# REGULATION OF BLOOD PLATELET FUNCTION BY THE AGC FAMILY OF PROTEIN KINASES.

AHMED A ABURIMA

SUBMITTED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF BIOMEDICAL SCIENCES

HULL YORK MEDICAL SCHOOL

2010

# Abstract

Upon vascular injury, platelets aggregate at the site of blood vessel injury to form a hemostatic plug maintaining the physiological integrity of the vascular system. Platelets respond to a variety of extracellular stimuli to undergo a rapid aggregation response, releasing active granule contents and leading to a rapidly growing thrombus. During the adhesion, activation, and aggregation of platelets at an injured site, the endothelium responds by limiting the size and growth of the hemostatic plug or thrombus, or even reversing platelet reactivity. These responses are defined as endothelial thromboregulation. There are three primary (and functionally independent) pathways during the early stages of thromboregulation by which the endothelium controls platelet reactivity (1) nitric oxide (NO); (2) prostacyclin ($PGI_2$); and (3) the ecto-nucleotidase CD39. NO and $PGI_2$ stimulate signalling cascades that result in the activation of the AGC family of Ser/Thr protein kinases (PKA, PKG and PKC). Once activated these kinase blunt platelet function through the phosphorylation of signalling proteins requested for activation. In this study, the role of AGC family kinases and their signaling cascades in regulating platelet function was assessed. The experimental data produced during this study demonstrate new insights in to the regulation of these kinases in platelets. More specifically it was found that

1. Peroxynitrite, a derivative of NO, regulated platelet function and particularly cytoskeletal rearrangement through PKC-dependent phosphorylation of $VASP^{Ser239/157}$
2. NO-mediated signalling in platelets had a requirement for PKC.
3. Multiple forms of PKA are present in platelets, which are differentially localised.
4. The potential regulation of platelet function by PKA is mediated through A-kinase anchoring proteins.
5. Lipid rafts may play an important role in platelet regulation by NO and PKG.

In summary, this studies present insights of the factors regulating AGC kinases in blood platelets.

# Publications

**Aburima, A.**, Riba, R., Naseem, K.M. (2010). "Peroxynitrite causes phosphorylation of vasodilator stimulated phosphoprotein through PKC-and PKG dependent mechanisms." Platelets. (2010) – *in press.*

Roberts W., S, Magwenzi, **Aburima A.**, and Naseem KM. Thrombinspondin-1 induces platelet activation through CD36-dependent inhibition of the cAMP/Protein kinase A signalling cascade. Blood. (2010) – *submitted.*

Roberts, W., A. Michno, **Aburima, A.**, Naseem, K.M. "Nitric oxide inhibits von Willebrand factor-mediated platelet adhesion and spreading through regulation of integrin alpha(IIIb)beta and myosin light chain." J Thromb. Haemost. (2009) 7: 2106-15.

Riba, R., B. Patel, **Aburima, A.**, Naseem, K.M. "Globular adiponectin increases cGMP formation in blood platelets independently of nitric oxide." J Thromb. Haemost. (2008). 6: 2121-31.

# Published abstracts

**Aburima A.A.**, Riba R., Naseem K.M. Nitric oxide induces vasodilator-stimulated phosphoprotein phosphorylation by protein kinase G-dependent and independent mechanisms: role of protein kinase C. Journal of Thrombosis and Haemostasis 2007; Volume 5, Supplement 2.

# Oral presentations

Peroxynitrite mediated phosphorylation of vasodilator phosphoprotein. **9th UK NO FORUM, (2008). King's College London.**

# Posters

Inhibition of platelet aggregation by peroxynitrite is associated with phosphorylation of vasodilator-stimulated phosphoprotein. **10$^{th}$ UK platelet meeting, (2008). University of Bradford.**

Platelet inhibition by peroxynitrite - a role for AMP activated protein kinase? **8th UK NO FORUM, (2007). University of Bradford.**

# Acknowledgments

I thank God almighty for giving me the strength to accomplish this work. I thank my family, my loving and caring parents for their love, patience and support all over the years, even when messed up so badly (sorry!!). I miss them heaps. I am deeply indebted to my supervisors Prof. K. Naseem and Dr R. Riba whose help, stimulating suggestions and encouragement made this PhD possible. I have been impossibly lucky to have met people in England who made me feel less alone, so a huge thank you. I thank all my friends for their encouragement whilst I am away and their friendship whilst at home. I would like to express my gratitude to all lab members Dr Wayne Roberts, Katie Wraith, Jonathan Wake and the three musketeers Simba (formerly known as Frank), Tanzeel (the Nerd) and Zaher (Mr. Muscles) for their friendship, support and ECL. Also, a zillion thank you to all volunteers and blood donors. Finally, I'm thankful for my scholarship and financial support from my country, **Libya**, which gave me the possibility to complete this work.

*Table of contents*

# Table of figures

# Abbreviations

| | |
|---|---|
| $\alpha_2\beta_1$ | Integrin alpha 1 beta 2 |
| $\alpha_{IIb}\beta_3$ | Integrin alpha IIb beta 3 |
| α-pY | Anti-phosphotyrosine |
| Ab | Antibody |
| Abs | Absorbance |
| AC | Adenylyl cyclase |
| ACD | Acid citrate dextrose |
| ADP | Adenosine diphosphate |
| AMP | Adenosine 5'-monophosphate |
| AMPk | AMP-activated protein kinase |
| AICAR | 5-aminoimidazole-4-carboxamide-1-β-d-ribofuranoside |
| APS | Ammonium persulfate |
| Apy | Apyrase |
| ATP | Adenosine triphosphate |
| BAPTA-AM | 1,2-bis (o-Aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetra(acetoxymethyl) ester |
| BSA | Bovine serum albumin |
| $Ca^{2+}$ | Calcium |
| CaM | Calmodulin |
| cAMP | Cyclic adenosine 3',5'-monophosphate |
| cGMP | Cyclic guanosine 3',5'-monophosphate |
| DAG | Diacylglycerol |

| | |
|---|---|
| DMSO | Dimethyl sulfoxide |
| ECL | Enhanced chemiluminescence |
| ECM | Extracellular matrix |
| EDTA | Ethylenediamine tetraacetic acid |
| EGTA | Ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid |
| eNOS | Endothelial nitric oxide synthase |
| FAD | Flavin adenine dinucleotide |
| FcR Y-chain | Fc receptor gamma-chain |
| $Fe^{2+}$ | Ferrous ion |
| FMN | Flavin adenine mononucleotide |
| GMP | Guanosine 5'-monophosphate |
| GP VI | Glycoprotein VI |
| GPIb-IX-V | Glycoprotein Ib-IX-V |
| GR | Glutathione reductase |
| GSNO | S-nitrosoglutathione |
| GSH | Glutathione |
| GSSG | Glutathione disulfide |
| $H_2O_2$ | Hydrogen peroxide |
| Hb | Haemoglobin |
| Hb-$O_2$ | Oxy-haemoblobin |
| HEPES | N-(2-Hydroxyethyl) piperayine-N'-(2-ethanesulfonic acid) |

| | |
|---|---|
| $HNO_2$ | Nitrous acid |
| HRP | Horseradish peroxidase |
| IB | Immunoblot |
| Ig | Immunoglobulin |
| IgG | Immunoglobulin G |
| Indo | Indomethacin |
| iNOS | Inducible nitric oxide synthase |
| $IP_3$ | Inositol (1,4,5)-triphosphate |
| IRAG | IP3 receptor associated PKG I substrate |
| ITAM | Immunoreceptor tyrosine-based activation motif |
| MAPK | Mitogen-activated protein kinase |
| MβCD | Methyl beta-cyclodextrin |
| $Mg^{2+}$ | Magnesium |
| MIDAS | Metal ion-dependent adhesion site |
| $Mn^{2+}$ | Manganese |
| mNOS | Mitochondrial nitric oxide synthase |
| MoAb | Monoclonal antibody |
| $NADP^+$ | Nicotinamide adenine dinucleotide phosphate (oxidised form) |
| NADPH | Nicotinamide adenine dinucleotide phosphate (reduced form) |
| nNOS | Neuronal nitric oxide synthase |
| NO | Nitric oxide |

| | |
|---|---|
| $NO_2$ | Nitrogen dioxide |
| $NO_2^-$ | Nitrite |
| $NO_3^-$ | Nitrate |
| NOS | Nitric oxide synthase |
| $O_2^{\bullet-}$ | Superoxide anion |
| $OH^{\bullet}$ | Hydroxyl ion |
| $ONOO^-$ | Peroxynitrite |
| PBS | Phosphate buffered saline |
| PDE | Phosphodiesterase |
| $PGE_1$ | Prostaglandin $E_1$ |
| $PGI_2$ | Prostacyclin $I_2$ |
| PI3 kinase | Phosphoinositol 3 kinase |
| PKA | Protein kinase A |
| PKC | Protein kinase C |
| PKG | Protein kinase G |
| $PLA_2$ | Phospholipase $A_2$ |
| PLCβ | Phospholipase C beta |
| PLCγ2 | Phospholipase C gamma 2 |
| PMSF | Phenyl methyl sulphonyl fluoride |
| PRP | Platelet rich plasma |
| PVDF | Polyvinylidene difluoride |
| RGDS | Arginine-glycine-aspartic acid-serine |

| | |
|---|---|
| SDS | Sodium dodecyl sulphate |
| SDS-PAGE electrophoresis | Sodium dodecyl sulphate-polyacrylamide gel |
| SEM | Standard error of the mean |
| sGC | Soluble guanylyl cyclase |
| SH2 | Src homology 2 domain |
| TBS | Tris buffered saline |
| TEMED | N,N,N',N'-Tetramethyl ethylenediamine |
| $TxA_2$ | Thromboxane $A_2$ |
| VASP | Vasodilator stimulated phosphoprotein |
| vWF | von Willebrand factor |
| WCL | Whole cell lysate |
| Wort | Wortmannin |
| WP | Washed platelets |
| 4G-10 | Phosphotyrosine |

# Chapter 1 INTRODUCTION

## 1.1 Overview.

Despite impressive medical advances that have led to diminished cardiovascular death rates in some countries over the past 20 years, cardiovascular disease (CVD) remains the leading cause of death in developed countries (Jamison *et al.*, 1991). This promises to worsen as a result of aging populations, increasing obesity, type II diabetes epidemic, sedentary lifestyle, and continued abuse of alcohol and tobacco. The pathology that underlies most CVD is atherosclerosis, a chronic inflammatory process, which is multifactorial in origin. At the cellular level atherosclerosis involves endothelial cells, neutrophils, platelets, numerous cytokines and chemical messengers (Packard *et al.*, 2008). The importance of platelets in the thrombotic process is demonstrated by the clinical success of anti-platelet drugs such as aspirin and clopidogrel in reducing CVD mortality (Weiss, 2003; Weiss *et al.*, 1967; Zucker *et al.*, 1968).

## 1.2 Platelet production and structure.

### 1.2.1 Megakaryocytes.

Megakaryocytes are highly specialized precursor cells that function solely to produce and release platelets into the circulation. Like any other cells in blood, megakaryocytes develop from hematopoietic stem cells, which in adults, reside primarily in bone marrow (Golde, 1991; Ogawa, 1993). Megakaryocytes, which can be distinguished by the expression of CD61 (integrin $\beta_3$) and elevated levels of CD41 (integrin $\alpha_2$) (Vainchenker *et al.*, 1988), undergo endomitosis and become polyploidy through repeated cycles

of DNA replication without cell division (Ebbe *et al.*, 1965; Odell *et al.*, 1968). After the process of endomitosis is completed, the megakaryocytes begin a maturation stage in which the cytoplasm rapidly fills with platelet-specific proteins, organelles, and membrane systems that will ultimately be subdivided and packaged into platelets. The production of approximately 35 million platelets per liter per day is the end process of thrombopoiesis (Harker *et al.*, 1969).

### 1.2.2 Platelets.

Platelets are the smallest of the many types of cells in circulating blood. In the quiescent state, platelets are discoid and have a smooth, rippled surface averaging only 2.0 to 5.0 μm in diameter, 0.5 μm in thickness, and having a mean cell volume of 6 to 10 femtoliters (Bessis *et al.*, 1973). The normal platelet count is in the range of 150-350 $x10^9$ platelets/L. However individuals with platelet count as low as 10 x $10^9$ platelets/L tend to exhibit only occasional major spontaneous bleeds, although they are at considerable risk of bleeding during major trauma (Hoffbrand *et al.*, 2005). Their shape and small size enables platelets to be pushed to the edge of vessel walls during blood flow, placing them next to the endothelium and in the right place to respond to vascular damage.

Platelets are anucleated, which is consistent with their short lifespan of 10 days and their acute role in haemostasis. Hence platelets lack nucleus, they cannot adapt to different situations by protein synthesis, although there is some evidence for residual protein synthetic capacity from messenger RNA (mRNA) carried over from megakaryocytes (Jandrot-Perrus *et al.*, 2000).

### 1.2.3 Platelet structure.

Plasma membrane is coated with a layer of lipids, sugars, and proteins termed *glycocalyx*, the overall appearance does not differ from other cell types. However, it is exceptionally complex in composition, distribution, and function, incorporating a high number of glycoproteins and lipid rafts (Behnke, 1968). Uncharged phospholipids, such as phosphatidylcholine and sphingomyelin, are mainly present in the outer leaflet of the bilayer, whereas the inner leaflet contains the negatively charged aminophospholipids phosphatidylserine (PS) and phosphatidylethanolamine (PE) (Schroit *et al.*, 1991). During platelet activation, this distribution becomes disrupted, phospholipids are scrambled, and PS and PE become exposed on the cell surface (Bevers *et al.*, 1983).which facilitates the activation of the coagulation cascade (Zwaal *et al.*, 1998).

Surface-Connected Canalicular System (*SCCS),* also called the *open canalicular system*, is part of the surface plasma membrane weaving through the entire platelet cytoplasm. SCCS functions as internal reservoir of membrane that facilitates platelet spreading, filopodia formation and granule release (JG, 1974). The dense tubular system (DTS) are believed to be residual smooth endoplasmic reticulum from the megakaryocyte and is the site for numerous metabolic processes including thromboxane generation (JG, 1974) **(figure 1.1)**.

Immediately below the membrane is the platelet cytoskeleton. The discoid shape of platelets and their ability to contract and spread depend on this cytoplasmic framework of monomers, filaments, and tubules. Critical

components of the cytoskeleton are, from the plasma membrane inward, a spectrin based skeleton that is adherent to the cytoplasmic side of the plasma membrane (Fox *et al.*, 1988), a microtubule coil that runs along the perimeter of the disc and hence lines the thin axis of the cell, and a rigid network of crosslinked actin filaments, crosslinked by filamin, which provides the major membrane–cytoskeletal connection linking actin to the cytoplasmic tail of the GPIbα chain of GPIb-IX-V complex (Kenney *et al.*, 1985). Platelets contain a single microtubule that is approximately 100 μm in length, that spiraled into a coil sits in the cytoplasm, just beneath the plasma membrane, along the thin edge of each disc. Each microtubule is composed of 13 stacks of αβ-tubulin subunits, each arranged in linear head-to-tail aggregates called protofilaments *(*Michelson, 2006*)*.

In addition to the tubular systems, platelets contain three main types of storage granules: α-granules, dense granules, and lysosomes the contents of α-granules and dense granules each are released **(Table 1.1)** upon activation. Platelets also contain mitochondria and glycogen storage, providing the energy required for their short activation.

| α granules | Dense granules |
|---|---|
| Fibrinogen | Adenosine diphosphate ADP |
| Factor V | Adenosine triphosphate ATP |
| P-selectin | Serotonin |
| vWF | Calcium |
| Thrombospondin | |

***Table 1.1. Major Platelet Granular Constituents Secreted with Activation.***

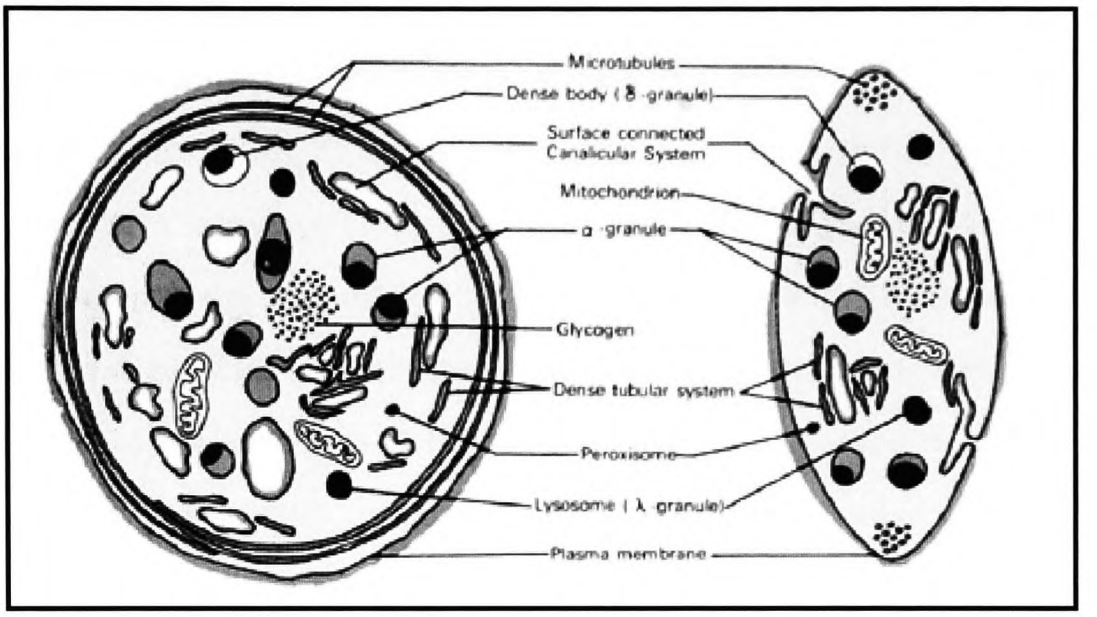



***Figure 1.1. Schematic diagram of platelet structure.***

*Courtesy of (Bentfeld-Barker et al., 1982)*

## 1.3 Physiological roles of platelets.

The major function of platelets is to arrest blood loss after vascular damage, a process termed haemostasis. Among the first, and still most compelling, evidence that platelets are crucial for human haemostasis is that platelet transfusion can restore haemostatic competence to individuals with low platelet counts (Duke, 1910). In normal conditions, platelets flow in blood vessels in a quiescent state, prevented from unnecessary activation by endothelium-derived prostacyclin ($PGI_2$) and nitric oxide (NO), whose net effect is to suppress the intracellular signaling needed for platelet activation by rising cyclic adenosine mono-phosphate (cAMP) and cyclic guanosine mono-phosphate (cGMP) respectively (Brass, 2003). As a further barrier to platelet activation, endothelial cells express ecto-ADPase (CD39) on their luminal surface. CD39 can hydrolyze small quantities of the platelet agonist adenosine diphosphate (ADP), which is released from damaged red blood cells and activated platelets, thus preventing the ADP from activating additional platelets (Marcus *et al.*, 1997). Upon vascular damage, platelets first interact with elements of the blood vessel wall and subsequently with other platelets. In order to perform these functions platelets possess numerous cell surface receptors and are rich in signaling proteins allowing them to respond to changes in their environment.

Platelet receptors determine the activity of platelets with a wide range of agonists and adhesive proteins. These receptors are broadly classified and some of those have become research disciplines in their own right. Listing all of these receptors is behind the scope of this introduction. However, three distinct families of surface glycoproteins which signal through Src family

tyrosine kinases are known to be present. (1) ITAM receptors, GPVI and FcγRIIA. (2) Integrins include $\alpha_{IIb}\beta_3$ and $\alpha_2\beta_1$ (3) leucine-rich repeat receptors include GPIb-IX-V complex (Hoffbrand *et al.*, 2005). Platelets also express G-Protein Coupled Receptors (GPCR). GPCRs are so-named because they are physically associated with heterotrimeric G proteins, α, β, and γ. (Wong *et al.*, 1990). All GPCRs share a common structure of seven transmembrane-spanning domains, with an extracellular N-terminus and an intracellular C-terminus (Kroeze *et al.*, 2003). GPCRs include $P_2Y_{1/12}$ , ADP receptor, and $PAR_1$ and $PAR_4$ , thrombin receptors (Woulfe, 2005).

## 1.4 Haemostasis.

Platelet plug formation occurs in three overlapping stages; initiation, extension, and perpetuation **(figure 1.2)**. Initiation may occur when circulating platelets are captured and then activated by exposed collagen and von Willebrand factor (vWF), allowing the accumulation of a platelet monolayer that will subsequently support thrombin generation and the formation of platelet aggregates. This is made possible by the presence of receptors on platelet surface that can bind to collagen [integrin $\alpha_2\beta_1$ and glycoprotein (GPVI)] and vWF (GPIbα and $\alpha_{IIb}\beta_3$). Extension occurs when additional platelets are recruited into the initial monolayer. Key to this is the presence of platelet receptors that can respond rapidly to soluble agonists such as thrombin, ADP, and thromboxane $A_2$ ($TxA_2$).

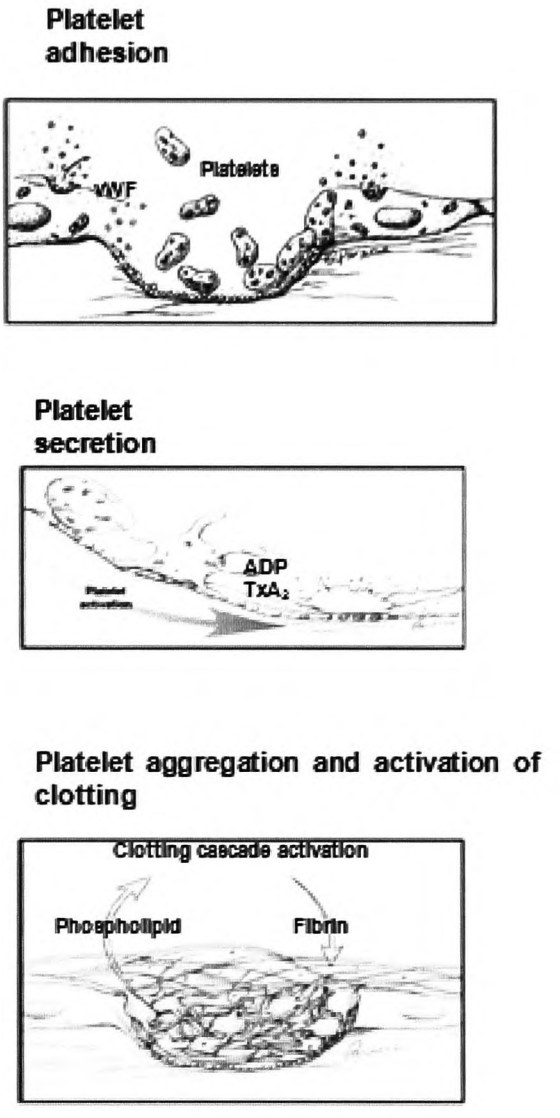



***Figure 1.2. Overview of the three main platelet functions, adhesion, secretion and aggregation. ADP, adensosine diphosphate. $TxA_2$, thromboxane. vWF, von Willebrand factor.***

*Courtesy of (Kickler, 2006).*

## 1.4.1 Platelet activation and adhesion.

The extracellular matrix contains numerous proteins that are thrombogenic, that is, they trap and activate platelets. Of these proteins, collagen and von Willebrand factor (vWF) are the most important.

### 1.4.1.1 Role of von Willebrand factor *(VWF)* in platelet activation.

Endothelial damage exposes the extracellular matrix protein collagen, which is a potent platelet agonist. Following exposure to collagen, platelets rapidly adhere, spread, become active, and then aggregate (Rauterberg *et al.*, 1993), during which platelets change their shape from discoid to spherical with the extrusion of the pseudopodia (JG, 1974). This interaction of collagen with platelets is both direct and indirect. Under the high shear stress conditions found in small arteries, von Willebrand factor (vWF), which binds to newly exposed collagen fibers, is required to capture flowing platelets (Savage *et al.*, 1996), via the GPIb-IX-V complex on the platelet surface or to the integrin $\alpha_{IIb}\beta_3$ in its activated conformation. The importance of GPIb-IX-V interaction with vWF in normal haemostasis is documented by the severe bleeding disorders derived from the lack of either GPIb-IX-V (Bernard-Soulier syndrome) (Clemetson *et al.*, 1982), or vWF (von Willebrand disease) (Von Willebrand, 1926).

In addition to mediate the initial platelet arrest on damaged vessel wall and participate in thrombus formation, GPIb-IX-V interaction with vWF promotes $\alpha_{IIb}\beta_3$ activation and aggregation (Chow *et al.*, 1992; Sakariassen *et al.*, 1986).

### 1.4.1.2 Role of collagen in platelet activation.

Collagens represent up to 40% of the total protein of the vessel wall, forming an insoluble scaffold which is essential for tissue integrity and which provides a surface for the attachment of other matrix constituents and for the adhesion of vascular cells (Farndale *et al.*, 2004b). At least 25 different types of collagen exist, a number of which, including major widely distributed types I, III, IV, V and VI, occur in the vessel wall (Barnes *et al.*, 1999). Collagen contains three polypeptide (α) chains, displaying an extended polyproline-II conformation, a right handed supercoil and a one-residue stagger between adjacent chains (Brodsky *et al.*, 2005). The three α chains are held together by interchain hydrogen bonds, and each polypeptide chain has a repeating Gly-X-Y triplet in which glycyl residues occupy every third position and the X and Y positions are frequently occupied by proline and 4-hydroxyproline, respectively (Kadler *et al.*, 2007), with Gly–Pro–Hyp (GPO) as the most frequent, forming about 10% of the primary structure of collagen types I and III (Baum *et al.*, 1999).

The binding of collagen, via GPO repeat sequences, to GPVI on the platelet surface causes the clustering of GPVI and its associated FcRγ-chain, a trans-membrane protein containing an immunoceptor tyrosine activation motif (ITAM). This leads to the phosphorylation of the γ-chain by tyrosine kinases in the Src family, creating a tandem phosphotyrosine motif that is recognized by the SH2 domain of spleen associated tyrosine kinase (Syk) (Gross *et al.*, 1999b) **(figure1.3)**. Association of Syk with GPVI/γ-chain activates Syk and leads to the phosphorylation and activation of the $\gamma_2$ isoform of phospholipase C ($PLC\gamma_2$) via the adaptor protein, SLP-76 (Gross

*et al.*, 1999a). Studies using Syk deficient mice have demonstrated a pivotal role for Syk in signalling downstream of ITAM receptors (Turner *et al.*, 2000). PLC$\gamma_2$ in turn hydrolyzes PI-4,5-P2 to produce 1,4,5-IP3 and diacylglycerol (DAG), raising the cytosolic- free $Ca^{2+}$ concentration within the adherent platelets by releasing $Ca^{2+}$ stores from within the dense tubular system and activating protein kinase C (PKC) (Brass, 2003).

In resting platelets the cytosolic $Ca^{2+}$ concentration is maintained at approximately 100nM by limiting $Ca^{2+}$ influx and by pumping $Ca^{2+}$ out of the cytosol across the plasma membrane or into the dense tubular system. This creates a steep $Ca^{2+}$ gradient across the plasma membrane. Once formed, 1, 4 , 5-IP3 releases $Ca^{+2}$ from the dense tubular system, which in turn opens $Ca^{2+}$ influx channels in the plasma membrane, extracellular $Ca^{2+}$ then pours in, following its concentration gradient, increasing the cytosolic $Ca^{2+}$ concentration to up to 1μM (Michelson, 2006), depending on the potency of the agonist. The rising $Ca^{2+}$ concentration in activated platelets is undoubtedly a critical trigger for numerous events, such as the $Ca^{2+}$ - dependent activation of the Ras family member, $Rap_{1b}$, which has been shown to be an important contributor to signaling pathways upon the activation of $\alpha_{IIb}\beta_3$ (Bertoni *et al.*, 2002; Chrzanowska-Wodnicka *et al.*, 2005), and $Ca^{2+}$-dependent reorganization of the actin cytoskeleton via activation of myosin light chain kinase (MLCk) downstream of $G_q$ family members (Wettschureck *et al.*, 2002), and some isoforms of PKC (Pula *et al.*, 2006; Tabuchi *et al.*, 2003).

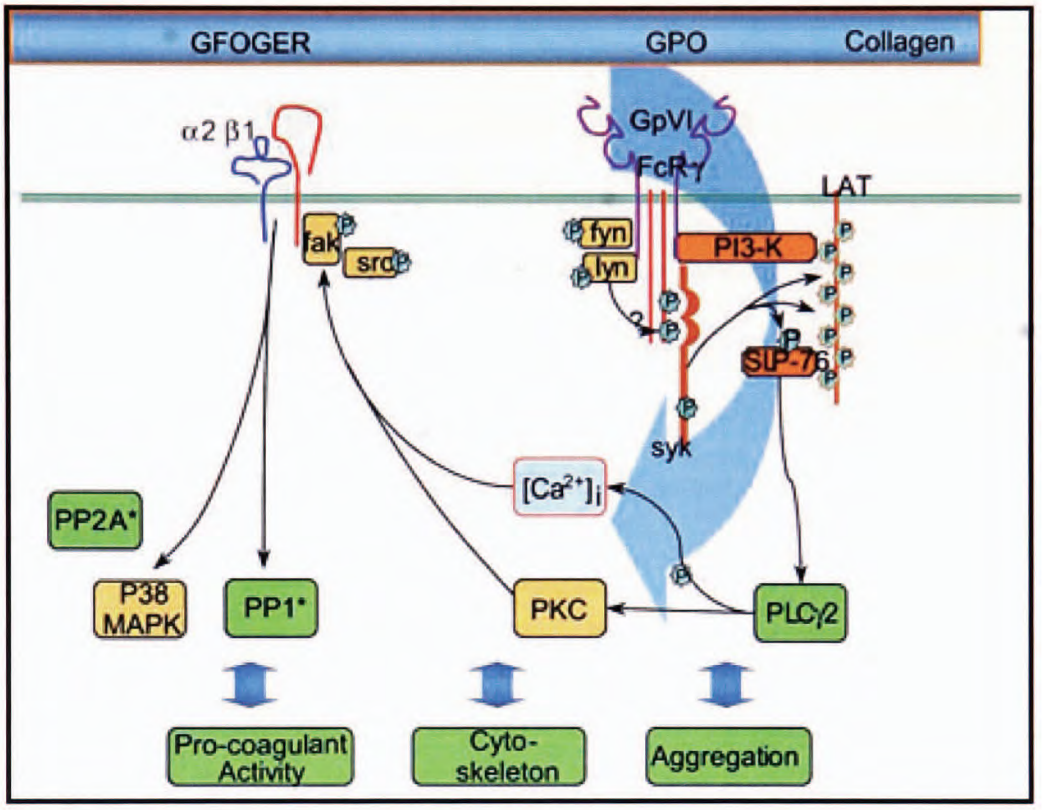



***Figure 1.3. Signaling through collagen receptors.***

***The major axis of collagen signaling is through GPVI, directed towards mobilization of Ca2+, like all strong platelet agonists. A series of adapter proteins mediate this process, with the multiply phosphorylated LAT acting as a crucial docking site upon which a signaling complex can assemble.*** *Courtesy of (Farndale et al., 2004a).*

Recent studies addressed the question of how $\alpha_2\beta_1$ and the GPVI/FcRγ complex are involved in collagen signaling. For example, Zhen (Zheng *et al.*, 2001) reconstituted the GPVI–FcRγ complex in RBL- 2H3 cells and found that the complex-expressing cells had strong adhesive and signaling responses to convulxin (Cvx), a snake venom protein that is a GPVI-specific agonist (Polgar *et al.*, 1997), and weak responsiveness to collagen- related peptide (CRP) but no response to collagen, suggesting that the direct binding of platelets to collagen should be mediated by $\alpha_2\beta_1$ rather than by the GPVI–FcRγ complex. The adhesion of platelets to GFOGER peptides is accompanied by the tyrosine phosphorylation of several proteins, including Src, Syk, SLP-76 and $PLC_{\gamma 2}$, which are also involved in GPVI signaling, and $Ca^{2+}$-dependent spreading (Inoue *et al.*, 2003). P38 MAP kinase, ILK, Rac and PAK have also been implicated downstream of $\alpha_2\beta_1$ ligation (Stevens *et al.*, 2004; Sundaresan, 2003; Suzuki-Inoue *et al.*, 2001). On the other hand Nieswandt and colleagues (Nieswandt *et al.*, 2001), performed functional studies using $\beta_1$-null or VI deficient mouse platelets and indicated that GPVI–collagen interaction is an essential prerequisite for integrin-mediated firm adhesion followed by platelet thrombus formation. Research focusing on the stimulation of tyrosine kinase signaling in platelets in suspension indicated initially that integrin $\alpha_2\beta_1$ does not engage in outside-in signaling (Hers *et al.*, 2000).

Following adhesion and activation of platelets, additional platelets from the blood are recruited into the growing platelet plug. This is made possible by the release of soluble agonists mainly thrombin, which is generated locally

from prothrombin once tissue factor has been exposed (a process facilitated by the negatively charged phospholipids on the surface of activated platelets), ADP, along with ATP, is stored within platelet dense granules and secreted upon platelets activation, epinephrine, and $TxA_2$.

### 1.4.2 Platelet shape change.

When platelets adhere to the subendothelial matrix they undergo a series of shape changes, first rounding, then projecting filopodia, and finally spreading (Allen *et al.*, 1979). The shape change starts with disassembly of the existing actin filament network followed by reorganization of the actin into new structures in different locations within the cell (Bearer, 1995). This actin reorganization is regulated by the interplay between many different actin binding proteins, of which gelsolin and vaso-dilator-stimulated phosphoprotein (VASP) are among the most abundant (Laurent *et al.*, 1999; Loscalzo *et al.*, 2002).

Gelsolin, which is activated by the elevated $Ca^{2+}$ concentrations, binds actin filament and sever it and cap the newly formed barbed ends. The rising $PIP_2$ levels inactivate both gelsolin and CapZ, removing them from the filament plus ends. Addition at the barbed end is facilitated by mainly profilin, which acts to shuttle actin subunits to actin filament barbed ends, causing the activated platelet to extend lamellipodia and filopodia. Once the $PIP_2$ signal subsides, the barbed ends are recapped primarily by CapZ, rendering them stable against depolymerization and locking the platelet into its spread form (Alberts *et al.*, 2002; Michelson, 2006) **(figure 1.4)**.

In resting platelets, VASP binds to and stabilizes actin filaments, preventing them from being disassembled by severing. Upon activation, VASP releases the filaments, which are then rapidly severed, causing the platelet to lose its discoid shape and round up (Bearer *et al.*, 2000).

While platelets can adhere to damaged endothelial cells, their principle adhesive surface is the extracellular matrix (ECM), which becomes exposed in injured vessels and offers a panoply of ligands for platelet adhesion receptors. Within this context, integrin adhesion receptors play critical roles in platelet function (Ruggeri, 2002a). Integrins are a family of heterodimeric proteins, composed of non-covalently associated α and β-subunits. Each subunit consists of a large extracellular domain, a single-span transmembrane domain, and a short cytoplasmic domain (or tail) composed of roughly 20–60 amino acids (Hynes, 2002).

Integrins bind to insoluble ligands (E.G. collagen fibrils) and link them to the intracellular cytoskeleton. In addition to forming these physical linkages, integrins regulate cell growth, survival, and differentiation (Hynes, 1992). Talin, an abundant cytosolic protein, is capable of linking integrins to the actin cytoskeleton either directly or indirectly via its interactions with vinculin and α-actinin (Burridge *et al.*, 1996; Otey *et al.*, 1990; Rees *et al.*, 1990). Talin cleavage by calpain, which itself becomes activated as a consequence of increases in cytosolic $Ca^{2+}$, is detected within activated platelets (Inomata *et al.*, 1996; Martel *et al.*, 2001).

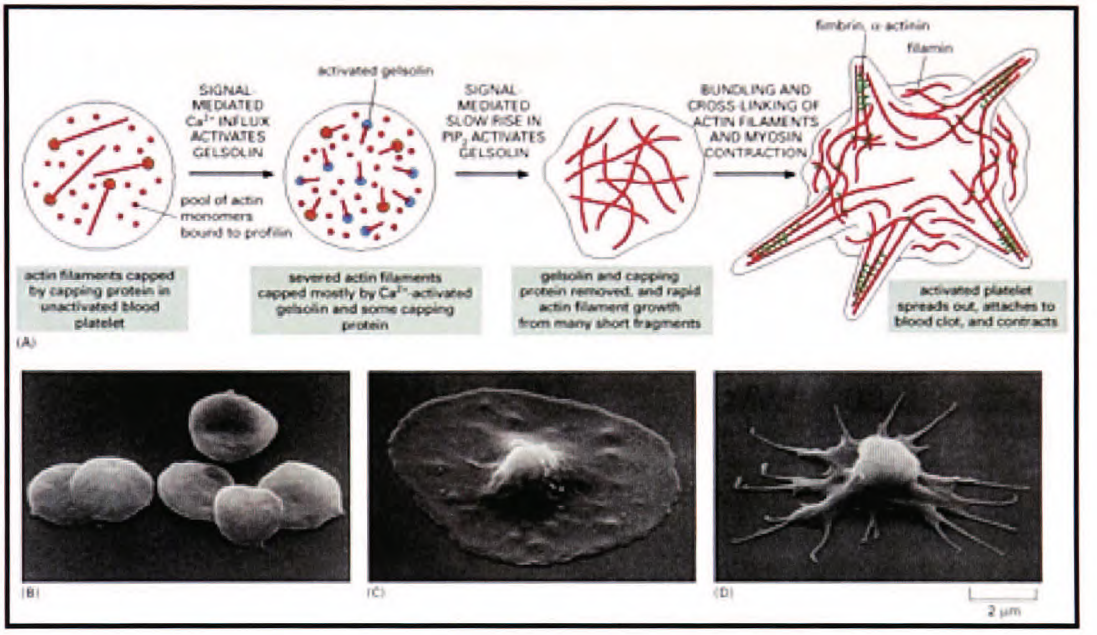



***Figure 1.4. Platelet shape change.***

***(A) Platelet activation is a controlled sequence of actin filament severing, uncapping, elongation, recapping, and cross-linking that creates a dramatic shape change in the platelet. (B) Scanning electron micrograph of platelets prior to activation. (C) An activated platelet with its large spread lamellipodia. (D) An activated platelet at a later stage than the one shown in C, after myosin II-mediated contraction.*** *Courtesy of (Alberts et al., 2002).*

### 1.4.3 Platelet secretion.

Platelet secretion is a mechanism to amplify the activation response and recruit additional platelets to the site of clotting. Secretion involves reorganization of the actin structure, the movement of granules into close physical apposition with the plasma membrane, granule-plasma membrane fusion, and release of intracellular contents (Flaumenhaft *et al.*, 2005; White, 1974), a process that occurs through a SNARE proteins-dependent mechanism, and tightly regulated by intracellular $Ca^{2+}$ levels and activated PKC (Konopatskaya *et al.*, 2009b; Schraw *et al.*, 2003). The degranulation acts to increase the bioavailability of factors required for platelet activation including $Ca^{2+}$, ADP and fibrinogen.

### 1.4.4 Role of soluble agonists in platelet activation.

A number of soluble agonists that are released from platelets or generated at the site of vascular damage act to amplify platelet activation. These include ADP, $TxA_2$ and thrombin. The importance of these agonists is explained by the clinical success of aspirin and clopidogrel, which target $TxA_2$ generation and ADP, respectively, in reducing CVD mortality (Harker *et al.*, 1998; Herbert *et al.*, 1998)

#### 1.4.4.1 Role of ADP in platelet activation.

ADP is stored in platelets dense granules and released upon platelet activation, and is also released from damaged red blood cells. ADP activates platelets by G-protein coupled receptors GPCRs termed $P_2Y_1$ and $P_2Y_{12}$ , which are coupled to $G_q$ and $G_i$ respectively (Daniel *et al.*, 1998; Dorsam *et al.*, 2004; Hechler *et al.*, 1998; Jin *et al.*, 1998; Kamae *et al.*, 2006).

Interaction of ADP with the $P_2Y_1$ $G_{\alpha q}$-coupled receptor leads to intracellular $Ca^{2+}$ release as well as RhoA activation, leading to thromboxane $A_2$ generation and platelet shape change (Kunapuli *et al.*, 2003). Further, $P_2Y_{12}$ activation by ADP initiates $G_i$ protein signaling leading to the inhibition of adenylyl cyclase and the potentiation of dense granule secretion (Kunapuli *et al.*, 2003). Some evidence exists that $P_2Y_{12}$ signaling leads to an augmentation of $P_2Y_1$-induced $Ca^{2+}$ signaling, although $P_2Y_{12}$ signaling does not seem to initiate $Ca^{2+}$ mobilization independently of $P_2Y_1$ (Hardy *et al.*, 2004).

### 1.4.4.2 Role of thromboxane in platelet activation.

Thromboxane ($TxA_2$) is derived from arachidonic acid (AA), which is cleaved from membrane phospholipids by the enzymatic activity of $PLA_2$ upon platelet activation. AA is further catalyzed by cyclooxygenase (COX) to a labile intermediate peroxides, $PGG_2$, which are further reduced to the corresponding alcohol, $PGH_2$, by the enzyme's hydroperoxidase (HOX) activity. $PGH_2$ is subject to further metabolism by thromboxane-A synthase to thromboxane $A_2$ (Diczfalusy *et al.*, 1977; Hsu *et al.*, 1999; Needleman *et al.*, 1976).

Platelets only have the A-type $TxA_2$ receptor (Hirata *et al.*, 1991). The $TxA_2$ receptor is coupled to signal transduction via several G proteins including $G_q$, and $G_{12/13}$ (Offermanns *et al.*, 1994), which activate phospholipase C to increase intracellular calcium and activates PKC-dependent pathways, which facilitate platelet aggregation, whereas $G_{12}/G_{13}$-mediated Rho/Rho-kinase-dependent regulation of myosin light chain phosphorylation participates in

receptor-induced platelet shape change (Klages *et al.*, 1999). $TxA_2$ receptor agonists induce tyrosine phosphorylation of several signaling proteins, including Syk (Maeda *et al.*, 1995).

**1.4.4.3 Role of thrombin in platelet activation.**

The serine protease, thrombin, is the end product of the plasma coagulation cascade of sequential Zymogen-to-Thrombin steps that requires the assembly of a prothrombinase complex comprised of prothrombin, coagulation factor Xa, calcium ions, and the active cofactor Va on the surface of a cellular phospholipid membrane (Mann *et al.*, 1988). Thrombin acts via cell surface Protease Activated Receptors (PARs). Four PARs (PAR-1, -2, -3, -4) are identified, of which $PAR_1$ and $PAR_4$ are identified in human. mouse platelets express $PAR_3$ and $PAR_4$, however signaling appears to be mediated solely through $PAR_4$ (Sambrano *et al.*, 2001).

PARs are G-protein coupled receptors, which couples to $G_{q\alpha}$ and $G_{12\alpha}$ / $G_{13\alpha}$, and activated by a proteolytic cleavage in an extracellular loop by thrombin. Once activated, this leads to the activation of $PLC_\beta$, PI 3-kinase, and the monomeric G proteins, Rho, Rac, and $Rap_1$, and also causing increase in the cytosolic $Ca^{2+}$ concentration and inhibiting cAMP formation. This process is supported by released ADP and $TxA_2$, which bind in turn to their GPCRs on the platelet surface (Brass, 2003).

One of the properties that set thrombin receptors apart from most other G-protein-coupled receptors is their inability to be activated by thrombin more than once. This is thought to be due in part to receptor phosphorylation and in part to the apparent inability of thrombin to reactivate cleaved receptors

(Brass *et al.*, 1994; Ishii *et al.*, 1994). Binding studies have identified a high affinity binding site for thrombin on $GPIb_{\alpha}$ (Harmon *et al.*, 1986) that overlap vWF binding site (Andrews *et al.*, 1999). Deletion of the extracellular domain of $GPIb_{\alpha}$ or blockade of the thrombin-binding site decreases platelet responses to, and Platelets from Bernard Soulier syndrome patients show an impaired response to thrombin (De Candia *et al.*, 2001; De Marco *et al.*, 1991). In addition, the GPIb-IX-V complex has a platelet-specific thrombin substrate, GPV, that is cleaved very early during thrombin-induced platelet aggregation (Berndt *et al.*, 1981). However, efforts to demonstrate any signaling significance were not entirely successful (Ramakrishnan *et al.*, 2001).

### 1.4.5 Platelet aggregation.

The capacity of platelets to form a thrombus depends on their ability to aggregate. At a molecular level, platelet aggregation is mediated by a specific receptor on the platelet surface, $\alpha_{IIb}\beta_3$, through what is commonly known as "inside-out" signaling. Inside-out signaling can be initiated by the engagement of various adhesion or G-protein coupled receptors, which are coupled to second messengers such as $Ca^{2+}$, nucleotides, phospholipases, and protein kinases (Nieswandt *et al.*, 2003). For example, PKC, phosphatidylinositol 3–kinase (PI3k), and $Rap_{1b}$ have been implicated as intermediates in promoting inside-out signalling, however the identities and activities of the relevant effectors of these enzymes remain unclear (Soriani *et al.*, 2006) (Chrzanowska-Wodnicka *et al.*, 2005), Integrin linked kinase (ILK), a serine/threonine kinase downstream of PI3k that interacts with the

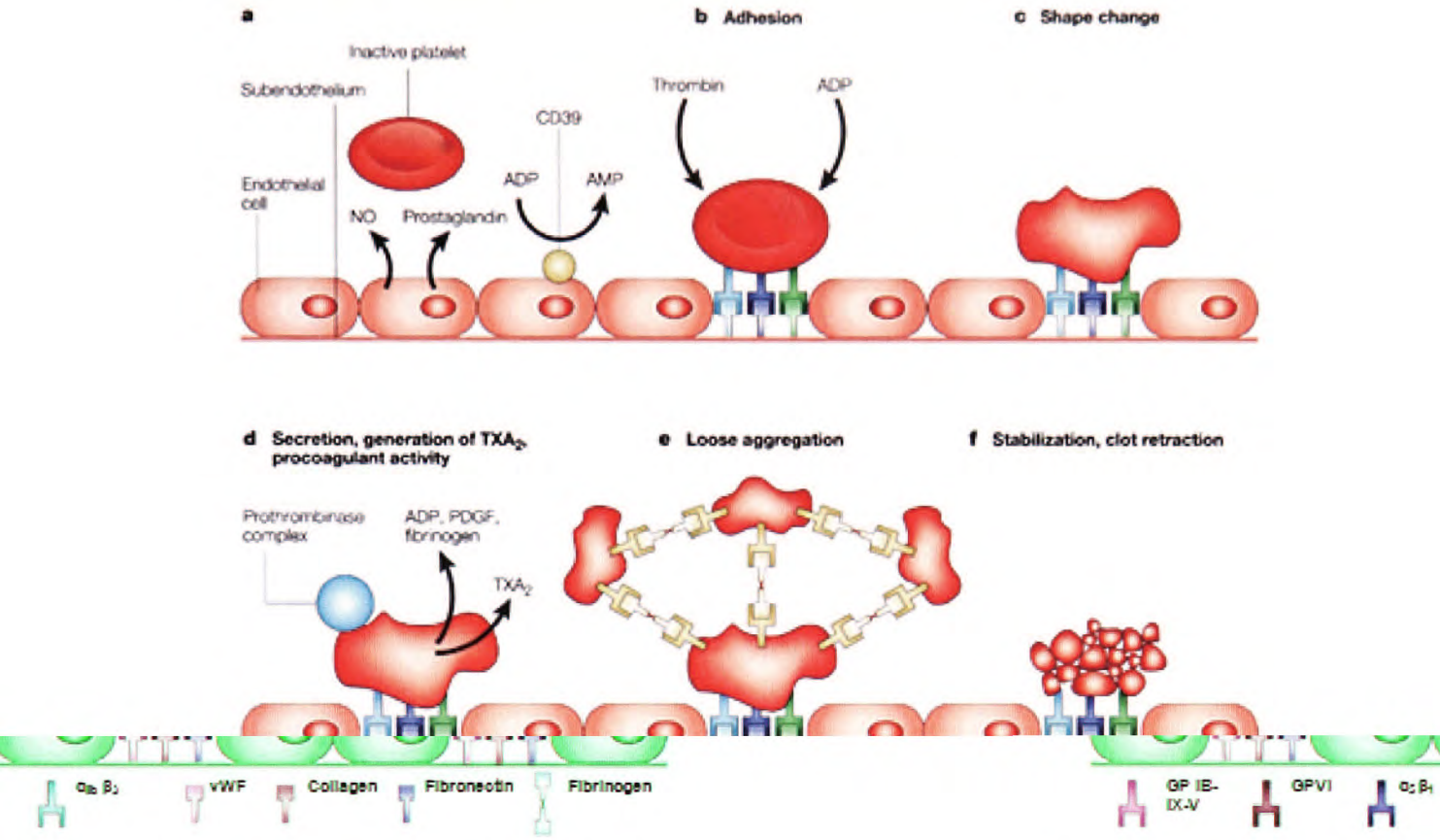
cytoplasmic tails of $\beta_1$ and $\beta_3$ integrin subunits (Hannigan *et al.*, 1996); thus, ILK in platelets is probably important for both outside-in and inside-out signaling by the integrins $\alpha_2\beta_1$ and $\alpha_{IIb}\beta_3$ (Stevens *et al.*, 2004; Yamaji *et al.*, 2002).

Once activated $\alpha_{IIb}\beta_3$ binds to its major physiological ligand, the plasma protein fibrinogen, or vWF to promote the formation of stable platelet/platelet bridges and prevent premature disaggregation. Occupancy of $\alpha_{IIb}\beta_3$ causes integrin microclustering (Buensuceso *et al.*, 2003; Loftus *et al.*, 1984) which appears necessary for tyrosine kinase dependent "outside-out" signaling (Hato *et al.*, 1998). Src kinases which is constitutively bound to the $\beta_3$ cytoplasmic tail becomes activated (Arias-Salgado *et al.*, 2003; Obergfell *et al.*, 2002) . Syk is recruited to the $\beta_3$ tail and become activated by Src (Obergfell *et al.*, 2002; Woodside *et al.*, 2001). Several substrates are phosphorylated downstream including SLP-76, ADAP, c-Cbl (molecular adaptors), and Vav (a Rac GTPase), that are implicated in signalling to the actin cytoskeleton (Miranti *et al.*, 1998; Obergfell *et al.*, 2001).

In contrast to GPVI signalling, the mechanism of activation of Syk by integrin $\alpha_{IIb}\beta_3$, which lack an ITAM, is controversial (Shattil *et al.*, 1998). It was originally proposed that integrin $\alpha_{IIb}\beta_3$ signalling proceeds independently of receptor tyrosine phosphorylation (Woodside *et al.*, 2002). However, a subsequent study provided evidence that the phosphotyrosine-binding capacity of Syk is required for activation by integrins, possibly via an unidentified ITAM-containing protein (Abtahian *et al.*, 2006). Indeed, it has recently been shown that the low affinity Fc receptor, FcγRIIA, couples integrin $\alpha_{IIb}\beta_3$ to downstream signaling events in human platelets (Boylan *et*

*al.*, 2008). It thus seems that $\alpha_{IIb}\beta_3$ signals through both ITAM-dependent and ITAM-independent regulation of Syk.

After platelet activation and aggregation have occurred in response to a vascular lesion, processes take place that consolidate the stability of the forming thrombus. An example of the advantage of binding to fibrinogen is anchoring aggregated platelets to the site of vascular injury, thus preventing downstream embolization under the effects of flow (Ni *et al.*, 2000).




*agram illustrates the role of platelets in*

*Figure 1.5. Schematic dia*

*thrombus formation.*

*e usually kept in an inactive state by PGI2*

*lothelial cells. Endothelial cells also express*

*ch inhibits platelet activation by converting*

*ites where the blood vessel wall has been*

*re to the exposed subendothelium through*

*gen, von Willebrand factor and fibronectin*

*platelets,GPVI, GPIb-IX-V and integrin α5β1,*

*and ADP cause platelets to change into an*

*Activated platelets secrete ADP, platelet-*

*d fibrinogen from storage granules in the*

*(a) Circulating platelets are*

*and NO released by the end*

*CD39 on their surface, whi*

*ADP into AMP. (b, c) At si*

*injured, the platelets adher*

*interactions between collag*

*and their receptors on the*

*respectively. Both thrombin*

*active conformation. (d)*

*derived growth factor, and*

***platelet, and thromboxane A2 (TxA2), produced by immediate biosynthesis. ADP and TxA2 cause circulating platelets to change shape and become activated. (e) $\alpha I_{Ib}\beta_3$ receptors on the surface of activated platelets bind fibrinogen, leading to the formation of fibrinogen bridges between the platelets, resulting in platelet aggregation. This, and the simultaneous formation of a fibrin mesh (not shown), lead to the formation of a platelet thrombus. (f)Clot retraction then leads to formation of a stable thrombus*** *(Bhatt et al., 2003).*

## 1.4.6 Blood coagulation.

Blood coagulation is the last step of homeostasis, and the primary defense system of vasculature. Vascular injury exposes collagen and leads to the release of tissue factor III, which with the aid of $Ca^{2+}$ activates factor VII, thus initiating the extrinsic pathway (Mackman, 2004). Factor XII from active platelets, is activated by collagen (van der Meijden *et al.*, 2009), which in turn activates factor XI, thus initiating the intrinsic pathway. Both active factor VII and active factor XI will promote cascade reactions, eventually activating factor X. Active factor X, along with factor III, factor V, $Ca^{2+}$, and platelet thromboplastic factor ($PF_3$), activate prothrombin activator. Prothrombin activator converts prothrombin to thrombin. Thrombin then converts fibrinogen to fibrin. Fibrin initially forms a loose mesh, but then factor XIII causes the formation of covalent cross links, which convert fibrin to a dense aggregation of fibers (Lorand *et al.*, 1964). (TAFI), Thrombin-activatable fibrinolysis inhibitor, protects the fibrin clot from proteolysis and subsequent degradation (Bajzar *et al.*, 1995). The propagation phase thus stems blood loss by providing a stable fibrin clot. Platelets and red blood cells become caught in this mesh of fiber, thus the formation of a blood clot.

## 1.5 Regulation of platelet function.

Platelets circulate in a quiescent state as long as the endothelium remains intact. Once vessel wall is injured, platelets adhere to the exposed subendothelial components and undergo sequence of events, which lead to a haemostatic plug. It is important that platelets are kept in the quiescent state in normal conditions to allow the processes of haemostasis and thrombus formation to remain balanced.

The blood vessels are lined by the endothelium, a group of cells that provide a physical barrier between the blood circulation and the surrounding tissues. The endothelium also produces a series of mediators which control blood flow and haemostasis as a whole. As well as acting as an antithrombotic surface, the endothelium produces numerous antithrombotic mediators which prevent platelet aggregation and promote fibrinolysis. Ectonucleotidases (CD39) and proteoglycans (heparin sulphates) are expressed on the cell surface and prevent platelet adhesion and thrombin activity (Olson *et al.*, 1994). Prostacyclin ($PGI_2$), prostaglandin ($PGE_2$) (Moncada *et al.*, 1976; Moncada *et al.*, 1977b; Whittaker *et al.*, 1976) and nitric oxide (NO) (Ignarro *et al.*, 1987b; Moncada *et al.*, 1988a; Moncada *et al.*, 1988b) are released into the lumen and antagonise platelet aggregation and adhesion as well as causing vessel dilation.

### 1.5.1 Nitric oxide.

Nitric oxide (NO) is a gaseous messenger that functions as both a critical cytotoxic agent and an essential signaling molecule. Historically the toxicity NO has long been accepted. However, with almost 100,000 references

demonstrating biological roles for NO listed in PubMed, it may be difficult to remember how controversial was the initial proposal that NO was a biological molecule. It is well established now that NO and natriuretic peptides (NPs) play an important role in cardiovascular health and disease (D'Souza *et al.*, 2004; Garbers *et al.*, 1999; Ignarro, 2002; Lloyd-Jones *et al.*, 1996). NO and ANP relax small arteries and arterioles resulting in decreased blood pressure, and NO prevents acute vasoconstriction and thrombosis.

In circulation an intact endothelium releases NO continuously in response to blood flow to inhibit platelet adhesion to endothelium (De Graaf *et al.*, 1992). Activated platelets also release NO in nanomolar concentrations (Zhou *et al.*, 1995), to prevent further platelet adhesion and aggregation to growing thrombus (Freedman *et al.*, 1999).

### 1.5.1.1 Nitric oxide production.

NO is biosynthesized by a family of enzymes called nitric oxide synthase (NOS). The three members of the enzyme are endothelial NOS (eNOS), inducible NOS (iNOS), and neuronal NOS (nNOS). A mitochondrial NOS (mtNOS) has also been reported elsewhere (Ghafourifar *et al.*, 1999). Generally, NOS catalyzes a reaction between L-arginine with oxygen, in a $Ca^{2+}$ bound to calmodulin ($Ca^{2+}$/CaM) -dependent manner, forming L-citrulline and releasing NO (Marletta *et al.*, 1988a). The enzyme has an absolute requirement for the electron donor NADPH, the electron carriers $FADH^{+}$ and $FMNH^{+}$, the cofactors tetra-hydrobiopterin ($BH_4$), zinc, haem (Moncada *et al.*, 1991).

In cells where eNOS is in caveolae, it is likely that binding to caveolin-1 may negatively regulate its function. On stimulation with calcium-mobilizing agonists such as bradykinin, acetylcholine, or vascular endothelial growth factor (VEGF, Akt, once recruited to the plasma membrane, is phosphorylated on threonine-308 (by the serine/threonine kinase PDK-1) and/or calmodulin dependent protein kinase kinase (CaMKK) and on serine-473 (by the serine/threonine kinase PDK2) resulting in its ability to directly activate eNOS by phosphorylate on serine-1179 (Fulton *et al.*, 2001). Evidences suggest that the shear stress acting on the endothelium is responsible for flow-induced NO release. Responses to shear stress include increases in ionic conductances (Lansman *et al.*, 1987), intracellular levels of $Ca^{2+}$ (Shen *et al.*, 1992), and $IP_3$ (Bhagyalakshmi *et al.*, 1992). Again phosphorylation by Akt occurs, resulting in NO release.

Synthesis of NO from L-arginine is now known to occur in vascular endothelial cells (Palmer *et al.*, 1988), macrophages (Hibbs *et al.*, 1988; Kwon *et al.*, 1989; Marletta *et al.*, 1988b), neutrophils (McCall *et al.*, 1989; Salvemini *et al.*, 1989), brain synaptosomes (Knowles *et al.*, 1989), adrenal glands (Palacios *et al.*, 1989) and a number of other tissues (Moncada *et al.*, 1989). In addition, eNOS and iNOS have been described to be expressed in platelets, however, data concerning expression, regulation, and function of eNOS and iNOS in platelets are highly controversial (Gkaliagkousi *et al.*, 2007; Naseem *et al.*, 2008). In contrast to these publications, data from others (Gambaryan *et al.*, 2008; Özüyaman *et al.*, 2005), clearly demonstrated that human and mouse platelets do not express any

functionally active NOS protein, indicating that endothelial cell-derived NO is the major activator of platelet sGC.

### 1.5.1.2 Nitric oxide bioavailability.

A decrease in NO bioavailability has been implicated to play a major role in the generation of atherosclerosis (Radomski *et al.*, 1995). However, it remains unclear whether NO production is down regulated or impaired. *In vivo* the bioavailability of NO is regulated by oxygenated haemoglobin ($HbO_2$) and myoglobin ($MbO_2$), which NO reacts with to form nitrate ($NO_3$) (Huang *et al.*, 2001). However, due to the abundance of red blood cells (RBC) in circulations, an NO-bioavailability export theory has been proposed. According to this theory, NO enters the RBC and preferentially binds with the free heme on Hb to form heme-nitrosylHb (HbNO) rather than being oxidized by $O_2$- conjugated heme. HbNO then transfers the conjugated NO to b-93Cys to form *S*-nitrosoHb. NO bioactivity is then exported out of RBCs through the anion exchange protein, band 3 (or AE1) (Gow *et al.*, 1999). NO can react with superoxide ($O_2^{\bullet-}$) to form peroxynitrite ($ONOO^-$) (Crane *et al.*, 2005), thus reducing the amount of bioavailable NO.

### 1.5.1.3 Mechanisms underlying the biological actions of nitric oxide.

#### 1.5.1.3.1 Nitric oxide-sensitive soluble guanylyl cyclase.

NO as a free radical with a half-life of about 10 seconds, diffuses readily across cellular compartments (RMJ *et al.*, 1991). Once inside platelets, NO binds to the haem group of soluble guanylyl cyclase (sGC).

sGC is a heterodimer formed by dimerization of either an $\alpha_1$- or $\alpha_2$-subunit to the $\beta_1$- subunit, and these dimers have indistinguishable enzymatic activity (Russwurm *et al.*, 2002). In platelets, only the $\alpha_1$ and $\beta_1$ subunits of sGC are expressed (Mergia *et al.*, 2006). Activation of sGC is initiated by NO binding to the sixth coordinating position of the heme iron which leads to the breakage of the histidine–iron bond yielding a five-coordinated nitrosyl–haem complex with an absorption maximum at 399 nm (Gerzer *et al.*, 1981). The change in haem conformation is transduced to the catalytic cGMP forming domain resulting in the up to 200-fold activation of the enzyme. This is evident by replacing haem by the haem precursor protoporphyrin IX, which stimulates sGC independently of NO indicating that protoporphyrin IX, due to the lack of the central iron, is able to mimic the conformation of NO-bound heme (Ignarro *et al.*, 1982). The finding is compatible with the assumption that the release of the histidine–iron bond is required for stimulation of sGC. Ultimately the increases in the catalytic activity of sGC increases intracellular cyclic guanosine 5-monophosphate (cGMP) levels formed from $Mg^{2+}$-GTP (Radomski *et al.*, 1990). cGMP then binds to phosphodiesterases (PDE), ion-gated channels, and cGMP-dependent protein kinases (PKG). These effectors can regulate several physiological functions including vasodilatation, platelet aggregation and neurotransmission (Munzel *et al.*, 2003; Sanders *et al.*, 1992; Warner *et al.*, 1994).

At any given time, the intracellular concentration of cGMP results from the balance between synthesis and degradation. The catabolism of cGMP is mediated by phosphodiesterases (PDEs), a large group of enzymes consisting of at least 11 different families (Bender *et al.*, 2006). PDEs

hydrolyze the 3′ phosphoester bond of cyclic nucleotides, converting them into biologically inactive 5′ nucleotide metabolites. Currently, only three PDEs have definitely been shown to be expressed in platelets, cGMP-stimulated $PDE_2$, cGMP-inhibited $PDE_3$, and the cGMP-binding, cGMP-specific $PDE_5$ (Haslam *et al.*, 1999). Whereas $PDE_2$ hydrolysis both cGMP and cAMP with similar affinities, $PDE_3$ preferentially hydrolysis cAMP. $PDE_3$ activity is increased by a direct PKA-catalyzed phosphorylation (Macphee *et al.*, 1988), and inhibited by the binding of cGMP. Therefore, cGMP can potentially decrease (via $PDE_2$) or enhance (via $PDE_3$) a cAMP response. $PDE_5$ is highly specific for cGMP hydrolysis (Schwarz *et al.*, 2001a).

**1.5.1.3.2 Nitric oxide-insensitive soluble guanylyl cyclase.**

In addition to NO, carbon monoxide (CO) can bind to the sGC haem and weakly activate the protein (Stone *et al.*, 1994). The binding of CO leads to the formation of a 6-coordinate $Fe^{II}$-CO complex with the histidine–iron bond remaining intact (Friebe *et al.*, 1996; Stone *et al.*, 1994), and a 2–4-fold increase in the rate of cGMP production. This activation is significantly lower than the 100–400-fold increase in cGMP production observed with NO. Other compounds also have been reported to activate sGC include organic nitrates (Obergfell *et al.*, 2001). Recently a new mechanism of NO-independent tyrosine phosphorylation of the sGC β-subunit and activation of sGC was described in platelets stimulated with vWF (Gambaryan *et al.*, 2008), and GP VI (Riba *et al.*, 2008). In a similar manner, it has been shown that sGC exist in NO-insensitive (haem-oxidized or haem-free) form under physiological

conditions, and in increased levels in certain disease conditions (Stasch *et al.*, 2006).

## 1.5.1.4 Role of nitric oxide in platelet function.

### 1.5.1.4.1 cGMP-dependent mechanism of regulation.

NO plays an important protective role in vascular haemostasis by suppressing thrombosis, atherosclerosis, and proliferation of vascular smooth muscle cells (Ignarro *et al.*, 2004). In blood vessels, the intact endothelium releases NO to inhibit platelet adhesion to the endothelium (De Graaf *et al.*, 1992; Ignarro, 1989), platelet activation and platelet aggregation (Azuma *et al.*, 1986). The vasodilatory effect of NO leads to smooth muscle relaxation (Furchgott *et al.*, 1980).

In platelets NO inhibits agonist-evoked calcium mobilization from intracellular stores by phosphorylation of $IP_3$ receptor-associated protein (IRAG) and calcium entry via store-operated calcium channels, but not via ADP-activated calcium channels (Geiger *et al.*, 1994; Moro *et al.*, 1996). The binding affinity of $\alpha_{IIb}\beta_3$ for fibrinogen is also decreased by cGMP-dependent inhibition of phosphoinositide 3-kinase (PI3k) activation (Pigazzi *et al.*, 1999), and by phosphorylation of focal adhesion vasodilator-stimulated phosphoprotein (VASP) (Horstrup *et al.*, 1994). NO also inhibits PLC-dependent activation of PKC (Schwarz *et al.*, 2001a), while $Rap_1$, which is required for normal integrin $\alpha_{IIb}\beta_3$ signalling in platelets (Chrzanowska-Wodnicka *et al.*, 2005), has also been identified as a substrate for NO (Danielewski *et al.*, 2005). Indirectly, cGMP can also increase intracellular cAMP levels by inhibiting the degradation of cAMP by $PDE_3$ **(figure 1.6)**.

Platelet secretion, in which dense granules, α-granules, and lysosomes are released, is a very complex process regulated by multiple intracellular signaling systems. Cytoskeletal protein reorganization, increase of cytosolic $Ca^{2+}$ concentration, and activation of protein kinase C (PKC) are essential for platelet secretion (Elzagallaai *et al.*, 2001). Activation of PKG inhibits platelet secretion by reducing both cytosolic $Ca^{2+}$ concentration and PLC-dependent activation of PKC (Schwarz *et al.*, 2001b).

Mice deficient for PKG show impaired NO/cGMP-dependent dilations of large and small arteries indicating that the vasorelaxant effects of NO, NPs and other cGMP elevating agents are mediated, at least in part, via activation of PKG (Koeppen *et al.*, 2004; Pfeifer *et al.*, 1998; Sausbier *et al.*, 2000; Weber *et al.*, 2007). Phosphorylation of IRAG by PKGIβ inhibits IP3-induced $Ca^{2+}$ release from intracellular stores in transfected COS cells and in smooth muscle cells (Ammendola *et al.*, 2001; Geiselhöringer *et al.*, 2004; Schlossmann *et al.*, 2000). PKGI activates large-conductance $Ca^{2+}$-activated maxi-K+ (BKCa) channels (Robertson *et al.*, 1993; Sausbier *et al.*, 2000), thereby reducing $Ca^{2+}$ influx.

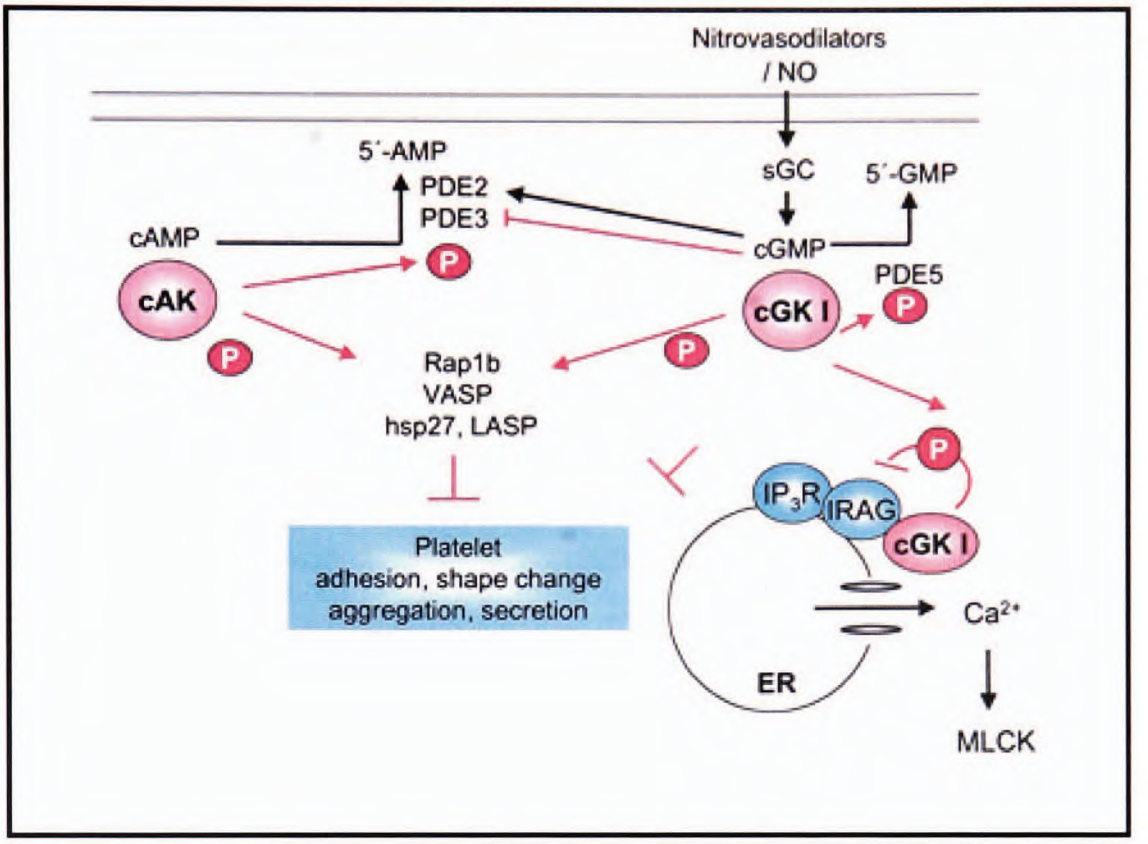



***Figure 1.6. Schemetic diagram of NO signaling in platelets.***

***cGKI-mediated inhibition of platelet activation by phosphorylation (P) of substrate proteins ($IP_3$ receptor, the small GTPase Rap $_{1b}$, vasodilator-stimulated phosphoprotein [VASP], heat shock protein [hsp]27, and the cGMP hydrolyzing phosphodiesterase, $PDE_5$) by inhibition of G-protein-coupled (Gq/Gi) receptor complexes and by inhibition of cAMP hydrolysis by $PDE_3$. ABP indicates actin-binding protein; AC, adenylate cyclase; cAK, cAMP-dependent protein kinase; EDRF, endothelium-derived relaxing factor; G, G-protein; GP, glycoprotein; $IP_3R$, $IP_3$ receptor; sGC, soluble guanylate cyclase; and $TXA_2$, thromboxane $A_2$.*** *Courtesy of Munzel and colleagues (Munzel et al., 2003).*

#### 1.5.1.4.2 cGMP-independent mechanism of regulation.

In literature the primary mechanisms described for NO as a signaling molecule appear to be cGMP-mediated through regulation of intracellular $Ca^{+2}$ levels. However, other cGMP-independent effects have also been described (Oberprieler *et al.*, 2007), such as nitration (Balafanova *et al.*, 2002a; Marcondes *et al.*, 2006a), modification of cellular or plasma proteins by S-nitrosylation of cystein residues forming S-nitrosothiols (Hanafy *et al.*), peroxynitrite generation (Crane *et al.*, 2005). Generally, the inhibitory effects of NO synchronize with those of prostacyclin, an arachidonic acid metabolite released by endothelial cells.

Excess production of the free radicals NO and superoxide ($O_2^{\bullet-}$) is related to cell and tissue pathology (Beckman, 1996; Freeman *et al.*, 1982). Unraveling the mechanisms by which these moderately reactive radicals disrupt biomolecular structure and function has been challenging due to both their transient nature and the potential multiplicity of cellular and extracellular target molecules. Substantial progress was made when a hypothesis was elaborated in the early 1990s, proposing that the pathways of NO and $O_2^{\bullet-}$ dependent molecular damage can merge into a common route involving the formation of peroxynitrite anion (Beckman, 1990; Radi *et al.*, 1991b).

## 1.5.2 Peroxynitrite.

Peroxynitrite is a strong oxidant and nitrating agent which affects the function of a number of cells and proteins and has been implicated to play a role in a variety of disease states, such as atherosclerosis (Rubbo *et al.*, 2000; Rubbo *et al.*, 2005), hypoxia (Cooper *et al.*, 2000; Nonami, 1997), diabetes (Pacher

*et al.*, 2006), and neurodegenerative disorders (Guix *et al.*, 2005; Moncada *et al.*, 2006). However, endogenously produced peroxynitrite has also been described to play a role in cell signaling under physiological conditions through the nitration of tyrosine residues (Balafanova *et al.*, 2002b; Marcondes *et al.*, 2006c; Naseem *et al.*, 2000).

### 1.5.2.1 Reactive oxygen species in biological systems.

Free radicals can be defined as molecules or molecular fragments containing one or more unpaired electrons in atomic or molecular orbitals (Halliwell *et al.*, 2007). Molecular oxygen ($O_2$) has a unique electronic configuration and is itself a radical. The addition of one electron to $O_2$ forms the superoxide anion radical ($O_2^{\bullet -}$). This process is mediated by enzymes such as NAD (P) H oxidases and xanthine oxidase (Droge, 2002). $O_2^{\bullet -}$ in turn is converted by $O_2^{\bullet -}$ scavenger superoxide dismutase (SOD) to $H_2O_2$ , which catalyzed to $H_2O$ by reduced glutathione (GSH) (Choi *et al.*, 2004) **(figure 1.7)**. In addition, under conditions such as the absence of L-arginine or BH4, eNOS can undergo a process called eNOS uncoupling, whereby eNOS catalyzes an uncoupled NADPH oxidation leading to the formation of $O_2^{\bullet -}$, instead of NO (Papapetropoulos *et al.*, 1999).

Reactive oxygen species (ROSs) are mostly generated by reduced nicotinamide adenine dinucleotide (phosphate) (NAD(P)H) oxidase in phagocytes, and has bactericidal function in these cells (Droge, 2002). Within the vessel wall, endothelial cells, vascular smooth muscle cells, and fibroblasts express nonphagocytic NAD(P)H oxidase isoforms that produce mostly intracellular ROSs involved in cellular signaling (Lassegue *et al.*,

2003) Here, generated ROSs act as second messengers in control of different physiologic responses such as gene expression, apoptosis, and proliferation (Droge, 2002).

In platelets the presence of NAD(P)H oxidase subunits has been shown by several groups(Seno *et al.*, 2001). ROSs may regulate platelet function by decreasing NO bioavailability because ROSs scavenge platelet or endothelium-derived nitric oxide (NO)(Chakrabarti *et al.*, 2004; Clutton *et al.*, 2004). ROSs are also involved in the regulation of $\alpha_{IIb}\beta_3$ activation without affecting the NO/cGMP pathway, granule secretion, and platelet shape change (Begonja *et al.*, 2005).

### 1.5.2.2 Generation of peroxynitrite.

Cells of the immune system produce both $O_2^{\bullet-}$ and NO during the oxidative burst triggered during inflammatory processes. While $O_2^{\bullet-}$ serves a signaling role in its own right, the increase in $O_2^{\bullet-}$ production during pathological scenarios such as ischemia-reperfusion, leads to the reaction of $O_2^{\bullet-}$ with NO. This has a number of significant consequences. Firstly, by reacting with $O_2^{\bullet-}$, the amount of bioavailable NO is reduced, thus reducing the beneficial effect of NO in the vasculature. Secondly, the reaction of $O_2^{\bullet-}$ and NO leads to the formation of peroxynitrite (Beckman *et al.*, 1996b; Bruckdorfer, 2005; DarleyUsmar *et al.*, 1996)

Peroxynitrite is a strong reactive nitrogen species, formed from the reaction of the radicals $O_2^{\bullet-}$ with NO at a much faster rate, $6.7 \times 10^9$ $M^{-1} \cdot s^{-1}$ (Huie *et al.*, 1993), than with SOD ~$2 \times 10^9$ $M^{-1} \cdot s^{-1}$. Thus when both $O_2^{\bullet-}$ and NO levels are in the high nanomolar range, the former reaction will generate

peroxynitrite (Ray *et al.*, 2005). It has also been suggested that peroxynitrite can also be formed by the reaction of nitroxyl anion ($NO^-$) with $O_2$ at a slower rate ($5.7 \times 10^9\ M^{-1} \cdot s^{-1}$) than that of NO with $O_2^{\bullet-}$ (Hogg *et al.*, 1996) **(figure 1.7).** The reaction of NO with oxymyoglobin and oxyhemoglobin leads to nitrate formation (Doyle *et al.*, 1981). It has been proposed that this occurs through the formation of an intermediate peroxynitrite (Herold, 1998; Ignarro, 1990; Wade *et al.*, 1996)

Peroxynitrite is remarkably stable at alkaline pH. This stability is due to it folds into a stable *cis*-conformation where the negative charge is localized over the entire molecule (Tsai *et al.*, 1994). Peroxynitrite decomposes to yield $OH^-$ and $NO_2^-$. This however is likely to become relevant only at acid pH because at neutral pH the proton-catalyzed decay is too slow to compete with biotargets such as $CO_2$, biothiols (RSH) and hemoproteins (Augusto *et al.*, 2004) **(figure1.8)**.

The decomposition of peroxynitrite can be affected by the concentration and the nature of the buffer. At low phosphate concentrations the $pK_a$ can decrease from 6.8 to 6.5 (Kissner *et al.*, 1997), and in the presence of HEPES, CAPS, CAPSO, or ammonia the apparent $pK_a$ increases to values near 8 (Beckman *et al.*, 1996a; Koppenol, 1999). Furthermore, reaction of peroxynitrite with the buffers can occur in some cases (Gadelha *et al.*, 1997). Physiological concentrations of peroxynitrite *in vivo* have been estimated to be ~50 μM (Dairou *et al.*), although 500 μM concentrations have been found within phagolysosomes of activated macrophages (Stachowiak *et al.*, 1998). Importantly in the context of platelet function, endothelial cells have the

potential to generate peroxynitrite (Altup *et al.*, 2001).The half-life of peroxynitrite is short (~10-20ms), but sufficient to cross biological membranes, diffuse one to two cell diameters (Denicola *et al.*, 1998).

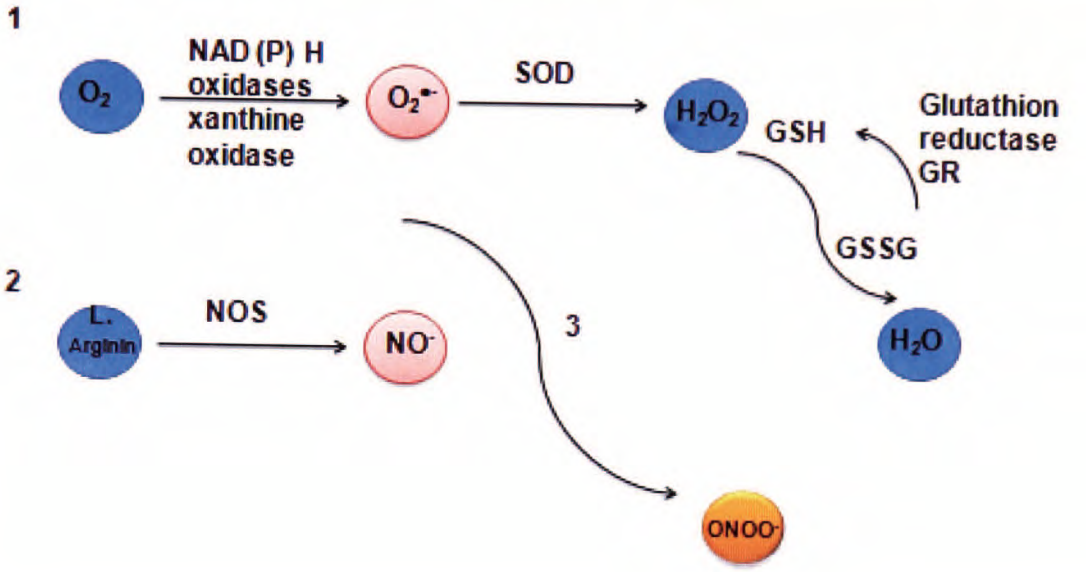



***Figure 1.7. Schematic diagram represents reactive oxygen species generation and consumption (1), NO production (2), and peroxynitrite generation (3).***

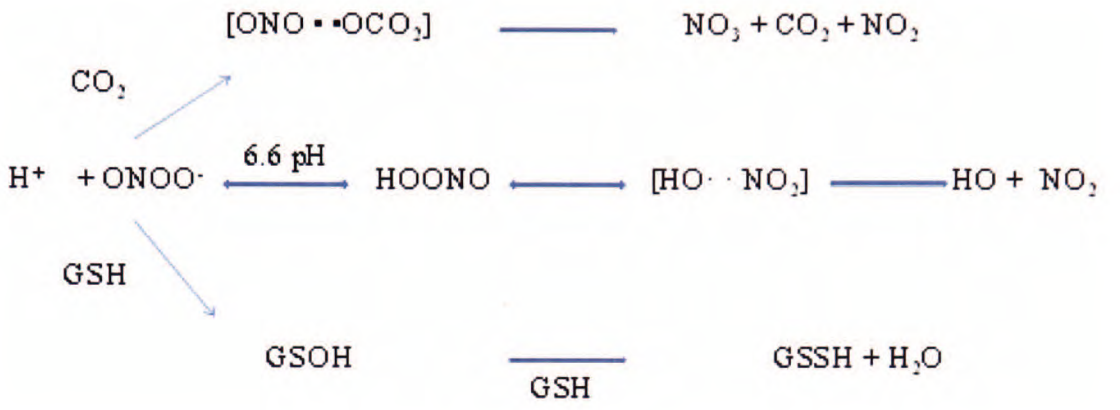



***Figure 1.8. Schematic diagram of decomposition pathways of peroxynitrite.***

***RSH, P-Fe$^{(III)}$ and $CO_2$ are anticipated to be the most important peroxynitrite biotargets because of their high biological concentrations and rapid reaction rate with the oxidant (k~102-106 $M^{-1}$. $s^{-1}$). These reactions greatly reduce the half-life of peroxynitrite (from s to ms) and the targets are usually oxidized by two-electron mechanisms. An important exception is the reaction with the biologically ubiquitous $CO_2$ that produces 65% $NO_3$-and 35% $CO_3^{\bullet}$ and $\bullet NO_2$.*** *(Augusto et al., 2004)*

### 1.5.2.3 Pathophysiological roles of peroxynitrite.

In red blood cells and platelets, peroxynitrite has been shown to diffuse into the cytoplasm through HCO3-/Cl- ion channel (Boulos *et al.*, 2000b; Denicola *et al.*, 1998). Peroxynitrite alters protein structure and function by reacting with metal centers, the fastest known for peroxynitrite (Alvarez *et al.*, 2003), and various amino acids in the peptide chain.

Peroxynitrite can cause DNA strand breaks (King *et al.*, 1993), which have been detected both in isolated DNA (Salgo *et al.*, 1995b) and in cells exposed to exogenous peroxynitrite (Salgo *et al.*, 1995a). Mechanistically, the strand breaks seem to arise both from sugar damage and from base modification (Burney *et al.*, 1999). The formation of strand breaks has been shown to activate poly (ADP-ribose) synthetase (PARS), leading to NAD+ consumption followed by energy depletion (Szabó *et al.*, 1997). Peroxynitrite also target lipids (Radi *et al.*, 1991a), mitochondria (Cassina *et al.*, 1996) and cell receptors (Newman *et al.*, 2002). Whether peroxynitrite has a physiological or pathological effect depends entirely on the concentration and target. In platelets, it is believed that high concentrations, in excess of 150μM, peroxynitrite acts as a platelet agonist by stimulating aggregation. At lower concentrations or in the presence of plasma, peroxynitrite acts as a platelet inhibitor (Low *et al.*, 2002). The mechanisms underlying these processes are unknown.

The ability of peroxynitrite to modulate cell signaling has also been demonstrated. Nitration of tyrosine residues, one of the major reactions of peroxynitrite, can impair signaling processes depending on tyrosine phosphorylation in number of cells such as T lymphocytes (Brito *et al.*, 1999).

Other studies have indicated that peroxynitrite promoted rather than inhibited tyrosine phosphorylation in red blood cells (Maccaglia *et al.*, 2003), and endothelial cells (Zou *et al.*, 2003), possibly by irreversible inhibition of phosphotyrosine phosphatases (PTPs) (Takakura *et al.*, 1999a). In red blood cells, peroxynitrite has been shown to activate Src Kinase lck via cysteine oxidation, whereas another Src Kinase, lyn, was activated through a mechanism involved the inhibition of Tyr527 binding to the SH2 domain, which maintain Src in its inactive state (Mallozzi *et al.*, 2001a). In endothelial cells, AMP-activated protein kinase (AMPk) has also been shown to be activated by peroxynitrite (Zou *et al.*, 2002).

peroxynitrite has been shown to trigger apoptosis in cardiomyocytes (Arstall *et al.*, 1999) as well as endothelial (Dickhout *et al.*, 2005) and vascular smooth muscle cells (Li *et al.*, 2004), induce decrease in spontaneous contractions of cardiomyocytes (Ishida *et al.*, 1996), and depending on the environment can stimulate or inhibit platelet aggregation (Moro *et al.*, 1994; Nowak *et al.*, 2002). In contrast with these reports, others have found that peroxynitrite can mediate a number of physiological processes that may be beneficial and can potentially result in cellular protection. For example, peroxynitrite produces vascular relaxation in isolated dog and human coronary arteries (Ku *et al.*, 1995; Liu *et al.*, 1994). Additionally, (Wu *et al.*, 1994), have demonstrated that peroxynitrite relaxes pulmonary arteries in vitro. Therefore, it is still unclear whether peroxynitrite is a physiological or pathological mediator.

## 1.5.3 Prostacyclin.

### 1.5.3.1 Synthesis of prostacyclin.

Prostaglandin $I_2$ ($PGI_2$) or prostacyclin is a derivative of the C-20 unsaturated fatty acid arachidonic acid (5,8,11,14-eicosatetraenoic acid) (Kobayashi *et al.*, 2000), and has a half-life of about 3 min (Cho *et al.*, 1978). The biosynthesis of prostacyclin takes a place in endothelial cells as a part of the arachidonic acid metabolic pathway. Arachidonic acid, which is present in the walls of arteries and veins in several species (Dusting *et al.*, 1977; Gryglewski *et al.*, 1976; Johnson *et al.*, 1976), including man (Moncada *et al.*, 1977a), is released by phospholipase $A_2$ upon activation of the enzyme by an increase in intracellular $Ca^{2+}$ concentration. Arachidonic acid is further metabolized by cyclooxygenase (COX), and (ii) 5-lipoxygenase (Parente *et al.*, 2003), to form prostaglandin $G_2$ ($PGG_2$), which is then converted by prostacyclin synthase into prostacyclin ($PGI_2$).

$PGI_2$ is a potent vasodilator, antithrombotic, and antiplatelet agent that mediates its effects through a specific membrane-bound receptor, the prostacyclin receptor (IP receptor), which belongs to the prostanoid family of G protein-coupled membrane receptors (GPCR) (Narumiya *et al.*, 1999). IP receptor is expressed on platelets, smooth muscle cells, the atrium and ventricle of the heart, arteries mostly abundant in the aorta, but no IP receptors in veins (Dutta-Roy *et al.*, 1987; Jones *et al.*, 1997; Nakagawa *et al.*, 1994; Narumiya *et al.*, 1999; Smyth *et al.*, 2002). The prostaglandins are not necessarily specific for an individual receptor. The binding pocket of the IP receptor can accommodate the cyclopentane rings of $PGI_2$, $PGE_1$, and $PGE_2$ (Smyth *et al.*, 2002).

### 1.5.3.2 Adenylyl cyclase.

Prostacyclin mediates its biological effects through the activation of the enzyme adenylyl cyclase. In mammals, there are at least nine isoforms of adenylate cyclase ($AC_1$-$AC_9$) with $AC_2$, $AC_3$, and $AC_7$ identified in platelets to date (Hellevuo *et al.*, 1995; Katsel *et al.*, 2003; Smit *et al.*, 1998). The approximately 120 kDa glycoprotein AC isoforms share a primary structure which consists of a small cytoplasmic N terminal domain (N), followed by two transmembrane regions, $M_1$ and $M_2$ (each contain six predicted membrane-spanning helices), and two cytoplasmic regions, $C_1$ and $C_2$ (Feinstein *et al.*, 1991). $M_1$ and $M_2$ domains could serve as a membrane voltage sensor that conformationally regulates adenylate cyclase (Dessauer *et al.*, 1996). The $C_1$ and $C_2$ regions are subdivided into $C_{1a}$ and $C_{1b}$; and $C_{2a}$ and $C_{2b}$. The $C_{1a}$ and $C_{2a}$ are well conserved, homologous to each other, contain all of the catalytic apparatus (Feinstein *et al.*, 1991), and heterodimerize with each other in solution (Whisnant *et al.*, 1996; Yan *et al.*, 1996) **(figure 1.9)**.

In addition to their (ACs) ability to respond to Gαs and to forskolin (Seamon *et al.*, 1981), the different isoforms can receive signals from a variety of sources, including other G proteins, e.g. Gαi and GβΥ, protein kinases (PKA, PKC), and calmodulin (CaM) kinase), phosphatases (calcineurin), calcium, and $Ca^{2+}$/CaM, and these isoforms are able to support and integrate differential regulatory pathways through cross-talk with other signal transduction systems (Defer *et al.*, 2000).

Binding of prostacyclin to its receptor induces a signaling cascade through coupling to the heterotrimeric G protein $G_{\alpha s}$ (Kobayashi *et al.*, 2000; Wise *et al.*, 2000), which then stimulates adenylate cyclase, assumed to be located

in the dense tubular system (González-Utor *et al.*, 1992), and leads to an increase in cAMP levels (Gorman *et al.*, 1977).

Like all of the GTPase switch proteins, the G protein α-subunits bind GTP and adopt an active conformation in which they modulate effector proteins until signalling is terminated by the action of an intrinsic GTPase activity and reassociation with the GβY complex (Hamm *et al.*, 1996).

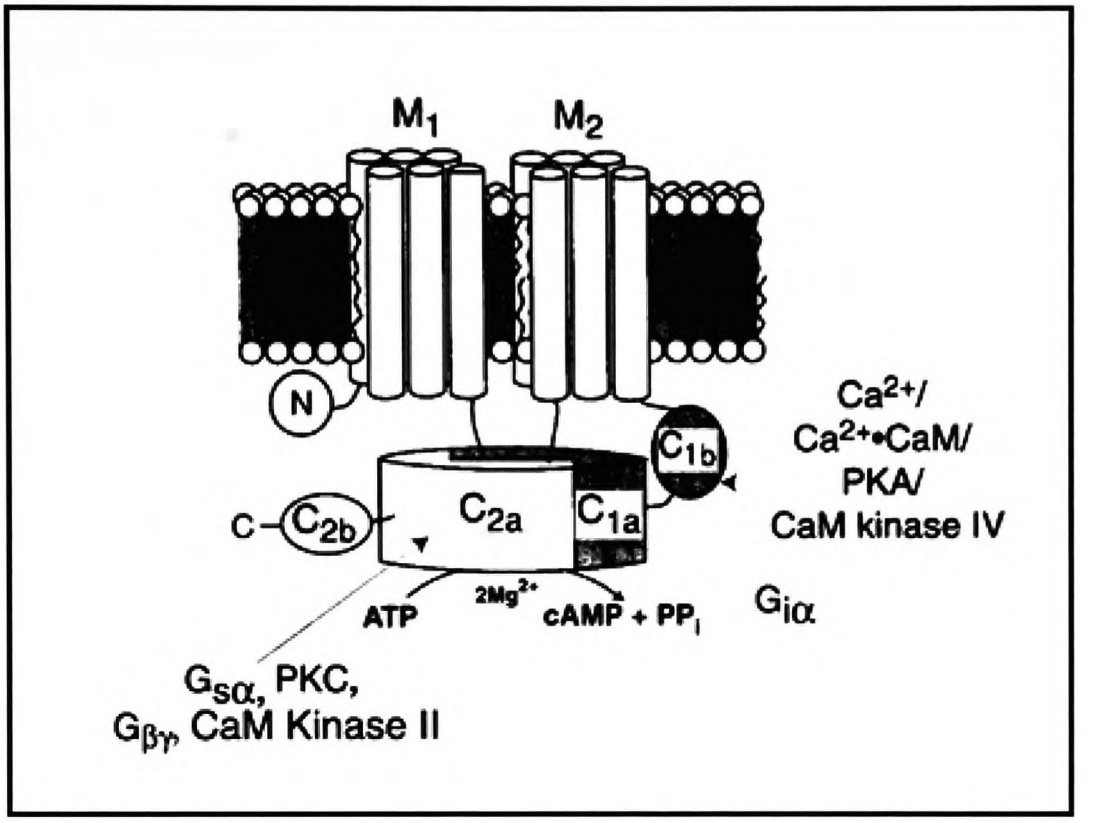



***Figure 1.9. Structure of adenylyl cyclase.***

***The M 1 and M 2 domains are each predicted to contain six transmembrane helices. C la and C2a form a pseudosymmetric heterodimer that represents the catalytic core of the enzyme. The active site is formed within their domain interface. The domains with which regulators are known to primarily interact are indicated. The N, C 1 b and C2b domains are variable among adenylyl cyclases and their structure and location with respect to the membrane and catalytic core are unknown.*** *Courtesy of (Tesmer et al., 1998).*

Increased cAMP production activates phospho kinase A (PKA) (Siess, 1989). PKA is a serine/threonine kinase composed of a homodimer regulatory subunit ($PKA_R$) and two catalytic subunits ($PKA_c$). Cooperative binding of cAMP molecules to four nucleotide binding site on $PKA_R$ causes a reversible dissociation of $PKA_c$ and thus PKA activation (Johnson *et al.*, 2001). PKA activation causes the phosphorylation of several key proteins, such as myosin light chain kinase (MLCK) (Hathaway *et al.*, 1981), the platelet inositol 1,4,5-triphosphate receptor ($IP_3$) (Cavallini *et al.*, 1996), and VASP (Aszodi *et al.*, 1999).

Phosphorylated MLCK is inactive and has a reduced affinity for calmodulin which then reduces the amount of phosphorylated myosin (Conti *et al.*, 1981). The effect of this is a decreased platelet contractile activity, including secretion, and a decreased association of myosin with the platelet cytoskeleton, since only the phosphorylated form of myosin can bind to actin (Fox *et al.*, 1982).

As with NO, VASP phosphorylation by $PGI_2$ closely correlates with platelet inhibition (Halbrugge *et al.*, 1990b; Walter, 1989), and platelets deficient in VASP exhibit enhanced agonist-induced activation of P-selectin expression and fibrinogen binding to GPIIb-IIIa integrin (Aszodi *et al.*, 1999; Hauser *et al.*, 1999).

Synergism between NO and $PGI_2$ signaling is evident not only on cellular levels but also on the vasculature system as a whole. in a study conducted by Murata et al. (Murata *et al.*, 1997), found that while IP-deficient mice lack the hypotensive response to the synthetic IP agonist cicaprost, their basal blood pressure and heart rate were not different from those of control

animals. This is in contrast to what was observed in mice lacking NO (Huang *et al.*, 1995). Mice deficient in the endothelial type of NO synthase showed elevated basal blood pressure. These results indicate that the $PGI_2$ and IP system does not work constitutively in regulation of the systemic circulation, and more likely works on demand in response to local stimuli. This can also explain the abundant expression of IP receptors in aorta (Nakagawa *et al.*, 1994), and its absence in veins (Narumiya *et al.*, 1999).

## 1.6 AGC protein kinases.

Protein kinases are key regulatory enzymes that change the properties of a substrate by attaching a phosphate group to Ser, Thr or Tyr residues. The term AGC kinase was coined by Steven Hanks and Tony Hunter (Hanks *et al.*, 1995) in 1995 to define the subgroup of Ser/Thr protein kinases that based on sequence alignments of their catalytic kinase domain, were most related to cAMP-dependent protein kinase (PKA), cGMP-dependent protein kinase (PKG) and protein kinase C (PKC). It is now appreciated that the AGC family contains 60 of the 518 human protein kinases (Manning *et al.*, 2002), which have been highly conserved throughout eukaryotic evolution.

Fourteen AGC kinase domain structures have been determined to date, all of which show the proto typical bilobal kinase fold that was first described for PKA (Knighton *et al.*, 1991). In the bilobal kinase fold, an amino-terminal small lobe (known as N-lobe) and a carboxy-terminal large lobe (known as C-lobe) sandwich one molecule of ATP, which serves as the phosphate donor during phosphorylation (Pearce *et al.*, 2010).

## 1.6.1 Protein kinase A (PKA).

cAMP-dependent protein kinase (PKA) was one of the first to be discovered (Walsh *et al.*, 1968), it was the first to be sequenced (Shoji *et al.*, 1981), and then cloned (MCKNIGHT, 1986). PKA is a serine/threonine kinase that phosphorylates a variety of substrate proteins and is involved in the regulation of many different intracellular events. PKA consists of an R (regulatory) subunit dimer that associates with two C (catalytic) subunits to form and ($R_2C_2$) kinase (Francis *et al.*, 1994; Taylor *et al.*, 1990). Four different isoforms ($RI_\alpha$, $RI_\beta$, $RII_\alpha$, and $RII_\beta$) of the R subunit have been identified, of which $RI_\alpha$ and $RII_\alpha$ are the most ubiquitously expressed in cells and tissues (Stokka *et al.*, 2006).

Cooperative binding of cAMP molecules to four nucleotide binding site on $PKA_R$ causes a reversible dissociation of $PKA_c$ and thus PKA activation **(Figure 1.10)** (Johnson *et al.*, 2001). The active enzyme is then free to phosphorylate target substrates within its vicinity. *In vivo* the binding affinity of RII to cAMP is relatively much lower of RI (Edelman *et al.*, 1987; Taylor *et al.*, 1992); thus RI and RII subunits decode cAMP signals that differ in duration and intensity. $PKA_{RI}$ responds transiently to weak cAMP stimulation, whereas $PKA_{RII}$ is activated by high and persistent cAMP signals (Feliciello *et al.*, 2001).

Compartmentalization of PKA favours the localized action by placing it in close proximity to a subset of its target substrates. this is achieved through protein-protein interactions of the R subunit with AKAPs (A-Kinase-anchoring proteins), a divers family of scaffolding proteins that target PKA to distinct subcellular compartments and towards specific substrates (Wong *et al.*,

2004). AKAPs are classified according to their ability to bind PKA inside cells (Colledge *et al.*, 1999).

Each AKAP contains at least two functional motifs. The conserved PKA-binding motif forms an amphipathic helix of 14-18 residues that interacts with hydrophobic determinants located in the extreme N-terminal docking and dimerization (D/D) domain of the regulatory subunit dimer of PKA (Carr *et al.*, 1991; Newlon *et al.*, 1997).

Some AKAPs bind RI subunits, although with affinities lower than those determined for RII (Herberg *et al.*, 2000). Many AKAPs also possess targeting domains that mediate AKAP attachment to the cytoskeleton and/or intracellular organelles (Glantz *et al.*, 1993). Beside PKA, various AKAPs bind proteins such as phosphatases, protein kinase C, and heterotrimeric G proteins, suggesting that AKAPs function as scaffolding proteins to integrate different signaling pathways (Coghlan *et al.*, 1989; Suzuki *et al.*, 1999).

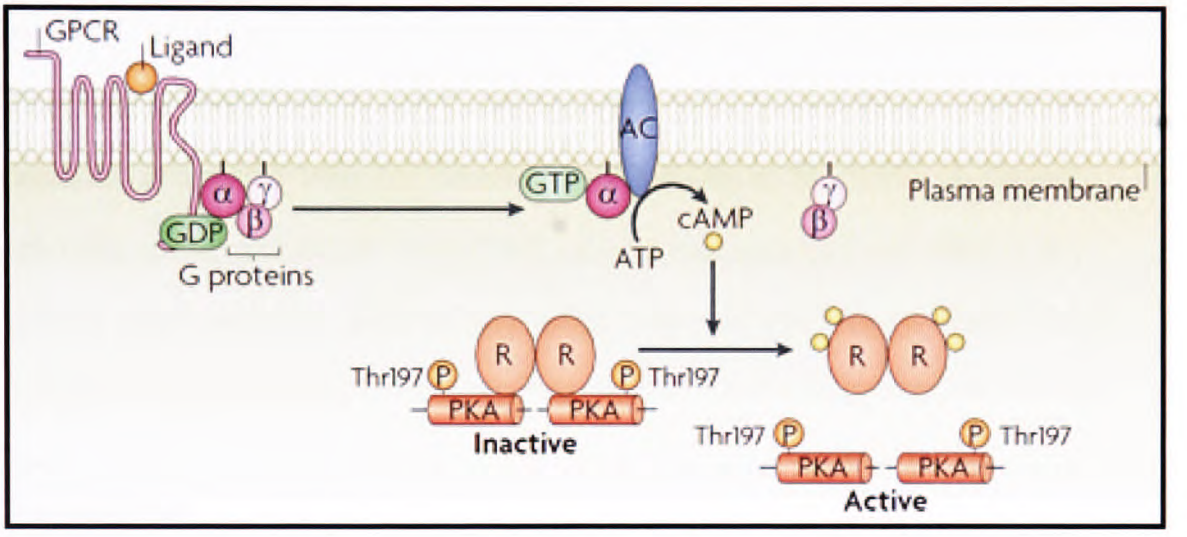



***Figure 1.10. Structure of PKA and mechanism of activation.***

***Ligands such as prostacyclin bind and activate G protein-coupled receptors (GPCRs). This causes the α-subunit of heterotrimeric G proteins to dissociate from the βγ subunits, associate with adenylate cyclase (Ac) and induce the production of cyclic AMP. cAMP binds to the regulatory (R) subunits of cAMP-dependent protein kinase (PKA), causing a conformational change that releases the active catalytic (C) subunits.*** *Courtesy of (Pearce et al., 2010).*

## 1.6.2 Protein kinase G (PKG).

Protein kinase G (PKG) is cyclic nucleotide-dependent kinase, which exists as two isoforms that are regulated by a different second messenger to PKA, cGMP. This is produced by soluble guanylyl cyclases, activated downstream of nitric oxide. In contrast to PKA, PKG forms homodimers, and its cGMP-binding domains are located in the same polypeptide as the catalytic domain. The soluble PKG type I exists in two isoforms (type Iα and Iβ) generated by separate promoters from the same gene (Orstavik *et al.*, 1997). A distinct, primarily membrane-bound form (PKG type II) was originally identified in and cloned from epithelial cells of the small intestine (De Jonge, 1981; DE JONGE *et al.*, 1994).

The enzymes have a rod like structure. They are composed of two functional domains: a regulatory (R) domain and a catalytic (C) domain. The regulatory domain is further subdivided into the N-terminal domain and the cGMP binding domain. Interaction of PKG with cGMP leads to a conformational change in PKG, relieving the inhibitory effect of a pseudosubstrate motif (Hofmann *et al.*, 2009) **(figure 1.11)**. The role of activation segment phosphorylation in controlling the activity of PKG has been poorly studied, although it has been reported that mutation of the activation segment residue (Thr516) inhibits PKG kinase activity(Browning *et al.*, 2000). PKG mediates many of the smooth muscle relaxation effects triggered by nitric oxide69. PKG is also thought to have overlapping effects with PKA and might also be involved in regulating long-term potentiation (Zhuo *et al.*, 1994).

Human platelets express only $PKG_{I\beta}$, whereas mouse platelets additionally express a small amount of $PKG_{I\alpha}$ (Antl, 2006). The $PKG_I$ concentration in

human platelets (3.65μM holoenzyme, equivalent to 14.6μM cGMP-binding sites) is higher than that in any other cell type examined (Eigenthaler *et al.*, 2005). The important role of $PKG_I$ inhibition of platelet activation in vitro and in vivo has been conclusively demonstrated in $PKG_I$-deficient ($PKG_I$ KO) murine platelets (Massberg *et al.*, 1999). In vitro activation of platelet $PKG_I$ by membrane-permeable cGMP analogs and NO donors inhibited agonist-induced serotonin release, shape change, and aggregation in wild-type platelets, but not $PKG_I$ KO mouse platelets. In $PKG_I$ KO mouse platelets, expression and functional activity of PKA is not altered, and there is also no cross-activation of PKA by cGMP. The reverse, activation of $PKG_I$ by cAMP seems also not to occur in platelets, indicating that the cAMP and cGMP signaling cascades inhibit platelet activation independently of each other (Massberg *et al.*, 1999). In vivo studies using $PKG_I$ KO mice showed that platelet $PKG_I$, but not endothelial or smooth muscle $PKG_I$, is essential to prevent intravascular adhesion and aggregation of platelets after ischemia, and a defect due to PKG loss was not compensated by the cAMP/PKA system (Massberg *et al.*, 1999).

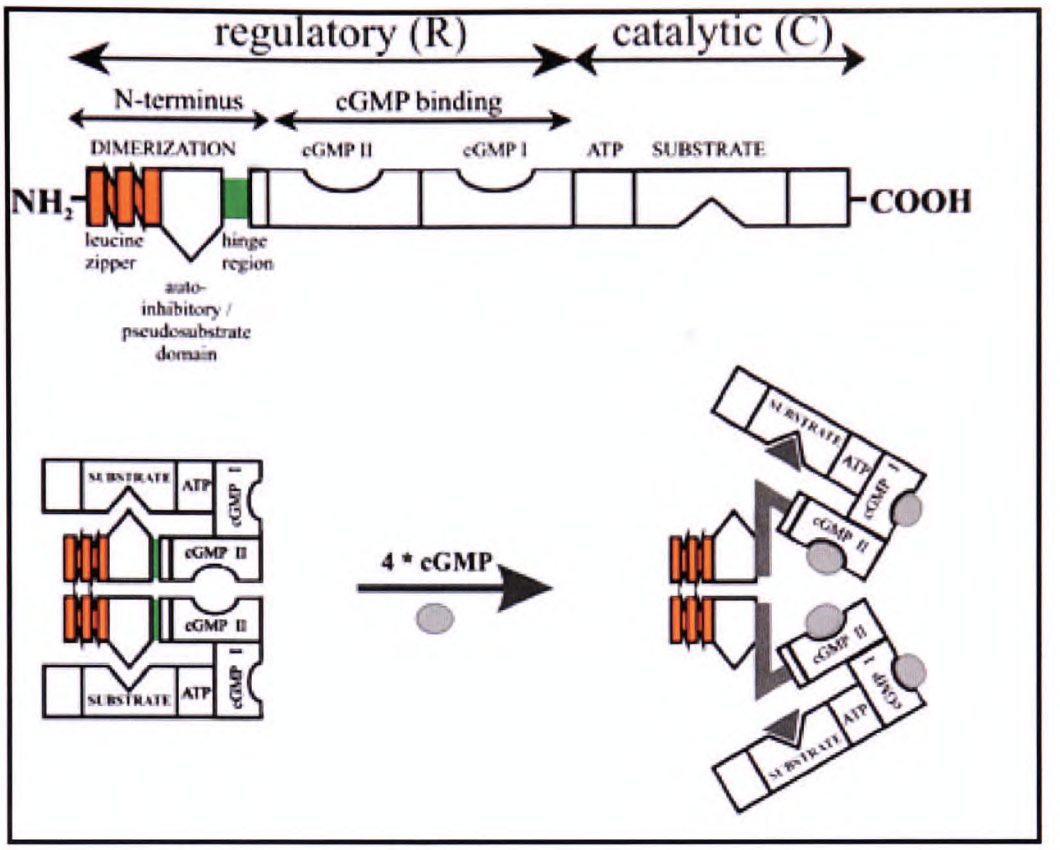



***Figure 1.11. Structure of PKG and mechanism of activation.***

***Occupation of both binding sites induces a large change in secondary structure. Binding of cGMP to both sites in the R-domain releases the inhibition of the catalytic centre by the N-terminal autoinhibitory/ pseudosubstrate domain and allows the phosphorylation of serine/threonine residues in target proteins.*** *Courtesy of (Hofmann et al., 2009).*

## 1.6.3 Protein kinase C (PKC).

Protein kinase C (PKC) was originally identified as a phospholipid-dependent and diacylglycerol-stimulated protein kinase activity. Early studies demonstrated that PKC is activated in vivo by the receptor-induced second messenger diacylglycerol or direct treatment of cells with tumor promoting phorbol esters; this quickly established PKC as a key regulator of growth, differentiation, cell survival, neurotransmission, and carcinogenesis (Kishimoto *et al.*, 1985; Nishizuka, 1995). In addition, many key steps in platelet activation and aggregation are regulated by members of protein kinase C (PKC) family **(figure 1.12)**.

PKC isoforms are a serious of serine/threonine kinases, which are subdivided into three subclasses, conventional (α, βI, βII, γ), novel (δ, ε, η/L, θ) and atypical (ζ, ιλ) (Mellor *et al.*, 1998). Conventional and novel PKCs are allosterically regulated by diacylglycerol (DAG), which binds to the C1 domain **(figure 1.13)**. Conventional isozymes are under additional control by $Ca^{2+}$, which binds to the C2 domain and promotes its interaction with anionic phospholipids. Although novel PKCs contain this domain, the $Ca^{2+}$ binding pocket lacks essential aspartate residues involved in coordinating $Ca^{2+}$ and thus does not bind $Ca^{2+}$. Atypical PKCs contain a single membrane-targeting module, the C1domain, but the ligand-binding pocket is compromised so that it is unable to bind diacylglycerol (Newton, 2003).

Generation of the lipid second messenger, DAG, results in the recruitment of most protein kinase C isozymes from the cytosol, where they are maintained in an inactive conformation, to the membrane, where it adopts a

conformation in which the pseudosubstrate is out of the active site, thus exposing the activation loop phosphorylation site. This conformation is essential to target protein kinase C for phosphorylation by PDK-1 (Dutil *et al.*, 2000; Mosior *et al.*, 1995).

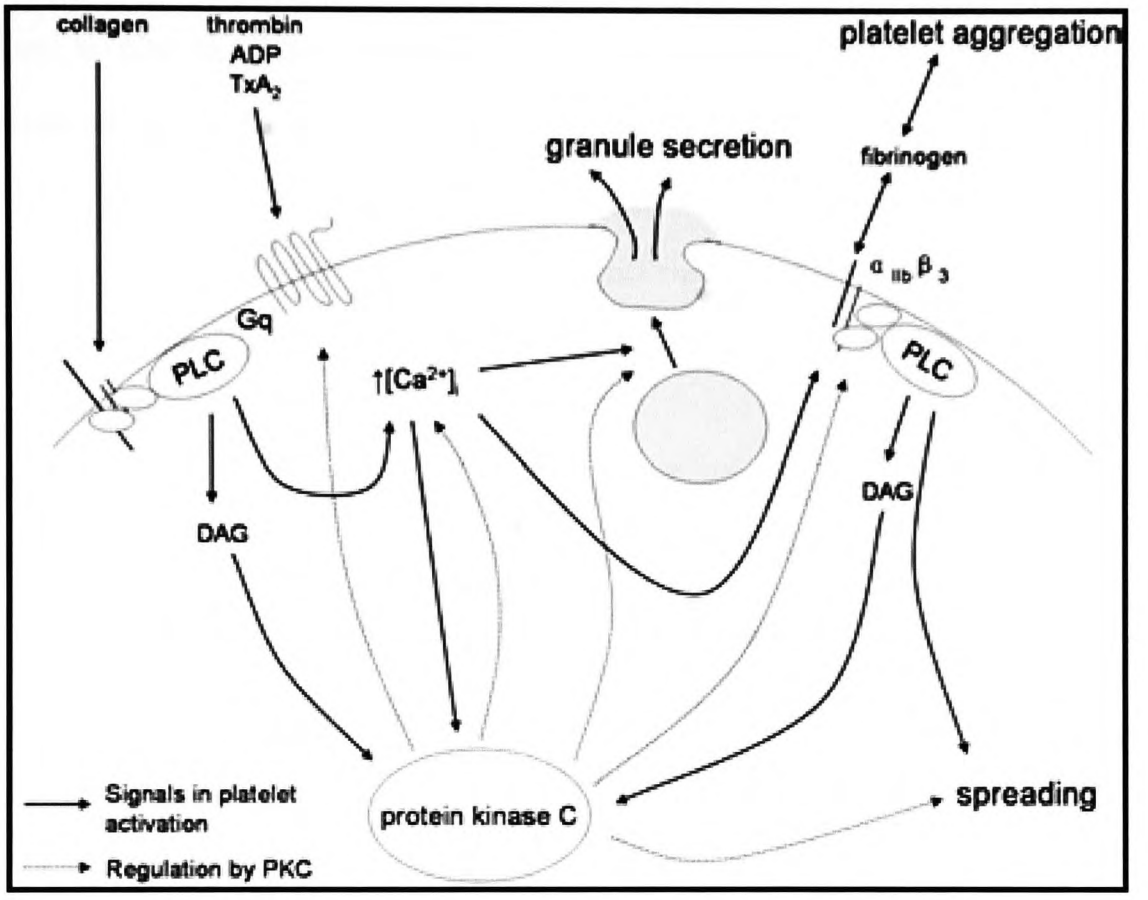



***Figure 1.12. Protein kinase C is a key regulator of platelet function.***

***Adhesion to collagen, or stimulation by soluble agonists such as thrombin, ADP or $TxA_2$, activates numerous intracellular signalling molecules, especially PLC, resulting in a rise in $[Ca^{2+}]$ and activation of PKC. PKC regulates many platelet responses to stimulation, such as granule secretion, aggregation and spreading.*** *Courtesy of (Harper et al., 2007).*

Under various conditions PKC colocalizes with actin microfilaments. This may in part be dependent upon interactions with actin binding proteins. However, particular PKCs may also interact directly. For example, $PKC_{\beta II}$ binds directly to F-actin but not to monomeric G actin (Blobe *et al.*, 1996). PKCε localizes with and directly binds actin (Prekeris *et al.*, 1996). *In vitro*, this interaction activates PKCε and, consistent with this, alters its protease sensitivity (Prekeris *et al.*, 1998).

Broadly speaking, in platelets evidence from knock-out mice modules suggest that different PKC isoforms play different roles. For example Buensuceso and colleagues (Buensuceso *et al.*, 2005), demonstrated the recruitment of $PKC_{\beta}$ to $\alpha_{IIb}\beta_3$ integrin during platelet interaction with either soluble or immobilized fibrinogen. Another study using knock-out mice has shown a novel role for $PKC_{\delta}$ in inhibiting collagen-induced aggregation (Pula *et al.*, 2006). Still further $PKC_{\alpha}$ may regulate granule secretion and thrombus formation (Konopatskaya *et al.*, 2009a). Furthermore (Pears *et al.*, 2008), demonstrated an impaired aggregation and secretion, but not spreading, in response to collagen and CRP (at low and moderate concentrations) in PKCε KO mice.

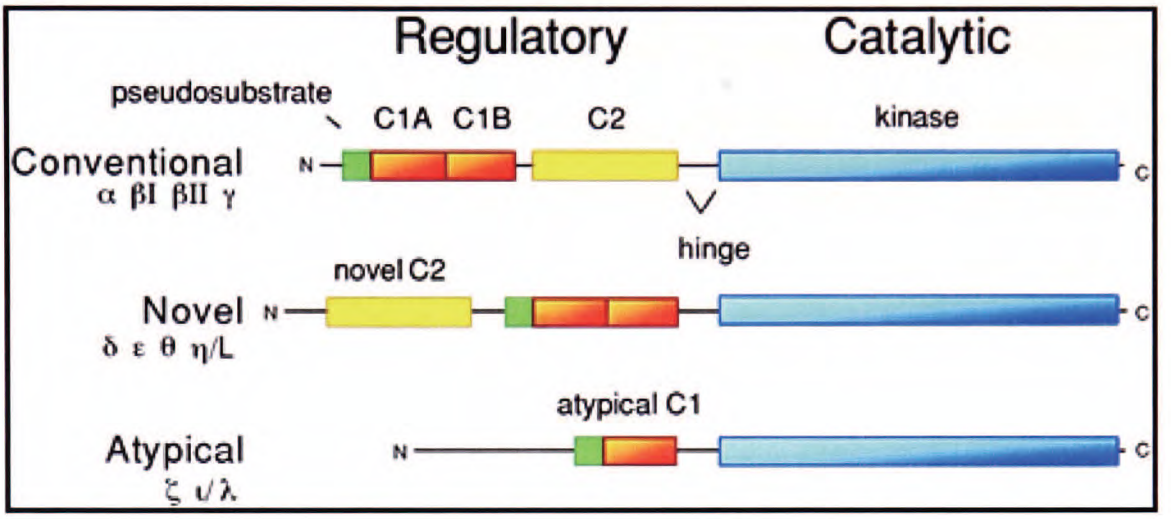



***Figure 1.13. Schematic showing the domain structure of the conventional, novel, and atypical subclasses of PKC.***

***Indicated are the pseudosubstrate, C1 and C2 domains in the regulatory moiety, and the carboxyl-terminal kinase domain****. Courtesy of (Barry et al., 2001).*

**Aims of study**

Over the last 10 years, platelet- and endothelial-derived NO has become a great focus for scientists in understanding the role of platelets in thrombosis and haemostasis. However, the beneficial actions of NO in regulating platelet function have frequently been overshadowed by the poorly documented negative effects mainly derived from the generation of peroxynitrite. Regulation of platelet function by NO is largely PKG-dependent. PKG is a serine/threonine protein kinase which is dependent on cyclic GMP and catalyzes the phosphorylation of serine or threonine residues of proteins. PKG belongs to the AGC family of serine/threonine protein kinases.

The aim of this study is to investigate the inhibitory actions of peroxynitrite on human platelets and whether AGC protein kinases are involved in peroxynitrite-mediated inhibition of platelet function. In addition, the regulation of AGC protein kinases by NO and $PGI_2$ in human platelets will be investigated.

This will be achieved by:

- To study the effects of peroxynitrite on intracellular signaling in human platelets.
- To examine the role of AGC protein kinases in peroxynitrite-mediated signaling in human platelets.
- To investigate intracellular compartmentation and signaling of AGC protein kinases with a greater emphasis on PKA.

# CHAPTER 2 METHODS

# 2. Methods.

## 2.1 Methods for the study of platelet function.

### 2.1.1 Isolation and preparation of human blood platelets.

Blood was drawn from consented volunteers, who claimed not to have taken any medication known to interfere with platelet function, using a 21-gauge butterfly needle with minimal stasis. The initial 5ml of whole blood were discarded to avoid the use of artifactually activated platelets. Platelet rich plasma (PRP) was prepared by centrifugation of whole blood at 200g for 20min at 25˚C using acid-citrate dextrose buffer (ACD) **(Appendix I-1)** as an anticoagulant. PRP was relocated to fresh centrifuge tubes and centrifuged at 800g for 12min at 25˚C in the presence of prostaglandin $E_1$ ($PGE_1$) (50ng/ml). The resulting platelet poor plasma (PPP) supernatant was discarded and washed platelets (WP) were prepared by suspending the platelet pellet in 1 ml of Tyrode's buffer **(Appendix I-1).** In some cases, Tyrode's buffer contained ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N'-teraacetic acid (EGTA) 1mM to provide platelets with non-aggregatory conditions or apyrase (1U/ml) and indomethacin (10μM) to abrogate the effect of ADP and $TXA_2$ , respectively. WP were left to recover from $PGE_1$ treatment for a minimum of 1h before starting experimental procedures. This method was adapted from (Vargas *et al.*, 1982)

Alternatively, PRP were treated with citric acid 0.3mM at a ratio of (20ul:1ml PRP) to lower the pH to 6.4. PRP were centrifuged at 800g for 12min at 25˚C. The pellet were suspended in washing buffer **(see Appendix I)** and

centrifuged 800g for 12min at 25°C. WP were resuspended in 1 ml Tyrode's buffer. In some experiments, phosphate buffer was used instead of Tyrode's buffer.

### 2.1.2 Determination of platelet count.

WP (5µl) were diluted 1:100 in Ammonium oxalate (1% w/v) and mixed well. This platelet suspension was then applied to a Neubauer cell counting chamber, which was then left to rest for 15 minutes. The number of platelets was counted on both sides of the cell counting chamber. The platelet count was corrected for dilutions and volumes and expressed as platelets/ml. All final platelet concentrations were calculated using this value **(Appendix I-2)**.

### 2.1.3 Preparation of platelet whole cell lysates.

WP ($5x10^8$platelets/ml) were prepared as described in **section 2.1.1**. For the preparation of lysates, WP were transferred to aggregometer cuvettes, preheated to 37°C for 1min and treated as with agonists or inhibitors with stirring. For longer stimulation times, sample was stirred for a maximum of 3min and then left under non-stirred conditions for the reminder of the incubation period. To terminate reaction Laemmli sample (2x) buffer **(Appendix I-6)**, was added at a ratio of 1:1 and gently mixed. All samples were stored on ice until the completion of the experiment. Sample were then boiled for 5min and stored at -20°C until required.

### 2.1.4. Measurement of protein concentration.

Aliquots, which were prepared for protein assay analysis, were subjected to a DC protein assay kit (Amersham Biosciences, UK). The assay is based on

the Lowry assay (Lowry *et al.*, 1951), and uses the reaction between protein and copper in an alkaline medium, followed by the reduction of Folin reagent by the copper-treated protein. All samples and bovine serum albumin (BSA) standards were diluted 1:2 in sterile PBS and applied to a micro titer plate in triplicate. Protein concentrations of samples were determined by comparing the absorbencies of each sample to the standard curve of known BSA concentrations using a wavelength of 750nm in a multiplate reader **(Appendix I-3)**.

### 2.1.5 Turbidimetric measurement of platelet aggregation.

Turbidimetric aggregation is a robust and reproducible technique that was initially described by Born (Born *et al.*, 1963). The assay is based on the principal of light transmission through a platelet suspension which is detected by a photocell. Suspended platelets cause light scattering and reduce the proportion of light passing through the suspension. The increase in light transmission is directly proportional to the degree of platelet aggregation. The aggregometer was calibrated using WP as 0% aggregation and Tyrode's buffer as 100% aggregation. Platelet aggregation measurement was used as a tool to assess the effect of various buffers on platelet function. WP were prepared using Tyrode's buffer and diluted to a final concentration of $3x10^8$platelets/ml **(Appendix I-4)**.

### 2.1.6 Analysis of platelet adhesion using fluorescence microscopy.

To be able to assess platelet adhesion quantitatively, platelets were left to adhere to immobilized collagen, permeabilised with triton X100 (0.1%) and the F-actin stained with Phalloidin-TRITC. Phalloidin is a fungal toxin that binds to the polymeric and oligomeric forms of actin, thus strongly stabilising actin filaments. This property of the agent can be used to deliver and fix fluorescent conjugates such as Tetramethyl Thodamine Iso-Thiocyaniate (TRITC) into the cell. TRITC has an excitation wavelength of 540-545nm and an emission wavelength of 570-573nm which makes it suitable to visualize using a fluorescence microscope.

WP ($2x10^7$platelets/ml) were prepared as described in **section 2.1.1**. When required, aliquots (500µl) of WP were incubated with inhibitors for up to 20min at 37°C prior to the addition of agonists. Immediately following the addition of agonists/antagonist, WP aliquots (100µl) were added to the microscope slides. Platelets were left to adhere at 37°C for 30 min before removing all unbound platelets with PBS. Adherent platelets were then fixed using para-formaldehyde (4%) (100µl), adding it to each area and left for 15min at room temperature. Excess para-formaldehyde was then washed off using PBS and adherent platelets were permeabilised using Triton-X100 (0.3%) in PBS for 7 min. Platelets were then incubated with phalloidin-TRITC (50µg/ml) for 40min at room temperature protected from light. Excess phalloidin-TRITC was then washed off using PBS and microscope slides covered with a glass cover slip. Adherent platelets were visualised using a

Olympus fluorescent microscope with an x60 oil immersion objective (Roberts *et al.*, 2008).

### 2.1.7 Quantitation of platelet adhesion.

In order to quantify the amount of adherent platelets, images of ten random fields of view were taken of each sample for each experiment to calculate the total number of adherent platelets /0.1mm$^2$.

### 2.1.8 Preparation of peroxynitrite and prostacyclin.

Peroxynitrite is a cell-permeable strong biological oxidizing agent that reacts with DNA, membrane phospholipids, sulfhydryl groups, and tyrosine. Peroxynitrite, synthesized from isoamyl nitrite and hydrogen peroxide, was purchased from Calbiochem. Peroxynitrite stock (170mM) was diluted in 0.1mM NaOH to give a final concentration of 100μM.

Prostacyclin, a potent platelet inhibitor, was purchased from Sigma. Stock was diluted in 100% ethanol to give a final dilution on 100nM.

## 2.2 Measurement of platelet cGMP concentrations.

The haem containing enzyme sGC is the major Intracellular receptor for NO. Once inside platelets, NO interacts with the haem group of β subunit of sGC, leading to its activation and to the conversion of GTP to cGMP (Radomski *et al.*, 1990). Thus, the measurement of cGMP concentration is a measure of sGC activation. cGMP concentrations were measured using a cGMP Enzyme Immunoassay Biotrak (EIA) system. This is a simple, sensitive method for measuring cGMP in biological samples at a range of 0.05 to 100

picomoles/ml. The method is based on the competitive binding of cGMP in the sample and a radioiodinated derivative of cGMP ([I-125] cGMP), for a highly specific antibody. The amount of labeled cGMP found in the complex decreases with increasing concentration of unlabeled cGMP in the sample. Separation of antibody bound cGMP from free cGMP is achieved through a precipitating antibody incorporated in the reagent system. Determination of the unknown is made by the comparison with a standard curve constructed in the same fashion. For the assay, WP ($3X10^8$) were isolated as described in **section 2.1.1** and incubated with zaprinast (10μM) for 20min, to inhibit PDEs activity, before the addition of peroxynitrite. Reaction was stopped by the addition of dodecyltrimethyl ammonium bromide (0.5%); this reaction hydrolyses cell membrane and release intracellular cGMP. **(Appendix I-5)**.

## 2.2.1 Enzyme-immunoassay procedure.

Within this assay several control wells were used including blanks to determine the background signal of the assay, and to determine the non-specific binding (NSB) of the conjugate to the secondary donkey anti-rabbit antibody. These were run in addition to the standards and unknown samples. All samples were run in duplicate. First, a series of standards containing 0 to 512fmol/ml cGMP were prepared by serial dilutions in glass test tube. Samples prepared for cGMP measurements in platelets suspension were thawed and centrifuged at 15000g for 5min to sediment insoluble debris. The resulting supernatants, containing extracted cGMP, were used in the assay. All standards and samples were acetylated by addition of acetylation reagent (1:10) using a mixture of acetic anhydride (1 volume) and triethylamine (2

volumes). Aliquots (50µl) of all acetylated standards and unknown samples were transferred into the appropriate wells in the presence of antiserum (100µM). Plates were incubated at 4°C for 2h followed by addition of HRP-labelled cGMP conjugate (100µl). After 1h incubation, plates were emptied by inversion and were blotted on tissue to remove any residual buffer before addition of the room temperature equilibrated enzyme substrate, TMB (200µl), into the wells. Plates were mixed on a microplate shaker for 30min at room temperature and then the colorimetric reaction was stopped by addition of sulphuric acid (1M) (100µl). The absorbance was read in a microplate reader at 450nm within 30min. This protocol was followed according to manufacturer's instruction.

### 2.2.2 Analysis of cGMP data.

The mean of all duplicate samples was calculated and the mean absorbance (Abs) of the NSB wells was subtracted from all other values. The percent bound (%B/Bo) for each standard (std) and unknown lysates was calculated as follows:

%B/Bo = (std or sample Abs-NBS Abs)/(zero std Abs-NBS Abs) x100

A standard curve was generated by plotting the %B/Bo (y axis) against cGMP expressed in fmol/$10^8$ (x axis).

## 2.3 Analysis of phosphorylation based protein signaling in platelets.

### 2.3.1 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) is a method used to separate proteins according to their size by utilising the properties of a polyacrylamide gel. Sodium dodecyl sulphate (SDS) is a detergent which affects hydrophobic molecules thus leading to the dissolution of cell membranes. SDS also binds to polypeptides in a constant weight ratio. In this process, the intrinsic charge of polypeptides becomes negligible when compared to the negative charges contributed by SDS. Thus, polypeptides after treatment become a linear structure possessing a uniform charge density that is same net negative charge per unit length. Mobility of these proteins will be a linear function of the logarithms of their molecular weights. Polyacrylamide is a polymer of acrylamide monomers which turns into a gel once the polymer is formed. When an electric current is applied, the negatively charged proteins will move through the polyacrylamide gel towards the positive electrode (anode). Due to the polyacrylamide tunnel structure, large proteins will travel slower through the gel than small proteins (Laemmli, 1970a; Shapiro *et al.*, 1967).

Gradient gels are prepared by pouring two resolving acrylamide solutions (10% and 18%) at a pH of 8.8 using a linear gradient former and a peristaltic

pump. This give high degree of resolution, as the low-percentage acrylamide gel at the top enables resolution of high-molecular-mass proteins, and higher-percentage gel at the bottom ensures resolution of low-molecular-mass proteins. The stacking gel is a large pore polyacrylamide gel (3%) at pH 6.8, lies on top of the resolving gel and contains the loading wells. The lower pH and lower acrylamide percentage of the stacking gel allows the proteins to form a tight band which ensures that all proteins reach the higher percentage resolving gel at the same time.

### 2.3.2 Procedures for SDS-PAGE.

Sample preparation for gel electrophoresis requires the addition of SDS and a reducing agent. Adapted from work which was originally conducted by Laemmli (Laemmli, 1970b), the Laemmli sample buffer contains SDS as well as 2-mercaptoethanol.

### 2.3.3. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis method.

Depending on the protein under investigation, a 10% or a 10-18% gradient polyacrylamide was selected. For detailed gel oompositions please refer to the **Appendix I-7**. The resolving gel was poured with aid of a gradient mixer and a peristaltic pump and left to set at room temperature for approximately 1h. Once polymerised, a 3% stacking gel was poured on top of the resolving gel and a well-forming comb inserted immediately. The stacking gel was left to set for approximately 20min at room temperature before removing the well-forming comb and washing all wells with running buffer **(Appendix I-7)**.

The wells of the gel were then loaded with aliquots of the protein samples (10-15µg), and in one well an aliquot (10µl) of biotinylated protein standard. Using a molecular weight protein standard increased the accuracy when estimating the molecular weight of specific proteins after immunoblotting. Gels were subjected to 120V for 2.5h.

## 2.4 Immunochemical investigation of platelet proteins.

Antibodies (immunoglobulins) are heavy plasma proteins consisting of two identical heavy chains and two identical lights chains which are connected by disulfide bonds. Together these polypeptide chains form a 'Y'-structure with two antigen binding sites. An antigen is defined as a foreign body which induces the activation of the immune response and the production of antibodies. It is possible to create antibodies to react with specific antigens which can then be used for biochemical applications such as Western blotting or immunoprecipitation.

### 2.4.1 Immunoprecipitation.

Immunoprecipitation (IP) is the technique of precipitating a protein containing the antigen using an antigen-specific antibody. Using this technique, it is also possible to immunoprecipitate a complex of proteins attached to the antigen-bearing protein. Once the antibody binds its antigen, the antibody-antigen complex can be precipitated out of solution using insoluble antibody-binding proteins, such as Protein A or Protein G. These in turn are coupled to

sepharose beads which can easily be isolated out of a solution. Immunoprecipitation partially purifies and isolates a protein under investigation, allowing more detailed and precise study, but can also aid in the identification potential interacting proteins. Once a protein or protein complex has been isolated, the resulting sample can be processed using SDS-PAGE and Western blotting **(figure 2.1)**.

### 2.4.1.1 Preparation of Protein A/G sepharose beads.

An aliquot of Protein A/G sepharose beads (300µl) was relocated to a fresh eppendorf tube and centrifuged at 13000g for 10sec. The resulting supernatant was removed from the bead pellet and $TBS_{T(0.1\%)}$ (75µl) was used to wash the bead pellet before centrifuging at 13000g for 10sec. This washing step was repeated twice before carefully removing the remaining supernatant. The bead pellet is then weighed and a 50% (w/v) suspension in $TBS_{T(0.1\%)}$ prepared.

### 2.4.1.2 Immunoprecipitation of platelet proteins.

Sample preparations for WP were processes as described in section **2.2.1**, although in this case ice-cold lysis buffer containing a cocktail of phosphatase and protease inhibitors was used **(Appendix I-8)**.

Sepharose beads were blocked with BSA (1mg/ml) in PBS or samples were pre-cleared by mixing lysates with protein sepharose beads. Preclearing minimizes non-specific binding of non-antigen bearing proteins to sepharose beads. For Syk and AMPk, 1µg of antibody was added to each 25µl of beads and left to mix for 3 hours at 4C° with rotation. 300-400µg of lysates per sample were added to the antibody/bead mixture and left to mix overnight at

4C° with rotation. Subsequently the samples were centrifuged at 13000g for 1min to produce a pellet of protein sepharose beads bound to the antibody-antigen complex. The remaining supernatant was relocated to fresh eppendorfs for immunoblotting analysis or discarded. The bead pellet was washed once with lysis buffer and twice with $TBS_{T(0.1\%)}$ before removing the supernatant and adding Laemmli sample buffer (65µl) to the pellet. Samples were then boiled for 5min to ensure complete separation of the Protein A sepharose bead-antibody-antigen complex. Protein A sepharose beads were then pelleted using pulse centrifugation for 10s and the supernatant containing the antibody and antigen-bearing protein was loaded directly on a SDS-PAGE gel and processes as described earlier.

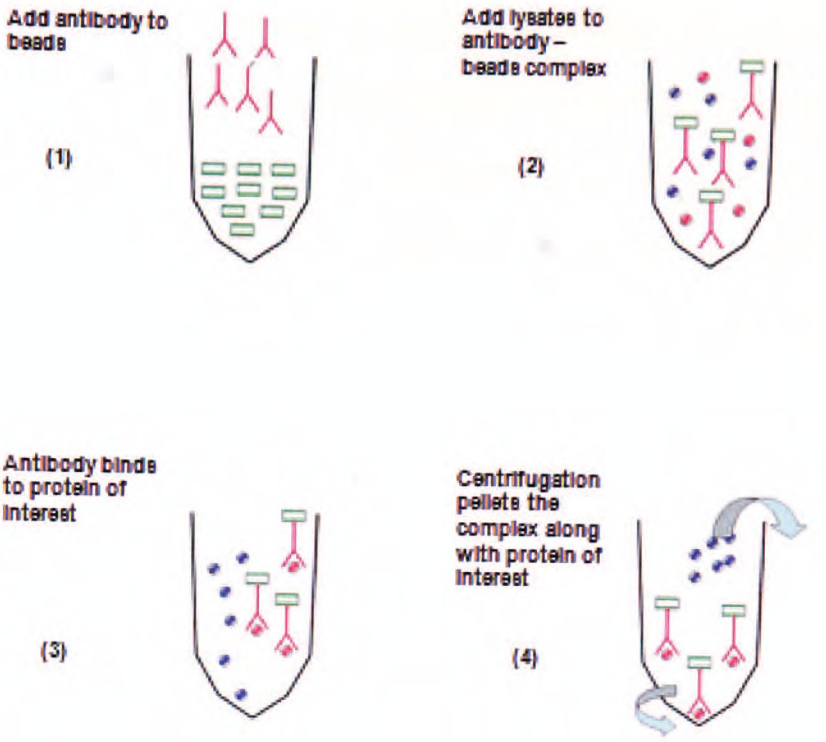



***Figure 2.1. Schematic diagram of immunoprecipitation.***

### 2.4.1.3. Cross-linking the antibody to protein G/A agarose beads.

Crosslinking an antibody to Protein A or G beads results in a permanent affinity support with the antibody properly oriented to bind the target antigen (Kaboord *et al.*, 2008). A common homobifunctional crosslinker such as Dimethyl pimelinediimidate dihydrochloride (DMP), which has carboxyl group at both ends, reacts with primary amines on both the antibody and the Protein A or G molecules in the pH range 7.0-10.0 to form amidine bonds, preventing loss of the antibody during antigen elution. This method is a good choice when the molecular weight for the protein of interest is the same or close to that for heavy or light chain **(figure 2.2)**.

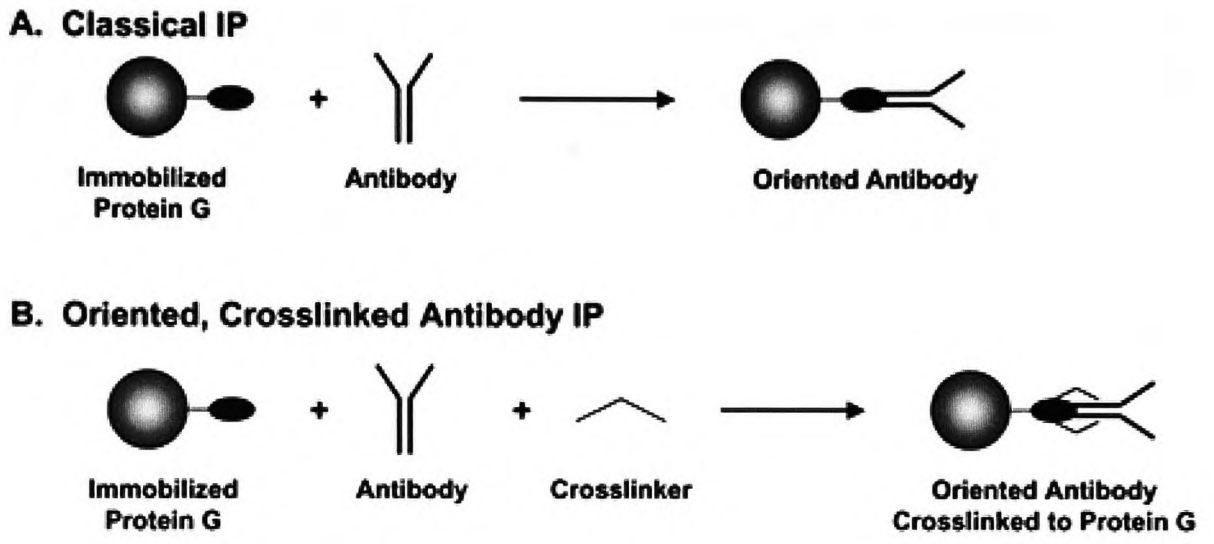



*Figure 2.2. Strategies preparing cross-linked antibody IP.*

25µl of beads were aliquoted into small eppendorfs, and washed with 400 µl PBS with rotation overnight at 4°C. After washing beads with 500ul D-buffer, beads were incubated with antibody. For Csk and PKA, 5µg of antibody was added to each 25µl of beads and left to mix for 3 hours at 4C° with rotation. Subsequently the samples were centrifuged at 13000g for 1min to produce a pellet of protein sepharose beads bound to the antibody-antigen complex. After washing pellet with PBS, beads were incubated with 500ul of DMP (13mg/ml) for 30min at 4°C. Subsequently After spinning samples, beads were washed with 500 µl D-buffer, samples were incubated with 2$^{nd}$ and 3$^{rd}$ DMP aliquots.

To quench excess DMP, beads were washed twice with 500µl Q-buffer **(Appendix I-10)** and once with PBS with rotation for 5 min at 4°C. To remove un-cross-linked antibody, beads were washed twice with 500µl 1M glycine pH 3 with rotation for 10 min.

To elute bound antigen, 10µl of 1M Tris-HCL, pH 9.5, placed into new eppendorfs receiving the eluted antigen. The Tris buffer will serve to immediately neutralize the low pH eluent, minimizing exposure of antigen to low pH conditions. 190µl of 0.1M glycine, pH 2.8, elution buffer was added to samples, mixed several times before transferring the mixture into the receiving tubes. Tubes then were centrifuged for 1 min, and the eluted buffer containing the antigen was saved in new eppendorfs to analysis with Western blot as in **figure 2.3**. This is protocol was adopted from (Clark) and (Kaboord *et al.*, 2008).

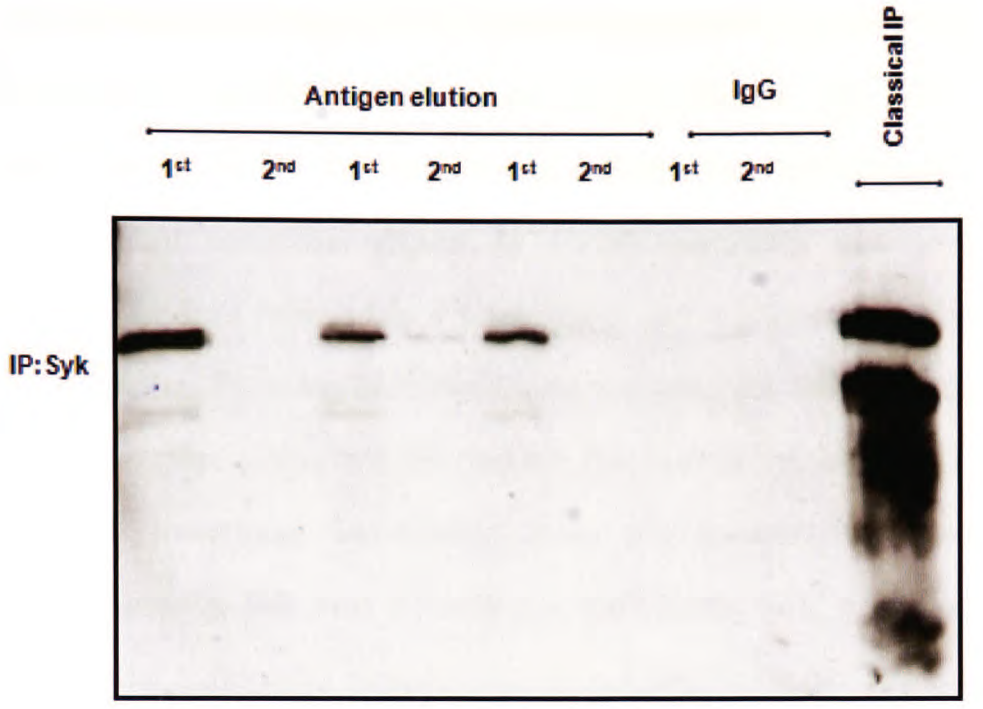



***Figure 2.3. Comparison of immunoprecipitation results between classical and cross-linked approaches***

***WP ($5x10^8$) were lysed with lysis buffer (1:1). Syk kinase was immunopreciptaed from lysates either using classical IP method (see methods) or by cross linking anti-Syk antibody to protein A sepharose beads first. Proteins were eluted with 0.1M glycine, pH 2.8. 2x Laemmli buffer were then added. Proteins were separated on SDS-gel and immunoblotted with anti-Syk antibody.***

## 2.4.2. Immunoblotting.

Immunoblotting is a method used to transfer separated proteins from a gel to the surface of a nitrocellulose paper or polyvinylidene difluoride (PVDF) (Towbin *et al.*, 1979). The immobilized proteins are accessible to interact with different antibodies (figure 4). PVDF membrane was pre-wet in methanol for 3min followed by a 10min wash with $dH_2O$ and finally stored in transfer buffer. Following SDS-PAGE, the resolving gel was separated from the casting glass plates and inserted into the transfer cassette together with the PVDF membrane, wet blotting paper, and sponges. The assembled transfer cassette was then inserted into the transfer tank, which was filled with transfer buffer, and placed in an ice box to prevent overheating during transfer. A constant voltage (100v) was applied for 2.5h **(figure 2.4)**.

Following protein transfer, the transfer cassette was dissembled and the PVDF membrane washed with $TBS_{T(0.1\%)}$ for 5min before immersing the membrane in 10% (w/v) BSA containing $TBS_{T(0.1\%)}$ for 30min at room temperature to block unoccupied protein binding sites on the membrane. This was followed by immersion of the membrane in the primary antibody solution at 4˚C overnight with gentle agitation. Primary antibody solutions (2% w/v BSA in $TBS_{T(0.1\%)}$) were either prepared on the day or stored at 4˚C for reuse by adding 0.1% (w/v) sodium azide to prevent bacterial contamination. Membranes were then washed 2 times 10min in $TBS_{T(0.1\%)}$ before incubation with secondary antibody solutions (2% v/w BSA in $TBS_{(T0.1\%)}$) for 1h at room temperature. Secondary antibody solutions also contained HRP-conjugated anti-biotin antibody (1:1000) for the detection of the biotinylated protein standard. Western blotting membranes were then

washed 4 to 6 times15min in $TBS_{T(0.1\%)}$ before immersing them in enhanced cemiluminescence (ECL) solution **(Appendix I-10)** for 90sec with gentle agitation protected from light. Membranes were then transferred to an exposure cassette and Bio Max film and developer solutions were used to visualise the immunoblot. All films were washed extensively with $dH_2O$ after processing. For all experiments involving the anti-nitrotyrosine antibody, fat free milk (5%) was used to block membranes instead of BSA.

Densitometry analysis was

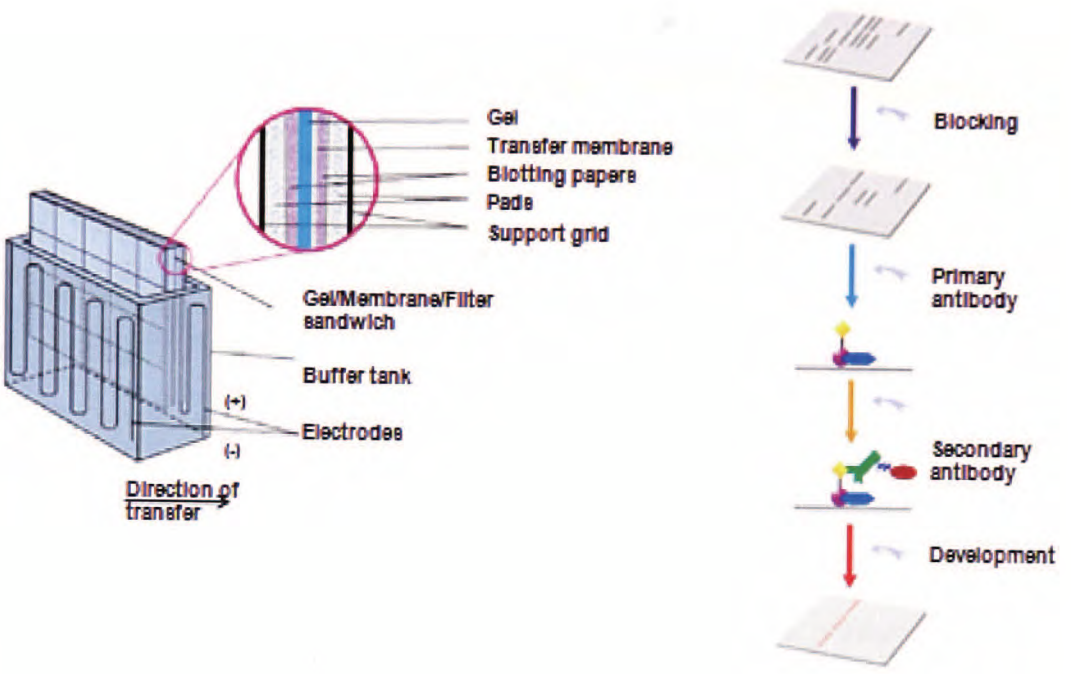



***Figure 2.4. Schematic diagram of immunoblotting.***

### 2.4.3. Stripping and reprobing of membranes.

A stripping procedure was used to remove the primary and secondary antibodies used during the first immunoblotting procedure. The membrane could then be "reprobed" with second combination of primary and secondary antibodies.

PVDF membranes were incubated with a stripping solution **(see Appendix)** at 80°C for 20 min. Membranes were then washed repeatedly in $TBS_{T(0.1\%)}$ to remove all traces of stripping solution before directly applying the desired primary antibody solution as described in section.

## 2.5 Subcellular fractionation of human platelets.

This method was used to separate the cytosolic fraction from the membrane fraction of the cell using ultracentrifugation. WP (5-7x $10^9$) were prepared as described earlier and incubated with fractionation buffer in a ratio of 1:1 **(Appendix I-11)**. The suspension was frozen rapidly in liquid nitrogen and thawed, which was repeated 5 times. Sonication was avoided as such harsh methods may lead to artificial dissociation of proteins that might be loosely attached to cell membrane. After freezing and thawing, lysates were ultracentrifuged at100000g for 60 min at 4°C. The pellet, which is the cytoskeleton fraction, was suspended in pellet buffer. Protein concentration in each fraction was measured using Bradford assay (Lowry *et al.*, 1951). Laemmli buffer was added to each of the fractions, which were then processed for electrophoresis and immunoblotting. This is a modified method from (Hall *et al.*, 2007).

## 2.6. The isolation of membrane lipid rafts.

Lipid rafts are regions in the membrane bilayer enriched with cholesterol and sphinoglipids that are highly ordered in comparison to the rest of the cell membrane (Simons *et al.*, 1997). As a consequence, lipid rafts can be isolated as a low-density, insoluble fraction after low-temperature non-ionic detergent extraction (Brown *et al.*, 1992).

The method used for lipid rafts isolation is a combination of sedimentation and flotation, achieved by using a density of sucrose gradient that straddles the density of particles concerned. On centrifugation, the particles move to an area of iso-density (Smyth, 1996) **(figure 2.5)**.

WP (450µl of $9x10^8$) were solubilised with an equal volume of ice-cold Raft lysis buffer **(see Appendix I-12)** and placed on ice for 30 min. Samples were then mixed with an equal volume of an ice-cold 80% sucrose and placed at the bottom of polyallomer ultraclear ultracentrifuge tubes (Beckman). Successive volumes of 30% (5 ml) and 5% (5 ml) sucrose solutions were consecutively layered upon the solubilised sample. The tubes were centrifuged at 200000g at 4°C for 18 hr. 1 ml fractions were sequentially removed from the top of the gradient and analysed by Western blotting. This is a modified method from (Lee *et al.*, 2006).

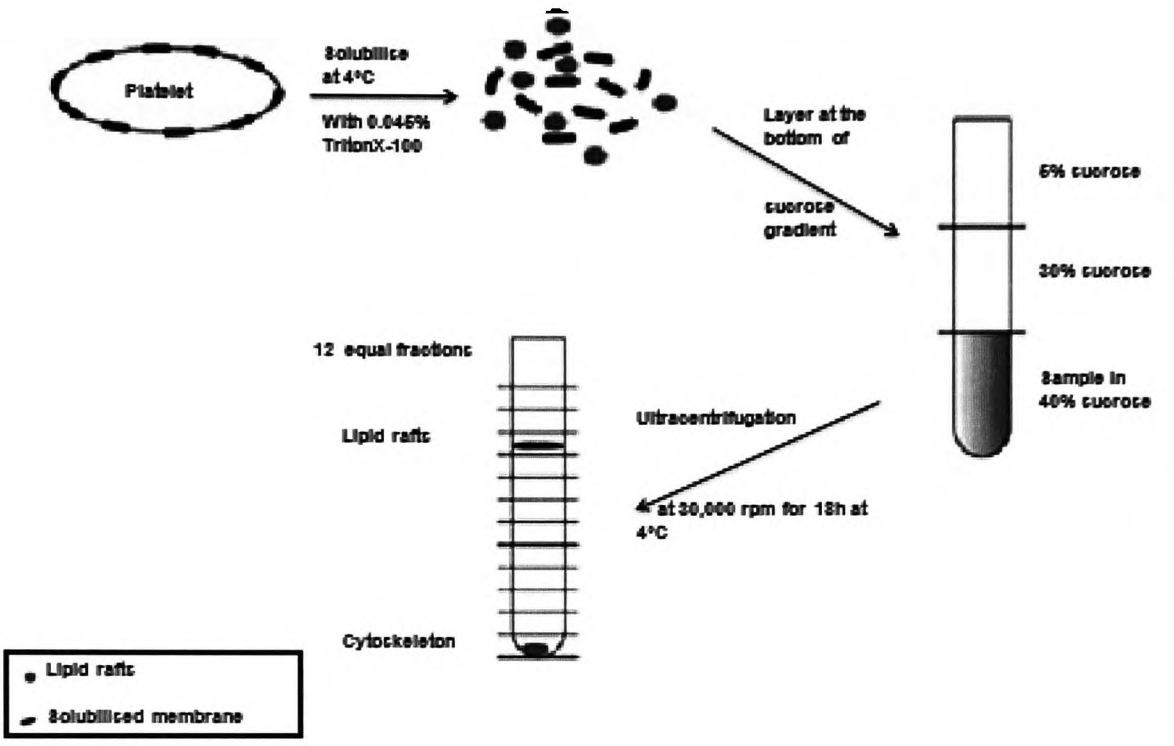



*Figure 2.5. Schematic diagram of isolation of lipid rafts.*

## 2.7 Phospho-flow analysis.

Flow cytometry has become an indispensable tool in clinical and basic immunological research due to its ability to distinguish subsets in heterogeneous populations of cells. Surface staining may be an effective means of characterizing cells; however it does not provide information about the functional responses of those cells to stimuli that are immediately reflective of intracellular events (Marodi *et al.*, 2001). Phospho-flow analysis is based on the premise that the phosphorylation state of an intracellular protein that correlates with its biological status (McCubrey *et al.*, 2000). As part of this project, preliminary experiments were performed to apply this methodology to the study of platelet signaling events.

### 2.7.1 Sample preparation.

WP were prepared as in **section 2.1.1.** Samples, containing 2.5 $X10^7$ platelets/ml, were treated with agonists or inhibitors as desired in cuvettes at 37C° with stirring. Samples were fixed for 10mins with a pre-warmed Fix buffer (BD Biosciences) at a ratio of 1:1+10, for example (100:110). Samples were then centrifuged at 13000g/10mins at 4C°. Fix buffer was aspirated and cells were permeabilised by resuspending in 300µl pre-cooled Perm buffer (BD Biosciences), and samples were stored at -80°C until further analysis.

### 2.7.2 Fluorescent cell barcoding (FCB).

Flow cytometry allows high-content, multiparameter analysis of single cells, making it a promising tool for drug discovery and profiling of intracellular signaling. In fluorescent cell barcoding (FCB), each sample is labeled with a

different signature, or barcode, of fluorescence intensity and emission wavelengths, and mixed with other samples before antibody staining and analysis by flow cytometry. The advantage of using such cell-based multiplexing technique is reducing antibody consumption and acquisition time (Krutzik *et al.*, 2006).

The fluorescent bar-coding of samples utilized a 96 well plate to add a specific ratio of dye to each sample or treatment. In these experiments to two dyes used were Pacific Blue (PcBlue) and Pacific orange (PcOrange). In the first instance a serial dilution of each dye was performed. For PcBlue 4 – 0.027µg/ml and for PcOrange 40 -0.27µg/ml). The dyes, 10µl of each, were then added to the wells of a 96-well plate as shown below.

| | | | |
|---|---|---|---|
| 4µg\40µg (sample1) | 1µg\40µg (sample2) | 0.25µg\40µg | 0.027µg\40µg |
| 4µg\10µg | 1µg\10µg | 0.25µg\10µg | 0.027µg\10µg |
| 4µg\2.5µg | 1µg\2.5µg | 0.25µg\2.5µg | 0.027µg\2.5µg |
| 4µg\0.27µg | 1µg\0.27µg | 0.25µg\0.27µg | 0.027µg\0.27µg (sample16) |

This ensured that each well had an individual ratio of PcBlue to PcOrange. Samples were centrifuged at 13000g/5mins at 4°C, the Perm buffer was aspirated and pellet resuspended in PBS (180µl). An individual platelet sample was then added to each of the wells, which meant that each sample had an individual fluorescent label or "barcode". Samples were incubated with the dyes for 30min at RT. For PcBlue and PcOrange controls, 190µl unstained sample were incubated with 10µl of the highest concentrations. In

this was each sample has an individual fluorescent signature of PcBlue and PcOrange based on the concentration of each dye as shown **(figure 2. 6.)**.

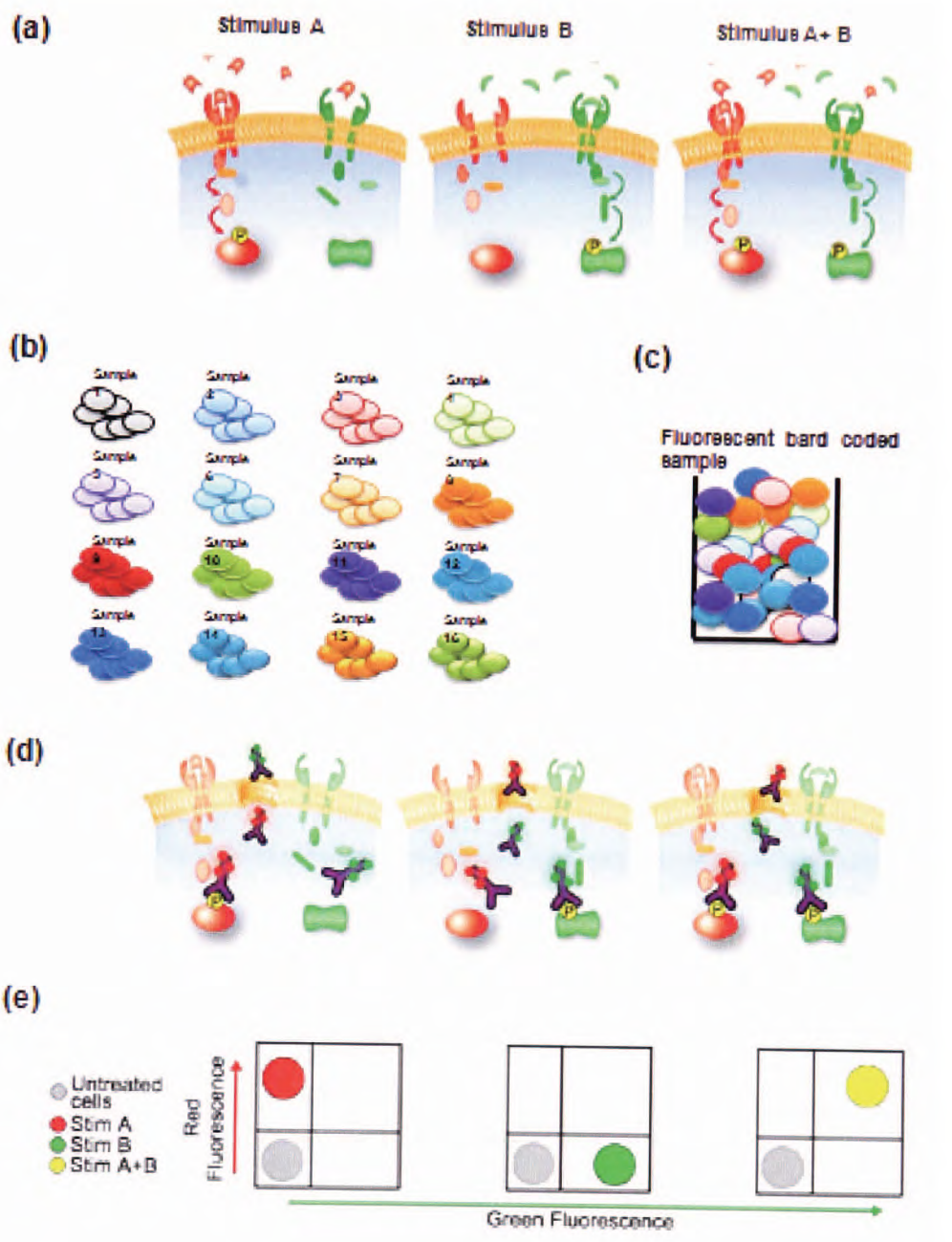



***Figure 2.6. Phosphoprotein staining technique for phospho flow analysis. Samples are treated with different stimulus (agonists, inhibitors, drugs, ect), fixed and permeabilised (a). Samples are then bar coded with a serial dilution of PcBlue and PcOrange (b). Samples are combined (c), and stained with fluorochrome-conjugated phospho-specific antibodies (d). Finally cells are analyzed with a flow cytometer.***

### 2.7.3 Antibody-antigen conjugates.

Subsequently, samples were centrifuged in a plate centrifuge at 1300g/5mins at 4°C, and pellet was washed twice with flow wash buffer **(Appendix I-13)**. After second spin, bar-coded samples were combined by resuspending pellet in first well in 200µl flow wash buffer, which then used to resuspend pellet in second well and so on, till all pellets had been collected. Final volume of bar-coded sample was adjusted according to the number of antibodies which would be used.

For PcBlue and PcOrange controls, 100µl flow wash buffer and 50µl of unstained samples were added to each.

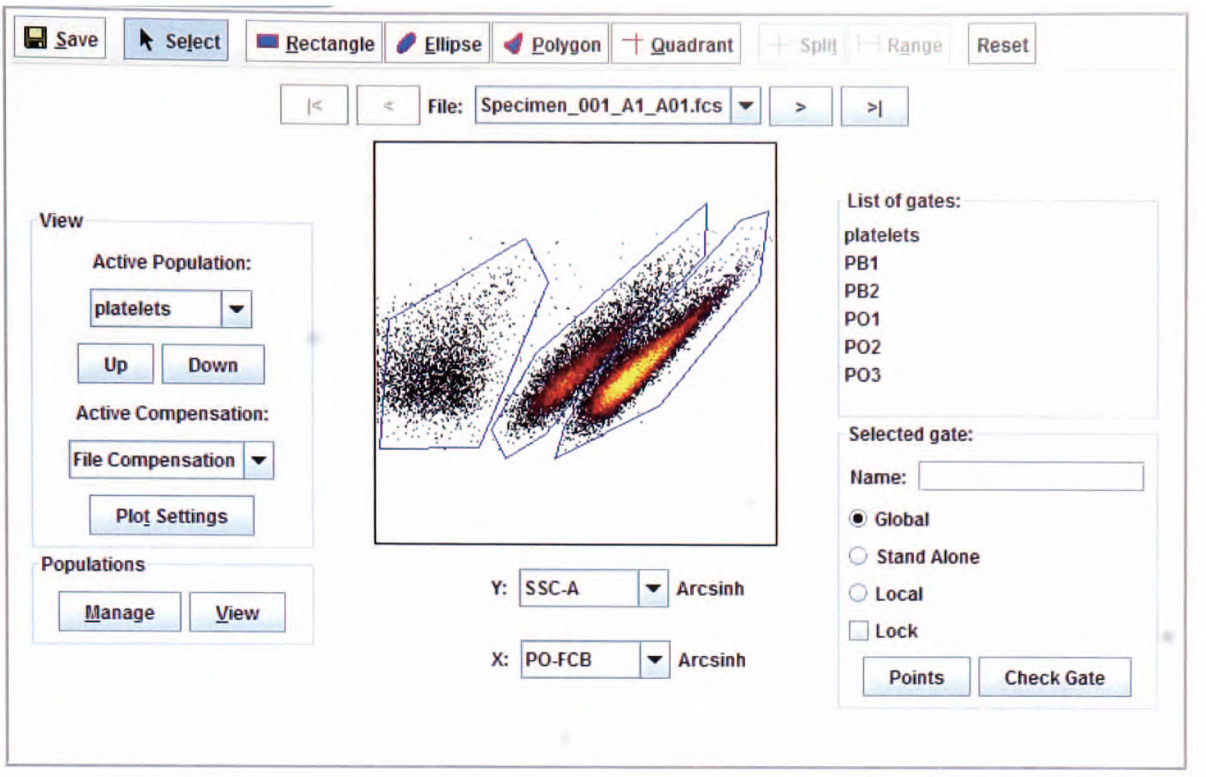
Conjugated primary antibodies at optimal titration in 20µl of flow wash buffer were added to new 96-well plate. To these 80µl of bar-coded samples were incubated with antibodies for 30mins in the dark at RT. Subsequently, 100µl of flow wash buffer were then added and 96-well plate was centrifuged in a plate centrifuge at 1300g/5mins at 4°C. Un-bound antibodies were discarded by inverting the 96-well plate. 150µl of flow was added to each sample and assembled for analysis on flow cytometry. Data were further analyzed on phospho-flow software, Cytobank, courtesy of Stanford University (USA).

For un-conjugated primary antibodies, (bar-coded samples+ primary antibody) were incubated with secondary conjugated antibodies at a ratio of 1:8000 for 30mins in the dark at RT. and wash step was repeated.

### 2.7.4 Samples analysis with Cytobank.

This is analysis software that was developed by a team in Stanford University (USA) and enables users to build data layout using details from flow experiments. Data are first uploaded to the program; events recorded by flow cytometry are gated into population according to the intensity degree of PcBlue and PcOrange **(figure2.6).**

Samples were then assigned to the population corresponding to the intensity of PcBlue and PcOrange staining. For example, control sample corresponds with PcBlue 1 and PcOrange 1 staining **(figure2.7)**. After assigning samples to populations, samples are then analyzed for phospho-antibodies. Data are presented using one of the templates such as Histograms, Overlays or Heatmaps **(figure2.8).**



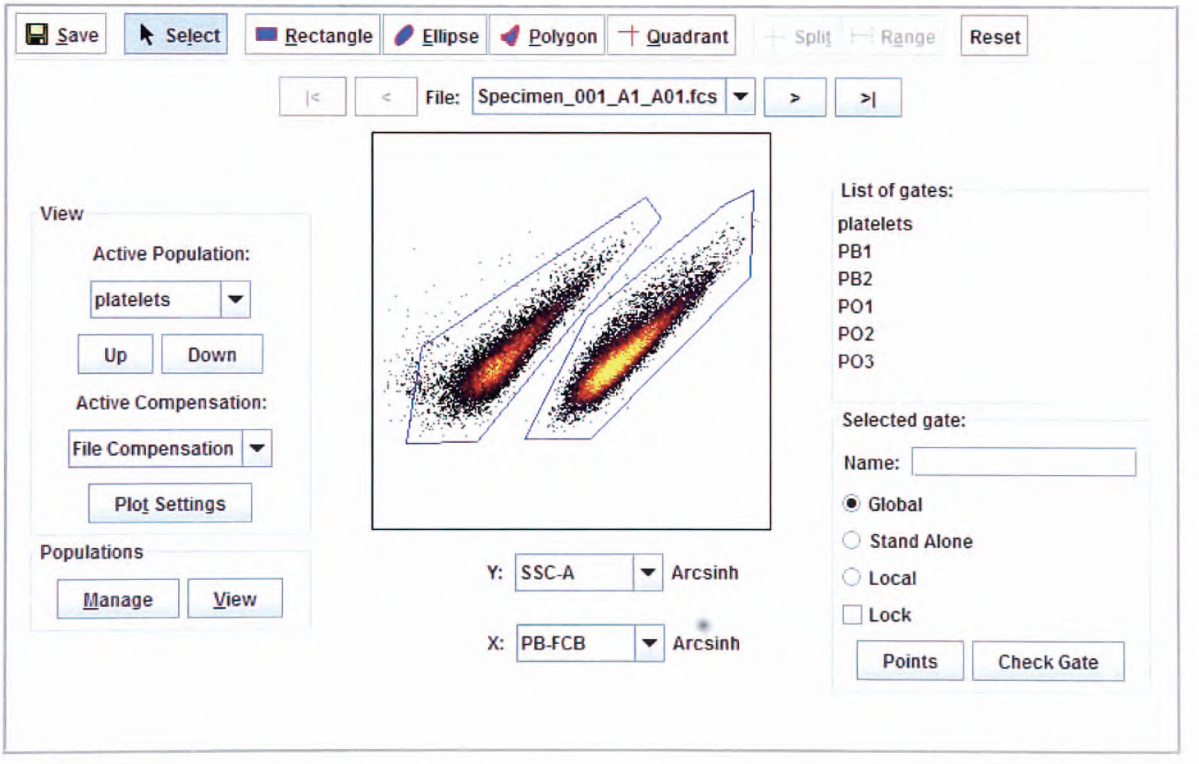
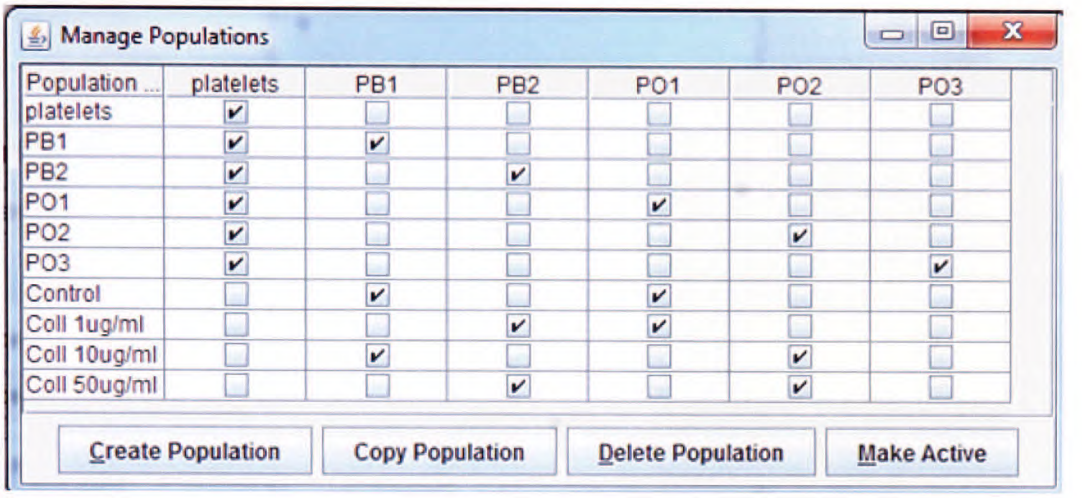




***Figure 2.7. Example of FCB using PcOrange and PcBlue staining.***



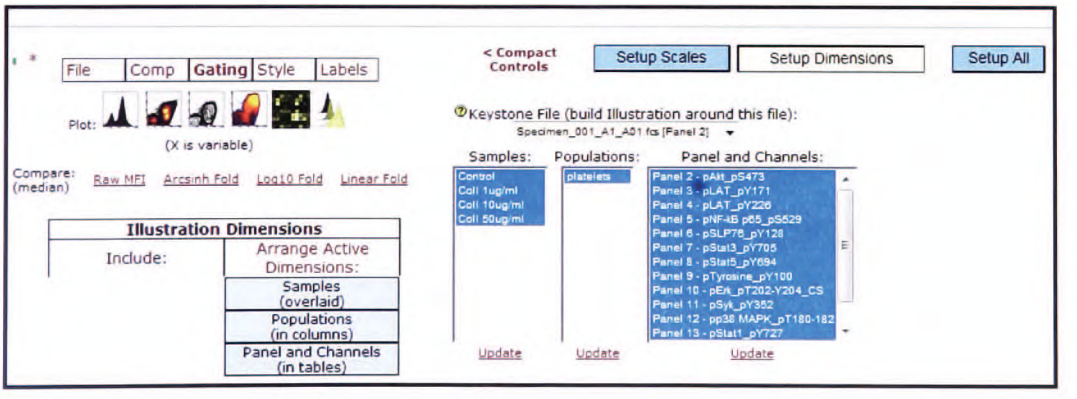




***Figure 2.8. Assigning samples to populations.***

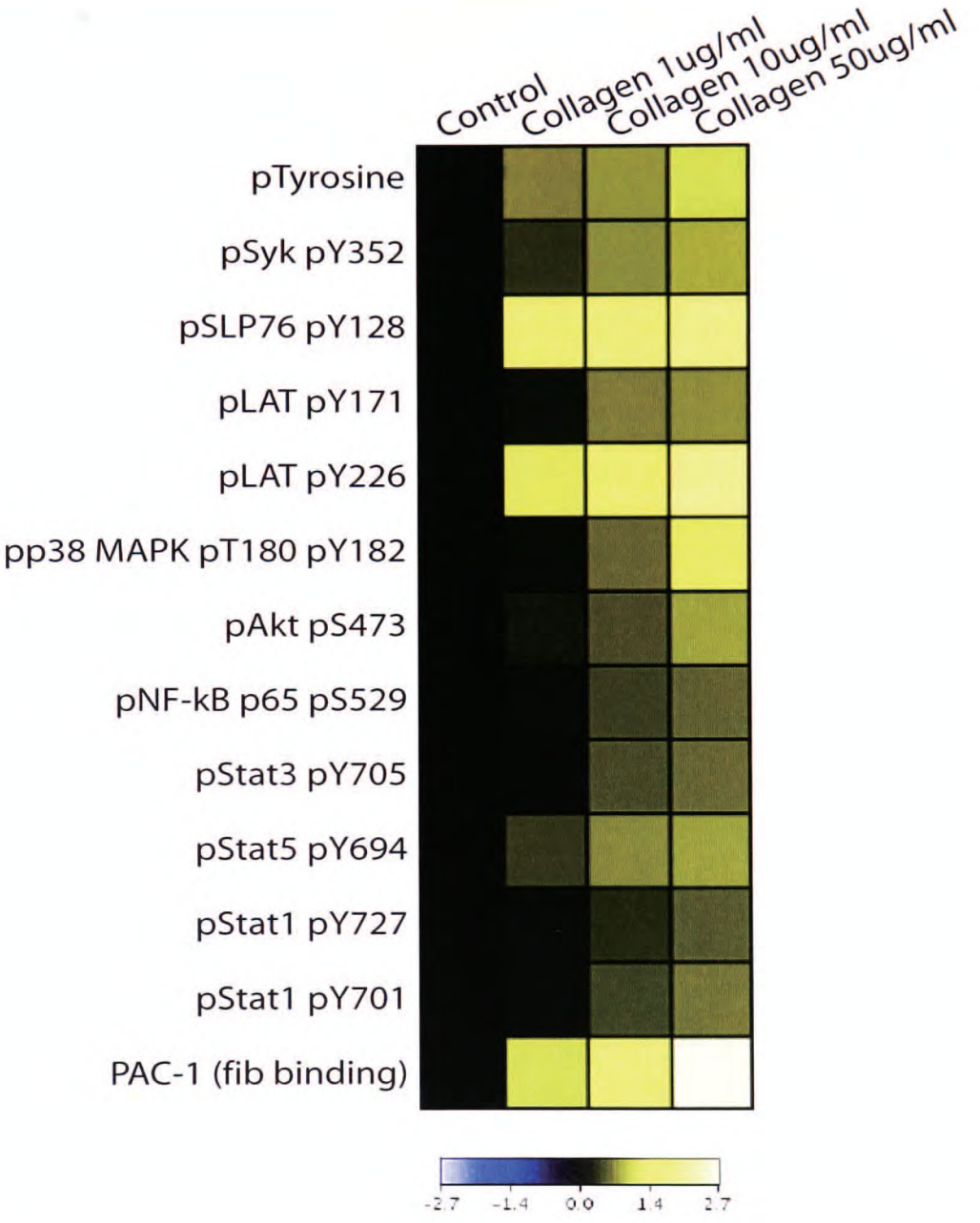



***Figure 2.9. An example of heatmap view of phospho-flow analysis of WP stimulated with collagen in a dose-dependent manner.***

## 2.8. Statistical analysis.

Aggregation experiments are expressed as % aggregation. Western blot analysis is conducted using densitometry software (ImageJ) on gels where blots are representative of more than two independent experiments .Results are expressed as means ± SEM for the number of experiments indicated. Where appropriate, Student's *t*-test was used to compare specific groups.

# CHAPTER 3. REGULATION OF PLATELET FUNCTION BY PEROXYNITRITE.

# ABSTRACT

**INTRODUCTION:** Peroxynitrite ($ONOO^-$), a strong oxidant, is formed in a reaction limited rate between nitric oxide (NO) and superoxide ($O_2^{\bullet-}$). Despite the early discovery for peroxynitrite, its "physiopathological" significance is still controversial. Peroxynitrite alters protein structure and function by reacting with metal centers and amino acid residues. It has been proposed that *in vivo* the conversion of NO to peroxynitrite is critical factor determining the outcome of ON signaling. In this study, we examined the effect of peroxynitrite on platelet function.

**METHODS:** Washed Platelets (WP) treated with peroxynitrite or decomposed peroxynitrite (DPN). Real time platelet responses were analyzed using a light-scattering aggregometer. Signaling mechanisms were studied using Western blotting.

**RESULTS:** peroxynitrite inhibited platelet aggregation through cGMP-dependent and independent mechanisms depending on the conditions. In the presence of HEPES buffer, peroxynitrite inhibited aggregation through the activation of sGC. The cGMP-independent mechanism observed for peroxynitrite, in phosphate buffer seems to be at least in part nitration-dependent. Peroxynitrite induced dose- and time-dependent increase in VASP-phosphorylation at $Ser^{157/239}$. Use of PKA and PKC inhibitors revealed that phosphorylation of VASP was mediated primarily by PKC not PKA.

**CONCLUSION:** This study shows for the first time that VASP is phosphorylated by peroxynitrite in a manner that does not require cGMP.

# 3. Introduction.

## 3.1 Peroxynitrite.

Peroxynitrite ($ONOO^-$), the reaction product of nitric oxide (NO) with superoxide ($O_2^{\bullet-}$), is a strong oxidant and nitrating agent. Peroxynitrite oxidizes sulfhydryl groups (Radi *et al.*, 1991b), mediates nitration of tyrosine residues (Ischiropoulos *et al.*, 1992), and induces lipid peroxidation (Radi *et al.*, 1991a). Peroxynitrite has been implicated to play a role in a variety of disease states, such as atherosclerosis (Rubbo *et al.*, 2000; Rubbo *et al.*, 2005), hypoxia (Cooper *et al.*, 2000; Nonami, 1997), diabetes (Pacher *et al.*, 2006), and neurodegenerative disorders (Guix *et al.*, 2005; Moncada *et al.*, 2006). However, endogenously produced peroxynitrite has also been described to play a role in cell signaling under physiological conditions (Balafanova *et al.*, 2002b; Marcondes *et al.*, 2006c; Naseem *et al.*, 2000). Although several studies have investigated the effect of peroxynitrite on platelets (Boulos *et al.*, 2000b; Brown *et al.*, 1998a; Low *et al.*, 2002; Naseem *et al.*, 1997; Rusak *et al.*, 2006), the signaling pathway by which peroxynitrite effects platelet function is still controversial.

**Aims of study**

- To establish the effect of exogenous peroxynitrite on platelet.
    - Platelet functional response is investigated by measuring platelet aggregation in response to peroxynitrite, and to agonists after treatment with peroxynitrite.
    - Platelet functional response is also assessed by quantifying platelet adhesion to immobilized collagen after treatment with peroxynitrite.
    - Platelet signaling response is investigated by immunoblotting of peroxynitrite-treated platelet proteins with anti-phosphotyrosine and anti-phospho VASP antibodies.

**Materials and methods of study**

- Peroxynitrite **(Method 2.1.8)**, 1,2-bis-(o-aminophenoxy) ethane-tetra-acetic acid tetra-(acetoxymethyl) ester (BAPTA-AM), L-NG-nitro-L-argininemethyl ester (L-NAME), N5-(1-Iminoethyl)-L-ornithine (L-NIO), 1H-[1,2,4]oxadiazole[4,3-a]quinoxalin-1-one (ODQ), were all purchased from Calbiochem (Nottingham, UK). For complete list of chemicals and antibodies **(Appendix II/III)**.

**Methods of study**

- Platelet aggregation.
- Immunoblotting.
- Measurement of cGMP formation.

## 3.2 Results.

## 3.2 The regulation of platelet functions by peroxynitrite.

### 3.2.1 Determination of buffering condition for use for peroxynitrite.

Stabilization of the peroxynitrite solutions requires the presence of NaOH (1.2M) to maintain it in its anionic form. Therefore it was important to establish the most appropriate conditions for the use of peroxynitrite in platelets to ensure that any observed effects are not pH mediated. It has been shown previously in our laboratory that peroxynitrite is more stable in 0.1M sodium phosphate buffer than in Tyrode's buffer (N. Oberprieler, University of Bradford, thesis 2007). However, platelets lack the response to agonists in this buffer since 0.1M sodium phosphate buffer works as a sink for calcium. The buffering capacity of Tyrode's buffer in response to peroxynitrite was established. In **table 3.1**, it can be observed that beyond 100 μM, peroxynitrite caused a significant increase in pH. Thus for further experimentation peroxynitrite (up to 100μM) was used for platelet studies.

| PN (μM) | pH of Tyrode's buffer |
|---|---|
| 1000 | 11.3 |
| 500 | 9.6 |
| 200 | 8 |
| 100 | 7.5 |
| 50 | 7.4 |
| 20 | 7.4 |
| 10 | 7.4 |
| 0 | 7.4 |

***Table 3.1. The effect of peroxynitrite concentrations on the pH of Tyrode's buffer.***

*Peroxynitrite at indicated concentrations was added to Tyrode's buffer. Changes in Tyrode's buffer pH were recorded with a pH meter.*

## 3.2.2 The influence of peroxynitrite on platelet signaling.

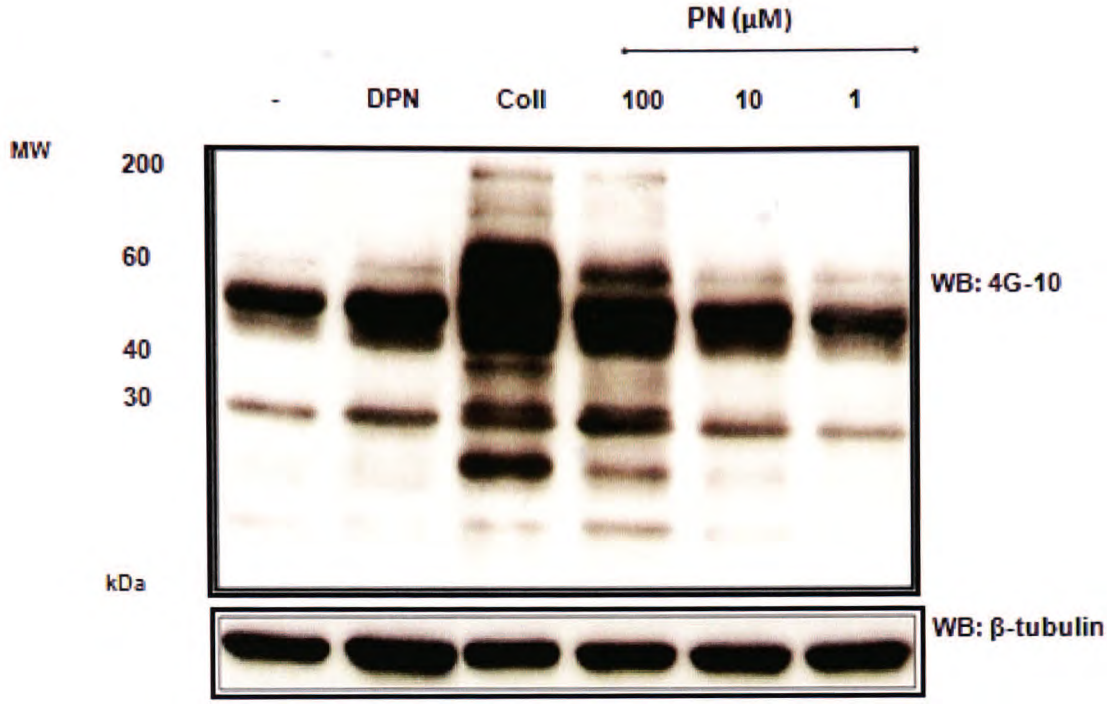
Platelet response is initiated by a number of biochemical signaling cascades. In first instance we examined the influence of peroxynitrite on platelet signaling cascades.

### 3.2.2.1 The influence of peroxynitrite on tyrosine phosphorylation.

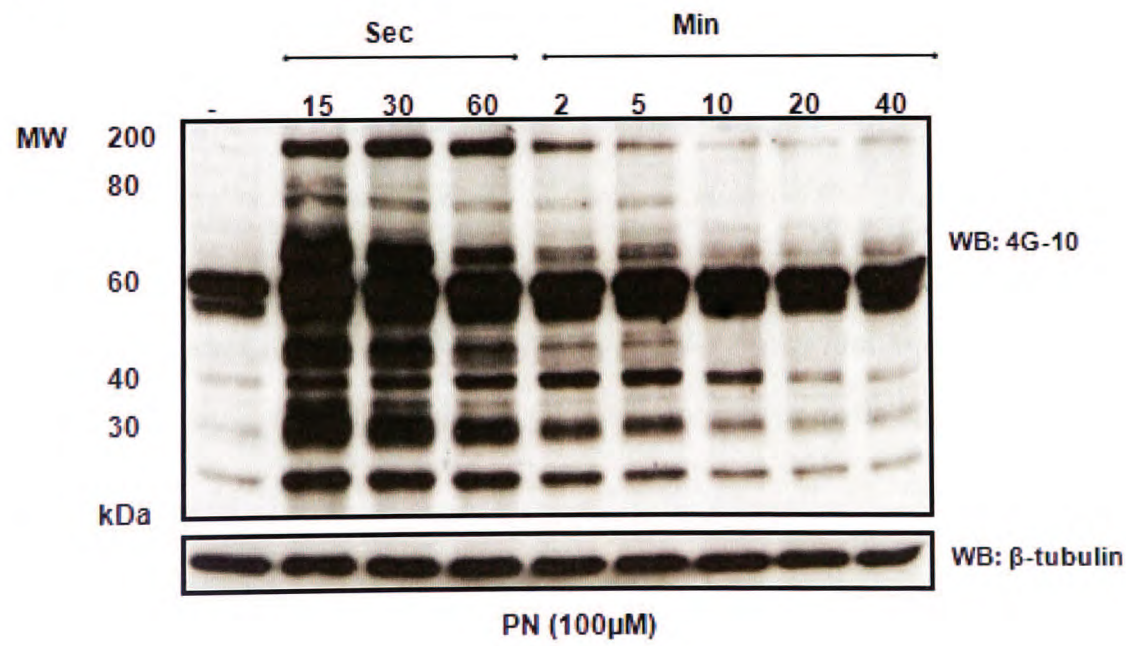
Peroxynitrite has been demonstrated to inhibit the activity of protein tyrosine phosphatases (Takakura *et al.*, 1999b). Due to this it was essential to examine the effect of peroxynitrite on tyrosine phosphorylation in platelets. Basal tyrosine phosphorylation levels were observed in resting platelets and platelets exposed to decomposed peroxynitrite (DPN). However, when WP were treated with peroxynitrite (1-100μM) for 3 minutes, there was a concentration-dependent increase in a number of tyrosine phosphorylated proteins, which was most evident using 100μM peroxynitrite. Although level of tyrosine phosphorylation in platelets stimulated with a physiological agonist, collagen, was sustained and more robust after 3 minutes than that caused by peroxynitrite **(figure 3.1a)**.

To investigate the reversibility of peroxynitrite-induced tyrosine phosphorylation, the time-dependent effect of peroxynitrite (100μM) on tyrosine phosphorylation was tested. Tyrosine phosphorylation was rapid with maximal phosphorylation in response to peroxynitrite was observed at 15sec, which was maintained for up to 60sec before declining to basal after 10min **(figure 3.1b)**.

(a)


PN (μM)
-
DPN
Coll
100
10
1
MW
200
60
40
30
kDa
WB: 4G-10
WB: β-tubulin

(b)


Sec
Min
-
15
30
60
2
5
10
20
40
MW
200
80
60
40
30
kDa
WB: 4G-10
WB: β-tubulin
PN (100μM)

(c)

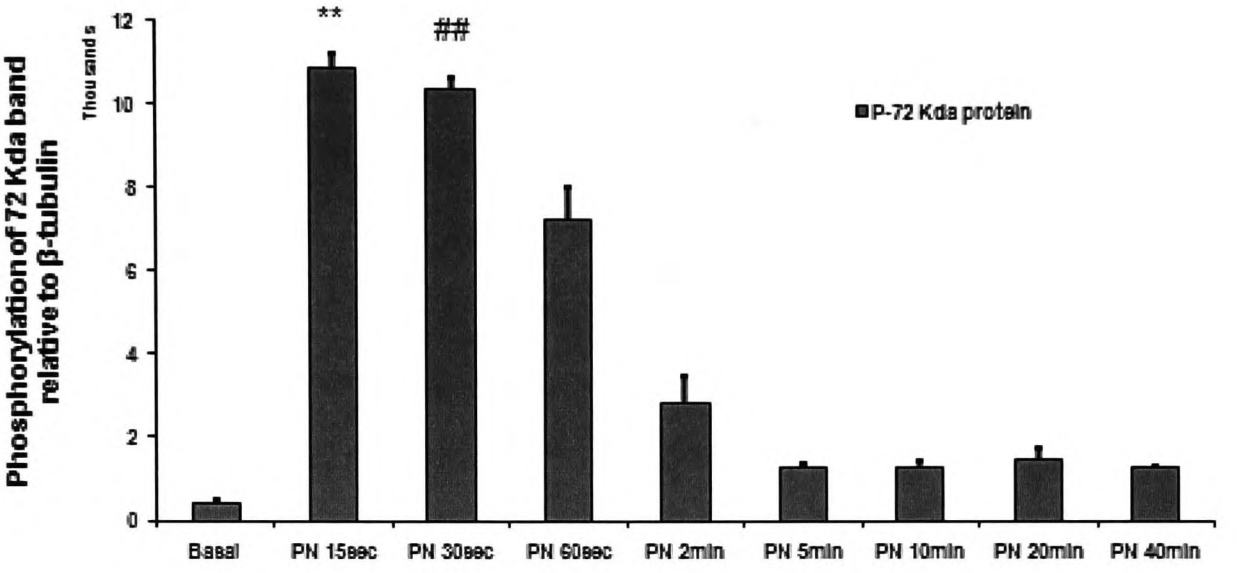


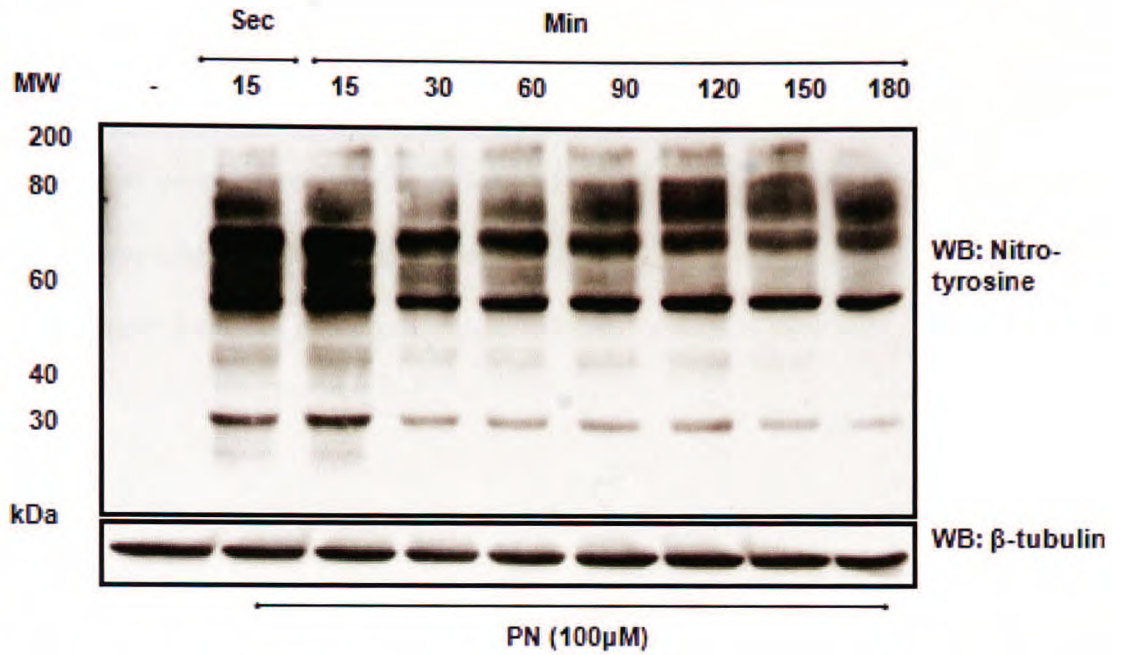

***Figure 3.1. Peroxynitrite induces tyrosine phosphorylation in platelets.***

*WP ($5x10^8$) treated with peroxynitrite for 3 minutes in a dose* ***(a)*** *and time* ***(b)*** *–dependent manner. Reactions were stopped with an equal volume of 2xLaemmli buffer. Proteins were separated in 10–18% gradient gels. Tyrosine phosphorylation was evaluated with anti-phosphotyrosine; membranes were stripped and reprobed with anti β-tubulin for equal loading.* ***(c)*** *Densitometric analysis of three independent immunoblot assays of an increase in 72Kda protein tyrosine phosphorylation in response to peroxynitrite. $^{**}P/^{\#\#}P < 0.01$ vs. basal sample. Data = mean±SEM*

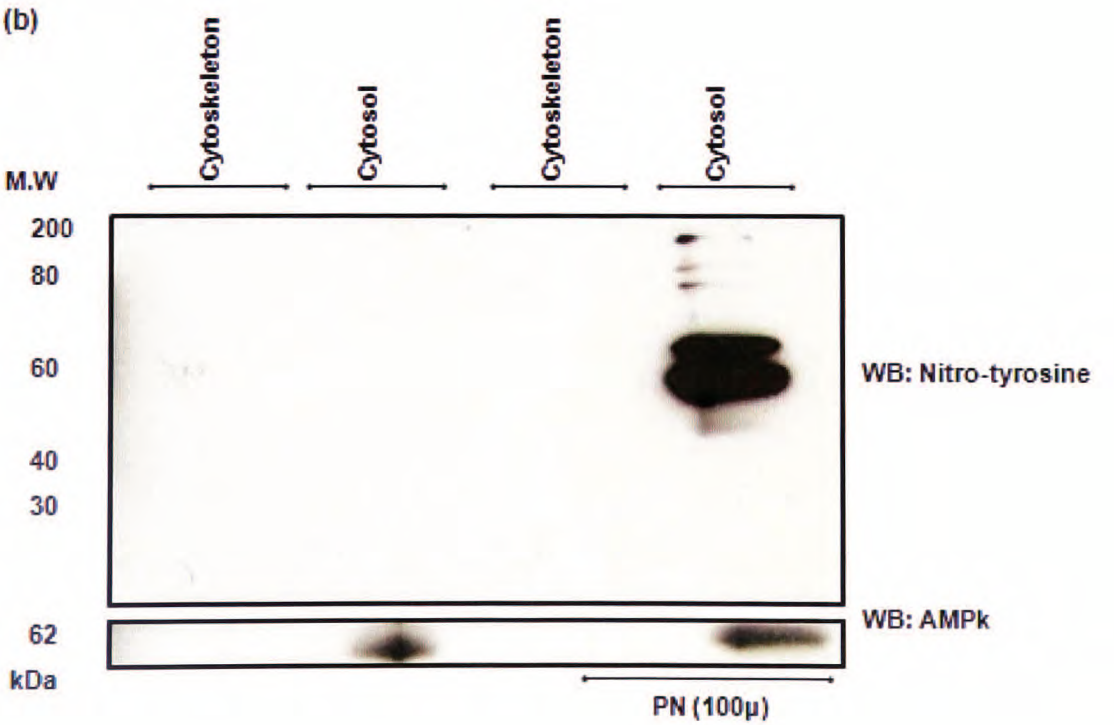
### 3.2.2.2 The influence of peroxynitrite on tyrosine nitration.

Peroxynitrite is a well known endogenous nitrating agent (Ischiropoulos *et al.*, 1992). The ability of peroxynitrite (100μM) to induce protein nitration was also investigated. Tyrosine nitration, the addition of a nitro (-NO2) group adjacent to the hydroxyl group on the aromatic ring of tyrosine residues (Gow *et al.*, 2004), was not detectable in resting platelets. However, the addition of peroxynitrite (100μM) induced a robust increase in tyrosine nitrated proteins **(figure 3.2a)**. Nitration was observed over a whole range of molecular weight with the heaviest nitrated bands observed at 30, 60 and 70 kDa. Nitration was rapid with modified proteins observed within 15 sec of exposure to the nitrating agent. In contrast to peroxynitrite induced tyrosine phosphorylation, nitration was maintained over a three hour time course. The exception was a band at 40 kDa, which began to decline within 30 min, but was still slightly nitrated after 3 hours. In order to assess where in the cell nitration was taken place. Platelets were fractionated after treatment with peroxynitrite for 1 min, interestingly, nitrated proteins were only found in the cytosolic fraction, as indicated by the presence of AMPk which is known to be a cytosolic enzyme. These data indicate that peroxynitrite is able to enter the cell **(figure 3.2b)**.

(a)




(b)




**Legend: overleaf**

***Figure 3.2. Peroxynitrite causes tyrosine nitration in the cytosolic***

*WP ($5x10^8$) treated with peroxynitrite 100μM for up to 180 min* ***(a)****. WP ($1x10^9$) were treated with peroxynitrite (100μM) for 1 min and fractionated by ultracentrifugation. Pellet and supernatants* ***(b)****. Proteins were subjected to SDS page electrophoresis, immunoblotted with anti-nitro tyrosine antibody. Membrane was stripped and reprobed with anti-AMPKα antibody as a control for cytosolic fraction. Blots are representative of one experiment.*

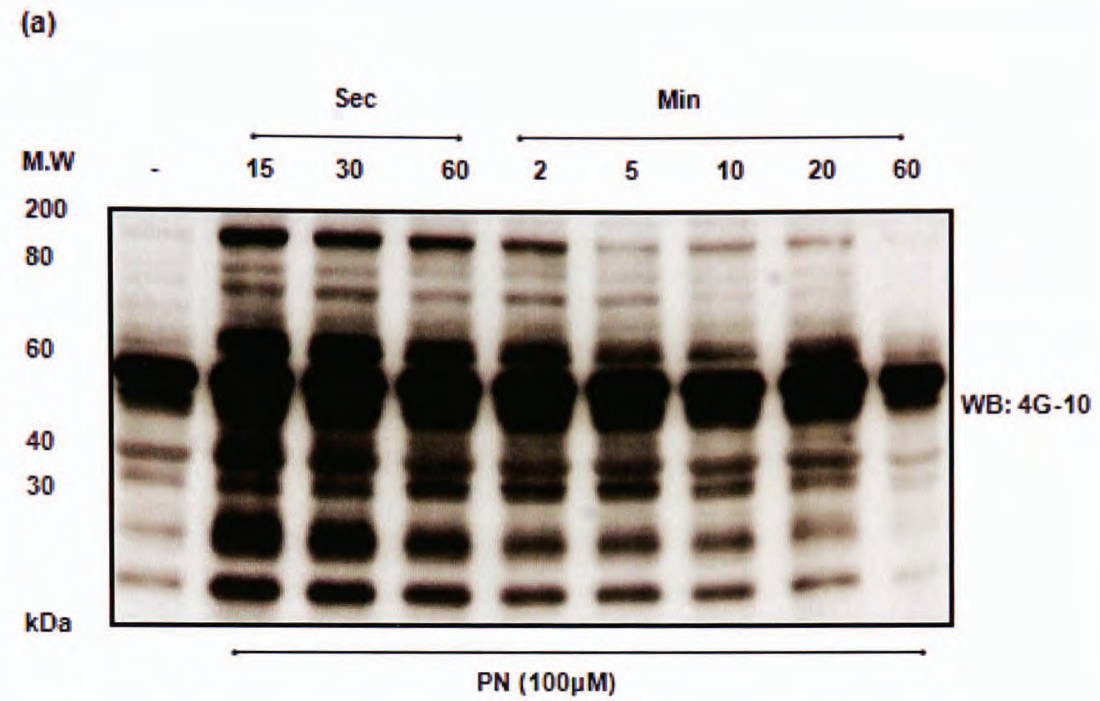
### 3.2.2.3. The role of secondary mediators on peroxynitrite-induced tyrosine phosphorylation.

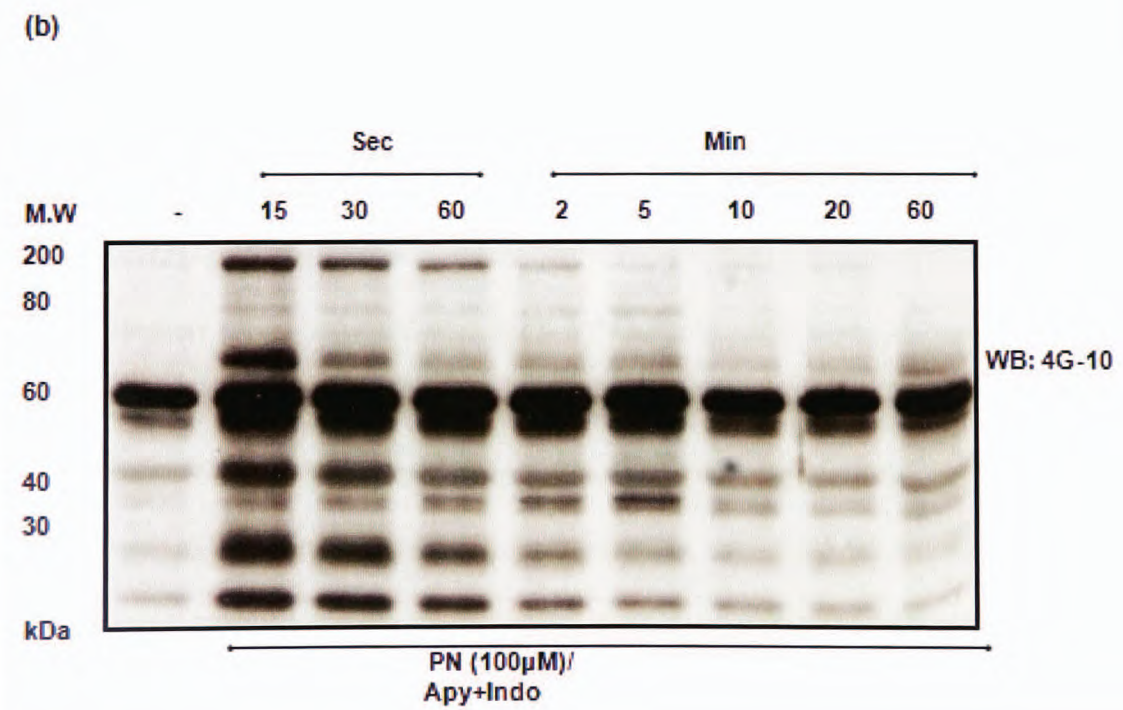
Having observed that peroxynitrite increases tyrosine phosphorylation, the mechanism of action was investigated using a series of pharmacological inhibitors. WP were preincubated with apyrase (1U/ml), ADP scavenger, and indomethacin (10μM), to block $TxA_2$ production, prior to exposure to peroxynitrite (100μM). This was to determine whether tyrosine phosphorylation caused by peroxynitrite was secondary mediator-dependent. **Figure 3.3** shows that peroxynitrite -induced tyrosine phosphorylation was reduced but not abolished under conditions where ADP and $TxA_2$ were absent. Indeed most phosphorylated bands were only slightly affected **(figure 3.3b).** However, tyrosine phosphorylation level was maintained over a longer period in the absence of apyrase and indomethacin **(figure 3.3a)**, indicating that peroxynitrite-induced tyrosine phosphorylation occurred through an ADP/$TxA_2$-dependent and independent mechanisms.

In erythrocytes, peroxynitrite has been shown to activate Src kinase (Mallozzi *et al.*, 2001b; Serafini *et al.*, 2005). In platelets, Src kinase activation leads to calcium mobilization and activation of number of downstream signaling cascades such as PLC and PKC. To assess whether peroxynitrite-induced tyrosine phosphorylation was Src family kinase dependent, WP were preincubated with, PP2 (20μM), Src family kinase inhibitor, RO31-8220 (10μM), PKC inhibitor and BAPTA (20μM), intracellular $Ca^{2+}$ chelator. The results from these experiments demonstrate that tyrosine phosphorylation of all proteins except 45kDa was abrogated in the presence of PP2 and

BAPTA, while RO31-8220 had no effect **(figure 3.4a)**. This indicates that tyrosine phosphorylation caused by peroxynitrite occur in a manner that is mediated by Src kinase activation and mobilization of calcium but independent of PKC activation.

We have also investigated the effect of nitration induced by peroxynitrite on tyrosine phosphorylation. In the presence of EGCG (100μM) (nitration scavenger), peroxynitrite failed to cause tyrosine nitration. However, peroxynitrite induced tyrosine phosphorylation was increased in the presence of EGCG **(figure 3.4b).** This could be accounted by the fact that tyrosine nitrated proteins are masked and once nitration is catalyzed by EGCG, more tyrosine residues are accessible for phosphorylation.


(a)
Sec
Min
M.W
-
15
30
60
2
5
10
20
60
200
80
60
40
30
kDa
WB: 4G-10
PN (100μM)


(b)
Sec
Min
M.W
-
15
30
60
2
5
10
20
60
200
80
60
40
30
kDa
WB: 4G-10
PN (100μM)/
Apy+Indo

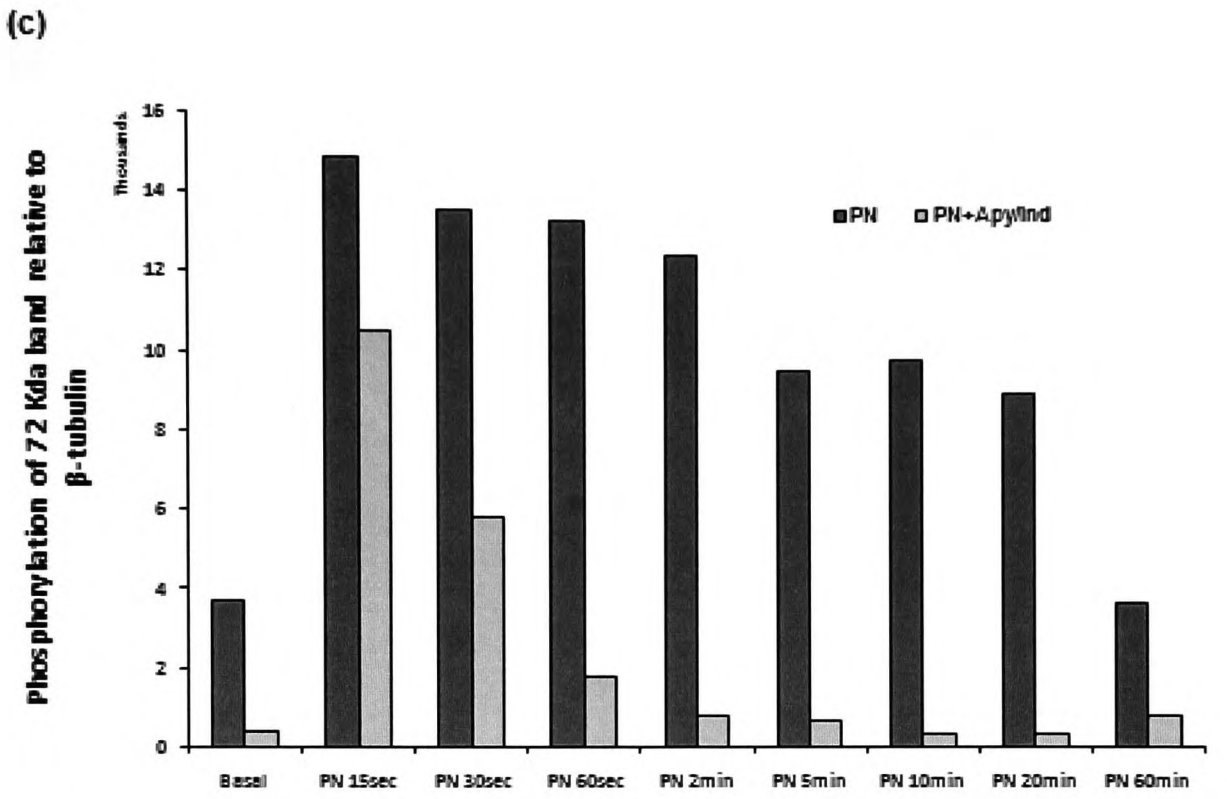
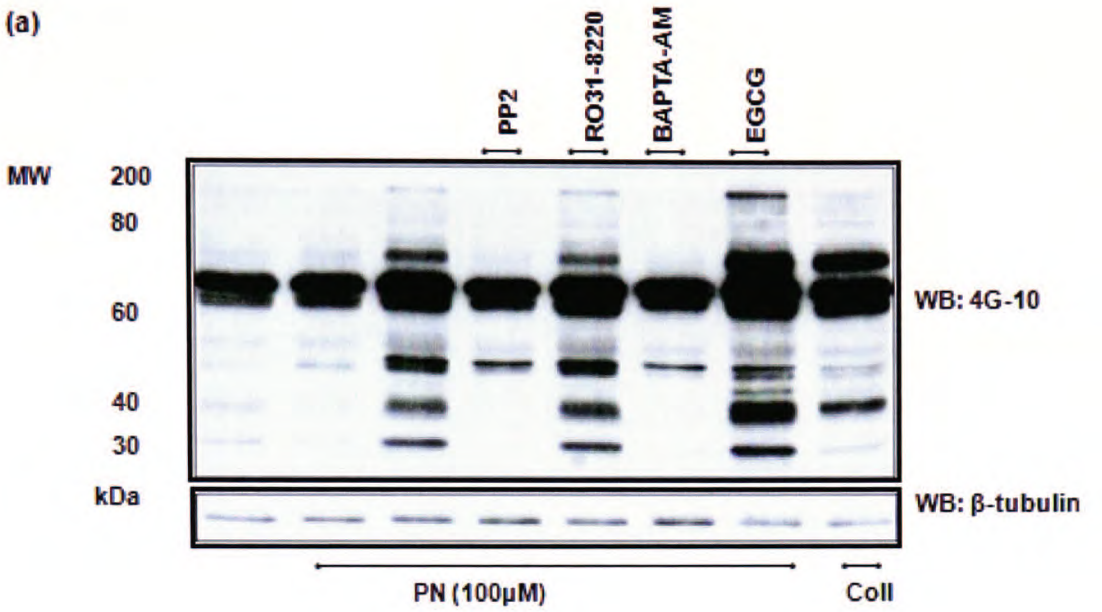
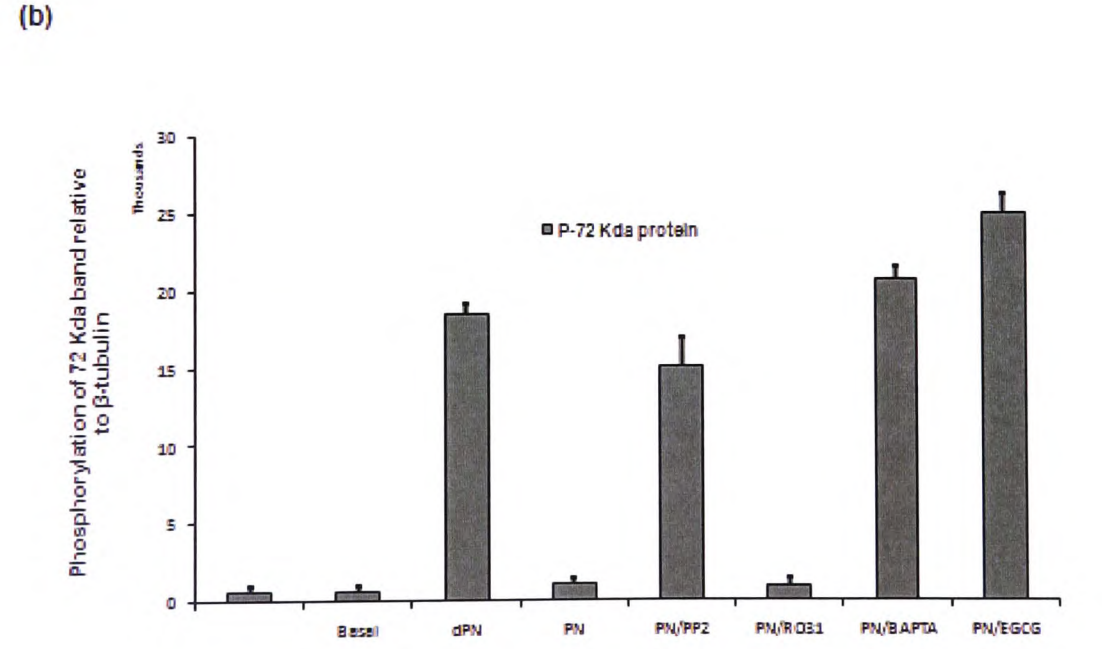



***Figure 3.3. Peroxynitrite-induced tyrosine phosphorylation is enhanced by secretion***

*WP ($5x10^8$) pre-incubated without* ***(a)*** *or with* ***(b)*** *apyrase (1U/ml), and indomethacin (10μM) for 20min, before treatment with peroxynitrite 100 μM for indicated times. Reactions were stopped with an equal volume of 2xLaemmli buffer. Proteins were separated in 10–18% gradient gels Tyrosine phosphorylation and nitration was evaluated with anti-phosphotyrosine. Blots are representative of 2 independent experiments.* ***(c)*** *Densitometric analysis of three independent immunoblot assays of an increase in 72Kda protein tyrosine phosphorylation in response to peroxynitrite. $^{**}P/^{\#\#}P < 0.01$ vs. basal sample. Data = mean±SEM*


(a)
PP2
RO31-8220
BAPTA-AM
EGCG
MW
200
80
60
40
30
kDa
WB: 4G-10
WB: β-tubulin
PN (100μM)
Coll


(b)
Phosphorylation of 72 Kda band relative to β-tubulin
thousands
P-72 Kda protein
30
25
20
15
10
5
0
Basal
dPN
PN
PN/PP2
PN/RO31
PN/BAPTA
PN/EGCG

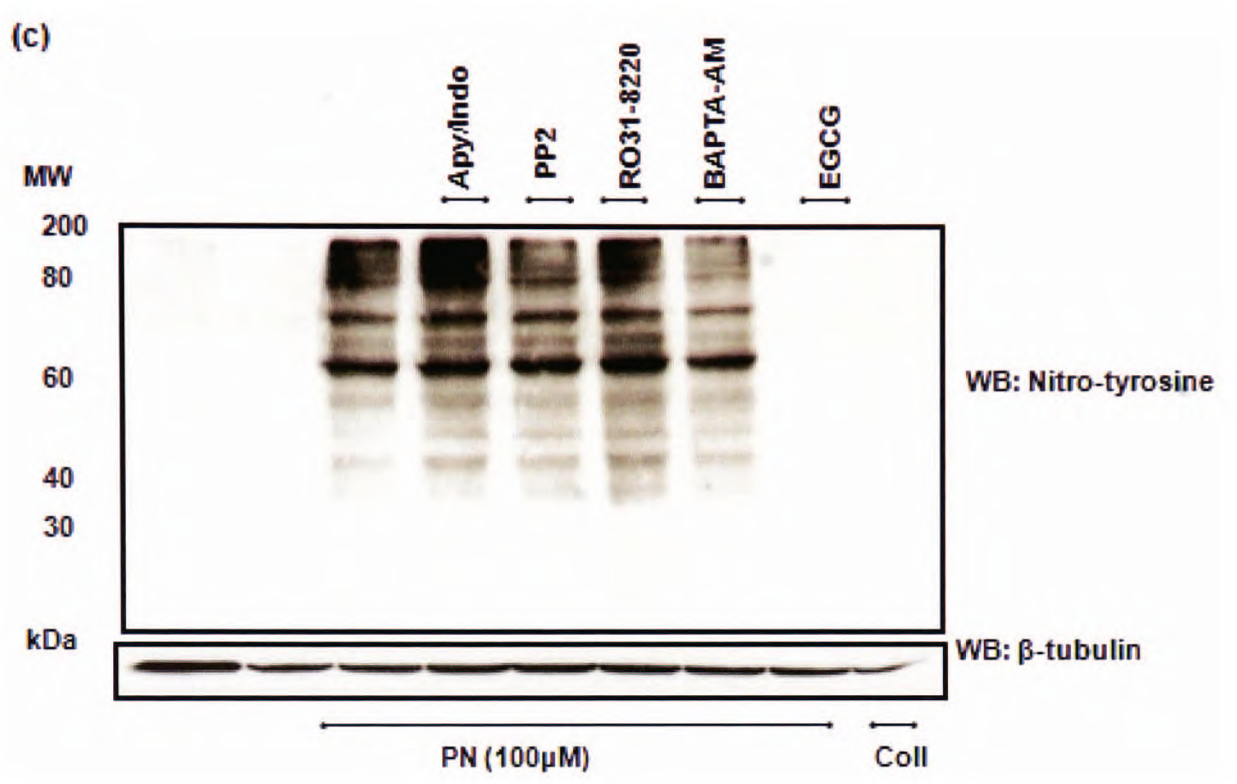



***Figure 3.4. Peroxynitrite-induced tyrosine phosphorylation is Src and calcium dependent.***

*WP (5x108) were preincubated with Src kinase inhibitor [PP2 (20μM]),PKC inhibitor [Ro31-8220 (10μM)], intracellular $Ca^{2+}$ chelator [BAPTA-AM (20μM)], or nitration inhibitor [EGCG (100μM)] in the presence of apyrase (1U/ml), and indomethacin (10μM) for 20min. WP were treated with 100μM peroxynitrite or decomposed peroxynitrite as indicated for 1min. Proteins were separated in 10–18% gradient gels. Tyrosine phosphorylation* ***(a)*** *with densitometric analysis (**b**) and nitration* ***(c)*** *was evaluated with anti-phosphotyrosine, and anti-nitrotyrosine antibody, receptively. Blots are representative of 3 independent experiments.*

## 3.3.3 The influence of peroxynitrite on platelet aggregation.

### 3.3.3.1 Peroxynitrite inhibits platelet aggregation in a dose-dependent manner.

Having examined peroxynitrite signaling cascades, we next measured the functional effects of peroxynitrite. Peroxynitrite was added to WP in aggregation tubes with continuous stirring and platelet aggregation response was monitored using an aggregometer. Peroxynitrite at 100μM failed to cause platelet aggregation when compared to the physiological agonist collagen; however, higher concentrations of peroxynitrite (700 μM), which has been suggested to occur *in vivo* (Stachowiak *et al.*), did induce a small level of aggregation **(figure 3.5).** Thus, for further experiments, peroxynitrite was used at concentrations of 100μM or less.

We next examined the effect of peroxynitrite on agonist-induced platelet aggregation. WP were first treated with peroxynitrite (5-100μM) for 30sec, and then stimulated with collagen (0.2μg/ml). **Figure 3.6** show that collagen caused 43% aggregation, which was reduced to 40%, 16%, and 7% in a concentration-dependent manner to peroxynitrite 5, 20, and 100 μM, respectively. Decomposed peroxynitrite (100μM), prepared by exposing an aliquot of peroxynitrite to room temperature prior to experiment, had no effect on the platelets response to collagen.

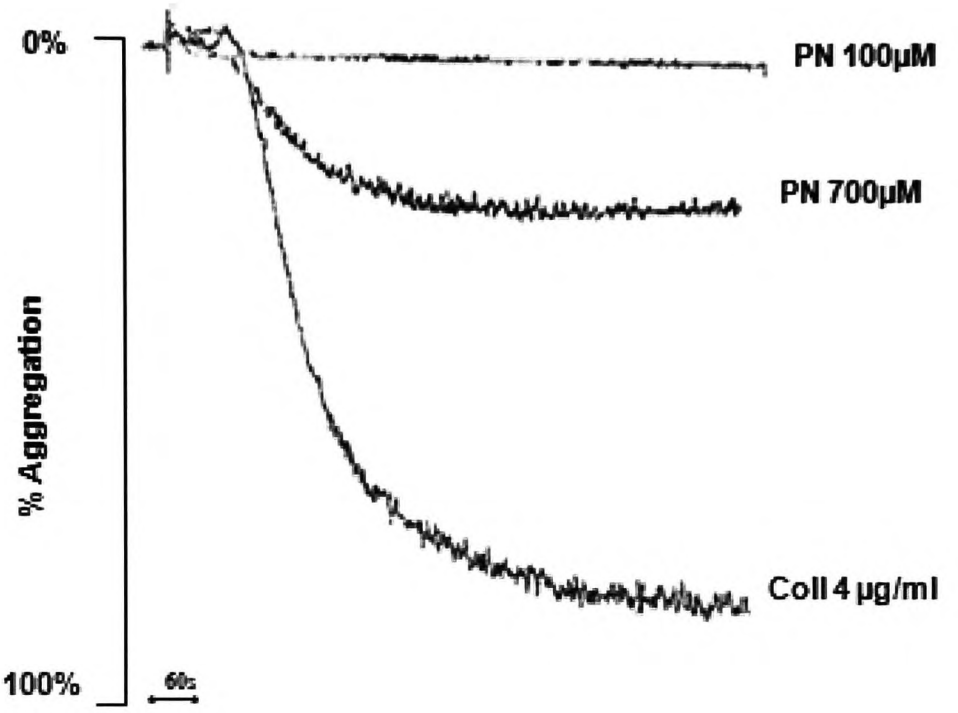
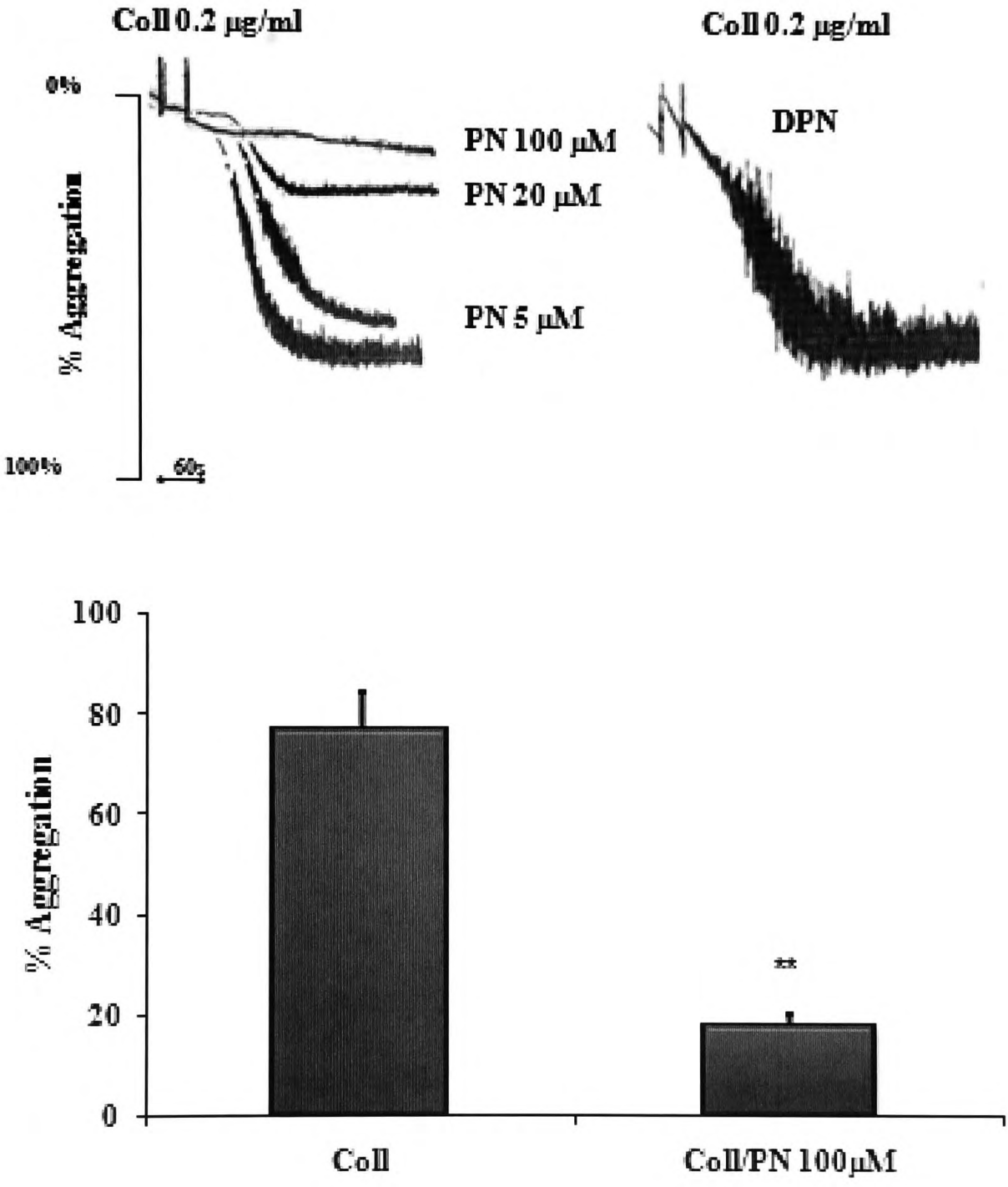



***Figure 3.5. Low peroxynitrite concentration (100µM) does not induce platelet aggregation.***

*WP ($3x10^8$) were treated with either peroxynitrite or collagen at the indicated concentrations. Platelet response was recorded for the indicated time and expressed as % of aggregation. Traces are representative of one experiment.*




**Legend: overleaf**

***Figure 3.6. Peroxynitrite causes platelets inhibition in dose-dependent manner.***

*WP ($3x10^8$) were stimulated with collagen (0.2µg/ml) or pretreated first with peroxynitrite at the indicated concentrations prior to stimulation with collagen. Response was recorded for the indicated time and expressed as % of aggregation. Graph represents data of 4 independent experiments. Data = mean±SEM. **P < 0.01 vs. collagen sample.*

### 3.3.3.2 Peroxynitrite inhibits platelet aggregation partially in a time dependent manner.

We next examined whether the inhibitory effect of peroxynitrite on platelet aggregation was reversible over time. WP were incubated with peroxynitrite (100μM) for over 2 hours, aliquots of WP platelets were then stimulated with collagen at the different time intervals and platelet aggregation response to collagen was monitored. **Figure 3.7** show that after 1 min incubation peroxynitrite was able to cause 80% inhibition of collagen induced aggregation. The level of inhibition was reduced to 60% after 10 min, and declined further to 57% by 20 min. At 40 min inhibition was 40%, but this was maintained for over 2hours (longest time tested).

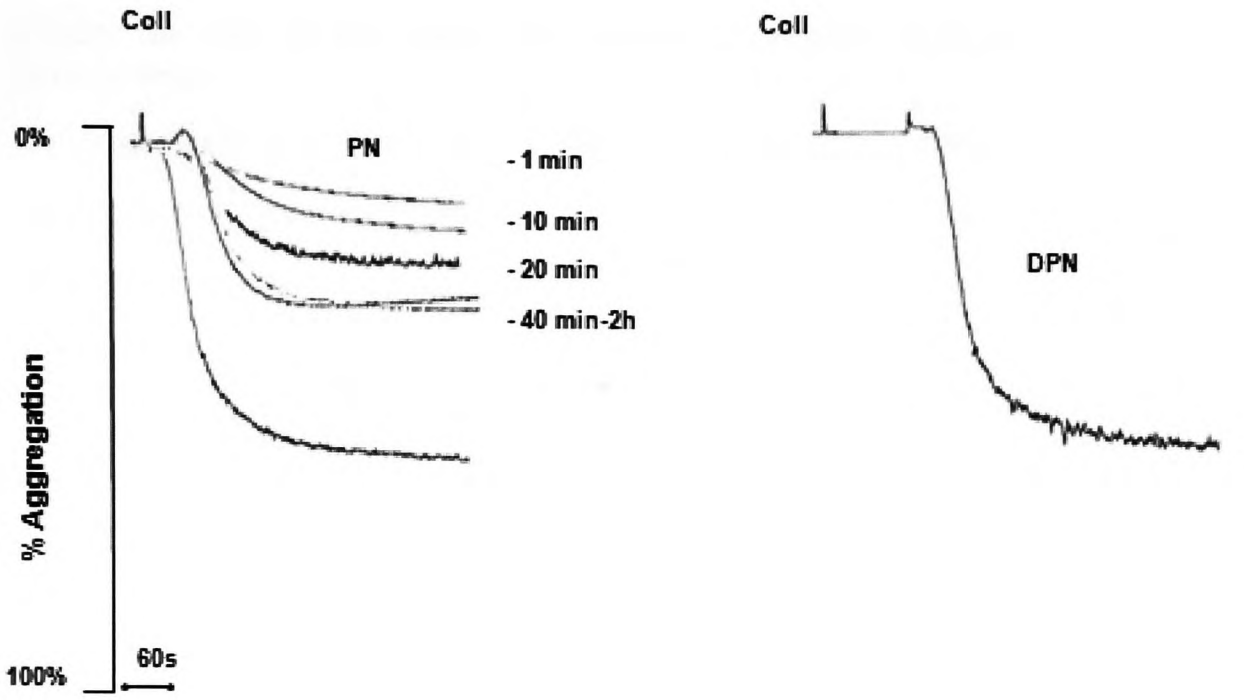



***Figure 3.7. Peroxynitrite inhibits platelets in a time-dependent manner.***

*WP ($3x10^8$) incubated with peroxynitrite (100μM) for over 2 hours. Aliquots were taken out at the indicated time intervals, and then stimulated with collagen 4μg/ml for 5min. Aggregation trace was recorded using dual channel aggregometer and expressed as % aggregation. Traces are representative of two experiments.*

## 3.3.4 Investigation the mechanism of platelet aggregation inhibition by peroxynitrite.

### 3.3.4.1 The role of Src kinase in platelet aggregation inhibition by peroxynitrite.

Our data suggests that peroxynitrite induces tyrosine phosphorylation in a Src-dependent mechanism. Although some Src family kinases (SFKs) are required for platelet aggregation by collagen, platelets possess at least 5 isoforms (Lyn, Fgr, Fyn, Yes, and Src) (Stenberg *et al.*, 1997). It is possible some of these isoforms have inhibitory roles (Chari *et al.*, 2009). Thus we aimed to investigate whether Src plays a role in the inhibitory effect of peroxynitrite on platelet aggregation.

We first evaluated the role of Src family kinases inhibition by PP2 by measuring platelet aggregation in response to collagen. consistent with others (Suzuki-Inoue *et al.*, 2003), PP2 abolished collagen induced aggregation **(figure 3.8a).** Next platelets were stimulated with an agonist, thrombin, which is known to induce platelet aggregation independently of Src kinases (Hughan *et al.*, 2007). **Figure 3.8b** shows that peroxynitrite in the presence of Src inhibitor was still able to inhibit platelet aggregation induced by thrombin, while Src inhibitor had no effect on platelet response to thrombin. This data demonstrate that peroxynitrite inhibits platelet aggregation in a manner that does not require Src kinase, and the inhibitory effect of peroxynitrite is not specific to platelet aggregation to collagen.

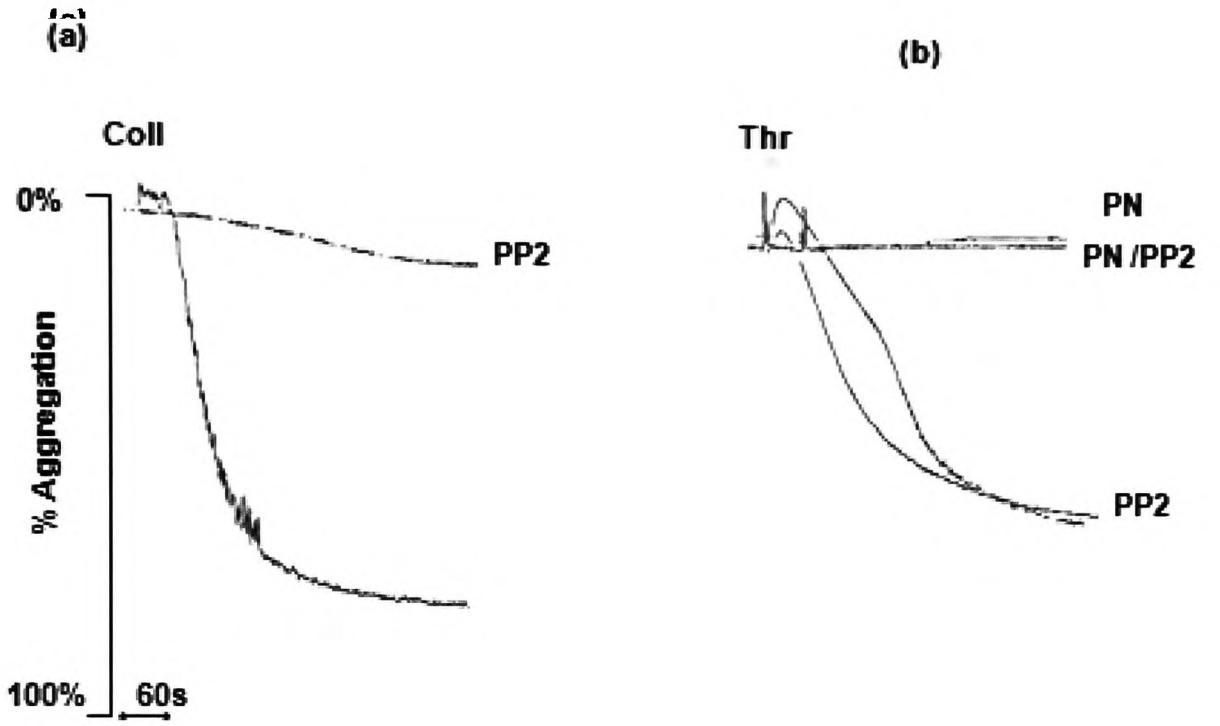



***Figure 3.8. The role of Src in the inhibitory action of peroxynitrite.***

*WP ($3x10^8$) pretreated with Src kinase inhibitor [PP2 (20μM)] were treated peroxynitrite (100μM) prior to stimulation with collagen (4 μg/ml)* ***(a)*** *or Thrombin 0.04 u/ml* ***(b)****. Response was recorded for 5min and expressed as % aggregation. Traces are representative of two experiments.*

### 3.3.4.2 The role of protein nitration in platelet aggregation inhibition by peroxynitrite.

In **figure 3.2** we showed that peroxynitrite caused robust increase in tyrosine nitration which was abolished by EGCG **(figure 3.4b). T**hus the role of nitration was also investigated in inhibition of platelet aggregation by peroxynitrite.

WP were incubated with EGCG (100μM) for 20min prior to being exposed to peroxynitrite 100μM for 30sec, and stimulation with collagen. **Figure 3.9** shows that peroxynitrite, as expected, reduced platelet aggregation in response to collagen. However in presence of EGCG, the inhibitory effect of peroxynitrite was significantly reduced. in agreement with others (Deana *et al.*, 2003), EGCG by itself reduced platelet response to collagen. Surprisingly, in the presence of EGCG, peroxynitrite also caused platelets to aggregate slightly. This data suggest that inhibition of platelet aggregation by peroxynitrite is in part mediated by tyrosine nitration, and that tyrosine nitration caused by peroxynitrite might play a protective role in a way that platelets are less responsive to stimulation.

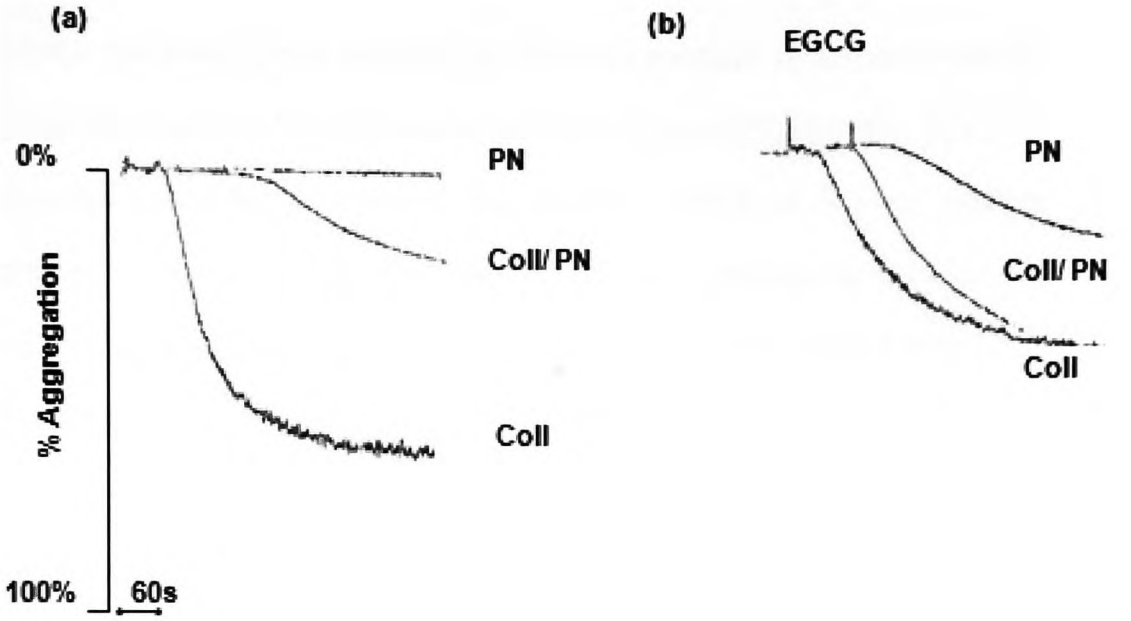



***Figure 3.9. Peroxynitrite inhibits platelets at least in part in a nitration dependent manner.***

*WP ($3x10^8$) stimulated with collagen (4µg/ml) alone or pretreated with peroxynitrite (100µM), or exposed to peroxynitrite alone* ***(a)****. WP ($3x10^8$) preincubated with nitration inhibitor [EGCG (100µM)] prior to stimulation with collagen (4µg/ml) alone, or in presence of peroxynitrite 100 µM. or exposed to peroxynitrite alone* ***(b)****. Aggregation trace was recorded using dual channel aggregometer and expressed as % aggregation. Traces are representative of two experiments.*

### 3.3.4.3 The role of soluble guanylyl cyclase in platelet aggregation inhibition by peroxynitrite.

Soluble guanylyl cyclase (sGC) is a key enzyme of NO/cGMP inhibitory pathway. We investigated whether peroxynitrite mediate inhibition of platelet aggregation via sGC. We first evaluated the ability of sGC inhibitor, ODQ, by measuring its ability to reverse the inhibitory effect of NO on platelet aggregation. Consistent with the study of Moro and colleagues (Moro *et al.*, 1996), ODQ (20μM) reversed the inhibitory effect of NO **(figure 3.10a)** Here NO reduced platelet aggregation to 15 ± 3.1 %, which was recovered to 70 ± 8 % by ODQ ($P < 0.05$). Next platelets were stimulated with collagen after treatment with peroxynitrite (100μM/30sec) in the presence of ODQ. **Figure 3.10b** shows that peroxynitrite significantly reduced platelet aggregation in response to collagen from 74 ± 13.1% to 27. ± 4.7 % ($P < 0.05$). However in the presence of ODQ, the ability of peroxynitrite to inhibit aggregation was diminished, with aggregation remains at 65 ± 16.5 %. In contrast, L.NIO (1mM) had no effect on the inhibitory action of peroxynitrite 28% ±8.5 ($P < 0.05$).

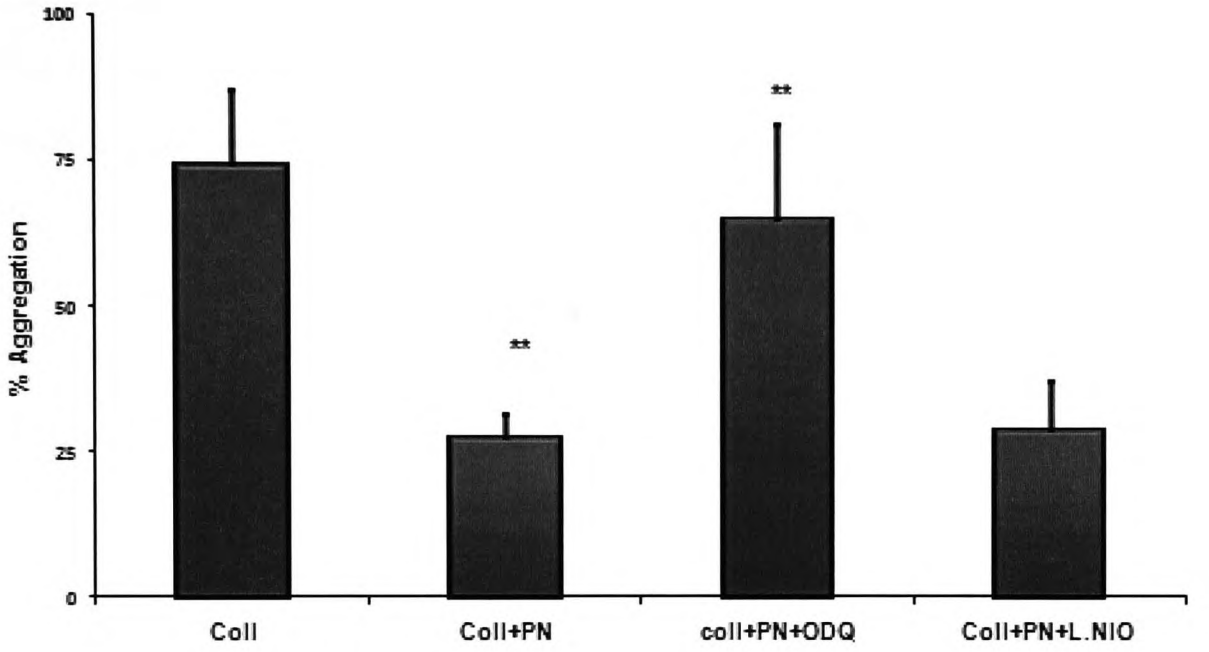



***Figure 3.10. Peroxynitrite inhibits platelet aggregation partially in guanylyl cyclase -dependent manner***

*WP ($3x10^8$) preincubated with sGC inhibitor [ODQ (20μM)] or eNOS inhibitor [ L.NIO (1mM)] for 20 minutes. WP were then treated with peroxynitrite (100μM) and stimulated with collagen (4μg/ml), and platelet response was recorded with Born aggregometer and expressed as % aggregation. Platelets incubated with ODQ were tested with GSNO (10μM) prior to experiment as a positive control. A graph represents data from three individual experiments. Data = mean±SEM. **P < 0.01 vs. collagen sample.*

### 3.3.4.4 NO-dependent and independent effects, the influence of buffering conditions.

It is possible that the buffering conditions used in experimental protocols could influence the actions of peroxynitrite. Schmidt and colleagues (Schmidt *et al.*, 1998), investigated a number of buffers for the NO-like biological activity of peroxynitrite, their data suggested that peroxynitrite may react with HEPES in Tyrode's buffer to produce an NO donor, a reaction which does not take a place with phosphate buffer. In light of their findings we examined whether buffer composition influenced peroxynitrite regulation of platelets. WP were re-suspended in phosphate buffer, and the previous experiment **(figure 3.10)** was repeated. **Figure 3.11** show that peroxynitrite still inhibited platelet aggregation indeed by collagen. However, ODQ did not reverse the inhibitory action of peroxynitrite. This suggests that the NO-dependent effect of peroxynitrite is entirely dependent on the experimental conditions. Because Tyrode's buffer appeared to cause the release of NO, phosphate buffer was used as the main buffer for any experiment involves the use of peroxynitrite.

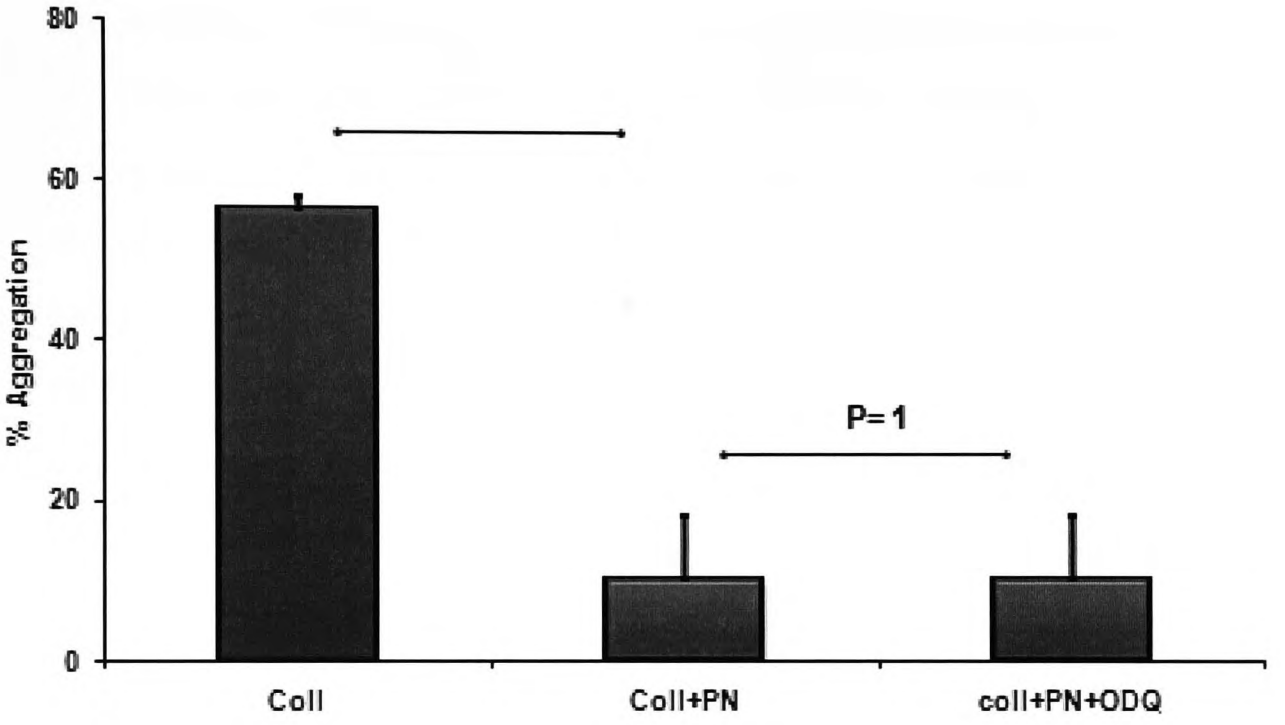



***Figure 3.11. Nitric oxide-dependent inhibition by peroxynitrite is dependent on the experimental conditions***

*WP were then treated with peroxynitrite (100μM/30sec) and stimulated with collagen (4μg/ml) and platelet response was recorded with Born aggregometer and expressed as % aggregation. Platelets incubated with ODQ were tested with GSNO (10μM) prior to experiment as a positive control. The graph represents data from 3 individual experiments. Data = mean±SEM. *P < 0.05 vs. collagen sample. P=1 collagen/PN vs. collagen/PN+ODQ.*

### 3.3.4.5 Peroxynitrite induced sGC activation and cGMP formation.

It is widely accepted that cGMP elevation in platelets occurs in response to NO-mediated activation of sGC, and that cGMP formation in response to platelet activation is NO-sensitive. However, No-independent regulation of sGC activity by vWF (Gambaryan *et al.*, 2008), and by adiponectin (Riba *et al.*, 2008), has been described. We investigated the contribution of peroxynitrite in the formation of cGMP in platelets under condition of phosphate buffer.

Peroxynitrite (100μM) significantly increased cGMP production over basal levels. Where the cGMP level was 1579.5 ± 76.5 fmol per $10^8$ platelets compared to basal 214.4 ± 82.4 ($P < 0.05$). Pre-incubation of platelets with ODQ (20μM) abolished the ability of peroxynitrite to stimulate cGMP production (261.3 ± 76,5 fmol per $10^8$ platelets) **(figure 3.12)**. However, in the presence of EGCG (100μM) tyrosine nitration inhibitor, the ability of peroxynitrite to stimulate cGMP production was unaffected (1264.1 ± 174.7 fmol per $10^8$ platelets). Although the increase of cGMP production by peroxynitrite was significant, it was significantly less than that produced by GSNO (10μM). It was surprising that peroxynitrite increased cGMP formation but its inhibitory effects on platelet aggregation were independent of ODQ. This is perhaps due to the fact that the basal concentration of cGMP is less than one-tenth of the concentration of cGMP binding site on PKG, and several-fold increases in cGMP levels are capable of stimulating only a small fraction of PKG (Schwarz *et al.*, 2001b). This is best highlighted comparing the potency of platelet inhibition by GSNO (0.1μM), which produced the same level of cGMP as that of peroxynitrite **(figure 3.13)**, and also failed to

inhibit aggregation. Thus while peroxynitrite may increase cGMP generation, it is not sufficient to account for inhibition of platelet aggregation.

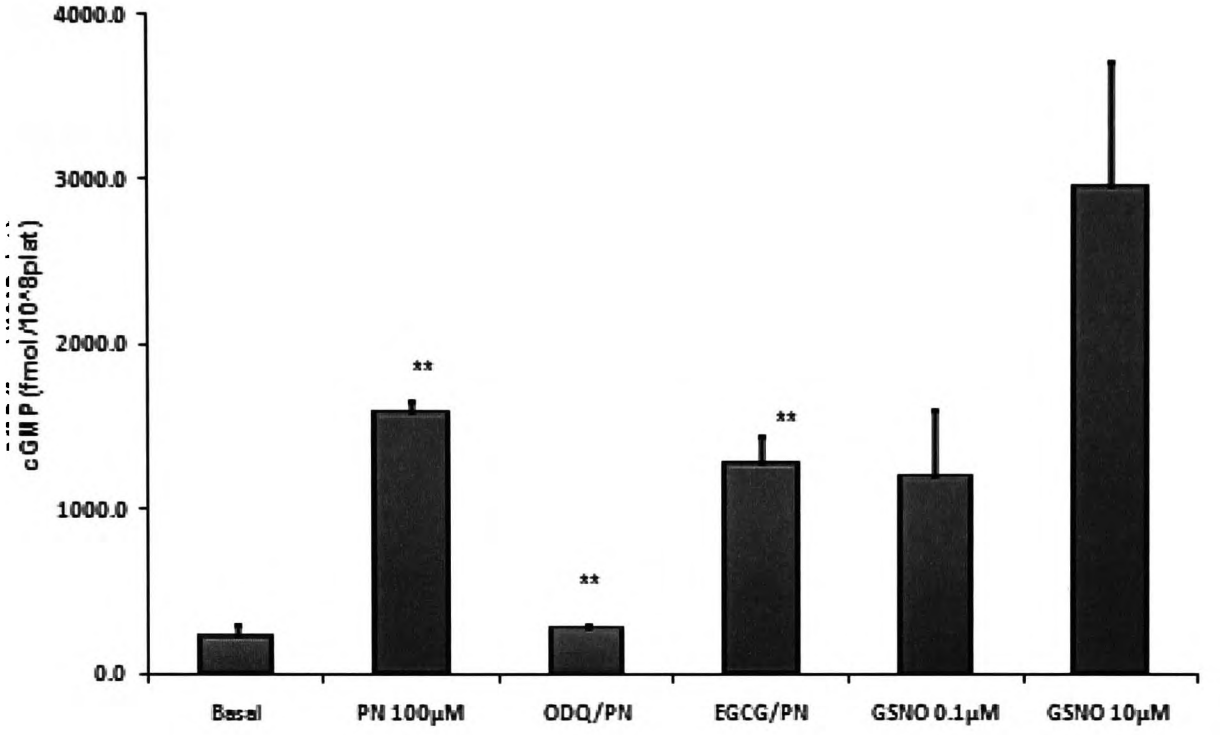



***Figure 3.12. Peroxynitrite increase cGMP production in platelets.***

*WP ($3x10^8$) pretreated with EGCG (100µM) or ODQ (20µM) for 20min prior to treatment with peroxynitrite (100µM) for 1min. Platelets were lysed and total cGMP concentration was measured using a competitive enzyme immunoassay as described in methods. The graph represents data from 4 independent experiments. Data = mean±SEM. **P < 0.01 vs. basal platelet sample.*

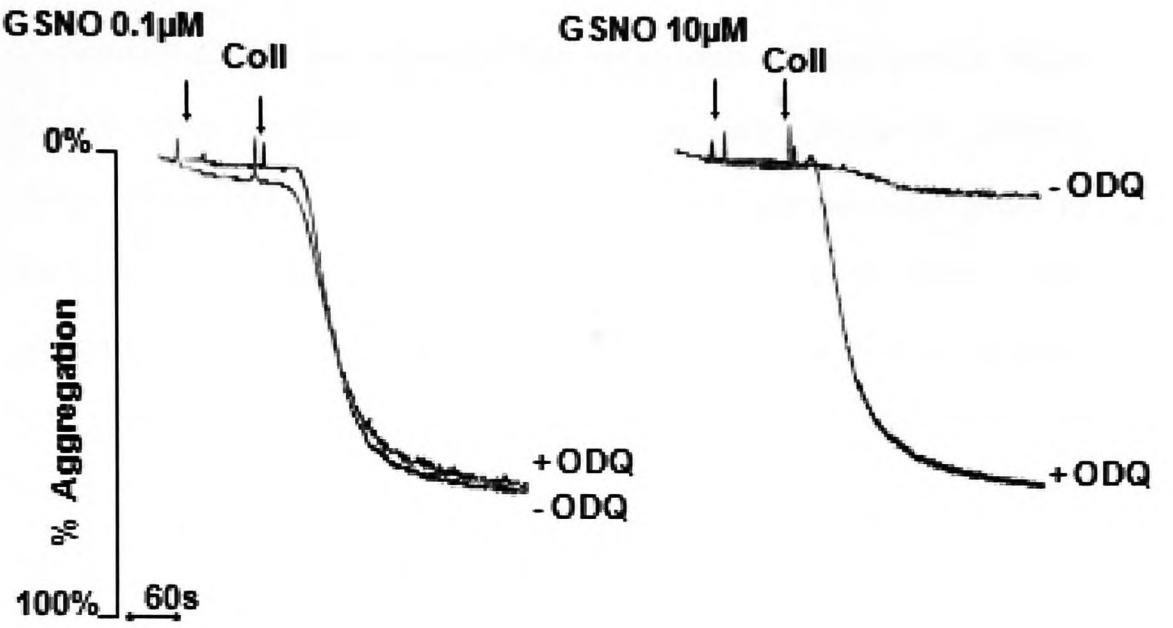



***Figure 3.13. Nitric oxide-dose dependent effect on inhibition of platelet aggregation.***

*WP ($3x10^8$) pretreated with ODQ (20µM) for 20min. WP were stimulated with collagen (2µg/ml) or pretreated first with the indicated doses of GSNO for 1min, and platelet response was recorded with Born aggregometer and expressed as % aggregation. Traces are representative of one experiment.*

### 3.3.5 The role of vasodilator stimulated phosphoprotein (VASP) in regulation of platelet function by peroxynitrite.

In section **3.3.3.1**, it was observed that peroxynitrite inhibited platelet shape change. Under physiological conditions such as upon adhesion to collagen, shape change allows filopodia to be formed which are then superseded by the sustained lamellipodia of the spread platelet (Pula *et al.*, 2006). VASP promotes filopodia formation and therefore shape change by allowing linear actin polymerization (Barzik *et al.*, 2005). In the next set of experiments, we tested whether peroxynitrite is targeting filopodia formation by inhibiting VASP and ultimately actin polymerization. To address this hypothesis, we examined the effect of peroxynitrite on platelet adhesion and spreading on immobilized collagen under static conditions. Platelets adhered to immobilized collagen (10μg/ml) seemed to spread consistent with filopodia and lamellipodia formation. Peroxynitrite-treated platelet spreading and adhesion was significantly reduced by 79 ± 20 % ($P < 0.05$). While adhesion of platelets treated with decomposed peroxynitrite was slightly affected by 18 ± 13 % **(figure 3.14a)**.

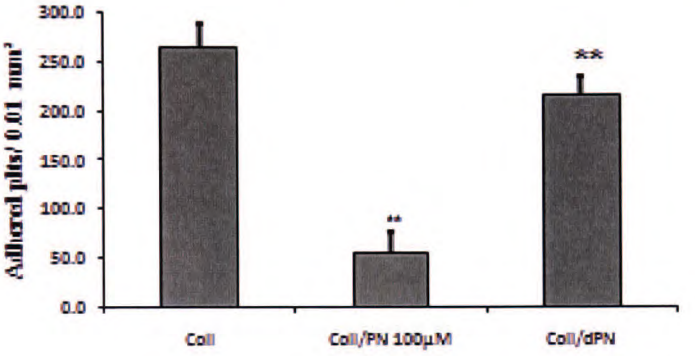
Platelet aggregation and adhesion are associated with platelet shape change. This is mediated by alterations to assembly and disassembly of actin cytoskeleton. Since peroxynitrite inhibited platelet spreading we examined whether influencing actin remodeling may be important for platelet inhibition by peroxynitrite. VASP is an actin associated protein whose phosphorylation is associated with reducing cytoskeleton remodeling in

platelets. Immunoblotting of proteins from adherent platelets to collagen show no increase in VASP phosphorylation. However, when platelet were pretreated with peroxynitrite, there was an increase in VASP phosphorylation. Pretreatment with decomposed peroxynitrite had no effect on VASP phosphorylation **(figure 3.14b)**.

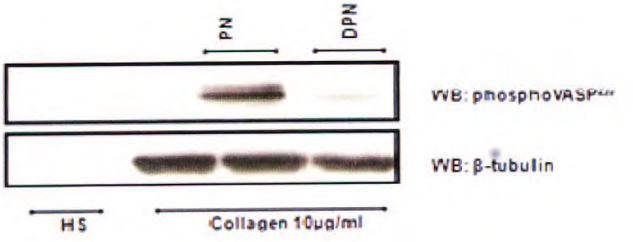
Treatment of washed platelets with peroxynitrite (5-100μM) for 1min led to a concentration-dependent increase in VASP phosphorylation of both $Ser^{239}$ and $Ser^{157}$ **(Figure 3.15a)**. Phosphorylation at both sites was observed at concentrations as low as 5μM and was maximal at 100μM (highest concentration tested). The incubation of platelets with decomposed peroxynitrite failed to induce phosphorylation, indicating that the observed effects were independent of potential change in pH or due to the effects of decomposition products.

Peroxynitrite-mediated $VASP^{Ser239}$ phosphorylation was observed within 15sec and declined to almost basal levels after 90mins. On the other hand, $VASP^{Ser157}$ phosphorylation was delayed till after 60sec of exposure to peroxynitrite and declined much faster by 60mins. These data indicate that peroxynitrite activates a number of AGC kinase family, since these kinases are the only known regulators of VASP phosphorylation on these sites.

(a)

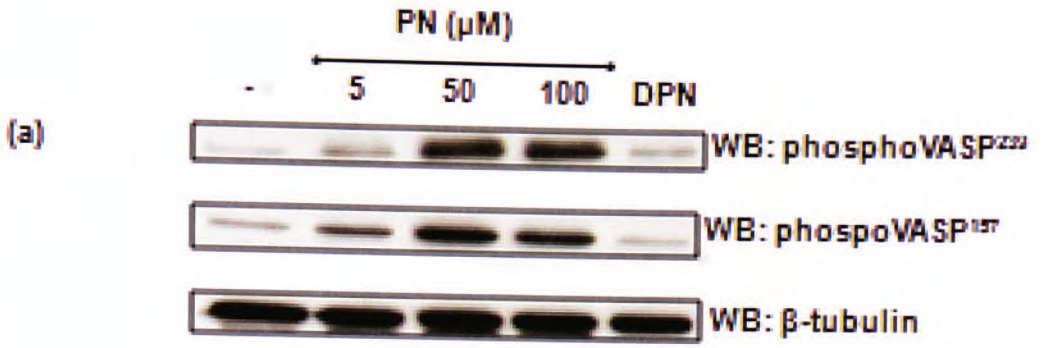

(b)

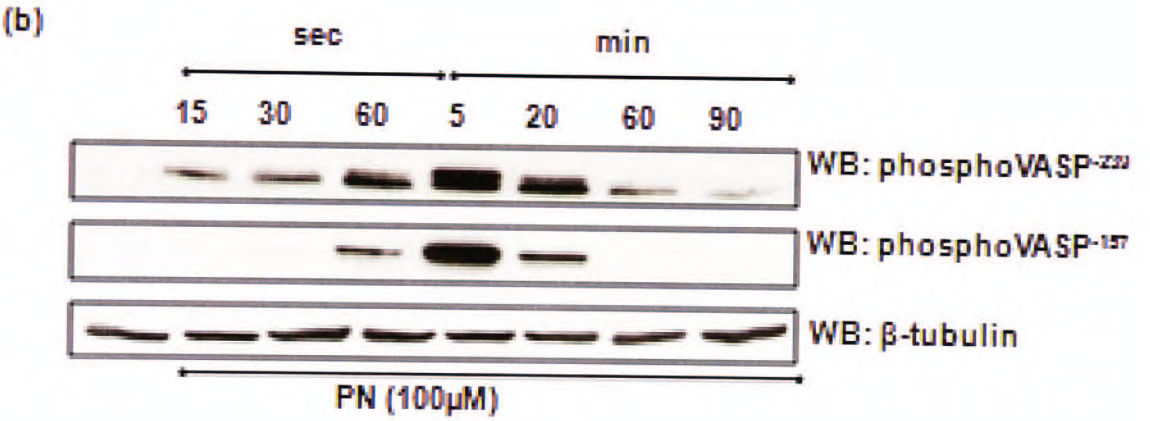



**Legend: overleaf**

***Figure 3.14. Peroxynitrite inhibits platelets adhesion to collagen.***

*WP ($3x10^7$/ml) were adhered to collagen (10µg/ml) coated microscope slides for 30 min in the presence or absence of peroxynitrite (100µM) or decomposed peroxynitrite, as indicated. Platelets were stained using TRITC-conjugated phalloidin and pictures were obtained using a fluorescent microscope (x60, oil immersion. Pictures represent results from 10 fields of view/sample of 3 independent experiments performed in duplicate. Experiments described were repeated 3 times with the results quantified as the number of adherent platelets/0.01mm$^2$. Data=mean±SEM. **P < 0.01 vs. collagen sample* ***(a)****. WP ($5x10^8$/ml) were adhered to either human serum (HS) or collagen (10µg/ml) for 30min in the presence or absence of peroxynitrite (100µM) or decomposed peroxynitrite. Reaction was stopped by adding 2x Laemmli buffer equal amount of proteins were loaded into SDS-PAGE Proteins (15µg) were separated in 10–18% gradient gels and immunoblotted for anti-phospho-VASP$^{Ser239}$,Membranes were then stripped and reprobed with anti-β-tubulin .*




**Legend: overleaf**

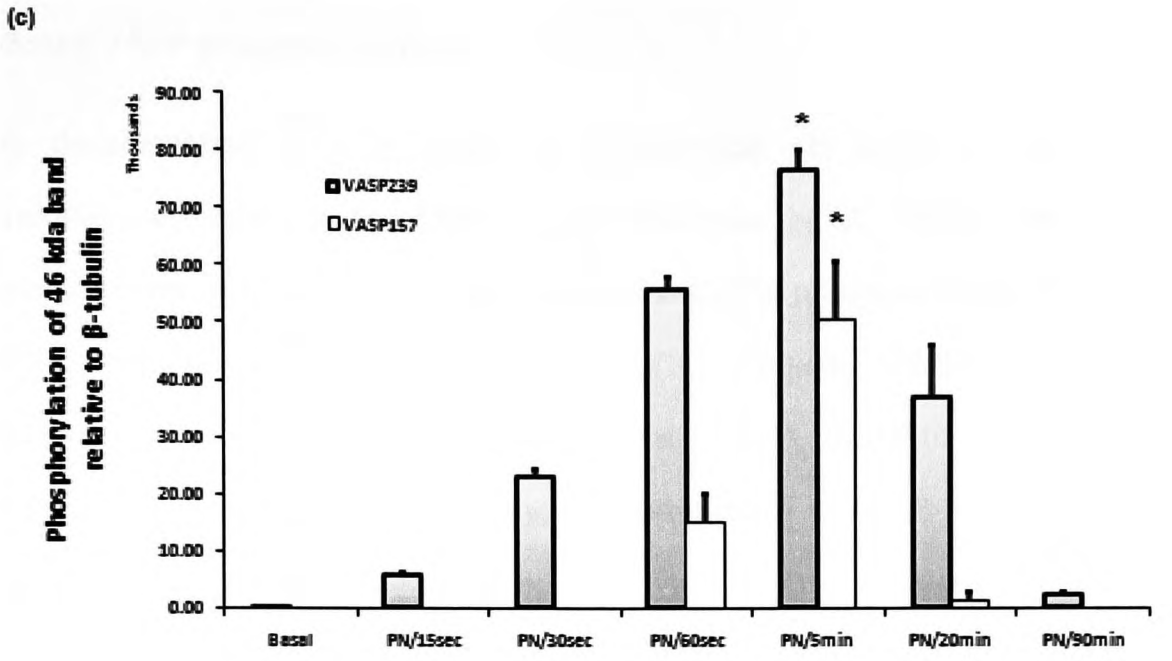


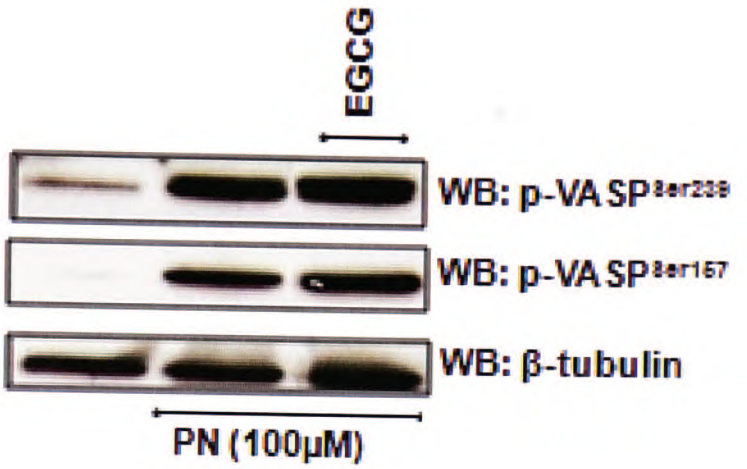

***Figure 3.15. Peroxynitrite induces a time- and concentration-dependent increase in VASP phosphorylation at both ser$^{157/239}$.***

*WP (3x10$^8$/ml) were treated with **(a)** peroxynitrite (0-100μM) for 1min or 100μM for the indicated time **(b)** with stirring before termination of the reaction with an equal volume of 2X Laemmli buffer. Proteins (15μg) were separated in 10–18% gradient gels and immunoblotted for anti-phospho-VASP$^{Ser239}$, or anti-phospho-VASP$^{Ser157}$. Membranes were then stripped and reprobed with anti-β-tubulin. Graph is representative of densitometric analysis of 7 independent experiments **(c)**. Data=mean±SEM. *P < 0.05 vs. Basal sample.*

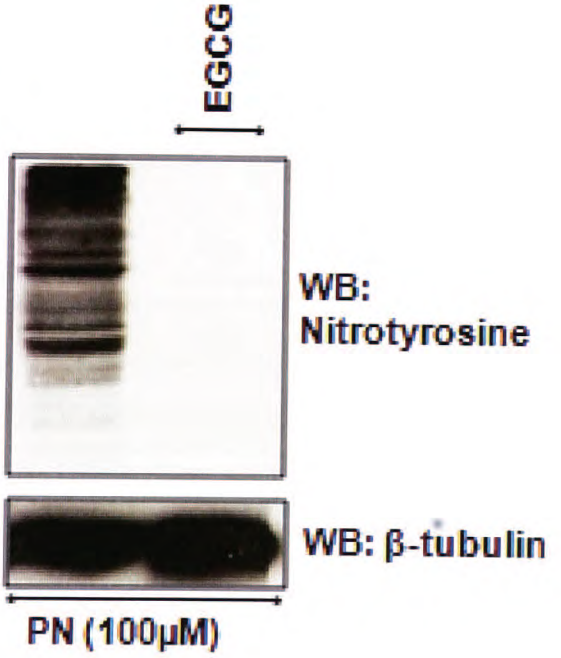
### 3.3.5.1 The role of nitration and oxidation in regulating peroxynitrite-induced VASP phosphorylation.

The decomposition of peroxynitrite at physiological pH leads to the generation of nitrating and oxidizing agents (Beckman *et al.*, 1996b), we assessed whether these agents were responsible for the phosphorylation of VASP. Preincubation of platelets with EGCG (100μM), inhibitor of peroxynitrite-induced nitration (Schroeder *et al.*, 2001), failed to inhibit phosphorylation of VASP in response to peroxynitrite (100μM) **(figure 3.16ai)**. In contrast, EGCG abolished peroxynitrite-induced nitration of platelet proteins **(figure 3.16aii)**. To confirm that our observations were not due to the presence of residual $H_2O_2$ (Kirsch *et al.*, 1998), the experiments were repeated in the presence of extracellular catalase. The presence of catalase (300 units/ml) had no effect on peroxynitrite-induced phosphorylation of $VASP^{Ser239}$, while $H_2O_2$ (100μM) alone failed to increase phosphorylation **(figure 3.16bi)**. Similarly, the antioxidant hydroxyl scavenger mannitol (10-100mM) failed to influence phosphorylation of VASP alone or in response to peroxynitrite **(figure 3.16bii)**. Thus, under these conditions nitration and oxidation mechanism were not responsible for peroxynitrite-induced effects.

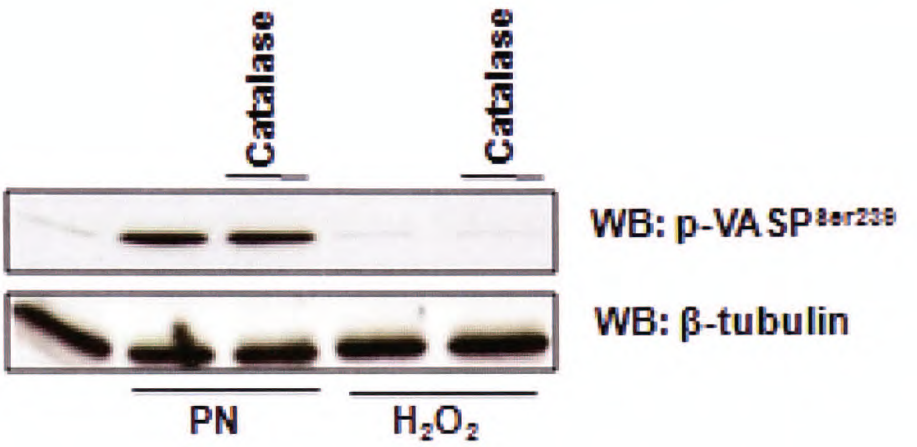
a (i)


EGCG
WB: p-VASP ser239
WB: p-VASP ser157
WB: β-tubulin
PN (100μM)

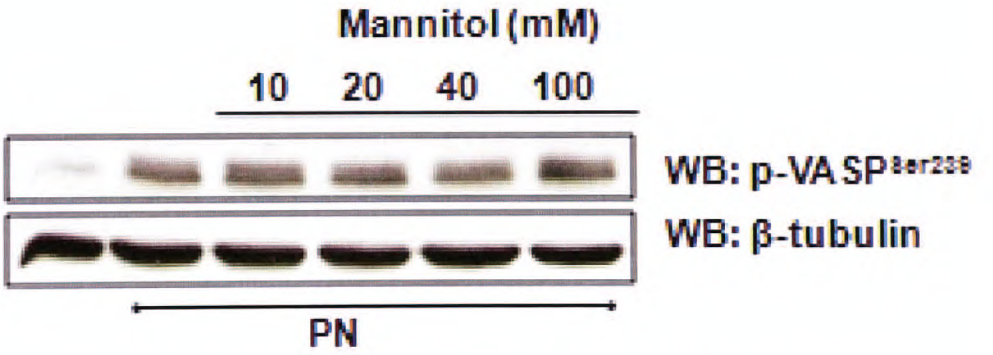
a (ii)


EGCG
WB:
Nitrotyrosine
WB: β-tubulin
PN (100μM)

b (i)




b (ii)




**Legend: overleaf**

***Figure 3.16. Peroxynitrite-induced phosphorylation of VASP does not require nitration.***

***(a)*** *WP ($3x10^8$/ml) were treated with peroxynitrite (100μM) for 1min in the presence and absence of nitration inhibitor [EGCG (100μM)]. Reactions were terminated by the addition of with an equal volume of 2xLaemmli buffer. Proteins (15μg) were separated in 10–18% gradient gels and immunoblotted for* ***(i)*** *anti-phospho-VASP$^{Ser239}$ and anti-phospho-VASP$^{Ser157}$, or* ***(ii)*** *anti-nitrotyrosine.* ***(b)*** *Platelets ($3x10^8$/ml) were treated with $H_2O_2$ (100μM) for 1 min in* ***(i)*** *presence and absence of catalase (300U/ml) or* ***(ii)*** *mannitol (10-100 mM). Reactions were terminated by the addition of with an equal volume of 2X Laemmli buffer. Proteins (15μg) were separated in 10–18% gradient gels and immunoblotted for anti-phospho-VASP$^{Ser239}$. Blots are representative of 4 independent experiments.*

### 3.3.5.2 The role of Src kinases and intracellular calcium in regulating peroxynitrite-induced VASP phosphorylation.

To further investigate the mechanisms responsible for peroxynitrite-mediated phosphorylation of VASP, a series of inhibitors were used. Peroxynitrite has been reported to activate Src kinase in red blood cells (Minetti *et al.*, 2002), and we have shown it is the case in platelets **(figure 3.4 and 3.5).** However, the presence of the Src family kinase inhibitor, PP1 (20μM), had no effects on peroxynitrite-induced VASP phosphorylation in platelets. In contrast, the intracellular calcium chelator, 2-bis (2-aminophenoxy) ethane-N,N,N,N',N'-tetraacetic acid (BAPTA-AM; 20μM), ablated phosphorylation of VASP at both sites **(Figure 3.18).** Thus, phosphoVASP occurs in a Src family kinase-independent in a manner but requires $Ca^{2+}$ mobilization.

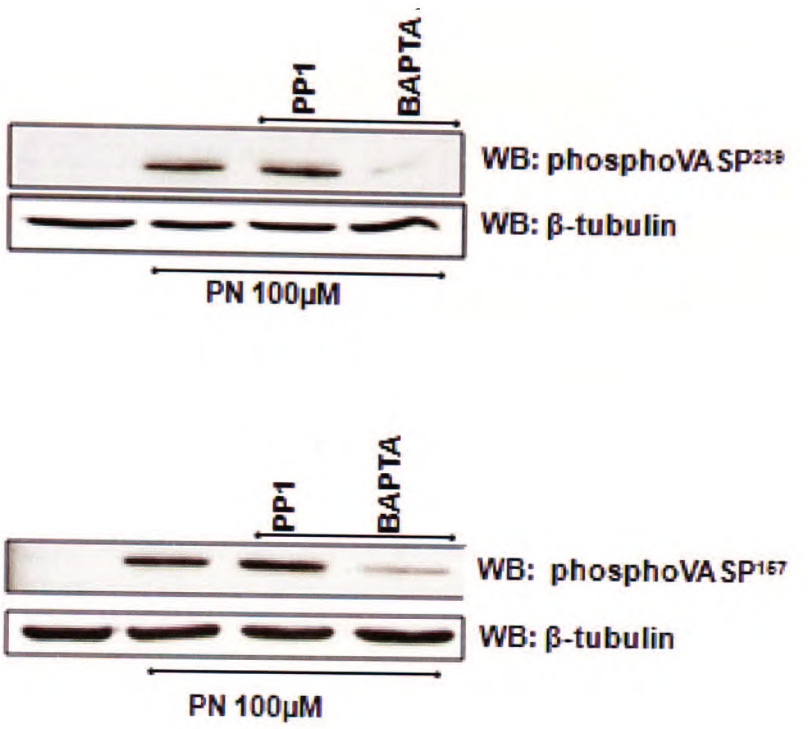



***Figure 3.17. Peroxynitrite-induced phosphorylation of VASP requires $Ca^{2+}$ mobilisation but not Src kinases.***

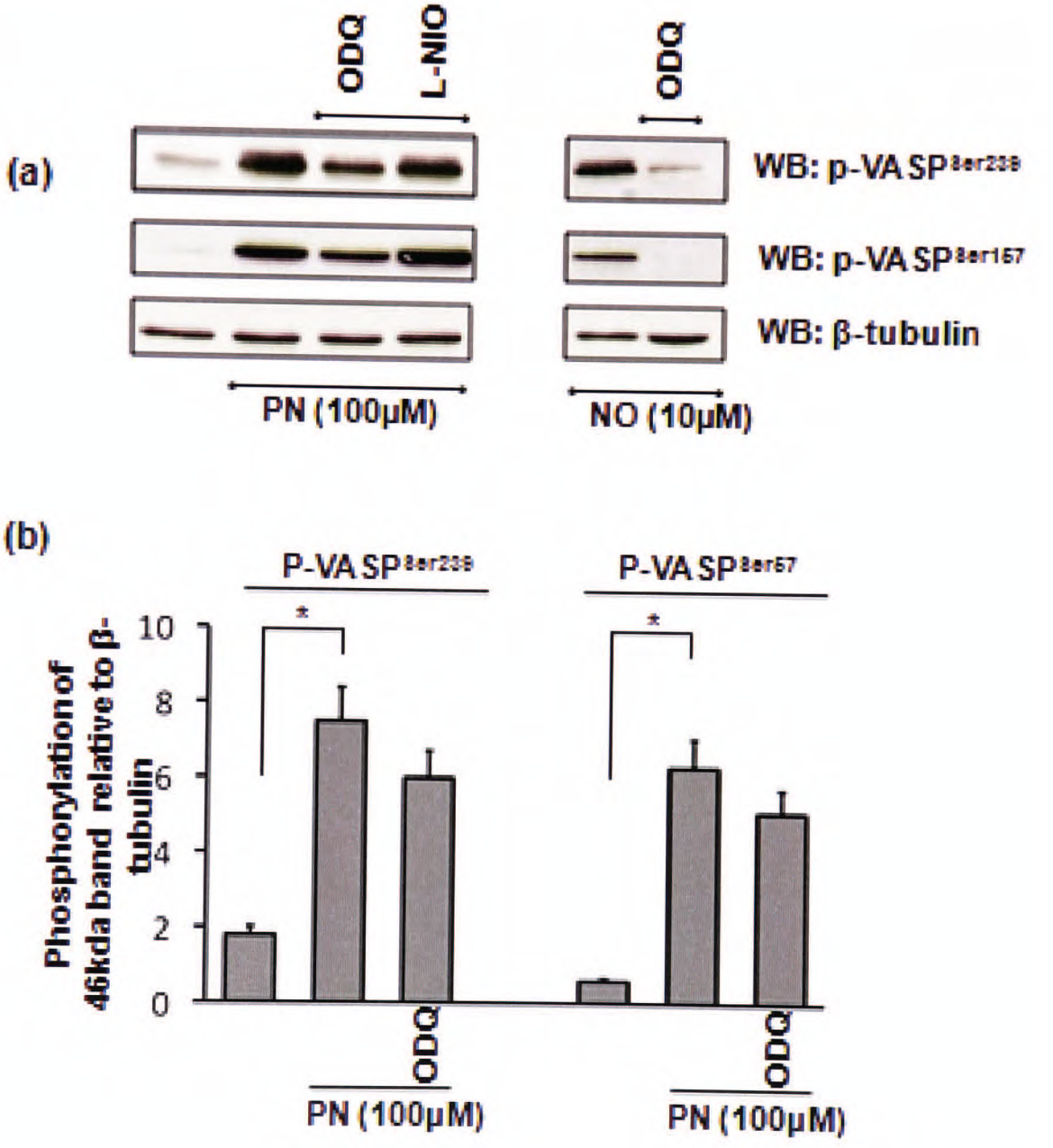
*WP ($3x10^8$/ml) were treated with peroxynitrite (100μM) in presence and absence of Src kinase inhibitor [PP1 (20μM)] or intracellular $Ca^{2+}$ chelator [BAPTA (20μM)]. Reactions were stopped with an equal volume of 2xLaemmli buffer. Proteins (15μg) were separated in 10–18% gradient gels and immunoblotted for anti-phospho-$VASP^{Ser239/157}$. Blots are representative of 3 independent experiments.*

## 3.3.6 The role of AGC family kinases in regulating peroxynitrite-induced VASP phosphorylation.

### 3.3.6.1 Protein kinase G (PKG) and protein kinase A (PKA).

In platelets and other cell types $VASP^{Ser239}$ phosphorylation has been shown to be a useful monitor for PKG activity (Halbrugge *et al.*, 1990a; Smolenski *et al.*, 1998). On the other hand, phosphorylation of $VASP^{Ser157}$ is predominantly dependent on protein kinase A (Butt *et al.*, 1994).

In the next series of experiments the potential role of PKG and PKA in peroxynitrite -mediated phosphorylation of VASP was investigated. Since a NOS type activity in blood platelets was described (Patel *et al.*, 2006), we examined its potential role in phosphorylation of VASP in response to peroxynitrite. Preincubation of platelets with the NOS inhibitor (L-NIO) had no effect on peroxynitrite-mediated phosphorylation of VASP. In the presence of the sGC inhibitor ODQ (20μM), the phosphorylation of $VASP^{Ser239/157}$ induced by peroxynitrite was reduced, although this was not significant, indicating that cGMP-dependent phosphorylation of VASP played only a minor role in the actions of peroxynitrite **(Figure 3.18a)**. Consistent with these observations peroxynitrite caused a modest increase in cGMP formation. In contrast, ODQ reduced NO (10μM) mediated phosphorylation of $VASP^{Ser239}$ to basal levels and abolished phosphorylation of $VASP^{Ser157}$ consistent with its ability to induce large increases in GMP.



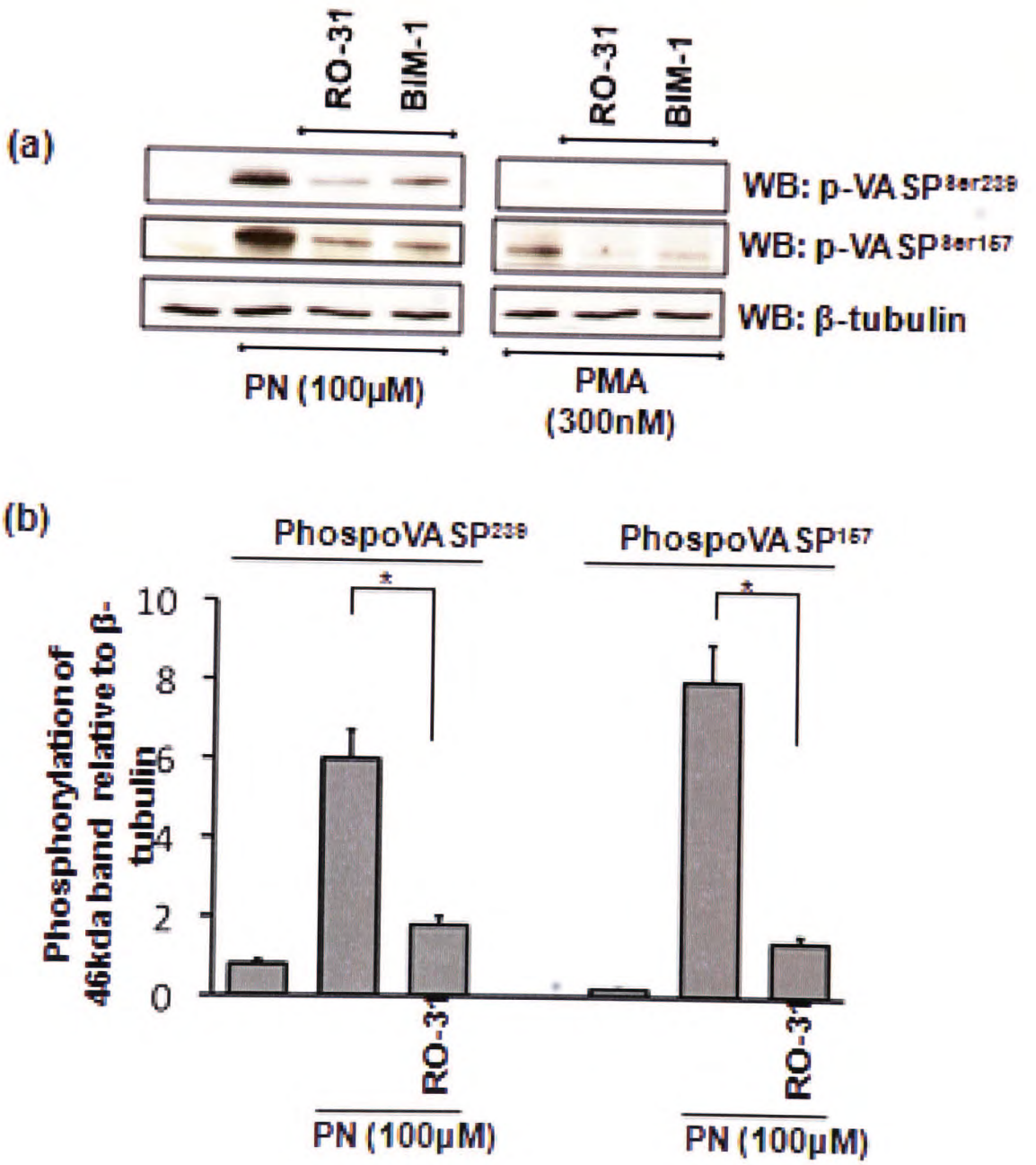

**Legend: overleaf**

***Figure 3.18. The role of cGMP in VASP phosphorylation stimulated by peroxynitrite.***

*WP ($3x10^8$/ml) were treated with peroxynitrite (100μM) or NO (10μM) for 1min in the presence and absence of sGC inhibitor [ODQ (20μM)] or eNOS inhibitor [L-NIO (1mM)]. Reactions were terminated by the addition of with an equal volume of 2X Laemmli buffer. Proteins (15μg) were separated in 10–18% gradient gels and immunoblotted for anti-phospho-VASP$^{Ser239/157}$. Blots are representative of three independent experiments, and Densitometric analysis of three independent immunoblot assays. *P < 0.05 vs. basal sample.*

### 3.3.6.2 Protein kinase C (PKC).

In addition to PKG and PKA, PKC has also been reported to phosphorylate VASP$^{Ser157}$ (Chitaley *et al.*, 2004), but not VASP$^{Ser239}$. *In* vitro, PMA [a potent diacylglycerol (*DAG*) *analog*] that acts as a PKC activator serves as a useful tool for studying the role of PKC in cells. A functional PKC activation was verified by incubation of platelets with PMA or peroxynitrite in the presence and absence of PKC inhibitors followed by determination of VASP$^{Ser239/157}$ phosphorylation. Consistent with the report by Wentworth and colleagues (Wentworth *et al.*, 2006), PMA (300nM/ml) caused a significant increase in phosphoVASP$^{157}$ but had not effects on phosphoVASP$^{Ser239}$. The formation of phosphoVASP$^{Ser157}$ in response to PMA was abolished by the two structurally distinct PKC inhibitors RO31-8220 (10μM) and BIM-I (10μM). Strikingly, preincubation with the PKC inhibitors caused significant inhibition of peroxynitrite-induced phosphorylation of VASP on both sites **(Figure 3.19a)**. Since this suggested a role for PKC in signaling events initiated by peroxynitrite, we examined PKC activity using an antibody that recognizes phosphorylation of the preferred PKC consensus phosphorylation motif: RXXS/TXRX (Pearson *et al.*, 1991). Here we observed low level of phosphorylation in untreated platelets. However peroxynitrite (100μM) induced phosphorylation of proteins with apparent molecular weights of 40 and 100kDa **(Figure3.19bi)**. These phosphorylation events seemed to occur in parallel to VASP phosphorylation occurring within 15sec before declining to basal after 60min. However it is important to note that this activation is modest in comparison to that observed with PMA of thrombin (1U/ml) **(Figure 3.19bii)**.


(a)
RO-31
BIM-1
RO-31
BIM-1
WB: p-VASP ser239
WB: p-VASP ser157
WB: β-tubulin
PN (100μM)
PMA
(300nM)
(b)
PhospoVASP239
PhospoVASP157
Phosphorylation of 46kda band relative to β-tubulin
10
8
6
4
2
0
*
*
RO-31
RO-31
PN (100μM)
PN (100μM)

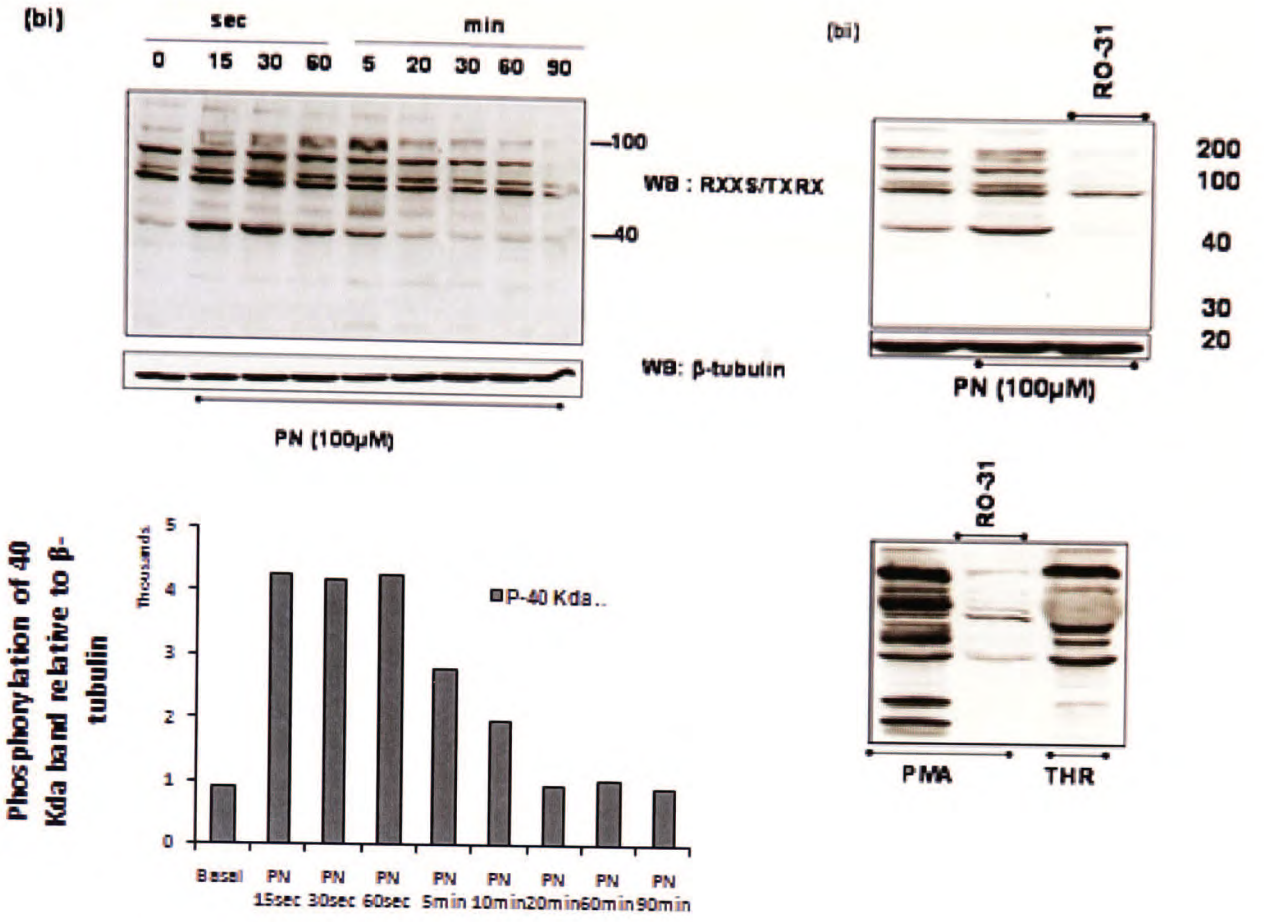



***Figure 3.19. The role of PKC in VASP phosphorylation stimulated by peroxynitrite.***

*WP ($3x10^8$/ml) were treated with peroxynitrite (100μM) for 1 min or PMA (300nM) for 5min in presence and absence of PKC inhibitor [Ro31-8220 (10μM) or BIM I (10μM)]* ***(a)****. Reactions were stopped with an equal volume of 2xLaemmli buffer. Proteins (15μg) were separated in 10–18% gradient gels and immunoblotted for anti-phospho-VASP$^{Ser239/157}$. Blots are representative of three independent experiments, and densitometric analysis of 3 independent immunoblot assays.* ***(bi)*** *Platelets ($3x10^8$/ml) were treated with peroxynitrite (100μM) for up to 60min, Thrombin (1U/ml) or PKC*

*activator [PMA 300nM] in presence and absence of RO31-8220 before termination of the reactions with an equal volume of 2xLaemmli buffer. The samples were processed as in* ***(a)*** *except membranes were probed with an anti-phospho PKC substrate antibody. Blot is representative of three independent experiments. *P < 0.05 vs. basal sample.*

### 3.3.7 AMP-activated protein kinase (AMPk).

AMP-activated protein Kinase (AMPk), a sensor of cellular energy status that is sensitive to the AMP: ATP ratio, is found in all eukaryotic cells, including *Giardia lamblia*, which does not have mitochondria, nucleoi, or peroxisomes (Adam, 2000). (Adam, 2000). AMPk is a heterotrimeric complex consisting of a catalyting α-subunit, regulatory β and γ subunits (Hardie *et al.*, 2003). The γ-subunit contains two AMP binding sites and one a tightly bound, non-exchangeable AMP. The α-subunit contains an N-terminal kinase domain and a C-domain that involved in complex formation. The β- subunit contains Glycogen-Binding Site (GBS), and studies show that high cellular glycogen represses activation of AMPk in muscles in vivo (Wojtaszewski *et al.*, 2002), suggesting that AMPk can act as glycogen sensor as well as AMP:ATP sensor.

AMPk can be activated by any stimuli that change AMP: ATP balance, this includes metabolic stress, oxidative stress, hypoxia, and glucose deprivation. Despite the relatively early discovery of AMPk, its signaling pathway is poorly understood especially in platelets.

Other protein targets which may play role in platelet inhibition by peroxynitrite were also investigated. Recently it has been shown that AMPk can be activated by peroxynitrite in endothelial cells (Zou *et al.*, 2002). This was evidenced by an increase in AMPkα $Thr^{172}$ phosphorylation as well as increased $Ser^{92}$ phosphorylation of acetyl-coenzyme A carboxylase (ACC), a downstream target of AMPk. AMPk activation by peroxynitrite has not been shown in platelets. In fact, little is known about AMPk and its signaling pathway in platelets.

### 3.3.7.1 Investigation the presence of AMPk and its substrates in platelets.

Given the limited data regarding the presence and/or the role of AMPk and ACC proteins in platelets, initial experiments sought to determine the presence of those proteins in platelets. In the first instance the relative amount of AMPk and specificity of anti-AMPk antibody was evaluated by comparing platelet lysates with endothelial cell lysates. Importantly, the presence of AMPk in endothelial cells is well established (Chen *et al.*, 1999; Nagata *et al.*). Immunoblotting experiments revealed that AMPk was highly expressed in platelets and was indistinguishable in terms of molecular weight under the same experimental conditions used from that in endothelial cells **(figure 3.20a)**.

Acetyl-coenzyme A carboxylase (ACC) is phosphorylated downstream of AMPk and has been used as a marker of AMPk activation (Chen *et al.*, 2000). While biochemical studies suggested that ACC is present in platelets (Philip W et al., 1969), no studies have demonstrated actual protein. Whole cell lysates from platelets and endothelial cells immunoblotted with an antibody that, according to manufacturer (Cell Signaling), detects all isoforms of ACC. **Figure 3.20b/c** shows that in endothelial cells there is a protein band at 280 kDa, which corresponds to the one from manufacturer on the left, this band is not present in platelets. Since ACC was to be used as a marker of AMPk activity, it lack of detection prevented the pursuit of these experiments.

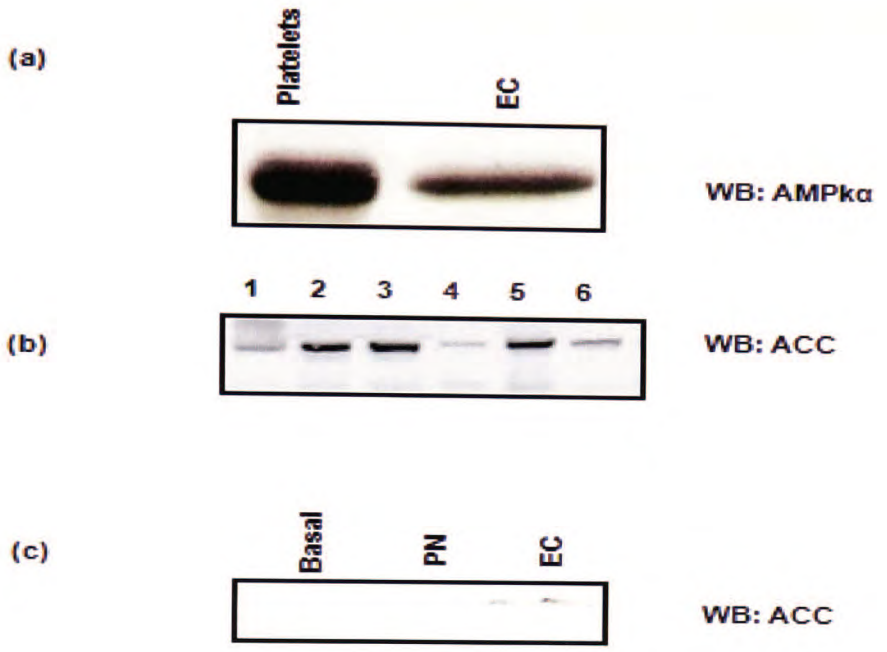



***Figure 3.20. Detecting the presence AMPk and Acetyl-CoA Carboxylase in platelets.***

*20µg of whole cell lysates from WP and endothelial cells (EC) were resolved on 10-18% SDS PAGE, Immunoblotted with anti-AMPkα* ***(a)****. Western blot analysis of cell extracts from various cell lines, using Acetyl-CoA Carboxylase adopted from cell signaling. 1(NIH/3T3), 2(293), 3(HCC78), 4(C6), 5(PAE), 6(CHO).* ***(b)****, 20mg/ml of whole cell lysates analysis of WP, basal, and treated with 100µM Peroxynitrite, compared to HUVECs* ***(c)****, resolved in 10-18% SDS PAGE, immunoblotted with rabbit mAB against ACC, as directed by manufacturer. Blot is representative of one experiment.*

### 3.3.7.2 The influence of peroxynitrite on AMPk phosphorylation.

AMPk is phosphorylated on threonine 172 residues by LKB1, and is used as a marker of enzyme activation. Since AMPk is present in platelets and phosphorylated in response to peroxynitrite in endothelial cells (Zou *et al.*, 2003), we examined its phosphorylation state of platelet AMPk was investigated

In unstimulated platelets, AMPk was found to be basally phosphorylated; however this was increased by addition of peroxynitrite (100μM). Phosphorylation of AMPk was time-dependent, increased phosphorylation was observed at 30secs and maintained for up to 90secs before declining back to basal **(figure 3.21).**

### 3.3.7.3 The mechanism underlying peroxynitrite-induced AMPk phosphorylation.

Previously we have shown that platelets signal through Src kinases **(figure 3.4)**, PKC **(figure 3.19**), intracellular calcium **(figure 3.17)**, and nitration **(figure 3.10)**. Therefore we used a series of inhibitors to determine which one of these pathways was important for peroxynitrite-mediated phosphorylation of AMPk. Inhibition of calcium mobilization and nitration using BAPTA (10μM) and EGCG (100μM) respectively, reduced but did not inhibit AMPk phosphorylation. In contrast, inhibition of Src kinases and PKC using PP2 (10μM) and RO31-8220 (10μM) respectively, failed to affect AMPk phosphorylation induced by peroxynitrite. This data suggest that peroxynitrite induces tyrosine phosphorylation and AMPk activation by two different mechanisms **(figure 3.22a)**. In addition, peroxynitrite caused AMPk

tyrosine nitration, which was inhibited in presence of EGCG. On the other hand, decomposed peroxynitrite and AICAR failed to induce tyrosine nitration **(figure 3.22b)**.

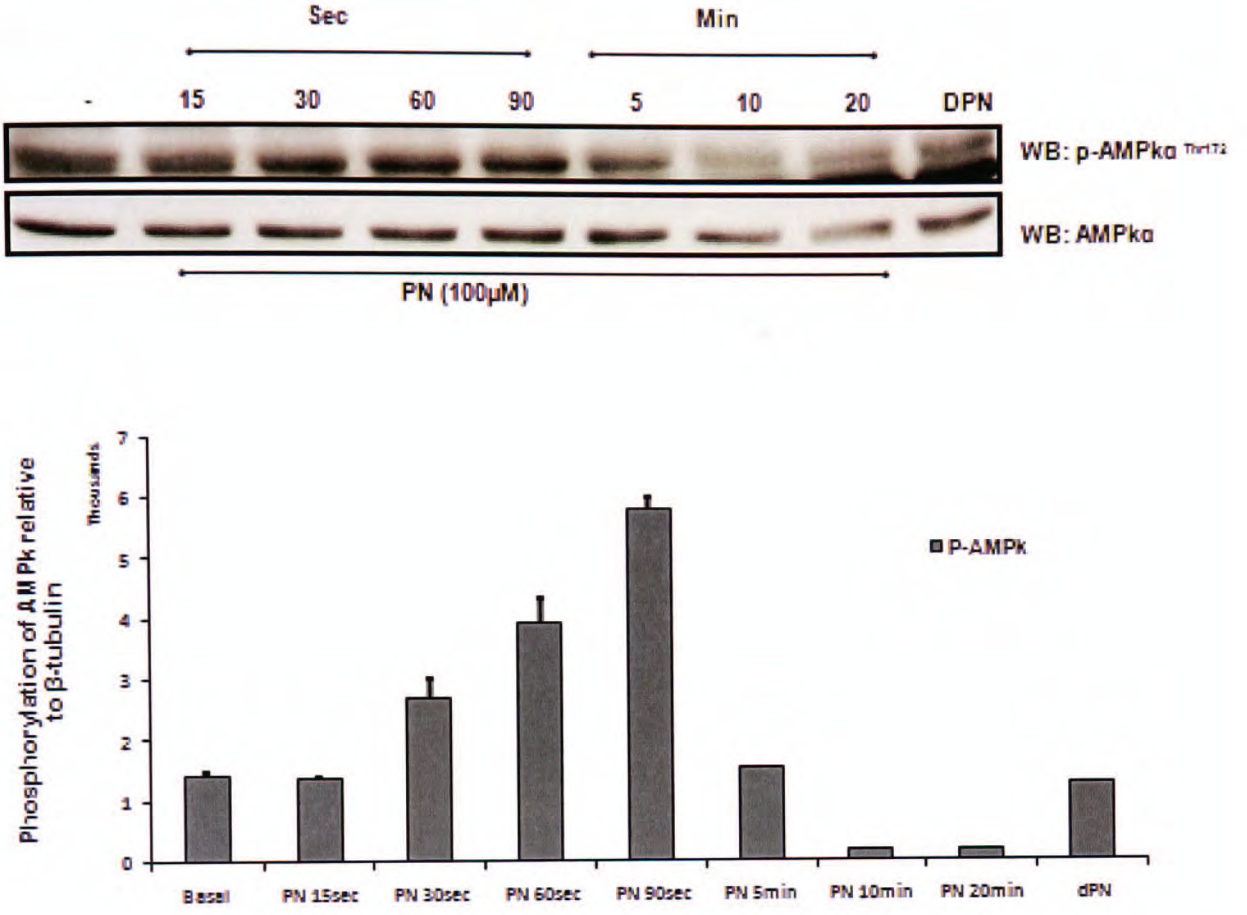
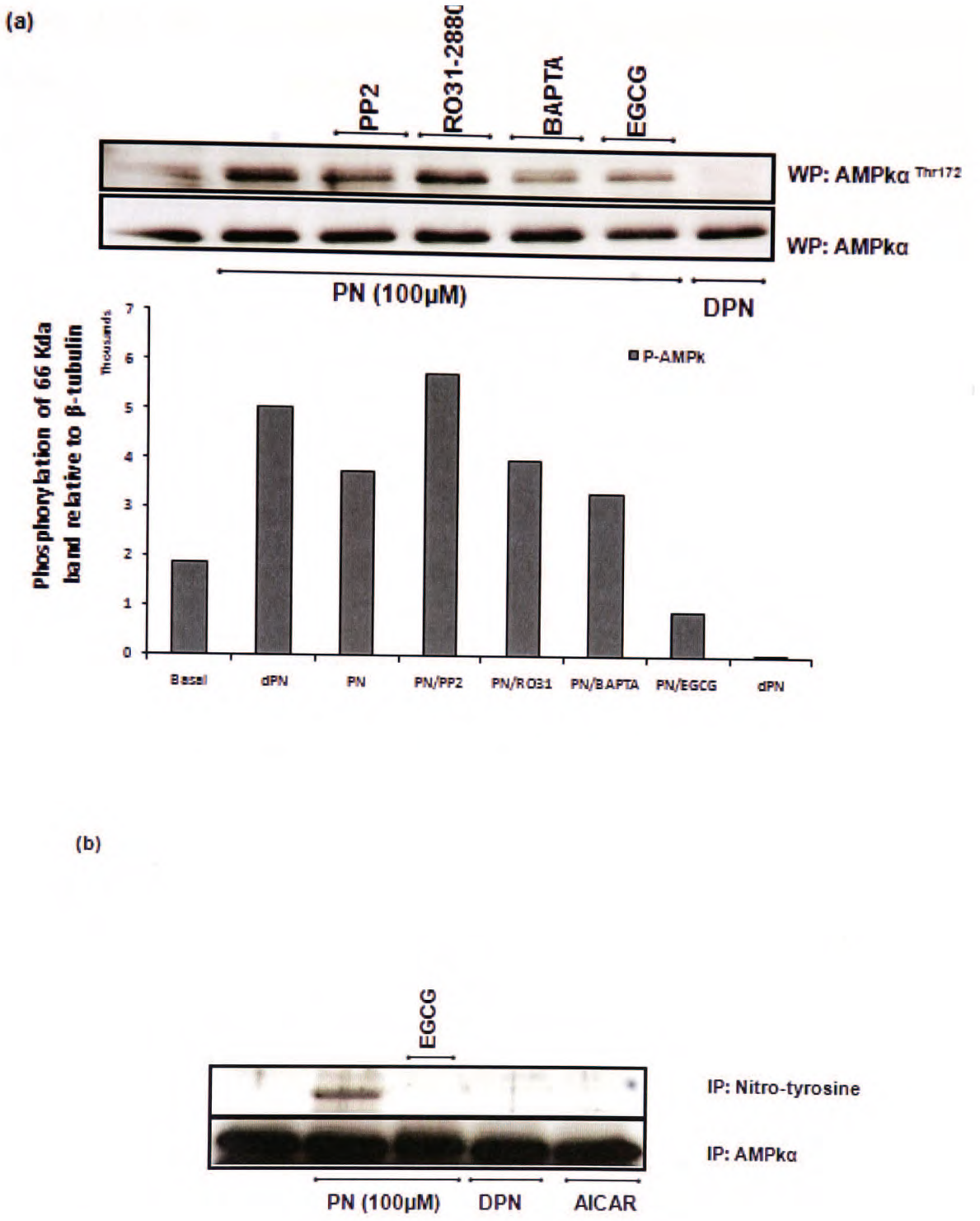



***Figure 3.21. AMPk is activated in response to peroxynitrite.***

*WP ($3x10^8$) treated with peroxynitrite or decomposed peroxynitrite 100μM for up to 20m. Whole cell lysates were resolved in 10-18% gradient SDS-PAGE and immunoblotted using anti-phospho AMPkα$^{Thr172,}$ membranes were stripped and reprobed with anti-AMPkα. The graph represents densitometric analysis of 3 independent immunoblot assays.*




**Legend: overleaf**

***Figure 3.22. Role of secondary mediators and protein kinases in peroxynitrite-mediated AMPk phosphorylation.***

*WP ($3x10^8$) treated with peroxynitrite or decomposed peroxynitrite 100μM, for 1min, in presence, or absence of Src kinase inhibitor [PP2 (20μM)], PKC inhibitor [ Ro31-8220 (10μM)], intracellular $Ca^{2+}$ chelator [ BAPTA-AM (20μM)], or nitration inhibitor [EGCG (100μM)]* ***(a)*** *whole cell lysates resolved on 10-18%SDS PAGE, immunoblotted with anti-AMPkα$^{Thr\ 172}$, and anti-AMPkα antibodies. WP ($7x10^8$) treated with AMPk activator [AICAR (500μM)], peroxynitrite or decomposed peroxynitrite 100μM for 1min in presence or absence of EGCG (100μM), Lysate were immunopreciptaed with anti-AMPkα antibody, and blotted with anti-nitrotyrosine* ***(b)****. Blots are representative of one experiment.*

### 3.3.7.4 AMPk inhibits platelet aggregation.

It has been suggested that peroxynitrite reduces platelet aggregation and secretion in part by inhibition of mitochondrial energy production (Rusak *et al.*, 2006). We have shown earlier that peroxynitrite increased AMPk phosphorylation, and since AMPk is "an energy sensor" of cells, it represented a potential target for peroxynitrite mediated inhibition of aggregation. We investigated the significance of AMPk activation in platelets. 5-aminoimidazole-4-carboxamide-1-β-d-ribofuranoside (AICAR) is a cell-permeable adenosine analogue that can be phosphorylated to ZMP, an AMP analogue and known AMPK activator (Hardie *et al.*, 1997; Zhou *et al.*, 2001). The incubation of platelets with AICAR did not induce platelet activation. However, when AICAR-treated platelets were stimulated with collagen (0.4μg/ml) we found a significant reduction in aggregation from 73.75 % ± 10.7 to 27.5 % ± 9.04 ($P < 0.05$) **(figure 3.23 a/b)**.

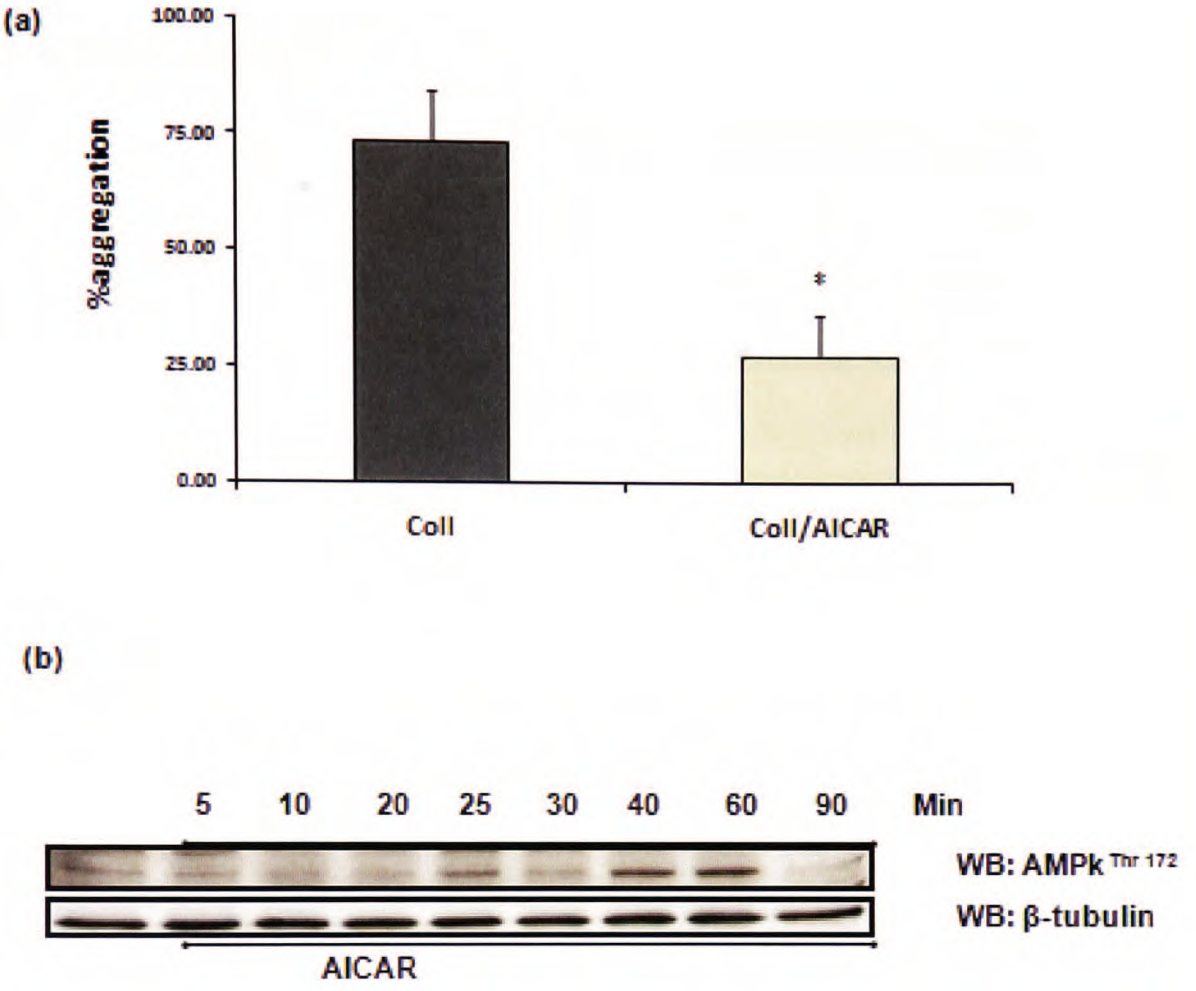



***Figure 3.23. AMPk activation inhibits platelets aggregation.***

*WP ($3x10^8$) untreated, or pre-incubated first with AMPk activator [AICAR (100μM)] were stimulated with collage and response was recorded for 5min and expressed at % aggregation. Graph representing data from 3 independent experiments. Data=mean±SEM. *P < 0.05 vs. collagen sample* ***(a).*** *WP ($3x10^8$) incubated with AMPk activator [AICAR (100μM)] for indicated time points. lysates were subject to SDS-PAGE and immunoblotted with anti-$AMPk^{thr172}$* ***(b).***

## 3.3.8 Discussion.

The primary source of NO in the vascular system is the endothelium (Ignarro *et al.*, 1987a). However, oxidative stress, a risk factor for several cardiovascular disorders, interferes with the NO/sGC/cGMP signalling pathway through scavenging of NO and formation of the strong intermediate oxidant, peroxynitrite (Pryor *et al.*, 1995). Peroxynitrite is a potent nitrating and oxidizing species, which can induce differential effects on platelet function depending on the concentration. At higher concentrations (>200μM), peroxynitrite can have potentially activatory effects such as increasing $Ca^{2+}$ mobilization, nitration of the platelet inhibitory receptor PECAM-1 (Newman *et al.*, 2002). While at lower concentrations (<100μM), peroxynitrite modulates platelet function by reducing energy metabolism, cyclooxygenase activity and inhibiting aggregation-induced phosphotyrosine signaling events (Boulos *et al.*, 2000a; Low *et al.*, 2002; Lufrano *et al.*, 2003). However, the precise mechanism underlying its potential inhibitory effects are unclear.

The ability of peroxynitrite to regulate platelet function was initially demonstrated by aggregation. In agreement with others (Naseem *et al.*, 1995; Yin *et al.*, 1995), exogenously administrated peroxynitrite caused a concentration and time-dependent inhibition of platelet aggregation. In the first instance the ability of peroxynitrite to inhibit platelet aggregation through the cGMP/PKG pathway was tested. When platelets were incubated with the sGC inhibitor ODQ (Moro *et al.*, 1996), the inhibitory effects of NO were completely abolished, consistent with its role as a potent activator of cGMP.

Surprisingly, the inhibitory actions of peroxynitrite were also ablated by ODQ, with is inconsistent with its role as a weak activator of sGC. There has been reports that peroxynitrite may react with HEPES based buffers to produce compounds that have NO like activity (Kirsch *et al.*, 1998; Moro *et al.*, 1995). In our experimental conditions, when a phosphate buffer was used instead of Tyrode's buffer, which contained HEPES, the apparent cGMP-mediated inhibitory effect of peroxynitrite on platelet aggregation was not recovered by ODQ. When the ability of peroxynitrite to activate sGC in Phosphate buffer was tested, peroxynitrite induced a small but significant increase in cGMP formation. This increase was completely blocked by ODQ, and was nitration-independent. This is consistent with its classification as a modest activator of sGC compared to GSNO. Indeed, the modest levels of cGMP produced by peroxynitrite stimulation were not sufficient to have any functional effects on platelets. This was confirmed by comparisons with the NO donor GSNO, which at 100nM caused a similar increase in cGMP formation but this was insufficient to induce inhibition of collagen-mediated platelet aggregation. Thus under conditions, that prevent the formation of secondary NO-donors the inhibitory effects of peroxynitrite are independent of cGMP. It is possible that previous studies highlighting cGMP dependency were due to either the composition of the platelet resuspenesion buffer or the presence of plasma where reactions between peroxynitrite and glutathione can form S-nitrosothiols that are potential cable of release NO (Van der Vliet *et al.*, 1994).

Platelet aggregation is a method to analyse the functional response of platelets (Born *et al.*, 1963), but does not provide information of the signal

transduction mechanisms that regulate platelet function. Therefore, we began to examine whether peroxynitrite could cause inhibition of aggregation through signaling events that were independent of cGMP. In agreement with (Mondoro *et al.*, 1997; Naseem *et al.*, 1997), we showed that peroxynitrite induced tyrosine phosphorylation and nitration of a number of platelet proteins in a time and concentration-dependent manner. This tyrosine phosphorylation was potentiated by the release of secondary mediators, namely ADP and $TxA_2$, and un-like tyrosine nitration, was totally mediated by a Src-dependent mechanism, but independent of nitration. Tyrosine nitration of proteins has long been viewed as a footprint of peroxynitrite, however, nitration may occur in physiological conditions and this has been demonstrated to be the case in platelets (Naseem *et al.*, 2000; Sabetkar *et al.*, 2002). As expected peroxynitrite caused nitration of platelet proteins, which was maintained for up to 60min. Interestingly, one protein band did decrease over time suggesting the presence of "denitration" mechanisms. Indeed, putative denitrase activity was demonstrated in several publications (Gow *et al.*, 1996; Kamisaki *et al.*, 1998; Kuo *et al.*, 1999; Kuo *et al.*, 2002). This activity was monitored by the decreased intensity of nitrotyrosine immune reactive bands in Western blots. This has also been described in platelets previously although the nature of this mechanism remains to be elucidated (Naseem *et al.*, 1997). However, peroxynitrite (100μM), same dose used to inhibit platelet aggregation, caused platelet aggregation when EGCG was added to platelets prior to treatment with peroxynitrite; thus, it is possible that nitration is a protective mechanism. The explanation for these data is complicated since on one hand peroxynitrite caused activation of

stimulatory pathways, but functionally the aggregation was inhibited. It is possible that while some activatory pathways are switched on, the nitration over rides these activatory effects, so the "net balance" is inhibition. Certainly, inhibition of aggregation is only partially reversed with time, since after 40mins exposure to peroxynitrite collagen mediated inhibition was stilled blunted. Furthermore blocking nitration with EGCG partially prevented the inhibitory actions of peroxynitrite. However, the precise mechanism by which nitration regulates platelet function is still poorly understood and requires further investigation.

The inhibition of aggregation by peroxynitrite was associated with an abolition of platelet shape changes, indicating that it may target the cytoskeletal rearrangement required for function. In this context a recent report demonstrated NO-mediated nitration of α-actinin (Marcondes *et al.*, 2006b), presumably through generation of intracellular peroxynitrite, can regulate platelet adhesion suggests that proteins regulating the platelet cytoskeleton may be potential targets of peroxynitrite signaling. Upon platelet adhesion to collagen, filopodia are transiently formed and then superseded by the sustained lamellipodia of the spread platelet (Frojmovic *et al.*, 1990). Our data showed an inhibition of platelet adhesion and spreading to immobilised collagen of platelets exposed to peroxynitrite. In platelets, vasodilator-stimulated phosphoprotein (VASP) promotes filopodia formation by allowing linear actin polymerization (Barzik *et al.*, 2005), and phosphorylation of VASP correlates with inhibition of platelets (Aszodi *et al.*, 1999). VASP, which regulates actin-myosin interactions, and whose activity is regulated by reversible phosphorylation induced by PKA, PKC and PKG.

We therefore explored whether VASP was a target for peroxynitrite and showed for the first time that low concentrations of peroxynitrite can induce rapid phosphorylation of VASP on both major phosphorylation sites. We found that phosphorylation was induced by concentrations as low as 5μM, although it should be noted that the rapid decomposition of peroxynitrite at physiological pH would suggest that phosphorylation may be induced at much lower concentrations. It has been established for some time that peroxynitrite has the capacity to modulate signal transduction systems through several different mechanisms including the nitration of protein tyrosine residues and oxidation of cysteine thiols. To investigate the mechanism of signal transduction leading to phosphorylation of VASP by peroxynitrite we used a series of pharmacological inhibitors. Peroxynitrite undergoes oxidation reactions, but the presence of mannitol had no effect on the phosphorylation of VASP. Protein nitration can both activate and inhibit phosphotyrosine-dependent signaling leading to altered functional responses. However, the presence of epicatechin, which inhibits nitration but not oxidation reactions of peroxynitrite, failed to influence phosphorylation of VASP indicating a mechanism independent of 3-nitrotyrosine. Peroxynitrite can activate phosphotyrosine-dependent signaling independently of nitration. In erythrocytes peroxynitrite activates Src family kinases, Hck and Lyn (Minetti *et al.*, 2002), and Lyn plays a key role in platelet function. Peroxynitrite did induced tyrosine phosphorylation in a Src family kinase-dependent manner, but it had no effects on VASP phosphorylation

It is widely accepted that activation of PKG via cGMP elevating agents is the main upstream signalling pathway of VASP phosphorylation on $Ser^{239}$. NO,

through its ability to elevate cGMP and activate both PKG and PKA, is a more potent stimulator of VASP phosphorylation than Peroxynitrite. The use of the NOS inhibitor L-NIO did not influence VASP phosphorylation and therefore it is unlikely that Peroxynitrite increased platelet-derived NO availability, which we have previously shown to phosphorylate VASP (Riba *et al.*, 2006). Our data indicated that PKG plays only a minor role in peroxynitrite-mediated phosphorylation of VASP. Importantly, we also saw no significant effect of PKA inhibitors on VASP phosphorylation.

In the absence of a major role for PKA or PKG, we next studied PKC. To our surprise the PKC inhibitors induced a significant and almost total reduction in VASP phosphorylation at both sites indicating a major role for PKC. Indeed consistent with data from other cell types we found that PKC was activated by peroxynitrite. Since the intracellular $Ca^{2+}$ cheater, BAPTA, ablated phosphorylation, we also propose that peroxynitrite leads to the release of $Ca^{2+}$ from internal stores and causes the activation of a $Ca^{2+}$-dependent is form of PKC. The latter point suggests that a conventional PKC is form (α, β or γ) is responsible. Our data contrast with that of Wentworth et al who found that PKC only phosphorylated VASP at serine$^{157}$ and not serine$^{239}$. The reason for this unclear but probably reflects different experimental conditions. In the former study, the authors used both PMA and thrombin, both of which have an established mechanism for activation of PKC isoforms. In contrast, peroxynitrite may be less specific and have a plethora of effects in the cell. In other cell types, peroxynitrite has been shown to activate nitration-dependent and independent activation of phosphotyrosine-dependent signalling events, PKB, PKC, MAPKs. Furthermore, the pattern of activation of these individual

pathways differs depending on the type of cell, concentration of peroxynitrite and duration of exposure. Further studies will be required to establish the functional significance of peroxynitrite-mediated phosphorylation of VASP and PKC isoform(s) responsible.

Resting platelets rely predominantly on the anaerobic glycolysis of blood-borne glucose as a major source of energy (Akkerman, 1978), despite the presence of mitochondria and glycogen particles within these cells. During platelet activation the requirements for glycolysis raises by approximately 3 fold (Sorbara *et al.*, 1997). It has also been suggested that peroxynitrite reduces platelet aggregation and secretion in a manner mediated in part by inhibition of mitochondrial energy production (Rusak *et al.*, 2006). The mechanism underlying this observation was unclear. However, it had been demonstrated that in endothelial cells peroxynitrite could regulate the enzyme AMPk. ATP is able to act as a donor of high-energy phosphate, Likewise, with adenylate kinase; ADP can accept high-energy phosphate to form ATP. This ATP/ADP cycle occurs at a very rapid rate, since the total ATP/ADP pool is extremely small and sufficient to maintain an active tissue for only a few seconds (Murray *et al.*, 2003). Fundamentally, AMPk is a sensor of cellular energy status that is sensitive to changes in AMP: ATP ratio. AMPk, once activated, phosphorylates several downstream substrates, the overall effect of which is to switch off ATP-consuming pathways (e.g. fatty acid synthesis and cholesterol synthesis) and to switch on ATP-generating pathways (e.g. fatty acid oxidation and glycolysis) (Hardie *et al.*, 1998). We hypothesised that AMPk may be a target for peroxynitrite in

platelets and could in part underpin its ability to inhibit aggregation and adhesion. In platelets little is known about the role of AMPk. Although Fleming and colleagues (Fleming *et al.*, 2003) suggested that AMPk activation by 5-aminoamidazole 4-carboxamide ribonucleoside (AICAR) increases platelet cyclic GMP levels and attenuates platelets activation. Using immunoblotting we found AMPk was expressed at high levels in platelets and under these conditions was indistinguishable from that in endothelial cells. Although its downstream target, ACC, was not detected under these conditions. Incubation of platelet with a pharmacological inhibitor of AMPk, AICAR, induced phosphorylation of AMPk, which attenuated platelet aggregation in response to collagen and thrombin. Interestingly, peroxynitrite also induced AMPk phosphorylation in a time-dependent manner. Peroxynitrite-mediated AMPk phosphorylation was partially $Ca^{2+}$ and nitration dependent but Src independent. This data demonstrate that intracellular calcium release, and subsequently AMPk activation, is mediated in a manner that is independent of Src kinase activation. These preliminary data indicated that AMPk might play an important role in the regulation of platelet function. Unfortunately, during this progress of this study it became apparent that the specificity of AICAR and the purported AMPk inhibitor, compound C, may have a plethora of none specific effects (Emerling *et al.*, 2007). In the absence of these tools we were unable to continue these experiments.

## 3.3.9 Conclusion.

The effects of peroxynitrite are not simply the accumulation of random damage to cells as specific responses determine how cells behave *in vivo.* There is a biological specificity to the effects of oxidants, and given the localization and the level of peroxynitrite generation in both physiological and pathological conditions, it will be of an interest to re-view peroxynitrite contribution to haemostasis.

# CHAPTER 4. INVESTIGATION OF THE ROLE OF PROTEIN KINASE C (PKC) IN NITRIC OXIDE (NO) SIGNALING.

# ABSTRACT

**INTRODUCTION:** Endothelial derived nitric oxide (NO) is a short-lived secondary messenger, which acts as an endogenous platelet inhibitor. The primary signalling events underlying the inhibitory actions of NO occur through cyclic guanosine monophosphate (cGMP), dependent activation of protein kinase G (PKG). Once activated PKG phosphorylates vasodilator-stimulated phosphoprotein (VASP), an actin binding protein that plays a major role in negatively regulating adhesive events in platelets. It has been shown that PKC can also phosphorylate VASP, leading to inhibition of platelet function. In the present study we used immunoprecipitation combined with immunoblotting techniques to examine the potential role of PKC in NO-mediated inhibition of platelet function.

**RESULTS:** NO induced a time- and concentration-dependent phosphorylation of VASP on serine$^{157/239}$. As expected this was blocked by the PKG inhibitor Rp-8-pCPT-cGMPS. However, the presence of PKC inhibitors BIM I (bisindolylmaleimide I) and RO-31-8220, but not inhibitors of Src kinases or phosphoinositide 3-kinase-dependent kinase also blocked NO-mediated phosphorylation of VASP serine$^{157/239}$. In contrast PKC inhibitors had no effect on VASP phosphorylation induced by the direct PKG activator 8- bromoguanosine 3',5' cyclic monophosphate (8-Bromo-cGMP; a PDE-resistant cGMP analogue), indicating that the effects of PKC lay upstream of cGMP formation and PKG. To further understand the potential role of PKC in NO-mediated signaling we examined PKC activity using a phospho-PKC substrate antibody. NO caused a modest but significant increase in PKC substrate phosphorylation that was sensitive to inhibitors of PKC, suggesting a role for NO in activating PKC.

**CONCLUSION:** These data indicate that PKC may regulate NO signaling upstream of cGMP, possibly via NO-sGC axis, or may affect cGMP bioavailability via regulating cyclic nucleotide phosphodiesterases (PDEs).

# 4. Introduction.

## 4.1 Protein Kinase C (PKC).

Protein kinase Cs are members of the extended AGC (protein kinases A, G, and C) family of differentially expressed serine/threonine kinases implicated in a diverse array of cellular functions. Human platelets express predominantly 4 isoforms of PKC α, β, δ and θ. The PKC family has long been known to positively regulate a number of platelet processes. Using biochemical approaches, PKCα has been identified as an essential factor in positively regulating α-granule and dense-granule secretion in platelets (Yoshioka *et al.*), as well as platelet aggregation (Tabuchi *et al.*, 2003). However, using genetic and pharmacological approaches, PKCδ has been shown to play a negative role in regulating filopodia formation and platelet aggregation in response to collagen through a functional interaction with the actin regulatory protein VASP (Crosby *et al.*, 2003; Pula *et al.*, 2006), and SHIP-1 (Chari *et al.*, 2009). In the previous section, peroxynitrite was demonstrated to activate a PKC isoform. Since peroxynitrite also increased cGMP levels in platelets, the relationship between cGMP-elevating agents and PKC was explored.

**Aims of study**

- To determine the effect of cGMP-elevating agents such as NO on PKC activation in platelets.
    - PKC activation will be investigated by immunoblotting of proteins from NO-treated platelets with an antibody that recognizes phosphorylation of PKC substrates.
- To investigate the role of PKC in NO-mediated signaling and NO-mediated inhibition of platelet function.
    - NO signaling in platelets will be investigated by looking at NO-mediated VASP phosphorylation in the presence of PKC inhibitors.
    - Inhibition of platelet aggregation by NO will be assessed in the presence of PKC inhibitors.

**Materials and methods of study**

- 8-Bromo-cGMP and RO 31-8220 were purchased from Calbiochem (Nottingham, UK). GSNO was obtained from Tocris Bioscience (Bristol, UK). For complete list of chemicals and antibodies **(Appendix II/III)**.

**Methods of study**

- Platelet aggregation.
- Immunoblotting.

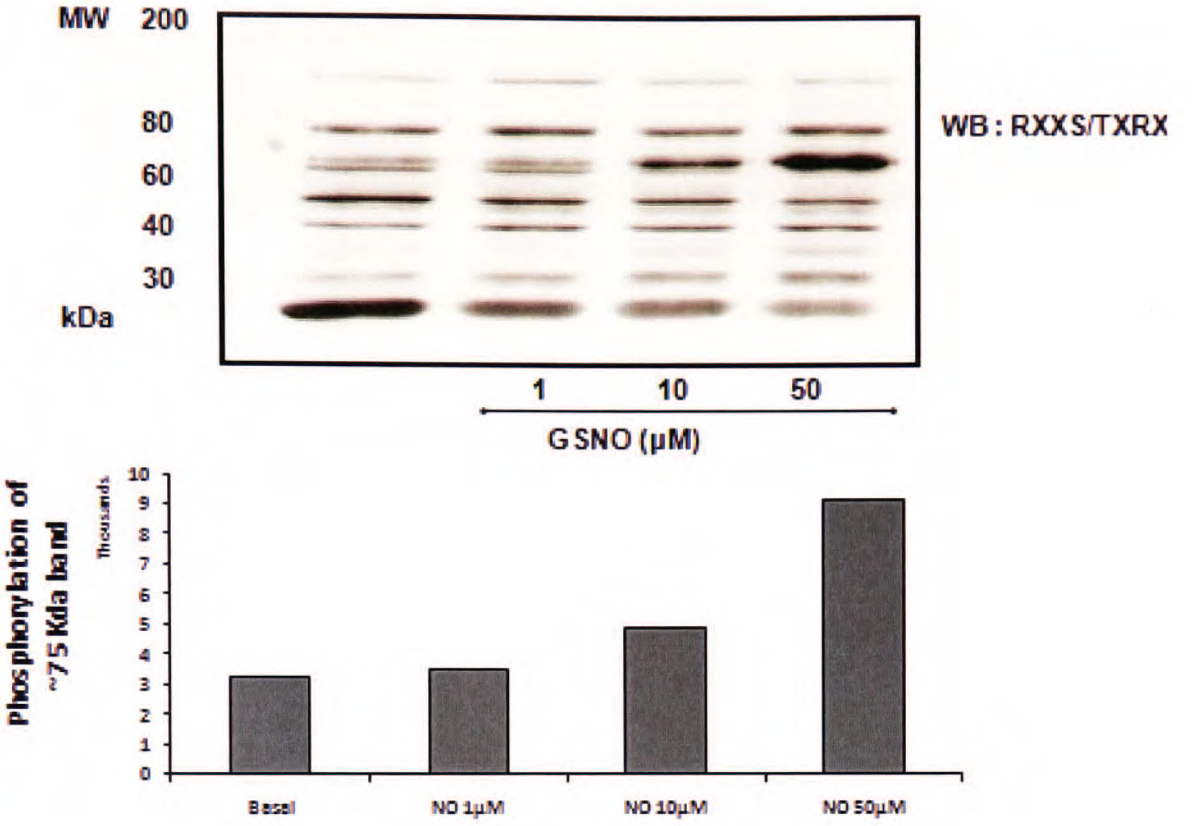
## 4.2 Results.

### 4.2.1 Nitric oxide activates PKC in platelets.

NO has been shown to activate PKC in kidney and cardiac cells (Liang *et al.*, 1999; Yoshida *et al.*, 1999), and cause nitration of PKC in platelets (Balafanova *et al.*, 2002a). By using an antibody that recognizes phosphorylation of the preferred PKC consensus phosphorylation motif [RXXS/TXRX (Pearson *et al.*, 1991)], we were able to investigate whether treatment of platelets with NO or 8-Bromo-cGMP, a cGMP analogue, activate PKC.

Treatment of WP with GSNO (0-50μM) led to a concentration-dependent increase in phosphorylation of one potential PKC substrate with an apparent molecular weight of ~75kDa **(figure 4.1)**. Phosphorylation was initiated at 10μM GSNO and maximal at 50μM. Phosphorylation occurred within 60 sec and was maintained for up to 120 sec. Importantly, phosphorylation of this substrate was blocked by PKC inhibitor RO31-8220 (10μM).

(a)


MW
200
80
60
40
30
kDa
WB : RXXS/TXRX
1
10
50
G SNO (µM)
Phosphorylation of ~75 Kda band
Thousands
10
9
8
7
6
5
4
3
2
1
0
Basal
NO 1µM
NO 10µM
NO 50µM

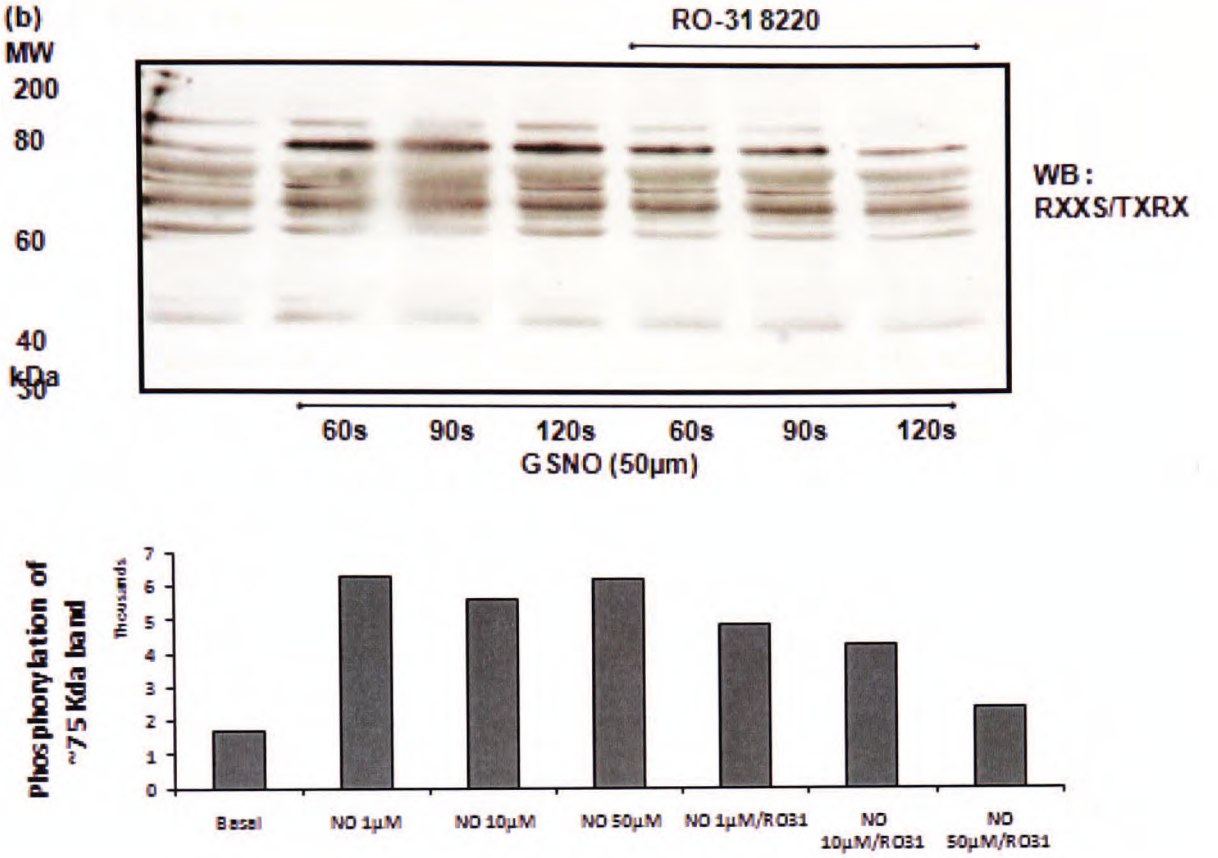



***Figure 4.1. Nitric oxide and 8-Br-cGMP activate PKC.***

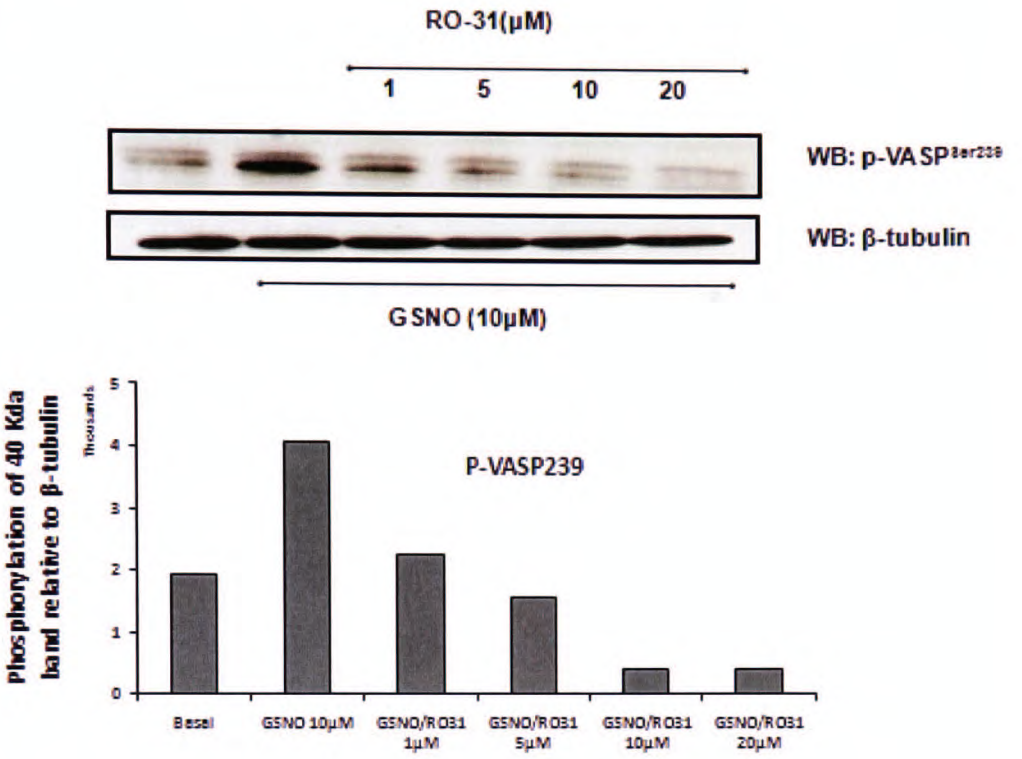
*WP ($3x10^8$) treated with GSNO (1, 10, and 50µM for 1min)* ***(a)****, or pre-incubated first with PKC inhibitor, RO31-8220 (10µM) for 20min before treatment with GSNO (50µM* ***(b)****. Reactions were stopped with an equal volume of 2xLaemmli buffer. Proteins (15µg) were separated in 10–18% gradient gels and immunoblotted with anti-PKC substrate antibody. Blots are representative of two experiments.*

## 4.2.2 PKC is required for Nitric oxide-mediated VASP phosphorylation.

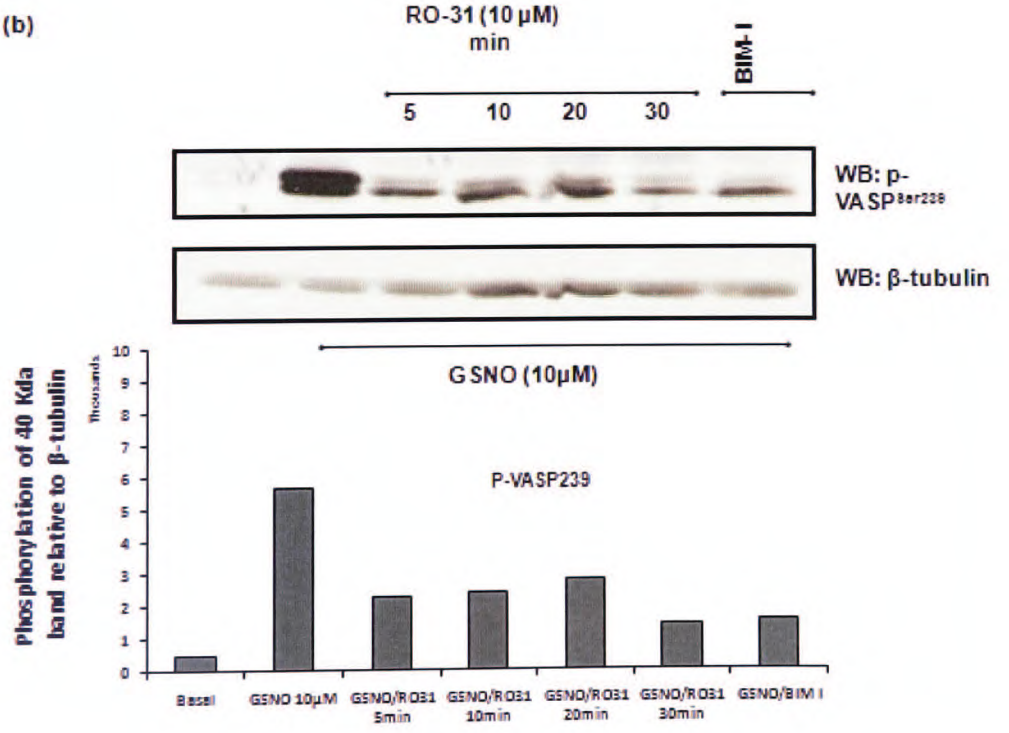
We have previously in **figure 4.1** suggested that PKC is a substrate for NO. Balafanova and colleagues demonstrated that PKC localization is regulated by NO (Balafanova *et al.*, 2002a), while others suggest that NO negatively regulates PKC activity (Gopalakrishna *et al.*, 1993). In this study, utilizing the widely used pharmacological inhibitors of PKC we assessed the potential role of PKC in NO-mediated signaling. According to the manufacturer (*Calbiochem*), RO31-2880 is a cell-permeable pharmacological inhibitor selective for isotypes of PKC at concentrations 100- to 1000-fold below its known effects on other intracellular signaling molecules such as PKA and $Ca^{2+}$/calmodulin-dependent protein kinase. First, we tested whether RO-31-8220 has any affect on NO-induced VASP phosphorylation. To our surprise, RO-31-8220 (10μM) inhibited VASP phosphorylation by GSNO (10μM), in a time and dose-dependent manner. Also, by using structurally distinct PKC inhibitor, BIM I, the inhibition of NO-mediated VASP phosphorylation was maintained. By using two structurally distinct PKC inhibitors this data confirm that NO signals through PKC **(figure 4.2)**.

To insure that these observations were not specific to GSNO, the experiments were repeated with other structurally distinct NO donors. DPTA-NONOate induced VASP phosphorylation only at a concentration of 10μM; nevertheless, VASP phosphorylation was abolished in the presence of PKC inhibitor **(figure 4.3)**.

(a)




(b)




**Legend: overleaf**

***Figure 4.2. Nitric oxide-mediated VASP$^{Ser239}$ is PKC-dependent.***

*WP ($3x10^8$) pre-incubated with PKC inhibitor, RO31-8220/20min, at indicated concentrations before treatment with GSNO (10μM) for 1min* ***(a)****. WP ($3x10^8$) pre-incubated with PKC inhibitor, RO31-8220 (10μM), for indicated time or BIM I (10μM) before treatment with GSNO (10μM) for 1min* ***(b)****. Reactions were stopped with an equal volume of 2xLaemmli buffer. Proteins (15μg) were separated in 10–18% gradient gels and immunoblotted for anti-phospho-VASP$^{Ser239}$. Blots are representative of two experiments.*

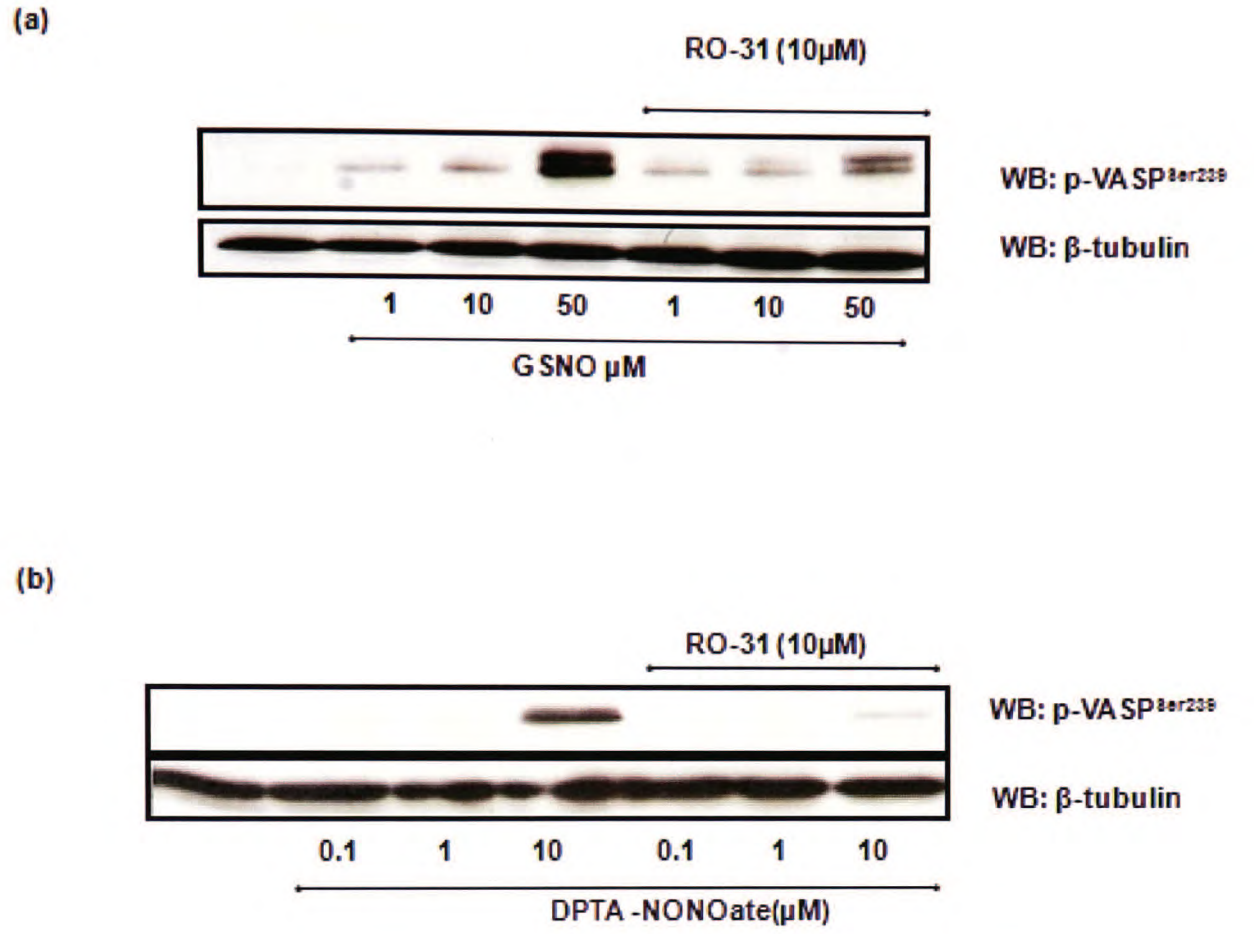



***Figure 4.3. Nitric oxide- mediated VASP$^{Ser239}$ phosphorylation (using DPTA-NONOate and GSNO) is PKC-dependent.***

*WP ($3x10^8$) pre-incubated with PKC inhibitor, RO31-8220 (10μM), for 20min before treatment with increasing concentrations of GSNO* ***(a)****, or DPTA-NONOate* ***(b)*** *for 1min. Reactions were stopped with an equal volume of 2xLaemmli buffer. Proteins (15μg) were separated in 10–18% gradient gels and immunoblotted for anti-phospho-VASP$^{Ser239}$. Blots are representative of two and one experiment, respectively.*

### 4.2.3 PKC is required for PKG but not PKA mediated phosphorylation of VASP by nitric oxide.

The effect of PKC inhibitor (RO31-8220) on NO-mediated VASP phosphorylation has been established in the previous figures. We next investigated whether other cyclic nucleotide-dependent kinases were also affected by RO31-8220. In platelets, cAMP levels are elevated in response to $PGE_1$ (Gorman *et al.*, 1977), which lead to VASP phosphorylation on $Ser^{157}$ (Aszodi *et al.*, 1999). Also, the increase in cGMP levels by NO inhibits $PDE_3$, leading to the increase in cAMP levels (Schwarz *et al.*, 2001a). We examined whether PKC played a role in PKA mediated VASP phosphorylation. As expected **(figure 4.4)**, treatment of WP with $PGE_1$ (50ng/ml) caused increase in $VASP^{Ser157}$ phosphorylation, which was slightly inhibited by RO31-8220 (10μM). However, within the same experiment in the presence of RO31-8220, NO-mediated $VASP^{Ser157}$ phosphorylation was completely abolished. This data demonstrate the specificity of RO31-8220 toward NO signaling.

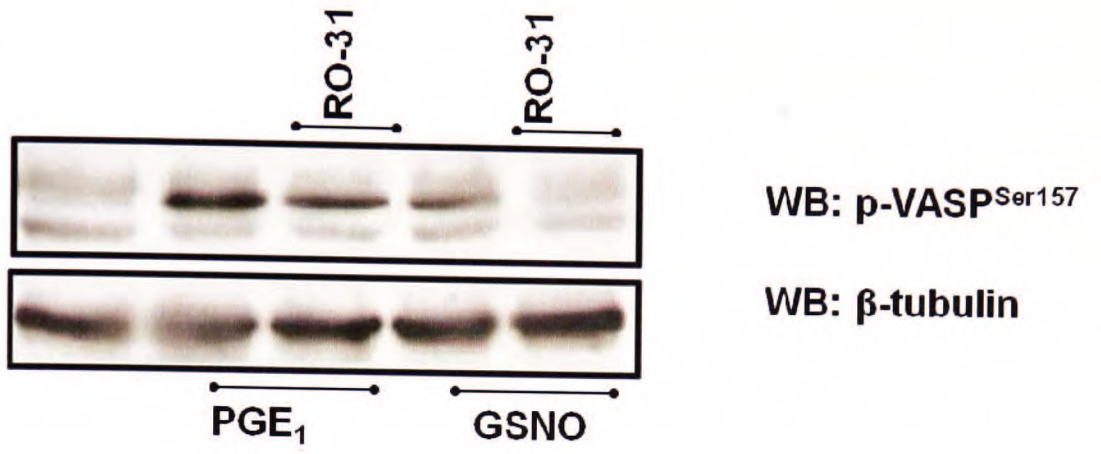


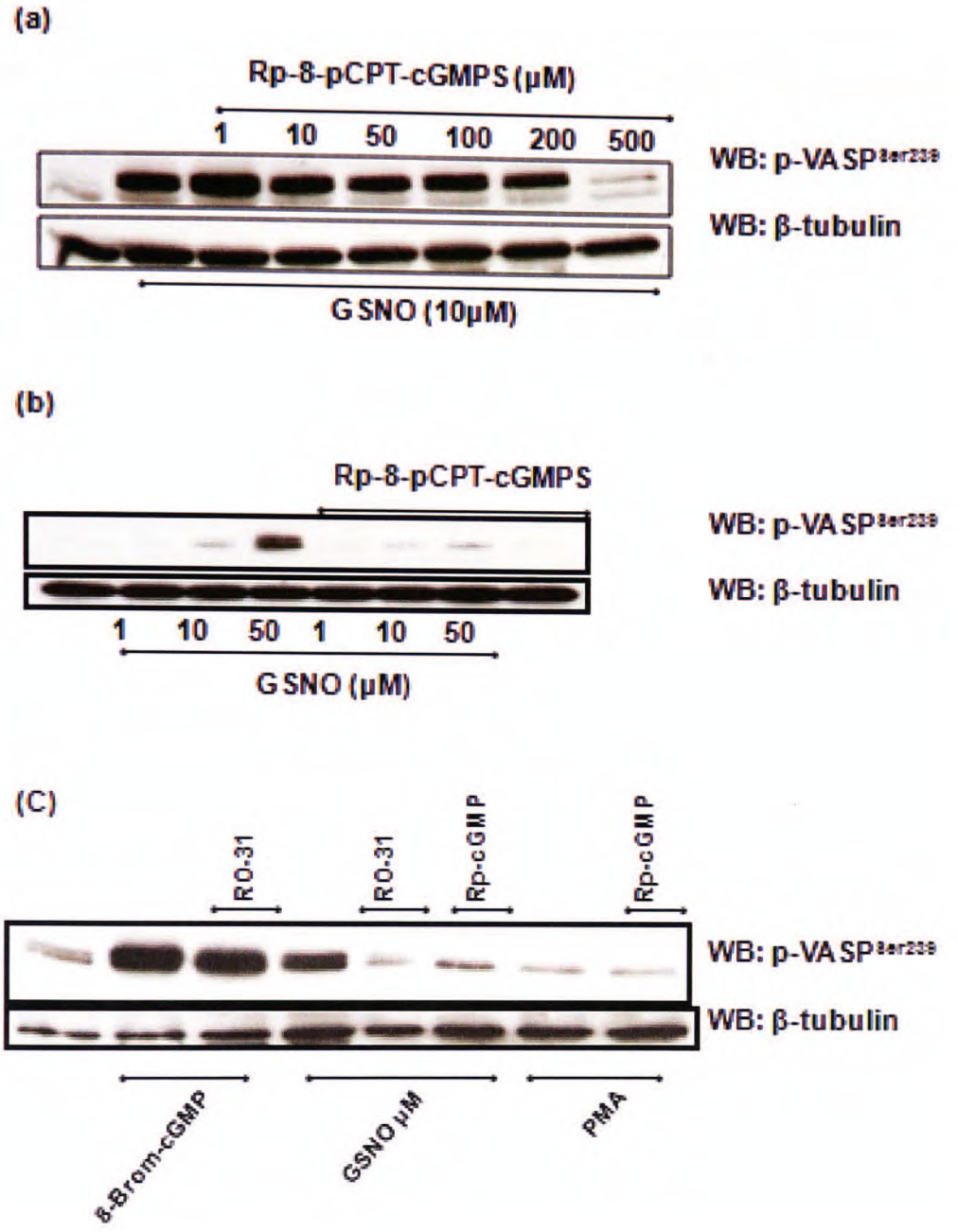

***Figure 4.4. RO31-8220 inhibits cGMP, but not cAMP mediated VASP$^{Ser157}$ phosphorylation.***

*WP ($3x10^8$) pre-incubated with PKC inhibitor, RO31-8220 (10μM), for 20min before treatment with increasing concentrations of GSNO (10μM for 1min), or $PGE_1$ (50ng/ml for 3min). Reactions were stopped with an equal volume of 2xLaemmli buffer. Proteins (15μg) were separated in 10–18% gradient gels and immunoblotted for anti-phospho-VASP$^{Ser157}$. Blot is representative of a single experiment.*

### 4.2.4 PKC is required for nitric oxide but not 8 Bromo-cGMP mediated phosphorylation of VASP.

Since NO mediated its effects through sGC and PKG, experiments were performed to determine whether PKC may lie in this pathway. To further assess the role of PKC in NO signaling, we needed first to use PKG inhibitors. The specificity of PKG inhibitors has previously been questioned by others (Gambaryan *et al.*, 2004). Thus prior to further experiments, the PKG inhibitor, RP-8-pCPT-cGMPs (biomol) was characterized. In **figure 4.5**, RP-8-pCPT-cGMPs affectively inhibited NO-mediated VASP phosphorylation only at 500μM concentration.

To further assess the role of PKC in NO signaling, we tested the ability of RO31-8220 to inhibit 8-Bromo-cGMP (1mM), a PDE resistant cGMP analog. Incubating WP with 8-Bromo-cGMP caused a robust increase in VASP phosphorylation which was not affected by RO31-8220 but inhibited in the presence of RP-8-pCPT-cGMP. However, at the same time RO31-8220 did inhibit NO-mediated VASP phosphorylation. This data suggests that either PKC signaling is upstream sGC or that PKC is regulating PDE/s following NO treatment; thus 8 Bromo-cGMP signaling was not affected **(figure 4.5c)**.




**Legend: overleaf**

***Figure 4.5. Nitric oxide-mediated VASP$^{Ser239}$ is PKC and PKG-dependent.***

*WP ($3x10^8$) pre-incubated with increasing concentrations of PKG inhibitor, Rp-8-pCPT-cGMPS, for 20min before treatment with GSNO (10μM for 1min)* ***(a)****. WP ($3x10^8$) pre-incubated with PKG inhibitor [Rp-8-pCPT-cGMPS (500μM) for 20min] before treatment with increasing concentrations of GSNO for 1min* ***(b)****. WP ($3x10^8$) pre-incubated with PKC inhibitor, RO31-8220 (10μM), or Rp-8-pCPT-cGMPS (500μM) for 20min before treatment with GSNO (10μM for 1min), 8-Bromo-cGMP (1mM/ml for 15min), or PMA (300nM for 5min)* ***(c)****. Reactions were stopped with an equal volume of 2xLaemmli buffer. Proteins (15μg) were separated in 10–18% gradient gels and immunoblotted for anti-phospho-VASP$^{Ser239}$. Blots are representative of two experiments.*

### 4.2.5 PDE activation reverses the inhibitory effect of RO31-8220.

We hypothesized that PKC activation following treatment of platelets with NO may lead to inhibition of PDEs, presumably $PDE_5$, in order to keep cGMP levels high enough to activate PKG. To test this theory, we incubated platelets with a non-specific PDE inhibitor (IBMX 100μM) in the presence or absence of RO31-8220. As seen in **figure 4.6**, GSNO-stimulated a robust increase in VASP phosphorylation which was inhibited by RO31-8220 (10μM). In contrast, IBMX did not significantly influence VASP phosphorylation induced by GSNO. Further IBMX alone did not increase VASP phosphorylation. However, the ability of RO31-8220 to inhibit GSNO-mediated VASP phosphorylation was reduced if platelets were preincubated with IBMX. This data suggest that PKC may be switching off PDE/s in platelets following treatment with NO, and that treatment of platelets with IBMX compensated for PKC activity which was inhibited by RO31-8220. *Blot is representative of one experiment.*

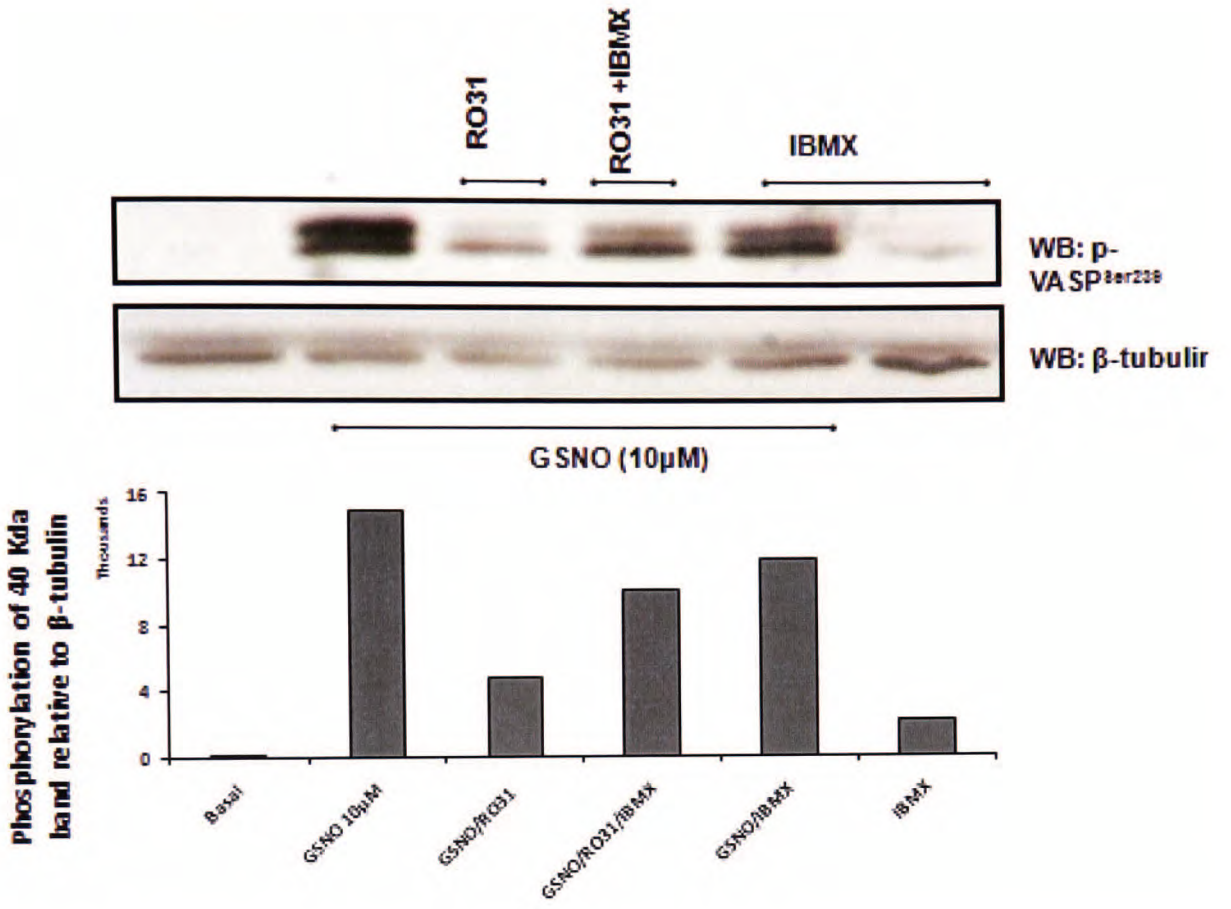



***Figure 4.6. Protein kinase C negatively regulates PDEs.***

*WP ($3x10^8$) pre-incubated with PKC inhibitor, RO31-8220 (10µM) alone or with PDEs inhibitor IBMX (100µM) for 20min before treatment with GSNO (10µM for 1min). Reactions were stopped with an equal volume of 2xLaemmli buffer. Proteins (15µg) were separated in 10–18% gradient gels and immunoblotted for anti-phospho-VASP$^{Ser239}$. Blot is representative of a single experiment.*

### 4.2.6 Inhibition of platelet aggregation by nitric oxide is PKC/VASP-independent.

The physiological functional significance of PKC in the regulation of platelet function by NO was investigated using platelet aggregation. Collagen induced platelet aggregation was inhibited by the presence of RO31-8220, where aggregation was reduced from 85.5 ± 0.5 to 46.5 ± 6.5 %, consistent with a mechanism that is PKC dependent and independent (Atkinson *et al.*, 2001). GSNO also caused an inhibition of aggregation from 85.5 ± 0.5 to 67 ± 9 %. However, the addition of NO to RO31-8220 treated platelets had a further inhibition (85.5 ± 0.5 to 21.5 ± 1.5 %), when compared to when the inhibitors were used alone. These data indicates that NO-mediated inhibition of aggregation were not PKC dependent **(figure 4.7)**.

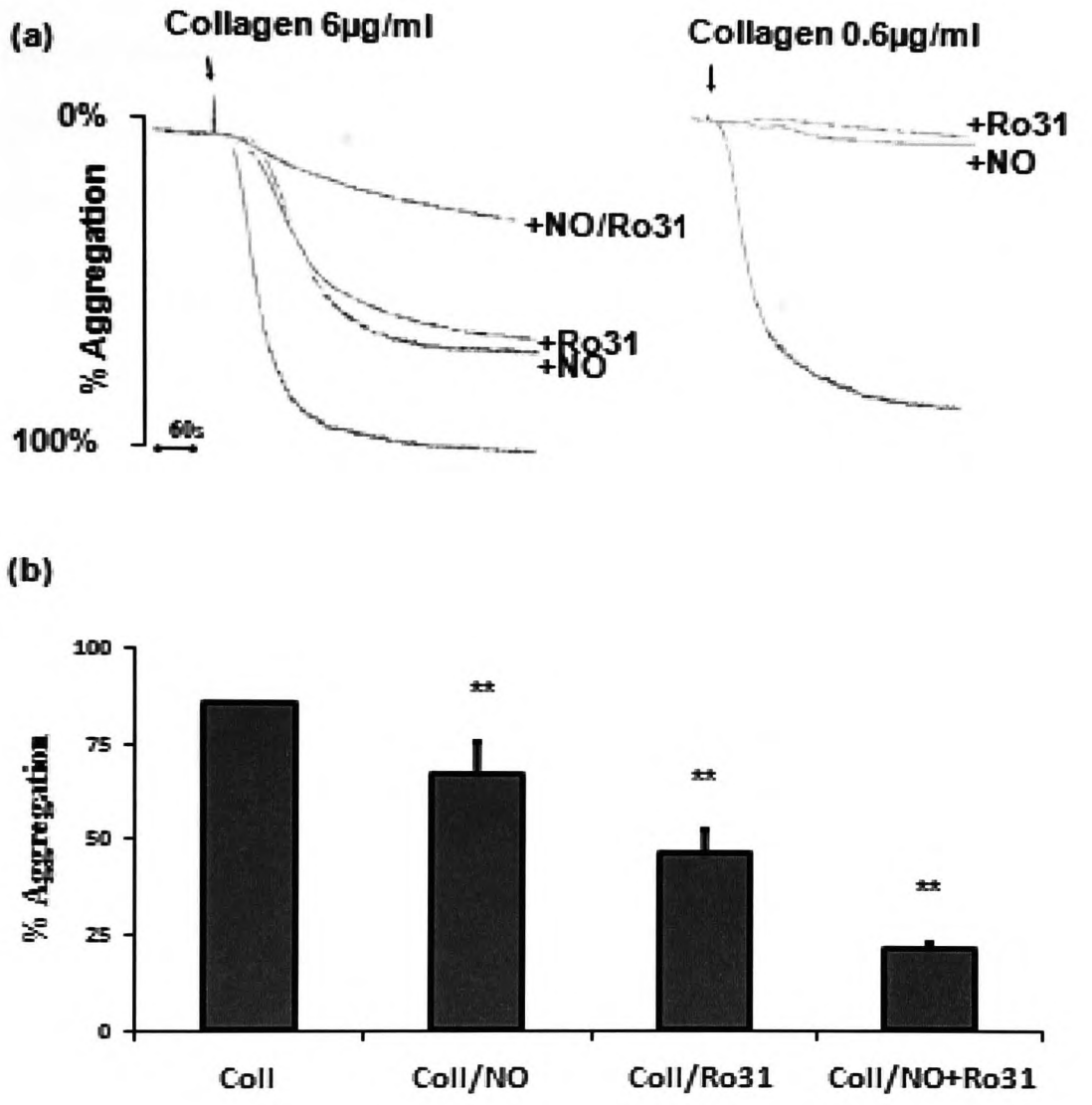



***Figure 4.7. Nitric oxide inhibits platelet aggregation independently of PKC.***

*WP ($3x10^8$) stimulated with collagen at indicated doses, or pre-incubated first with PKC inhibitor [RO-31 2880 (10µM for 20min)] or GSNO (10µM for 1min) before stimulation. Response was recorded for 5min and expressed as % aggregation. Data of 3 independent experiments as in* ***(a)*** *are quantified in a graph* ***(b)****. (n=3) P value compared to basal. **P < 0.01 vs. collagen sample.*

## 4.3 Discussion.

It is well established that PKC is required for platelet activation; however, the specific function of each PKC isoforms remains unclear. More recently it has been suggested that some isoforms such as $PKC_{\delta}$ may play an inhibitory role (Pula *et al.*, 2006). Since NO has been shown to regulate PKC in kidney cells (Liang *et al.*, 1999), its role in NO signaling cascade in platelets was investigated.

The use of genetically modified animals to define these functions is essential. However differences in regulation of some PKC isoforms between species are a drawback. For example different roles of PKCε between human and mice platelets have been described (Pears *et al.*, 2008). Also the presence of some isoforms such as $PKC_{\varepsilon}$ in human platelets is still controversial (Buensuceso *et al.*, 2005; Pears *et al.*, 2008). Using pharmacological inhibitors is of a great benefit when proper concentrations are carefully applied. In a study carried out by F.S London (London, 2003), the author concluded a regulatory role for PKC in thrombin-mediated prothrombinase activity. However, the use of high concentrations of RO31-8220 (100μM), without the use of proper controls leaves the outcome of the study questionable. Our study demonstrates a selective role for PKC in NO signaling in human platelets. The effect of PKC was tested using two structurally distinct inhibitors and the use of inhibitors from more than a source.

In our study we used pharmacological inhibitors at concentrations that are known to regulate PKC (Davis *et al.*, 1989). To our surprise the PKC inhibitor

RO31-8220 (10μM), reduced NO-mediated phosphorylation of VASP$^{Ser239/157}$. The vast majority of the work was done with GSNO, but it is important to notice that DPTANONOate-mediated VASP phosphorylation was also blocked. These data are important since they suggest that the role of PKC is related to NO rather than to a specific donor. A reduction of NO-mediated VASP phosphorylation could be accounted for by (1) inhibition of sGC, (2) increased cGMP degradation, (3) Inhibition of PKG. Therefore experiments were performed to assess which aspect of these pathways PKC could potentially modulate. By means of widely used inhibitors for PKC, we were able to establish investigate the role for PKC in NO signaling.

Our first piece of evidence demonstrated that NO-mediated VASP$^{Ser239/157}$ phosphorylation was blocked in conditions where PKC activity was inhibited with RO31-8220 or BIM I, two structurally distinct inhibitors. Since VASP is also phosphorylated by PKA in response to elevations in cAMP, the role of PKC in this pathway was also evaluated. $PGE_1$-mediated phosphorylation was not significantly affected by inhibition of PKC, suggesting that the direct activation of cAMP/PKA cascade does not involve PKC. However, NO increases cAMP indirectly through cGMP-mediated inhibition of $PDE_{3A}$ (Schwarz *et al.*, 2001a). The data that PKC inhibition does not influence $PGE_1$-mediated phospho VASP$^{Ser157}$ but abolish NO-mediated phospho VASP$^{Ser157}$ may also suggest that the role of PKC in NO-mediated signaling is related to its ability to regulate cGMP formation and ultimately cAMP levels. Our data also suggests that PKC regulates cGMP-mediated PKG activation. To further assess this, we next examined whether PKC regulates

cGMP formation and/or PKG activity. Platelets were incubated with RP-8-pCPT-cGMP (PKG inhibitor) and RO31-8220 prior to treatment with 8-Bromo-cGMP, a cGMP analog that directly activates PKG and bypass sGC activation and cGMP formation.

Using this approach, we were able to confirm that PKG and PKC played role in NO signaling. However, only PKG inhibitor blocked the effect of 8-Bromo-cGMP, where the PKC inhibitor had no effect. These data indicate that PKC lies upstream of PKG in the NO signaling cascade.

Since inhibition of PKC influenced the ability of NO to induce VASP phosphorylation, it suggested that PKC must be activated by NO. To test this, a phospho-(Ser) PKC substrate antibody that detects endogenous levels of cellular proteins only when phosphorylated at serine residues surrounded by Arg or Lys (Cell Signaling), the preferable phosphorylation site of PKC (Kishimoto *et al.*, 1985; Pearson *et al.*, 1991) was used. Treatment of platelets with NO and 8-Bromo-cGMP resulted in a modest increase in the phosphorylation of a protein of ~80kDa. The identity of this protein is unknown and requires identification. However, the overall effect of NO was modest when compared to that by PMA or thrombin. This increase in phosphorylation by NO and PMA was equally inhibited by RO31-8220. On the other hand, blocking sGC activation by ODQ has no effect on PMA-mediated PKC activation indicating that PKC activation and signaling does not require cGMP. Our data contradict that of (Murohara *et al.*, 1995), since these authors suggested that NO down-regulates PKC activity. Here a slight increase in PKC activity in the membrane fraction of feline platelets, in

conditions where eNOS was blocked, assuming that any increase in PKC activity would be due to the inhibition of NO production. The reason for this unclear but probably reflects differences between species or perhaps between endogenous NO and exogenous NO.

It is possible that PKC could influence PDE activity regulating cGMP levels. We hypothesized that PKC may inhibit PDE activity following sGC activation by NO. To test this theory we used IBMX as a general PDE inhibitor. IBMX was able partially to reverse the inhibitory effect of RO31-8220 on NO-mediated VASP phosphorylation. Treatment of platelets with IBMX alone did not increase VASP phosphorylation, this is because several folds increase in cGMP are needed to activate a small proportion of PKG (Schwarz *et al.*, 2001a). These data suggest that upon PKC activation by NO, PKC is negatively regulating PDE as suggested by others (Bian *et al.*, 1998; Bian *et al.*, 2000; Udovichenko *et al.*, 1994), in order to keep cGMP levels high enough to activate PKG.

The biological significance of PKC activation by NO was suggested by ischemic preconditioning (IPC) studies, where the initial ischemic stimulus by NO induced selective translocation of 2 novel PKC isoforms (ε/η) from cytosolic to the particulate fraction and the inhibition of PKCε resulted in abrogation of late IPC (Ping *et al.*, 1999). To assess the physiological relevance of PKC activation in response to NO in platelets, we investigated the inhibitory effect of NO on platelet aggregation in conditions where PKC was blocked. Consistent with several studies, NO caused inhibition of platelet aggregation induced by collagen and that PKC inhibition by RO31-

8220 blocked platelet aggregation in response to collagen by 50%, as did NO. However, in the presence of both RO31-8220 and NO there was an additive inhibitory effect on platelets aggregation. If NO mediated inhibition of aggregation required PKC then the inhibitory effects if NO would have been lost under these conditions. This suggests that inhibition of platelet aggregation by NO was independent of PKC.

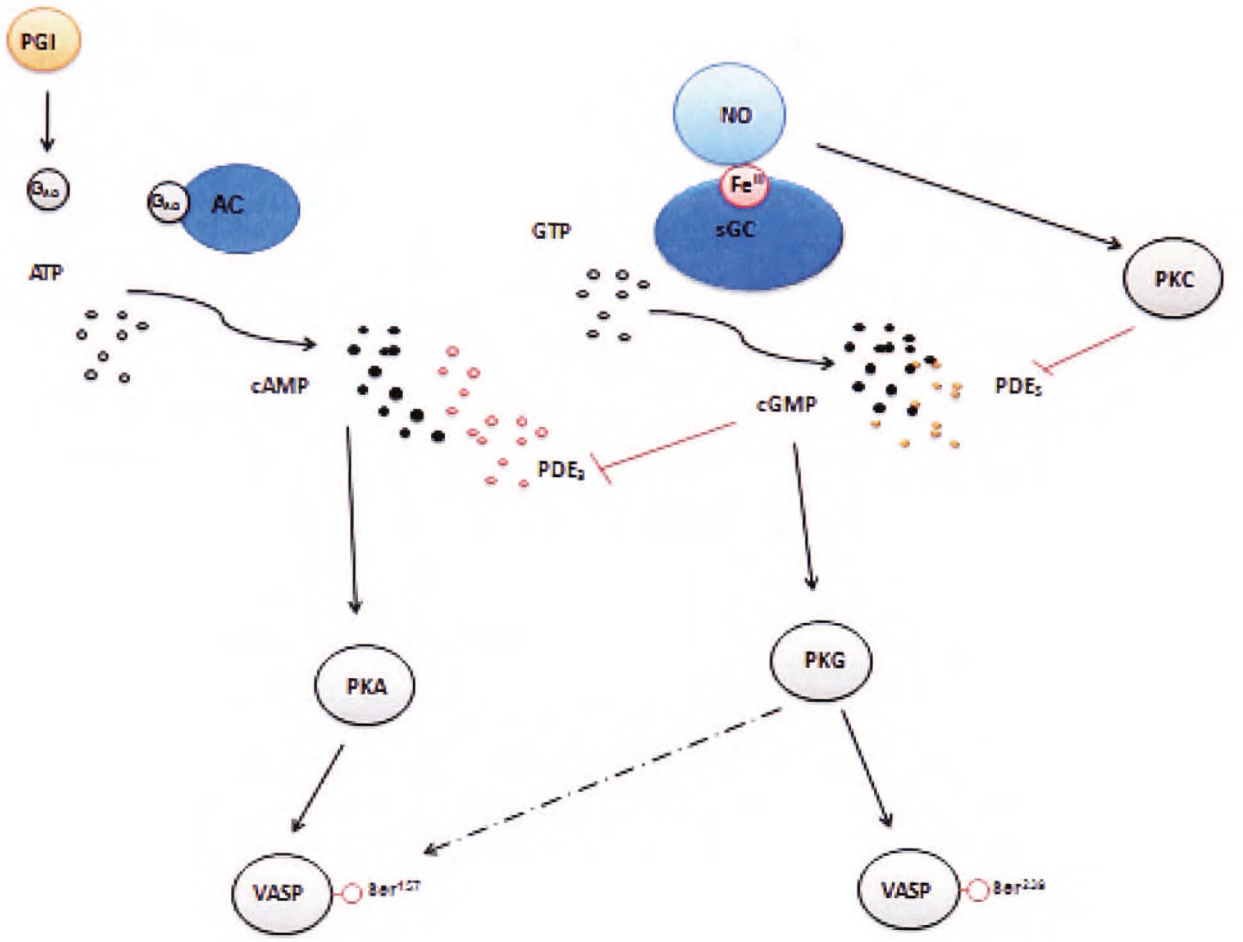



***Figure 4.8. Schematic diagram of PKC-mediated nitric oxide signaling in platelets.***

*NO synthesized by NOS diffuses into platelets, activates sGC which leads to cGMP formation. NO also activates a PKC isoform, which in turn transiently inhibits $PDE_5$, in order to keep cGMP levels several folds high to activate PKG.*

## 4.4 Conclusion.

PKC has long been viewed as a key kinase for platelet activation, however, recent studies suggest a negative regulatory role for PKC. Our preliminary data also suggest PKC may negatively regulate PDE, allowing rapid increase in cGMP formation following sGC activation by NO.

***Future work***

The following experiments to be conducted to reach more conclusive results

- cGMP formation measurement. WP will be incubated with RO31-8220, BAPTA-AM prior to treatment with NO donor. Intracellular cGMP will be measured in the lysates.

- To assess which PKC isoform mediates NO signaling; WP will be incubated with GÖ6976, rottlerin, BAPTA-AM prior to treatment with NO donor. VASP phosphorylation status will be determined.

# CHAPTER 5. DYNAMICS OF PROTEIN KINASE A (PKA) AND PROTEIN KINASE G (PKG) SIGNALING CASCADES IN PLATELETS. ROLE OF LIPID RAFTS.

# ABSTRACT

**INTRODUCTION:** Synergism between NO and $PGI_2$ signaling is most evident in platelets via activation of protein kinase G (PKG) and protein kinase A (PKA), respectively. While only $PKGI_\beta$ isoform is believed to be present in platelets, four isoforms of PKA (PKA $RI_\alpha$, $RI_\beta$, $RII_\alpha$, and $RII_\beta$) are identified in other cells. Lipid rafts are microdomains within the plasma membrane that are rich in cholesterol and sphingolipids, and have been implicated in the stimulatory mechanisms of platelet agonists. We sought to determine the importance of subcellular localization of elements of the NO/cGMP/PKG and $PGI_2$/cAMP/PKA signaling cascade, with particular emphasis on lipid rafts

**METHODS:** Platelets were separated into particulate and soluble fractions by ultracentrifugation, while lipid rafts were isolated by sucrose density gradient ultracentrifugation. The presence of specific proteins was detected by immunoblotting. To examine the function of lipid rafts, platelets were treated with methyl-β-cyclodextrin (MβCD).

**RESULTS:** Both PKA $_{RI}$ and PKA $_{RII}$ were found in platelets in both fractions of the cell. In contrast, only PKA $_{RI}$ was partially located in lipid rafts. Importantly, depletion of cholesterol by MβCD had little effect on $PGI_2$ signaling in platelets. Immunoprecipitation experiments revealed that soluble guanylyl cyclase (sGC) and heat shock protein-90 (HSP-90) were associated in a complex in the non raft fraction. However, disruption of lipid rafts abolished NO-mediated VASP phosphorylation.

**CONCLUSION:** The combined results suggest that lipid rafts play an important role in NO signaling in platelets. While PKA isoforms have a non-redundant role in $PGI_2$ signaling in platelets.

# 5. Introduction.

## 5.1 Lipid rafts and platelets.

It is now established that the localization of kinases and substrates within distinct cellular compartments is important for signaling events to take place. In particular, membrane associated protein complexes are required to transduce extracellular stimuli. Within the cellular membrane structure, lipids are asymmetrically distributed over the exoplasmic and cytoplasmic leaflets of the membrane (Van Meer, 1989). The lipids contained within the cellular plasma membrane include glycerophospholipids, glycosphingolipids, and sterols. Lipid rafts consist of sphingolipids and cholesterol, which can move through the more liquid-disordered phase of the membrane containing glycerophospholipids (Morley *et al.*, 2001). Because of their lipid constituents, lipid rafts have also been referred to as *glycosphingolipid-enriched membrane microdomains* or *(GEMs)*. The lipid rafts are also characterized by their resistant to solubilisation in non-ionic detergents at low temperatures (Brown *et al.*, 1998b). Lipid rafts were estimated to have a mean diameter of 44nm that occupy 35% of cell surface (Prior *et al.*, 2003). However, they have the capacity to coalesce into larger rafts (Kono *et al.*, 2002). Lipid rafts are not only found at the plasma membrane, but also as part of the internal membrane of granules, Golgi complex and even phagosomes (Dermine *et al.*, 2001; Gkantiragas *et al.*, 2001).

In platelets, a variety of specific proteins are concentrated in raft domains, including many glycophosphatidylinositol-anchored proteins, Src family kinases, linker for activation of T cells (LAT) (Ezumi *et al.*, 2002), $G_{\alpha i}$ protein

(Quinton *et al.*, 2005), GPVI and GPIb-IX-V complex (Shrimpton *et al.*, 2002). $\alpha_{IIb}\beta_3$ on the other hand does not utilize lipid rafts to initiate signaling or support platelet aggregation (Wonerow *et al.*, 2002a).

Also in platelets, Heijnen and colleagues (Heijnen *et al.*, 2003), demonstrated that upon interaction with fibrinogen, cholesterol accumulated at the tips of filopodia and at the leading edge of spreading cells, and that stimulation with thrombin receptor activating peptide (TRAP) resulted in a similar redistribution of cholesterol towards filopodia. The adhesion-dependent raft aggregation was accompanied by concentration of the tyrosine kinase c-Src and CD63 in these domains, whereas in contrast to (Shrimpton *et al.*, 2002), glycoprotein Ib (GPIb) was not selectively targeted to the raft clusters. While components of platelet activatory cascades are bow thought to be localized to rafts which is important to their function. Little is known about whether components of the inhibitory cascades require rafts for effective function.

## Aims of study

The aim of this study is to assess the role of lipid rafts in the dynamics of PKA and PKG signaling cascades. This is achieved using a combination of ultracentrifugation, immunoblotting and functional assays.

## Materials of study

- Prostacyclin ($PGI_2$), prostaglandin ($PGE_1$) and methyl beta cyclodextrin (MβCD) were all purchased from Sigma (UK). For complete list of chemicals and antibodies **(Appendix II/III)**.

## Methods of study

- Platelet aggregation.
- Lipid rafts isolation.
- Immunoblotting.
- Phosphoflow cytometry.

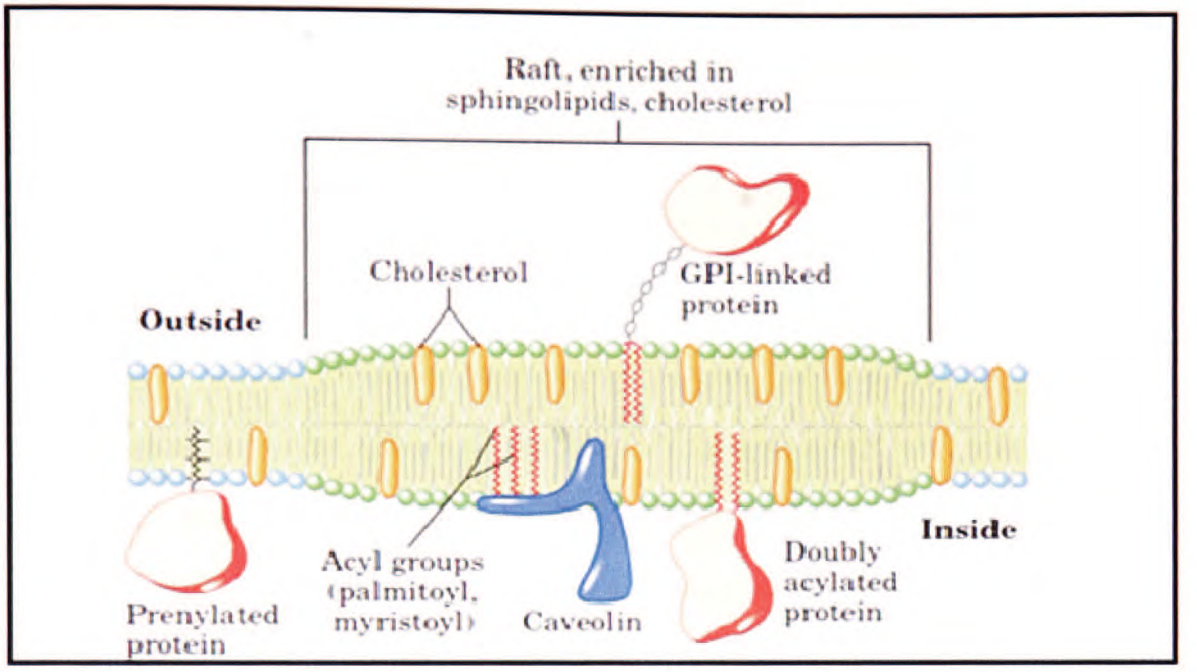



***Figure 5.1. Schematic diagram of lipid rafts.***

***Proposed structure and organization of a lipid raft microdomain in the plasma membrane. Sphingolipids, which include both sphingomyelin and glycosphingolipids, associate with cholesterol to form a more tightly packed domain. The regions rich in phosphatidylcholine and other glycerol-based phospholipids are less densely packed, and form fluid regions outside the raft microdomains. Lipid rafts are enriched in GPI-anchored proteins and enzymes at their external surface, and acylated proteins, such as tyrosine kinases of the Src family at the cytoplasmic surface. Transmembrane integral proteins are generally excluded from rafts, and are found in the more fluid phospholipid-rich regions of the membrane.***

## 5.2 Results

### 5.2.1 Protein composition of cytoskeleton and cytosolic fractions from unstimulated platelets and prostacyclin treated platelets.

In order to investigate signaling compartmentalization in platelets, first, the presence of proteins known to be attached to the cytoskeleton or soluble fractions was assessed. Consistent with previous studies, subcellular fractionation of untreated platelets revealed that, In agreement with others, Integrin $\beta_3$ (Obergfell *et al.*, 2002), Src (Courtneidge *et al.*, 1980) and LAT (Tanimura *et al.*, 2006) were all found in the particulate fraction of the cell, while SLP-76 (Boerth *et al.*, 2000) and the majority of PLCγ (Billah *et al.*, 1980) were found in the cytosolic fraction **(figure 5.2a)**.

In addition, the distribution of proteins known to be associated with $PGI_2$ signaling was assessed. $PKA_{RII}$ was found in both fractions but was more abundant in the cytosolic fractions. $PKA_{RI}$ was also present in both fractions but more evenly disturbed **(figure 5.2b)**. Csk, a known target for PKA in T-cells, was largely present in the cytosolic fractions, and although Csk does not possess a membrane anchoring motif, a proportion was found associated with the cytoskeletal fraction. These data confirm the presence of both PKA isoforms in platelets.

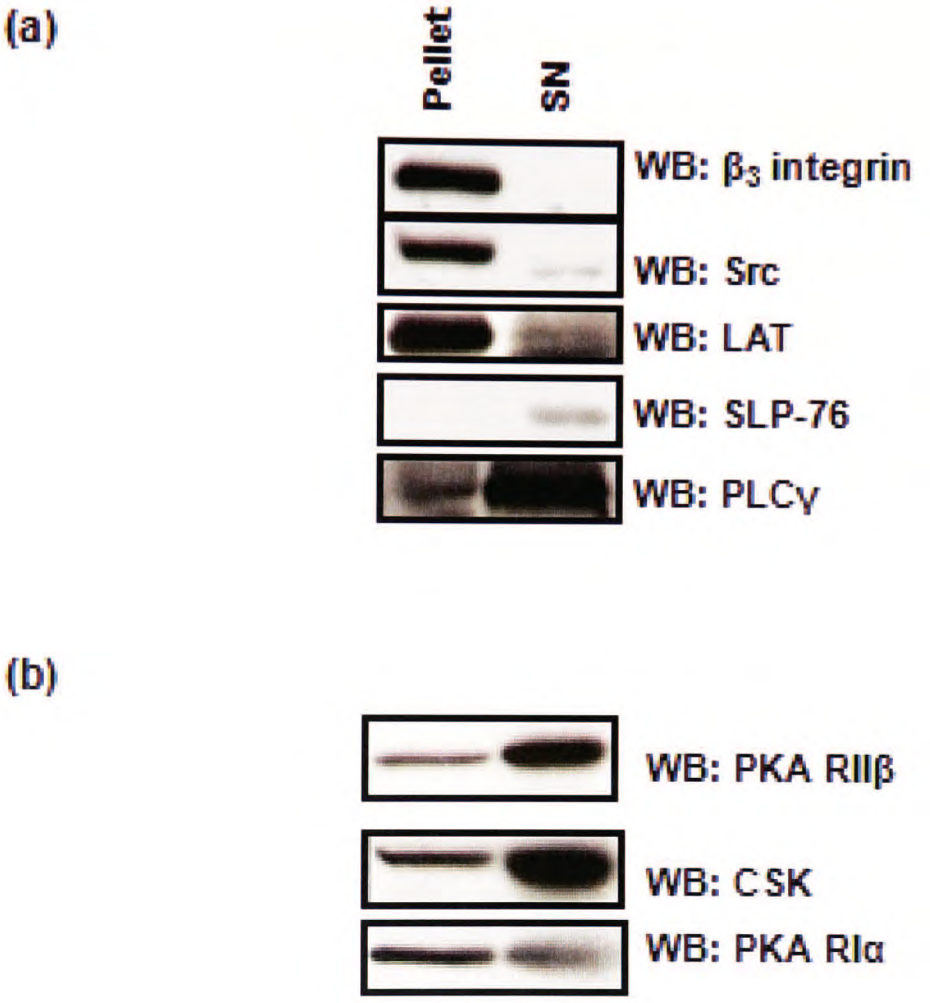



***Figure 5.2. Localization PKA signaling components in platelets.***

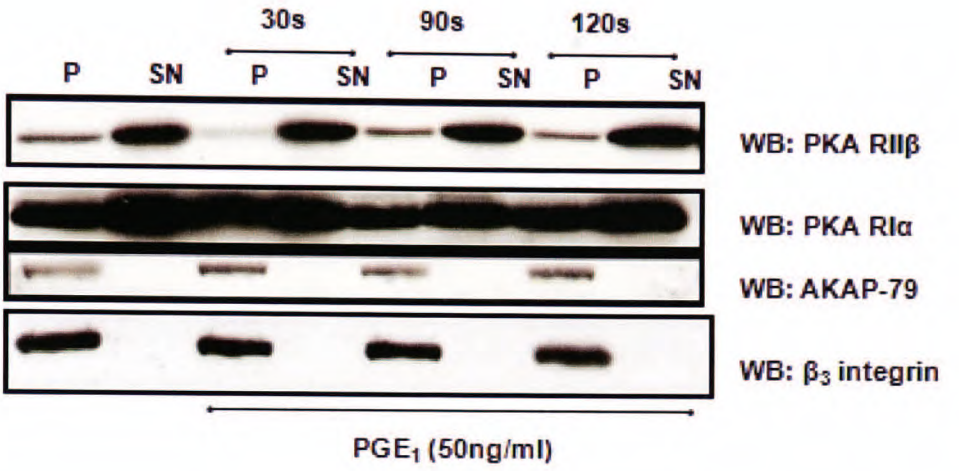
*WP ($5x10^8$) were added to fractionation buffer (1:1). Cells were subjected to freeze-thaw cycles. Lysate was centrifuged first at 3000g/5min. supernatant was further subjected to ultracentrifugation. 10µg of supernatants and cytoskeleton fractions were loaded on SDS-PAGE followed by Western blot. Membrane blotted with indicated antibodies. n=4.*

### 5.2.2 Protein kinase A dynamics and localization of protein kinase A substrates.

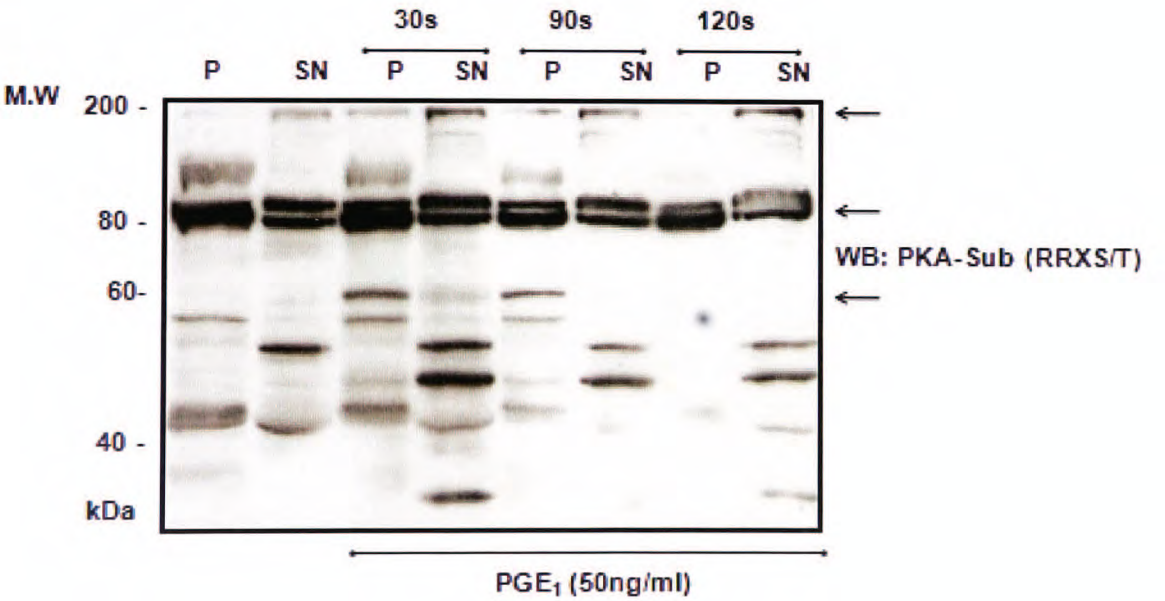
Having established the presence of PKA isoforms in platelets, subcellular localization of proteins was investigated upon activation of GPCRs by $PGI_2$ or $PGE_1$. Western blot analyses of fractionated platelets after treatment with $PGE_1$ or [$PGI_2$ **(not shown)**] revealed a transient translocation of $PKAR_{II\beta}$ from cytoskeletal to cytosolic fractions. The small amount of $PKA_{RII\beta}$ present in the membrane fraction was completely lost after 30sec, but had returned to basal by 90sec. In contrast, the relative proportion of $PKA_{RI\alpha}$ in each fraction remained constant after treatment with $PGE_1$ **(figure 5. 3a)**. Since there seemed to be PKA isoforms in each fraction of the cell, it was likely that there were PKA substrates in each cellular fraction. To test this, platelets were treated with $PGE_1$ (50ng/ml), fractionated and blotted for phospho PKA substrates. Immunoblotting revealed a distinct difference in distribution of PKA substrates between cytoskeletal and cytosolic fraction. For instance, phosphorylation of proteins that contained the phospho PKA substrate motif in untreated platelets, specially a doublet of 90 and 100kDa was phosphorylated basally and after treatment in both fractions. In the particulate fraction, proteins of 55 and 45kDa were basal phosphorylated, while in soluble fraction a 50kDa was basally phosphorylated. In addition, a protein of ~200kDa was highly phosphorylated at the cytosolic fractions than in the particulate fractions. While one protein of ~65kDa was only present phosphorylated at the cytosolic fraction within 30sec and declined back to basal after 90sec of treatment with $PGE_1$. Another protein in the particulate fraction at ~50kDa was phosphorylated within 30 sec of treatment with $PGE_1$.

This phosphorylation was maintained for 2min (longest time tested) **(figure 5. 3b)**.

(a)




(b)




**Legend: overleaf**

***Figure 5.3. PKA dynamics in platelets.***

*WP ($5x10^8$) were treated with $PGE_1$ (50ng/ml) for 90sec, (1:1) fractionation buffer was added to stop reaction. Cells subjected to freeze-thaw cycles. Lysate was centrifuged first at 3000g/5min. supernatant was further subjected to ultracentrifugation then subjected to subcellular fractionation. 10µg of supernatants and cytoskeleton fractions were loaded on SDS-PAGE followed by Western blot. Membrane blotted with indicated antibodies. n=3*

### 5.2.3 Detergent-sensitive localization of LAT in lipid rafts from unstimulated platelets.

Combination of lipid raft isolation and subcellular fractionation would allow a more complete picture of the temporal regulation of plasma membrane-based signaling complex. In the absence of a raft-deficient animal model, biochemical approaches using non-ionic detergents such as Triton X-100 and Brij 98 are being deployed to study rafts. Lipid rafts are insoluble in non-ionic detergents and because of their high lipid content, they float at a low density during gradient centrifugation (Simons *et al.*, 1997).

Since isolation of proteins associated with lipid rafts and preservation of lipid rafts structure depends on protein/detergent ratio. Initially, the ability of Triton X-100 to isolate and preserve lipid rafts in platelets and associated proteins was assessed. The purity of the raft fractions are estimated using platelet proteins that are known to be found in rafts or excluded from them. In this case, distribution of LAT was used as a marker for lipid rafts fraction, while the soluble fraction contained non-lipid rafts associated proteins was identified by the presence of $\beta_3$ integrin (Lee *et al.*, 2006; Wonerow *et al.*, 2002a).

It has been established by others (Lee *et al.*, 2006), that lipid rafts fractions are found at the interface between 5-36% sucrose. However, 36% sucrose was replaced by 30% sucrose for clearer separation of fractions. In the first instance, lipid rafts were isolated using a range of concentrations of Triton X-100, the proteins separated by SDS-PAGE and immunoblotted. Upon lipid raft isolation in the presence of low concentrations of Triton X-100 (0.025%)

**(figure 5.4a)**, a high proportion of LAT was recovered in the lipid rafts fraction. However a proportion of $\beta_3$ integrin was also recovered in the same fractions, indicating incomplete solubilisation of cell membrane. As the concentration of Triton X-100 increased (0.04%), the amount of $\beta_3$ integrin recovered in lipid rafts fractions decreased **(figure 5.4b)**. At Triton X100 (0.045%), integrin $\beta_3$ was redistributed to the soluble fractions **(figure 5.4c)**, while LAT remained in the raft fraction. At slightly higher concentrations of Triton X100 (0.05%) there was a complete redistribution of LAT to the soluble fraction **(figure 5.4d)**. Thus, Triton X100 (0.045%) allowed the optimal redistribution of LAT to lipid raft fractions and $\beta_3$ integrin to the soluble fraction. these conditions were used for future experiments.

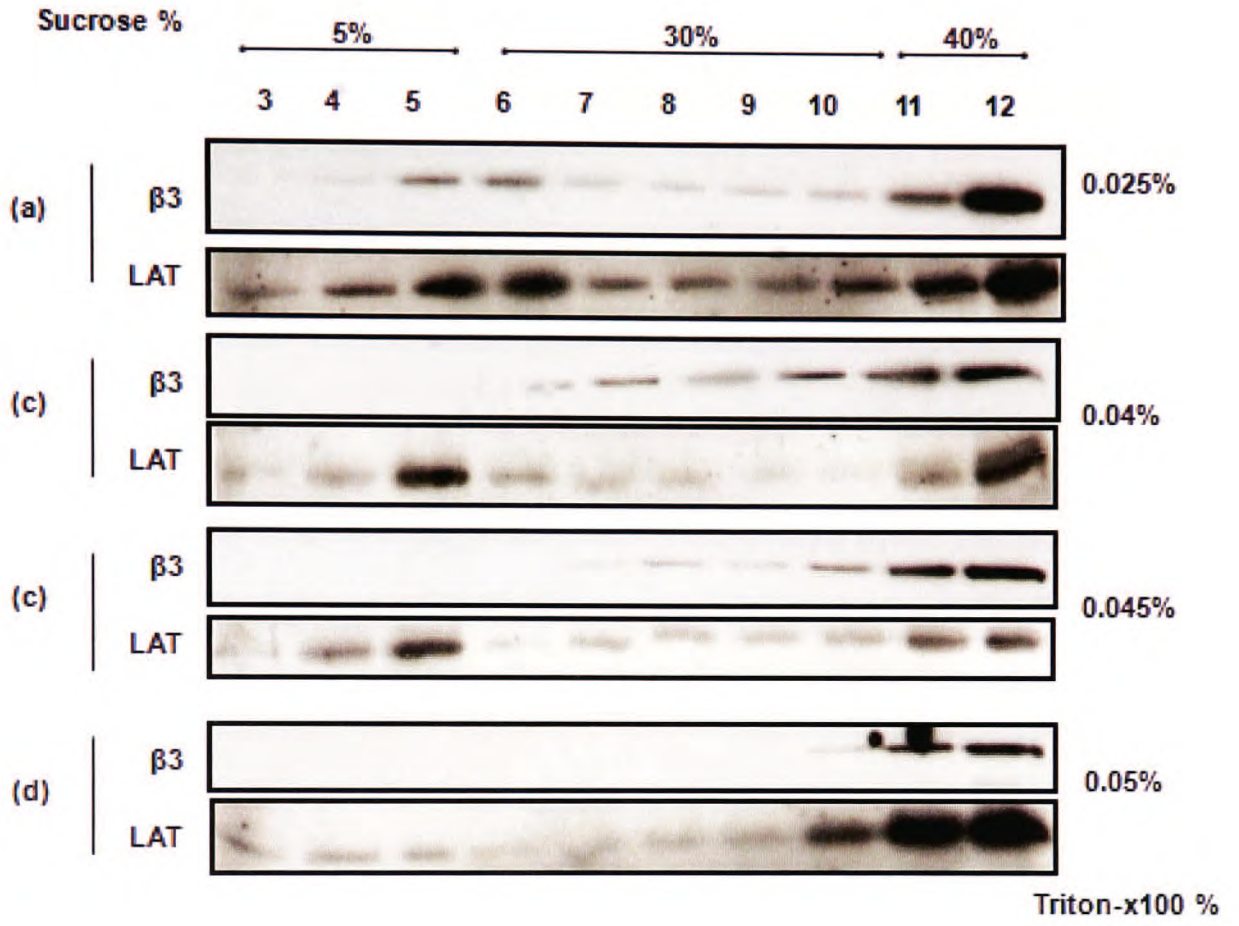



***Figure 5.4. Optimizing conditions for lipid rafts isolation.***

*450µl of WP ($1x10^9$) were lysed with a lysis buffer containing the indicated concentrations of Triton-X100. Mixture was mixed with 80% sucrose (1:1) and loaded at the bottom of polyethen clear tube. 5ml of 30% sucrose was layered on top followed by 5ml 5% sucrose. Tubes were spun at 200000 g for 18hrs at 4 °C. Starting from top, 12 fractions (1ml each) were collected. Laemmle buffer was added to reach 1x. Fractions from 3 to -12 were loaded on SDS-PAGE followed by Western blot. Membranes were blotted with anti-$\beta_3$ integrin for soluble fraction or anti-LAT for insoluble fraction. n=1*

### 5.2.4 The role of lipid rafts in localizing PKA in unstimulated platelets.

Having established that Triton X-100 (0.045%) was to be used, lipid rafts and soluble fractions were characterised further by Western blotting for proteins known to be or potentially involved in $PGI_2$ signaling. Under basal conditions $PKA_{RII}$ were detected in the soluble fractions but not rafts. In contrast, a proportion of $PKA_{RI}$ were found to be distributed in lipid rafts and the remainder in soluble fractions **(figure 5.5)**. Csk (C terminal Src kinase), which is known to be a substrate for PKA signaling (Vang *et al.*, 2001), was only found in the soluble fractions. These data establish for the first time the differential localization of PKA isoforms in lipid rafts in platelets.

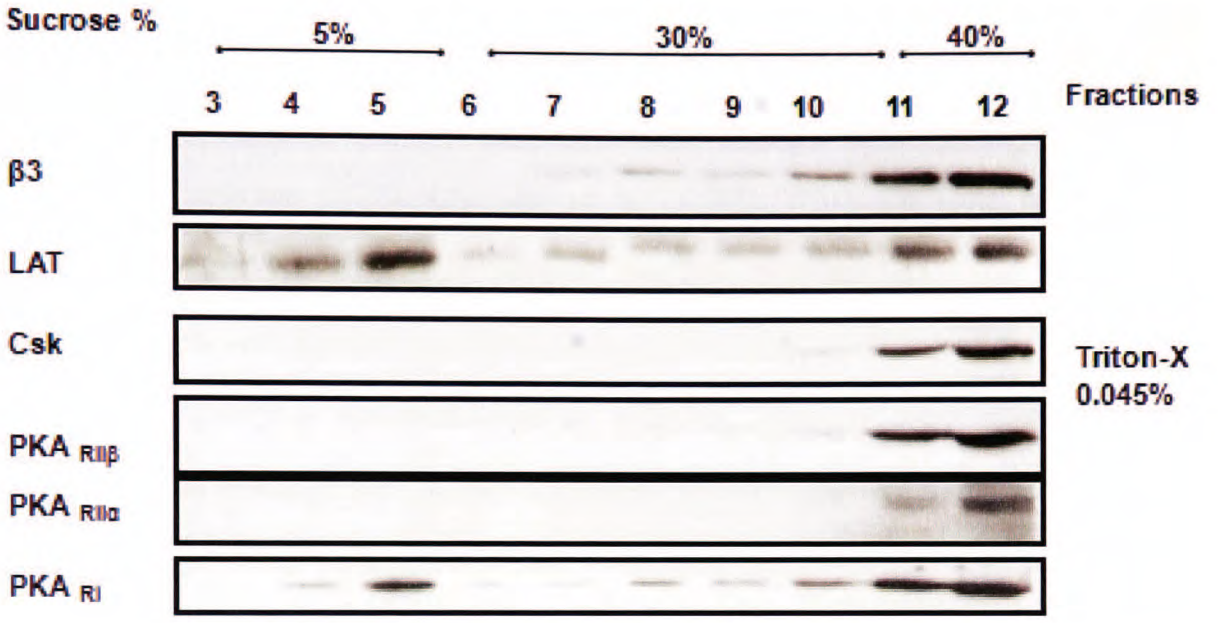



***Figure 5.5. PKARI is present in lipid rafts and soluble fraction.***

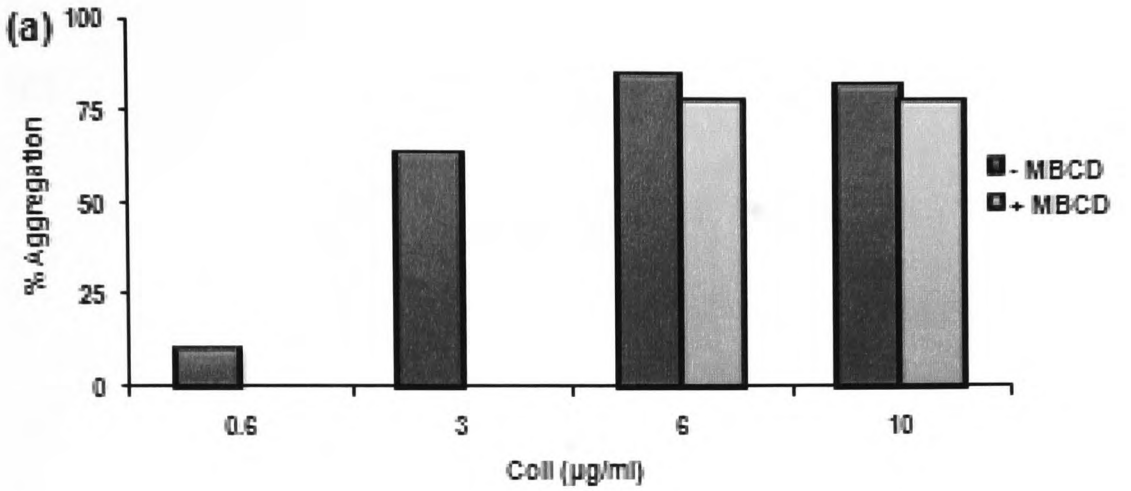
*450µl of WP ($1x10^9$) were lysed with a lysis buffer containing 0.045% Triton-X100. Mixture was mixed with 80% (1:1) sucrose and loaded at the bottom of polyethen clear tube. 5ml of 30% sucrose was layered on top followed by 5ml 5% sucrose. Tubes were spun at 200000 g for 18hrs at 4 °C. Starting from top, 12 fractions (1ml each) were collected. Laemmle buffer was added to reach 1x. Fractions from 3 to -12 were loaded on SDS-PAGE followed by Western blot. Membranes were blotted with anti-$PKAR_{IIα}$, $PKAR_{IIβ}$, $PKA_{RI}$, or CSK antibody. n=1*

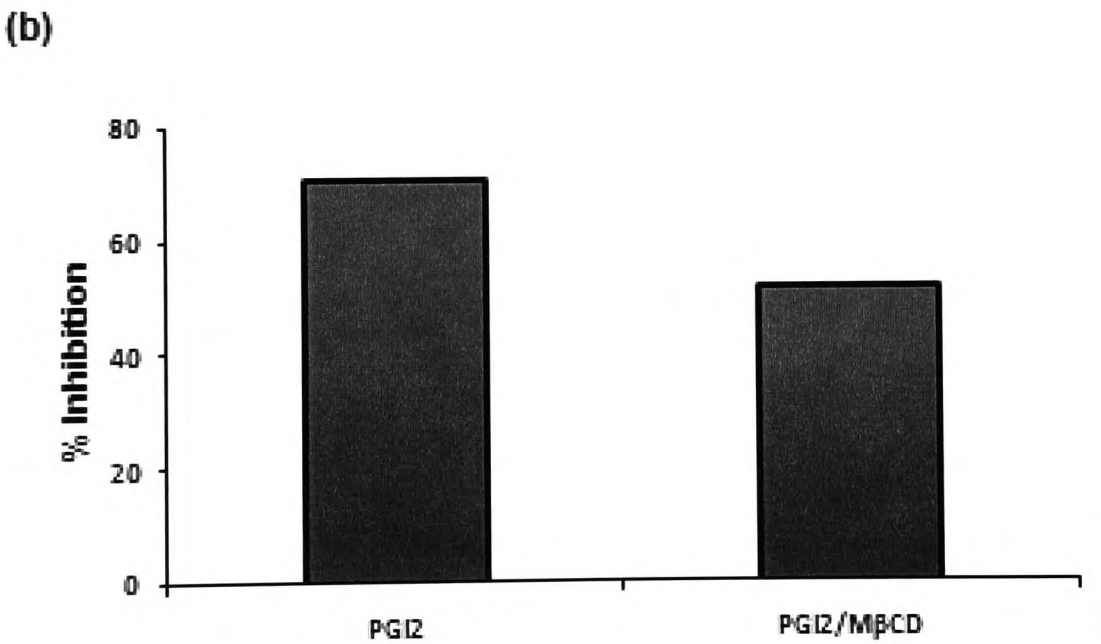
### 5.2.5 The effect of cholesterol depletion on inhibition of platelet aggregation by prostacyclin.

To assess the significance of PKA isoform distribution between lipid rafts and soluble fractions, the integrity of lipid rafts was disrupted by depleting cholesterol from cell membrane using methyl-β cyclodextrin (MβCD) as a cholesterol-depleting agent (Christian *et al.*, 1997). When platelets were incubated with MβCD (5mM) for 60min, their response to stimulation with collagen at low and medium concentrations (0.3 and 3μg/ml) was lost **(figure 5. 6a)**. Using higher concentrations of collagen, platelets were able to overcome the effect of MβCD and aggregation was close to control levels (78% in the presence of MβCD compared to 82% in the absence of MβCD).

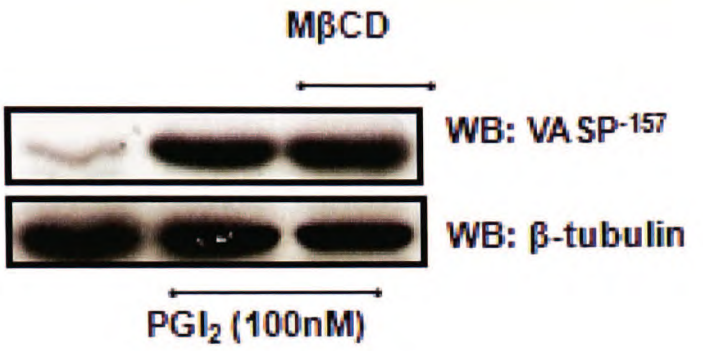
If the presence of $PKA_{RI}$ in lipid rafts was important for VASP phosphorylation, disruption of lipid rafts with MβCD would be expected to abolish or at least inhibit $PGI_2$-mediated VASP phosphorylation. Treatment of platelets with $PGI_2$ (100nM/90sec) predictably led to an increase in $VASP^{Ser157}$ phosphorylation. When platelets were cholesterol depleted, $PGI_2$-mediated $VASP^{Ser157}$ phosphorylation was not affected **(figure 5. 6b)**.

To further investigate the significance of lipid rafts in PKA signaling, the inhibitory effect of $PGI_2$ on platelet aggregation under conditions of cholesterol depletion was also investigated. Treatment of WP with $PGI_2$ prior to stimulation with collagen reduced platelet aggregation by 70%. However, under conditions of lipid rafts disruption by MβCD, $PGI_2$ -mediated inhibition of platelet aggregation was slightly reduced to 51.5% **(figure 5. 6c)**.

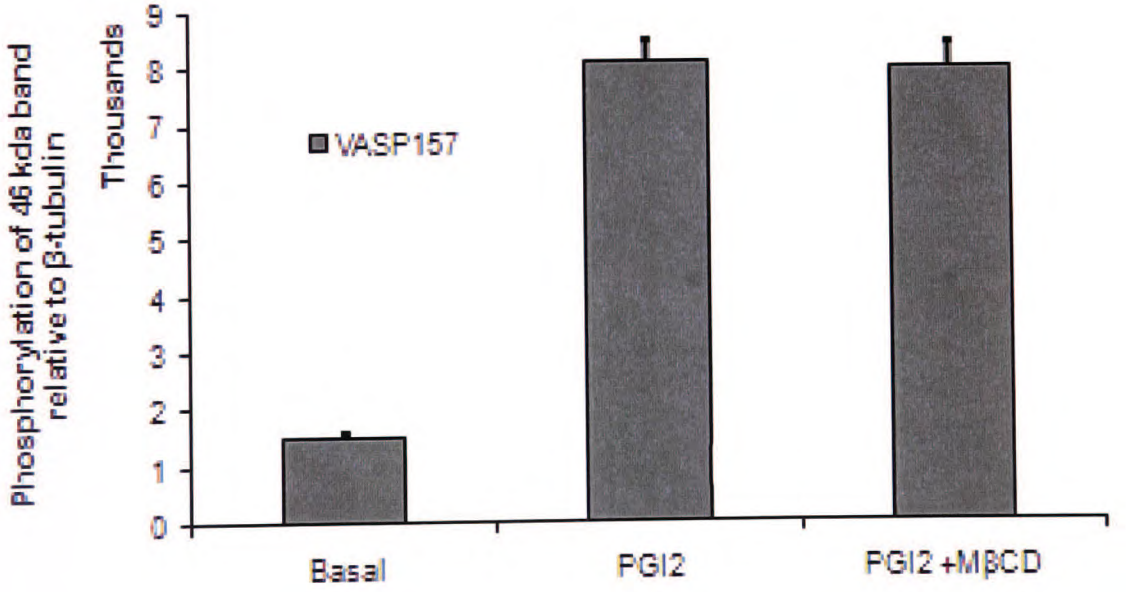

(a)
100
75
50
25
0
% Aggregation
0.6
3
6
10
Coll (µg/ml)
- MBCD
+ MBCD


(b)
80
60
40
20
0
% Inhibition
PGI2
PGI2/MβCD

(c)




(d)




**Legend: overleaf**

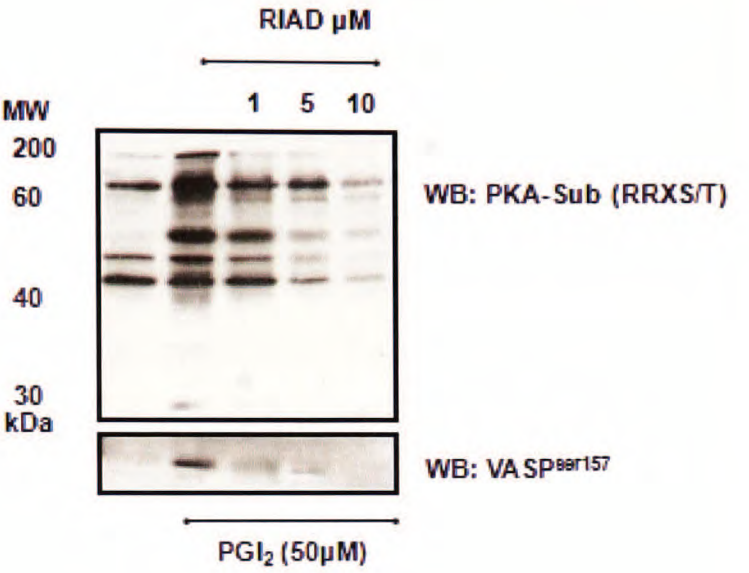
***Figure 5.6. The effect of cholesterol depletion on prostacyclin signaling.*** *WP ($3x10^8$) were depleted of cholesterol by preincubation with MβCD (5mM/1hr). WP were stimulated with collagen and aggregation response was recorded* ***(a)****. WP ($3x10^8$) treated with $PGI_2$ prior to stimulation with collagen and data represented as % of platelet inhibition (n=2)* ***(b)****. WP ($3x10^8$) treated with $PGI_2$ (100nM/90sec), reactions were stopped with an equal volume of 2xLaemmli buffer. Proteins were separated in 10–18% gradient gels and immunoblotted for anti-phospho- phospho$^{ser157}$ (n=4)* ***(c)****. Densitometry analysis of 4 independent immunoblot assays* ***(d)****. Data=mean±SEM.*

### 5.2.6 The role of AKAPs in PKA signaling in blood platelets.

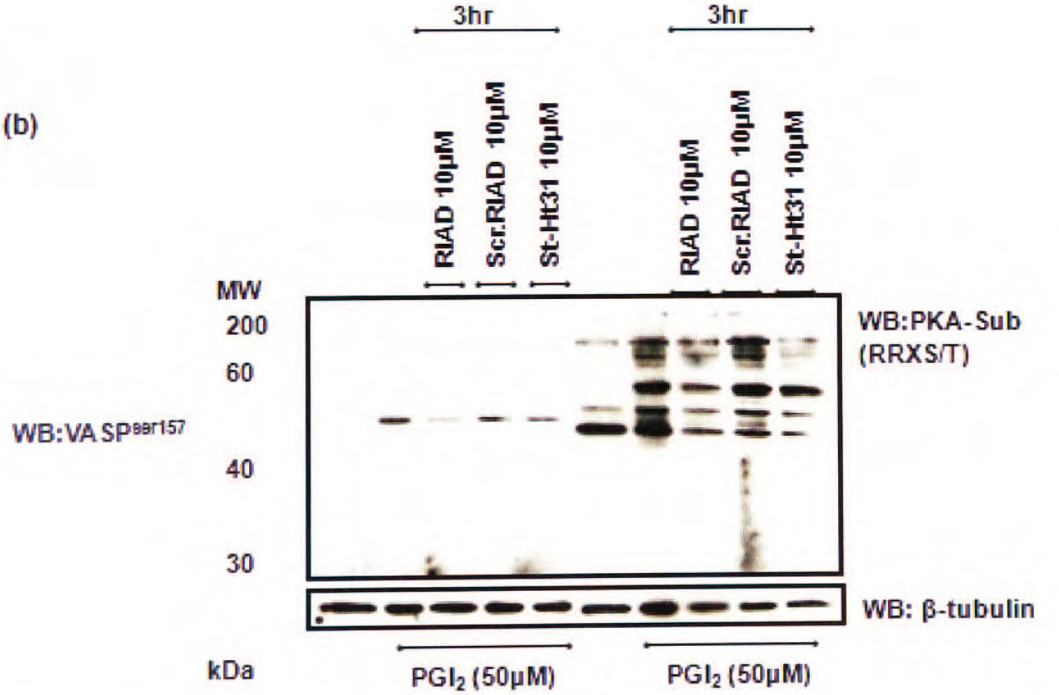
Control of specificity in cAMP signaling is achieved by AKAPs, which assemble PKA into multiprotein signaling complexes (Carlson *et al.*, 2006). We hypothesized that this could contribute to the localization of PKA isoforms in platelets. To assess this we performed preliminary experiments with three different peptides that have been reported to disrupt AKAP-PKA binding. RIAD ($R_I$-anchoring disruptor) *(LEQYANQLADQIIKEATEK (5-carboxyfluorescein)-CONH2)*, and StHt-31 (AKAP-$R_{II}$ anchoring disruptor) *(N-stearate-DLIEEAAS RIVDAVIEQVKAAGAY)* (Burton *et al.*, 1997; Gold *et al.*, 2006; Herberg *et al.*, 2000; Stokka *et al.*, 2006).

Western blot of platelets revealed that treatment with $PGI_2$ (50μM) induced increase in $VASP^{ser157}$ phosphorylation and phospho PKA substrate phosphorylation, as evidenced by using an antibody that recognizes phosphorylation of PKA substrates. Preincubation of platelets with RIAD led to inhibition of $PGI_2$-mediated $VASP^{Ser157}$ phosphorylation and phospho PKA substrate activity in a concentration dependent manner, with maximal effect observed at 10μM **(figure 5. 7a)**. In order to determine the specificity of the RIAD effect, the experiment was repeated with a scrambled version of RIAD *(IEKELAQQYQNADAITLEK (5-carboxyl fluorescein)-CONH2)*. Importantly scrambled RIAD peptide had only a minor effect on $PGI_2$- mediated $VASP^{ser157}$ phosphorylation and phospho PKA substrate activity. Preincubation of platelets with StHt-31 had less inhibitory effect on $PGI_2$-mediated signaling than RIAD **(figure 5. 7b)**.

(a)




(b)



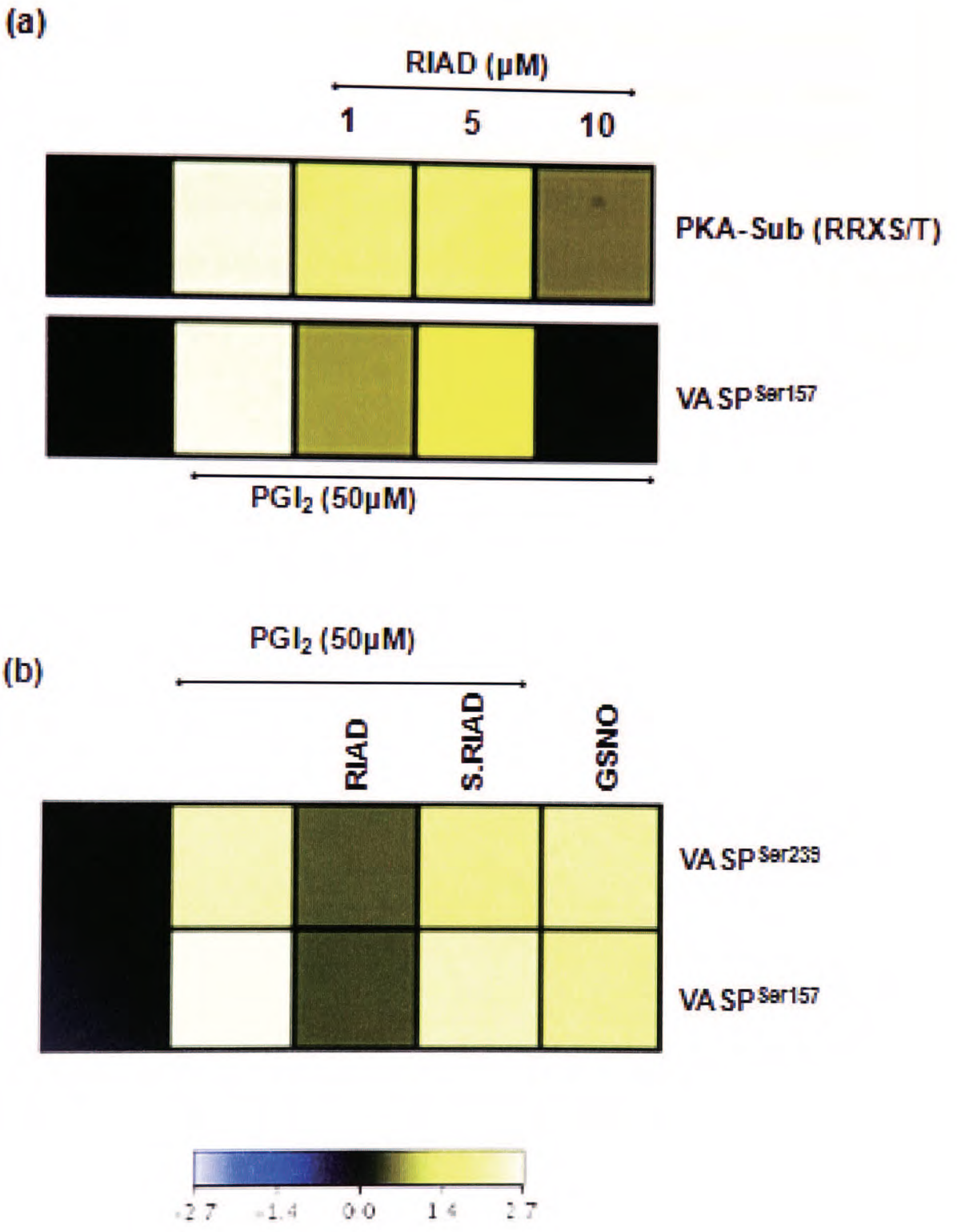

**Legend: overleaf**

***Figure 5.7. AKAP disruption causes inhibition of PKA signaling.***

*WP ($2.5x10^7$) pre-incubated with RIAD (1-10μM) for 3hrs before treatment with $PGI_2$ 50μM for 90sec) (a). WP ($2.5x10^7$) pre-incubated with RIAD, scrambled RIAD (10μM) or StHt-31 (10μM0 for 3hrs. before treatment with $PGI_2$ 50μM for 90sec (b). Reactions were stopped with 5xLaemmli buffer. Proteins were separated in 10–18% gradient gels and immunoblotted for anti- phospho$^{ser157}$ or PKA substrate antibody. n=1*

### 5.2.7 Disruption of AKAP –RI causes inhibition of $PGI_2$-mediated phosphorylation of VASP and PKA substrates: analysis using phospho-flow.

In these experiments, we compared data obtained from analyzing samples treated with $PGI_2$ in the presence and absence of AKAPs disrupting peptides by Western blot, with those obtained by using phosphoflow. Samples from **figure 5.7** were split into two; one set was analyzed by Western blot while the other half was analyzed with phosphoflow. **Figure 5.8a** shows that platelets treated with $PGI_2$ show a significant increase in $VASP^{Ser157}$ phosphorylation, and also increase in phosphorylation of PKA substrates. Preincubation of platelets with RIAD led to inhibition of phospho PKA substrate and VASP phosphorylation in a dose dependent manner. Interestingly, RIAD was much more effective against VASP compared to overall PKA substrates, suggesting some potential target specificity of $PKA_{RI}$. Disruption of AKAP/RI tethering by RIAD (10μM) inhibits $PGI_2$-mediated phosphorylation of $VASP^{Ser239/157}$ **(figure 5. 8b)** while S.RIAD had no effect. This data demonstrate the specificity and accuracy not only of results obtained with RIAD, but also of results obtained with phosphoflow.




**Legend: overleaf**

***Figure 5.8. AKAP disruption causes inhibition of PKA signaling.***

*WP ($2.5x10^7$) pre-incubated with RIAD (1-10μM) for 3hrs before treatment with $PGI_2$ 50μM for 90sec)* ***(a)****. WP ($2.5x10^7$) pre-incubated with RIAD, scrambled RIAD (10μM) for 3hrs. before treatment with $PGI_2$ 50μM for 90sec)* ***(b)****. Reactions were stopped by with Fix buffer. Samples were permeabilised and analyzed with phosphoflow using fluorescent antibodies against VASP $^{Ser239/157}$ and anti-PKA substrates antibody. n=1*

### 5.2.8 Localization of soluble guanylyl cyclase in unstimulated platelets (subcellular fractionation).

NO diffuse freely across cell membrane to reach its receptor, sGC, which raises intracellular cGMP levels. sGC has been purified from various tissues from the cytosolic fractions (Theilig *et al.*, 2001). However, NO has a very short half-life (Lancaster, 1997), and is approximately nine times more soluble in a hydrophobic environment than in water (Shaw *et al.*, 1977). This results, at least *in vitro*, in an NO gradient with high concentrations at or near membranes and a lower concentration in the aqueous environment (Malinski *et al.*, 1993). This suggests that it is not the cytosol, but instead cellular membranes, that may be the preferred site of NO action. We therefore examined the subcellular distribution of the $sGC_{\alpha 1}$ and $sGC_{\beta 1}$ subunits of heterodimeric sGC in platelets by subcellular fractionations.

Analysis of unstimulated platelets revealed that the majority of sGC is found in the soluble fraction consistent with its classification as a cytosolic enzyme. However, a substantial proportion of total $sGC_{\alpha 1}$ and $sGC_{\beta 1}$ was also detectable in the particulate fraction **(figure 5. 9a)**. In addition, HSP-90, a protein thought to stabilize sGC structure (Nedvetsky *et al.*, 2007; Papapetropoulos *et al.*, 2005), was found to be physically associated in a complex with both $sGC_{\alpha 1}$ and $sGC_{\beta 1}$. The accuracy of sGC and HSP-90 localization was confirmed by blotting for integrin $\beta_3$ and $PLC_{\gamma 2}$ **(figure 5. 9b)**.

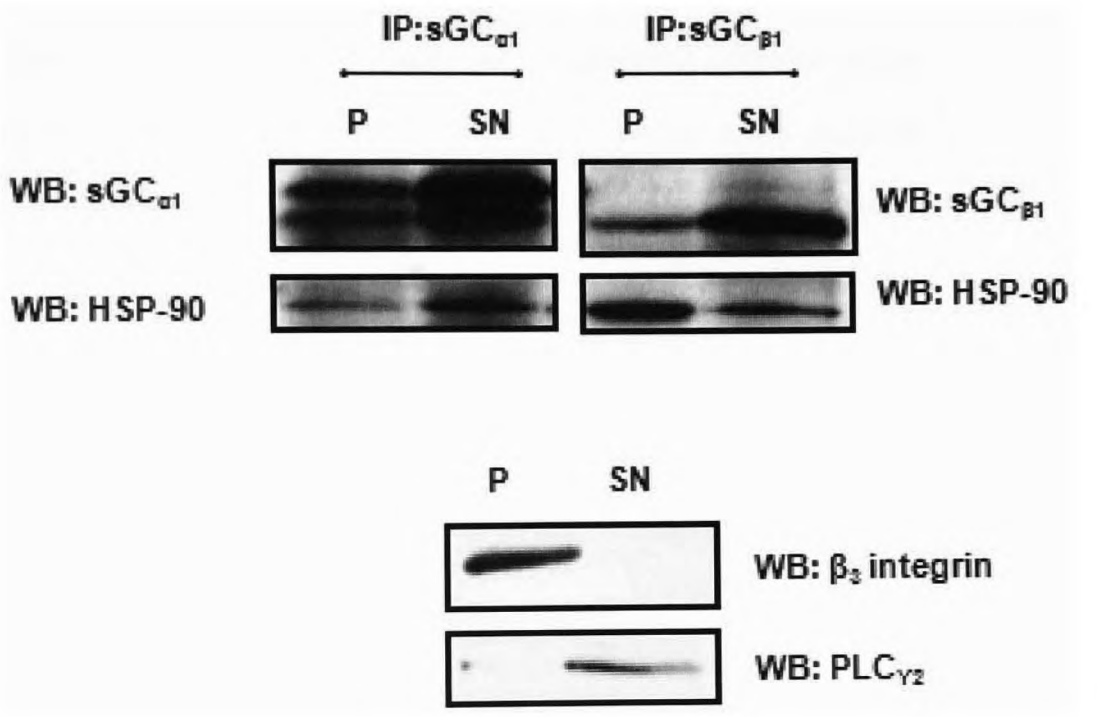



***Figure 5.9. Soluble guanylyl cyclase and heat shock protein-90 are present in soluble and particulate fractions of platelets.***

*WP ($5x10^8$) were added to fractionation buffer (1:1). Cells subjected to freeze-thaw cycles. Lysate was centrifuged first at 3000g/5min. supernatant was further subjected to ultracentrifugation then subjected to subcellular fractionation. 10µg of supernatants and cytoskeleton fractions were loaded on SDS-PAGE followed by Western blot. Membrane blotted with indicated antibodies. n=1*

## 5.2.9 Localization of soluble guanylyl cyclase and heat shock protein-90 in unstimulated platelets (lipid rafts isolation).

In the previous section **5.2.9** we have shown that proportion of $sGC_{\alpha 1/\beta 1}$ were located in the particulate fraction. Next we investigated whether these isoforms are located within lipid rafts. Analysis of unstimulated platelets shows that $sGC_{\alpha 1}$ and $sGC_{\beta 1}$ were localized in the soluble fractions, furthermore, HSP-90 was also physically associated with $sGC_{\alpha 1}$ and $sGC_{\beta 1}$ **(figure5. 10a/b)**.

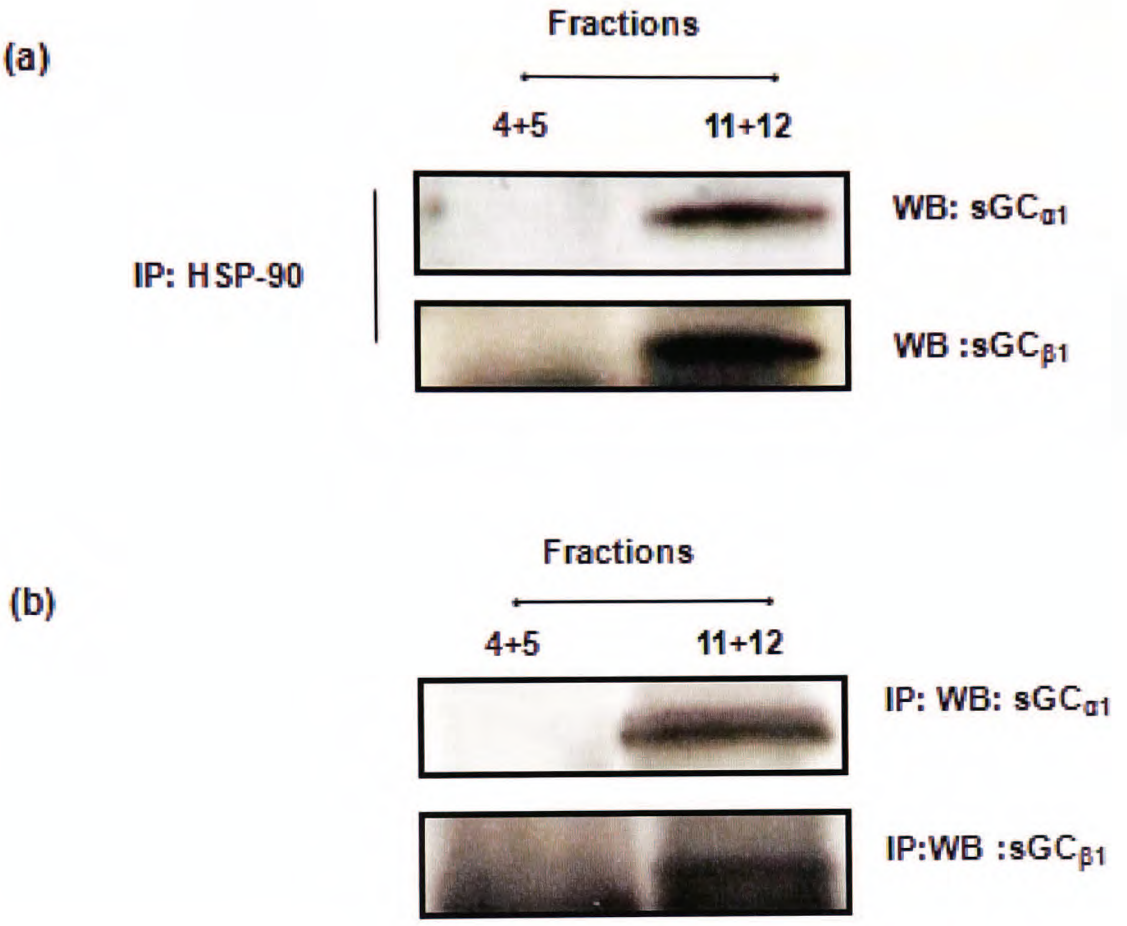


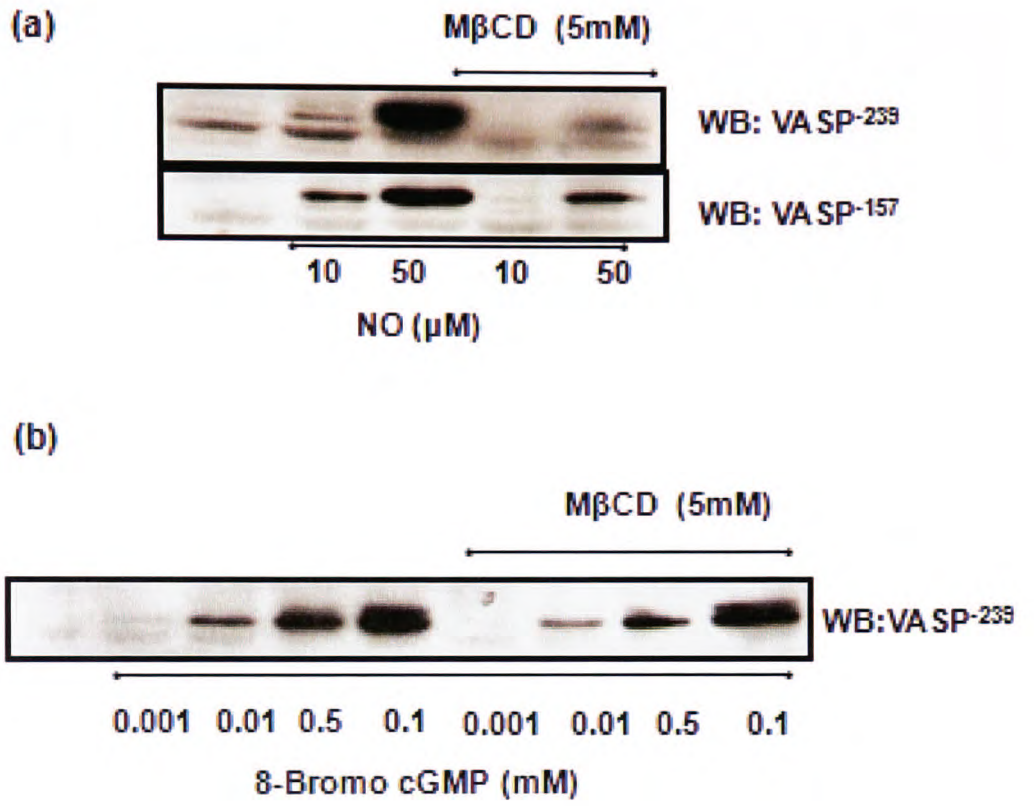

***Figure 5.10. Soluble guanylyl cyclase and heat shock protein-90 are present in soluble fractions of platelets.***

*450µl of WP (1x10$^9$) were lysed with a lysis buffer containing 0.045% Triton-X100. Mixture was mixed with 1:1. As in figure (1), HSP-90 was immunopreciptaed from fractions (4+5) and (11+12)* ***(a)****. Or sGC$_{\alpha 1}$ and sGC$_{\beta 1}$ were immunopreciptaed from fractions (4+5) and (11+12)* ***(b)****. Proteins were loaded on SDS-PAGE followed by Western blot. Membranes were blotted with anti-sGC$_{\alpha 1}$, or sGC$_{\beta 1}$ antibody. n=1*

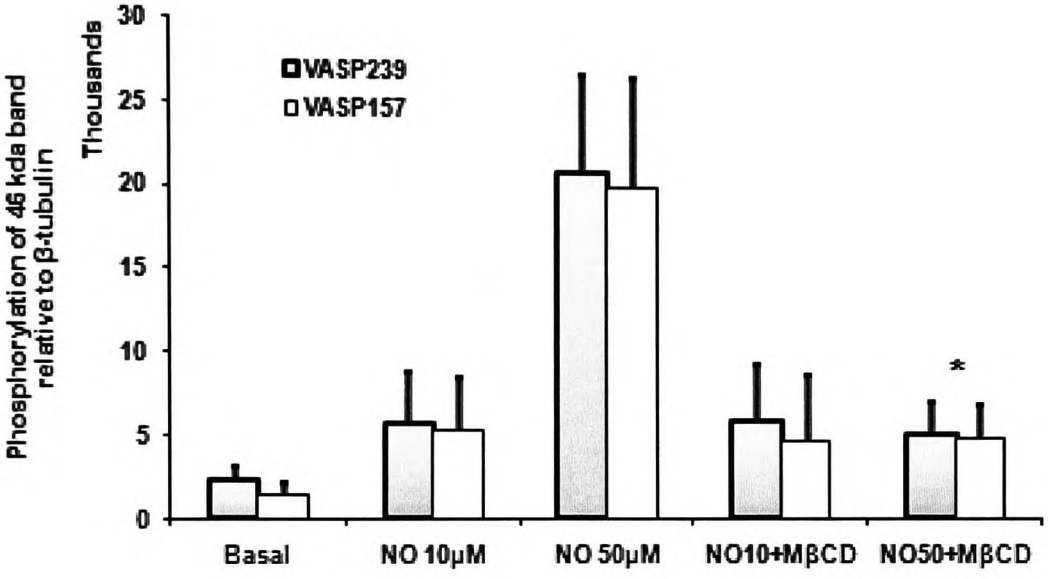
### 5.2.10 The effect of cholesterol depletion on inhibition of platelet aggregation by nitric oxide.

Having established that $sGC_{\alpha 1/\beta 1}$ were not associated with lipid rafts, we sought to determine the effect of membrane cholesterol depletion on PKG signaling. When platelets were incubated with MβCD (5mM) for 60min, the level of NO-induced $VASP^{Ser239/157}$ phosphorylation was reduced **(figure 5. 11a)**. On the other hand, there was no difference in level of VASP phosphorylation in platelets treated with cGMP analogue, 8-Bromo-cGMP under conditions of lipid rafts disruption **(figure 5. 11b)**.

Treatment WP with GSNO for 1 min prior to stimulation with collagen caused inhibition of platelet aggregation by 77.5 ± 14.5%. However, under conditions of lipid rafts disruption by MβCD, the ability of GSNO to inhibit platelet aggregation in response to collagen was significantly reduced to 14.5 ±9.2% ($P<0.05$) **(figure 5. 11c)**.


(a)
MβCD (5mM)
WB: VASP-239
WB: VASP-157
10 50 10 50
NO (μM)
(b)
MβCD (5mM)
WB:VASP-239
0.001 0.01 0.5 0.1 0.001 0.01 0.5 0.1
8-Bromo cGMP (mM)

(c)




**Legend:overleaf**

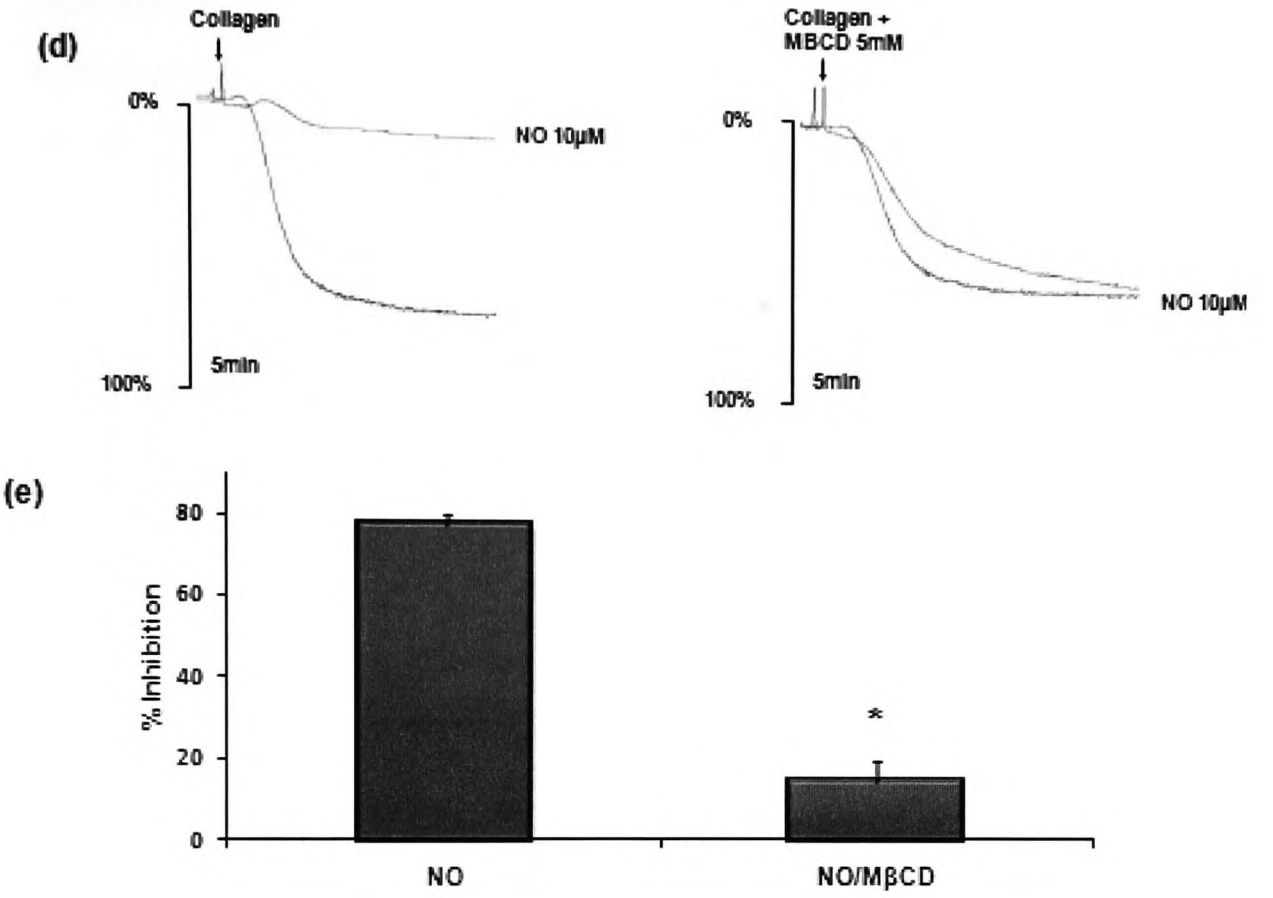
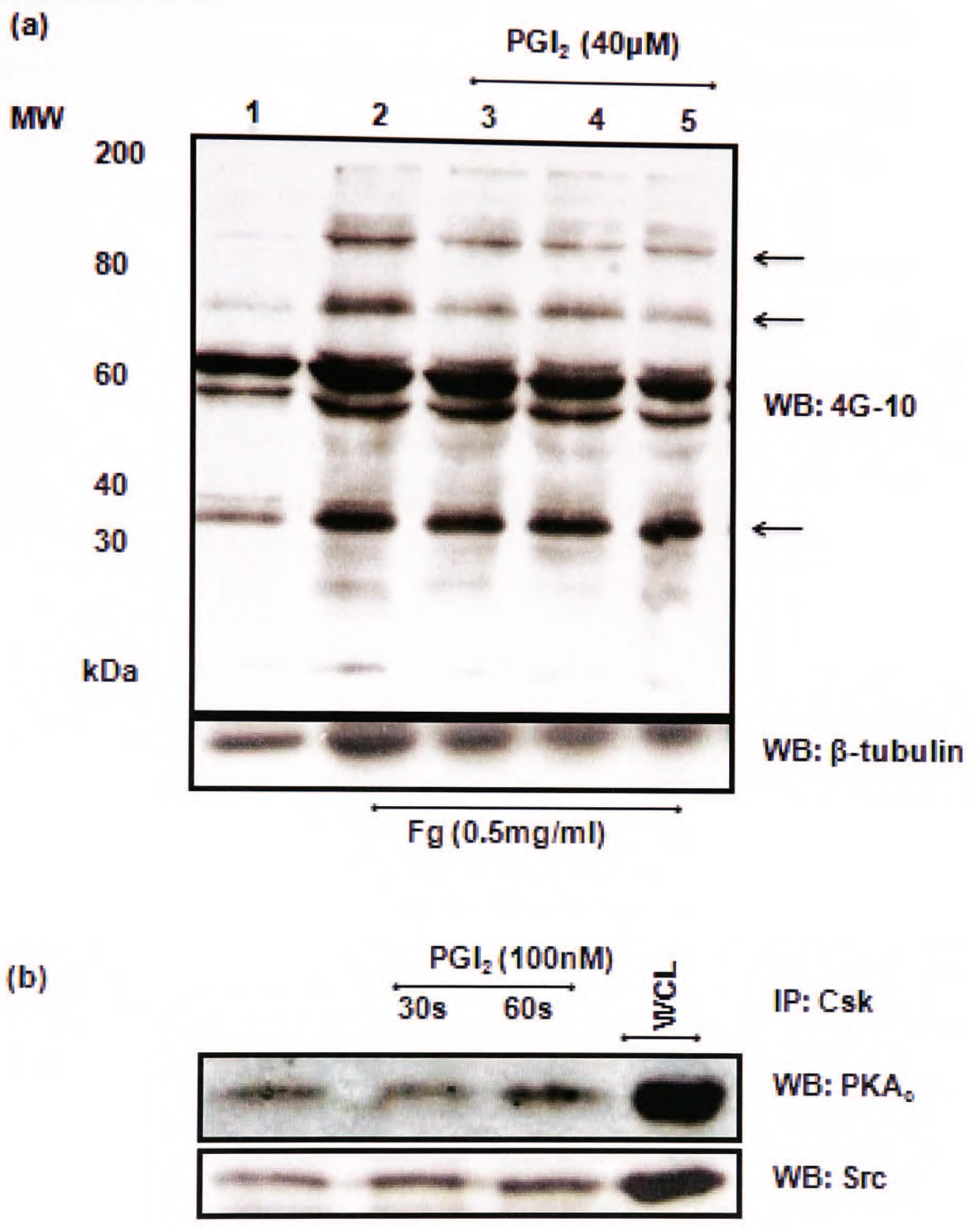



***Figure 5.11. The effect of cholesterol depletion on nitric oxide signaling.*** *WP ($3x10^8$) were depleted of cholesterol by preincubation with MβCD (5mM/1hr). WP were treated with either GSNO (10 and 50µM/1min)* ***(a)****, 8-Bromo-cGMP at indicated concentrations for 15min* ***(b)*** *and reactions were stopped with an equal volume of 2xLaemmli buffer. Proteins were separated in 10–18% gradient gels and immunoblotted for anti-phospho-VASP$^{ser239}$ or phospho$^{ser157}$. Densitometric analysis of 3 independent immunoblot assays* ***(c)****. WP ($3x10^8$) stimulated with collagen or NO prior to stimulation with collagen and aggregation response were recorded* ***(d)****. Graph representative of 3 independent experiments* ***(e)****. Data=mean±SEM. $^*P<0.05$.*

### 5.2.11 Prostacyclin inhibits outside-in signaling in platelets.

Src-family kinases (SFKs) play initiating and critical roles in signaling through GPVI and integrin $\alpha_{IIb}\beta_3$ receptors on platelets. The enzymatic activities of SFKs are regulated by tyrosine phosphorylation, with the phosphorylation of a conserved tyrosine in the activation loop generating an active form of the enzyme, and phosphorylation of a conserved tyrosine in the C-terminal tail resulting in an intramolecular binding event causing inactivation (Harrison, 2003; Sicheri *et al.*, 1997; Xu *et al.*, 1997). Activation loop phosphorylation is mediated by the SFKs themselves, while the C-terminal Src kinase (Csk) and family member Chk are responsible for the inhibitory phosphorylation (Veillette *et al.*, 2002). In T-cells, Csk has been identified as a PKA substrate (Vang *et al.*; Vang *et al.*). Since it has been shown that Csk is associated with Src kinase and integrin $\beta_3$ in a complex and Csk is a PKA substrate in T-cells (Obergfell *et al.*, 2002), we investigated whether PKA targets integrin $\alpha_{IIb}\beta_3$- downstream signaling. We examined this by adding $PGI_2$ at different times relative to the stimulation of platelets with fibrinogen. Stimulation of WP with fibrinogen (0.5mg/ml) for 10min caused a significant increase in tyrosine phosphorylation at ~ 30, 70 and 95 kDa **[figure 5. 12a (lane 2)]**. Treatment of WP with $PGI_2$ (40μM) for 5mins prior to stimulation with fibrinogen reduced tyrosine phosphorylation of protein(s) at ~70k and 95 kDa **[figure 5. 12a (lane 3)]**. Simultaneous treatment addition of $PGI_2$ and fibrinogen to WP caused a modest inhibition of tyrosine phosphorylation of the same protein(s) **[figure 5. 12a (lane 4)]**. Furthermore, treatment of WP with $PGI_2$ 5min after stimulation with fibrinogen also caused inhibition of tyrosine phosphorylation

**[figure 5. 12a (lane 5)]**. In addition, examination of Csk immune precipitates from untreated platelets and $PGI_2$ –treated platelets showed a time-dependent increase in association of Src kinase and PKA catalytic subunit with Csk **(figure 12. 5b)**. Due to the close approximately of the molecular weight of Csk (50kDa) and heavy chain (50kDa), it was difficult to distinguish between the Csk and the heavy chain on Western blot membranes.




**Legend: overleaf**

***Figure 5.12. Prostacyclin downregulates outside in signaling in platelets.***

*WP ($5X10^8$) stimulated with fibrinogen (0.5 mg/ml) for 10min, or treated with $PGI_2$ (40μM) 90sec prior stimulation with fibrinogen (-90sec), simultaneously (0sec) or 90sec after stimulation with fibrinogen (+90sec) Samples were loaded on SDS-PAGE followed by Western blot. Membrane blotted with anti phosphotyrosine* ***(a)****. WP ($7X10^8$) were treated with $PGI_2$ (100nM) for 30 and 60sec. Csk was immunopreciptated from lysates and samples were loaded on SDS-PAGE followed by Western blot. Membrane blotted with $PKA_c$ antibody, stripped and reprobed with Src antibody* ***(b)****. n=1*

## 5.3 Discussion.

In living cells, changes in the molar ratio of cholesterol/phospholipid of cell membranes induced by a change in cholesterol content affect a number of important membrane properties, including permeability, transport functions, membrane enzyme activities, the availability of membrane components as substrates, conformation of membrane proteins, and exposure of proteins (Aloia, 1983; Shinitzky, 1984; Yeagle, 1985). These alterations are mediated either by the change in membrane cholesterol content itself or by a concomitant change in membrane fluidity. Elegant studies have highlighted the importance of lipid rafts in platelet response to agonists (Bodin *et al.*, 2005; Bodin *et al.*, 2003; Lee *et al.*, 2006; Shrimpton *et al.*, 2002; Wonerow *et al.*, 2002b). The role of lipid rafts in GPCR-mediated signaling via $G_{\alpha i}$ protein (ADP) has also been demonstrated (Ostrom *et al.*, 2004). However, little is known about the role of lipid rafts in signaling downstream AC and sGC.

### 5.3.1 Subcellular localization of PKA.

PKA is a heterodimer composed of two regulatory and two catalytic subunits. The regulatory ($R_{I\alpha}$, $R_{I\beta}$, $R_{II\alpha}$, $R_{II\beta}$) and catalytic domains ($C_{\alpha}$, $C_{\beta}$,$C_{\gamma}$) are differentially expressed giving rise to different isoforms of the haloenzyme (Tasken *et al.*, 2004). PKA isoforms are categorized by their R-subunit giving rise to two main classes of isozymes type I and type II PKA. While the presence of PKA isoforms have been identified and studied in numerous cell types, little is known about their presence in platelets. Data from this thesis confirms for the first time that both isoforms of PKA are present in platelets,

although their subcellular distribution showed differences. $PKA_{RII}$ was found to be present in particulate and cytosolic fractions of platelet, but was more abundant in the cytosolic fraction. In contrast $PKA_{RI}$ was more evenly distributed between each fraction. In platelets, activation of cAMP signaling by prostacyclin involves binding to IP receptors [a G protein-coupled receptor (GPCR)], which through $G_s$ proteins regulates one of several isoforms of adenylyl cyclase leading to generation of cAMP. However, the pools of cAMP generated are determined by the localization of receptors and shaped by phosphodiesterases. It is feasible that a cAMP gradient elicited by a distinct receptor is specifically organized to follow a distinct route of PKA signaling by reaching and activating single isoform of PKA to mediate a biological effect. To this end treatment of platelets with $PGE_1$ or [$PGI_2$ **(not shown)**] led to transient delocalization of $PKA_{RII}$ to cytosolic fractions while $PKA_{RI}$ localization was unchanged. Consistent with these findings we found a distinctive distribution of PKA substrates between particulate and cytosolic fractions after stimulation with PGE1. Although it is not clear which PKA isoform(s) target which downstream substrates, these data suggest that PKA isoforms may mediate their effects in discrete locations of the cell.

In order to try and examine this issue we tried to evaluate the role of Csk, a substrate of PKA in T-cells. Shattil and colleagues (Obergfell *et al.*, 2002), demonstrated that Csk is constitutively associated with integrin $\beta_3$ in resting platelets, and released upon fibrinogen binding. In platelets Csk was found associated with Src kinase, and though to regulate Src activation by phosphorylation of the C-terminal inhibitory tyrosine reside on Src; causing it to fold back in its an active confirmation (Hirao *et al.*, 1997; Okada *et al.*,

1991). Other Csk binding proteins including Cbp/PAG and Paxillin family members are tyrosine phosphorylated following platelet activation, creating a binding site for the SH2 domain of Csk that brings it into proximity with SFKs (Rathore *et al.*, 2007). Critically the release of Csk upon fibrinogen binding allows the activation of $\alpha_{IIb}\beta_3$-mediated outside signaling. In immune cells, Csk phosphorylation on serine residues by PKA leads to Csk activation which in turn cause inhibition of Src kinase (Vang *et al.*, 2001; Yaqub *et al.*, 2003), a signaling cascade which leads to dysfunctional T-cells and defective immune response (Tasken, 2009). Thus it was possible that phosphorylation of Csk by PKA may represent a potential target for the regulation of platelets. Subcellular localization of proteins in platelets under resting conditions showed that high proportion of Csk was recruited to the cytosolic fraction, whereas a smaller proportion was found in the particulate fraction. Since Csk does not contain any posttranslational modifications for membrane attachment (Rafnar *et al.*, 1998). Interestingly stimulation of cells with $PGE_1$ showed that Csk localized to the same fraction as PKARII and with the same kinetics. Studies suggested that prostacyclin inhibits integrin $\alpha_{IIb}\beta_3$ activation (inside-out signaling) via inhibition of VASP anti-capping activity (Siess *et al.*, 1989; Wise *et al.*, 2000). Out preliminary data revealed that treatment of platelets with prostacyclin inhibits integrin $\alpha_{IIb}\beta_3$-mediated tyrosine phosphorylation (outside-in signaling). PKA catalytic subunit was physically associated in a complex with Csk in platelets. Upon treatment of platelets with $PGI_2$, slightly higher proportion of PKA catalytic subunit was associated with Csk. We hypothesis that cAMP levels increase following prostacyclin treatment, leading to activation of PKA, which in turn activates

Csk. Active Csk forms a complex with Src and negatively regulates integrin $\alpha_{IIb}\beta_3$. However, further experimentation is required to characterize this potential mechanism for platelet regulation by cAMP/PKA signaling.

In this study we have also demonstrated a distinct role for lipid rafts in regulating $PGI_2$ signaling, the most studied inhibitory pathways in platelets. Lipid raft isolation data revealed that $PKA_{RI}$ was redistributed in lipid rafts and non-lipid rafts domains, whereas $PKA_{RII}$ was localized only in non-rafts fraction. This unique distribution of $PKA_{RI}$ and $PKA_{RII}$ is not cell specific as our data matches those obtained by others in T-cells (Carlson *et al.*, 2006). In order to examine whether lipid rafts affected signalling by PKA we used, methyl-β-cyclodextrin, to disrupt rafts. Consistent with previous studies, depletion of cholesterol, the main component of lipid rafts, from platelet cell membrane caused inhibition of platelet response to collagen, thrombin and vWF (Lee *et al.*, 2006; Shrimpton *et al.*, 2002). Cholesterol depletion modestly but significantly reversed the inhibitory effect of $PGI_2$ on platelet aggregation. However, it failed to influence $PGI_2$-mediated VASP phosphorylation. These data could suggest that lipid rafts are important for platelet inhibition by $PGI_2$, but it is independent of VASP phosphorylation, and the modest loss of the inhibitory effect of $PGI_2$ could be attributed to a loss in another inhibitory pathway such as inhibition of $Ca^{2+}$ mobilization.

### 5.3.2 The role of AKAPs in PKA-mediated signaling in platelets.

In cells, additional factors such as localization, accessibility of the phosphorylation sites and concentration of protein kinases or phosphatases and their activators or inhibitors might affect VASP phosphorylation. For example, although $Ser^{157}$ is quantitatively phosphorylated by PKG *in vitro*, the endogenous nitric oxide (NO)– cGMP–PKG pathway maximally phosphorylates ~50% of the available $Ser^{157}$ sites in human platelets and fibroblasts (Reinhard *et al.*, 2001).

Eukaryotic cells express multiple forms of PKA regulatory subunit isoforms, of which RI and RII are widely present in cells and tissue. PKA catalytic subunits share common kinetic features and substrate specificity (Edelman *et al.*, 1987; Taylor *et al.*, 1992); Therefore it is likely that localization of PKA isoforms determines their substrate specificity. PKA isoforms are localized through interaction with a family of structurally distinct but functionally homologous A kinase anchoring proteins (AKAPs). These proteins are grouped into RI and RII specific AKAPs depending on which isoforms of PKA they interact with. Critically in a number of cell types AKAPs act to focus PKA isoforms to distinct substrates thereby allowing specificity of PKA signaling. Having identified both isoforms of PKA in platelet we wished to understand they played redundant or non-redundant roles. However, the inhibitors used to evaluate PKA signaling have well-documented non-specific actions (Davies *et al.*, 2000; Gambaryan *et al.*, 2004). Therefore a different approach was used. A peptide that disrupted PKAI-AKAP interactions was used to try

and evaluate the effects of PKAI (Carlson *et al.*, 2006). Although it is noteworthy that the use of RIAD only delineates cAMP signaling events that depend on anchoring of $PKA_{RI}$ to an AKAP rather than all $PKA_{RI}$.

Western blot data of WP incubated with RIAD (RI-anchoring disruptor) prior to treatment with $PGI_2$ revealed almost complete inhibition of $VASP^{Ser157}$, however phosphorylation of PKA substrates was less affected. Data with RIAD was reproducible and the concentration ranged from 1μM to 10μM, consistent with other cell types (Carlson *et al.*, 2006). Crucially results obtained by RIAD have to be compared with the negative control Sr.RIAD. To confirm these findings we used alternative methodology, phosphoflow. Pretreatment of platelets with RIAD led to a dose-dependent inhibition of $PGI_2$-mediated $VASP^{Ser157}$ phosphorylation. While RIAD almost completely abolished $PGI_2$-mediated VASP phosphorylation, RIAD had less effect on $PGI_2$-mediated PKA substrates phosphorylation. In addition, preliminary platelet aggregation data show the ability of RIAD to reverse the inhibitory effect of $PGI_2$ and $PGE_1$ on platelets stimulated with collagen **(not shown)**. These data mirrored the data obtained from Western blotting analysis. Together, these data suggest a non-redundant role for PKA isoforms in a manner that PKA substrate phosphorylation is mediated by $PKA_{RI}$ and $PKA_{RII}$ isoforms.

### 5.3.3 New insights into nitric oxide signaling in platelets.

Subcellular localization data of unstimulated platelets show that $sGC_{\alpha 1}$ and $sGC_{\beta 1}$ are unevenly distributed between particulate and cytosolic fractions,

where the majority of $sGC_{\alpha1/\beta1}$ are present in the cytosolic fraction, a proportion of the enzyme was found in the particulate fraction - Subcellular distribution of sGC receptors may reflect a physical compartmentation of the signal transduction cascade, rather than regulation of their sensitivity to NO. However, this is still controversial (Theilig *et al.*, 2001; Wykes *et al.*, 2004). The presence of sGC in lipid rafts was also investigated. Our data also reveal that $sGC_{\alpha1}$ and $sGC_{\beta1}$ are localized in the non-raft fractions of the cell. Importantly, in support to our previous observations (Riba *et al.*, 2008), HSP-90 was found to be colocalized in a complex with both sGC isoforms. This is consistent with its classification as a stabilizer of sGC (Nedvetsky *et al.*; Papapetropoulos *et al.*). Interestingly, disruption of lipid rafts by cholesterol depletion compromised the inhibitory effect of NO on platelet aggregation. In addition, in conditions of cholesterol depletion only NO-mediated $VASP^{Ser239}$ phosphorylation was abolished but not $VASP^{Ser157}$ phosphorylation. 8-Bromo-cGMP-mediated VASP phosphorylation was not affected by cholesterol depletion. This is an important observation as it confirms that sGC/PKG-mediated signaling cascade is disrupted upon cholesterol depletion, while PKA signaling cascade is less affected. Our results, however, contradicts those of another study (Miersch *et al.*, 2008), as the author demonstrated a decrease in NO diffusion in conditions where cholesterol levels were increased, and an increase in NO-mediated VASP phosphorylation in conditions of cholesterol depletion. It is noteworthy that since $PGI_2$-mediated VASP phosphorylation was maintained under conditions of cholesterol depletion it indicates that VASP localization was not affected, which rules out delocalization of VASP as the reason for the partial

loss of the inhibitory effect of NO. It is possible that if PKG is either located in or recruited to lipid rafts and that loss of raft structure disrupts the formation of complexes important for PKG mediated signaling. It would also be important to ascertain whether cGMP formation requires competent lipid rafts. Thus cholesterol deletion is required for NO signaling but not that by 8-Bromo-cGMP-mediated VASP phosphorylation. This disparity required further investigation.

## 5.4 Conclusion.

Protein kinase A (PKA) is a key regulatory enzyme that, on activation by cAMP, modulates a wide variety of cellular functions. PKA isoforms type RI and type RII possess different structural features and biochemical characteristics, resulting in non redundant function. However, how different PKA isoforms expressed in the same cell manage to perform distinct functions on activation by the same soluble intracellular messenger, cAMP, remains to be established.

***Future work***

The work to be carried out will attempt to reinforce the preliminary data that has already been accumulated which has indicated a possible role for lipid rafts in mediating PKA and PKG signaling.

- To show the effect of lipid rafts isolation on cyclic nucleotides formation.

- To assess this, Intracellular cAMP and cGMP formation will be measured in conditions of cholesterol depletion.

- To show that the effect is not due to internalization of IP receptors.
    - To tackle this, surface expression of IP receptors after cholesterol depletion will be assessed using flow cytometry.

- To test the ability of other detergents to reproduce data obtained from Triton X-100.
    - To test this, other detergents will be tested as a mean for lipid rafts isolation. Lurbol WX and CHAPS as milder detergents to preserve rafts that might be solubilized by Triton X-100. Brij98 will be used for lipid rafts isolation at 37°C.

- To assess the possible recruitment of sGC and/or SHP-90 to lipid rafts.
    - To demonstrate this, WP will be treated with NO donors in time course. Distribution of $sGC_{\alpha 1/\beta 1}$ and HSP-90 will be determined by immunoprecipitation from rafts and non-rafts fractions.

- To investigate whether the inhibitory effect of $PGI_2$ on other platelet functional aspects such as adhesion and calcium mobilization is mediated by $PKA_{RI}$, $PKA_{RII}$ or both.

- To investigate whether PKA intersects Src kinases activation by targeting Csk.

- To assess this WP will be treated with $PGI_2$ before stimulation with fibrinogen+$Mn^{2+}$, Src kinase family SKF (Lyn, Fyn and Src) will be immunopreciptated and status of tyrosine phosphorylation on the activatory site will be determined. SKF association with Csk will be assessed as well.

# CHAPTER 6. GENERAL DISCUSSION.

# 6. General discussion.

The adhesion and activation of blood platelets at sites of vascular damage is essential for haemostasis, but can also initiate thrombosis. Vascular injury leads to exposure of prothrombotic extracellular matrix (ECM) proteins like von Willebrand factor (vWF) and collagen, which trap and activate platelets. Activation of platelets leads to the release of soluble platelet agonists, adenosine diphosphate (ADP) and thromboxane $A_2$ ($TXA_2$), which act in a paracrine fashion to further enhance platelet function and ensure rapid haemostasis(Ruggeri, 2002b). In healthy or undamaged blood vessels platelet activation is counterbalanced by negative signaling cascades that modulate excessive activation. This is achieved primarily through endothelial-derived nitric oxide (NO) and prostaglandin ($PGI_2$). The biological effects of NO and $PGI_2$ are mediated through the formation of cyclic nucleotides, cyclic guanosine 3',5'monophosphate (cGMP) and cyclic adenosine 3',5'monophosphate (cAMP). The elevated cyclic nucleotide concentrations activate protein kinase G (PKG) and PKA, which in turn blunt platelet activation(Schwarz *et al.*, 2001b). More recently PKC another member of the AGC family of protein kinases has also been shown to inhibit platelet activation. Although in this case it was independent of cyclic nucleotides. Of particular relevance to the resent study was NO, which is a major regulator of platelet function and signals through both PKA and PKG. On the other hand, accumulated experimental evidence suggests that secondary oxidants derived from NO are rather responsible for cytoxicity and associated tissue injury. In particular, there has been a key interest in the

role of peroxynitrite, a powerful oxidative and nitrating agent formed *in vivo* through the reaction of NO with superoxide.

In this study, evidence was presented that peroxynitrite inhibits platelet aggregation, adhesion and spreading to collagen. The data in the field has often been contradictory, with both activatory and inhibitory actions shown. To some degree this is exemplified in our studies where peroxynitrite, induced tyrosine phosphorylation and the release of secondary platelet agonists, but also caused functional inhibition. The reasons for this are unclear but must reflect the inhibitory actions of peroxynitrite are quantitatively more important than the activatory effects. The inhibitory effects of peroxynitrite have also proved controversial, however data produced in this thesis may go some way to explaining it. The cGMP-dependent effects of peroxynitrite on platelets were completely dependent upon the presence of HEPES in the resuspenesion buffer. Since this is widely used by many groups for analysis of platelet function, it is likely to have a direct influence of platelet regulation by peroxynitrite. Importantly in the absence of significant cGMP formation we found that inhibition of platelet function by peroxynitrite was mediated, at least in part, by the direct phosphorylation of VASP. This occurred through PKC rather than PKA or PKG. Therefore it is possible to speculate that is a compensatory mechanism for the loss of NO. The formation of peroxynitrite diminishes the antiplatelet effects of NO, however, the formation of peroxynitrite activates an alternative pathway in platelets PKC, which results in the same outcome, that is, the phosphorylation of VASP and therefore inhibition of platelet function.

The actin cytoskeleton is required for many important processes during haemostasis and blood vessel preservation. Therefore, the function of actin cytoskeleton must be tightly regulated. VASP functions as an anti-capping protein that binds and regulates the actin cytoskeleton. As a regulator of actin ultrastructure, VASP is involved in crucial platelet functions, such as shape change, adhesion and aggregation. The molecular function of VASP at the extremity of lamellipodia and filopodia is that VASP can bind the barbed end of actin filaments and protect them from being capped by capping proteins. Three phosphorylation sites were identified on VASP; $Ser^{157}$, $Ser^{239}$, $Thr^{274}$, all of which can be phosphorylated by either PKA or PKG. In addition, PKC has also been reported to phosphorylate VASP at $Ser^{157}$.

VASP phosphorylation has different consequences. Phosphorylation of VASP results in down regulation of its filament bundling and anti-capping activities (Harbeck *et al.*, 2000). VASP phosphorylation by cAMP and cGMP-elevating agents inhibits $\alpha_{IIb}\beta_3$ receptor and fibrinogen binding (Horstrup *et al.*, 1994). VASP phosphorylation could therefore represent a general negative feed-back regulatory mechanism for the control of platelet aggregation. The production of NO and prostacyclin in endothelial cells and their release in the bloodstream negatively regulate the activity of platelets and prevent thrombosis by increasing intracellular cGMP and cAMP levels, respectively.

It is noteworthy that Clopidogrel, an antiplatelet therapy for the prevention of adverse consequences during coronary intervention, is a $P_2Y_{12}$ antagonist

that inhibits the $G_i$ protein-dependent signaling, potentiates the cAMP/PKA (Schwarz *et al.*, 1999). Cilostazol, a cyclic nucleotide phosphodiesterase (PDE) inhibitor used for the treatment of chronic peripheral arterial occlusion and stroke, also enhances the phosphorylation of VASP at both $Ser^{157}$ and $Ser^{239}$ (Sudo *et al.*, 2003) et al. 2003). Dipyridamole, another PDE inhibitor that in combination with aspirin is very effective in preventing recurrent stroke, amplifies the NO/cGMP-dependent phosphorylation of VASP (Aktas *et al.*, 2003).

In living cells, NO exerts its effect primarily through a sGC/cGMP-dependent mechanism. In platelets, activation of PKG leads to numerous phosphorylation events that blunt platelet activation. However, PKG phosphorylates and activates $PDE_5$ to engineer a feedback mechanism shaping the cGMP response (Mullershausen *et al.*, 2003). In this study evidence was presented that NO caused a modest increase in PKC activation and that NO mediates VASP phosphorylation in a manner that requires PKC. The presence of PDE activator (IBMX) reversed NO-mediated VASP phosphorylation close to control levels. Our data presented indirect evidences that NO-mediated VASP phosphorylation may occur in a manner that requires inhibition of PDE via PKC.

Since NO activates PKA, PKC and PKG in platelets it indicates the potential for the presence of multiple cyclic nucleotide signaling pathways. The downstream signaling, regulation and physiological importance of these individual pathways are unclear. Thus, whilst it is clear that cyclic nucleotides inhibit a number of physiological processes required for platelet activation, a co-ordinated understanding of how these pathways interact, achieve target

specificity and respond to different stimuli is unknown. In other cells, enzymes that generate, propagate and terminate AGC kinase signaling are organized into restricted cellular domains facilitating formation of distinct pools of cyclic nucleotides that aid target specificity. This "compartmentalization" of cyclic nucleotide signaling has not been described in platelets and in the final part of the work preliminary experiments were performed to begin to address this issue. Two related plasma membrane domains that compartmentalize GPCR signaling complexes are lipid rafts and caveolae. Unlike platelets, caveolae have been described in other cells such as endothelial cells (Spisni *et al.*, 2001), and cardiac myocytes (Feron *et al.*, 1996). In addition, the localization of individual PKA isoforms is further regulated by A-kinase anchoring proteins (AKAPs).

In our study, PKA isoform localization and dynamics were presented for the first time in platelets. Lipid raft domains were found to be critical for signaling through NO and $PGI_2$. Although NO signaling cascade was more dependent on lipid rafts integrity as VASP phosphorylation in response to NO was inhibited under condition of lipid rafts disruption, VASP phosphorylation in response to $PGI_2$ or $PGE_1$ was not affected. Further studies however are required to investigate the role of lipid rafts in inhibition of platelet adhesion and calcium mobilization by NO and prostacyclin.

The importance of AKAPs in regulating PKA signaling in platelets was presented for the first time in this study. Subcellular fractionation data revealed that PKA substrates were uniquely distributed between cytosolic and particulate compartments. This strongly suggested a separation of PKA substrates into distinct regions of the cell and therefore PKA must also be

differentially distributed. Our data suggest a non-redundant role for PKA isoforms signaling in platelets, since phosphorylation of the major PKA substrates was inhibited by the loss of $PKA_{RI}$ interactions with AKAPs. This was assessed by synthetic peptides that disrupt AKAP-RI tethering. These data are preliminary, but first to demonstrate a regulatory role for AKAPs in PKA signaling in platelets. It will be important in the future to determine if AKAPS allow PKA isoforms to interact with specific down stream substrates.

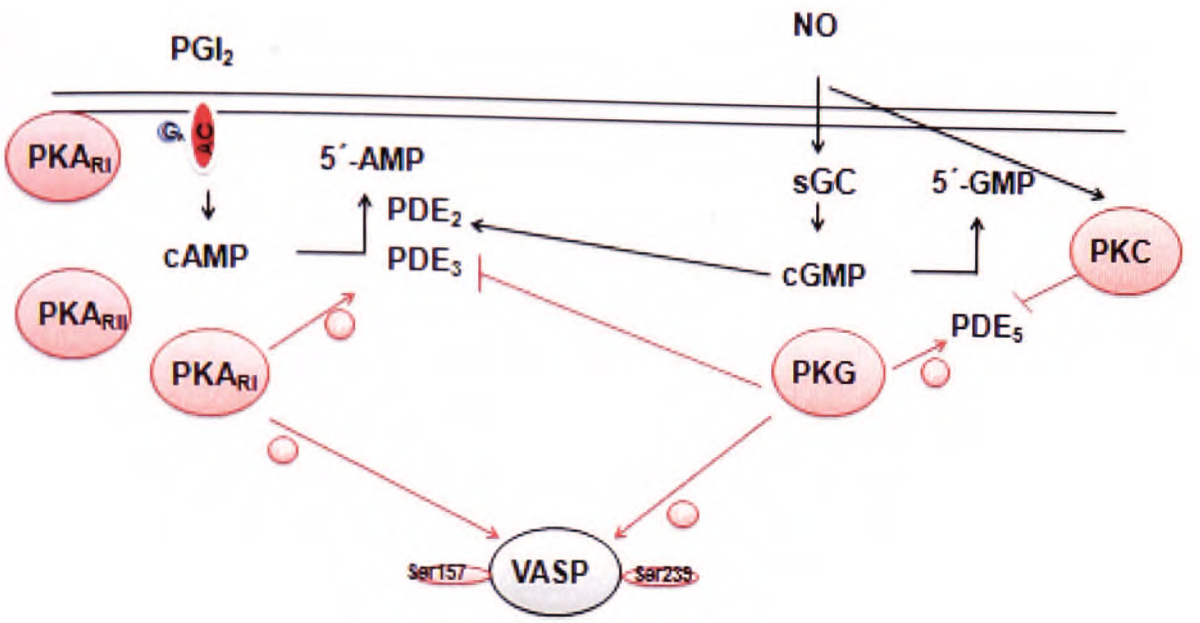



*Figure 6 1:. Schematic diagram of regulation of PKG and PKA signaling in platelets.*

*NO crosses plasma membrane and activates sGC, which lead to an increase in cGMP levels. NO also activates PKC, which may down regulate $PDE_5$ allowing sufficient increase in cGMP levels to activate PKG. Upon activation by NO, sGC may subsequently localize to lipid rafts, hence lipid rafts disruption inhibit NO-mediated $VASP^{Ser239}$ phosphorylation but not 8-Bromo-cGMP-mediated $VASP^{Ser239}$ phosphorylation. $PGI_2$ activates AC via $G_s$ proteins coupled to IP receptors, which leads to an increase in cAMP levels. Increased cAMP*

***levels activate $PKA_{RI/RII}$ isoforms. $PKA_{RI}$ is the main isoform activating VASP on Ser157 residues. Because $PKA_{RI}$ is localized in lipid rafts and non rafts domains. Lipid rafts disruption has little effect on $PKA_{RI}$-mediated $VASP^{Ser157}$ phosphorylation.***

# Appendix I

### *1- Isolation and preparation of human blood platelets.*

### *$PGE_1$ method.*

Buffers

- Acid-citrate dextrose (ACD): Glucose (113mM), Tri-sodium-citrate (29mM), NaCl (72mM), citric acid (3mM), pH 6.4
- Tyrode's buffer: NaCl (137mM), KCl (2.7mM), $MgCl_2$ (1mM), Glucose (5.6mM), $NaH_2PO_4$ (3.3mM), HEPES (20mM), pH 7.4

Equipment

- Butterfly-21 Venisystems ..... Abbot Laboratories
- Falcon Tubes (15 and 50ml) ..... Falcon, Becton Dickinson
- Centrifuge

### *pH method.*

Buffers

- 0.3M citric acid, pH 6.5
- Wash buffer: Citric acid (0.036M), EDTA (0.1M), Glucose (0.005M), KCl (0.005M), NaCl (0.09M)

Equipment

- Butterfly-21 Venisystems ..... Abbot Laboratories
- Falcon Tubes (15 and 50ml) ..... Falcon, Becton Dickinson
- Centrifuge

***2- Determination of platelet concentration.***

Buffers

- Ammonium oxalate: Ammonium oxalate (1% w/v) in $dH_2O$

Equipment

- Improved Neubauer cell counter

  Inverted light microscope

***3- Measurement of protein concentration.***

Buffers

- Tyrode's buffer: NaCl (137mM), KCl (2.7mM), $MgCl_2$ (1mM), Glucose (5.6mM), $NaH_2PO_4$ (3.3mM), HEPES (20mM), pH 7.4 : lysis buffer containing phosphatase and protease inhibitors: NaCl (150mM), Tris base (10mM), EDTA (1mM), EGTA (10mM), ( 1:1).

Assay kit

- DC protein assay kit..... Bio-Rad

Equipment

- Costar 96-well cell culture plate..... Corning Incorporated
- Multiplate reader with 750nm filter

***4- Measurement of platelet aggregation.***

Buffers

- Tyrode's buffer: NaCl (137mM), KCl (2.7mM), $MgCl_2$ (1mM), Glucose (5.6mM), $NaH_2PO_4$ (3.3mM), HEPES (20mM), pH 7.4
- Phosphate buffer: NaCl (137mM), KCl (2.7mM), $NaH_2PO_4$ (4.2mM), NaHCO3 (11.9mM).

Equipment

- Aggregation Module-Dual Channel..... Payton
- Aggregation cuvettes

***5- cGMP Enzymeimmuno Assay Biotrak System.***

Buffers

- Assay buffer: containing on dilution 0.05M sodium acetate buffer (pH 5.8), 0.02% (w/v) BSA and 0.5% (w/v) preservative.
- Standard: reconstituted by addition of lysis reagent 1 (2.5ml) to give a concentration of cGMP (10.24pmol/ml).
- cGMP antibody: reconstituted by addition of lysis reagent 2 (11ml).
- HRP-labelled cGMP conjugate: reconstituted by addition of diluted assay buffer (11ml).
- Wash buffer: containing on dilution 0.01M phosphate buffer (pH7.5) and 0.05% (v/v) Tween-20.
- 3,3',5',5'-Tetramethylbenidine (TMB)/hydrogen peroxide substrate
- Acetic anhydride
- Triethylamine.

- Lysis reagent 1: containing dodecyltrimethylammonium bromide.
- Lysis buffer A: this buffer was prepared from lysis reagent 1 and contains 0.5% solution of dodecyltrimethylammonium bromide.
- Lysis reagent 2: containing chemicals to sequester the key components of the lysis reagent 1 and ensures cGMP is free for the analysis.
- Sulphuric acid (1M): 1ml of sulphuric acid was diluted in 19ml of $dH_2O$
- Microplate: 96 wells plate coated with donkey anti-rabbit IgG.

Equipment

- Glass test tubes
- Glass aggregation cuvettes
- Microplate shaker
- Plate reader

***6- Analysis of phosphorylation based signaling in platelets.***

***Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).***

***Sample preparation.***

Buffers

- Laemmli sample buffer (2x): Tris base (50mM), SDS (4% w/v), Glycerol (20% v/v), bromophenol blue (trace), 2-mercaptoethanol (5% v/v) pH 6.8

Equipment

- Aggregation Module-Dual Channel..... Payton
- Aggregation cuvettes

### *7- Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)*

***Method.***

Buffers

- Buffer 1: Tris base (0.5M), SDS (0.4% w/v), pH 8.8
- Buffer 2: Tris base (1.5M), SDS (0.4% w/v), pH 6.8
- Ammonium persulfate (APS): APS (10% w/v) in $dH_2O$
- Running buffer: Tris base (25mM), Glycine (192mM), SDS (0.1% w/v)

Equipment

- Miniprotean 3 Cell..... Bio-Rad (UK)
- Gradient mixer ..... Bio-Rad (UK)
- Peristaltic pump
- Butterfly-21 Venisystems ..... Abbot Laboratories
- Plastic tubing
- Biotin-protein ladder..... Cell Signaling Tech. (UK)

***Gradient gel compositions for 1.5mm casting plates.***

| Compound | 3% stacking gel | 10% resolving gel | 18% resolving gel |
|---|---|---|---|
| **dH2O** | 4.87ml | 1.418ml | 0,708ml |
| **Acrylamide 30%** | 0.75ml | 1.182ml | 1.961ml |
| **Buffer I** | --- | 0.886ml | 0.886ml |
| **Buffer II** | 1.87ml | --- | --- |
| **APS 10%** | 75µl | 18µl | 18µl |
| **TEMED** | 10µl | 2µl | 2µl |

***10% gel compositions for 1.5mm casting plates.***

| Compound | 3% stacking gel | 10% resolving gel |
|---|---|---|
| dH2O | 4.87ml | 6.48ml |
| Acrylamide 30% | 0.75ml | 5.3ml |
| Buffer I | --- | 4ml |
| APS 10% | 75µl | 65µl |
| TEMED | 10µl | 5.3µl |

***8- Immunochemical investigation of platelet proteins.***

***Immunoprecipitation.***

***Classical method.***

Buffers

- lysis buffer containing phosphatase and protease inhibitors: NaCl (150mM), Tris base (10mM), EDTA (1mM), EGTA (10mM), Igepal (1% v/v), PMSF (1mM), Aprotinin (5ug/ml), Leupeptin (5ug/ml), Pepstatin (0.5ug/ml), $Na_3VO_4$ (2.5 mM), pH 7.4
- Tris buffered saline containing Tween (0.1%): NaCl (100mM), Tris base (10mM), Tween 20 (0.1% v/v), pH 7.4
- Laemmli sample buffer (2x): Tris base (50mM), SDS (4% w/v), Glycerol (20% v/v), bromophenol blue (trace), 2-mercaptoethanol (5% v/v) pH 6.8

Equipment

- Rotator
- Microcentrifuge

### *9- Cross-linking method.*

Buffers

- BPS: see different protocol.
- Dilution buffer: 1 mg/ml BSA in PBS
- Cross linking reagent: Dimethyl pimelimidate (DMP) Sigma D-8388. Stock concentration 13 mg/ml DMP in W-Buffer.
- Wash buffer: Triethanolamine Sigma T-1377 Stock concentration 0.2 M triethanolamine in PBS (3.04 ml triethanolamine per 100 ml buffer).
- Quenching buffer: Ethanolamine Sigma E-9508 Stock concentration 50 mM ethanolamine in PBS (311.7 ul per 100 ml).
- Elution reagent: 1 M glycine (Add conc. HCl to correct pH to pH3)

### *10- Immunoblotting.*

Buffers

- Transfer buffer: Tris base (25mM), Glycine (192mM), methanol (20% v/v)
- Tris buffered saline containing Tween (0.1%): NaCl (100mM), Tris base (10mM), Tween 20 (0.1% v/v), pH 7.4
- Stripping buffer: SDS (2% w/v), 2-mercaptoethanol (5% v/v) in Tris buffered saline containing Tween (0.1%)
- ECL 1: Luminol (250mM), p-coumaric acid (90mM), Tris base (100mM, pH 8.5), in 100ml using $dH_20$
- ECL 2: Tris base (100mM, pH 8.5), 64µl of $H_2O_2$ (30%), in 100ml using $dH_2O$
- ECL 1 and ECL 2 were mixed fresh at a ratio of 1:1 before use.

- Developing solution: diluted 1:5 prior to use in $dH_2O$
- Fixing solution: diluted 1:5 prior to use in $dH_2O$

Equipment

- Hybond-P PVDF membrane ..... Amersham Pharmacia Biotech
- Mini Trans-Blot elctroph. transfer cell ..... Bio-Rad (UK)
- Exposure cassette ..... Sigma Ltd (Poole, UK)
- Hyper film..... Amersham Biosciences (UK)
- Microplate shaker

***11- Subcellular fractionation.***

Buffers

- Fractionation lysis buffer: Sucrose (320mM), HEPES (4mM), Protease Inhibitors cocktail, pH 7.4.
- Pellet buffer: Tris-HCL (10mM), NaCl (158mM), EGTA (1mM), SDS (0.1% w/v), Sodium deoxycholate (1% w/v), Protease Inhibitors cocktail, pH 7.2.

Equipment

- 1.5 ml eppendorf
- Ultracentrifuge
- Liquid nitrogen

***12- Lipid rafts isolation.***

Buffers

- Tris-base (20mM), NaCl (100mM), sodium pyrophosphate (60mM), sodium glycerophosphate (20mM), sodium azide (0.02% w/v), triton X-100 (0.045%), Protease Inhibitors cocktail, pH 8.0.

Equipment

- Thin wall tubes, ultraclear (ultracentrifuge tubes). Beckman coulter. 344059
- Ultracentrifuge

***13- Phosphoflow studies.***

Buffers

- Perm Buffer III ..... (BD Biosciences, 558050).
- Fix Buffer I ..... (BD Biosciences, 557870).
- Flow-washing buffer (PBS 392.4ml, FCS 4ml, 10% sodium azide 3.6ml).

Equipment

- 96-well plate
- Centrifuge
- Flow Cytometer

# Appendix II

### *Inhibitors/Activators*

| Compound | Concentration | Target |
|---|---|---|
| AICAR | 100μM-1mM | AMPk |
| RO31-8220 | 10μM | PKC |
| Indomethacin | 10μM | $TxA_2$ |
| Apyrase | 1μ/ml | ADP |
| PN | 100μM | VASP |
| PP2 | 20μM | Src |
| PP1 | 20μM | Src |
| BAPTA | 20μM | Intracellular $Ca^{2+}$ |
| EGTA | 1mM | Extracellular $Ca^{2+}$ |
| EGCG | 100μM | Nitration |
| Wortmannin | 100nM | PI3k |
| ODQ | 20μM | sGC |
| L.NIO | 1mM | eNOS |
| GSNO | 1mM | sGC |
| Forsklin | 10μM | AC |
| H89 | 100nM | PKA |
| $PGE_1$ | 50ng | PKA |
| RIAD | 10μM | RI binding AKAPs |
| Super AKAP | 10μM | RII binding AKAPs |
| StHt-31 | 10μM | RII binding AKAPs |
| $PGI_2$ | 50μM | PKA |
| KT 5720 | 10μM | PKA |
| PMA $dH_2O$ | 300nM | PKC |
| MβCD | 5mM | Lipid rafts |

***Chemicals list.*** All other chemicals were from Sigma

| Chemical | Company |
|---|---|
| Acrylamide/Bis Solution 30% | Biorad |
| BAPTA-AM | Calbiochem |
| BIM-I | Calbiochem |
| Citric Acid | BDH AnalaR |
| Collagen Reagent Horm | Nycomed (AXIS SHIELD UK) |
| GSNO | Tocris |
| Guanosine 3'-5'-cyclic monophosphate 8-bromo-sodium salt | Calbiochem |
| H-89 dihydrochloride (protein kinase A inhibitor) | Calbiochem |
| ODQ | Calbiochem |
| PN | Calbiochem |
| PKA inhibitot (PKI) | Calbiochem |
| PP1 (scr kinase inhibitos) | Biomol |
| PP2 (Src kinase inhibitor) | Calbiochem |
| PP3 (4-amino-7-phenylpyrazol[3,4-d]pyrimidine) | Calbiochem |
| Ro 31-8220 mesylate | TOCRIS |
| Ro-31-8220 (PKC inhibitor) | Calbiochem |
| Spermine NONOate | Axxora |
| StHt-31 | Invitrogen |
| RIAD | Kind gift from Prof. Tasken |
| Scr. RIAD | Kind gift from Prof. Tasken |
| PcBlue | BD Biosciences |
| Pc Orange | BD Biosciences |

# Appendix III

***Antibody list***

| Antibody | Company |
|---|---|
| Anti-rabbit IgG:HRP | Amersham |
| Anti-AMPK and ACC Ab sampler kit | Cell signalling |
| Anti-Biotin-protein ladder | Cell signalling |
| Anti-CSK antibody | BD Transduction Labs |
| Anti-guanylyl cyclase a1, soluble (sGC) | Sigma |
| Anti-Hsp90 (Clone 68) | BD Transduction Labs |
| Anti-integrin b3 | Cemfret analytics |
| Anti-LAT | Upstate |
| Anti-mouse Ig HRP | Amersham |
| Anti-phospho-AMPK(thr172) Ab | Cell signalling |
| Anti-phospho-AMPK(thr172) Ab | Upstate |
| Anti-phospho-PKA substrate (RRXS/T) | New england bio-labs |
| Anti-phosphotyrosoine clone 4G10 | Upstate |
| Anti-phospho-VASP (Ser 157) | Cell Signalling |
| Anti-Phospho-Vasp (Ser $^{239}$) | Cell signalling |
| Anti-PKA | BD Transduction Labs |
| Anti-PKC substrate phospho Ser | Cell signalling |
| Anti-PLCγ2 (Q20) | Santa Cruz |
| Anti-rat IgG:HRP | Amersham |
| Anti-SLP76 | Upstate |
| Anti-Syk mAb | Santa Cruz Biotechnology |
| Anti-β-tubulin | Upstate |

# References


Abtahian F, Bezman N, Clemens R, Sebzda E, Cheng L, Shattil S, *et al.* (2006). Evidence for the requirement of ITAM domains but not SLP-76/Gads interaction for integrin signaling in hematopoietic cells. *Molecular and Cellular Biology* **26**(18): 6936.

Adam R (2000). The Giardia lamblia genome. *International journal for parasitology* **30**(4): 475-484.

Akkerman JW (1978). Regulation of carbohydrate metabolism in platelets. A review. *Thromb Haemost* **39**(3): 712-724.

Aktas B, Utz A, Hoenig-Liedl P, Walter U, Geiger J (2003). Dipyridamole enhances NO/cGMP-mediated vasodilator-stimulated phosphoprotein phosphorylation and signaling in human platelets: in vitro and in vivo/ex vivo studies. *Stroke* **34**(3): 764.

Alberts B, Bray D, Lewis J, Raff M, Roberts K, Watson J (2002). Molecular biology ofthe cell. *New York: Garland Science.*

Allen R, Zacharski L, Widirstky S, Rosenstein R, Zaitlin L, Burgess D (1979). Transformation and motility of human platelets: details of the shape change and release reaction observed by optical and electron microscopy. *Journal of Cell Biology* **83**(1): 126.

Aloia R (1983). *Membrane fluidity in biology.* edn. Academic Press.

Altup S, Demiryürek A, Ak D, Tungel M, k K (2001). Contribution of peroxynitrite to the beneficial effects of preconditioning on ischaemia-induced arrhythmias in rat isolated hearts. *European journal of pharmacology* **415**(2-3): 239-246.

Alvarez B, Radi R (2003). Peroxynitrite reactivity with amino acids and proteins. *Amino Acids* **25**(3): 295-311.

Ammendola A, Geiselhöringer A, Hofmann F, Schlossmann J (2001). Molecular Determinants of the Interaction between the Inositol 1, 4, 5-Trisphosphate Receptor-associated cGMP Kinase Substrate(IRAG) and cGMP Kinase Ibeta. *Journal of Biological Chemistry* **276**(26): 24153-24159.

Andrews RK, Shen Y, Gardiner EE, Dong JF, Lopez JA, Berndt MC (1999). The glycoprotein Ib-IX-V complex in platelet adhesion and signaling. *Thromb Haemost* **82**(2): 357-364.

Antl M (2006). IRAG mediates NO/cGMP-dependent inhibition of platelet aggregation and thrombus formation. *Blood.*

Arias-Salgado E, Lizano S, Sarkar S, Brugge J, Ginsberg M, Shattil S (2003). Src kinase activation by direct interaction with the integrin cytoplasmic domain. *Proceedings of the National Academy of Sciences of the United States of America* **100**(23): 13298.

Arstall M, Sawyer D, Fukazawa R, Kelly R (1999). Cytokine-mediated apoptosis in cardiac myocytes: the role of inducible nitric oxide synthase induction and peroxynitrite generation. *Circulation research* **85**(9): 829.

Aszodi A, Pfeifer A, Ahmad M, Glauner M, Zhou X, Ny L, *et al.* (1999). The vasodilator-stimulated phosphoprotein (VASP) is involved in cGMP-and cAMP-mediated inhibition of agonist-induced platelet aggregation, but is dispensable for smooth muscle function. *The EMBO Journal* **18**(1): 37.

Atkinson B, Stafford M, Pears C, Watson S (2001). Signalling events underlying platelet aggregation induced by the glycoprotein VI agonist convulxin. *European Journal of Biochemistry* **268**(20): 5242-5248.

Augusto O, Bonini M, Trindade D (2004). Spin trapping of glutathiyl and protein radicals produced from nitric oxide-derived oxidants. *Free radical biology and medicine* **36**(10): 1224-1232.

Azuma H, Ishikawa M, Sekizaki S (1986). Endothelium-dependent inhibition of platelet aggregation. *British journal of pharmacology* **88**(2): 411.

Bajzar L, Manuel R, Nesheim M (1995). Purification and characterization of TAFI, a thrombin-activable fibrinolysis inhibitor. *Journal of Biological Chemistry* **270**(24): 14477.

Balafanova Z, Bolli R, Zhang J, Zheng Y, Pass J, Bhatnagar A, *et al.* (2002a). Nitric Oxide (NO) Induces Nitration of Protein Kinase C (PKC ), Facilitating PKC Translocation via Enhanced PKC -RACK2 Interactions. *Journal of Biological Chemistry* **277**(17): 15021.

Balafanova Z, Bolli R, Zhang J, Zheng Y, Pass JM, Bhatnagar A, *et al.* (2002b). Nitric oxide (NO) induces nitration of protein kinase Cepsilon (PKCepsilon ), facilitating PKCepsilon translocation via enhanced PKCepsilon -RACK2 interactions: a novel mechanism of no-triggered activation of PKCepsilon. *J Biol Chem* **277**(17): 15021-15027.

Barnes M, Farndale R (1999). Collagens and atherosclerosis. *Experimental gerontology* **34**(4): 513-525.

Barry O, Kazanietz M (2001). Protein kinase C isozymes, novel phorbol ester receptors and cancer chemotherapy. *Current pharmaceutical design* **7**(17): 1725-1744.

Barzik M, Kotova T, Higgs H, Hazelwood L, Hanein D, Gertler F, *et al.* (2005). Ena/VASP proteins enhance actin polymerization in the presence of barbed end capping proteins. *Journal of Biological Chemistry* **280**(31): 28653.

Baum J, Brodsky B (1999). Folding of peptide models of collagen and misfolding in disease. *Current opinion in structural biology* **9**(1): 122-128.

Bearer E (1995). Cytoskeletal domains in the activated platelet. *Cell Motility and the Cytoskeleton* **30**(1): 50-66.

Bearer E, Prakash J, Manchester R, Allen P (2000). VASP protects actin filaments from gelsolin: an in vitro study with implications for platelet actin reorganizations. *Cell Motility and the Cytoskeleton* **47**(4): 351-364.

Beckman J (1990). Ischaemic injury mediator. *Nature* **345**(6270): 27.

Beckman J (1996). The physiological and pathological chemistry of nitric oxide. *Nitric Oxide: Principles and Actions* **21**.

Beckman J, Koppenol W (1996a). Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly. *American Journal of Physiology- Cell Physiology* **271**(5): C1424.

Beckman JS, Koppenol WH (1996b). Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly. *Am J Physiol* **271**(5 Pt 1): C1424-1437.

Begonja AJ, Gambaryan S, Geiger J, Aktas B, Pozgajova M, Nieswandt B, *et al.* (2005). Platelet NAD(P)H-oxidase-generated ROS production regulates {alpha}IIb{beta}3-integrin activation independent of the NO/cGMP pathway. *Blood* **106**(8): 2757-2760.

Behnke O (1968). An electron microscope study of the megacaryocyte of the rat bone marrow. I. The development of the demarcation membrane system and the platelet surface coat. *Journal of ultrastructure research* **24**(5): 412.

Bender A, Beavo J (2006). Cyclic nucleotide phosphodiesterases: molecular regulation to clinical use. *Pharmacological reviews* **58**(3): 488.

Bentfeld-Barker M, Bainton D (1982). Identification of primary lysosomes in human megakaryocytes and platelets. *Blood* **59**(3): 472.

Berndt MC, Phillips DR (1981). Interaction of thrombin with platelets: purification of the thrombin substrate. *Ann N Y Acad Sci* **370**: 87-95.

Bertoni A, Tadokoro S, Eto K, Pampori N, Parise L, White G, *et al.* (2002). Relationships between Rap1b, affinity modulation of integrin alpha IIbbeta 3, and the actin cytoskeleton. *Journal of Biological Chemistry* **277**(28): 25715.

Bessis M, Weed R (1973). Living blood cells and their ultrastructure.

Bevers E, Comfurius P, Zwaal R (1983). Changes in membrane phospholipid distribution during platelet activation. *Biochimica et Biophysica Acta (BBA)-Biomembranes* **736**(1): 57-66.

Bhagyalakshmi A, Berthiaume F, Reich K, Frangos J (1992). Fluid shear stress stimulates membrane phospholipid metabolism in cultured human endothelial cells. *Journal of vascular research* **29**(6): 443-449.

Bhatt D, Topol E (2003). Scientific and therapeutic advances in antiplatelet therapy. *Nature Reviews Drug Discovery* **2**(1): 15-28.

Bian J, Wang H, Zhang W, Wong T (1998). Effects of -opioid receptor stimulation in the heart and the involvement of protein kinase C. *British journal of pharmacology* **124**(3): 600.

Bian J, Zhang W, Pei J, Wong T (2000). The role of phosphodiesterase in mediating the effect of protein kinase C on cyclic AMP accumulation upon kappa-opioid receptor stimulation in the rat heart. *Journal of Pharmacology and Experimental Therapeutics* **292**(3): 1065.

Billah M, Lapetina E, Cuatrecasas P (1980). Phospholipase A2 and phospholipase C activities of platelets. Differential substrate specificity, Ca2+ requirement, pH dependence, and cellular localization. *Journal of Biological Chemistry* **255**(21): 10227.

Blobe G, Stribling D, Fabbro D, Stabel S, Hannun Y (1996). Protein kinase C II specifically binds to and is activated by F-actin. *Journal of Biological Chemistry* **271**(26): 15823.

Bodin S, Soulet C, Tronchere H, Sie P, Gachet C, Plantavid M, *et al.* (2005). Integrin-dependent interaction of lipid rafts with the actin cytoskeleton in activated human platelets. *Journal of Cell Science* **118**(4): 759.

Bodin S, Viala C, Ragab A, Payrastre B (2003). A critical role of lipid rafts in the organization of a key FcgammaRIIa-mediated signaling pathway in human platelets. *Thromb Haemost* **89**(2): 318-330.

Boerth N, Sadler J, Bauer D, Clements J, Gheith S, Koretzky G (2000). Recruitment of SLP-76 to the membrane and glycolipid-enriched membrane microdomains replaces the requirement for linker for activation of T cells in T cell receptor signaling. *Journal of Experimental Medicine* **192**(7): 1047.

Born G, Cross M (1963). The aggregation of blood platelets. *The Journal of physiology* **168**(1): 178.

Boulos C, Jiang H, Balazy M (2000a). Diffusion of peroxynitrite into the human platelet inhibits cyclooxygenase via nitration of tyrosine residues. *J Pharmacol Exp Ther* **293**(1): 222-229.

Boulos C, Jiang H, Balazy M (2000b). Diffusion of peroxynitrite into the human platelet inhibits cyclooxygenase via nitration of tyrosine residues. *Journal of Pharmacology and Experimental Therapeutics* **293**(1): 222.

Boylan B, Gao C, Rathore V, Gill JC, Newman DK, Newman PJ (2008). Identification of FcgammaRIIa as the ITAM-bearing receptor mediating alphaIIbbeta3 outside-in integrin signaling in human platelets. *Blood* **112**(7): 2780-2786.

Brass L (2003). Thrombin and Platelet Activation*. *Chest* **124**(3 suppl): 18S.

Brass L, Pizarro S, Ahuja M, Belmonte E, Blanchard N, Stadel J, *et al.* (1994). Changes in the structure and function of the human thrombin receptor during receptor activation, internalization, and recycling. *Journal of Biological Chemistry* **269**(4): 2943.

Brito C, Naviliat M, Tiscornia A, Vuillier F, Gualco G, Dighiero G, *et al.* (1999). Peroxynitrite inhibits T lymphocyte activation and proliferation by promoting impairment of tyrosine phosphorylation and peroxynitrite-driven apoptotic death. *The Journal of Immunology* **162**(6): 3356.

Brodsky B, Persikov A (2005). Molecular structure of the collagen triple helix. *Fibrous proteins: coiled-coils, collagen and elastomers*: 301.

Brown A, Moro M, Masse J, Cramer E, Radomski M, Darley-Usmar V (1998a). Nitric oxide-dependent and independent effects on human platelets treated with peroxynitrite. *Cardiovascular research* **40**(2): 380.

Brown D, London E (1998b). Functions of lipid rafts in biological membranes. *Annual Review of Cell and Developmental Biology* **14**(1): 111-136.

Brown D, Rose J (1992). Sorting of GPI-anchored proteins to glycolipid-enriched membrane subdomains during transport to the apical cell surface. *Cell(Cambridge)* **68**(3): 533-544.

Browning D, McShane M, Marty C, Ye R (2000). Nitric oxide activation of p38 mitogen-activated protein kinase in 293T fibroblasts requires cGMP-dependent protein kinase. *Journal of Biological Chemistry* **275**(4): 2811.

Bruckdorfer R (2005). The basics about nitric oxide. *Mol Aspects Med* **26**(1-2): 3-31.

Buensuceso C, de Virgilio M, Shattil S (2003). Detection of integrin alpha IIbbeta 3 clustering in living cells. *Journal of Biological Chemistry* **278**(17): 15217-15224.

Buensuceso C, Obergfell A, Soriani A, Eto K, Kiosses W, Arias-Salgado E, *et al.* (2005). Regulation of outside-in signaling in platelets by integrin-associated protein kinase C {beta}. *Journal of Biological Chemistry* **280**(1): 644.

Burney S, Caulfield J, Niles J, Wishnok J, Tannenbaum S (1999). The chemistry of DNA damage from nitric oxide and peroxynitrite. *Mutation Research-Fundamental and Molecular Mechanisms of Mutagenesis* **424**(1-2): 37-49.

Burridge K, Chrzanowska-Wodnicka M (1996). Focal adhesions, contractility, and signaling. *Annual Review of Cell and Developmental Biology* **12**(1): 463-519.

Burton K, Johnson B, Hausken Z, Westenbroek R, Idzerda R, Scheuer T, *et al.* (1997). Type II regulatory subunits are not required for the anchoring-dependent modulation of Ca2+ channel activity by cAMP-dependent protein kinase. *Proceedings of the National Academy of Sciences of the United States of America* **94**(20): 11067.

Butt E, Abel K, Krieger M, Palm D, Hoppe V, Hoppe J, *et al.* (1994). cAMP- and cGMP-dependent protein kinase phosphorylation sites of the focal adhesion vasodilator-stimulated phosphoprotein (VASP) in vitro and in intact human platelets. *J Biol Chem* **269**(20): 14509-14517.

Carlson CR, Lygren B, Berge T, Hoshi N, Wong W, Tasken K, *et al.* (2006). Delineation of type I protein kinase A-selective signaling events using an RI anchoring disruptor. *Journal of Biological Chemistry* **281**(30): 21535.

Carr D, Stofko-Hahn R, Fraser I, Bishop S, Acott T, Brennan R, *et al.* (1991). Interaction of the regulatory subunit (RII) of cAMP-dependent protein kinase with RII-anchoring proteins occurs through an amphipathic helix binding motif. *Journal of Biological Chemistry* **266**(22): 14188.

Cassina A, Radi R (1996). Differential inhibitory action of nitric oxide and peroxynitrite on mitochondrial electron transport. *Archives of Biochemistry and Biophysics* **328**(2): 309-316.

Cavallini L, Coassin M, Borean A, Alexandre A (1996). Prostacyclin and sodium nitroprusside inhibit the activity of the platelet inositol 1, 4, 5-trisphosphate receptor and promote its phosphorylation. *Journal of Biological Chemistry* **271**(10): 5545.

Chakrabarti S, Clutton P, Varghese S, Cox D, Mascelli M, Freedman J (2004). Glycoprotein IIb/IIIa inhibition enhances platelet nitric oxide release. *Thrombosis research* **113**(3-4): 225-233.

Chari R, Kim S, Murugappan S, Sanjay A, Daniel JL, Kunapuli SP (2009). Lyn, PKC-delta, SHIP-1 interactions regulate GPVI-mediated platelet-dense granule secretion. *Blood* **114**(14): 3056-3063.

Chen Z, McConell G, Michell B, Snow R, Canny B, Kemp B (2000). AMPK signaling in contracting human skeletal muscle: acetyl-CoA carboxylase and NO synthase phosphorylation. *American Journal of Physiology-Endocrinology And Metabolism* **279**(5): E1202.

Chen Z, Mitchelhill K, Michell B, Stapleton D, Rodriguez-Crespo I, Witters L, *et al.* (1999). AMP-activated protein kinase phosphorylation of endothelial NO synthase. *FEBS letters* **443**(3): 285-289.

Chitaley K, Chen L, Galler A, Walter U, Daum G, Clowes A (2004). Vasodilator-stimulated phosphoprotein is a substrate for protein kinase C. *FEBS letters* **556**(1-3): 211-215.

Cho M, Allen M (1978). Chemical stability of prostacyclin (PGI2) in aqueous solutions. *Prostaglandins* **15**(6): 943.

Choi Y, Kim Y, Lee K, Kim B, Kim D (2004). Protective effect of epigallocatechin gallate on brain damage after transient middle cerebral artery occlusion in rats. *Brain research* **1019**(1-2): 47-54.

Chow T, Hellums J, Moake J, Kroll M (1992). Shear stress-induced von Willebrand factor binding to platelet glycoprotein Ib initiates calcium influx associated with aggregation. *Blood* **80**(1): 113.

Christian A, Haynes M, Phillips M, Rothblat G (1997). Use of cyclodextrins for manipulating cellular cholesterol content. *The Journal of Lipid Research* **38**(11): 2264.

Chrzanowska-Wodnicka M, White G (2005). Rap1b is required for normal platelet function and hemostasis in mice. *Journal of Clinical Investigation* **115**(3): 680-687.

Clark P Immunoprecipitation with Antibody Cross-Linked to Protein G-Agarose Beads July 7, 2000.

Clemetson K, McGregor J, James E, Dechavanne M, Lüscher E (1982). Characterization of the platelet membrane glycoprotein abnormalities in Bernard-Soulier syndrome and comparison with normal by surface-labeling techniques and high-resolution two-dimensional gel electrophoresis. *Journal of Clinical Investigation* **70**(2): 304.

Clutton P, Miermont A, Freedman J (2004). Regulation of endogenous reactive oxygen species in platelets can reverse aggregation. *Arteriosclerosis, thrombosis, and vascular biology* **24**(1): 187.

Coghlan V, Penino B, Howard M, Langeberg L, Hicks J, Gallatin W, *et al.* (1989). Association of protein kinase A and protein phosphatase 2B with a common anchoring protein. *Proc. Natl. Acad. Sci. USA* **86:** 8946.

Colledge M, Scott J (1999). AKAPs: from structure to function. *Trends in Cell Biology* **9**(6)**:** 216-221.

Conti M, Adelstein R (1981). The relationship between calmodulin binding and phosphorylation of smooth muscle myosin kinase by the catalytic subunit of 3': 5'cAMP-dependent protein kinase. *Journal of Biological Chemistry* **256**(7)**:** 3178.

Cooper CE, Davies NA (2000). Effects of nitric oxide and peroxynitrite on the cytochrome oxidase K(m) for oxygen: implications for mitochondrial pathology. *Biochim Biophys Acta* **1459**(2-3)**:** 390-396.

Courtneidge S, Levinson A, Bishop J (1980). The protein encoded by the transforming gene of avian sarcoma virus (pp60src) and a homologous protein in normal cells (pp60proto-src) are associated with the plasma membrane. *Proceedings of the National Academy of Sciences* **77**(7)**:** 3783.

Crane M, Rossi A, Megson I (2005). A potential role for extracellular nitric oxide generation in cGMP-independent inhibition of human platelet aggregation: biochemical and pharmacological considerations. *British journal of pharmacology* **144**(6)**:** 849.

D'Souza S, Davis M, Baxter G (2004). Autocrine and paracrine actions of natriuretic peptides in the heart. *Pharmacology and Therapeutics* **101**(2)**:** 113-129.

Dairou J, Atmane N, Rodrigues-Lima F, Dupret J (2004). Peroxynitrite Irreversibly Inactivates the Human Xenobioticmetabolizing Enzyme Arylamine N-Acetyltransferase 1 (NAT1) in Human Breast Cancer Cells. *Journal of Biological Chemistry* **279**(9)**:** 7708.

Daniel JL, Dangelmaier C, Jin J, Ashby B, Smith JB, Kunapuli SP (1998). Molecular basis for ADP-induced platelet activation. I. Evidence for three distinct ADP receptors on human platelets. *J Biol Chem* **273**(4)**:** 2024-2029.

Danielewski O, Schultess J, Smolenski A (2005). The NO/cGMP pathway inhibits Rap 1 activation in human platelets via cGMP-dependent protein kinase I. *Thromb Haemost* **93**(2)**:** 319-325.

DarleyUsmar V, Halliwell B (1996). Blood radicals - Reactive nitrogen species, reactive oxygen species, transition metal ions, and the vascular system. *Pharmaceutical Research* **13**(5)**:** 649-662.

Davies S, Reddy H, Caivano M, Cohen P (2000). Specificity and mechanism of action of some commonly used protein kinase inhibitors. *BIOCHEMICAL JOURNAL-LONDON-* **351**(1): 95-105.

Davis P, Hill C, Keech E, Lawton G, Nixon J, Sedgwick A, *et al.* (1989). Potent selective inhibitors of protein kinase C. *FEBS letters* **259**(1): 61-63.

De Candia E, Hall S, Rutella S, Landolfi R, Andrews R, De Cristofaro R (2001). Binding of thrombin to glycoprotein Ib accelerates the hydrolysis of Par-1 on intact platelets. *Journal of Biological Chemistry* **276**(7): 4692.

De Graaf J, Banga J, Moncada S, Palmer R, De Groot P, Sixma J (1992). Nitric oxide functions as an inhibitor of platelet adhesion under flow conditions. *Circulation* **85**(6): 2284.

De Jonge H (1981). Cyclic GMP-dependent protein kinase in intestinal brushborders. *Advances in cyclic nucleotide research* **14:** 315.

DE JONGE H, LOHMANN S, WALTER U (1994). Cloning, expression, and in situ localization of rat intestinal cGMP-dependent protein kinase II. *Proc. Nati. Acad. Sci. USA* **91:** 9426-9430.

De Marco L, Mazzucato M, Masotti A, Fenton J (1991). Function of glycoprotein Ib alpha in platelet activation induced by alpha-thrombin. *Journal of Biological Chemistry* **266**(35): 23776.

Deana R, Turetta L, Donella-Deana A, Don M, Brunati A, De Michiel L, *et al.* (2003). Green tea epigallocatechin-3-gallate inhibits platelet signalling pathways triggered by both proteolytic and non-proteolytic agonists. *Thrombosis and haemostasis* **89**(5): 866.

Defer N, Best-Belpomme M, Hanoune J (2000). Tissue specificity and physiological relevance of various isoforms of adenylyl cyclase. *American Journal of Physiology- Renal Physiology* **279**(3): 400.

Denicola A, Souza J, Radi R (1998). Diffusion of peroxynitrite across erythrocyte membranes. *Proceedings of the National Academy of Sciences of the United States of America* **95**(7): 3566.

Dermine J, Duclos S, Garin J, St-Louis F, Rea S, Parton R, *et al.* (2001). Flotillin-1-enriched lipid raft domains accumulate on maturing phagosomes. *Journal of Biological Chemistry* **276**(21): 18507.

Dessauer C, Gilman A (1996). Purification and characterization of a soluble form of mammalian adenylyl cyclase. *Journal of Biological Chemistry* **271**(28): 16967.

Dickhout J, Hossain G, Pozza L, Zhou J, Lhotak S, Austin R (2005). Peroxynitrite causes endoplasmic reticulum stress and apoptosis in human

vascular endothelium: implications in atherogenesis. *Arteriosclerosis, thrombosis, and vascular biology* **25**(12): 2623.

Diczfalusy U, Falardeau P, Hammarström S (1977). Conversion of prostaglandin endoperoxides to C17-hydroxy acids catalyzed by human platelet thromboxane synthase. *FEBS letters* **84**(2): 271.

Dorsam RT, Kunapuli SP (2004). Central role of the P2Y12 receptor in platelet activation. *J Clin Invest* **113**(3): 340-345.

Doyle M, Hoekstra J (1981). Oxidation of nitrogen oxides by bound dioxygen in hemoproteins. *Journal of inorganic biochemistry* **14**(4): 351.

Droge W (2002). Free radicals in the physiological control of cell function. *Physiol Rev* **82**(1): 47-95.

Duke W (1910). The relation of blood platelets to hemorrhagic disease: Description of a method for determining the bleeding time and coagulation time and report of three cases of hemorrhagic disease relieved by transfusion. *JAMA* **55**(14): 1185.

Dusting G, Moncada S, Vane J (1977). Prostacyclin (PGX) is the endogenous metabolite responsible for relaxation of coronary arteries induced by arachindonic acid. *Prostaglandins* **13**(1): 3.

Dutil EM, Newton AC (2000). Dual role of pseudosubstrate in the coordinated regulation of protein kinase C by phosphorylation and diacylglycerol. *J Biol Chem* **275**(14): 10697-10701.

Dutta-Roy A, Sinha A (1987). Purification and properties of prostaglandin E1/prostacyclin receptor of human blood platelets. *J Biol Chem* **262**(26): 12685-12691.

Ebbe S, Stohlman Jr F (1965). Megakaryocytopoiesis in the rat. *Blood* **26**(1): 20.

Edelman A, Blumenthal D, Krebs E (1987). Protein serine/threonine kinases. *Annual review of biochemistry* **56**(1): 567-613.

Eigenthaler M, NOLTE C, HALBRÜGGE M, WALTER U (2005). Concentration and regulation of cyclic nucleotides, cyclic-nucleotide-dependent protein kinases and one of their major substrates in human platelets. *European Journal of Biochemistry* **205**(2): 471-481.

Elzagallaai A, Rose S, Brandan N, Trifaro J (2001). Myristoylated alanine-rich C kinase substrate phosphorylation is involved in thrombin-induced serotonin release from platelets. *British Journal of Haematology* **112**(3): 593-602.

Emerling B, Viollet B, Tormos K, Chandel N (2007). Compound C inhibits hypoxic activation of HIF-1 independent of AMPK. *FEBS letters*.

Ezumi Y, Kodama K, Uchiyama T, Takayama H (2002). Constitutive and functional association of the platelet collagen receptor glycoprotein VI-Fc receptor gamma-chain complex with membrane rafts. *Blood* **99**(9): 3250.

Farndale R, Sixma J, Barnes M, De Groot P (2004a). The role of collagen in thrombosis and hemostasis. *Journal of Thrombosis and Haemostasis* **2**(4): 561-573.

Farndale RW, Sixma JJ, Barnes MJ, de Groot PG (2004b). The role of collagen in thrombosis and hemostasis. *J Thromb Haemost* **2**(4): 561-573.

Feinstein P, Schrader K, Bakalyar H, Tang W, Krupinski J, Gilman A, *et al.* (1991). Molecular cloning and characterization of a Ca2+/calmodulin-insensitive adenylyl cyclase from rat brain. *Proceedings of the National Academy of Sciences* **88**(22): 10173.

Feliciello A, Gottesman M, Avvedimento E (2001). The biological functions of A-kinase anchor proteins. *Journal of Molecular Biology* **308**(2): 99-114.

Feron O, Belhassen L, Kobzik L, Smith T, Kelly R, Michel T (1996). Endothelial nitric oxide synthase targeting to caveolae. Specific interactions with caveolin isoforms in cardiac myocytes and endothelial cells. *Journal of Biological Chemistry* **271**(37): 22810.

Flaumenhaft R, Dilks J, Rozenvayn N, Monahan-Earley R, Feng D, Dvorak A (2005). The actin cytoskeleton differentially regulates platelet {alpha}-granule and dense-granule secretion. *Blood* **105**(10): 3879.

Fleming I, Schulz C, Fichtlscherer B, Kemp B, Fisslthaler B, Busse R (2003). AMP-activated protein kinase (AMPK) regulates the insulin-induced activation of the nitric oxide synthase in human platelets. *THROMBOSIS AND HAEMOSTASIS-STUTTGART-* **90**(5): 863-871.

Fox J, Boyles J, Berndt M, Steffen P, Anderson L (1988). Identification of a membrane skeleton in platelets. *Journal of Cell Biology* **106**(5): 1525.

Fox J, Phillips D (1982). Role of phosphorylation in mediating the association of myosin with the cytoskeletal structures of human platelets. *J Biol Chem* **257**(8): 4120-4126.

Francis SH, Corbin JD (1994). Structure and function of cyclic nucleotide-dependent protein kinases. *Annu Rev Physiol* **56**: 237-272.

Freedman J, Sauter R, Battinelli E, Ault K, Knowles C, Huang P, *et al.* (1999). Deficient platelet-derived nitric oxide and enhanced hemostasis in mice lacking the NOSIII gene. *Circulation research* **84**(12): 1416.

Freeman B, Crapo J (1982). Biology of disease: Free radicals and tissue injury. *Laboratory investigation; a journal of technical methods and pathology* **47**(5): 412.

Friebe A, Schultz G, Koesling D (1996). Sensitizing soluble guanylyl cyclase to become a highly CO-sensitive enzyme. *The EMBO Journal* **15**(24): 6863.

Frojmovic M, Longmire K, van de Ven TG (1990). Long-range interactions in mammalian platelet aggregation. II. The role of platelet pseudopod number and length. *Biophys J* **58**(2): 309-318.

Fulton D, Gratton J, Sessa W (2001). Post-translational control of endothelial nitric oxide synthase: why isn't calcium/calmodulin enough? *Journal of Pharmacology and Experimental Therapeutics* **299**(3): 818.

Furchgott R, Zawadzki J (1980). The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature* **288**(5789): 373-376.

Gadelha F, Thomson L, Fagian M, Costa A, Radi R, Vercesi A (1997). Ca2+-independent permeabilization of the inner mitochondrial membrane by peroxynitrite is mediated by membrane protein thiol cross-linking and lipid peroxidation. *Archives of Biochemistry and Biophysics* **345**(2): 243-250.

Gambaryan S, Geiger J, Schwarz U, Butt E, Begonja A, Obergfell A, *et al.* (2004). Potent inhibition of human platelets by cGMP analogs independent of cGMP-dependent protein kinase. *Blood* **103**(7): 2593.

Gambaryan S, Kobsar A, Hartmann S, Birschmann I, Kuhlencordt P, Muller-Esterl W, *et al.* (2008). NO-synthase-/NO-independent regulation of human and murine platelet soluble guanylyl cyclase activity. *Journal of Thrombosis and Haemostasis* **6**(8): 1376-1384.

Garbers D, Dubois S (1999). The molecular basis of hypertension. *Annual review of biochemistry* **68**(1): 127-155.

Geiger J, Nolte C, Walter U (1994). Regulation of calcium mobilization and entry in human platelets by endothelium-derived factors. *American Journal of Physiology- Cell Physiology* **267**(1): C236.

Geiselhöringer A, Werner M, Sigl K, Smital P, Wörner R, Acheo L, *et al.* (2004). IRAG is essential for relaxation of receptor-triggered smooth muscle contraction by cGMP kinase. *The EMBO Journal* **23**(21): 4222.

Gerzer R, Böhme E, Hofmann F, Schultz G (1981). Soluble guanylate cyclase purified from bovine lung contains heme and copper. *FEBS letters* **132**(1): 71.

Ghafourifar P, Schenk U, Klein S, Richter C (1999). Mitochondrial Nitric-oxide Synthase Stimulation Causes Cytochromec Release from Isolated Mitochondria. *Journal of Biological Chemistry* **274**(44): 31185.

Gkaliagkousi E, Ritter J, Ferro A (2007). Platelet-derived nitric oxide signaling and regulation. *Circulation research* **101**(7): 654.

Gkantiragas I, Brugger B, Stuven E, Kaloyanova D, Li X, Lohr K, *et al.* (2001). Sphingomyelin-enriched microdomains at the Golgi complex. *Molecular Biology of the Cell* **12**(6): 1819.

Glantz S, Li Y, Rubin C (1993). Characterization of distinct tethering and intracellular targeting domains in AKAP75, a protein that links cAMP-dependent protein kinase II beta to the cytoskeleton. *Journal of Biological Chemistry* **268**(17): 12796.

Gold M, Lygren B, Dokurno P, Hoshi N, McConnachie G, Taskén K, *et al.* (2006). Molecular basis of AKAP specificity for PKA regulatory subunits. *Molecular cell* **24**(3): 383-395.

Golde D (1991). The stem cell. *Scientific American* **265**(6): 86.

González-Utor A, Sanchez-Aguayo I, Hidalgo J (1992). Cytochemical localization of K+-dependent p-nitrophenyl phosphatase and adenylate cyclase by using one-step method in human washed platelets. *Histochemistry and Cell Biology* **97**(6): 503-507.

Gopalakrishna R, Chen Z, Gundimeda U (1993). Nitric oxide and nitric oxide-generating agents induce a reversible inactivation of protein kinase C activity and phorbol ester binding. *Journal of Biological Chemistry* **268**(36): 27180.

Gorman R, Bunting S, Miller O (1977). Modulation of human platelet adenylate cyclase by prostacyclin (PGX). *Prostaglandins* **13**(3): 377.

Gow A, Duran D, Malcolm S, Ischiropoulos H (1996). Effects of peroxynitrite-induced protein modifications on tyrosine phosphorylation and degradation. *FEBS letters* **385**(1-2): 63-66.

Gow A, Farkouh C, Munson D, Posencheg M, Ischiropoulos H (2004). Biological significance of nitric oxide-mediated protein modifications. *American Journal of Physiology- Lung Cellular and Molecular Physiology* **287**(2): L262.

Gow A, Luchsinger B, Pawloski J, Singel D, Stamler J (1999). The oxyhemoglobin reaction of nitric oxide. *Proceedings of the National Academy of Sciences of the United States of America* **96**(16): 9027.

Gross BS, Lee JR, Clements JL, Turner M, Tybulewicz VL, Findell PR, *et al.* (1999a). Tyrosine phosphorylation of SLP-76 is downstream of Syk following

stimulation of the collagen receptor in platelets. *J Biol Chem* **274**(9): 5963-5971.

Gross BS, Melford SK, Watson SP (1999b). Evidence that phospholipase C-gamma2 interacts with SLP-76, Syk, Lyn, LAT and the Fc receptor gamma-chain after stimulation of the collagen receptor glycoprotein VI in human platelets. *Eur J Biochem* **263**(3): 612-623.

Gryglewski R, Bunting S, Moncada S, Flower R, Vane J (1976). Arterial walls are protected against deposition of platelet thrombi by a substance (prostaglandin X) which they make from prostaglandin endoperoxides. *Prostaglandins* **12**(5): 685.

Guix FX, Uribesalgo I, Coma M, Munoz FJ (2005). The physiology and pathophysiology of nitric oxide in the brain. *Prog Neurobiol* **76**(2): 126-152.

Halbrugge M, Friedrich C, Eigenthaler M, Schanzenbacher P, Walter U (1990a). Stoichiometric and reversible phosphorylation of a 46-kDa protein in human platelets in response to cGMP-and cAMP-elevating vasodilators. *Journal of Biological Chemistry* **265**(6): 3088.

Halbrugge M, Walter U (1990b). Analysis, purification and properties of a 50,000-dalton membrane-associated phosphoprotein from human platelets. *J Chromatogr* **521**(2): 335-343.

Hall KJ, Jones ML, Poole AW (2007). Coincident regulation of PKCdelta in human platelets by phosphorylation of Tyr311 and Tyr565 and phospholipase C signalling. *Biochem J* **406**(3): 501-509.

Halliwell B, Gutteridge J (2007). *Free radicals in biology and medicine*. edn. Oxford University Press Oxford.

Hamm HE, Gilchrist A (1996). Heterotrimeric G proteins. *Curr Opin Cell Biol* **8**(2): 189-196.

Hanafy K, Krumenacker J, Murad F NO, nitrotyrosine, and cyclic GMP in signal transduction. *Medical science monitor: international medical journal of experimental and clinical research* **7**(4): 801.

Hanks S, Hunter T (1995). Protein kinases 6. The eukaryotic protein kinase superfamily: kinase (catalytic) domain structure and classification. *The FASEB Journal* **9**(8): 576.

Hannigan G, Leung-Hagesteijn C, Fitz-Gibbon L, Coppolino M, Radeva G, Filmus J, *et al.* (1996). Regulation of cell adhesion and anchorage-dependent growth by a new 1-integrin-linked protein kinase.

Harbeck B, Hüttelmaier S, Schlüter K, Jockusch B, Illenberger S (2000). Phosphorylation of the vasodilator-stimulated phosphoprotein regulates its interaction with actin. *Journal of Biological Chemistry* **275**(40): 30817-30825.

Hardie D, Carling D, Carlson M (1998). The AMP-activated/SNF1 protein kinase subfamily: metabolic sensors of the eukaryotic cell? *Annual review of biochemistry* **67**(1): 821-855.

Hardie DG, Carling D (1997). The AMP-activated protein kinase--fuel gauge of the mammalian cell? *Eur J Biochem* **246**(2): 259-273.

Hardie DG, Scott JW, Pan DA, Hudson ER (2003). Management of cellular energy by the AMP-activated protein kinase system. *FEBS Lett* **546**(1): 113-120.

Hardy AR, Jones ML, Mundell SJ, Poole AW (2004). Reciprocal cross-talk between P2Y1 and P2Y12 receptors at the level of calcium signaling in human platelets. *Blood* **104**(6): 1745-1752.

Harker L, Finch C (1969). Thrombokinetics in man. *Journal of Clinical Investigation* **48**(6): 963.

Harker L, Marzec U, Kelly A, Chronos N, Sundell I, Hanson S, *et al.* (1998). Clopidogrel inhibition of stent, graft, and vascular thrombogenesis with antithrombotic enhancement by aspirin in nonhuman primates. *Circulation* **98**(22): 2461.

Harmon J, Jamieson G (1986). The glycocalicin portion of platelet glycoprotein Ib expresses both high and moderate affinity receptor sites for thrombin. A soluble radioreceptor assay for the interaction of thrombin with platelets. *Journal of Biological Chemistry* **261**(28): 13224.

Harper M, Poole A (2007). Isoform-specific functions of protein kinase C: the platelet paradigm. *Biochemical Society Transactions* **35**(5): 1005-1008.

Harrison S (2003). Variation on an Src-like theme. *Cell* **112**(6): 737-740.

Haslam R, Dickinson N, Jang E (1999). Cyclic nucleotides and phosphodiesterases in platelets. *Thrombosis and haemostasis* **82**(2): 412-423.

Hathaway D, Eaton C, Adelstein R (1981). Regulation of human platelet myosin light chain kinase by the catalytic subunit of cyclic AMP-dependent protein kinase.

Hato T, Pampori N, Shattil S (1998). Complementary roles for receptor clustering and conformational change in the adhesive and signaling functions of Integrin alpha IIbbeta 3. *Journal of Cell Biology* **141**(7): 1685.

Hauser W, Knobeloch K, Eigenthaler M, Gambaryan S, Krenn V, Geiger J, *et al.* (1999). Megakaryocyte hyperplasia and enhanced agonist-induced platelet activation in vasodilator-stimulated phosphoprotein knockout mice. *Proceedings of the National Academy of Sciences* **96**(14): 8120.

Hechler B, Leon C, Vial C, Vigne P, Frelin C, Cazenave JP, *et al.* (1998). The P2Y1 receptor is necessary for adenosine 5'-diphosphate-induced platelet aggregation. *Blood* **92**(1): 152-159.

Heijnen H, Van Lier M, Waaijenborg S, Ohno-Iwashita Y, Waheed A, Inomata M, *et al.* (2003). Concentration of rafts in platelet filopodia correlates with recruitment of c-Src and CD 63 to these domains. *Journal of Thrombosis and Haemostasis* **1**(6): 1161-1173.

Hellevuo K, Berry R, Sikela J, Tabakoff B (1995). Localization of the gene for a novel human adenylyl cyclase (ADCY7) to chromosome 16. *Human genetics* **95**(2): 197-200.

Herberg F, Maleszka A, Eide T, Vossebein L, Tasken K (2000). Analysis of A-kinase anchoring protein (AKAP) interaction with protein kinase A (PKA) regulatory subunits: PKA isoform specificity in AKAP binding. *Journal of Molecular Biology* **298**(2): 329-339.

Herbert J, Dol F, Bernat A, Falotico R, Lale A, Savi P (1998). The antiaggregating and antithrombotic activity of clopidogrel is potentiated by aspirin in several experimental models in the rabbit. *Thrombosis and haemostasis* **80**(3): 512-518.

Herold S (1998). Kinetic and spectroscopic characterization of an intermediate peroxynitrite complex in the nitrogen monoxide induced oxidation of oxyhemoglobin. *FEBS letters* **439**(1-2): 85-88.

Hers I, Berlanga O, Tiekstra M, Kamiguti A, Theakston R, Watson S (2000). Evidence against a direct role of the integrin a2b1 in collagen-induced tyrosine phosphorylation in human platelets. *European Journal of Biochemistry* **267**(7): 2088-2097.

Hibbs JB, Jr., Taintor RR, Vavrin Z, Rachlin EM (1988). Nitric oxide: a cytotoxic activated macrophage effector molecule. *Biochem Biophys Res Commun* **157**(1): 87-94.

Hirao A, Hamaguchi I, Suda T, Yamaguchi N (1997). Translocation of the Csk homologous kinase (Chk/Hyl) controls activity of CD36-anchored Lyn tyrosine kinase in thrombin-stimulated platelets. *The EMBO Journal* **16**(9): 2342.

Hirata M, Hayashi Y, Ushikubi F, Yokota Y, Kageyama R, Nakanishi S, *et al.* (1991). Cloning and expression of cDNA for a human thromboxane A2 receptor.

Hoffbrand A, Catovsky D, Tuddenham E (2005). *Postgraduate haematology*. edn. Wiley-Blackwell.

Hofmann F, Bernhard D, Lukowski R, Weinmeister P (2009). cGMP regulated protein kinases (cGK). *Handb Exp Pharmacol* **191:** 137-162.

Hogg N, Singh R, Kalyanaraman B (1996). The role of glutathione in the transport and catabolism of nitric oxide. *FEBS letters* **382**(3)**:** 223-228.

Horstrup K, Jablonka B, Honig-Liedl P, Just M, Kochsiek K, Walter U (1994). Phosphorylation of focal adhesion vasodilator-stimulated phosphoprotein at Ser157 in intact human platelets correlates with fibrinogen receptor inhibition. *European Journal of Biochemistry* **225**(1)**:** 21-27.

Hsu P, Tsai A, Kulmacz R, Wang L (1999). Expression, purification, and spectroscopic characterization of human thromboxane synthase. *Journal of Biological Chemistry* **274**(2)**:** 762.

Huang K, Han T, Hyduke D, Vaughn M, Van Herle H, Hein T, *et al.* (2001). Modulation of nitric oxide bioavailability by erythrocytes. *Proceedings of the National Academy of Sciences of the United States of America* **98**(20)**:** 11771.

Huang P, Huang Z, Mashimo H, Bloch K, Moskowitz M, Bevan J, *et al.* (1995). Hypertension in mice lacking the gene for endothelial nitric oxide synthase.

Hughan SC, Hughes CE, McCarty OJ, Schweighoffer E, Soultanova I, Ware J, *et al.* (2007). GPVI potentiation of platelet activation by thrombin and adhesion molecules independent of Src kinases and Syk. *Arterioscler Thromb Vasc Biol* **27**(2)**:** 422-429.

Huie R, Padmaja S (1993). The reaction of NO with superoxide. *Free Radical Research* **18**(4)**:** 195-199.

Hynes R (2002). Integrins bidirectional, allosteric signaling machines. *Cell* **110**(6)**:** 673-687.

Hynes R (1992). Integrins: versatility, modulation, and signaling in cell adhesion. *Cell* **69**(1)**:** 11-25.

Ignarro L (1989). Biological actions and properties of endothelium-derived nitric oxide formed and released from artery and vein. *Circ Res* **65**(1)**:** 1-21.

Ignarro L (1990). Biosynthesis and metabolism of endothelium-derived nitric oxide. *Annual review of pharmacology and toxicology* **30**(1)**:** 535-560.

Ignarro L (2002). NITRIC OXIDE AS A UNIQUE SIGNALING MOLECULE IN THE. *Journal of physiology and pharmacology* **53**(4): 503-514.

Ignarro L, Buga G, Wood K, Byrns R, Chaudhuri G (1987a). Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. *Proceedings of the National Academy of Sciences* **84**(24): 9265.

Ignarro L, Napoli C (2004). Novel features of nitric oxide, endothelial nitric oxide synthase, and atherosclerosis. *Current Atherosclerosis Reports* **6**(4): 281-287.

Ignarro L, Wood K, Wolin M (1982). Activation of purified soluble guanylate cyclase by protoporphyrin: IX. *Proc. Natl Acad. ScL USA* **79:** 2870-2873.

Ignarro LJ, Buga GM, Wood KS, Byrns RE, Chaudhuri G (1987b). Endothelium-Derived Relaxing Factor Produced and Released from Artery and Vein Is Nitric-Oxide. *Proceedings of the National Academy of Sciences of the United States of America* **84**(24): 9265-9269.

Inomata M, Hayashi M, Ohno-Iwashita Y, Tsubuki S, Saido T, Kawashima S (1996). Involvement of calpain in integrin-mediated signal transduction. *Archives of Biochemistry and Biophysics* **328**(1): 129-134.

Inoue O, Suzuki-Inoue K, Dean W, Frampton J, Watson S (2003). Integrin {alpha} 2 {beta} 1 mediates outside-in regulation of platelet spreading on collagen through activation of Src kinases and PLC {gamma} 2. *Journal of Cell Biology* **160**(5): 769.

Ischiropoulos H, Zhu L, Chen J, Tsai M, Martin J, Smith C, *et al.* (1992). Peroxynitrite-mediated tyrosine nitration catalyzed by superoxide dismutase. *Archives of Biochemistry and Biophysics* **298**(2): 431.

Ishida H, Ichimori K, Hirota Y, Fukahori M, Nakazawa H (1996). Peroxynitrite-induced cardiac myocyte injury. *Free radical biology and medicine* **20**(3): 343-350.

Ishii K, Chen J, Ishii M, Koch W, Freedman N, Lefkowitz R, *et al.* (1994). Inhibition of thrombin receptor signaling by a G-protein coupled receptor kinase. Functional specificity among G-protein coupled receptor kinases. *Journal of Biological Chemistry* **269**(2): 1125.

Jamison D, Mosley W (1991). Disease control priorities in developing countries: health policy responses to epidemiological change. *American journal of public health* **81**(1): 15.

Jandrot-Perrus M, Busfield S, Lagrue A, Xiong X, Debili N, Chickering T, *et al.* (2000). Cloning, characterization, and functional studies of human and mouse glycoprotein VI: a platelet-specific collagen receptor from the immunoglobulin superfamily. *Blood* **96**(5): 1798.

JG W (1974). shape change. *Thromb Diath Haemorrh* **74;60:159–171**

Jin J, Daniel JL, Kunapuli SP (1998). Molecular basis for ADP-induced platelet activation. II. The P2Y1 receptor mediates ADP-induced intracellular calcium mobilization and shape change in platelets. *J Biol Chem* **273**(4): 2030-2034.

Johnson D, Akamine P, Radzio-Andzelm E, Madhusudan M, Taylor S (2001). Dynamics of cAMP-dependent protein kinase. *Chemical reviews* **101**(8): 2243.

Johnson R, Morton D, Kinner J, Gorman R, McGuire J, Sun F, *et al.* (1976). The chemical structure of prostaglandin X (prostacyclin). *Prostaglandins* **12**(6): 915-928.

Jones F, Qian Y, Wong H, Chan H, Yim A (1997). Prostanoid action on the human pulmonary vascular system. *Clinical and Experimental Pharmacology and Physiology* **24**(12): 969-972.

Kaboord B, Perr M (2008). Isolation of Proteins and Protein Complexes by Immunoprecipitation. *METHODS IN MOLECULAR BIOLOGY-CLIFTON THEN TOTOWA-* **424:** 349.

Kadler K, Baldock C, Bella J, Boot-Handford R (2007). Collagens at a glance. *Journal of Cell Science* **120**(12): 1955.

Kamae T, Shiraga M, Kashiwagi H, Kato H, Tadokoro S, Kurata Y, *et al.* (2006). Critical role of ADP interaction with P2Y receptor in the maintenance of alphabeta activation: association with Rap1B activation. *J Thromb Haemost* **4**(6): 1379-1387.

Kamisaki Y, Wada K, Bian K, Balabanli B, Davis K, Martin E, *et al.* (1998). An activity in rat tissues that modifies nitrotyrosine-containing proteins. *Proceedings of the National Academy of Sciences of the United States of America* **95**(20): 11584.

Katsel P, Tagliente T, Schwarz T, Craddock-Royal B, Patel N, Maayani S (2003). Molecular and biochemical evidence for the presence of Type III adenylyl cyclase in human platelets. *Platelets* **14**(1): 21-33.

Kenney D, Linck R (1985). The cystoskeleton of unstimulated blood platelets: structure and composition of the isolated marginal microtubular band. *Journal of Cell Science* **78**(1): 1.

Khan W, Blobe G, Halpern A, Taylor W, Wetsel W, Burns D, *et al.* (1993). Selective regulation of protein kinase C isoenzymes by oleic acid in human platelets. *Journal of Biological Chemistry* **268:** 5063-5063.

Kickler T (2006). Platelet biology-an overview. *Transfusion Alternatives in Transfusion Medicine* **8**(2): 79.

King P, Jamison E, Strahs D, Anderson V, Brenowitz M (1993). 'Footprinting'proteins on DNA with peroxonitrous acid. *Nucleic acids research* **21**(10): 2473.

Kirsch M, Lomonosova E, Korth H, Sustmann R, de Groot H (1998). Hydrogen peroxide formation by reaction of peroxynitrite with HEPES and related tertiary amines. Implications for a general mechanism. *Journal of Biological Chemistry* **273**(21): 12716.

Kishimoto A, Nishiyama K, Nakanishi H, Uratsuji Y, Nomura H, Takeyama Y, *et al.* (1985). Studies on the phosphorylation of myelin basic protein by protein kinase C and adenosine 3': 5'-monophosphate-dependent protein kinase. *Journal of Biological Chemistry* **260**(23): 12492.

Kissner R, Nauser T, Bugnon P, Lye P, Koppenol W (1997). Formation and properties of peroxynitrite as studied by laser flash photolysis, high-pressure stopped-flow technique, and pulse radiolysis. *Chem. Res. Toxicol* **10**(11): 1285-1292.

Klages B, Brandt U, Simon M, Schultz G, Offermanns S (1999). Activation of G12/G13 results in shape change and Rho/Rho-kinase-mediated myosin light chain phosphorylation in mouse platelets. *Journal of Cell Biology* **144**(4): 745.

Knighton D, Zheng J, Ten Eyck L, Ashford V, Xuong N, Taylor S, *et al.* (1991). Crystal structure of the catalytic subunit of cyclic adenosine monophosphate-dependent protein kinase. *Science* **253**(5018): 407.

Knowles RG, Palacios M, Palmer RM, Moncada S (1989). Formation of nitric oxide from L-arginine in the central nervous system: a transduction mechanism for stimulation of the soluble guanylate cyclase. *Proc Natl Acad Sci U S A* **86**(13): 5159-5162.

Kobayashi T, Ushikubi F, Narumiya S (2000). Amino acid residues conferring ligand binding properties of prostaglandin I and prostaglandin D receptors. Identification by site-directed mutagenesis. *Journal of Biological Chemistry* **275**(32): 24294.

Koeppen M, Feil R, Siegl D, Feil S, Hofmann F, Pohl U, *et al.* (2004). cGMP-dependent protein kinase mediates NO-but not acetylcholine-induced dilations in resistance vessels in vivo. *Hypertension* **44**(6): 952.

Kono H, Suzuki T, Yamamoto K, Okada M, Yamamoto T, Honda Z (2002). Spatial raft coalescence represents an initial step in Fc gamma R signaling. *J Immunol* **169**(1): 193-203.

Konopatskaya O, Gilio K, Harper M, Zhao Y, Cosemans J, Karim Z, *et al.* (2009a). PKC regulates platelet granule secretion and thrombus formation in mice. *The Journal of Clinical Investigation* **119**(2): 399.

Konopatskaya O, Gilio K, Harper MT, Zhao Y, Cosemans JM, Karim ZA, *et al.* (2009b). PKCalpha regulates platelet granule secretion and thrombus formation in mice. *J Clin Invest* **119**(2): 399-407.

Koppenol W (1999). Chemistry of peroxynitrite and its relevance to biological systems. *Metal ions in biological systems* **36:** 597.

Kroeze W, Sheffler D, Roth B (2003). G-protein-coupled receptors at a glance. *Journal of Cell Science* **116**(24): 4867.

Krutzik P, Nolan G (2006). Fluorescent cell barcoding in flow cytometry allows high-throughput drug screening and signaling profiling. *Nature Methods* **3**(5): 361.

Ku D, Liu S, Dai J (1995). Coronary vascular and antiplatelet effects of peroxynitrite in human tissues. *Endothelium* **3**(4): 309-319.

Kunapuli SP, Dorsam RT, Kim S, Quinton TM (2003). Platelet purinergic receptors. *Curr Opin Pharmacol* **3**(2): 175-180.

Kuo W, Kanadia R, Shanbhag V, Toro R (1999). Denitration of peroxynitrite-treated proteins by 'protein nitratases' from rat brain and heart. *Molecular and cellular biochemistry* **201**(1): 11-16.

Kuo W, Kocis J, Webb J (2002). Protein denitration/modification by Escherichia coli nitrate reductase and mammalian cytochrome P-450 reductase. *Front Biosci* **7:** a9-a14.

Kwon NS, Nathan CF, Stuehr DJ (1989). Reduced biopterin as a cofactor in the generation of nitrogen oxides by murine macrophages. *J Biol Chem* **264**(34): 20496-20501.

Laemmli U (1970a). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**(5259): 680-685.

Laemmli UK (1970b). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**(259): 680-685.

Lancaster J (1997). A tutorial on the diffusibility and reactivity of free nitric oxide. *Nitric Oxide* **1**(1): 18-30.

Lansman J, Hallam T, Rink T (1987). Single stretch-activated ion channels in vascular endothelial cells as mechanotransducers?

Lassegue B, Clempus R (2003). Vascular NAD (P) H oxidases: specific features, expression, and regulation. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology* **285**(2): 277.

Laurent V, Loisel T, Harbeck B, Wehman A, Grobe L, Jockusch B, *et al.* (1999). Role of proteins of the Ena/VASP family in actin-based motility of Listeria monocytogenes. *Journal of Cell Biology* **144**(6): 1245.

Lee FA, van Lier M, Relou IA, Foley L, Akkerman JW, Heijnen HF, *et al.* (2006). Lipid rafts facilitate the interaction of PECAM-1 with the glycoprotein VI-FcR gamma-chain complex in human platelets. *J Biol Chem* **281**(51): 39330-39338.

Li J, Li W, Su J, Liu W, Altura B, Altura B (2004). Peroxynitrite induces apoptosis in rat aortic smooth muscle cells: possible relation to vascular diseases. *Experimental Biology and Medicine* **229**(3): 264.

Liang M, Knox F (1999). Nitric oxide activates PKCalpha and inhibits Na+-K+-ATPase in opossum kidney cells. *American Journal of Physiology- Renal Physiology* **277**(6): 859.

Liu S, Beckman J, Ku D (1994). Peroxynitrite, a product of superoxide and nitric oxide, produces coronary vasorelaxation in dogs. *Journal of Pharmacology and Experimental Therapeutics* **268**(3): 1114.

Lloyd-Jones M, DM, Bloch M, KD (1996). The vascular biology of nitric oxide and its role in atherogenesis. *Annual review of medicine* **47**(1): 365-375.

Loftus J, Albrecht R (1984). Redistribution of the fibrinogen receptor of human platelets after surface activation. *Journal of Cell Biology* **99**(3): 822.

London F (2003). The protein kinase C inhibitor RO318220 potentiates thrombin-stimulated platelet-supported prothrombinase activity. *Blood* **102**(7): 2472.

Lorand L, Konishi K (1964). ACTIVATION OF THE FIBRIN STABILIZING FACTOR OF PLASMA BY THROMBIN. *Arch Biochem Biophys* **105**: 58-67.

Loscalzo J, Schafer A (2002). *Thrombosis and hemorrhage*. edn. Lippincott Williams & Wilkins.

Low SY, Sabetkar M, Bruckdorfer KR, Naseem KM (2002). The role of protein nitration in the inhibition of platelet activation by peroxynitrite. *FEBS Lett* **511**(1-3): 59-64.

Lowry O, Rosebrough N, Farr A, Randall R (1951). Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**(1): 265-275.

Lufrano M, Balazy M (2003). Interactions of peroxynitrite and other nitrating substances with human platelets: the role of glutathione and peroxynitrite permeability. *Biochem Pharmacol* **65**(4): 515-523.

Maccaglia A, Mallozzi C, Minetti M (2003). Differential effects of quercetin and resveratrol on Band 3 tyrosine phosphorylation signalling of red blood cells. *Biochemical and biophysical research communications* **305**(3): 541-547.

Mackman N (2004). Role of tissue factor in hemostasis, thrombosis, and vascular development. *Arteriosclerosis, thrombosis, and vascular biology* **24**(6): 1015.

Macphee C, Reifsnyder D, Moore T, Lerea K, Beavo J (1988). Phosphorylation results in activation of a cAMP phosphodiesterase in human platelets. *Journal of Biological Chemistry* **263**(21): 10353.

Maeda H, Inazu T, Nagai K, Maruyama S, Nakagawara G, Yamamura H (1995). Possible involvement of protein-tyrosine kinases such as p72syk in the disc-sphere change response of porcine platelets. *Journal of Biochemistry* **117**(6): 1201.

Malinski T, Taha Z, Grunfeld S, Patton S, Kapturczak M, Tomboulian P (1993). Diffusion of nitric oxide in the aorta wall monitored in situ by porphyrinic microsensors. *Biochemical and biophysical research communications(Print)* **193**(3): 1076-1082.

Mallozzi C, Di Stasi M, Minetti M (2001a). Peroxynitrite-dependent activation of src tyrosine kinases lyn and hck in erythrocytes is under mechanistically different pathways of redox control. *Free radical biology and medicine* **30**(10): 1108-1117.

Mallozzi C, Di Stasi MA, Minetti M (2001b). Peroxynitrite-dependent activation of src tyrosine kinases lyn and hck in erythrocytes is under mechanistically different pathways of redox control. *Free Radic Biol Med* **30**(10): 1108-1117.

Mann K, Jenny R, Krishnaswamy S (1988). Cofactor proteins in the assembly and expression of blood clotting enzyme complexes. *Annual review of biochemistry* **57**(1): 915-956.

Manning G, Whyte D, Martinez R, Hunter T, Sudarsanam S (2002). The protein kinase complement of the human genome. *Science* **298**(5600): 1912.

Marcondes S, Cardoso M, Morganti R, Thomazzi S, Lilla S, Murad F, *et al.* (2006a). Cyclic GMP-independent mechanisms contribute to the inhibition of platelet adhesion by nitric oxide donor: A role for -actinin nitration. *Proceedings of the National Academy of Sciences* **103**(9): 3434.

Marcondes S, Cardoso MH, Morganti RP, Thomazzi SM, Lilla S, Murad F, *et al.* (2006b). Cyclic GMP-independent mechanisms contribute to the inhibition of platelet adhesion by nitric oxide donor: A role for {alpha}-actinin nitration. *Proc Natl Acad Sci U S A.*

Marcondes S, Cardoso MH, Morganti RP, Thomazzi SM, Lilla S, Murad F, *et al.* (2006c). Cyclic GMP-independent mechanisms contribute to the inhibition of platelet adhesion by nitric oxide donor: a role for alpha-actinin nitration. *Proc Natl Acad Sci U S A* **103**(9): 3434-3439.

Marcus A, Broekman M, Drosopoulos J, Islam N, Alyonycheva T, Safier L, *et al.* (1997). The endothelial cell ecto-ADPase responsible for inhibition of platelet function is CD39. *Journal of Clinical Investigation* **99**(6): 1351.

Marletta M, Yoon P, Iyengar R, Leaf C, Wishnok J (1988a). Macrophage oxidation of L-arginine to nitrite and nitrate: nitric oxide is an intermediate. *Biochemistry* **27**(24): 8706-8711.

Marletta MA, Yoon PS, Iyengar R, Leaf CD, Wishnok JS (1988b). Macrophage oxidation of L-arginine to nitrite and nitrate: nitric oxide is an intermediate. *Biochemistry* **27**(24): 8706-8711.

Marodi L, Goda K, Palicz A, Szabo G (2001). Cytokine receptor signalling in neonatal macrophages: defective STAT-1 phosphorylation in response to stimulation with IFN. *Clinical and Experimental Immunology* **126**(3): 456.

Martel V, Racaud-Sultan C, Dupe S, Marie C, Paulhe F, Galmiche A, *et al.* (2001). Conformation, localization, and integrin binding of talin depend on its interaction with phosphoinositides. *Journal of Biological Chemistry* **276**(24): 21217.

Massberg S, Sausbier M, Klatt P, Bauer M, Pfeifer A, Siess W, *et al.* (1999). Increased adhesion and aggregation of platelets lacking cyclic guanosine 3', 5'-monophosphate kinase I. *Journal of Experimental Medicine* **189**(8): 1255.

McCall TB, Boughton-Smith NK, Palmer RM, Whittle BJ, Moncada S (1989). Synthesis of nitric oxide from L-arginine by neutrophils. Release and interaction with superoxide anion. *Biochem J* **261**(1): 293-296.

McCubrey J, May W, Duronio V, Mufson A (2000). Serine/threonine phosphorylation in cytokine signal transduction. *Leukemia* **14**(1): 9-21.

MCKNIGHT G (1986). Isolation of cDNA clones coding for the catalytic subunit of mouse cAMP-dependent protein kinase. *Proc. Nati. Acad. Sci. USA* **83**: 1300-1304.

Mellor H, Parker P (1998). The extended protein kinase C superfamily. *Biochemical Journal* **332**(2): 281-292.

Mergia E, Friebe A, Dangel O, Russwurm M, Koesling D (2006). Spare guanylyl cyclase NO receptors ensure high NO sensitivity in the vascular system. *Journal of Clinical Investigation* **116**(6): 1731-1737.

Michelson AD (2006). Platelets, 2nd edition. *Academic Press.*

Miersch S, Espey M, Chaube R, Akarca A, Tweten R, Ananvoranich S, *et al.* (2008). Plasma membrane cholesterol content affects nitric oxide diffusion dynamics and signaling. *Journal of Biological Chemistry* **283**(27): 18513.

Minetti M, Mallozzi C, Di Stasi AM (2002). Peroxynitrite activates kinases of the src family and upregulates tyrosine phosphorylation signaling. *Free Radic Biol Med* **33**(6): 744-754.

Miranti CK, Leng L, Maschberger P, Brugge JS, Shattil SJ (1998). Identification of a novel integrin signaling pathway involving the kinase Syk and the guanine nucleotide exchange factor Vav1. *Curr Biol* **8**(24): 1289-1299.

Moncada S, Bolanos JP (2006). Nitric oxide, cell bioenergetics and neurodegeneration. *J Neurochem* **97**(6): 1676-1689.

Moncada S, Gryglewski R, Bunting S, Vane JR (1976). An enzyme isolated from arteries transforms prostaglandin endoperoxides to an unstable substance that inhibits platelet aggregation. *Nature* **263**(5579): 663-665.

Moncada S, Higgs E, Vane J (1977a). Human arterial and venous tissues generate prostacyclin (prostaglandin x), a potent inhibitor of platelet aggregation. *Lancet* **1**(8001): 18.

Moncada S, Higgs EA, Hodson HF, Knowles RG, Lopezjaramillo P, McCall T, *et al.* (1991). The L-Arginine - Nitric-Oxide Pathway. *Journal of Cardiovascular Pharmacology* **17:** S1-S9.

Moncada S, Higgs EA, Vane JR (1977b). Human arterial and venous tissues generate prostacyclin (prostaglandin x), a potent inhibitor of platelet aggregation. *Lancet* **1**(8001): 18-20.

Moncada S, Palmer RM, Higgs EA (1989). Biosynthesis of nitric oxide from L-arginine. A pathway for the regulation of cell function and communication. *Biochem Pharmacol* **38**(11): 1709-1715.

Moncada S, Palmer RM, Higgs EA (1988a). The discovery of nitric oxide as the endogenous nitrovasodilator. *Hypertension* **12**(4): 365-372.

Moncada S, Radomski MW, Palmer RM (1988b). Endothelium-derived relaxing factor. Identification as nitric oxide and role in the control of vascular tone and platelet function. *Biochem Pharmacol* **37**(13): 2495-2501.

Mondoro T, Shafer B, Vostal J (1997). Peroxynitrite-induced tyrosine nitration and phosphorylation in human platelets. *Free radical biology and medicine* **22**(6): 1055-1063.

Morley S, Bierer B (2001). The actin cytoskeleton, membrane lipid microdomains, and T cell signal transduction. *Advances in immunology* **77**: 1.

Moro M, Darley-Usmar V, Goodwin D, Read N, Zamora-Pino R, Feelisch M, *et al.* (1994). Paradoxical fate and biological action of peroxynitrite on human platelets. *Proceedings of the National Academy of Sciences* **91**(14): 6702.

Moro M, Darley-Usmar V, Lizasoain I, Su Y, Knowles R, Radomski M, *et al.* (1995). The formation of nitric oxide donors from peroxynitrite. *British journal of pharmacology* **116**(3): 1999.

Moro M, Russel R, Cellek S, Lizasoain I, Su Y, Darley-Usmar V, *et al.* (1996). cGMP mediates the vascular and platelet actions of nitric oxide: confirmation using an inhibitor of the soluble guanylyl cyclase. *Proc Natl Acad Sci USA* **93**(4): 1480-1485.

Mosior M, Newton A (1995). Mechanism of interaction of protein kinase C with phorbol esters. *Journal of Biological Chemistry* **270**(43): 25526.

Mullershausen F, Friebe A, Feil R, Thompson W, Hofmann F, Koesling D (2003). Direct activation of PDE5 by cGMP: long-term effects within NO/cGMP signaling. *Journal of Cell Biology* **160**(5): 719.

Munzel T, Feil R, Mulsch A, Lohmann S, Hofmann F, Walter U (2003). Physiology and pathophysiology of vascular signaling controlled by cyclic guanosine 3', 5'-cyclic monophosphate-dependent protein kinase. *Circulation* **108**(18): 2172.

Murata T, Ushikubi F, Matsuoka T, Hirata M, Yamasaki A, Sugimoto Y, *et al.* (1997). Altered pain perception and inflammatory response in mice lacking prostacyclin receptor. *Nature* **388**(6643): 678-682.

Murohara T, Parkinson S, Waldman S, Lefer A (1995). Inhibition of nitric oxide biosynthesis promotes P-selectin expression in platelets: role of protein kinase C. *Arteriosclerosis, thrombosis, and vascular biology* **15**(11): 2068.

Murray R, Granner D, Harper H, Mayes P, Rodwell V (2003). *Harper's illustrated biochemistry*. edn. McGraw-Hill Medical.

Nagata D, Mogi M, Walsh K AMP-activated protein kinase (AMPK) signaling in endothelial cells. *Journal of Biological Chemistry*.

Nakagawa O, Tanaka I, Usui T, Harada M, Sasaki Y, Itoh H, *et al.* (1994). Molecular cloning of human prostacyclin receptor cDNA and its gene expression in the cardiovascular system. *Circulation* **90**(4): 1643.

Narumiya S, Sugimoto Y, Ushikubi F (1999). Prostanoid receptors: structures, properties, and functions. *Physiological reviews* **79**(4): 1193.

Naseem KM, Bruckdorfer KR (1995). Hydrogen peroxide at low concentrations strongly enhances the inhibitory effect of nitric oxide on platelets. *Biochem J* **310 ( Pt 1)**: 149-153.

Naseem KM, Khan J, Jacobs M, Bruckdorfer KR (1997). Nitration of platelet cytosolic proteins by peroxynitrite. *Biochem Soc Trans* **25**(3): 397S.

Naseem KM, Low SY, Sabetkar M, Bradley NJ, Khan J, Jacobs M, *et al.* (2000). The nitration of platelet cytosolic proteins during agonist-induced activation of platelets. *FEBS Lett* **473**(1): 119-122.

Naseem KM, Riba R (2008). Unresolved roles of platelet nitric oxide synthase. *J Thromb Haemost* **6**(1): 10-19.

Nedvetsky P, Meurer S, Opitz N, Nedvetskaya T, Müller H, Schmidt H (2007). Heat shock protein 90 regulates stabilization rather than activation of soluble guanylate cyclase. *FEBS letters*.

Needleman P, Moncada S, Bunting S, Vane J, Hamberg M, Samuelsson B (1976). Identification of an enzyme in platelet microsomes which generates thromboxane A2 from prostaglandin endoperoxides. *Nature* **261**(5561): 558.

Newlon M, Roy M, Hausken Z, Scott J, Jennings P (1997). The A-kinase anchoring domain of type IIalpha cAMP-dependent protein kinase is highly helical. *Journal of Biological Chemistry* **272**(38): 23637.

Newman D, Hoffman S, Kotamraju S, Zhao T, Wakim B, Kalyanaraman B, *et al.* (2002). Nitration of PECAM-1 ITIM tyrosines abrogates phosphorylation and SHP-2 binding. *Biochemical and biophysical research communications* **296**(5): 1171-1179.

Newton A (2003). The ins and outs of protein kinase C. *METHODS IN MOLECULAR BIOLOGY-CLIFTON THEN TOTOWA-* **233**: 3-8.

Newton A (2001). Protein kinase C: structural and spatial regulation by phosphorylation, cofactors, and macromolecular interactions. *Chem. Rev* **101**(8): 2353-2364.

Ni H, Denis C, Subbarao S, Degen J, Sato T, Hynes R, *et al.* (2000). Persistence of platelet thrombus formation in arterioles of mice lacking both von Willebrand factor and fibrinogen. *Journal of Clinical Investigation* **106**(3): 385-392.

Nieswandt B, Brakebusch C, Bergmeier W, Schulte V, Bouvard D, Mokhtari-Nejad R, *et al.* (2001). Glycoprotein VI but not 2 1 integrin is essential for platelet interaction with collagen. *The EMBO Journal* **20**(9): 2120.

Nieswandt B, Watson SP (2003). Platelet-collagen interaction: is GPVI the central receptor? *Blood* **102**(2): 449-461.

Nishizuka Y (1995). Protein kinase C and lipid signaling for sustained cellular responses. *The FASEB Journal* **9**(7): 484.

Nonami Y (1997). The role of nitric oxide in cardiac surgery. *Surg Today* **27**(7): 583-592.

Nowak P, Wachowicz B (2002). Peroxynitrite-mediated modification of fibrinogen affects platelet aggregation and adhesion. *Platelets* **13**(5-6): 293-299.

Obergfell A, Eto K, Mocsai A, Buensuceso C, Moores S, Brugge J, *et al.* (2002). Coordinate interactions of Csk, Src, and Syk kinases with {alpha} IIb {beta} 3 initiate integrin signaling to the cytoskeleton. *Journal of Cell Biology* **157**(2): 265.

Obergfell A, Judd B, del Pozo M, Schwartz M, Koretzky G, Shattil S (2001). The Molecular Adapter SLP-76 Relays Signals from Platelet Integrin alpha IIbbeta 3 to the Actin Cytoskeleton. *Journal of Biological Chemistry* **276**(8): 5916.

Oberprieler NG, Roberts W, Riba R, Graham AM, Homer-Vanniasinkam S, Naseem KM (2007). cGMP-independent inhibition of integrin alphaIIbbeta3-mediated platelet adhesion and outside-in signalling by nitric oxide. *FEBS Lett* **581**(7): 1529-1534.

Odell T, Jackson C, Reiter R (1968). Generation cycle of rat megakaryocytes. *Exp Cell Res* **53**: 321.

Offermanns S, Laugwitz K, Spicher K, Schultz G (1994). G proteins of the G12 family are activated via thromboxane A2 and thrombin receptors in human platelets. *Proc Natl Acad Sci USA* **91**(2): 504-508.

Ogawa M (1993). Differentiation and proliferation of hematopoietic stem cells. *Blood* **81**(11): 2844.

Okada M, Nada S, Yamanashi Y, Yamamoto T, Nakagawa H (1991). CSK: a protein-tyrosine kinase involved in regulation of src family kinases. *Journal of Biological Chemistry* **266**(36): 24249.

Olson ST, Bjork I (1994). Regulation of thrombin activity by antithrombin and heparin. *Semin Thromb Hemost* **20**(4): 373-409.

Orstavik S, Natarajan V, Tasken K, Jahnsen T, Sandberg M (1997). Characterization of the human gene encoding the type I alpha and type I beta cGMP-dependent protein kinase (PRKG1). *Genomics* **42**(2): 311-318.

Ostrom R, Insel P (2004). The evolving role of lipid rafts and caveolae in G protein-coupled receptor signaling: implications for molecular pharmacology. *British journal of pharmacology* **143**(2): 235.

Otey C, Pavalko F, Burridge K (1990). An interaction between alpha-actinin and the beta 1 integrin subunit in vitro. *Journal of Cell Biology* **111**(2): 721.

Özüyaman B, Gödecke A, Küsters S, Kirchhoff E, Scharf R, Schrader J (2005). Endothelial nitric oxide synthase plays a minor role in inhibition of arterial thrombus formation. *Thrombosis and haemostasis* **93**(6): 1161-1167.

Pacher P, Szabo C (2006). Role of peroxynitrite in the pathogenesis of cardiovascular complications of diabetes. *Curr Opin Pharmacol* **6**(2): 136-141.

Packard R, Libby P (2008). Inflammation in atherosclerosis: from vascular biology to biomarker discovery and risk prediction. *Clinical chemistry* **54**(1): 24.

Palacios M, Knowles RG, Palmer RM, Moncada S (1989). Nitric oxide from L-arginine stimulates the soluble guanylate cyclase in adrenal glands. *Biochem Biophys Res Commun* **165**(2): 802-809.

Palmer RM, Ashton DS, Moncada S (1988). Vascular endothelial cells synthesize nitric oxide from L-arginine. *Nature* **333**(6174): 664-666.

Papapetropoulos A, Rudic R, Sessa W (1999). Molecular control of nitric oxide synthases in the cardiovascular system. *Cardiovascular research* **43**(3): 509.

Papapetropoulos A, Zhou Z, Gerassimou C, Yetik G, Venema R, Roussos C, *et al.* (2005). Interaction between the 90-kDa heat shock protein and soluble guanylyl cyclase: physiological significance and mapping of the domains mediating binding. *Molecular pharmacology* **68**(4): 1133.

Parente L, Perretti M (2003). Advances in the pathophysiology of constitutive and inducible cyclooxygenases: two enzymes in the spotlight. *Biochemical pharmacology* **65**(2): 153-159.

Patel B, Sharifi M, Milward AD, Oberprieler NG, Gibbins JM, Parkin S, *et al.* (2006). Platelet nitric oxide synthase is activated by tyrosine dephosphorylation: possible role for SHP-1 phosphatase. *J Thromb Haemost* **4**(11): 2423-2432.

Pearce L, Komander D, Alessi D (2010). The nuts and bolts of AGC protein kinases. *Nature Reviews Molecular Cell Biology* **11**(1): 9-22.

Pears CJ, Thornber K, Auger JM, Hughes CE, Grygielska B, Protty MB, *et al.* (2008). Differential roles of the PKC novel isoforms, PKCdelta and PKCepsilon, in mouse and human platelets. *PLoS One* **3**(11): e3793.

Pearson R, Kemp B (1991). Protein kinase phosphorylation site sequences and consensus specificity motifs: tabulations. *Methods in enzymology* **200:** 62.

Pfeifer A, Klatt P, Massberg S, Ny L, Sausbier M, Hirneiss C, *et al.* (1998). Defective smooth muscle regulation in cGMP kinase I-deficient mice. *The EMBO Journal* **17**(11): 3045.

Pigazzi A, Heydrick S, Folli F, Benoit S, Michelson A, Loscalzo J (1999). Nitric oxide inhibits thrombin receptor-activating peptide-induced phosphoinositide 3-kinase activity in human platelets. *Journal of Biological Chemistry* **274**(20): 14368.

Ping P, Takano H, Zhang J, Tang X, Qiu Y, Li R, *et al.* (1999). Isoform-selective activation of protein kinase C by nitric oxide in the heart of conscious rabbits: a signaling mechanism for both nitric oxide–induced and ischemia-induced preconditioning. *Circulation research* **84**(5): 587.

Polgar J, Clemetson J, Kehrel B, Wiedemann M, Magnenat E, Wells T, *et al.* (1997). Platelet activation and signal transduction by convulxin, a C-type lectin from Crotalus durissus terrificus (tropical rattlesnake) venom via the p62/GPVI collagen receptor. *Journal of Biological Chemistry* **272**(21): 13576.

Prekeris R, Hernandez R, Mayhew M, White M, Terrian D (1998). Molecular analysis of the interactions between protein kinase C- and filamentous actin. *Journal of Biological Chemistry* **273**(41): 26790.

Prekeris R, Mayhew M, Cooper J, Terrian D (1996). Identification and localization of an actin-binding motif that is unique to the epsilon isoform of protein kinase C and participates in the regulation of synaptic function. *Journal of Cell Biology* **132**(1): 77.

Prior I, Parton R, Hancock J (2003). Observing cell surface signaling domains using electron microscopy. *Science Signaling* **2003**(177).

Pryor W, Squadrito G (1995). The chemistry of peroxynitrite: a product from the reaction of nitric oxide with superoxide. *American Journal of Physiology-Lung Cellular and Molecular Physiology* **268**(5): 699.

Pula G, Schuh K, Nakayama K, Nakayama K, Walter U, Poole A (2006). PKC {delta} regulates collagen-induced platelet aggregation through inhibition of VASP-mediated filopodia formation. *Blood* **108**(13): 4035.

Quinton T, Kim S, Jin J, Kunapuli S (2005). Lipid rafts are required in Galpha (i) signaling downstream of the P2Y12 receptor during ADP-mediated platelet activation. *J Thromb Haemost* **3**(5): 1036-1041.

Radi R, Beckman J, Bush K, Freeman B (1991a). Peroxynitrite-induced membrane lipid peroxidation: the cytotoxic potential of superoxide and nitric oxide. *Archives of biochemistry and biophysics(Print)* **288**(2): 481-487.

Radi R, Beckman J, Bush K, Freeman B (1991b). Peroxynitrite oxidation of sulfhydryls. The cytotoxic potential of superoxide and nitric oxide. *Journal of Biological Chemistry* **266**(7): 4244.

Radomski M, Palmer R, Moncada S (1990). Characterization of the L-arginine: nitric oxide pathway in human platelets. *British journal of pharmacology* **101**(2): 325.

Radomski MW, Salas E (1995). Nitric oxide--biological mediator, modulator and factor of injury: its role in the pathogenesis of atherosclerosis. *Atherosclerosis* **118 Suppl:** S69-80.

Rafnar T, Peebles RS, Brummet ME, Catipovic B, Imani F, MacGlashan DW, *et al.* (1998). Stimulation of the high-affinity IgE receptor results in the tyrosine phosphorylation of a 60 kD protein which is associated with the protein-tyrosine kinase, Csk. *Mol Immunol* **35**(4): 249-257.

Ramakrishnan V, DeGuzman F, Bao M, Hall S, Leung L, Phillips D (2001). A thrombin receptor function for platelet glycoprotein Ib–IX unmasked by cleavage of glycoprotein V. *Proceedings of the National Academy of Sciences* **98**(4): 1823.

Rathore VB, Okada M, Newman PJ, Newman DK (2007). Paxillin family members function as Csk-binding proteins that regulate Lyn activity in human and murine platelets. *Biochem J* **403**(2): 275-281.

Rauterberg J, Jaeger E, Althaus M (1993). Collagens in atherosclerotic vessel wall lesions. *Current topics in pathology. Ergebnisse der Pathologie* **87:** 163.

Ray R, Shah A (2005). NADPH oxidase and endothelial cell function. *Clinical Science* **109:** 217-226.

Rees D, Ades S, Singer S, Hynes R (1990). Sequence and domain structure of talin.

Reinhard M, Jarchau T, Walter U (2001). Actin-based motility: stop and go with Ena/VASP proteins. *Trends in Biochemical Sciences* **26**(4): 243-249.

Riba R, Oberprieler NG, Roberts W, Naseem KM (2006). Von Willebrand factor activates endothelial nitric oxide synthase in blood platelets by a GPIb-dependent mechanism. *Journal of thrombosis and haemostasis* **12:** 2638 - 2644.

Riba R, Patel B, Aburima A, Naseem KM (2008). Globular adiponectin increases cGMP formation in blood platelets independently of nitric oxide. *J Thromb Haemost* **6**(12): 2121-2131.

RMJ M, Higgs E (1991). Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol Rev* **43**(2): 109-142.

Roberts W, Riba R, Homer-Vanniasinkam S, Farndale RW, Naseem KM (2008). Nitric oxide specifically inhibits integrin-mediated platelet adhesion and spreading on collagen. *J Thromb Haemost* **6**(12): 2175-2185.

Robertson B, Schubert R, Hescheler J, Nelson M (1993). cGMP-dependent protein kinase activates Ca-activated K channels in cerebral artery smooth muscle cells. *American Journal of Physiology- Cell Physiology* **265**(1): C299.

Rubbo H, Batthyany C, Radi R (2000). Nitric oxide-oxygen radicals interactions in atherosclerosis. *Biol Res* **33**(2): 167-175.

Rubbo H, O'Donnell V (2005). Nitric oxide, peroxynitrite and lipoxygenase in atherogenesis: mechanistic insights. *Toxicology* **208**(2): 305-317.

Ruggeri Z (2002a). Platelets in atherothrombosis. *Nature medicine* **8**(11): 1227-1234.

Ruggeri ZM (2002b). Platelets in atherothrombosis. *Nat Med* **8**(11): 1227-1234.

Rusak T, Tomasiak M, Ciborowski M (2006). Peroxynitrite can affect platelet responses by inhibiting energy production. *ACTA BIOCHIMICA POLONICA-ENGLISH EDITION-* **53**(4): 769.

Russwurm M, Koesling D (2002). Isoforms of NO-sensitive guanylyl cyclase. *Molecular and cellular biochemistry* **230**(1): 159-164.

Sabetkar M, Low SY, Naseem KM, Bruckdorfer KR (2002). The nitration of proteins in platelets: significance in platelet function. *Free Radic Biol Med* **33**(6): 728-736.

Sakariassen K, Nievelstein P, Coller B, Sixma J (1986). The role of platelet membrane glycoproteins Ib and IIb-IIIa in platelet adherence to human artery subendothelium. *British Journal of Haematology* **63**(4): 681-691.

Salgo M, Bermudez E, Squadrito G, Pryor W (1995a). Peroxynitrite causes DNA damage and oxidation of thiols in rat thymocytes [corrected]. *Archives of Biochemistry and Biophysics* **322**(2): 500.

Salgo M, Stone K, Squadrito G, Battista J, Pryor W (1995b). Peroxynitrite causes DNA nicks in plasmid pBR322. *Biochemical and biophysical research communications* **210**(3): 1025-1030.

Salvemini D, de Nucci G, Gryglewski RJ, Vane JR (1989). Human neutrophils and mononuclear cells inhibit platelet aggregation by releasing a nitric oxide-like factor. *Proc Natl Acad Sci U S A* **86**(16): 6328-6332.

Sambrano G, Weiss E, Zheng Y, Huang W, Coughlin S (2001). Role of thrombin signalling in platelets in haemostasis and thrombosis. *Nature* **413**(6851): 74-78.

Sanders K, Ward S (1992). Nitric oxide as a mediator of nonadrenergic noncholinergic neurotransmission. *American Journal of Physiology-Gastrointestinal and Liver Physiology* **262**(3): 379.

Sausbier M, Schubert R, Voigt V, Hirneiss C, Pfeifer A, Korth M, *et al.* (2000). Mechanisms of NO/cGMP-dependent vasorelaxation. *Circulation research* **87**(9): 825.

Savage B, Saldívar E, Ruggeri Z (1996). Initiation of platelet adhesion by arrest onto fibrinogen or translocation on von Willebrand factor. *Cell* **84**(2): 289-298.

Schlossmann J, Ammendola A, Ashman K, Zong X, Huber A, Neubauer G, *et al.* (2000). Regulation of intracellular calcium by a signalling complex of IRAG, IP3 receptor and cGMP kinase Ibeta. *Nature* **404**(6774): 197-201.

Schmidt K, Pfeiffer S, Mayer B (1998). Reaction of peroxynitrite with HEPES or MOPS results in the formation of nitric oxide donors. *Free radical biology and medicine* **24**(5): 859-862.

Schraw T, Lemons P, Dean W, Whiteheart S (2003). A role for Sec1/Munc18 proteins in platelet exocytosis. *Biochemical Journal* **374**(Pt 1): 207.

Schroeder P, Klotz LO, Buchczyk DP, Sadik CD, Schewe T, Sies H (2001). Epicatechin selectively prevents nitration but not oxidation reactions of peroxynitrite. *Biochem Biophys Res Commun* **285**(3): 782-787.

Schroit A, Zwaal R (1991). Transbilayer movement of phospholipids in red cell and platelet membranes. *BBA-Reviews on Biomembranes* **1071**(3): 313-329.

Schwarz U, Geiger J, Walter U, Eigenthaler M (1999). Flow cytometry analysis of intracellular VASP phosphorylation for the assessment of

activating and inhibitory signal transduction pathways in human platelets--definition and detection of ticlopidine/clopidogrel effects. *Thrombosis and haemostasis* **82**(3): 1145.

Schwarz U, Walter U, Eigenthaler M (2001a). Taming platelets with cyclic nucleotides. *Biochemical pharmacology* **62**(9): 1153-1161.

Schwarz UR, Walter U, Eigenthaler M (2001b). Taming platelets with cyclic nucleotides. *Biochem Pharmacol* **62**(9): 1153-1161.

Seamon K, Padgett W, Daly J (1981). Forskolin: unique diterpene activator of adenylate cyclase in membranes and in intact cells. *Proceedings of the National Academy of Sciences* **78**(6): 3363.

Seno T, Inoue N, Gao D, Okuda M, Sumi Y, Matsui K, *et al.* (2001). Involvement of NADH/NADPH oxidase in human platelet ROS production. *Thrombosis research* **103**(5): 399-409.

Serafini M, Mallozzi C, Di Stasi AM, Minetti M (2005). Peroxynitrite-dependent upregulation of SRC kinases in red blood cells: strategies to study the activation mechanisms. *Methods Enzymol* **396:** 215-229.

Shapiro AL, Vinuela E, Maizel JV, Jr. (1967). Molecular weight estimation of polypeptide chains by electrophoresis in SDS-polyacrylamide gels. *Biochem Biophys Res Commun* **28**(5): 815-820.

Shattil S, Brass L (1987). Induction of the fibrinogen receptor on human platelets by intracellular mediators. *Journal of Biological Chemistry* **262**(3): 992.

Shattil S, Cunningham M, Wiedmer T, Zhao J, Sims P, Brass L (1992). Regulation of glycoprotein IIb-IIIa receptor function studied with platelets permeabilized by the pore-forming complement proteins C5b-9. *Journal of Biological Chemistry* **267**(26): 18424.

Shattil S, Kashiwagi H, Pampori N (1998). Integrin signaling: the platelet paradigm. *Blood* **91**(8): 2645.

Shaw A, Vosper A (1977). Solubility of nitric oxide in aqueous and nonaqueous solvents. *Journal of the Chemical Society, Faraday Transactions 1* **73:** 1239-1244.

Shen J, Luscinskas F, Connolly A, Dewey Jr C, Gimbrone Jr M (1992). Fluid shear stress modulates cytosolic free calcium in vascular endothelial cells. *American Journal of Physiology- Cell Physiology* **262**(2): C384.

Shinitzky M (1984). Membrane fluidity and cellular functions. *Physiology of membrane fluidity* **1:** 1-51.

Shoji S, Parmelee D, Wade R, Kumar S, Ericsson L, Walsh K, *et al.* (1981). Complete amino acid sequence of the catalytic subunit of bovine cardiac muscle cyclic AMP-dependent protein kinase. *Proceedings of the National Academy of Sciences* **78**(2): 848.

Shrimpton C, Borthakur G, Larrucea S, Cruz M, Dong J, Lopez J (2002). Localization of the adhesion receptor glycoprotein Ib-IX-V complex to lipid rafts is required for platelet adhesion and activation. *Journal of Experimental Medicine* **196**(8): 1057.

Sicheri F, Moarefi I, Kuriyan J (1997). Crystal structure of the Src family tyrosine kinase Hck. *Nature* **385**(6617): 602-609.

Siess W (1989). Molecular mechanisms of platelet activation. *Physiol Rev* **69**(1): 58-178.

Siess W, Lapetina E (1989). Prostacyclin inhibits platelet aggregation induced by phorbol ester or Ca2+ ionophore at steps distal to activation of protein kinase C and Ca2+-dependent protein kinases. *Biochemical Journal* **258**(1): 57.

Simons K, Ikonen E (1997). Functional rafts in cell membranes. *Nature* **387**(6633): 569-572.

Smit M, Verzijl D, Iyengar R (1998). Identity of adenylyl cyclase isoform determines the rate of cell cycle progression in NIH 3T3 cells. *Proceedings of the National Academy of Sciences* **95**(25): 15084.

Smolenski A, Bachmann C, Reinhard K, Honig-Liedl P, Jarchau T, Hoschuetzky H, *et al.* (1998). Analysis and regulation of vasodilator-stimulated phosphoprotein serine 239 phosphorylation in vitro and in intact cells using a phosphospecific monoclonal antibody. *Journal of Biological Chemistry* **273**(32): 20029.

Smyth E, FitzGerald G (2002). Human prostacyclin receptor. *Vitamins and hormones* **65**: 149.

Smyth M (1996). Analytical Biochemistry David J. Holme and Hazel Peck, 2nd edn., Longman, Harlow, 1993 (ISBN 0-582-06694). xv+ 507 pp. Price£ 19.99. *Analytica Chimica Acta* **319**(3): 394-394.

Sorbara L, Davies-Hill T, Koehler-Stec E, Vannucci S, Horne M, Simpson I (1997). Thrombin-induced translocation of GLUT3 glucose transporters in human platelets. *Biochemical Journal* **328**(Pt 2): 511.

Soriani A, Moran B, de Virgilio M, Kawakami T, Altman A, Lowell C, *et al.* (2006). A role for PKCtheta in outside-in alpha (IIb) beta3 signaling. *J Thromb Haemost* **4**(3): 648-655.

Spisni E, Griffoni C, Santi S, Riccio M, Marulli R, Bartolini G, *et al.* (2001). Colocalization prostacyclin (PGI2) synthase–caveolin-1 in endothelial cells and new roles for PGI2 in angiogenesis. *Experimental Cell Research* **266**(1): 31-43.

Stachowiak O, Dolder M, Wallimann T, Richter C (1998). Mitochondrial creatine kinase is a prime target of peroxynitrite-induced modification and inactivation. *Journal of Biological Chemistry* **273**(27): 16694.

Stasch J, Schmidt P, Nedvetsky P, Nedvetskaya T, HS A, Meurer S, *et al.* (2006). Targeting the heme-oxidized nitric oxide receptor for selective vasodilatation of diseased blood vessels. *Journal of Clinical Investigation* **116**(9): 2552-2561.

Stenberg P, Pestina T, Barrie R, Jackson C (1997). The Src family kinases, Fgr, Fyn, Lck, and Lyn, colocalize with coated membranes in platelets. *Blood* **89**(7): 2384.

Stevens J, Jordan P, Sage T, Gibbins J (2004). The regulation of integrin-linked kinase in human platelets: evidence for involvement in the regulation of integrin alpha 2 beta 1. *Journal of Thrombosis and Haemostasis* **2**(8): 1443-1452.

Stokka A, Gesellchen F, Carlson C, Scott J, Herberg F, Taskén K (2006). Characterization of A-kinase-anchoring disruptors using a solution-based assay. *Biochemical Journal* **400**(Pt 3): 493.

Stone J, Marletta M (1994). Soluble guanylate cyclase from bovine lung: activation with nitric oxide and carbon monoxide and spectral characterization of the ferrous and ferric states. *Biochemistry* **33**(18): 5636.

Sudo T, Ito H, Kimura Y (2003). Phosphorylation of the vasodilator-stimulated phosphoprotein (VASP) by the anti-platelet drug, cilostazol, in platelets. *Platelets* **14**(6): 381-390.

Sundaresan PaF, R. W. (2003). Platelet p38 MAP kinase phosphorylation is required by α2β1 through Src family kinases and protein *Platelets* **13**: 361.

Suzuki-Inoue K, Inoue O, Frampton J, Watson S (2003). Murine GPVI stimulates weak integrin activation in PLC {gamma} 2-/-platelets: involvement of PLC {gamma} 1 and PI3-kinase. *Blood* **102**(4): 1367.

Suzuki-Inoue K, Yatomi Y, Asazuma N, Kainoh M, Tanaka T, Satoh K, *et al.* (2001). Rac, a small guanosine triphosphate-binding protein, and p21-activated kinase are activated during platelet spreading on collagen-coated surfaces: roles of integrin alpha 2beta 1. *Blood* **98**(13): 3708.

Suzuki M, Furuuchi K, Tonoki H, Ozaki T, Iizuka K, Murakami T, *et al.* (1999). A novel A-kinase anchoring protein in the heart interacts with G alpha 13. *Jpn Heart J* **40**(2): 199-208.

Szabó C, Ohshima H (1997). DNA damage induced by peroxynitrite: subsequent biological effects. *Nitric Oxide* **1**(5): 373-385.

Tabuchi A, Yoshioka A, Higashi T, Shirakawa R, Nishioka H, Kita T, *et al.* (2003). Direct demonstration of involvement of protein kinase Calpha in the Ca2+-induced platelet aggregation. *J Biol Chem* **278**(29): 26374-26379.

Takakura K, Beckman J, MacMillan-Crow L, Crow J (1999a). Rapid and irreversible inactivation of protein tyrosine phosphatases PTP1B, CD45, and LAR by peroxynitrite. *Archives of Biochemistry and Biophysics* **369**(2): 197-207.

Takakura K, Beckman JS, MacMillan-Crow LA, Crow JP (1999b). Rapid and irreversible inactivation of protein tyrosine phosphatases PTP1B, CD45, and LAR by peroxynitrite. *Arch Biochem Biophys* **369**(2): 197-207.

Tanimura N, Saitoh S, Kawano S, Kosugi A, Miyake K (2006). Palmitoylation of LAT contributes to its subcellular localization and stability. *Biochemical and biophysical research communications* **341**(4): 1177-1183.

Tasken K (2009). Waking up regulatory T cells. *Blood* **114**(6): 1136-1137.

Tasken K, Aandahl EM (2004). Localized effects of cAMP mediated by distinct routes of protein kinase A. *Physiol Rev* **84**(1): 137-167.

Taylor S, Knighton D, Zheng J, Ten Eyck L, Sowadski J (1992). Structural framework for the protein kinase family. *Annual review of cell biology* **8**(1): 429-462.

Taylor SS, Buechler JA, Yonemoto W (1990). cAMP-dependent protein kinase: framework for a diverse family of regulatory enzymes. *Annu Rev Biochem* **59**: 971-1005.

Tesmer J, Sprang S (1998). The structure, catalytic mechanism and regulation of adenylyl cyclase. *Current opinion in structural biology* **8**(6): 713-719.

Theilig F, Bostanjoglo M, Pavenstadt H, Grupp C, Holland G, Slosarek I, *et al.* (2001). Cellular distribution and function of soluble guanylyl cyclase in rat kidney and liver. *Journal of the American Society of Nephrology* **12**(11): 2209.

Towbin H, Staehelin T, Gordon J (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci U S A* **76**(9): 4350-4354.

Tsai J, Harrison J, Martin J, Hamilton T, van der Woerd M, Jablonsky M, *et al.* (1994). Role of Conformation of Peroxynitrite Anion (ONOO-) with Its Stability and Toxicity. *Journal of the American Chemical Society* **116**(9): 4115-4116.

Turner M, Schweighoffer E, Colucci F, Di Santo J, Tybulewicz V (2000). Tyrosine kinase SYK: essential functions for immunoreceptor signalling. *Immunology Today* **21**(3): 148-154.

Udovichenko I, Cunnick J, Gonzalez K, Takemoto D (1994). Functional effect of phosphorylation of the photoreceptor phosphodiesterase inhibitory subunit by protein kinase C. *Journal of Biological Chemistry* **269**(13): 9850.

Vainchenker W, Kieffer N (1988). Human megakaryocytopoiesis: in vitro regulation and characterization of megakaryocytic precursor cells by differentiation markers. *Blood reviews* **2**(2): 102.

van der Meijden PE, Munnix IC, Auger JM, Govers-Riemslag JW, Cosemans JM, Kuijpers MJ, *et al.* (2009). Dual role of collagen in factor XII-dependent thrombus formation. *Blood* **114**(4): 881-890.

Van der Vliet A, Smith D, O'neill C, Kaur H, Darley-Usmar V, Cross C, *et al.* (1994). Interactions of peroxynitrite with human plasma and its constituents: oxidative damage and antioxidant depletion. *Biochemical Journal* **303**(Pt 1): 295.

Van Meer G (1989). Lipid traffic in animal cells. *Annual review of cell biology* **5**: 247.

Vang T, Abrahamsen H, Myklebust S, Horejsi V, Tasken K (2003). Combined spatial and enzymatic regulation of Csk by cAMP and protein kinase a inhibits T cell receptor signaling. *Journal of Biological Chemistry* **278**(20): 17597-17600.

Vang T, Torgersen K, Sundvold V, Saxena M, Levy F, Skalhegg B, *et al.* (2001). Activation of the COOH-terminal Src kinase (Csk) by cAMP-dependent protein kinase inhibits signaling through the T cell receptor. *Journal of Experimental Medicine* **193**(4): 497.

Vargas J, Radomski M, Moncada S (1982). The use of prostacyclin in the separation from plasma and washing of human platelets. *Prostaglandins* **23**(6): 929.

Veillette A, Latour S, Davidson D (2002). Negative regulation of immunoreceptor signaling. *Annu Rev Immunol* **20**: 669-707.

Von Willebrand E (1926). Hereditar pseudohemofili. *Finska Lak-Sallsk Handl* **67**: 87-112.

Wade R, Castro C (1996). Reactions of Oxymyoglobin with NO, NO2, and NO2-under Argon and in Air. *Chem. Res. Toxicol* **9**(8): 1382-1390.

Walsh D, Perkins J, Krebs E (1968). An adenosine 3', 5'-monophosphate-dependant protein kinase from rabbit skeletal muscle. *Journal of Biological Chemistry* **243**(13): 3763.

Walter U (1989). Physiological role of cGMP and cGMP-dependent protein kinase in the cardiovascular system. *Rev Physiol Biochem Pharmacol* **113:** 41-88.

Warner T, Mitchell J, Sheng H, Murad F (1994). Effects of cyclic GMP on smooth muscle relaxation. *Advances in pharmacology (San Diego, Calif.)* **26:** 171.

Weber S, Bernhard D, Lukowski R, Weinmeister P, Worner R, Wegener J, *et al.* (2007). Rescue of cGMP kinase I knockout mice by smooth muscle specific expression of either isozyme. *Circulation research.*

Weiss H (2003). The discovery of the antiplatelet effect of aspirin: a personal reminiscence. *Journal of thrombosis and haemostasis: JTH* **1**(9): 1869.

Weiss H, Aledort L (1967). Impaired platelet-connective-tissue reaction in man after aspirin ingestion. *Lancet* **2**(7514): 495-497.

Wentworth JK, Pula G, Poole AW (2006). Vasodilator-stimulated phosphoprotein (VASP) is phosphorylated on Ser157 by protein kinase C-dependent and -independent mechanisms in thrombin-stimulated human platelets. *Biochem J* **393**(Pt 2): 555-564.

Wettschureck N, Offermanns S (2002). Rho/Rho-kinase mediated signaling in physiology and pathophysiology. *J Mol Med* **80**(10): 629-638.

Whisnant R, Gilman A, Dessauer C (1996). Interaction of the two cytosolic domains of mammalian adenylyl cyclase. *Proc Natl Acad Sci USA* **93**(13): 6621-6625.

White J (1974). Electron microscopic studies of platelet secretion. *Progress in hemostasis and thrombosis* **2**(0): 49.

Whittaker N, Bunting S, Salmon J, Moncada S, Vane JR, Johnson RA, *et al.* (1976). The chemical structure of prostaglandin X (prostacyclin). *Prostaglandins* **12**(6): 915-928.

Wise H, Wong Y, Jones R (2000). Prostanoid signal integration and cross talk. *Neurosignals* **11**(1): 20-28.

Wojtaszewski J, Jørgensen S, Hellsten Y, Hardie D, Richter E (2002). Glycogen-Dependent Effects of 5-Aminoimidazole-4-Carboxamide (AICA)-Riboside on AMP-Activated Protein Kinase and Glycogen Synthase Activities in RatSkeletal Muscle. *Diabetes* **51**(2): 284.

Wonerow P, Obergfell A, Wilde J, Bobe R, Asazuma N, Brdicka T, *et al.* (2002a). Differential role of glycolipid-enriched membrane domains in glycoprotein VI-and integrin-mediated phospholipase Cgamma2 regulation in platelets. *Biochemical Journal* **364**(Pt 3): 755.

Wonerow P, Obergfell A, Wilde JI, Bobe R, Asazuma N, Brdicka T, *et al.* (2002b). Differential role of glycolipid-enriched membrane domains in glycoprotein VI- and integrin-mediated phospholipase Cgamma2 regulation in platelets. *Biochem J* **364**(Pt 3): 755-765.

Wong S, Parker E, Ross E (1990). Chimeric muscarinic cholinergic: beta-adrenergic receptors that activate Gs in response to muscarinic agonists. *Journal of Biological Chemistry* **265**(11): 6219.

Wong W, Scott JD (2004). AKAP signalling complexes: focal points in space and time. *Nature Reviews Molecular Cell Biology* **5**(12): 959-970.

Woodside D, Obergfell A, Leng L, Wilsbacher J, Miranti C, Brugge J, *et al.* (2001). Activation of Syk protein tyrosine kinase through interaction with integrin cytoplasmic domains. *Current Biology* **11**(22): 1799-1804.

Woodside D, Obergfell A, Talapatra A, Calderwood D, Shattil S, Ginsberg M (2002). The N-terminal SH 2 Domains of Syk and ZAP-70 Mediate Phosphotyrosine-independent Binding to Integrin beta Cytoplasmic Domains. *Journal of Biological Chemistry* **277**(42): 39401-39408.

Woulfe D (2005). Platelet G protein-coupled receptors in hemostasis and thrombosis. *Journal of Thrombosis and Haemostasis* **3**(10): 2193-2200.

Wu M, Pritchard Jr K, Kaminski P, Fayngersh R, Hintze T, Wolin M (1994). Involvement of nitric oxide and nitrosothiols in relaxation of pulmonary arteries to peroxynitrite. *American Journal of Physiology- Heart and Circulatory Physiology* **266**(5): H2108.

Wykes V, Garthwaite J (2004). Membrane-association and the sensitivity of guanylyl cyclase-coupled receptors to nitric oxide. *British journal of pharmacology* **141**(7): 1087.

Xu W, Harrison SC, Eck MJ (1997). Three-dimensional structure of the tyrosine kinase c-Src. *Nature* **385**(6617): 595-602.

Yamaji S, Suzuki A, Kanamori H, Mishima W, Takabayashi M, Fujimaki K, *et al.* (2002). Possible role of ILK–affixin complex in integrin–cytoskeleton

linkage during platelet aggregation. *Biochemical and biophysical research communications* **297**(5): 1324-1331.

Yan S, Hahn D, Huang Z, Tang W (1996). Two Cytoplasmic Domains of Mammalian Adenylyl Cyclase Form a G-and Forskolin-activated Enzyme in Vitro. *Journal of Biological Chemistry* **271**(18): 10941.

Yaqub S, Abrahamsen H, Zimmerman B, Kholod N, Torgersen K, Mustelin T, *et al.* (2003). Activation of C-terminal Src kinase (Csk) by phosphorylation at serine-364 depends on the Csk-Src homology 3 domain. *Biochemical Journal* **372**(Pt 1): 271.

Yeagle P (1985). Cholesterol and the cell membrane. *BBA-Reviews on Biomembranes* **822**(3-4): 267-287.

Yin K, Lai P, Rodriguez A, Spur B, Wong P (1995). Antithrombotic effects of peroxynitrite: inhibition and reversal of aggregation in human platelets. *Prostaglandins* **50**(3): 169-178.

Yoshida K, Mizukami Y, Kitakaze M (1999). Nitric oxide mediates protein kinase C isoform translocation in rat heart during postischemic reperfusion. *BBA-Molecular Basis of Disease* **1453**(2): 230-238.

Zheng Y, Liu C, Chen H, Locke D, Ryan J, Kahn M (2001). Expression of the platelet receptor GPVI confers signaling via the Fc receptor gamma-chain in response to the snake venom convulxin but not to collagen. *Journal of Biological Chemistry* **276**(16): 12999-13006.

Zhou G, Myers R, Li Y, Chen Y, Shen X, Fenyk-Melody J, *et al.* (2001). Role of AMP-activated protein kinase in mechanism of metformin action. *Journal of Clinical Investigation* **108**(8): 1167-1174.

Zhou Q, Hellermann G, Solomonson L (1995). Nitric oxide release from resting human platelets. *Thrombosis research* **77**(1): 87.

Zhuo M, Hu Y, Schultz C, Kandel E, Hawkins R (1994). Role of guanylyl cyclase and cGMP-dependent protein kinase in long-term potentiation.

Zou M, Hou X, Shi C, Nagata D, Walsh K, Cohen R (2002). Modulation by peroxynitrite of AKt-and AMP-activated kinase-dependent serine phosphorylation of endothelial nitric oxide synthase. *Journal of Biological Chemistry*: 204512200.

Zou MH, Hou XY, Shi CM, Kirkpatick S, Liu F, Goldman MH, *et al.* (2003). Activation of 5'-AMP-activated kinase is mediated through c-Src and phosphoinositide 3-kinase activity during hypoxia-reoxygenation of bovine aortic endothelial cells. Role of peroxynitrite. *J Biol Chem* **278**(36): 34003-34010.

(1968). Inhibition of adenosine diphosphate-induced secondary aggregation and other platelet functions by acetylsalicylic acid ingestion. p 547.

Zwaal R, Comfurius P, Bevers E (1998). Lipid–protein interactions in blood coagulation. *BBA-Reviews on Biomembranes* **1376**(3): 433-453.