitor for the indicated time prior to the addition of bacteria.

4.6. Immunoprecipitation, SDS-PAGE

Platelet aggregation was measured as described and terminated by the addition of equal volume of stopping buffer. Samples were kept on ice and centrifuged at 8500g for 30 seconds. Pellets were lysed with 2X RIPA (2% Triton X 100, 2% sodium deoxycholate, 0.2% SDS, 316 mM NaCl, 2mM EGTA, 20mM Tris(pH 7.6), 1mM PMSF, 1mM sodium orthovanadate, 1 protease inhibitor tablet) and sonicated for 10 seconds. Samples were incubated for 30 minutes at 4^0C, centrifuged at 12000g for 10 minutes and the supernatant was retained while the pellet was discarded.

For immunoprecipitation studies, the samples were pre-cleared by incubating the lysates at 4^0C for 60 minutes with 30µl of Protein A beads (for PLCγ2). The samples were centrifuged at 5000g for 30 seconds and the supernatant retained.

For PLCγ2, 2.5µg/mL of anti-PLCγ2 rabbit polyclonal Ab was incubated with lysates for 18 hours at 4^0C and 30 µL of agarose beads were added for a further 60 minutes.

The beads were isolated via centrifugation and washed (X3) with 1X RIPA buffer (with 1X RIPA buffer (1% triton X 100, 1% sodium deoxycholate, 0.1 % SDS, 158 mM NaCl, 10 mM Tris (pH 7.2), 1mM PMSF, 1mM sodium orthovanadate). Proteins were eluted by boiling for 10 minutes in 35 µL of 1X reducing buffer and further separated by 10 or 12% SDS-PAGE (180 mV for 80 minutes at room temperature) and transferred to nitrocellulose membranes (100mV for 60 minutes at 4^0C). The membranes were blocked with Western Wash Buffer in 5% bovine serum albumin (BSA) for 4G10 while PLCγ2 was blocked in Tris buffered saline (TBS) containing 0.1% Tween (TBS-T) in 5% BSA for 90 minutes (1:1000 dilution in WWB

containing 3% BSA), anti-PLCγ2 rabbit polyclonal Ab (1:200 dilution in TBS-T containing 5% BSA) for 18 hours at 4^0C respectively.

Each membrane was then stripped for 20 minutes, using Restore Stripping Buffer (Thermo Scientific) and washed for 6x5 minute intervals with (TBS-Tween) and re-probed with the appropriate antibody.

4.7. Immunoblotting

Samples were collected in an identical manner as described in the previous section. Platelet aggregation was terminated by the addition of stopping buffer (120 µL of 500mM EDTA, 100µL of 200 mM sodium orthovanadate, 8.3 µL of staurosporine and 2.77 mL of water). Samples were spun at 8500 g for 30 seconds and the supernatant was discarded. Pellets were re-suspended in 75 µL of 1X RIPA lysis buffer (5 mL of 1X RIPA, 25 µL of PMSF and 25 µL sodium orthovanadate). Samples were sonicated for 10 seconds and boiled for 10 minutes. Proteins were separated by 10 or 12% SDS-PAGE (180 mV for 80 minutes at room temperature) and transferred to nitrocellulose membranes (100mV for 60 minutes at 4^0C). After completion of transfer, the blots were incubated for 90 min at room temperature in 5% skim milk for p38 and 5% BSA for $cPLA_2$. The blots were incubated overnight at 4°C with the primary antibody (1:1000 for anti-p38 and anti-$cPLA_2$), washed for 6x5 minute intervals with TBS-Tween the incubated for 1 hour at room temperature with the secondary antibody (1:2000). The blots were washed again for 6x5 minute intervals and immersed in a detection agent after which blots were exposed and developed on x-ray film. Each membrane was then stripped for 20 minutes, using Restore Stripping Buffer (Thermo Scientific) and washed for 6x5 minute intervals with (TBS-Tween) and re-probed with the appropriate antibody.

4.8. Statistical Analysis

All experimental results were analyzed using one tailed t-tests. Statistical significance was achieved at $p<0.05$.

5. RESULTS

5.1. 2017-78 induced platelet aggregation

S. sanguis has been shown to induce platelet aggregation *in vitro* as well as *in vivo* in a strain- and donor-dependent manner (Herzberg *et al.,* 1983). *S. sanguis* adheres to platelets and stimulates platelet aggregation. Previous studies in the laboratory have suggested roles for both IgG/FcγRIIa and vWF/GPIb/IX/V in platelet aggregation in response to *S. sanguis* 2017-78 (McNicol *et al.,* 2007; McNicol *et al.,* 2006; Pampolina and McNicol 2005). In addition it has been suggested that it is a specific region on the N-terminal domain of GPIbα which mediates the interaction between platelets and *S. sanguis*, resulting in platelet activation (Kerrigan *et al.,* 2002).

Platelets challenged with *S. sanguis* 2017-78 aggregated with a lag phase of (85.4 ± 40.1 sec; n=5), similar to that previously reported (Pampolina and McNicol, 2005) (Figure 5.1). Platelet aggregation was analyzed in 4 stages. The pre aggregation (lag phase) defines the time during which no aggregation occurs, but is a time during which bacteria and platelets are initially combined. The early aggregation phase represents the time during which aggregate formation commences and continues into the mid aggregation phase. The final stage of aggregation is full aggregation, at which time all of the aggregates have formed.

Studies show that *S. sanguis* expresses a protein referred to as PAAP that has homology with collagen (Erickson and Herzberg, 1990). The homology between PAAP and collagen, as well as the capacity of collagen to bind vWF suggest a potential role for vWF in *S. sanguis* induced platelet aggregation.

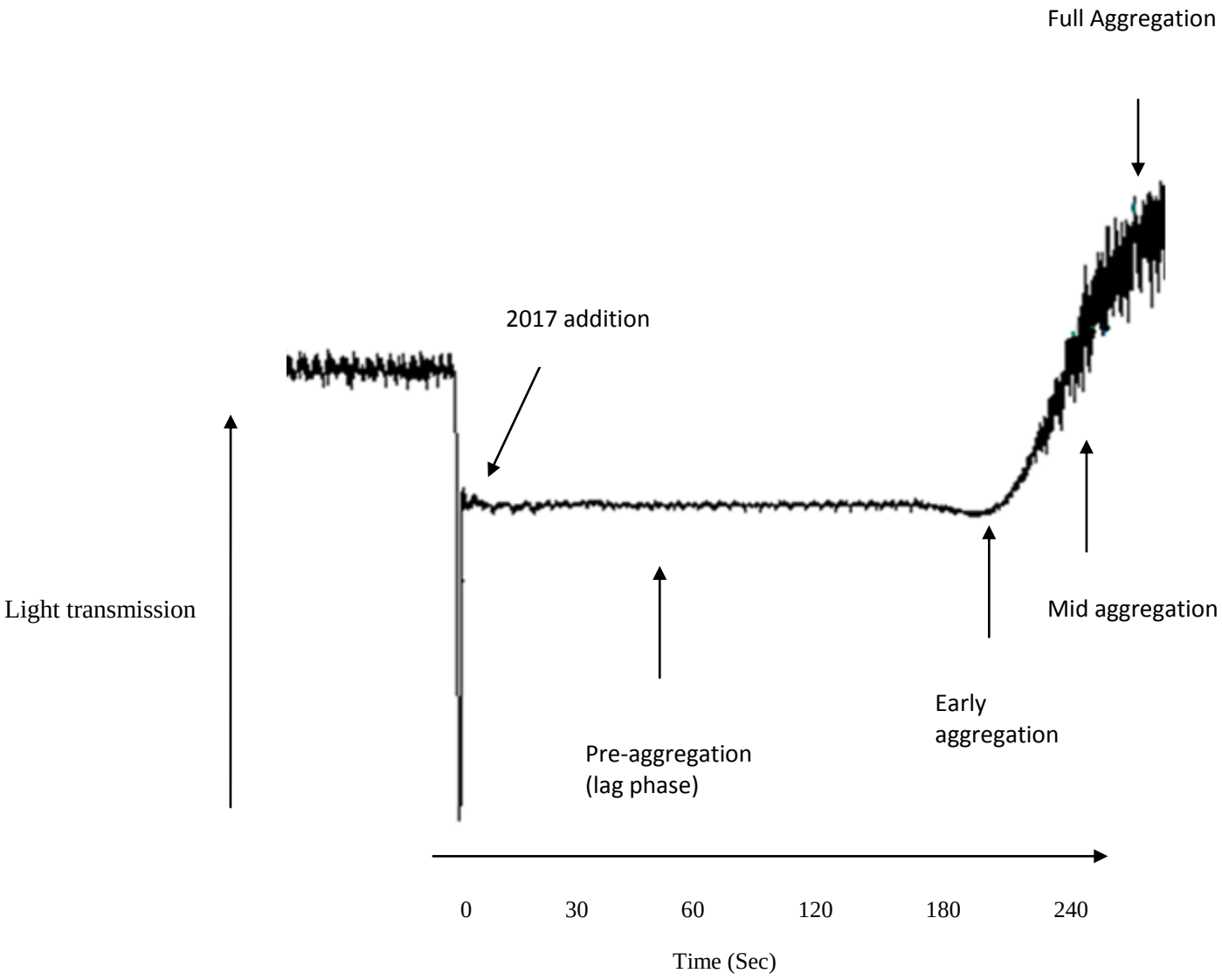


Figure 5.1. *S. sanguis* 2017-78 induced platelet aggregation time points

Platelets were stirred at 37°C prior to the addition of 2017-78. Aggregation was monitored as an increase in light transmission in an aggregometer with continuous stirring.

5.2. Effect of AN51 on platelet aggregation

In order to address the potential role of GPIb/IX/V in *S. sanguis* strain 2017-78-induced activation, the effects of an anti-GPIb Ab, AN51, were examined.

Pre-treatment (0.165 mg/ml; 5mins.) of platelets with AN51 significantly reduced the lag time to *S. sanguis* 2017-78-induced aggregation from 85.4 ± 40.1 seconds to 50.4 ± 19.7 seconds ($p<0.05$; n=5) (Figure 5.2). This differs from previous observations where AN51 totally blocked *S. sanguis*-induced aggregation (Kerrigan *et al.*, 2002). Previous studies have, however shown that aggregation in response to soluble agonists such as ADP, collagen type I or type III, thrombin or arachidonic acid is not inhibited by AN51 (Ruan *et al.*, 1981).

5.3. Effect of AN51 on PLCγ2 phosphorylation

There is increasing evidence that PLCγ2 is tyrosine phosphorylated, and thus activated, in response to the binding of vWF to GPIb/IX/V (Canobbio *et al.*, 2001; Wu *et al.*, 2001; Marshall *et al.*, 2002).

As previously shown (Pampolina and McNicol 2005), *S. sanguis* strain 2017-78 caused the phosphorylation of PLCγ2 (Figure 5.3).

Pre-treatment of platelets with AN51 (0.165 mg/ml; 5mins) significantly reduced this PLCγ2 phosphorylation (Figure 5.3).

Taken together these data are consistent with a complex role for GPIb/IX/V in platelet activation in response to *S. sanguis* strain 2017-78.

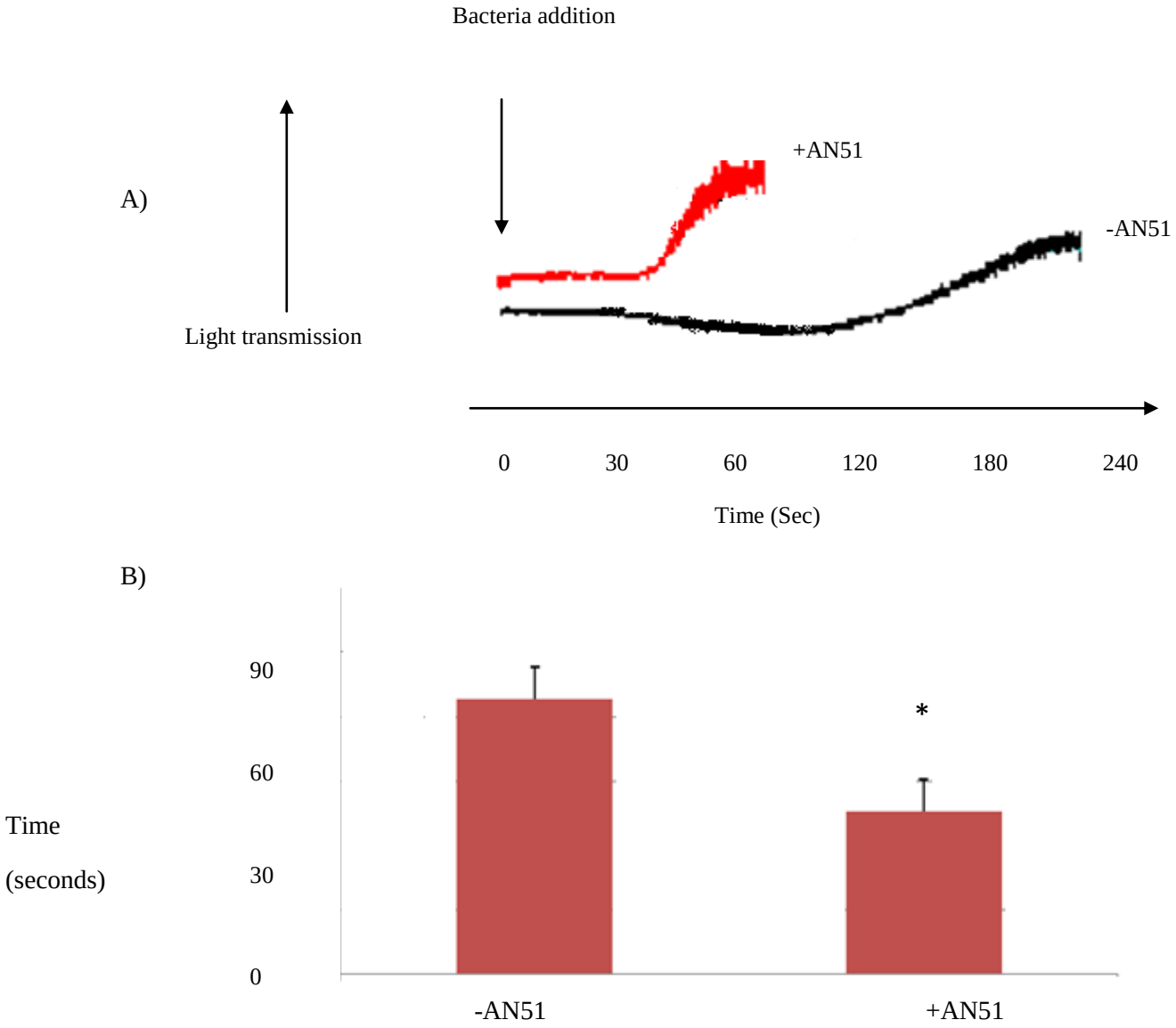


Figure 5.2. Effects of AN51 on *S. sanguis* 2017-78-induced platelet aggregation

A) Platelets were stirred in an aggregometer (37°C; 5mins) in the presence (+) or absence (-) of AN51. Platelets were challenged with *S. sanguis* 2017-78 and aggregation was monitored as an increase in light transmission. Tracing is representative of 5 experiments showing similar results. B) Platelets were stirred in an aggregometer (37°C; 5mins) in the presence (+) or absence (-) of AN51. Platelets were challenged with *S. sanguis* 2017-78 and aggregation was monitored as an increase in light transmission. Lag time was measured and data presented as mean±SEM of 5 experiments. *p <0 .05 compared to control.

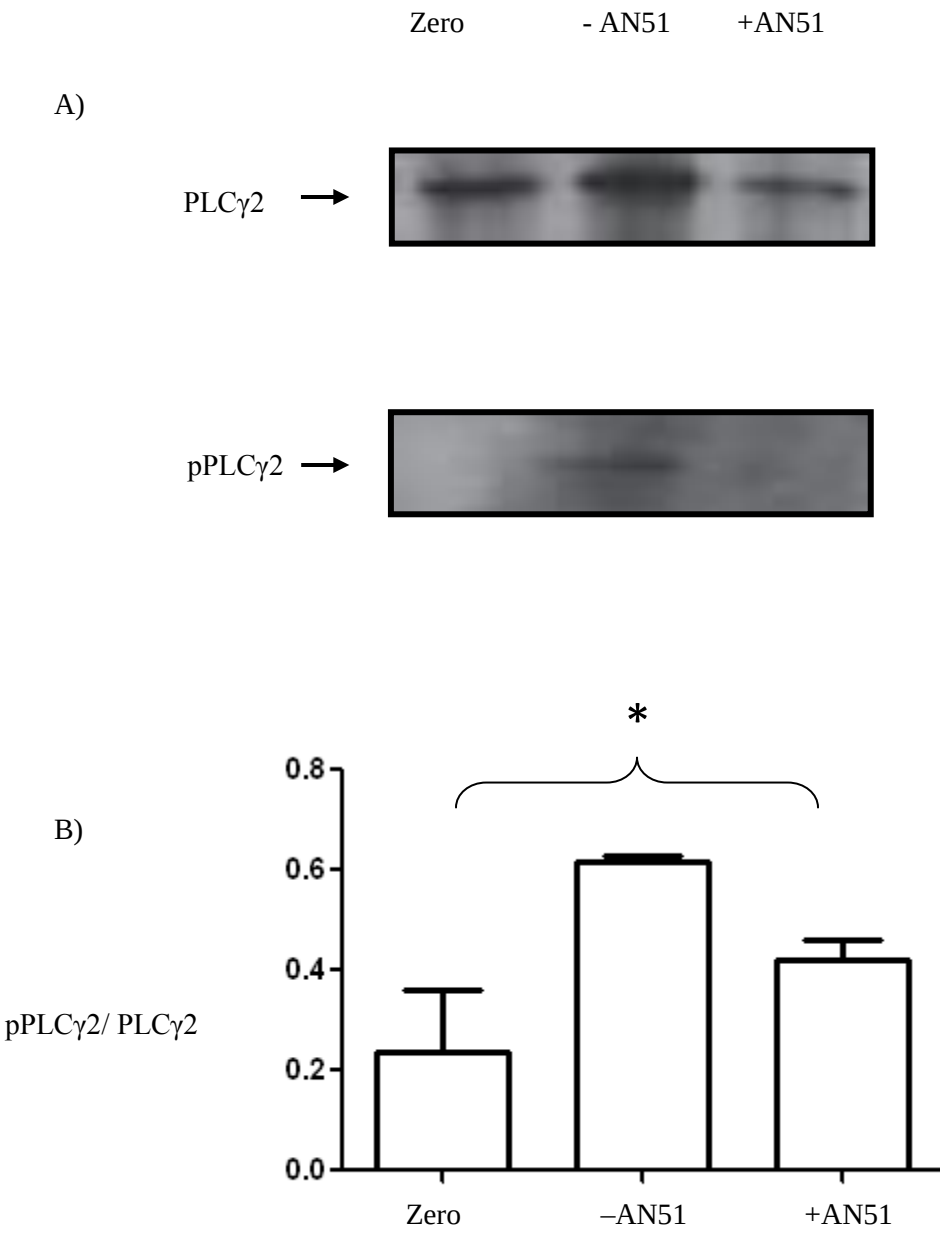


Figure 5.3. Effect of AN51 on PLCγ2 phosphorylation

A) Platelets were stirred in an aggregometer (37°C; 5mins) in the presence (+AN51) or absence (-AN51) of AN51 (0.165 mg/ml, 5 mins). Platelets were challenged with *S. sanguis* 2017-78 and aggregation was monitored as an increase in light transmission. PLCγ2 phosphorylation was detected by 4G10 (lower panel) and visualized by ECL. The blot was stripped and re-probed with an anti- PLCγ2 antibody (PLCγ2; upper panel) and visualized by ECL. PLCγ2 was isolated by immunoprecipitation. Blot is representative of 3 similar experiments B) Immunoblots were subjected to densitometry and results expressed as the phosphoPLCγ2/PLCγ2 ratio. Data presented as mean±SEM of 5 experiments. *p <0 .05 compared to control.

5.4. Effect of *S. Sanguis* 2017-78 on p38 phosphorylation

The activation of platelets by vWF through GPIb/IX/V is associated with p38 activation (Canobbio *et al* 2004). To date there is no information on the activation of p38 in *S. sanguis* 2017-78 induced platelet activation.

S. sanguis 2017-78 induced platelet aggregation was accompanied by the significant phosphorylation of p38 during early and mid aggregation (Figure 5.4). The levels of phosphorylation were not significant at the pre- and full aggregation phase (Figure 5.4). To determine a role for p38, the effects of the inhibitor SB203580 were examined.

5.5. Effect of SB203580 on platelet aggregation by *S. sanguis* 2017-78

Pre-treatment of platelets with SB203580 (10 mins, 6.5µM, n=4) inhibited *S. sanguis* 2017-78 induced platelet aggregation (Figure 5.5). A lower concentration of SB203580 (2 µM) inhibited *S. sanguis* 2017-78 induced platelet aggregation in 3 of 4 donors, consistent with the donor variability previously seen in response to various strains of *S. sanguis* (Herzberg *et al.,* 1983).

5.6. Effect of SB203580 on p38 phosphorylation

Pre-treatment of platelets with SB203580 (10 mins; 2 µM, 6.5 µM) inhibited 2017-78-induced p38 phosphorlyation (Figure 5.6). This is further evidence for a role for p38 in platelet activation by *S. sanguis* strain 2017-78.

Previous studies using soluble agonists such as thrombin and collagen have suggested that one function of p38 is the phosphorylation and activation of $cPLA_2$ (Kramer *et al.,* 1996). Thus the effect of 2017-78 on $cPLA_2$ phosphorylation was examined.

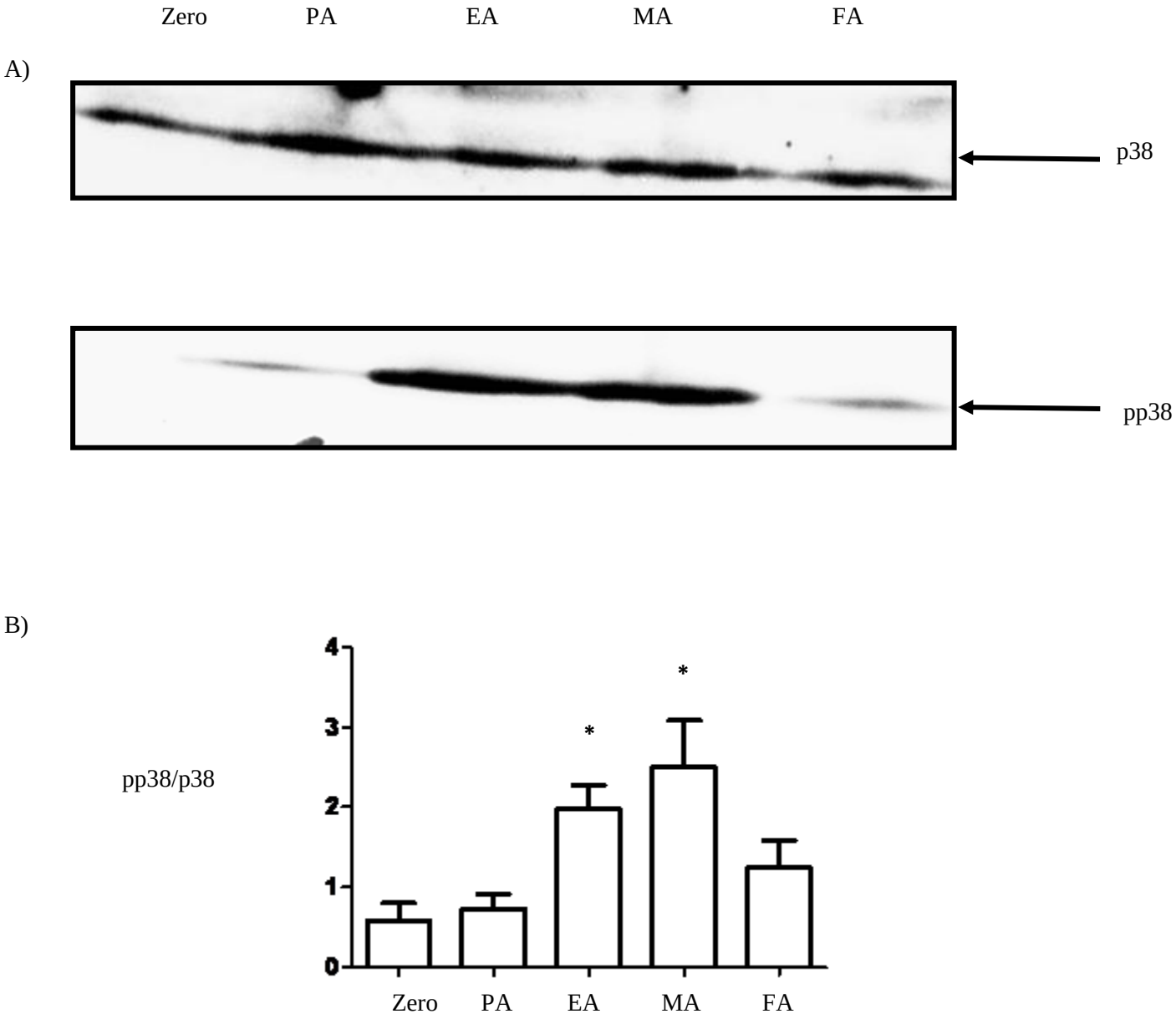


Figure 5.4. Platelet p38 phosphorylation induced by *S. sanguis* 2017-78

A) Platelets were stirred in an aggregometer (37°C). Platelets were challenged with *S. sanguis* 2017-78 and aggregation was monitored as an increase in light transmission. p38 phosphorylation was detected by anti-phosphospecific p38 antibody (pp38; lower panel) and visualized by ECL. The blot was stripped and re-probed with an anti-p38 antibody (p38; upper panel) and visualized by ECL. Blot is representative of 6 similar experiments B) Immunoblotted protein bands were measured by densitometry graphed according to phosphorylation ratios. Data presented as mean±SEM of 3 experiments. *$p < .05$ compared to control. (P.A. = pre aggregation, E.A. = early aggregation, M.A. = mid aggregation, F.A. = full aggregation).

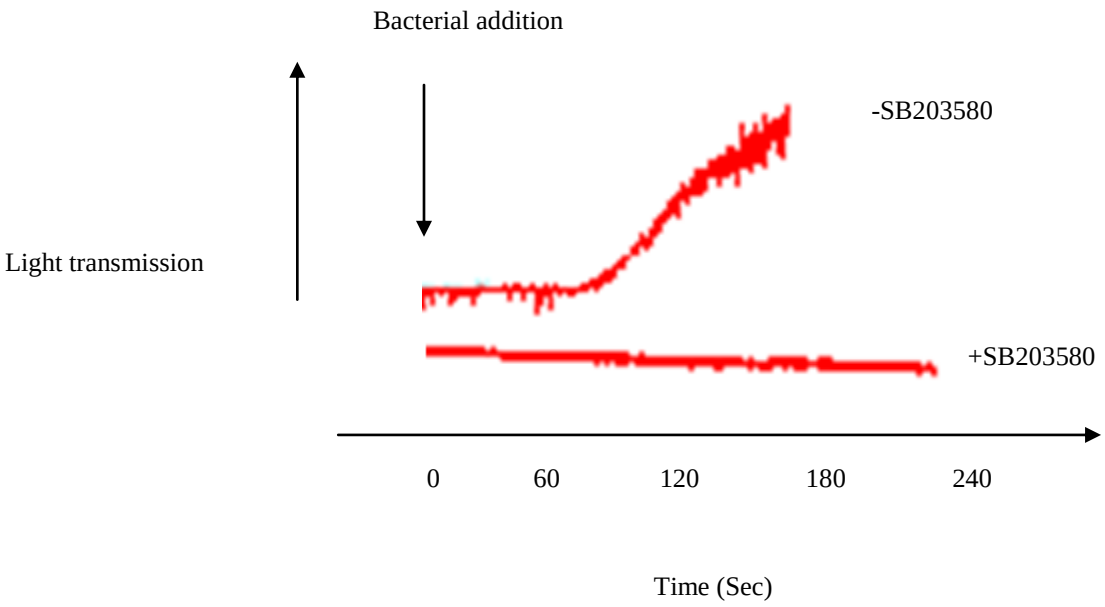


Figure 5.5. **Inhibitory effects of SB203580 on *S. sanguis* 2017-78 induced platelet aggregation**

A) Platelets were stirred in an aggregometer (37°C; 10mins; 6.5 µM) in the presence (+) or absence (-) of SB203580. Platelets were challenged with *S. sanguis* 2017-78 and aggregation was monitored as an increase in light transmission. Tracing is representative of 4 experiments showing similar results.

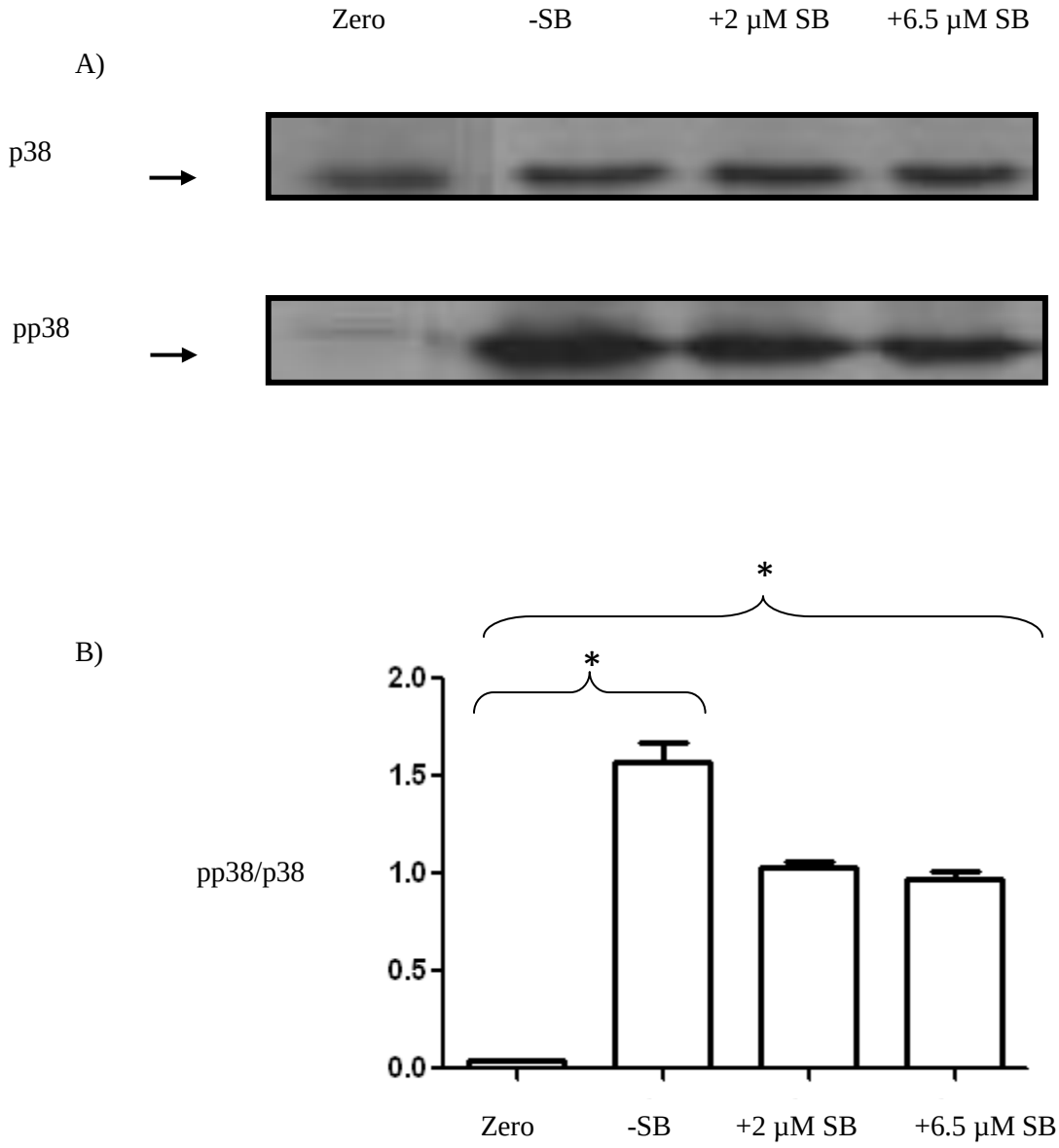


Figure 5.6. Effect of SB203580 on p38 phosphorylation with 2.0 µM and 6.5µM of SB203580

A) Platelets were stirred in an aggregometer (37°C; 10mins; 2 µM , 6.5 µM) in the presence (+) of or absence (-) of SB203580. Platelets were challenged with *S. sanguis* 2017-78 and aggregation was monitored as an increase in light transmission. p38 phosphorylation was detected by anti-phosphospecific p38 antibody (pp38; lower panel) and visualized by ECL. The blot was stripped and re-probed with an anti-p38 antibody (p38; upper panel) and visualized by ECL. Blot is representative of 3 similar experiments B) Immunoblotted protein bands were measured by densitometry graphed according to phosphorylation ratios. Data presented as mean±SEM of 3 experiments. *$p < .05$ compared to control.

5.7. Effect of SB203580 on $cPLA_2$ phosphorylation

S. sanguis strain 2017-78 caused the phosphorylation of $cPLA_2$ (Figure 5.7). Pre treatment of platelets with SB203580 (6.5 µM; 10 mins.), significantly reduced *S. sanguis* strain 2017-78 –induced $cPLA_2$ phosphorylation (Figure 5.7).

Thus this inhibition of $cPLA_2$ phosphorylation is consistent with findings of Kramer and colleagues who found that the inhibition of the p38 kinase prevented the proline-directed phosphorylation of $cPLA_2$ Kramer *et al* (1996).

5.8. Effect of aspirin on *S. sanguis* 2017-78 induced p38 phosphorylation

Aspirin, the cyclooxygenase inhibitor has been shown to inhibit platelet aggregation to ADP and collagen (Pulcinelli *et al.,* 2004) as a result of blocking COX thereby preventing the conversion of AA into TxA_2 (Helagson *et al.,* 2002). Therefore, the effects of aspirin on *S. sanguis* strain 2017-78-induced p38 phosphorylation were examined. The pre-treatment of platelets with aspirin (100µM, 20 mins) inhibited p38 phosphorylation at all stages during aggregation (Figure 5.8), consistent with a role for TxA_2 in the process.

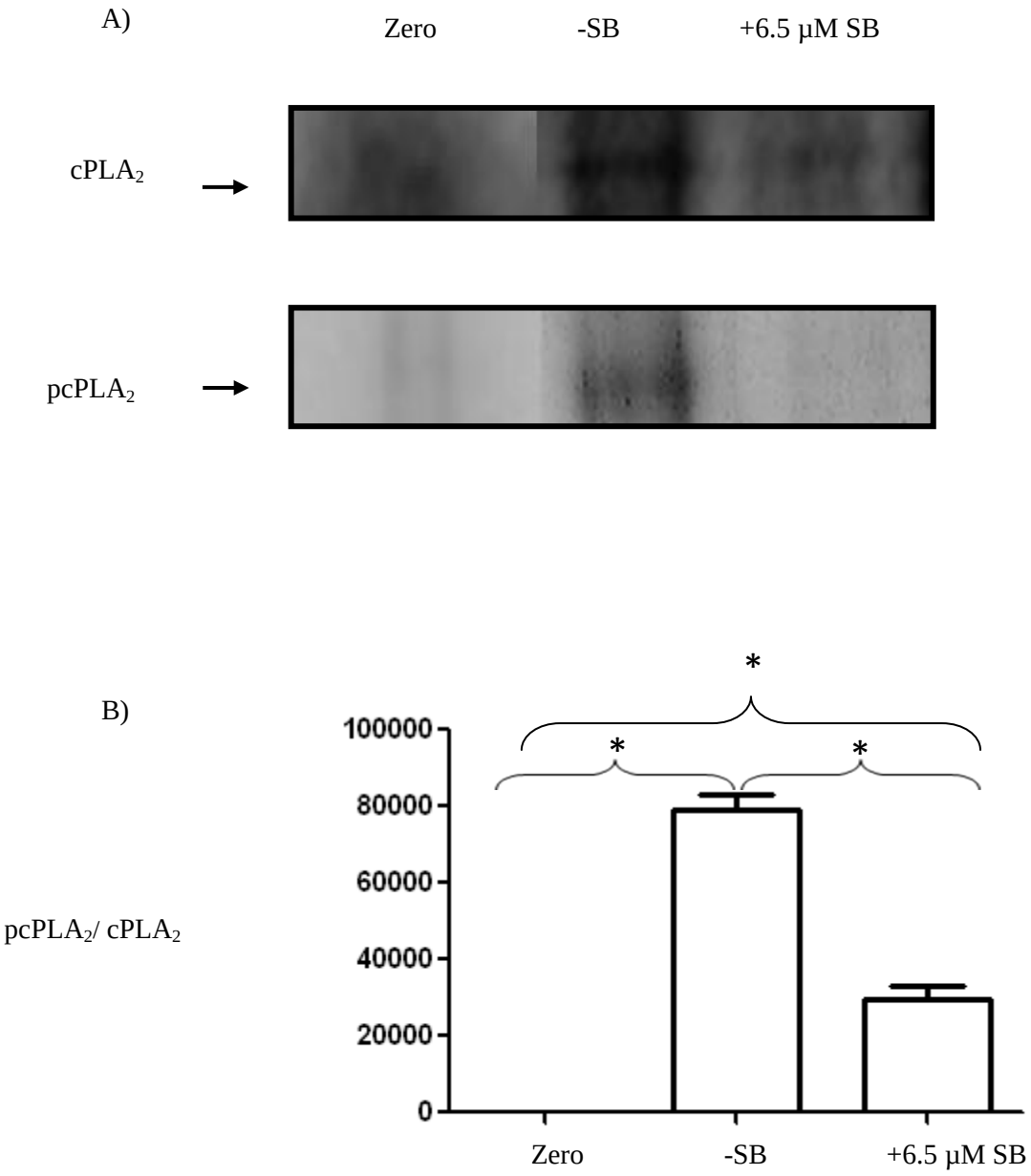


Figure 5.7. Effect of SB203580 on $cPLA_2$ phosphorylation

A) Platelets were stirred in an aggregometer (37°C; 10mins; 6.5 µM) in the presence (+) of or absence (-) of SB203580. Platelets were challenged with *S. sanguis* 2017-78 and aggregation was monitored as an increase in light transmission. $cPLA_2$ phosphorylation was detected by anti-phosphospecific $cPLA_2$ antibody ($pcPLA_2$; lower panel) and visualized by ECL. The blot was stripped and re-probed with an anti-$cPLA_2$ antibody ($cPLA_2$; upper panel) and visualized by ECL. Blot is representative of 4 similar experiments B) Immunoblotted protein bands were measured by densitometry graphed according to phosphorylation ratios. Data presented as mean±SEM of 3 experiments. *$p < .05$.

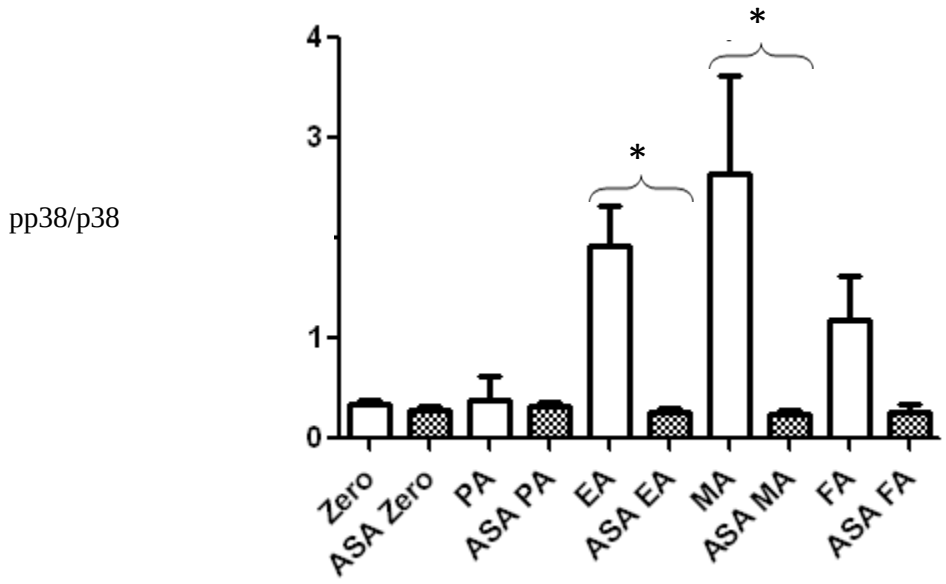


Figure 5.8. Effect of aspirin on platelet p38 phosphorylation

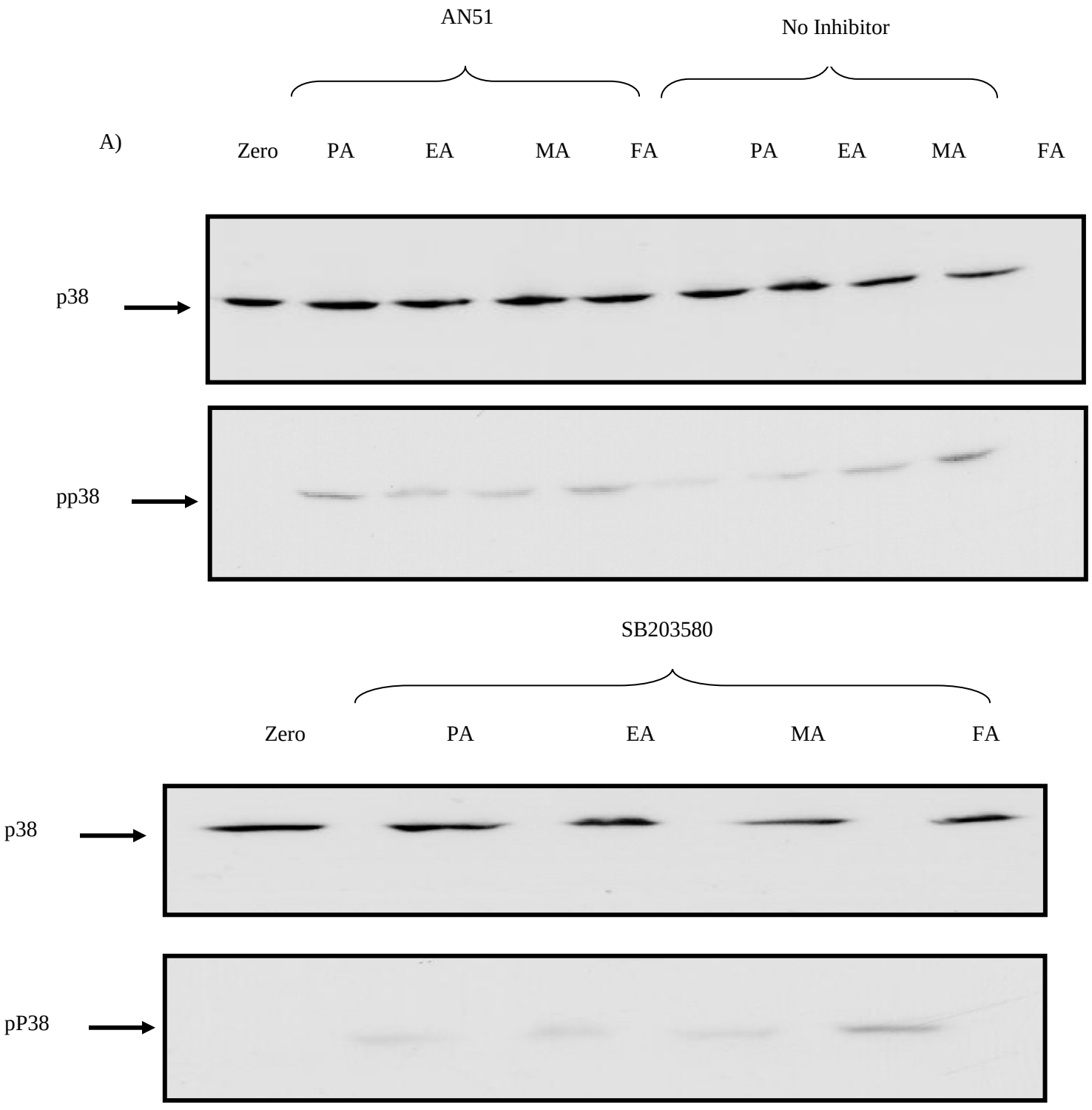
Data presented as mean±SEM of 3 experiments. *$p < .05$. (P.A. = pre aggregation, E.A. = early aggregation, M.A. = mid aggregation, F.A. = full aggregation, ASA = aspirinated platelets).

5.9. Effect of AN51 on *S. sanguis* 2017-78 induced p38 phosphorylation in aspirinated platelets

We examined the effects of pre-treating platelets with aspirin (10 mg/mL;20 mins.) and AN51 (.165 mg/ml, 5 min) on p38 phosphorylation in order to determine if there were any other contributing factors to p38 phosphorylation. Under these conditions there was significantly enhanced phosphorylation of *S. sanguis* strain 2017-78-induced p38 phosphorylation at the pre, early and mid stages of aggregation (n=3; Figure 5.9)

5.10. Effect of SB203580 on *S. sanguis* 2017-78 induced p38 phosphorylation in aspirinated platelets

A similar series of experiments were conducted to determine the effects of aspirin (10 mg/mL; 20 mins.) and SB203580 (10mins; 6.5 μM) on *S. sanguis* strain 2017-78-induced p38 phosphorylation. The pre-treatment of aspirinated platelets with SB203580 prior to stimulation with *S. sanguis* 2017-78 resulted in no significant enhancement of p38 phosphorylation (n=3; Figure 5.9).


AN51
No Inhibitor
A)
Zero
PA
EA
MA
FA
PA
EA
MA
FA
p38
pp38
SB203580
Zero
PA
EA
MA
FA
p38
pP38

B)

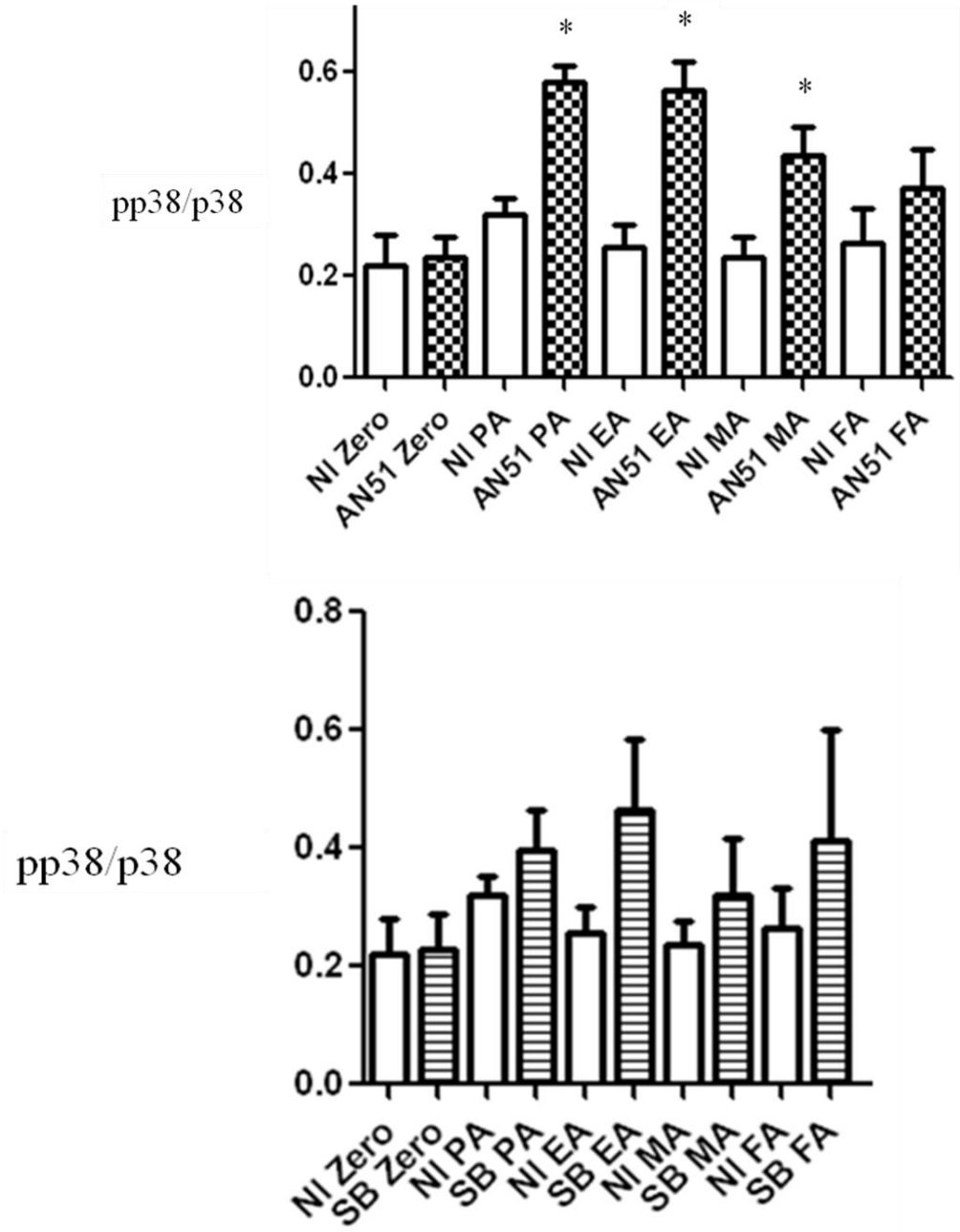


Figure 5.9. Effect of AN51 and SB203580 on *S. sanguis* 2017-78 induced p38 phosphorylation in aspirinated platelets

A) Aspirinated platelets were stirred in an aggregometer (37°C; 5 mins with AN51;10 mins with SB203580) in the presence AN51 (AN51) or SB203580 (SB) or without either (NI). Platelets were challenged with *S. sanguis* 2017-78 and aggregation was monitored as an increase in light transmission. p38 phosphorylation was detected by anti-phosphospecific p38 antibody (pp38; lower panel) and visualized by ECL. The blot was stripped and re-probed with an anti-p38 antibody (p38; upper panel) and visualized by ECL. Blot is representative of 3 similar experiments B) Immunoblotted protein bands were measured by densitometry graphed according to phosphorylation ratios. Data presented as mean±SEM of 3 experiments. *$p < .05$ compared to control. (P.A. = pre aggregation, E.A. = early aggregation, M.A. = mid aggregation, F.A. = full aggregation, ASA=aspirinated platelets).

6.0. DISCUSSION

Periodontitis is a bacterially-induced inflammatory disease (Wilson and Kornman, 2003) characterized by gingival bleeding, loosened teeth and a foul taste in the mouth (Coventry *et al.*, 2000). Oral pathogens, notably *Porphyromonas gingivalis* (*P. gingivalis*), *Bacteroides forsythus* and *Actinobacillus actinomycetemcomitans* and their metabolic by products accumulate on both teeth and gingival surfaces (Wilson and Kornman, 2003). These pathogens and their by-products, such as amines, sulphur compounds and fatty acids, accumulate within periodontal pockets, and subsequently pass through the epithelium and initiate an inflammatory response (Wilson and Kornman, 2003). In addition there is an increase in permeability which facilitates the entry of bacteria and their by-products into the blood; indeed bacteraemias occur more frequently in individuals with periodontal disease than in controls (Forner *et al.,* 2006). The severity of periodontitis depends on a variety of variables including oral hygiene, genetic predisposition, environmental factors, systemic health and prescription drugs (van Dyke, 2005).

There is a relationship between periodontal disease and systemic diseases (Engerbretson *et al.*, 1999; Garcia *et al.,* 2001). Of particular interest is the potential relationship between periodontal disease and cardiovascular diseases, notably atherosclerosis and its related conditions such as MI, stroke and peripheral arterial disease (Beck *et al.*, 1996; Scannapieco, 2003). Traditional risk factors for atherosclerosis are currently well understood however can only account for 50-70% (Haynes and Stanford, 2003) of cases of atherosclerosis and thus has led to research that has supported a correlation between periodontal disease and atherosclerosis (Research, Science and Therapy Committee of the American Academy of Periodontology, 1998). Many of these studies are compromised as the two diseases share multiple common risk factors (eg. diabetes, smoking) making it difficult to dissociate a cause and effect relationship. However

meta-analysis have suggested a small positive correlation between the two conditions (Janket *et al.,* 2003; Hujoel *et al.,* 2000; Emingil *et al.,* 2000).

Li and colleagues have proposed several potential mechanisms which may underlie the relationship between periodontitis and cardiovasculoar disease, each occurring as result of a bacteremia (Li *et al.,* 2000). It is possible that the bacteria or bacteria-derived lipopolysaccharide (LPS) stimulates an exaggerated host inflammatory, proatherogenic response (*e.g.* PGE_2, TNF-α, IL-1β) (Li *et al.,* 2000). Indeed many orally-derived bacteria have been localized within atherosclerotic lesions (Chiu, 1999; Haraszthy *et al.,* 2000). Alternatively LPS may directly act on the endothelium and promote atherosclerosis by recruiting inflammatory cells and stimulating the proliferation of vascular smooth muscle, vascular fatty degeneration, and intravascular coagulation (Li *et al.,* 2000). Finally, orally-derived micro-organisms may directly activate platelets (Fitzgerald *et al.,* 2006). As platelets have prothrombotic and proatherogenic actions (Michelson, 2007), this interaction may be involved both atherosclerosis and thrombosis.

A variety of organisms can stimulate platelets using a number of different mechanisms. For example platelet activation by the periopathogen *P.gingivalis* may occur directly or indirectly via bacterial fimbriae. These fimbriae trap and collect released outer membrane vesicles from the bacterium in close proximity to the platelet. These vesicles in turn stimulate platelet aggregation (Sharma *et al.,* 2000). Platelet activation by *S. sanguis* is a multi-step process involving adhesion and activation. The binding of *S. sanguis* to platelets is rapid, taking place within 15 seconds of addition, whereas aggregation is delayed (Herzberg *et al.,* 1983).

The mechanism of *S. sanguis*-induced platelet activation requires further elucidation. Gong and colleagues identified a protein termed Platelet Associated Activating Protein (PAAP)

on the surface of *S. sanguis* with significant homology to platelet activating domains on collagen and which they suggest interact with platelet collagen receptors to stimulate aggregation (Gong *et al* 1995).

S. sanguis **induced activation via GPIb/IX/V**

The platelet GPIb/IX/V complex is present on the platelet surface at numbers near 25000 copies per platelet (Modderman *et al.,* 1992). This receptor is made up of four transmembrane subunits; GPIbα is disulphide linked to GPIbβ and includes the covalently bonded GPIX and GPV (Canobbio *et al.*, 2001). The role of GPIb/IX/V in the platelet is to bind vWF at high shear rates. However it has also been shown to be variable as it can also bind to thrombin, clotting factors XI and XII in addition to glycoproteins such as P-selectin (Canobbio *et al.,* 2004).

Platelet aggregation in response to certain strains of *S. sanguis*-is inhibited by anti-GPIb antibodies (Kerrigan *et al*., 2002; Ford *et al.,* 1993). Additionally, platelets that were exposed to an enzyme (mocharagin) that cleaved GPIb at amino acid 282 failed to aggregate when challenged with *S. sanguis* (Kerrigan *et al*., 2002). Finally platelets from an individual with Bernard Soulier syndrome failed to aggregate in response to S. *sanguis* (Kerrigan *et al.,* 2002). Taken together these observations are consistent with platelet activation by *S. sanguis* depending upon the presence of GPIb, and indeed localising this interaction the N-terminal region of GPIb, a component of the vWF receptor (Kerrigan *et al*., 2002). Plummer and colleagues have shown that a glycoprotein, SrpA, on the surface of *S. sanguis* mediates the interaction with platelet GPIb (Plummer *et al* 2002). Strains of *S. sanguis* which lack SrpA had a significant decreased platelet binding capability as well as an increased lag time prior to platelet aggregation (Plummer *et al* 2002).

There is increasing evidence that GPIb/IX/V is not a passive adhesive receptor but acts to elicit intracellular signalling (Kerrigan *et al.,* 2002; Kroll *et al.,* 1991). The primary physiological ligand for GPIb/IX/V is vWF (Kerrigan *et al.,* 2002). The interaction between vWF and GPIb/IX/V results in the tyrosine phosphorylation of PLCγ2 (Canobbio *et al.,* 2001; Marshall *et al.*, 2002; Wu *et al.*, 2001). This in turn leads to phosphoinositide metabolism, calcium mobilization, activation of the platelet fibrinogen receptor and aggregation. In the present study we examined the effects of AN51, a blocking anti-GPIb/IX/V monoclonal Ab on platelet activation by *S. sanguis* strain 2017-78. AN51 failed to inhibit platelet aggregation in response to *S. sanguis* 2017-78; indeed aggregation was apparently accelerated, as defined by a shortened lag. This contrasts with the inhibition of platelet aggregation reported by a previous study using all strains of *S. sanguis* (Kerrigan *et al.*, 2002). As outlined below, this may reflect the inhibition, by AN51, of a negative regulatory process normally engaged in platelets leading to enhanced activation.

Previous studies have shown that *S. sanguis* strain 2017-78 causes the tyrosine phosphorylation of Syk, LAT and PLCγ2 (Pampolina and McNicol, 2005). In the current study *S. sanguis* 2017-78-induced PLCγ2 phosphorylation was confirmed, and, in addition, this phosphorylation was reduced by pre-treatment with AN51. This suggests that PLCγ2 activation lies downstream of GPIb/IX/V activation. The downstream activation of PLCγ2 after GPIb/IX/V activation is further confirmed by several studies that also showed GPIb/IX/V activation results in tyrosine phosphorylation of PLCγ2 (Marshall *et al.*, 2002; Wu *et al.,* 2001; Canobbio *et al.*, 2001).

S. sanguis induced activation of platelet MAP Kinases

Engagement of GPIb/IX/V with vWF has been shown to stimulate the rapid and sustained phosphorylation of the p38 member of the MAP kinase family (Canobbio *et al.,* 2004). In addition, agonists such as collagen, thrombin and ADP stimulate p38 (Saklatavala *et al.,* 1996; Canobbio *et al.,* 2004; Begonja *et al.,* 2007) and it has been suggested that p38 activation is likely upstream of $cPLA_2$ activity, with the resultant AA release and TxA_2 generation (Canobbio *et al.,* 2004). Indeed, the activation of platelet p38 coincides with $cPLA_2$ phosphorylation on Ser^{505} (Kramer *et al* 1996).

In the present study, platelet aggregation in response to *S. sanguis* 2017-78 was monitored at 4 stages; pre aggregation (PA), early aggregation (EA), mid aggregation (MA) and full aggregation (FA). Phosphorylation of p38 occurred during the early and mid phases of platelet aggregation but not during the pre and full aggregation stages, consistent with p38 contributing to specific stages of S. *sanguis*-induced platelet aggregation.

The p38 inhibitor SB203580 was used to further address the precise contribution of p38 activation to platelet aggregation. Previous studies have shown that SB203580 blocked platelet aggregation in response to low doses of collagen and AA, consistent with a role for p38 activation in platelet aggregation in response to these agonists (Borsch-Haubold *et al.,* 1998; Kramer *et al.,* 1995; Coleman *et al.,* 2004). SB203580 also inhibited the proline-directed phosphorylation of $cPLA_2$ in thrombin-stimulated platelets (Kramer *et al*, 1996). However SB203580 also prevented the conversion of endogenous AA to TxA_2 suggesting that SB203580 had an additional effect on cyclooxygenase-1 and/or thromboxane synthase which may account for the observed effects (Borsch Haunold *et al.,* 1998)

In the current study SB203580 inhibited S. *sanguis*-induced platelet aggregation in a concentration-dependent manner. The concentrations used in this study, 2 and 6.5µM were slightly higher than the IC_{50} of 0.7µM but similar to those used by BorschHaubold and colleagues, who inhibited collagen-induced aggregation by SB203580 (Borsch-Haubold *et al*., 1998).

Concurrent with the inhibition of aggregation, SB203580 also inhibited, in a concentration-dependent manner, the phosphorylation of both p38 and $cPLA_2$ in response to *S. sanguis* strain 2017-78. These data are consistent with p38 activation upstream of $cPLA_2$. In addition these data further demonstrate the critical role played by the AA/TxA_2 pathway in platelet activation in response to S. *sanguis* strain 2017-78.

The cyclooxygenase inhibitor aspirin, is a widely used inhibitor of platelet aggregation both *in vitro* and *in vivo* (Michelson, 2007). In the current study, pre-treatment of platelets with aspirin inhibited platelet aggregation in response to *S. sanguis* 2017-78. This is consistent with studies by Canobbio and colleagues who showed that cyclooxygenase inhibitors prevented platelet aggregation in response to vWF (Canobbio *et al.,* 2004).

Surprisingly however aspirin also had an inhibitory effect on p38 phosphorylation which coincided with the inhibition of platelet aggregation. This would suggest that inhibition of TxA_2 attenuated p38 phosphorylation, therefore placing p38 distal, rather than prior, to $cPLA_2$. This is consistent the activation of p38 in response to a thromboxane analogue (Saklatvala *et al* 1996). In addition the data may suggest that S. *sanguis* strain 2017-78-induced AA release, prior to TxA_2 synthesis, does not result from a $cPLA_2$-mediated pathway. Indeed platelets are known to possess an active diglyceride lipase (Bell *et al.,* 1979) which would liberate arachidonic acid

from diglyceride generated by aspirin-insensitive PLCγ2 activity. In the present study, in aspirinated platelets, SB203580 resulted in an increase in *S. sanguis*-induced p38 phosphorylation, suggesting that p38 is present downstream of thromboxane A_2. It is possible that increased phosphorylation seen may result from a competitive binding taking place between aspirin and SB203580, resulting in an overcompensation in p38 phosphorylation downstream of thromboxane A_2. A similar, clinically relevant, interaction takes place between aspirin and ibuprofen where ibuprofen competes for aspirin binding sites in platelet cyclooxygense and protects the platelet from the actions of aspirin. This can compromise the antithrombotic actions of aspirin.

Effect of aspirin on p38 activation in the presence AN51

To further address the inter-relationships between GPIb/IX/V, TxA_2 and p38 we examined the effects of AN51 on p38 phosphorylation in aspirinated platelets. Despite the blockade of GPIb/IV/V and cyclooxygenase, our results showed a significant enhancement of p38 phosphorylation in these platelets. This is consistent with the enhanced aggregation observed, in non-aspirinated platelets, treated with AN51 and challenged with 2017-78.

This increased phosphorylation can potentially be explained on the basis of the phosphorylation of an inhibitory complex PECAM, which acts to negatively regulate GPIb/IX/V. Rathore and colleagues showed that platelets lacking PECAM form much larger thrombi than the PECAM-containing platelets (Rathore *et al* 2003). They explain this negative regulation by PECAM 1 on the basis of an interaction with the GPIb/IV/V complex. They concluded that the binding of vWF to GPIb/IV/V led to the tyrosine phoshorylation of an ITAM bearing FcRγ chain and the initiation of a negative feedback loop that involves the tyrosine phosphorylation of

PECAM-1. Thus, their data support the enhanced phosphorylation of p38 as the engagement of GPIb stimulated the activation of a negative feedback loop involving PECAM-1. Hence blockade of GPIb with AN51 would remove the negative regulation and account for the enhanced aggregation observed in response to *S.sanguis* strain 2017-78.

The importance of these studies is that they provide greater insight into the platelet activation response to *S. sanguis* 2017-78. Accelerated aggregation to *S. sanguis* 2017-78 in AN51 pre-treated platelets has not been previously reported. Additionally, the inhibition of PLCγ2 phosphorylation as a result of blocking GPIb/IX/V shows consistency with previous research (Kerrigan *et al.,* 2002; Ozaki *et al.,* 2005) and further elucidates the events that follow GPIb/IX/V engagement. The demonstration of a timed response of p38 during aggregation further provided a switch like model to the activation of p38. Further, our demonstration of p38 inhibition by SB203580 displays consistency with results from research conducted by Saklatvala *et al* (1996) allowing us to go on to demonstrate the placement of p38 upstream of $cPLA_2$.

The pre-treatment of aspirinated platelets with SB203580 provided an opportunity to consider a competitive binding between inhibitors which resulted in an enhanced phosphorylation of p38 downstream of TxA_2. Additionally, the pre-treatment of aspirinated platelets with AN51 strengthened previous hypothesis put forth by Rathore *et al* (2003) that PECAM acts to inhibit the function of GPIb/IX/V inhibitors, establishing the context for a double negative scenario.

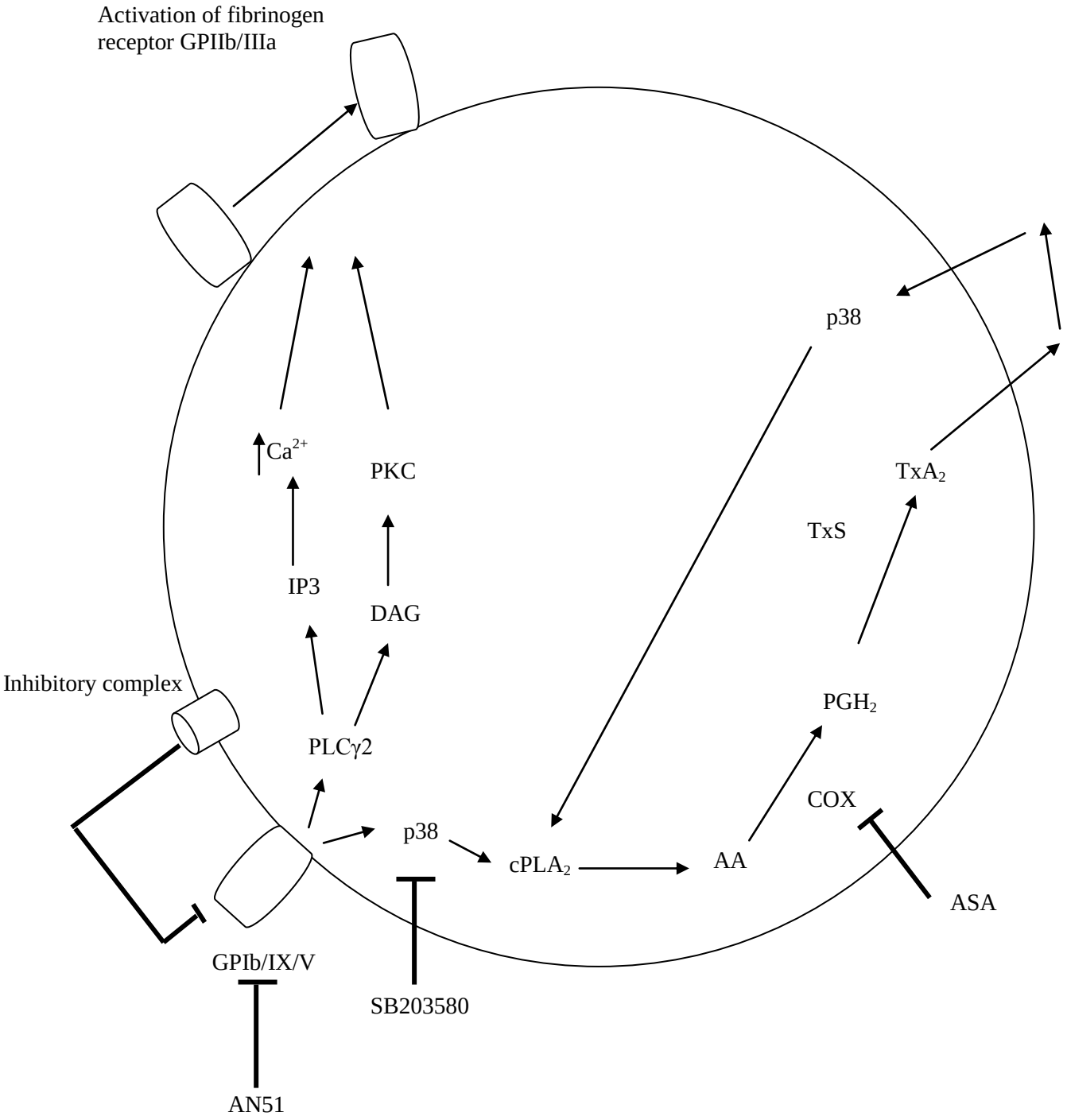


Figure 6.1. Intracellular signalling events involved in *S. sanguis* 2017-78 induced platelet activation

Engagement of GPIb/IX/V leads to PLCγ2 and p38 activation with the subsequent downstream events. In the current study p38 has been shown to be active downstream of TxA_2. The presence of an inhibitory action is also observed as a result of GPIb/IX/V engagement. As a result of blocking GPIb/IX/V with AN51, the effects of this inhibitory complex were prevented through the establishment of a "double negative" scenario.

7.0. References

Abrams CS. (2005). *Curr Opin Hematol* **12**: 401-104.

Anderson CL, Chacko GW, Osborne JM, Brandt JT. (1995). *Semin Thromb Hemost* **21**: 1 – 9.

Andoh A, Yoshida T, Yagi Y. (2006). *J Gastroenterol* **41**: 91-92.

Antonikov I, Alekseev O, Chernukh A. (1981). *Morphol Pathomorphol* **92**: 87-89.

Ardlie NG, Garrett JJ, Bell LK. (1986) *Thromb Res* **42**: 115–124.

Armstrong RA. (1996). *Pharmacol Ther* **72**: 171-191.

Barkalow K, Falet H, Italiano JE, van Vugt A, Carpenter CL, Schreiber AD, Hartwig JH. (2003). *Am J Cell Physiol* **285**: C797-C805.

Baumgartner HR, Haudenschild C. (1972). *Ann NY Acad Sci* **201**: 22-36.

Beck J, Garcia R, Heiss G, Vokonas PS, Offenbacher S. (1996). *J Periodontol* **67**: 1123-1137.

Begonja A, Geiger J, Rukoyatkina N, Rauchfuss S, Gambaryan S, Walter U. (2007). *Blood* **109**: 616-618.

Bell RL, Kennerly DA, Stanford N, Majerus PW. (1979). *Proc Natl Acad Sci* **76**: 3233-3241.

Berger G, Masse JM, Cramer EM. (1996). *Blood* **87**: 1385-1395.

Borsch-Haubold AG, Pasquet S, Watson SP. (1998). *J Biol Chem* **44**: 28766-28772.

Brass LF. (2003). *Chest* **124(3 suppl)**: 18S-25S.

Brown KK, Heitmeyer SA, Hookfin EB, Hsieh L, Buchalova M, Taiwo YO, Janusz MJ. (2008). *J Inflam* **5**: 1-8.

Canobbio I, Reineri S, Sinigaglia F, Baldiuni C, Torti M. (2004). *Thromb Haemost* **91**: 102-110.

Canobbio I, Bertoni A, Lova P, Paganini S, Hirsch E, Sinigaglia F, Balduini C, Torti M. (2001) *J Biol Chem* **276**: 26022-26029.

Chia JS, Lin YL, Lien HT, Chen JY. (2004). *Infect Immun* **72**: 2605-2617.

Chiu B. (1999). *Am Heart J* **138 (5 pt 2)**: S534-6.

Cobb MH. (1999). *Prog Biophys Mol Biol* **71**: 479-500.

Coleman LG, Polanowska-Grabowska RK, Marcinkiewicz, Gear A. (2004). *Blood* **104**: 380-389.

Cooney D, Phee H, Jacob A, Coggeshall K. (2001). *J Immunol* **167**: 844-854.

Coventry J, Griffiths G, Scully C, Tonetti M. (2000). *BMJ* **321**: 36-39.

Cowan D. (1981). *Br J Haematol* **49**: 425-434.

Cramer EM, Berger G, Berndt MC. (1994). *Blood* **84**: 1722-1730.

Cunningham JG, Meyer JE, Fox. (1996). *J Biol Chem* **271**: 11581-11587.

Curley DB, Wells V, Viscount H, Munro CL, Fenno JC, Taylor PF, Macrine FL. (1995). *Infect Immun* 63: 4669-4674.

Cutler L, Rodan G, Feinstein M. (1978). *Biochemica et Biophysics Acta* **542**: 357-371.

Daniel JL, Molish IR, Rigmaiden M, Stewart G. (1984). *J Biol Chem* **259**: 9826-9831.

Douglas CWI, Heath J, Hampton KK, Perston FE. (1993). *J Med Microbiol* **39**: 179-182.

Emingil G, Buduneli E, Aliyev A, Akili A, Atilla G. (2000). *J Periodontol* **71**: 1882-1886.

Engebretson SP, Lalla E, Lamster IB. (1999). *NY State Dent J* **65**: 30-32.

Erickson PR, Herzberg MC. (1990). *J Biol Chem* **268**: 1646-1649.

Febbraio M, Silverstein RL. (1990). *J Biol Chem* **265**: 18531-18537.

Fenton JW, Landis BH, Walz DA, Finlayson JL. (1979). *The Chemistry and Physiology of Human Plasma Proteins* pp. 151-183, Pergamon Press, New York.

Ferrel JE, Martin GS. (1988). *Mol Cell Biol* **8**: 3603-3610.

Fischer T, Gatling M, McCormick F, Dum C, White G. (1994). *J Biol Chem* 269: 17257-17261.

Fishkes H, Rudnick G. (1982). *J Biol Chem* **257**: 5687-5677.

Fitzgerald JR, Foster TJ, Cox D. (2006). *Nature* **4**: 445-457.

Ford I, Douglas CW, Preston FE, Lawless A, Hampton KK. (1993). *Br J Haematol* **84**: 95-100.

Ford I, Douglas CWI, Cox D, Rees DGC, Heath J, Preston FE. (1997). *Br J Haematol* **97**: 737-746.

Forner L, Larsen T, Kilian M, Holmstrup P. (2006). *Clin Perio* **33**: 401-407.

Fox JEB. (2001). *Thromb Haemost* **86**: 198-213

Garcia RI, Henshaw MH, Krall E. (2001). *Periodontol 2000* **25**: 21-36.

Gerrard JM, White JG, Rao GHR, Townsend D. (1976). *Am J Pathol* **83**: 283-298.

Gibbins, JM. (2004). *Cell Sc* **117**: 3415-3425.

Gogstad GO, Hagen I, Korsmo R, Solum NO. (1981). *Biochem Biophys Acta* **670**: 150-162.

Gong K, Wen D, Ouyang T, Rao A, Herzberg M. (1995). *Infect Immune* **63**: 3628-3633.

Gordon JL. (1976). *Platelets in Biology and Pathology* North Holland Publishing, New York. pp1-378.

Grinstein S, Furuya W. (1983). *J Biol Chem* **258**: 7876-7882.

Guy EJ, Stahl U, Lindqvist Y. (2009). *J Biol Chem* **284**: 19371-19379.

Han Y, Nurden A, Combrie R, Pasquet JM. (2003). *J Thromb Haemost* **1**: 2206-15.

Haraszthy VI, Zambon JJ, Trevisan M, Zeid M, Genco RJ. (2000). *J Periodontol* **71**: 1554-1560.

Harrison P, Cramer EM. (1993). *Blood Rev* **7**:52-62.

Hartleib J, Kohler N, Dickinson RB, Chatwal GS, Sixma JJ, Hartford OM, Foster TJ, Peters G, Kehrel BE, Herrmann M. (2000). *Blood* **96**: 2149-2156.

Hathaway DR, Adelstein RS (1979). PNAS **76**: 1653-1657.

Hausmann E, Raisz LG, Miller WA. (1970). *Science* **168**: 862-864.

Haynes WG, Stanford C. (2003). *Arterioscler, Thromb Vasc Biol* **23**: 1309.

Henn V, Slupsky JR, Grafe M, Anagnostopoulos I, Forster R, Muller-Berghaus G, Kroczek RA. (1998). *Nature* **391**: 591-594.

Hers I, Donath J, van Willigen G, Akkerman JW. (1998). *Arterioscler Thromb Vasc Biol* **18**: 404-414.

Herzberg MC, MacFariane GD, Gong K, Armstrong NN, Witt AR, Erickson PR, Meyer MW. (1992). *Infect Immun* **60**: 4809-4818.

Herzberg MC, Brintzenhofe KL, Clawson CC. (1983). *Infect Immun* **39**: 1457-1469.

Herzberg MC, Macfarlane GD, Liu P, Erickson PR. (1994). *In: Molecular Pathogenesis of Periodontal Disease.* Washington, DC: American Society for Microbiology, pp. 247-255.

Hollister MC, Weintraub JA. (1993). *J Dent Educ* **57**: 901-912.

Holmsen H. (1985). *Semin Hematol* **22**: 219-240.

Holmsen H. (1994). *Eur J Clin Invest* **24, Suppl 1**: 3-8.

Hujoel PP, Drangsholt M, Spiekerman C, DeRouen TA. (2000). *JAMA* **284**: 1406-1410.

Israels SJ, Gerrard JM. (1996). *In: Platelets: a practical approach.* (Watson SP, Authi KS, eds.) Oxford, UK: Oxford University Press, pp.279-297.

Iwai T. (2009) *J Periodontal Res* **44**: 689-94.

Jackson MR, Clagett P. (2001). *Chest* **119**: 2835-2995.

Jackson EC, McNicol A. (2010) *Thromb Res* **125**:147-51.

Jackson SP, Nesbitt WS, Kulkarni S. (2003). *Thromb Haemost* **1**: 1602-1612.

Janket S, Baird A, Chuang S, Jones JA (2003). *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* **95**: 559-569.

Jantzen HM, Milstone DS, Gousset L, Conley PB, Mortensen RM. (2001). *J Clin Invest* **108:**477–483.

Johnson CM. (1993). *Infect Dis Clin North Am* **7**: 21-36.

Kao K, Pizzo S, McKee P. (1979). *PNAS* **76**: 5317-5320.

Kerrigan SW, Douglas I, Wray A, Heath J, Byrne MF, Fitzgerald D, Cox D. (2002). *Blood* **100**: 509-516.

Koedam J, Cramer E, Briend E, Furie B, Furie BC, Wagner D. (1992). *J Cell Biol* **116**: 617-625.

Kramer RM, Roberts EF, Strifler BA, Johnstone EM. (1995). *J Biol Chem* **270**: 27395-27398.

Kramer RM, Roberts EF, Um SL, Borsch-Haubold AG, Watson SP, Fisher MJ. (1996). *J Biol Chem* **271**: 27723-9.

Kroll MH, Harris TS, Moake JL. (1991). *J Clin Invest* **88**: 1568–1573.

Kunapuli SP, Dorsam RT, Kim S, Quinton TM. (2003). *Curr Opin Pharmacol* **3**: 175-180.

Lee SB, Rao AK, Lee KH, Yang X, Bae YS, Rhee SG. (1996). *Blood* **88**: 1684-1691.

Legrand Y, Fauvel F, Gutman N, Muh JP, Tobelem G, Souchon H, Karniguian A, Caen JP. (1980). *Thromb Res* **63**: 656-664.

Li X, Kolltveit KM, Tronstad L, Olsen I. (2000). *Clin Microbiol Rev* **13**: 547-558.

Lian L, Wang Y, Draznin J, Eslin D, Bennett JS, Poncz M, Wu D, Abrams CS. (2005). *Blood* **106**: 110-117.

Libby P, Simon DI. (2001). *Circulation* **103**:1718-1720.

Lindmark E, Tenno T, Siegbahn A. (2000). *Arterioscler Thromb Vasc Biol* **20**: 2322-2328.

Lin LL, Wartmann M, Lin AY, Knopf JL, Seth A, Davis RJ. (1993) *Cell* **72**: 269-278.

Lockhart PB. (2000). *Periodontol 2000* **23**: 127-135.

Lourbakos A, Yuan YP, Jenkins AL, Travis J, Andrade-Gordon P, Santulli R, Potempa J, Pike RN. (2001). *Blood* **97**: 3790-3797.

Lowrance JH, Baddour LM, Simpson WA. (1990). *J Clin Invest* **86**: 7-13.

Lusis AJ. (2000). *Nature* **407**: 233-241.

Mackay HJ, Twelves CJ. (2007) *Nature Reviews* **7**: 554-562.

Marnett LJ, Rowlinson SW, Goodwin DC, Kalgutkar AS, Lanzo CS. (1999). *J Biol Chem* **274**: 22903-22906.

Marshall SJ, Asazuma N, Best D, Wonerow P, Salmon G, Andrews RK, Watson SP. (2002) *Biochem J* **361**: 297-305.

McNicol A, Israels SJ, Gerrard JM. (1993). *In: Recent Advances in Blood Coagulation* **6**: 17–49. Churchill Livingstone Ltd, Edinburgh, UK.

McNicol A, Gerrard JM. (1997). *Adv Mol Cell Biol* **18**: 1-29.

McNicol A, Israels SJ. (1999). *Thromb Res* **95**: 1-18.

McNicol A, Jackson EC. (2003) *Biochem Pharmacol* **65**:1243-50.

McNicol A, Zhu R, Pesun R, Pampolina C, Jackson EC, Bowden GH, Zelinski T. (2006). *Thromb Haemost* **95**: 288-93.

McNicol A, Eyer E, Jackson E, Israels S. (2007). *Thromb Haemost* **98**: 1382–1384.

Mealey BL. (1991) *Periodontol 2000* **21**: 197-209.

Micheslon AD. Platelets. (2007). Academic Press, pg: 319-377, 2005.

Michelson AD, Cattaneo M, Eikelboom JW, Gurbel SP, Kottke-Marchant K, Kunicki TJ, Pulcinelli FM, Cerletiss C, Rao AK. (2005). *Thromb Haemost* **3**: 1309-1311.

Miyakawa T, Maeda A, Yamazawa T, Hirose K, Kurosaki T, Lino M. (1999) .*EMBO J* **18**: 1303-1308.

Modderman PW, Admiraal LG, Sonnenberg A, von dem Borne AE. (1992). *J Biol Chem* **267**: 364-369.

Moog S, Mangin P, Lenain N, Strassel C, Ravanat C, Schuhler S, Freund M, Santer M, Kahn M, Nieswandt B, Gachet C, Cazenave JP, Lanza F. (2001). *Blood* **98**: 1038-1046.

Moreillon P, Que YA, Bayer AS. (2002). *Infect Dis Clin North Am* **16**: 297-318.

Morton LF, Peachey AR, Barnes MJ. (1989). *Biochem* J **258**: 157-163.

Murray R, FitzGerald G. (1989). *Biochem **86***: 124-128.

Myers RI. (2005). *Baylor University Medical Center Proceedings* **18**: 331-336.

Nadal F, Levy S, Grelac F, Caen J, Rosa J, Bryckaert M. (1997). *J Biol Chem* **272**: 22381-22384.

Narumiya S, Hirata N, Namba T, Hayashi Y, Ushikubi F, Sugimoto Y, Negishi M, Ichikawa A. (1993) *J Lipid Mediators* **6**: 155–161.

Newman PJ, Newman DK. (2003). *Arterioscler Thromb Vasc Biol* **23**:1-12.

Newton AC. (1995). *J. Biol Chem* **270**: 28495–28498

Nishizuka Y. (1992). *Science* **258**: 607–614.

O'Brien L, Kerrigan SW, Kaw G, Hogan M, Penades J, Litt D, Fitzgerald DJ, Foster TJ, Cox D. (2002). *Mol Microbiol* **44**: 1033-1044.

Ono K, Han J. (1999). *Cell Signal* **12**: 1-13.

Oury C, Toth-Zsamboki E, Vermylen J, Hoylaerts MF. (2002). *Blood* **99**: 2275-2276.

Ozaki Y, Asazuma N, Suzuki-Inoue K, Berndt MC. (2005). *Thromb Haemost* **3**: 1745-1751.

Pampolina C, McNicol A. (2005). *Thromb. Haemost.* **93**: 932-939.

Paul BZ, Daniel JL, Kunapuli SP. (1999). *J Biol Chem* **274**: 28293-300.

Pearson G, Robinson F, Gibson T, Xu B, Karandikar M, Berman K Cobb M. (2001). *Endocrine Reviews **22***: 153-183.

Persson RG, Persson RE. (2008). *Clin Periodontol* **35**: 362-379.

Pham K, Feik D, Hammond BF, Rams TE, Whitaker EJ. (2002). *Platelets* **13**: 21-30.

Polasek J. (1989). *Eur J Haematol* **Suppl. 50, 43**: 1-24.

Poole AW, Watson SP. (1995). *Brit J Pharamacol* **115**: 101-106.

Plummer C, Wu H, Kerrigan S, Meade G, Cox D, Douglas CW. (2005). *Br Haematol* **129**: 101-109.

Quinton TM, Kim S,Dangelmaier C, Dorsam RT, Jin J,Daniel JL, Kunapuli SP. (2002). *J Biochem* **368**: 535-543.

Ragab A, Bodin S, Viala C, Chap H, Payrastre B, Ragab-Thomas J. (2003). *J Biol Chem* **278**: 40923-40932.

Rathore V, Stapleton MA, Hillery CA, Montgomery RR, Nichols TC, Merricks EP, Newman

DK, Newman PJ. (2003). *Blood* **102**: 3658-3664.

Reed GL, Fitzgerald ML, Polgar J. (2000). *Blood* **96**: 3334-3342.

Research, Science and Therapy Committee of the American Academy of Periodontology. (1999). *J Periodontol* **70** : 457-470.

Rittenhouse SE, Allen CL. (1982). *J Clin Invest* **70**: 1216-1224.

Rivera J, Lozano ML, Núñez LN, Vicente V. (2009). *Ferrata Storti Foundation* **94**: 700-711.

Roberts DE, Bose R. (2002) *Ann N Y Acad Sci* **976**: 345–349.

Rolf MG, Brearley CA, Mahaut-Smith MP. (2001). *Thromb Haemost* **85**: 303-308.

Rosado JA, Sage SO. (2001) *J Biol Chem* **276**:15659-65.

Ruan C, Du X, Xi X, Castaldi PA, Berndt M. (1987). *Blood* **69**: 570-577.

Ruggeri ZM, dent JA, Saldivar E. (1999). *Blood* **94**: 172-178.

Saklatvala J, Rawlinson L, Waller R, Sarsfield S, Lee, J, Morton L, Barnes M, Farndale R. (1996). *J Biol Chem* **271**: 6586-6589.

Sandre RM, Shafran SD. (1996). *Clin Infect Dis* **22**: 276-286.

Santoro S, Walsh J, Staatz W, Baranski K. (1991. *Cell Regulat* **2**: 905-913.

Sarchielli P, Gallai V. (2001). *J Headache Pain* **2**: S61-S66.

Scannapieco FA. (2003). *Ann Periodontol* **8**: 38-53.

Scheld WM, Valone JA, Sande MA. (1978). *J Clin Invest* **61**: 1394-1404.

Schenk PW, Snaar-Jagalska BE. (1999). *Biochem Biophys Acta* **1449**: 1–24.

Sexton DJ, Spelman D. (2003). *Cardiol Clin* **21**: 273-282, vii-viii.

Sharma A, Novak EK, Sojar HT, Swank RT, Kuramitsu HK, Genco RJ. (2000). *Oral Microbiol*

Immunol **15**: 393-396.

Shattil SJ, Cooper RA. (1978). *Prog Hemost Thromb* **4**: 59-86.

Shattil SJ, Cunningham M, Wiedmer T, Zhao J, Sims PJ, Brass LF. (1992). *J Biol Chem* **267**: 18424-18431.

Shattil SJ, Kashiwagi H, Pampori N. (1998). *Blood* **91**: 2645-2657.

Silver JG, Martin AW, McBride BC. (1977) *J Clin Periodontol* **4**: 92-9.

Sims PJ, Wiedmer T. (2001). *Thromb Haemost* **86**: 266-275.

Slupsky JR, Kalbas M, Willuweit A, Henn V, Kroczek RA, Muller-Berghaus G. (1998). *Thromb Haemost* **80**: 1008-1014.

Socransky SS, Haffajee AD. (1992). *J Periodontol* **63**: 322-331.

Steckleberg JM, Wilson WR. (1993). *Infect Dis Clin North Am* **7**: 9-19.

Stenberg P, McEvr R, Shuma M, Jacques Y, Bainton D. (1985). *Cell Biol* **101**: 880-886.

Sullam PM, Valone FH, Mills J. (1987). *Infect Immun* **55**: 1743-1750.

Sullam PM, Hyun WC, Szollosi J, Dong J, Foss WM, Lopez JA. (1998). *J Biol Chem* **273**: 5331-5336.

Sun B, Li J, Kambayashi J. (1999) *Biochem Biophys Res Commun* **266**: 24– 27.

Tandon NN, Kralisz U, Jamieson GA. (1989). *J Biol Chem* **264**: 7576.

Tonetti MS, Claffey N. (2005). *J Clin Peiodont* **32**: 210-213.

Toth-Zsmaboski E, Oury C, Cornelissen H, De Vos R, Vermylen J, Hoylaerts MF. (2003). *J Biol Chem* **278**: 46661-46667.

Trybulec M, Kowalska MA, McLane MA, Silver L, Lu W, Niewiarowski S. (1993). *Proc Soc Exp Biol Med* **203**: 108-116.

Turgeon VL, Houenou LJ. (1997). *Brain Res Rev* **25**: 85-95.

U.S. Department of Health and Human Services. *Oral Health in America: A Report of the Surgeon General.* Rockville, MD: U.S. Department of Health and Human Services, National Institute of Dental and Craniofacial Research, National Institutes of Health, 2000:2-3.

Van Dyke TE. (2005). *J Int Acad Periodontol* **7**: 3-7.

Van Gorp ECM, Suharti C, ten Cate H, Dolmans WMV, van der Meer JWM, ten Cate JW, Brnadjes PM. (1999). *Infect Dis* **189**: 176-86.

Van Oost BA. (1986). *In: Platelet responses and metabolism II.* (Holmsen H, ed.) Boca Raton FL: CRC Press Inc., pp. 163-191.

Van Willigen G, Akkerman JW. (1991). *Biochem J* **273:** 115-120.

Van Willigen G, Akkerman JW. (1992). *Blood* **79**: 82-90.

Vettore M, Janket SJ, Baird AE, Chuang SK, Jones JA. (2003). *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* **95**: 559-569.

Wang F, Wang NS, Yan CG, Li JH, Tang LQ. (2007). *Clin Rheumatol* **26**: 768-771.

Watson SP, Reep B, McConnel RT, Lapetina EG. (1985). *Biochem J* **226**: 831-837.

Watson SP, McNally J, Shipman LJ, Godfrey PP. (1988). *Biochem J* **249**: 345-350.

Wilson TG, Korman KS, eds. (2003). *Fundamentals of Periodontics* (2nd Edition) pp.195-204. Quintessence Publishing Co., Inc. Chicago.

Wu Y, Suzuki-Inoue K, Satoh K, Asazuma N, Yatomi Y, Berndt MC, Ozaki Y (2001) *Blood* **97**: 3836-3845.