The Role of Scavenger Receptors in Oxidised Low-Density Lipoprotein induced Platelet Hyperactivity

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Abstract

Increased platelet hyperactivity is a common consequence of hyperlipidemia and is associated with increased risk of acute coronary syndromes. Platelet interaction with oxidised low-density lipoprotein (oxLDL) is believed to play a crucial role in platelet hyperactivity, although the mechanisms underpinning this are poorly understood. The aims of this study was to further understand the effects of oxLDL on platelet function. We performed a detailed characterisation of the conditions of LDL oxidation that generated a biologically active group of particles. Using this LDL preparation we show that oxLDL caused platelet degranulation, shape change and aggregation. This occurred through ligation to the receptor CD36, shown via pharmaceutical and knockout mouse data, with other evidence suggesting a supporting role for lectin-like oxidised LDL receptor 1 (LOX-1). OxLDL promoted adhesion to collagen, but not fibrinogen in human platelets, which was lost in murine platelets deficient in CD36. Interestingly, we found that whilst oxLDL did not promote thrombus formation on collagen, it reduced the effects of both cyclic guanosine monophosphate and cyclic adenosine signaling pathways on platelet thrombus formation. We next examined the mechanisms underpinning ability of oxLDL to alter platelet function. Using immunoblotting, we found a central role for tyrosine kinase signaling in oxLDL mediated activation of platelets. We showed inactivation of Src family kinases, spleen tyrosine kinase (Syk), phospholipase Cγ2 (PLCγ2) and phosphoinositide 3-kinase (PI3-K), using a range of established pharmaceutical inhibitors, prevented platelet spreading to oxLDL. We later focused on PLCγ2 in greater detail, showing the protein to be tyrosine phosphorylated in platelets in response to oxLDL. We attempted to confirm these results in PLCγ2 deficient mice, although these mice showed no altered response to the oxidised lipid KOdiA-PC, requiring further investigation. The findings of this report support previous findings of a tyrosine kinase dependent signalling pathway and offer novel insight into how oxLDL is able to affect platelet reactivity by modulation of the effects of endogenous inhibitors. In summary, this data could suggest that the primary

effect of oxLDL is to change the balance between platelet activatory and inhibitory pathways and therefore reduce the threshold for platelet activation.

Publications

Published articles

- Magwenzi, S., **Woodward, C.**, Wraith, K.S., Aburima, A., Raslan, Z., Jones, H., McNeil, C., Wheatcroft, S., Yuldasheva, N., Febbriao, M., Kearney, M. and Naseem K.M. (2015) Oxidized LDL activates blood platelets through CD36/NOX2–mediated inhibition of the cGMP/protein kinase G signaling cascade. *Blood*, 125(17), pp.2693-2703.
- Arman, M., Lindergard, G., Adams, Y., **Woodward, C**., Naseem, K., Higgins, M.K., Claessens, A., Ghumra, A., Raza, A., Rowe, J.A. (2016) Identification of a CD36-binding and platelet-activating malaria parasite adhesin for platelet-mediated clumping *Blood* (In submission)
- Berger, M., **Woodward, C.**, Wraith, K.S., Aburima, A., Raslan, Z., Febbriao, M. and Naseem, K.M. (2016) Dyslipidemia associated atherogenic oxidised lipids induce platelet hyperactivity through CD36 and PLCγ2 dependent reactive oxygen species generation. (In preparation)
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Oral Presentations

- Oxidised LDL activates platelets via a CD36 - PLCγ2 dependent pathway – Hull York Medical School Postgraduate Conference 2015, Hull, UK
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- Oxidised LDL activates platelets via a CD36 - PLCγ2 dependent pathway – International Atherosclerosis Association 2015, Amsterdam, The Netherlands
- Oxidised LDL activates platelets by causing tyrosine phosphorylation and PKC activation - Hull York Medical School Postgraduate Conference 2014, York, UK

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Abbreviations

αIIbβ3	Integrin alpha IIb beta 3 a
ACD	Acid citrate dextrose
ADP	Adenosine 5'-diphosphate
ApoB-100	Apolipoprotein B-100
APS	Ammonium persulfate
ATP	Adenosine 5'-triphosphate
BSA	Bovine serum albumin
Ca^{2+}	Calcium ion
cAMP	Cyclic adenosine monophosphate
CD36	Cluster of differentiation 36
cGMP	Cyclic guanosine monophosphate
CLESH	CD36 LIMP II Emp structural homology
CM	Chylomicrons
COX-1	Cyclooxygenase-1
CRP	Collagen related peptide
Cu^{2+}	Copper ion
CVD	Cardiovascular disease
DAG	1, 2 diacylglycerol
DMSO	Dimethyl sulfoxide
DTS	Dense tubular system
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethyleneglycoltetraacetic acid
FcR γ-chain	Fc receptor gamma chain
FITC	Fluorescein isothiocyanate
GP	Glycoprotein

GPIb-V-IX	Glycoprotein Ib-V-IX receptor complex
GPCR	G protein coupled receptor
GPVI	Glycoprotein VI
HDL	High density lipoproteins
HDS	High density solution
HEPES	N-(2-Hydroxyethyl) piperayine-N'-(2ethanesulfonic acid)
HSA	Human serum albumin
IDL	Intermediate density lipoproteins
IgG	Immunoglobulin G
IP_3	Inositol 1,5,4-trisphosphate
ITAM	Immunoreceptor tyrosine-based activation motif
LAT	Linker for activation of T cells
LDL	Low density lipoproteins
LOX-1	Lectin-like oxidised LDL receptor-1
LPA	Lysophosphatidic acid
MAPK	Mitogen activated protein kinases
MOPS	3-(N-Morpholino)propanesulfonic acid
nLDL	Native low density lipoprotein
NO	Nitric oxide
OCS	Open canalicular system
oxLDL	Oxidised low density lipoproteins
PAR1	Protease-activated receptor-1
PAR4	Protease-activated receptor-4

PC	Phosphatidylcholine
PDE	Phosphodiesterase
PGI_2	Prostacyclin
PI3-K	Phosphatidylinositol 3-kinase
PKA	Protein kinase A
PKG	Protein kinase G
PLA_2	Phospholipase A2
PLCβ	Phospholipase C beta
PLCγ2	Phospholipase C gamma 2
PMSF	Phenyl methyl sulphonyl fluride
PP2	AG 1879, 4-Amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine
PPP	Platelet poor plasma
PRP	Platelet rich plasma
PS	Phosphatidylserine
PSGL-1	P-selectin glycoprotein ligand 1
PVDF	Polyvinylidene fluoride
REM	Relative electrophoretic mobility
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM	Standard error of the mean
sGC	Soluble guanylyl cyclase
SH2	Src homology 2
SH3	Src homology 3

SLP-76	Src homology 2 domain-containing leukocyte protein of 76kDa
SNARE	Soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptor
SR-A	Scavenger receptor A
SR-BI	Scavenger receptor B-I
Syk	Spleen tyrosine kinase
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substances
TBS-T	Tris-buffered saline-tween
TEMED	N,N,N',N'-tetramethylethylenediamine
TSP-1	Thrombospondin-1
TXA_2	Thromboxane A_2
U73122	1-[6-[[(17β)-3-Methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]- 1H-pyrrole-2,5-dione
VLDL	Very low density lipoproteins
vWF	von Willebrand factor
WCL	Whole cell lysate
WHO	World Health Organisation

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Video 010 – Platelet incubation with oxLDL reduces the inhibitory effects of Prostacyclin

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“Get your head down…It’s belt and braces time.”

Khalid Naseem.

Author's declaration

I confirm that this work is original and that if any passage(s) or diagram(s) have been copied from academic papers, books, the internet or any other sources these are clearly identified by the use of quotation marks and the reference(s) is fully cited. I certify that, other than where indicated, this is my own work and does not breach the regulations of HYMS, the University of Hull or the University of York regarding plagiarism or academic conduct in examinations. I have read the HYMS Code of Practice on Academic Misconduct, and state that this piece of work is my own and does not contain any unacknowledged work from any other sources.

Chapter 1: Introduction

1.1 Cardiovascular Disease

Cardiovascular disease (CVD) is a multifactorial disorder with a range of pathologies affecting the heart, brain, kidneys and vasculature. CVD is currently the second largest cause of mortality in the UK, resulting in 160,000 deaths in 2014 and with a cost of £6.8 billion in England (Townsend et al., 2014). Whilst the incidence of CVD is decreasing in the UK, due to improved lifestyle choices and public health campaigns, the rate of CVD is increasing dramatically in developing countries, meaning that CVD is becoming a global problem. The pathology of CVD is complex, although known risk factors include sex, age, physical activity, smoking status, diet and obesity. However, elevated blood cholesterol levels are by far the strongest indicator of CVD risk (Yusuf et al., 2004) as illustrated by multiple epidemiological studies, reviewed in a recent article by Wong (Wong, 2014). In particular, consumption of fatty foods is strongly correlated with the formation of atherosclerotic plaques in blood vessels and the resulting myocardial infarction and/or stroke (Clarkson and Newburgh, 1926).

1.2 Atherosclerosis and the formation of arterial plaques

The onset and development of atherosclerosis has been at the forefront of recent biomedical research and can be characterised in four key stages: *Endothelial dysfunction*, *monocyte recruitment*, *plaque formation* and *plaque rupture* (Fig. 1.1). Atherosclerosis occurs at areas of the vasculature with disturbed blood flow, characterised by low shear stress and oscillating flow, demonstrated to occur within the coronary system – the area most at risk (Hopkins, 2013). Endothelial dysfunction creates a pro-inflammatory environment and permits the passage of LDL into the vessel wall where it becomes oxidised. Monocytes are recruited during this time to sites of inflammation and internalise excess LDL, forming lipid-rich 'foam' cells. Foam cell-rich regions begin to accumulate and trigger other pathophysiological processes, resulting in the formation of

an atherosclerotic plaque. Plaques are then prone to rupture over time, releasing their content and triggering thrombosis. This process is summarised below (Fig. 1.1):

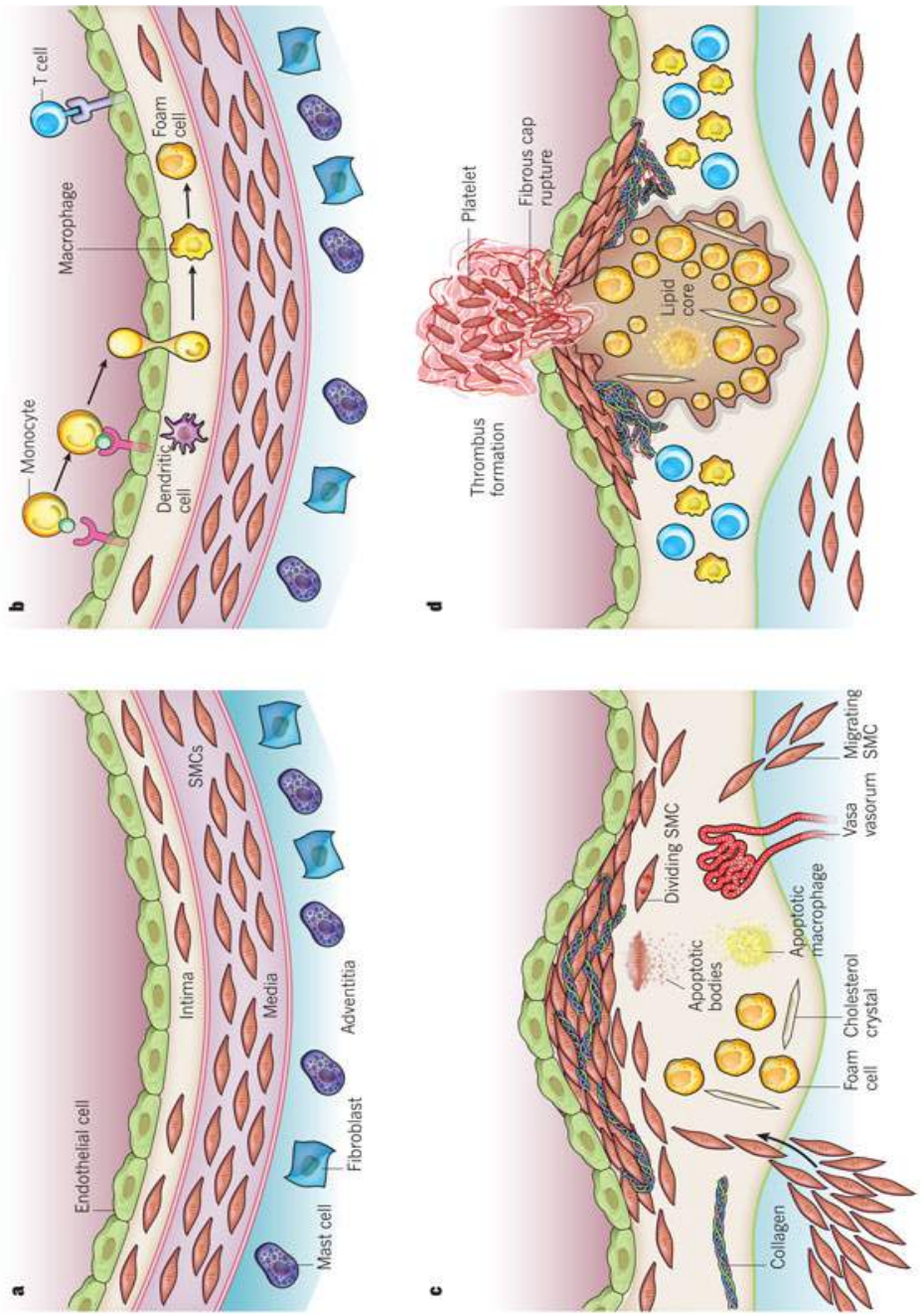


Figure 1.1 - Basic Diagram of Atherosclerosis. Panels a-d represent key events in the progression of atherosclerosis over time. A) X-section of blood vessels in a healthy state. B) Endothelial dysfunction causes migration of monocytes into the intima where they encounter oxidised LDL and become foam cells. C) Changes in the cytokine environment causes migration of SMCs, collagen deposition and cholesterol crystal formation, resulting in an atherosclerotic plaque. D) Foam cells necrose, releasing lipid contents and plaque ruptures, releasing the oxidised lipids and other plaque contents into circulation and triggering thrombosis. Taken from Libby et al. 2011.

1.2.1 Endothelial Dysfunction

The endothelium, comprised of endothelial cells (EC), plays an important part in healthy vasculature. EC are extremely active and secrete a plethora of agents that regulate vascular function and prevent atherosclerosis. Two of these agents, nitric oxide and prostacyclin, are of particular interest as they are known to control vascular tone and prevent formation of platelet aggregates. However, EC can lose these homeostatic properties, a process termed endothelial dysfunction, which is thought to play a key role in the initiation of atherogenesis (Hopkins, 2013). The progression of endothelium from a healthy to pathogenic state is observed in multiple conditions and include hypertension, hypercholesterolemia and diabetes (Sitia et al., 2010).

Endothelial cells are highly sensitive to the flow of blood within the vessel, with their growth and phenotype regulated by shear rates. Low shear, oscillating blood flow causes the endothelium to change from a quiescent state to a pro-inflammatory phenotype. This change results in increased ROS, cell turnover, cell-cell permeability and apoptosis (Pober and Sessa, 2007). In addition, activation of the transcription factor NF-κB causes elevated expression of cell surface adhesion molecules and decreased production and bioavailability of NO, generating a localised pro-thrombotic environment (Pober and Sessa, 2007). The activation of endothelium causes increased expression of adhesion molecules including ICAM-1 (Poston et al., 1992) and VCAM (Davies et al., 1993), resulting in increased monocyte and lymphocyte adherence and translocation into the intima. These changes result in the propagation of the atherosclerotic pathology process since leaky cell-cell junctions, formed as a result of apoptosis or cell division, allow LDL to enter the intima (Cancel et al., 2007; Dabagh et al., 2009).

Changes in EC function occur much earlier than the manifestation of clinical symptoms of atherosclerosis, with some evidence speculating that EC may begin as early as childhood. It is currently uncertain what triggers ECs to switch from a quiescent to a dysfunctional

phenotype, although known cardiovascular risk factors are believed to play a role (Deanfield et al., 2007).

1.2.2 Dyslipidemia and the role of plasma lipoproteins in atherogenesis

Elevated plasma lipids, particularly cholesterol in the form of low-density lipoprotein (LDL), are associated with increased CVD risk (Nissen et al., 2005). An increase in LDL is commonly associated with increased consumption of dietary fat in which it localises to the blood vessel walls in vulnerable regions of the vasculature.

Lipoproteins are spherical, lipid-rich particles, comprised of an 'outer shell', 'lipid core' and protein component that predominantly serve to transport lipids to the tissues. The outer shell is composed predominantly of a phospholipid monolayer, with traces of cholesterol and contains specialised apolipoproteins. These proteins facilitate recognition of particles by cell surface receptors or enzymes. The lipid core contains triglycerides and cholesterol esters, which are transferred to tissues during the lipoprotein's lifetime (Mahley et al., 1984). Several lipoprotein subtypes exist in humans to supply the body with lipids for normal cellular functions. The classification of lipoproteins is dependent on their relative density and is shown in Table 1. However, LDL is most strongly implicated in atherosclerosis and the resulting thrombosis event (Goldstein and Brown, 1977).

Lipoprotein	Size (nm)	Density (g/mL)
Chylomicron	100-1000	<0.95
VLDL	30-80	0.950 - 1.006
IDL	25-50	1.006 - 1.019
LDL	18-28	1.019 - 1.063
HDL	5-15	>1.063

Table 1.1 – Table describing the relative attributes of lipoproteins present within humans. Taken from Superko 2009.

Low-Density Lipoproteins

Clinical studies have attributed increased circulating LDL levels to risk of atherosclerotic cardiovascular disease. Concentrations of LDL below 1,000nmol/L are considered to be low, whilst an increase in risk is seen at 1,300nmol/L. Concentrations above 1600nmol/L are considered to have high risk of CVD (Otvos et al., 2011).

LDL are an intermediate size particle, ranging from 18-25nm and with a molecular weight of 2,300,000 Daltons. The density of LDL ranges from 1.019-1.063 g/mL (Gotto et al., 1986). The lipid contents of LDL are summarised in the table below (Table 1.2):

Lipid	Abundance (molecules per particle)	Function
Free cholesterol	600	Cellular metabolism, hormone production
Cholesterol Esters	1600	Waste product for detoxification
Phospholipids	700	Maintenance of LDL hydrophobic core
Triglycerides	185	Energy, membrane fluidity

Table 1.2 – Summary of the average lipid content per LDL particle. Adapted from Gotto Jr, Pownall et al. (1986).

In addition to their lipid content, the outer shell also contains a single apolipoprotein (Apo B-100). Apo B-100, one of the largest encoded proteins in the human genome, is comprised of 4536 amino acid residues.

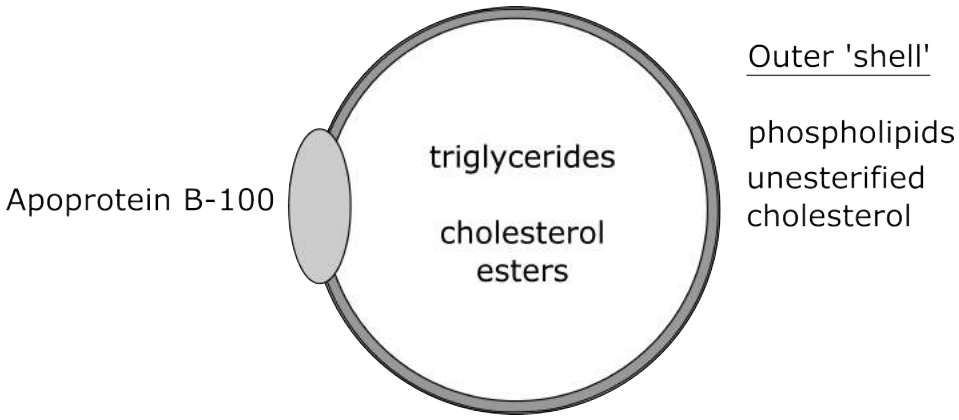


Figure 1.2 - Schematic of low-density lipoprotein. Cross-section of a LDL particle. The ApoB-100 protein covers a large area of the outer shell, allowing for recognition by the LDL receptor. The outer shell consists of phospholipids and unesterified cholesterol, whilst the LDL interior contains triglycerides and cholesterol esters.

Functionally, Apo B-100 is essential for the delivery of cholesterol by acting as a ligand for cells bearing the LDL-receptor (LDL-R). LDL-R is present on the cell membrane in clathrin-coated pits, which upon binding to LDL, cause endocytosis of LDL into the cell. Vesicles containing LDL fuse with lysosomes, causing the degradation of the LDL particle and allow fatty and amino acids to be utilised by the cell (Anderson et al., 1977). In patients with hypercholesterolemia, the amount of LDL increases either due to a lack of LDL-R, or mutation within the LDLR gene, increasing the risk of cardiovascular disease (Brown and Goldstein, 1975; Goldstein and Brown, 1975).

Due to the high proportion of tyrosine and lysine residues, Apo B-100 is particularly sensitive to direct oxidation or modification by lipid oxidation products (Tsimikas and Miller, 2011).

1.2.3 Oxidation of low density lipoproteins in atherogenesis

At sites of endothelial dysfunction, LDL can transverse the EC layer and accumulate in the *intima* layer of the blood vessel by binding to proteoglycans (Borén et al., 1998). Perlectan, the most abundant proteoglycan, is believed to play the largest role in LDL immobilisation as shown by genetic deletion (Vikramadithyan et al., 2004). Once retained in the intima, LDL undergoes chemical modification resulting in *oxidised* LDL, a lipoprotein with distinct biological function and central in the pathology of atherosclerosis. However, multiple oxidised species are produced during the oxidation process, with varying effects on the surrounding cells and environment (Levitan et al., 2010). The heterogeneity of oxidised species produced is dependent on numerous factors including oxidising agent, duration of oxidation, lipoprotein size, composition and antioxidant levels.

Minimally modified LDL (mmLDL), formed earlier in the oxidation process, features oxidised lipids yet contains an intact, unmodified ApoB-100 protein, and is therefore still recognised by the apoB/E receptor (Naseem et al., 1997), but not scavenger receptors. Polyunsaturated fatty acids present within the LDL particle, most notably arachidonic and linoleic acids are highly prone to oxidation by free radicals and enzymatic activity, initiating the peroxidation reaction (Fig. 3). This reaction results in the formation of

bioactive lipid molecules such as lysophosphatidylcholine, lysophosphatidic acid and sphingolipid products (Levitan et al., 2010). These have been shown to have a range of proinflammatory effects on cells including monocyte recruitment, cytokine upregulation and platelet activation (E. a. Podrez, 2002; Siess and Tigyi, 2004; Yeh et al., 2004).

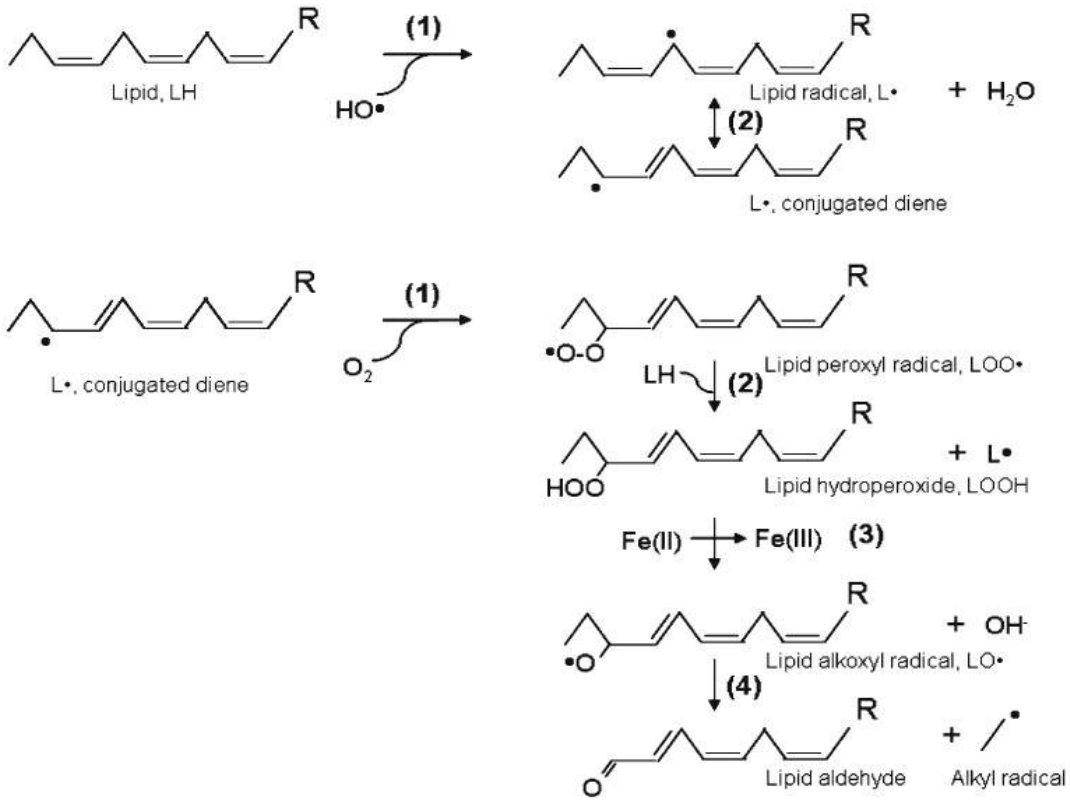


Figure 1.3 – Diagram illustrating lipid peroxidation leading to the formation of oxLDL. A lipid is encounters ROS, e.g. hydroxyl radical, forming a lipid radical, which rapidly undergoes electron rearrangement to form a conjugated diene. Upon oxygen addition, a lipid peroxyl radical is formed, which causes further hydrogen abstraction, resulting in the formation of lipid hydroperoxides. Under harsh oxidative conditions, lipid hydroperoxides can undergo homolysis by Fenton chemistry, forming another radical that can degrade forming lipid aldehydes and alkyl radicals, capable of binding to ApoB-100. Adapted from Potter et al. 2011.

Conversely, the generation of lipid aldehydes from the peroxidation reaction causes the formation of extensively oxidised LDL (oxLDL). Unlike mmLDL, oxLDL is no longer recognised by LDL-R due to the formation of adducts with tyrosine and lysine residues on ApoB-100. OxLDL also undergoes extensive lipid oxidation, with high levels of the lipid aldehydes 4-hydroxynonenal and malondialdehyde, oxysterols and isoprostanes (Levitan et al., 2010). The plethora of oxidised lipid products enables oxLDL to elicit a range of proinflammatory effects, including leukotriene production (Jiang et al., 2015), macrophage adhesion and activation (Leitinger and Schulman, 2013) and intimal thickening and collagen deposition (Bentzon et al., 2014). Cellular recognition of oxLDL is considered to be largely driven by scavenger receptors, an ancient family of receptors capable of binding to pathogen associated molecular patterns (Goldstein et al., 1979; Brown et al., 1979; Krieger, 1997). The role of scavenger receptors in atherogenesis and platelet function is explored in greater detail in later sections of this chapter.

LDL oxidation *in vivo* is attributed to *oxidative stress*, a condition where the regular redox balance is disrupted in a localised area (Esterbauer et al., 1991). Excessive reactive oxygen species (ROS) causes oxidation of fatty acyl chains, present in phospholipids and triglycerides, culminating in protein modification by forming covalent adducts with reactive aldehydes.

ROS are generated by multiple mechanisms *in vivo*. Low levels of ROS are beneficial to cells and act as common signalling molecules (Ray et al., 2012). It is for this reason that cells possess enzymes allowing them to produce ROS and contribute to the redox status of the environment. NADPH oxidase is present in multiple cell types and is most commonly associated with bactericidal action of leukocytes. NADPH oxidase generates superoxide directly from oxygen and is considered to have the largest contribution to lipid peroxidation within the atherosclerotic plaque, since turbulent blood flow is directly related to an NADPH oxidase subunit (gp91) gene expression (Meyer and Schmitt, 2000; Harrison et al., 2003). Two other enzymes, myeloperoxidase and lipoxygenase have also been implicated in LDL oxidation, by acting upon lipids present within the LDL particle

(Chen, 2003). Myeloperoxidase is normally found within neutrophils and macrophages and is present within the plaque. Using hydrogen peroxide as a substrate, MPO catalyses the reaction forming hypochlorous acid, a potent oxidising agent. Hypochlorous acid is able to cause protein modification on tyrosine residues, allowing the production of chlorotyrosine, nitrotyrosine and dityrosine residues (Ferrante et al., 2010; Sadowski et al., 2014). Moreover, like NADPH oxidase, myeloperoxidase is also able to generate reactive oxygen species (ROS) and reactive nitrogen species (RNS), affecting LDL oxidation further (Nicholls and Hazen, 2005). Lipoxygenases are enzymes which are able to directly oxidise fatty acids, including phospholipids and cholesterol esters, both present within LDL (Kuhn et al., 2015). Lipoxygenases are found at significantly higher levels in atherosclerotic plaques and are known to affect the localised redox environment (Folcik et al., 1995; Mertens and Holvoet, 2001). However, whilst much evidence exists to show lipoxygenases are pro-inflammatory, their specific role in LDL oxidation remains controversial due to the presence of anti-inflammatory lipid species produced and therefore may also be acting in a beneficial manner (Kuhn et al., 2015). It is generally considered that none of these forms of LDL oxidation occur in isolation *in vivo* and rather act as a cumulative 'oxidative environment', allowing the generation of oxLDL.

The presence of modified LDL in the intima alters the local environment, causing changes to the secreted chemical profile from activated EC, causing release of inflammatory factors including C-reactive protein, TNF-α and IFN-γ (Sprague and Khalil, 2009). Moreover, increased adhesion receptor expression causes the tethering and infilitration of T cells resulting in an early inflammatory response (Bentzon et al., 2014). Secretion of monocyte chemoattractant protein-1 by EC and smooth muscle cells causes recruitment of monocytes to the area of inflammation (Bentzon et al., 2014) and to enter the intima where they differentiate into macrophages and encounter oxLDL. Recognition of LDL, both native and modified, by LDL-R and scavenger receptors causes unregulated lipid uptake into the macrophages and causes their phenotype to change, hence described as 'foam cells' (Henriksen et al., 1981a). In addition, internalised LDL can also be modified

further due to the acidic conditions within the macrophage lysosome (Wen and Leake, 2007). Increased numbers of foam cells in the vessel manifests as a fatty streak- the earliest pathological legion in atherosclerosis.

1.2.4 Plaque formation and rupture

Over time, the areas of foam cell accumulation lead way to fibrous legions. These legions are characterised by deposition of collagen and other matrix proteins by fibroblasts and oxLDL internalisation by smooth muscle cells (Jimi et al., 1995; Rudijanto, 2007). The developing plaque contains almost every type of known immune cell (Hansson and Hermansson, 2011) which serves to both promote and limit atherosclerotic progression by the presence of pro- and anti-inflammatory cytokines and macrophages of the M1 and M2 phenotype. Interestingly, the immune response to LDL present within the plaque specifically targets non-oxidised epitopes (Hermansson et al., 2010), although further work is required to deduce the significance of this. Conversely, low concentrations of oxLDL have been shown to alter smooth muscle cell function by promoting smooth muscle cell proliferation (Kiyan et al., 2014). High concentrations of oxLDL are cytotoxic, leading to necrosis and release of further danger associated molecular patterns (Lordan et al., 2007).

Eventual foam cell death causes the oxidised lipids and breakdown products to accumulate and the plaque to increase in size and calcify, resulting in advanced legions (Hopkins, 2013). Over time, plaques can become more unstable and susceptible to haemorrhage and erosion, releasing oxLDL into the bloodstream and causing production of a thrombus. Various biological and mechanical factors have been shown to affect plaque stability. Vulnerable plaques have been shown to exhibit certain key features including a lipid core and thin fibrous cap, along with localised inflammation, neovascularisation and matrix remodelling (Halvorsen et al., 2008). However, it is worth noting that none of these factors has been shown to have a cause/effect relationship to subsequent rupture events. Recent studies have begun to describe patients as vulnerable, rather than plaques in order for better stratification (Fishbein, 2010).

The obstruction of blood vessels by the resulting thrombus can cause localised cell death by preventing the flow of oxygen and nutrients. Dependent on the location of the blockage, this may result in myocardial infarction or stroke (Bentzon et al., 2014).

The association between hyperlipidemia and CHD have been the subject of many studies over recent decades and is comprehensively reviewed in 'The Cholesterol Wars' book by Steinberg et al. Even as early as 1913, Anitschkow and Chalatow showed that increased cholesterol consumption in rabbits lead to fatty streak formation, mimicking human atherosclerosis (Steinberg, 2013). The link between increased dietary lipids and risk of CHD was shown in the 1960s by several epidemiological studies (KANNEL et al., 1961; Leren, 1968) and in 1984, the first lipid lowering agents (statins) were developed and shown to reduce CHD by 19% (AMA, 1984). Statins are a family of compounds that target the enzyme HMG-CoA reductase, a critical enzyme for the mevalonate metabolic pathway responsible for the production of isoprenoids. Once inhibited, the body is unable to synthesise cholesterol, causing a dramatic reduction in the amount of cholesterol in plasma (Armitage, 2007). At a similar time, the first definitive evidence was shown that inhibition of platelets by aspirin led to a reduction in thrombotic events (de Gaetano, 2001), tying the two together.

Evidence in recent years has shown that platelets play an important role in atherogenesis too by adhering to activated EC and releasing pro-inflammatory mediators including IL-1β and CD40L (Gawaz et al., 2005). However, what remains poorly understood are the effects of oxLDL/oxidised lipids on platelet function and whether this interaction can be prevented.

1.3 Blood platelets and their role in haemostasis and thrombosis.

Since platelets were first described by Bizzozero in 1881, they have been associated with haemostasis and the prevention of blood loss from sites of injury (de Gaetano, 2001). Platelets are highly specialised cells and possess a unique structure to enable them to fulfil this role. The evolution of the platelet from a common ancestral cell has resulted in platelets having additional functions alongside haemostasis, including involvement in the immune system, angiogenesis and cancer progression (Knighton et al., 1982; Semple et al., 2011; Menter et al., 2014).

1.3.1 Platelet production

Platelets are released from megakaryocytes, a haematopoietic stem cell derivative, which reside in the bone marrow niche. To date, the primary known role of the megakaryocyte is to produce platelets, although megakaryocytes have also been shown to modulate conditions within the haematopoietic stem cell niche (Day and Link, 2014). Platelet production begins with immature megakaryocytes undergoing a maturation process, resulting in endomitosis and nuclear polyploidy. Megakaryocyte maturation is a tightly regulated process, in which thrombopoetin (TPO), released from the liver and bone marrow binds directly to platelets via the mpl receptor, acting as a sink for the hormone. When levels of platelets are low, TPO levels are elevated and this serves to trigger platelet production (Kaushansky, 2006).

Protein and membrane synthesis occurs in abundance and the cell begins to re-organise its cytoskeleton, forming long extensions which transverse into local blood vessels via cell junctions between endothelial cells. From these cells, ‘proplatelets’ are released, which like the progenitor cell, mature to form functional platelets (Kaushansky, 1996; Machlus and Italiano, 2013).

1.3.2 Platelet ultrastructure

Platelets have evolved to be highly specialised in their role in haemostasis. Studies investigating platelet ultrastructure have shown that they differ significantly from other

cells, being much smaller, anucleate and containing multiple unique granules containing a variety of proteins and small compounds that support their role in haemostasis (Fig. 1.4).

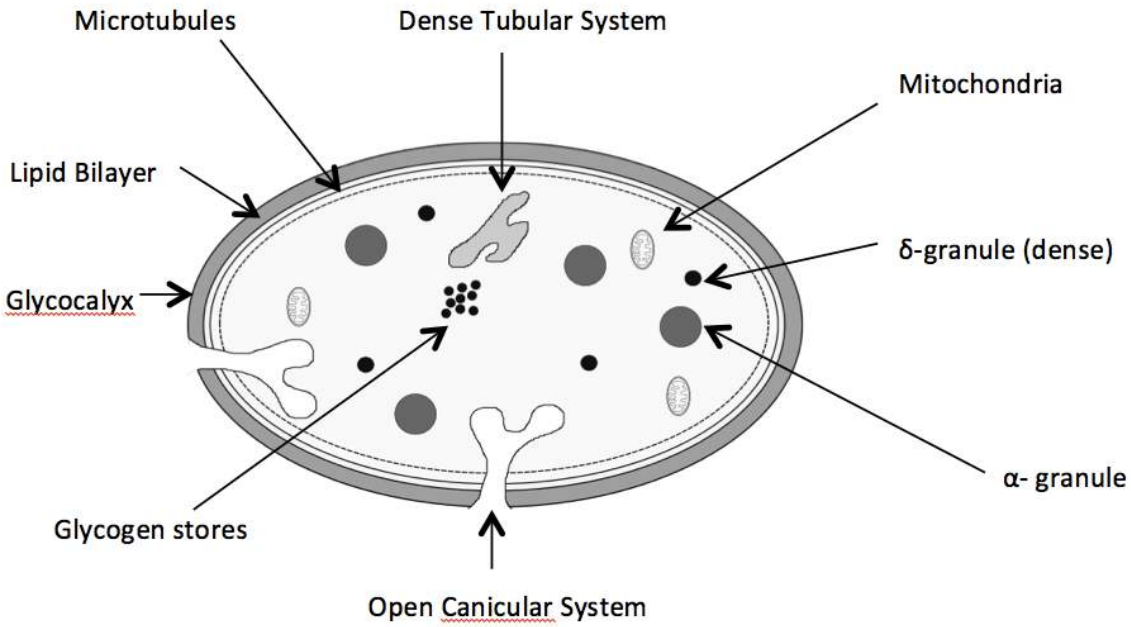


Figure 1.4 - Diagram of platelet structure, showing main structural features and sub-cellular contents. Ribosomes and Lysosomes were excluded for brevity. Not to scale.

Platelets have a complex exterior in comparison to other cells. The outermost layer is deemed the *glycocalyx*, a dynamic structure which many of the platelet glycoprotein receptors reside (White and Conard, 1973). Underneath the glycocalyx is the lipid bilayer, which is characteristic of most cells. However, a key aspect of the platelet lipid bilayer is the presence of phosphatidylserine, which accelerates blood clotting by allowing the conversion of prothrombin to thrombin (Rosing et al., 1985). The submembrane area contains a dynamic microtubule network for rapid platelet shape change. The additional membrane required for when platelets change from a discoid to activated, stellar configuration is kept in the open canicular system, which also serves to allow molecules from outside the platelet to enter (Zucker-Franklin, 1981), and in some cases, be processed and transported to other parts of the cell, including the plasma membrane (Escolar et al., 1989).

Platelets contain several well-characterised organelles essential for the normal function of the cell (Fig 1.4). Energy is provided by the metabolic activity of the mitochondria through oxidative phosphorylation of glycogen and fatty acids, and also contributes to platelet activation and apoptosis via generation of ROS (Garcia-Souza and Oliveira, 2014). Energy required for activation is also partially fuelled by the intracellular carbohydrate-based glycogen stores and a Dense Tubular System (DTS) that functions as a store for intracellular calcium ions (Ebbeling et al., 1992). In addition, platelets also possess ribosomes, which are believed to process mRNA inherited from the megakaryocyte. Recent studies have shown that platelets are able to actively translate mRNA to elicit specific responses, with altered expression profiles in disease (Rowley et al., 2012). However, unlike other cells, platelets (and megakaryocytes) possess unique granules, which upon activation release their contents by fusing to the plasma membrane. These granules are termed alpha granules (α-granules) and dense granules (δ-granules) and are classified by the size of the granule and contents (Golebiewska and Poole, 2015). In addition, platelets also contain lysosomes (γ-granules) that also release their contents upon activation, although the role and importance of this is less well defined (McNicol and Israels, 1999).

The release of platelet granules occurs in a tightly controlled way. Dense granules are released first and contain many small molecules to accelerate platelet aggregation (Golebiewska and Poole, 2015) (Table 1.3). In particular, δ-granules release ADP, a potent platelet agonist which promotes subsequent activation by other agonists including TxA_2. This is followed shortly after by release of α-granules containing adhesive proteins such as vWF and fibrinogen. Alpha granule release is traditionally associated with potentiating platelet activation, but also critical for platelet aggregation since almost 50% of the adhesion receptors GPVI and $\alpha_{IIb}\beta_3$ reside in these granules (Blair and Flaumenhaft, 2009). Interestingly, studies have shown that α-granules are not homogenous in their content, with some containing fibrinogen and others containing vWF (Sehgal and Storrie, 2007;

Italiano et al., 2008). However, it is currently unknown as to whether granule release is dependent on the stimuli and the degree of platelet activation required.

α-granules	**δ-granules**
Adhesive proteins – vWF, fibrinogen, fibronectin, vitronectin	*Vasoconstriction* – serotonin
Membrane proteins – $\alpha_{IIb}\beta_3$, GPIb, P-selectin	*Platelet activation* – ADP
Coagulation biochemistry – FV, FVIII	*Coagulation biochemistry* – Ca^{2+}
Fibrinolysis – plasminogen	*Energy* - ATP
Inhibition – thrombospondin-1, PAI-1, coagulation inhibitors	*Membrane proteins* - $\alpha_{IIb}\beta_3$, GPIb, P-selectin
Chemokines for leukocyte attraction – PF4, β-thromboglobulin	*Integrin regulation* - Ca^{2+}, Mg^{2+}
Cellular mitogens – PDGF, VEGF-A, VEGF-C	

Table 1.3 – Table showing contents of platelet granules. Alpha granules are more abundant within the platelet and constitute a greater proportion of secreted proteins which are heavily focused on haemostasis and wound healing. Dense granules are less abundant and feature essential non-protein factors for haemostasis. Adapted from McNicol & Israels 1999; Blair & Flaumenhaft 2009.

The specialised nature of the platelet enables it to become rapidly activated in the event of an injury and forms a 'platelet plug', which prevents excessive blood loss and microbial contamination. The formation of the platelet plug occurs in several well characterised, pre-defined stages: an initial adhesion stage, followed by shape change and secretion, resulting in additional platelet recruitment and aggregation. However, it is important to consider platelet plug formation a dynamic process, with multiple inputs and feedback mechanisms and not simply a strict, linear progression due to the numbers of platelets involved.

1.3.3 Platelet adhesion

In healthy vessels, platelets are located at the very periphery of blood flow and are directly in contact with endothelial cells and their anti-thrombogenic compounds. The

marginalisation of platelets to the vessel wall is dependent on laminar flow, in which the larger red blood cells force the smaller platelets away from areas of highest sheer stress (Aarts et al., 1984). Upon vessel injury, platelets are no longer in contact with endothelial derived inhibitory molecules and are exposed to thrombogenic extracellular matrix proteins including collagen, von Willebrand factor, laminin, fibronectin and proteoglycans (Broos et al., 2011; Clemetson, 2012). Platelets have an array of receptors on their surface which allow their interaction with these extracellular matrix proteins. Of these interactions it is the adhesion to exposed collagens which is considered to have the largest thrombogenic effect on the recruitment of platelets to sites of vascular injury (Savage et al., 1999; Nieswandt and Watson, 2003). The sheer conditions present within the injured vessel determine whether platelet adhesion is direct or indirect (Broos et al., 2011; Clemetson, 2012). Under low shear conditions, such as those found in veins and large arteries, platelets can directly bind collagen (Saelman et al., 1994). Platelet adhesion to collagen is believed to be due to GPVI and integrin α2β1, although the degree of synergy and/or compensation between these receptors is still unclear (Broos et al., 2011). Both receptors are expressed at high copy, with platelets expressing ~3600 copies of GPVI and 2000-4000 copies of α2β1 (Kauskot and Hoylaerts, 2012). Studies have shown that GPVI monomers only possess a weak affinity for collagen, binding to Gly-Pro-Hyp triplet residues, yet when dimerised, affinity strongly increases (Kehrel et al., 1998; Jung et al., 2012). GPVI is associated with the ITAM-bearing protein FcRγ chain and it is this protein that causes robust intracellular signalling, supporting strong adhesion (Varga-Szabo et al., 2008; Broos et al., 2011). Binding of the platelet to collagen causes GPVI clustering and triggers 'inside-out' signalling, activating α2β1. The activation of α2β1 is believed to aid platelet adhesion by stabilising the platelet and reinforcing interactions between GPVI and collagen (Stevens et al., 2004). Platelets adhered via GPVI and α2β1 begin the shape change process due to multiple activatory signalling pathways becoming triggered and cause changes resulting in the modulation of the cytoskeletal proteins actin and myosin.

Under high shear conditions, found in small arteries and arterioles, recruitment of fast-flowing platelets is dependent on the receptor complex GPIb-V-IX. High shear conditions are also present at sites of atherosclerosis and stenosis due to the narrowing of the vessel (Cunningham and Gotlieb, 2005). Upon vascular injury, circulating von Willibrand factor (vWF) binds to exposed collagen, serving to link the platelet to the exposed matrix. This interaction converts vWF to a form that can interact with platelets. The interaction between the GPIb-V-IX and vWF is transient, although it is sufficient to slow the flow of platelets over the injured surface and allows time for the formation of stronger, GPVI-mediated bonds (Varga-Szabo et al., 2008; Broos et al., 2011; Clemetson, 2012). The GPIb-V-IX receptor complex is extremely highly expressed, with reports stating ~50,000 copies per platelet (Saboor et al., 2013). Platelets are also able to bind vWF via the activated integrin $\alpha_{IIb}\beta_{3,}$ although this is considered to play a greater role in platelet aggregation than initial adhesion (Ma et al., 2007). Once adhered, activated platelets are able to release vWF from α-granules, propagating this process.

1.3.4 Platelet activation

Platelet adhesion to matrix proteins initiates an activation process that is dependent on complex intracellular signalling events and is often referred to as '*inside out signalling*'. Regardless of adhesion receptor involved (GPVI, GPIb-V-IX etc) the signalling pathways activated result in the activation of phospholipase Cγ2 downstream of a tyrosine-kinase fuelled cascade (Varga-Szabo et al., 2008; Broos et al., 2011). The end result of PLCγ2 activation is further propagation of the platelet activation process through the activation of Ca^{2+} dependent processes and a conformational change of Integrin $\alpha_{IIb}\beta_3$ from a low affinity to high affinity state. Collectively, this process is referred to as '*outside in signalling*'.

Under normal conditions, GPVI is constitutively bound to two Src-family kinases Fyn and Lyn (Suzuki-Inoue et al., 2002). This association is mediated via a SH3 domains present on Src kinases which allows binding to a proline rich region of the GPVI protein. Upon binding to collagen, GPVI becomes crosslinked and Fyn and Lyn come into contact with the ITAM

region of FcRγ, causing it to become tyrosine phosphorylated (Watson and Gibbins, 1998; Watson et al., 2005) (Fig. 1.5). The phosphorylated ITAM acts as a docking site for the tyrosine kinase, Syk, which binds the ITAM via an SH2 domain and undergoes autophosphorylation (Gibbins et al., 1996). Subsequently, Syk phosphorylates multiple targets causing the formation of a signalosome. The proteins LAT, Gads and SLP76 are crucial for correct signalosome assembly and regulate PLCγ2 activity. LAT phosphorylation acts as docking site for the SH2-domain of PLCγ2 and this interaction is supported by Gads and SLP76 (Watson et al., 2001; Watson et al., 2005). Another important protein involved in signal transduction is the lipid kinase PI3-K which is required for full activation of PLCγ2 (Pasquet et al., 1999). PI3-K is able to catalyse the conversion of PI-4,5P_2 into PI-3,4,5P_3, changing the relative levels of PIP_3 present within the membrane. Increased levels of PIP_3 support PLCγ2 activation by facilitating recruitment to the plasma membrane and signalosome complex via a PH-domain (Fig. 1.5). The activation of PI3-K also causes the activation of the substrate kinase Akt (also known as Protein kinase B) which plays a role in platelet activation by the phosphorylation of over 100 proteins. Notably, these include glycogen synthase kinase 3β (GSK3β) and extracellular signal related kinase (ERK) shown to have inhibitory and activatory roles respectively (Moroi & Watson, 2015).

Similarly, evidence exists showing that GPIb-V-IX can also cause platelet activation via a tyrosine kinase pathway. The ligation of vWF to GPIb-V-IX has been shown to cause phosphorylation of the ITAM bearing proteins FcγRIIa and FcRγ chain and this is likely to play a role in subsequent PLCγ2 activation in platelets (Canobbio et al., 2004). The activation of PLCγ2 causes the production of 1,2-diacylglycerol (DAG) and inositol 1,4,5 trisphosphate (IP_3) from phosphatidylinositol 4,5 bisphosphate. The production of DAG and IP_3 has significant consequences in the platelet, acting as messenger molecules, causing the platelet to undergo activation.

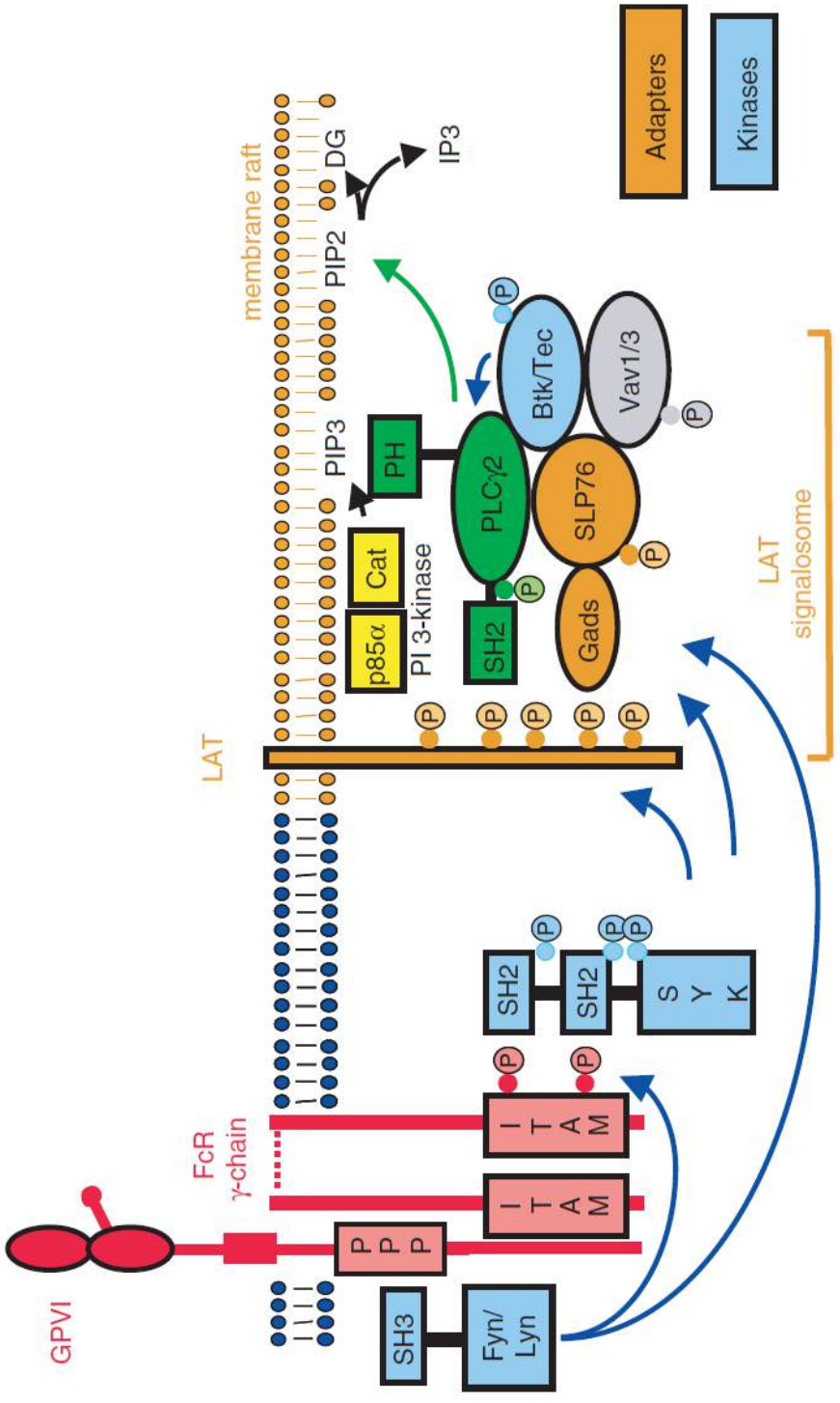


Figure 1.5 – Diagram showing the signalling cascade initiated from GPVI binding to collagen. Taken from Watson et al. 2005.

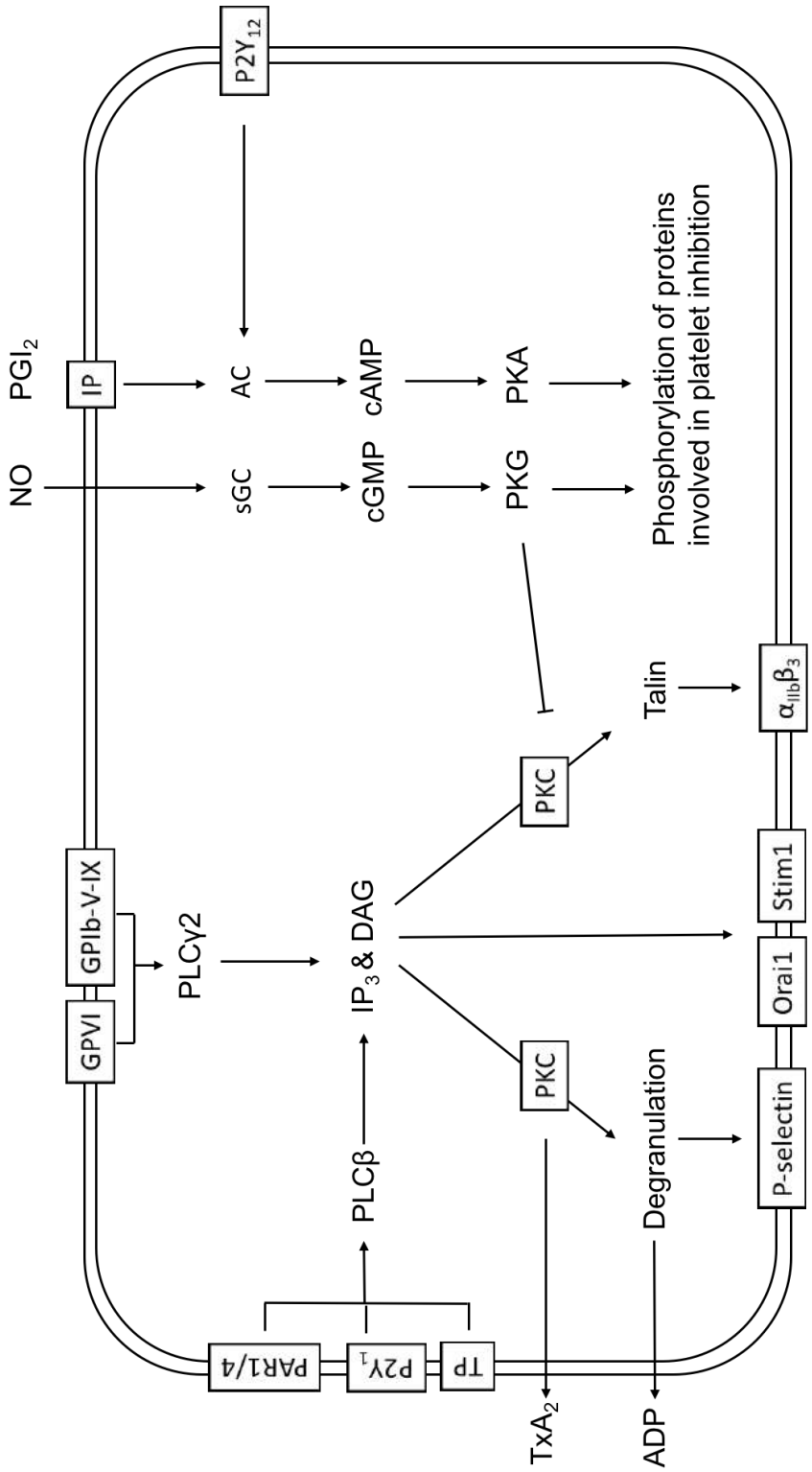


Figure 1.6 –Diagram providing an overview to the central activation and inhibitory signalling pathways in platelets. Adapted from *Broos, Feys et al. 2011*.

The generation of IP_3 by PLCγ2 allows activation of the IP_3 receptor, located on the DTS. This causes calcium ion efflux from the DTS into the cytoplasm as the receptor also acts as a calcium channel (Clemetson, 2012). The increase in cytosolic calcium results in the activation of the Ca^{2+} sensor Stromal interaction molecule 1 (STIM1), causing a conformational change (Lopez et al., 2016). This change serves to cluster and activate another Ca^{2+} selective ion channel, Calcium release-activated calcium channel protein 1 (Orai1), causing further calcium to enter the cell (Varga-Szabo et al., 2008; Jardín et al., 2008). Increased levels of intracellular calcium cause phosphotidylserine exposure, which acts as a site for prothrombinase complex formation, causing the conversion of prothrombin to thrombin, a potent platelet activator (Kodigepalli et al., 2015).

The production of DAG results in the activation of the serine/threonine protein kinase C (PKC). Multiple PKC isoforms exist in platelets (α, β, δ and θ) and all serve to phosphorylate multiple substrates involved in the platelet activation process, as highlighted by use of antibodies recognising the phosphorylation motif of PKC substrates (Poole et al., 2004; Harper and Poole, 2007). Proteins known to be phosphorylated by PKC include pleckstrin, myristoylated alanine-rich C kinase substrate (MARCKS) and SNARE proteins, although tying these phosphorylation events to a specific isoform remains controversial. Ultimately, the interplay between PKC and Ca^{2+} causes platelet shape change, spreading, granule release and consequently, aggregation (Broos et al., 2011) and is crucial for platelet function, with PKC inhibitors preventing all of these effects (Heemskerk et al., 2011).

Platelet activation also induces cytoskeletal rearrangement, causing the formation of filopodia and eventual spreading, with platelets adopting a ‘fried egg’ appearance. A microtubule coil, composed of tubulin monomers polymerised into a 3D structure, maintains the resting platelet shape and resides close to the plasma membrane. Upon calcium influx into the cytoplasm, the microtubule coil rapidly undergoes degradation resulting in a spherical platelet appearance (Fig. 1.7). This is due to activity by the enzyme gelsolin which also cleaves actin filaments ready for rearrangement and polymerisation

into new filaments, forming filopodia (Lind et al., 1982). Over time, the spaces between filopodia coalesce, forming laemellipodia and resulting in a fully spread morphology, allowing a single platelet to maximise its area covered (Hartwig et al., 1999).

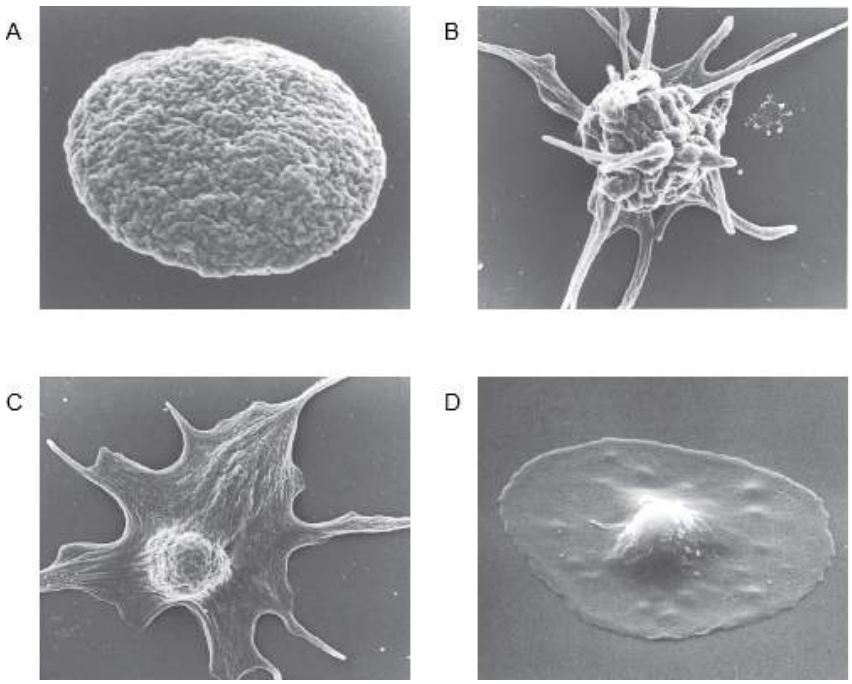


Figure 1.7 – EM images of platelet shape change. A – Quiescent, discoid platelet found under normal conditions. B – Early stage activated platelet, displaying laemellipodia and stellar appearance. C - Intermediate stage activated platelet, showing laemellipodia and limited filopodia formation. D – Fully spread platelet. Images taken from Michelson et al. 2013.

1.3.5 Platelet secretion and aggregation

As part of the shape change process, platelets undergo degranulation of intracellular granules to potentiate plug formation. Platelet granules contain multiple thrombogenic compounds, deemed secondary or soluble agonists, which act in both an autocrine and paracrine fashion in the local area. Activation of adjacent platelets by the existing platelet monolayer allows the thrombus to continue to grow and stabilise (Broos et al., 2011). Soluble platelet agonists include ADP, TxA_2 and thrombin, all of which act on platelets by binding to G-protein coupled receptors on the platelet surface. Similar to ITAM signalling cascades, multiple GPCRs are linked to a phospholipase isoform, PLCβ causing further Ca^{2+} mobilisation and PKC isoform activation (Clemetson, 2012).

TxA_2 is a product of arachidonic acid metabolism involving COX-1 and thromboxane synthase. It is produced during platelet activation and can diffuse out of the cell into the local environment. TxA_2 binds to the thromboxane receptors TPα and TPβ, which are coupled to both Gαq and $G\alpha_{12/13}$. Activation of these receptors causes further platelet activation via PLCβ activation or myosin light chain phosphorylation (Knezevic et al., 1993; Djellas et al., 1999). In contrast, ADP is released from δ-granules and binds to the purigenic receptors $P2Y_1$ and $P2Y_{12}$. Similar to TP, $P2Y_1$ is also coupled to Gαq whereas $P2Y_{12}$ is coupled to Gαi. Gαi is able to exert multiple effects within the cell including PI3-Kβ/AKT activation and inhibition of adenylyl cyclase, an important enzyme for the conversion of ATP to cAMP, a potent platelet inhibitor (Daniel et al., 1998; Broos et al., 2011). In addition to the release of secondary agonists, platelet aggregation is heavily dependent on localised generation of thrombin. Thrombin, a serine protease, is converted from an inactive *prothrombin* form to the active form via the enzyme complex prothrombinase which occurs at phosphotidylserine rich areas of the platelet membrane (Lentz, 2003). Platelets recognise thrombin by a specialised group of GPCRs called protease-activated receptors (PAR), of which PAR1 and PAR4 isoforms are present in platelets. The activation of PAR receptors occurs when thrombin cleaves the extracellular N-terminus of the receptor, displaying a new motif that can tether to the body of the receptor, eliciting a signalling response (Coughlin, 2000; Brass, 2003). PAR1 and PAR4 are coupled to multiple Gα proteins, including Gαq, Gα13 and Gαi and it is likely that this wide range of Gα proteins caused the potency of the thrombin response (Brass, 2003; Traynelis and Trejo, 2007).

The activation of integrin $\alpha_{IIb}\beta_3$ is one of the final stages of platelet activation and culminates in the formation of platelet aggregates. Integrin $\alpha_{IIb}\beta_3$ serves to bind fibrinogen, and to a lesser extent, vWF (Ma et al., 2007). Fibrinogen is a bivalent ligand which allows it to bind two separate $\alpha_{IIb}\beta_3$ molecules. The binding of fibrinogen acts as a molecular bridge to enable platelets to become coupled, causing rapid platelet aggregation.

Like many platelet activation processes, the change of the integrin from a 'closed', low affinity to an exposed, high affinity state relies on PKC and increased Ca^{2+}, triggering key protein phosphorylation and cytoskeletal rearrangement (Watson et al., 2005; Clemetson, 2012). The ligation of $\alpha_{IIb}\beta_3$ is also able to perpetuate activatory signalling further by inducing an ITAM dependent signalling pathway and the activation of PLCγ2 (Watson et al., 2001; Watson et al., 2005), which may also act to stabilise platelet-platelet interactions. Integrin activation occurs via activation of the enzyme talin-H, which serves to unclasp the cytoplasmic tail of β3. This unclasping event causes the release of the transmembrane domain, where it can interact with its substrate with high affinity (Ma et al., 2007).

It is important to consider the growing thrombus as a highly dynamic collection of cells rather than a static 'plug'. Growth of the thrombus is tightly regulated by several different factors including inhibitor bioavailability and shear stress (Brass et al., 2011). Platelets at the 'core' of the thrombus express high levels of P-selectin which is speculated to be key for later wound healing events since these cells are close to the site of injury (Golebiewska and Poole, 2015). The high degree of activation of these cells has been shown to be dependent on localised thombin generation and cell-cell contact (Brass et al., 2011). However, platelets present in the exterior of the thrombus ('shell') contains less activated platelets. These cells are less tightly packed, allowing infiltration of leukocytes and are dependent on activation via ADP signalling. This core/shell model allows chemotactic gradients to form such as CXCL-7 (also referred to as NAP-2) released from α-granules, allowing effective wound healing (Ghasemzadeh et al., 2013).

1.4 The modulation of platelet function

While platelet activation is critical for haemostasis, it must be tightly controlled in order to prevent pathological thrombosis. This is largely attributed to the endothelium-derived compounds, nitric oxide (NO) and prostaglandins, in particular prostacyclin (PGI_2). These compounds serve to prevent platelet activation under normal conditions, but also regulate thrombi size during platelet activation (Schwarz et al., 2001; Clemetson, 2012). NO and PGI_2 have similar effects on the platelet, acting to counteract activation signals by preventing of calcium influx into the cytoplasm and inhibition of integrin activation (Smolenski, 2012). These short lived mediators are continually released by the endothelium where they are easily accessible to platelets that are marginalised to the periphery of the vessel wall by the effects of shear stress.

NO is a free radical, gaseous messenger produced by nitric oxide synthase, present in the endothelial cells lining the vessel. It is capable of diffusing into the circulation, where it encounters platelets. Here, NO diffuses through the platelet membrane and is recognised by soluble guanylyl cyclase (sGC), which catalyses the conversion of GTP to cGMP (Mellion et al., 1981). In turn, the increased concentration of cGMP triggers the activation of protein kinase G (PKG). This serine/threonine kinase phosphorylates multiple substrates including IP_3R, IRAG, VASP and HSP27 on sites indicative of inhibition (Smolenski, 2012) preventing the platelet from activation, including inhibition of calcium efflux, cytoskeleton rearrangement, δ-granule secretion and integrin activation (Schwarz et al., 2001; Clemetson, 2012).

PGI_2 is a lipid messenger synthesised in endothelial cells in a similar fashion to TxA_2. It is a product of arachidonic acid metabolism, involving the enzymes COX-2 and prostacyclin synthase. Upon entry into the circulation, it is recognised by the prostacyclin receptor (IP), present on the platelet cell membrane. IP is also a GPCR, coupled to Gαs which stimulates adenylyl cyclase. Like sGC, adenylyl cyclase catalyses the conversion of ATP to cAMP, which in turn activates protein kinase A (PKA) (Narumiya et al., 1999). The effects of PKA include the inhibitory phosphorylation of IP_3R and myosin light chain kinase,

preventing calcium efflux and cytoskeletal rearrangement respectively, and prevention of integrin activation *(Schwarz, Walter et al. 2001)*. It is believed that the release of these short lived mediators are key regulators of platelet function as loss of their signalling through deletion of PKG or IP from platelets is associated with a prothrombotic phenotype (Massberg et al., 1999; Cheng et al., 2002).

Levels of cGMP and cAMP are tightly controlled within the platelet in order to allow activation when required. Modulation of cyclic nucleotides is performed by phosphdiesterases (PDE) – a group of enzymes able to catalyse the conversion of cGMP/cAMP to GMP/AMP respectively. Platelets express three members of the PDE family – PDE2, PDE3a and PDE5, with PDE3a reported to have the dominant role in cAMP modulation and PDE5 modulating cGMP. Under basal conditions, PDEs are active at low levels, causing high levels of intracellular cyclic nucleotides and active PKG/PKA. Conversely, platelet activation causes the phosphorylation of PDE, reducing cyclic nucleotide levels and activation of inhibitory proteins. Activation of PDE3a is reportedly caused by PKC/PKA activation (Hunter et al., 2009) whilst PDE5 activation is shown to be part of a negative feedback loop involving cGMP (Mullershausen et al., 2003; Smolenski, 2012).

1.5 Atherothrombosis

The collective process of plaque formation, rupture and the subsequent thrombotic event has been termed atherothrombosis and is associated with platelet hyperactivity. Platelet hyperactivity is a poorly defined term, but has been previously described in the context of acute MI as platelets requiring lower concentrations of agonists to induce an aggregation response and decreased sensitivity to inhibitory agents (Mueller et al., 1986). More crucially, the precise mechanisms underpinning this increased propensity to activate in the presence of oxLDL remain unclear. Early studies investigating platelet activity in patients with hyperlipoproteinemia showed that significantly reduced concentrations of agonists (epinephrine, ADP and collagen) were able to induce aggregation compared to healthy individuals (Carvalho et al., 1974). However, in the same study, platelet adhesiveness and clot retraction times remained comparable between groups. Later studies investigated platelet function in individuals with familial hypercholesterolemia (Aviram and Brook, 1982), also reporting platelet hypersensitivity to physiological agonists. Having shown that LDL was the likely causative agent, subsequent studies isolated LDL directly from the plasma of healthy individuals and oxidised it *in vitro*, showing definitively that oxLDL acts as a weak platelet activating stimulus (Zhao, Dierichs, Liu and Holling-Rauss, 1994; Naseem et al., 1997). Therefore, it is possible that platelet hyperactivity associated with dyslipidaemia may be caused by oxLDL either released from the plaque or present in circulation.

1.5.1 OxLDL mediated platelet activation

Evidence exists to show that platelets are able to bind and sequester oxLDL (Zhao, Dierichs, Liu and Berkes, 1994) . Early biochemical studies showed that platelets were able to bind oxLDL, but not acetylated LDL and that ligation of oxLDL induces platelet activation (Volf et al., 1999). Ligation of oxLDL by platelets has been supported with recent clinical studies which showed that patients with acute coronary syndromes have increased oxLDL bound to their platelets, inducing platelet adhesion to the endothelium (Stellos et al., 2012). Furthermore, platelets incubated with fluorescently dyed oxLDL

showed that oxLDL was able to bind to platelets and moreover was able to become internalised (Daub, Seizer, et al., 2010). Platelet interaction with oxLDL has been shown to induce a plethora of functional responses.

1.5.1.1 Induction of platelet adhesion and shape change

The ligation of oxLDL to platelets has been shown to trigger shape change. A central *in vivo* study showed that platelets adhere to endothelial cells at sites of atherogenesis in a hyperlipidaemic mouse model and moreover, speculated that oxLDL could be the causative agent (Massberg et al., 2002). Later *in vitro* studies showed definitively that immobilised oxLDL can cause platelet adhesion and spreading (Hartwich et al., 2002; Nergiz-Unal, Lamers, et al., 2011). Importantly, oxLDL was shown to be able to induce activatory signalling mechanisms and showed independence from secondary signalling mediators (Nergiz-Unal, Lamers, et al., 2011; Wraith et al., 2013).

1.5.1.2. Release of secondary mediators

OxLDL has been shown to trigger release of α-granule contents from platelets. One important protein, P-selectin has been shown to be expressed on platelets after oxLDL stimulation (Sener et al., 2011) and clinical studies show that this can be prevented by use of statins (Bruni et al., 2005; Puccetti et al., 2005). Many *ex vivo* reports have measured P-selectin expression on platelets using flow cytometry after treatment with oxLDL, finding α-granule secretion to occur following stimulation with this modified lipoprotein (Takahashi et al., 1998; Nergiz-Unal, Lamers, et al., 2011). Along with *in vitro* oxidatively modified LDL, a species of modified LDL with very similar characteristics to oxLDL, *electronegative LDL* isolated from ST wave-elevated myocardial infarction (STEMI) patients, also has the ability to induce α-granule secretion of platelets (Chan et al., 2013). In addition to P-selectin, platelets have also been shown to release CD147 from α-granules in response to oxLDL (Yang et al., 2013). CD147 is a known matrix metalloprotease (MMP) inducer and has been shown to be elevated on platelets in patients with coronary artery disease (Pennings et al., 2010), although the mechanism by

how it signals in platelets is unknown. The ability of oxLDL to induce P-selectin expression is significant since it is correlated with increased risk of atherosclerotic lesion development by adhering to the endothelium and recruiting leukocytes (Burger and Wagner, 2003; Badrnya et al., 2014) . In animal studies, mice deficient in CD62P delays fatty streak formation in a hyperlipidaemic model, indicating that whilst acting as a platelet agonist, oxLDL may also aids the onset of atherogenesis (Johnson et al., 1997).

Similar to α-granules, oxLDL can trigger release of δ-granules. Several *in vitro* studies have shown that platelets release serotonin, present in δ-granules, upon stimulation with oxLDL (Zhao et al., 1995; Yanai et al., 2007). Furthermore, a clinical study by Hara et al. showed that in patients with atherosclerotic cardiovascular disease, serum levels of serotonin were elevated above controls (Hara et al., 2004), indicating this may play an important role *in vivo*. ADP is another key platelet agonist released from δ-granules in response to oxLDL stimulation. An early study by Haserück et al. showed that lysophosphatidic acid, a lipid present within modified LDL species, was able to induce platelet aggregation, but this was lost if pre-treated with either apyrase or ADP receptor antagonists (Haserück et al., 2004). Like TxA_2, ADP secretion is required for oxLDL to induce platelet shape change and aggregation. Subsequent studies using oxLDL as a ligand echo these findings, stating that oxLDL pathogenicity is exacerbated by secretion of ADP (Korporaal et al., 2007; Nergiz-Unal, Lamers, et al., 2011; Wraith et al., 2013).

Unlike granule release, the ability of oxLDL to induce TxA_2 production remains less clear. Early studies have shown that oxLDL triggers TxA_2 synthesis via a phospholipase A_2 dependent mechanism (Mahfouz and Kummerow, 1998; Mahfouz and Kummerow, 2000). However, in studies where TxA_2 production is blocked by COX inhibitors, platelet aggregation in response to oxLDL does not change, indicating that TxA_2 is not involved (Ardlie et al., 1989; Weidtmann et al., 1995). Interestingly, LDL isolated from patients with metabolic syndrome or type 2 diabetes also induces the production of TxA_2 in platelets (Colas et al., 2011) and in patients with hyperlipidaemia, levels of the thromboxane

metabolite (TxB_2) were significantly elevated, a response which was lost when anti-platelet agents were present (Yamada et al., 1999). Whilst evidence suggests that oxLDL is able to induce platelet mediated TxA_2 production, the resulting TxA_2 may have far-reaching affects including acting upon platelets and the vasculature. This requires further work to fully understand the role TxA_2 plays in atherothrombosis.

1.5.1.3 OxLDL induced platelet aggregation

The ability of oxLDL to cause platelet aggregation remains ambiguous. It is likely that differences in the methodology of LDL oxidation and variability in the responses of healthy platelet donors has compounded this. LDL oxidised by hypocholorous acid yields a product modified preferentially on the protein constituent of LDL and has been shown to trigger aggregation of platelets alone (Volf et al., 2000) and enhance aggregation responses in combination with ADP (Coleman et al., 2004). For oxLDL produced by transition metal ions, there remains conflict between reports. Early studies showed that mmLDL was able to induce platelet aggregation, whereas fully oxLDL could not (Weidtmann et al., 1995; Naseem et al., 1997). However, more recent studies have showed oxLDL to induce mild amounts of platelet aggregation (Wraith et al., 2013) with the degree of aggregation induced increasing as the degree of oxidation increases (Korporaal et al., 2005). Several attempts have been made to isolate modified LDL direct from patients with disease and to examine the effects of this on healthy platelet function. Modified LDL from both diabetic patients and patients with metabolic syndrome can potentiate platelet aggregation to collagen, although the effects on the platelet-aggregating abilities of these modified LDL species alone was not investigated (Colas et al., 2011). Similarly, electronegative LDL was able to enhance platelet aggregation to ADP alone (Chan et al., 2013) indicating that platelets are hyperresponsive in these conditions. Together these data suggest that oxLDL can have a sensitising effect on platelets and serves to 'prime' them for activation. The nature of the mechanism that facilitates this is unclear given the wide variety of oxLDL species used in the various studies highlighted.

1.6 Scavenger receptors in platelets

The biological effects of oxLDL are though to be mediated through a group of scavenger receptors. These receptors are a diverse group of proteins involved in the recognition of pathogen-associated molecule patterns (PAMPs) and self-derived danger-associated molecular patterns (DAMPs). The human genome encodes a wide range of different scavenger receptors (Classes A-H), which are classified in groups depending on their multidomain structure and reviewed comprehensively in (Plüddemann et al., 2007). It is well established that platelets possess receptors from three classes of scavenger receptors- Class B, D and E (Valiyaveettil and Podrez, 2009; Ashraf and Gupta, 2011), with limited evidence for a fourth type- Class A (Korporaal et al., 2007). Of the scavenger receptors present, only the Class B receptors, CD36 and SR-BI are constitutively expressed on platelets with Class D (CD68) and Class E (LOX-1) receptors expressed upon platelet activation. CD36 is the most heavily implicated in the pathology process, with burgeoning evidence for the role of LOX-1 in endothelial cells and platelets.

1.6.1 CD36

CD36 is an 88 kDa transmembrane protein which is encoded by the gene *cd36* on chromosome 7 (7q11.2). It is part of the Class B scavenger receptor family which also includes Scavenger Receptor B1 and Lysosomal integral membrane protein 2. Expression levels of CD36 varying significantly within the population from 2,000 – 14,000 copies (Ghosh et al., 2011) and with a subset of the population, notably from Asian and African backgrounds (3-8%) not expressing the receptor at all, known as the Nak^a phenotype (Yamamoto et al., 1990; Curtis and Aster, 1996).

CD36 consists of a single peptide chain of 472 amino acids, which translocates to the plasma membrane. The receptor forms two transmembrane regions, a heavily glycosylated extracellular hairpin-like domain and two uncharacteristically short cytoplasmic tails. Despite this, CD36 is associated with the Src-family kinases (SFKs), Lyn, Fyn and Yes (Huang et al., 1991; Thorne et al., 2006) which are considered to be involved in the ability of CD36 to trigger intracellular signalling.

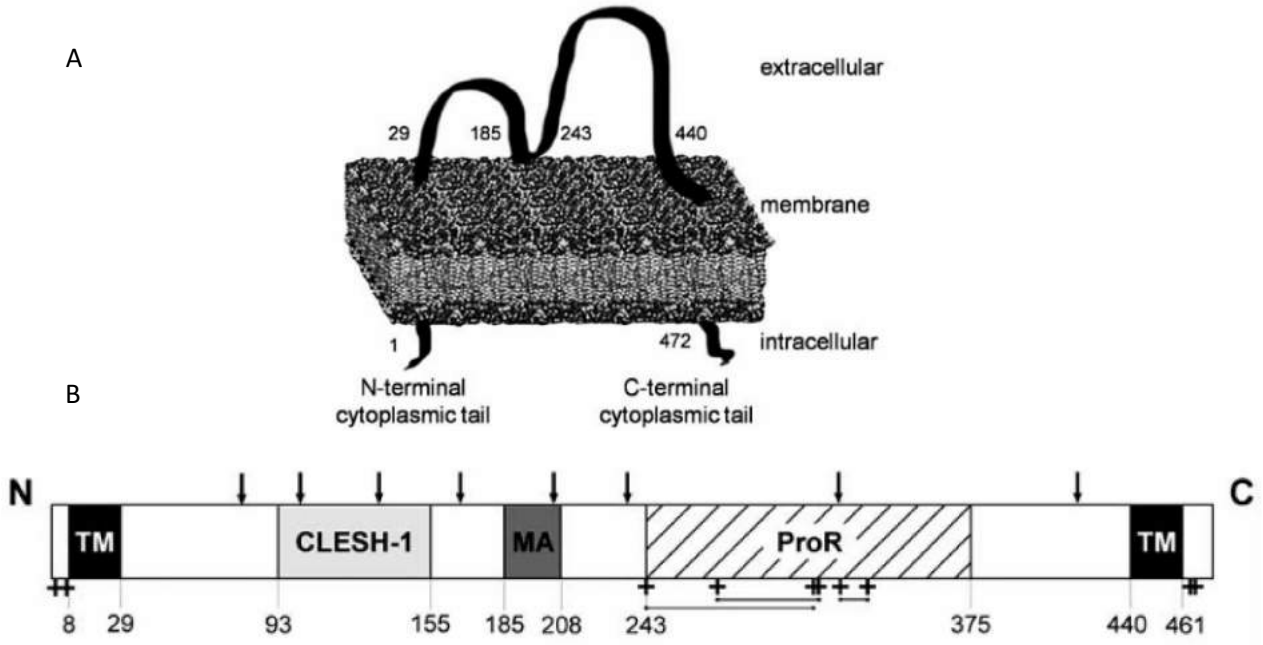


Figure 1.8 – Schematic of CD36. A – 3-dimensional representation of CD36 in relation to the plasma membrane. Numbers represent corresponding amino acid residues at each part of the protein. B – Detailed diagram of amino acid residues in the CD36 protein. Black arrows represent N-linked glycosylation sites and cross markers represent sites of cysteine residues and cysteine bridges. TM- transmembrane region, MA – membrane associated region, ProR – proline-rich regions. Adapted from Nergiz-Unal, Rademakers, et al. 2011.

The large hairpin loop contains a number of different binding motifs, enabling a wide range of ligands to bind to CD36 (Nergiz-Unal, Rademakers, et al., 2011). One of the earliest identified regions of CD36 is the CLESH-1 domain (CD36 LIMP II Emp structural homology-1) which was shown to bind TSP-1 in a calcium dependent manner (Pearce et al., 1995). Since its identification, it has been implicated in the binding of most ligands, with binding starting in the CLESH-1 domain and ending outside of it, as shown by use of synthetic peptides capable of binding to particular regions of the protein and mutations in the gene *Cd36* (Nergiz-Unal, Rademakers, et al., 2011). The role of the proline-rich domain is less well characterised, although it is hypothesised that it is required for suitable folding of the protein and is less likely to be involved with ligand binding (Hoosdally et al., 2009).

1.6.2.1 The function of CD36

CD36 has been implicated in a number of different processes including lipid and glucose metabolism, innate immunity and angiogenesis and sensory perception and is expressed in a range of different cell types, including leukocytes, smooth muscle cells, adipocytes and platelets (Silverstein and Febbraio, 2009; Nergiz-Unal, Rademakers, et al., 2011). Depending on ligand and cell type, ligation to CD36 can initiate numerous cellular effects, including metabolic changes, gene transcription and cytoskeletal rearrangement. This variety of cellular responses may also be in part due to the promiscuity of CD36 to act as a co-receptor for many other known receptors. These include toll-like receptors, tetraspanins and integrins (Miao et al., 2001; Triantafilou et al., 2006; Stewart et al., 2010). Particular interest surrounds the ability for CD36 to bind oxidised lipids. OxLDL was first shown to bind to macrophages, platelets and CHO cells transfected with CD36 in 1993 (Endemann et al., 1993), but it was not until 2007 that a definitive link between CD36 and atherothrombosis was made (Podrez et al., 2007). In this seminal paper, Podrez and colleagues showed that hyperlipidaemia was directly associated with platelet activation and that mice deficient in CD36 displayed a protective phenotype.

Ligand	CD36 Binding Motif	Reference
oxLDL	155–183	(Puente Navazo et al., 1996)
Oxidised phospholipids	157–171	(E. a. Podrez, 2002)
Fatty acids	127-279	(Hoebe et al., 2005; Martin et al., 2011)
Thrombospondin-1	93–155	(Stomski et al., 1992; Roberts et al., 2010)
Hexarelin	132-177	(Marleau et al., 2005)
Collagen	Unknown	(Tandon et al., 1989; Matsuno et al., 1996)
Plasmodium spp. Infected erthryocytes	145-183	(Eda et al., 1999; Patel et al., 2004)
Amyloid-β	Unknown	(Baranova et al., 2010)
Bacterial cell wall products	Unknown	(Hoebe et al., 2005)
Microparticles & Apoptotic cells	Unknown	(Ghosh et al., 2008)

Table 1.4 – CD36 ligands and known binding motifs on the CD36 protein.

1.6.2 CD36 Signalling in Platelets

Like multiple platelet receptors, CD36 is considered to signal in a tyrosine kinase dependent manner. Indeed studies in 1991 showed that Src family kinases, Lyn and Fyn, were constitutively associated with CD36 (Huang et al., 1991). Studies have shown that oxLDL ligation to platelets causes tyrosine phosphorylation of multiple proteins, including SFKs, Syk and PLCγ2 using the anti-phosphotyrosine antibody 4G10 (Wraith et al., 2013). Blocking of CD36 prevents tyrosine phosphorylation by oxLDL, implicating the role of CD36 further. It is generally considered that the association between CD36 and the Src-family kinases Fyn and Lyn propagates signalling in response to CD36 ligands, with the activated kinases shown to be associated with CD36 in response to oxLDL stimulation (Chen et al., 2008). However, it remains unclear whether CD36 monomers are able to transduce signalling changes or whether it requires co-receptors or an ITAM bearing protein in a similar fashion to other tyrosine kinase linked receptors. Evidence of CD36 clustering upon stimulation has been shown in macrophages using single molecule tracking (Jaqaman et al., 2011) and in endothelial cells, CD36 clustering in response to P. falciparum triggers SFK dependent signalling cascades (Davis et al., 2012). Canonical activation of SFKs causes phosphorylation and activation of Syk, with Syk inhibition preventing shape change and aggregation *in vitro* in response to oxLDL (Nergiz-Unal, Lamers, et al., 2011; Wraith et al., 2013).

OxLDL activation of Syk has been shown to cause phosphorylation of PLCγ2 (Wraith et al., 2013; Zimman et al., 2014). However, the consequences of PLCγ2 phosphorylation remain poorly understood. PLCγ2 activation serves to increase intracellular levels of DAG and IP_3 and studies have shown that oxLDL stimulation of platelets causes Ca^{2+} elevation (Coleman et al., 2004; Korporaal et al., 2005; Nergiz-Unal, Lamers, et al., 2011) and PKC activation (Coleman et al., 2004; Magwenzi et al., 2015), although these effects have not been thoroughly tied to the PLCγ2 isoform. OxLDL ligation to CD36 is able to cause the activation of NADPH oxidase resulting in ROS formation in a PKC dependent manner

(Assinger et al., 2010; Magwenzi et al., 2015). Conflicting evidence exists to show that oxLDL is able to induce activation of the platelet MAP kinases (Korporaal et al., 2007; Chen et al., 2008; Karimi and Rashtchizadeh, 2013), although their relative contribution to platelet activation in response to oxLDL is poorly understood. Furthermore, the role of CD36 in the activation of MAP kinases requires further investigation. Other noteworthy proteins phosphorylated and activated in response to oxLDL ligation to CD36 include the RhoGEF Vav, shown to be downstream of SFK which is associated with integrin activation and increased risk of atherothrombosis (Chen et al., 2011) and myosin light chain and myosin light chain kinase, promoting platelet shape change in response to oxLDL (Wraith et al., 2013). Whilst many proteins have been shown to become phosphorylated in platelets in response to oxLDL, the respective signalling pathways and functional consequences remain incompletely defined.

1.6.3 LOX-1

Since the discovery of LOX-1 in 1997 in endothelial cells, it has subsequently been discovered in macrophages, smooth muscle cells and platelets (Sawamura et al., 1997; Chen et al., 2001). LOX-1 is a 50kDa, Class E scavenger receptor that is activated by proinflammatory and proatherogenic stimuli, including TNF-α, IFN-γ, IL-1, Angiotensin II and endothelin 1 (Yoshimoto et al., 2011). It has been implicated in multiple pathogenic processes including endothelial cell activation/dysfunction, foam cell formation and smooth muscle cell migration and is at much higher abundance in atherosclerotic plaques. In addition, immunohistochemistry studies have detected LOX-1 in thrombi from human endarterectomy tissue samples from patients with unstable angina pectoris (Chen et al., 2001).

LOX-1 is a Type II integral membrane glycoprotein, containing a short N-terminus cytoplasmic domain, transmembrane domain, neck domain and extracellular C-type lectin-like domain (Ogura et al., 2009). Evidence suggests that LOX-1 functions as an oligomer which may enable the receptor to transduce intracellular signalling (Silver, 2002; Matsunaga et al., 2007), although the number of receptor copies present on platelets is

unknown. Like CD36, LOX-1 binds multiple ligands including modified LDL, C-reactive protein (CRP) and activated platelets (Xu et al., 2013). LOX-1 has recently been shown to bind electronegative LDL, which represents ~10% circulating LDL and can promote atherosclerosis (Chan et al., 2013). In platelets, LOX-1 is believed to be present in α-granules, although very little evidence exists to support this claim (Chan et al., 2013). Interestingly, blockade of LOX-1 reduces platelet aggregation in a time and dose dependent manner, indicating that LOX-1 may play a role in platelet aggregation in haemostasis (Marwali et al., 2007).

Very little is known about how LOX-1 signals in platelets; with the majority of signalling studies focusing on endothelial cells. In endothelial cells, LOX-1 signalling plays a major role, causing activation of PKC, inhibition of PI3-K and Akt and inhibition of MYPT1 (Xu et al., 2013). In platelets, LOX-1 has been associated with ADP dependent inside-out signalling, with blockage of PKC causing decreased integrin activation (Marwali et al., 2007) although very little information of the types of signalling cascades activated and the key proteins involved are known.

1.7 Project Aims

There is a growing body of historical evidence to link hyperlipidemia with elevated risk of atherothrombosis. This increased risk is now attributed, at least in part, to oxLDL priming platelets for activation and increasing the propensity of platelet activation and subsequent thrombus formation. The recognition of oxLDL is believed to be mediated by scavenger receptors capable of binding oxLDL and triggering intracellular signalling mechanisms causing functional effects to the platelet. CD36 has been the attention of the majority of studies investigating oxLDL-induced thrombosis although in recent years it has been believed that LOX-1 may also play a significant role in triggering activation. However, significant further study is required to fully elucidate its role.

Therefore, the aims of this study are to:

- Identify and confirm the effects of oxLDL on platelet function
- Determine the role of scavenger receptors in this process, namely CD36 and LOX-1
- Investigate the mechanisms underpinning scavenger receptor mediated platelet activation

Chapter 2: Materials & Methods

2.1 Chemicals and reagents

Bis-acrylamide and Trans-Blot Turbo® Transfer packs was purchased from Bio-Rad (Hemel Hempstead, UK) and Prestained molecular weight protein ladder was from New England Biolabs (Ipswich, UK). Phe-Pro-Arg-Chloromethylketone (PPACK), Dasatinib, prostacyclin and phospholipids KOdiA-PC and PAPC was purchased from Cambridge Bioscience Ltd (Cambridge, UK). 1-[6-[[(17β)-3- Methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1H-pyrrole-2,5-dione (U73122) was purchased from Tocris Bioscience (Bristol, UK). R406 was from Selleckchem (Suffolk, UK). 8-CPT-cGMP analogue was purchased from Biolog (Bremen, Germany). Tirofiban (Aggrastat; Iroko Pharmaceuticals, USA) was a kindly donated from the Haematology Department at Castle Hill Hospital (Hull, UK). Fluorescently labelled secondary antibodies were purchased from Li-Cor (Cambridge, UK) and VenaFlux™ biochips were purchased from Cellix (Dublin, Ireland).

CRP-XL was purchased from Prof. Richard Farndale, Department of Biochemistry at the University of Cambridge (Cambridge, UK). Collagen horm reagent was purchased from Nycomed (Zurich, Switzerland). Human α-thrombin, recombinant fibrinogen, bovine serum albumin (BSA), and all other reagents were purchased from Sigma-Aldrich (Poole, UK).

2.2 Antibodies

Antibody manufacturers and conditions used are detailed in the table below:

Antibody	Source	Species raised	Conditions
Anti-CD36 (FA6.152)	Santa Cruz	Rabbit	Receptor blocking (1µg/mL)
Anti-CD36 (H-300)	Santa Cruz (sc-9154)	Rabbit	IB: 1:1000
Anti-LOX-1	Gift from University of Leeds	Sheep	Receptor blocking (1µg/mL)
Anti-LOX-1	Abcam (ab60178)	Rabbit	IB: 1:250
Anti-phospholipase Cγ2 (Q-20)	Santa Cruz (sc-407)	Rabbit	IB: 1:1000 IP: 5µg
Anti-phosphoPKC substrate	Cell Signalling Technology (#6967S)	Rabbit	IB: 1:1000
Anti-phosphoSrc^{Y416}	Cell Signalling Technology (#6943S)	Mouse	IB: 1000
Anti-phosphotyrosine (4G10)	Merck Millipore (05-321)	Mouse	IB: 1:1000
Anti-Syk (4D10)	Santa Cruz (sc-1240)	Rabbit	IB: 1:1000 IP: 1µg
Anti-β-tubulin	Upstate (05-661)	Mouse	IB: 1:1000
Fluorescent secondary antibodies	Li-Cor	Mouse/Rabbit	IB: 1:15,000

Table 2.1 – Table of antibodies used and their respective conditions.

2.3 Isolation of platelets

To determine the effects of modified low-density lipoproteins on platelet function and signalling, it is important to be able to isolate platelets free of contamination of other cells, whilst keeping them in a non-activated state. In brief, a method adapted from Mustard et al., was used in which platelets were isolated from the rest of the blood using low speed centrifugation and re-suspended into a physiological buffer (Mustard et al., 1972), although the specific conditions varied from human to mouse. Whilst different

strategies exist to keep platelets inactive during the isolation, including use of prostaglandins and alterations to pH (Cazenave et al., 2004; Aburima et al., 2013), we chose to use an acid-based isolation method to avoid complication when investigating the effects of inhibitory pathways.

2.3.1 Human platelets

Blood was collected from healthy, adult volunteers who were drug-free and free of chronic illnesses for a minimum of 14 days prior to donation. Ethical approval was obtained from the Hull York Medical School Ethical Committee. Blood was taken from the antecubital fossa following a standardised venepuncture technique using a 21g butterfly needle and syringe. The first three millilitres of blood were discarded to exclude any platelets undergoing artifactual activation as part of the procedure. To prepare washed platelets, blood was collected into syringes containing acid-citrate-dextrose (ACD; 29.9mM sodium citrate, 113.8mM glucose, 72.6mM NaCl, 2.9mM citric acid, pH 6.5) at the ratio 5:1 (v/v) as an anticoagulant. Anticoagulated blood was centrifuged at 200*g* for 15 minutes and the uppermost layer containing platelet rich plasma (PRP) was removed. To prevent sequential platelet activation, PRP was treated with 0.3M citric acid to reduce plasma pH and centrifuged at 800 xg for 12 minutes. The resulting platelet pellet was resuspended in 4-6mL Wash buffer (36 mM citric acid, 10 mM EDTA, 5 mM glucose, 5 mM KCl, and 9 mM NaCl), depending on the pellet size, to further remove any plasma proteins present and then centrifuged once again. The washed platelet pellet was then resuspended in 1-2mL modified Tyrode's buffer (50mM NaCl, 5mM HEPES, 0.55mM NaH_2PO_4, 7mM $NaHCO_3$, 2.7mM KCl, 0.5mM $MgCl_2$ and 5.6mM glucose, pH 7.4) and allowed to warm up to 37^{o}c prior to use.

2.3.2 Murine platelets

Murine platelets were isolated via a similar process to human platelets. Mice were culled by CO_2 overdose and blood was drawn via cardiac puncture using a 25g needle and syringe containing ACD (200µL). After mixing in the syringe, blood was immediately transferred to an Eppendorf tube containing modified Tyrode's buffer (1mL) and mixed

again to dilute the blood. Diluted blood was spun at 100*g* for 5 minutes with no brake to separate PRP from the rest of the blood components. PRP was removed and replaced with modified Tyrode's buffer (300µL) and mixed. This was repeated twice and the collected PRP was treated with citric acid and spun again at 1000*g* for 6 minutes to pellet. Platelets were resuspended in modified Tyrode's buffer and counted before use.

2.3.3 Cell counting

Platelets were quantified using a Beckman Coulter Z1 Coulter Particle Counter. In brief, this procedure detects changes in electrical impedance. Cells suspended in a physiological buffer are passed through an aperture and disrupt the native electrolyte, which records a voltage pulse.

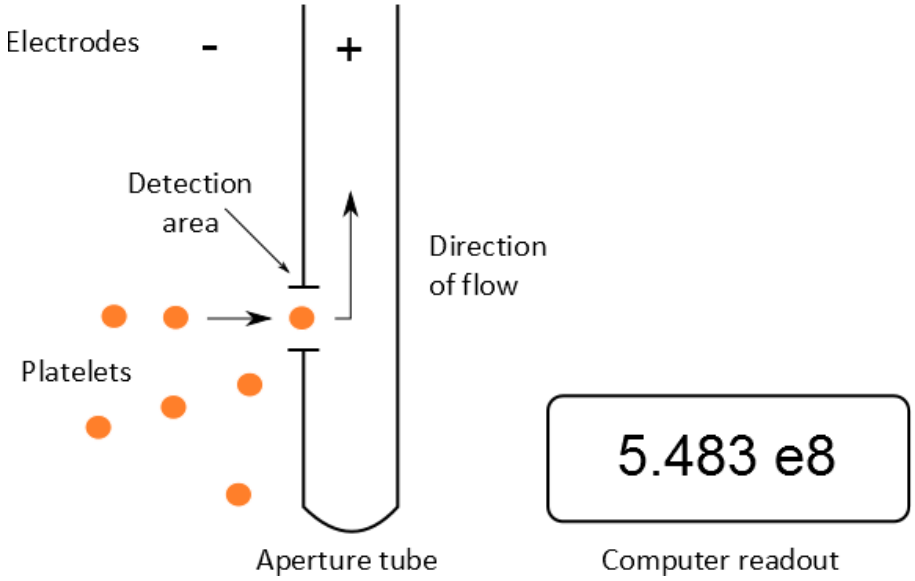


Figure 2.1 – Coulter Principle schematic. Platelets pass through a detector which measures changes in electrical impedance and counts the number of events within a specific volume of aspirated liquid. The digital computer readout screen provides the number of platelets per mL buffer.

2.4Methods for the isolation and oxidation of LDL

In order to study the effects of modified lipoproteins on platelets, LDL from plasma was isolated by ultracentrifugation. The isolated LDL was oxidatively modified using metal ions to simulate the pathological oxLDL particles present within the circulation. The degree of

oxidation was confirmed using multiple biochemical tests which measure both lipid and protein modification in response to oxidation.

2.4.1 Isolation of LDL by sequential ultracentrifugation

The isolation of lipoproteins using ultracentrifugation is a well-defined method, first described in 1955 (Havel et al., 1955). Blood was collected from healthy, drug-free donors into syringes containing sterile EDTA (150mM) to prevent blood clotting and reduce the likelihood of artificial oxidation. Upon thorough mixing within the syringe, blood was immediately transferred to chilled Falcon tubes (50 mL) and centrifuged at 1500*g* for 30 minutes to remove cells. Plasma was removed and the density was adjusted to 1.019g/mL using a high density salt solution (2.97M KBr, 2.62M NaCl, 297µM EDTA, pH. 7.4). Plasma was further treated to dialysis for 2 hours in a 1.019g/mL solution at 4^{o}C. Dialysed plasma was transferred to ultracentrifuge compatible tubes and centrifuged at 115,000*g* for 18 hours at 4^{o}c in order to separate chylomicrons and VLDL.

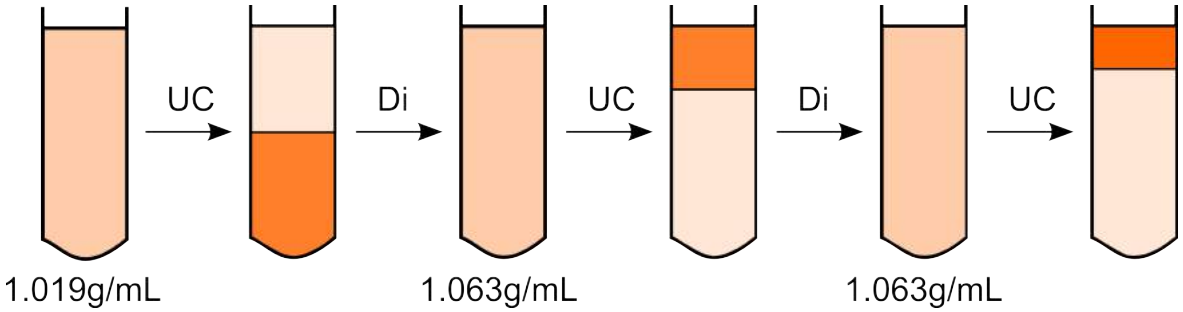


Figure 2.2 – Schematic of LDL isolation. Plasma was treated to a series of dialysis and ultracentrifugation stages in order to isolate LDL. Orange colouring represents the section of the ultracentrifugation tube which was isolated and dialysed to the density labelled below the following tube. UC – Ultracentrifugation stage, Di – Dialysis stage.

These lipoprotein species collected at the top of the tube and were removed using a Pasteur pipette. The remaining fraction was isolated, density adjusted to 1.063g/mL using HDS and was dialysed in a 1.063g/mL buffer for a further 2 hours. Subsequently, plasma was transferred into fresh ultracentrifugation tubes and spun again to separate the LDL from HDL. LDL collected at the top of the tube was distinguished from HDL by its bright orange colour. In order to concentrate LDL, isolated LDL was dialysed again against

1.063g/mL buffer and centrifuged at 115,000*g*. Harvested LDL was further dialysed against a phosphate buffer (140mM NaCl, 8.1mM Na_2HPO_4, 1.9mM NaH_2PO_4, 100µM EDTA, pH 7.4) for 24 hours to remove any residual salts and EDTA which may have inhibited the subsequent oxidation reaction. LDL was sterilised using a 0.22µm filter and stored at 4°c in a light-resistant Eppendorf tube.

2.4.2 Oxidation of LDL using Cu^{2+} ions

Whilst several different methods exist to oxidise LDL *in vitro*, we used transition metal ions as described in the method by Gerry et al. (Gerry et al., 2008). In brief, transition metal ions serve to catalyse the fissure of existing lipid hydroperoxides to result in the formation of lipid aldehydes and alkyl radicals that can modify protein residues (Stohs and Bagchi, 1995). Native LDL was dialysed in phosphate buffer (140mM NaCl, 8.1mM Na_2HPO_4, 1.9mM NaH_2PO_4, pH 7.4) for 4.5 hours at 4°C with 3 changes to ensure all EDTA was removed from the isolation stage. Native LDL in phosphate buffer was further dialysed in MOPS buffer (150mM NaCl, 10mM MOPS, pH 7.4) containing Chelex-100, a transition metal ion chelator for 24 hours, with 2 changes during this time. Having removed any residual factors that might impair the oxidation process, nLDL was dialysed in MOPS buffer containing $CuSO_4$ (10µM) with two buffer changes. The temperature of the buffer varied on the type of oxidised LDL required. LDL was oxidised at 4°C for hydroperoxide-rich oxLDL, whereas more extensively modified, oxysterol-rich oxLDL was produced by incubation at 37°C. LDL oxidation was stopped by addition of the chelating agent EDTA (1mM) and subsequent dialysis in phosphate buffer containing EDTA for 24 hours at 4°C until a final concentration of 100µM was reached. For storage, LDL was sterilised using a 0.22µm filter and stored at 4°c in a light-resistant Eppendorf tube.

2.4.3 Modified Lowry assay

In order to quantify the concentration of nLDL or oxLDL, the Lowry protein assay was used to measure the concentration of apoB100. BSA standard curve samples were prepared (0-100µg/mL) in distilled water and a LDL sample was diluted 1:10 in order to fit on the scale. The complete Lowry solution was prepared by mixing Lowry solution A (0.2M

Na_2CO_3, 0.1M NaOH, 5.7mM Sodium tartrate, 35mM SDS) and Lowry solution B (0.16M $CuSO_4$) in a ratio of 100:1 and was added to samples and left for ten minutes to ensure the formation of tetradentate copper-protein complexes in the alkaline environment provided. Folin-Ciocalteau's Phenol reagent was added to each sample, thoroughly mixed and the reaction allowed to take place for 5 minutes at 55^{o}C. During this step, Folin-Ciocalteau's Phenol reagent becomes reduced in relation to the amount of chelated copper complexes present and produces a blue-coloured product. Samples were transferred to a clear bottom 96 well plate and absorbance read at 650nm in a plate reader (Tecan).

2.4.4 Lipid hydroperoxide assay

In order to quantify the degree of LDL oxidation, lipid hydroperoxides were measured using the method described by el-Saadani (el-Saadani et al., 1989). nLDL and oxLDL (25µg) were diluted in deionised water (250µl) and added to 1mL measurement buffer (163mM KH_2PO_4, 37mM K_2HPO_4, 120mM KI, 0.2% Triton X-100, 0.15mM sodium azide, 0.01% w/v benzalkonium chloride, 10µM ammonium molybdate, pH 6), thoroughly mixed and left to incubate for 1 hour in the dark. During this time, the lipid peroxides present within the LDL enable the conversion of iodide to iodine within the measurement buffer causing a colour change. Samples were transferred to a clear bottom 96-well plate and absorbance was read at 365nm. All samples were made in triplicate and serial dilutions of hydrogen peroxide (0-200ng/mg protein) were used as a standard curve.

2.4.5 Relative electrophoretic mobility assay

Protein modification of ApoB100 is another important marker for LDL oxidation. Upon LDL oxidation, lipid hydroperoxides degrade forming aldehydes that can covalently modify lysine residues within the protein (Levitan et al., 2010). This protein modification causes the LDL particle to have a net negative charge and consequently can be exploited as a proxy for degree of oxidation.

Agarose gels were prepared (1% agarose dissolved in TAE buffer (40mM Tris base, 0.1% glacial acetic acid, 1mM EDTA, pH 8)) and loaded with nLDL and oxLDL (20µg). Gels were ran at 100V for 1 hour in TAE buffer. In order to visualise bands, gels were stained with Coomassie Blue solution (0.025% w/v Coomassie Brilliant Blue, 40% v/v methanol, 7% v/v glacial acetic acid) for 2 hours. Gels were then de-stained for 2 hours in De-stain Solution 1 (40% v/v methanol, 7% v/v glacial acetic acid) to remove the majority of background staining and transferred to De-stain solution 2 (5% v/v methanol, 7% v/v glacial acetic acid) overnight to help remove any residual background staining and prevent the gel from drying out. The following morning, the relative electrophoretic mobility value was calculated using the formula:

$$\mathrm{REM} = \frac{\text{Distance travelled by oxLDL (mm)}}{\text{Distance travelled by nLDL (mm)}}$$

2.5 Microscopy

2.5.1 Fluorescence microscopy

In order to visualise platelet adhesion and shape change responses, we employed fluorescence microscopy. Fluorescence microscopy works on the principle of staining macromolecules e.g. proteins, lipids, nucleic acids of interest with specific fluorescent markers (known as fluorophores/ fluorochromes) and imaging this using a camera and image capturing software. Fluorophores consist of a macromolecule capable of binding to a specific target and a fluorescent dye covalently bound (Fig. 2.3). Cells stained with one fluorophore can be imaged using a fluorescence microscope. In brief, a fluorescence microscope works by exciting the fluorophore using light energy causing a shift in photon energy levels ($S_0 \rightarrow S_1$) (Sanderson et al., 2014) (Fig. 2.4). Nanoseconds later, the excited particle relaxes, causing surplus energy to be released in the form a photon of a longer wavelength which is able to be detected.

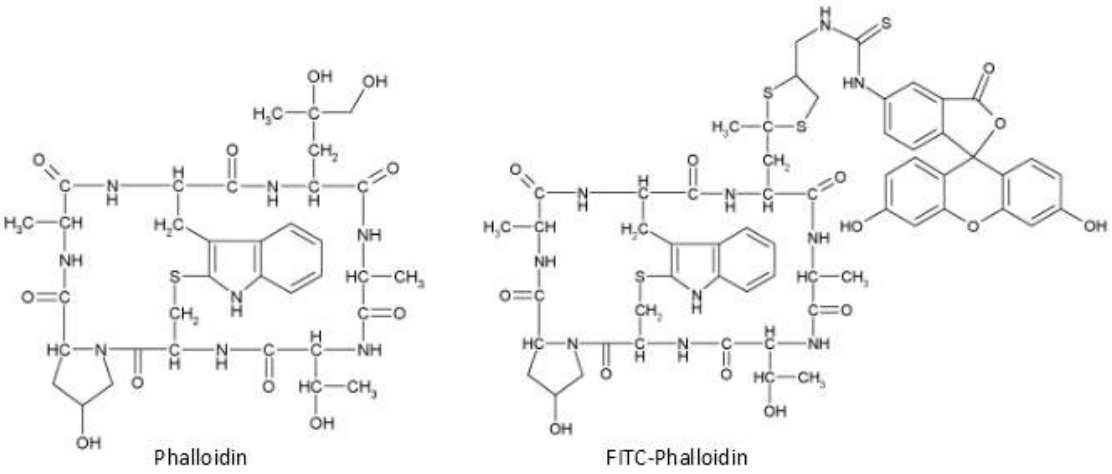


Figure 2.3 – Example of a fluorophore. Phalloidin, a potent inhibitor of actin polymerisation is conjugated to FITC via a covalent bond allowing staining and visualisation of cellular actin.

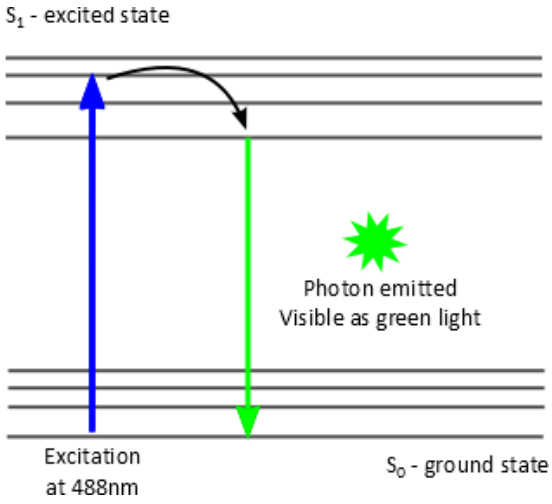


Figure 2.4 – Jablonski Diagram illustrating the principles of energy transfer of a molecule. In this example, a FITC-conjugated molecule is excited at 488nm before returning back to the ground state. In order to return back, it has to release excess energy in the form of a photon, which appears green. Adapted from Sanderson et al.

2.5.2 Platelet static adhesion assay

In a physiological setting, platelet adhesion is a crucial event in formation of the platelet plug. The platelet static adhesion assay is a microscopy-based technique, allowing visualisation of platelet adhesion and shape change in response to an agonist of choice. Moreover, this technique allows dissection of the signalling pathway involved by treatment of cells with pharmaceutical inhibitors or the use of platelets from mice deficient in specific proteins.

To provide an adherent surface for platelet adhesion, agents of interest including oxLDL, nLDL, fibrinogen and collagen were immobilised onto glass coverslips in a 24-well plate overnight at 4^{o}C. Residual unbound proteins were washed away with PBS and slides blocked with 5% human serum albumin for 30 minutes at room temperature to prevent platelet adhesion to exposed glass.

Washed platelets (5x10^{7}/mL) were treated with inhibitors if required prior to incubation for a maximum of 30 minutes and then left to settle on the coverslips for 60 minutes at 37^{o}C. Unbound platelets were washed away and cells fixed with 4% paraformaldehyde for 10 minutes. Fixed platelets were permeabilised with 0.3% Triton X-100 in PBS for 5 minutes before staining with TRITC-phalloidin for 60 minutes to enable visualisation. Coverslips containing the stained platelets were mounted onto glass slides using a small drop of Histomount® Mounting media and left overnight at 4^{o}C in the dark to allow the mounting media to set firm.

The following day, slides were imaged using a Zeiss Axio Vert.A1 inverted fluorescence microscope with Axiocam IC CCD camera. Images were captured using either x60 or x100 oil immersion lenses and analysed using ImageJ (NIH, USA). When imaging slides, a minimum of 8 random fields of view were selected by moving the image out of focus and moving to a different region and re-focusing. For platelet adhesion analysis, cells were counted and a mean average calculated per treatment type. For platelet spreading analysis, two methods were used. Using the ImageJ software, platelets and background space were converted into black or white pixels with the average size of pixels per

platelet used as a proxy for determining cell size and converted into μm^2. Alternatively, platelets were individually outlined and the process repeated with a minimum of 100 cells analysed per treatment where possible. Results calculated were shown as mean number of platelets ±SEM per $0.1mm^2$ for cell adhesion and mean surface area per platelet ±SEM for cell spreading.

2.5.3 *Ex vivo* thrombosis assay

Unlike the static platelet adhesion assay, the *ex vivo* thrombosis assay allows real-time detection of thrombus formation *ex vivo* at a user defined sheer rate. Like the static adhesion assay, an agonist is immobilised to a surface, but for *ex vivo* thrombosis assays, agonists are immobilised to channels (flow chambers) allowing the passage of blood. The flowing of blood through the channel causes platelets to become forced to the channel periphery due to the effects of sheer stress (Kroll et al., 1996), where they interact with the immobilised agonist. Two separate techniques were used in this project including glass capillary microslides and a microfluidic plate system.

Glass capillary microslides were coated with proteins of interest overnight at 4^oC, including collagen (50µg/mL), fibrinogen (1000µg/mL) and oxLDL (10-200µg/mL) and blocked the following day using BSA (10mg/mL) in PBS. Glass capillary microslides were inserted into plastic tubing and washed with 3mL PBS to remove any unbound proteins.

Blood was drawn into syringes containing PPACK (50µM) and $DiOC_6$ (0.5µM) to fluorescently label cells, gently mixed and allowed to stain for 10 minutes. Since all experiments were flowed for 2 minutes, 1.6mL blood was added to a reservoir supplying the tubing and the pump set to the correct flow rate to flow blood at the desired sheer. 3mL PBS was flowed through the system afterwards to remove any unbound cells and images were taken using an inverted microscope (Olympus IX71) and CCD camera (XM-10, Olympus).

Unlike the glass capillary microslides, the microfluidic (Cellix) system uses microscopic printed channels to minimise the amount of blood required per flow experiment.

VenaFlux™ (Cellix, Ireland) biochips were coated with agonists of choice overnight at 4°C and blocked the following day with BSA (10µg/mL). Blood (100µL) treated with PPACK (50µM) and $DiOC_6$ (0.5µM) was added to the injection port using a pipette and the software (VenaFlux™, Cellix) programmed with the desired flow rate and running time (2 minutes). PBS (100µL) was used to wash away any unbound cells and images taken using an inverted microscope (Olympus IX71) and CCD camera (XM-10, Olympus). Data was measured as percentage coverage of the field of view, with a minimum of 8 images captured across the length of the channel.

2.6 Platelet aggregometry

In order to assess the effects of different agonists and inhibitors on platelet aggregation, light-transmission aggregometry was used. Light-tranmission aggregometry works in principle by measuring changes in optical density of a platelet suspension when an agonist is introduced (Born and Cross, 1963). Platelet aggregation is conducted under stirring conditions and is able to measure very early events in the aggregation process, including shape change, primary aggregation and secondary aggregation in response to ADP and TxA_2 (Frontroth, 2013).

Washed platelets (2.5 $x10^8$/mL) were warmed to physiological temperatures prior to the experiment and placed in siliconised glass aggregation tubes (Chrono-Log) containing a small magnetic bar, which allowed stirring at a set rate of 1000 rpm. Platelets were subjected to stirring for 30 seconds prior to the experiment to set a baseline measurement and were stimulated with agonists for a minimum of 3 minutes. Aggregation traces were recorded using the platelet aggregometer (Chrono-Log) and appropriate software (Aggro/Link).

2.7 Flow cytometric analysis

Flow cytometry is a rapid, high-throughput method for the profiling of cells suspended in a solution. A particular population of cells can be investigated by using cell size and granularity as specific markers, as well as cell-type specific antigens and the

corresponding fluorescently labelled antibodies. For platelet analysis, FACS is able to measure a wide range of platelet activation markers including α-granule release (CD62P), integrin activation, platelet-leukocyte interactions and receptor expression/shedding.

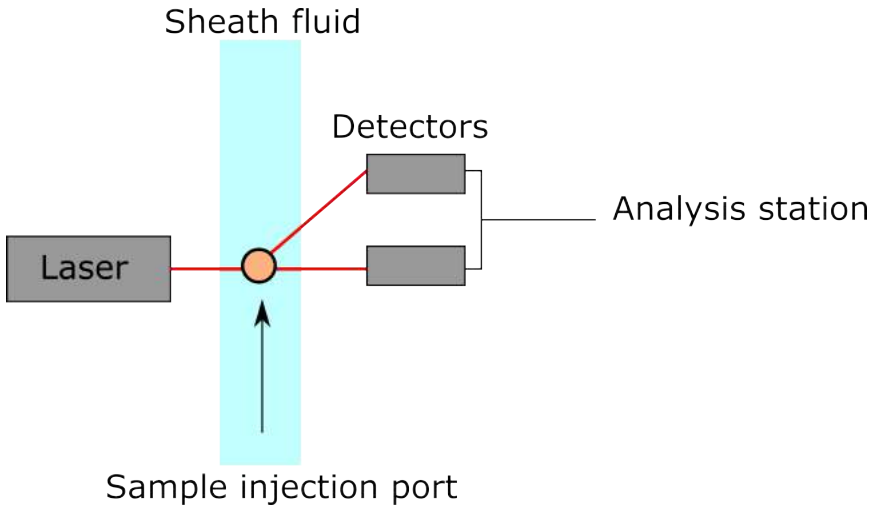


Figure 2.5 – Schematic of a Flow Cytometer. Cells are passed in single-file at high pressure through the sample injection port, where lasers are used to excite fluorophores attached to the cell surface and measure FSC and SSC. Multiple detectors measure photons of light emitted and convert this into electrical signals which are transmitted to the PC and interpreted by specialist software.

2.7.1 Whole blood analysis

Whole blood cytometry analysis was used to measure the degree of platelet activation on exposure to different agonists. Blood was taken into sodium citrate in the ratio 1:9 to prevent artificial platelet activation and clotting and gently mixed. Blood (5µL) was transferred into pre-prepared FACS tubes containing antibodies, agonists and modified Tyrode's buffer and mixed via gentle flicking. Blood platelets were stimulated for 20 minutes and fixed with 500µL formalsaline (0.2%). After 10 minutes, FACS tubes were loaded into the sample injection probe and 10,000 events measured in the platelet gate population. The platelet population was measured using the specific antibody CD42b.

2.7.2 Platelet-rich plasma analysis

For experiments using pharmaceutical inhibitors or to avoid the complicating effects of other cell populations, platelet-rich plasma was used instead of whole blood. Human or

murine blood was collected into syringes containing sodium citrate (1:9) and spun at 200 or 100*g* respectively, depending on the species for 5 minutes. PRP was removed and treated, if required, with inhibitors for 20 minutes. Treated PRP (5µL) was transferred into pre-prepared tubes containing antibodies, agonists and modified Tyrode's buffer and mixed via gentle flicking. For murine studies, modified Tyrode's buffer enriched with $CaCl_2$ (2mM) was used to counteract the effects of the anticoagulant. PRP was stimulated for 20 minutes and fixed with 500µL formalsaline (0.2%) for 10 minutes prior to signal detection. 10,000 events were measured and the purity of the PRP isolation checked via use of CD42b (>95% total cells).

2.8 Methods for the examination of protein signalling events

The intracellular environment is highly dynamic and often changes in response to different effectors. In order to measure changes to protein expression and post-translational modifications, SDS-PAGE and immunoblotting are used to correlate the response to a specific effector.

2.8.1 Sample preparation

In order to investigate changes in intracellular signalling, platelets were stimulated with agonists for a specific period of time. Washed platelets ($5x10^8$/mL) were stimulated in glass aggregation tubes under stirring conditions (1000 rpm) and were treated with EGTA (1mM), apyrase (2U/mL), indomethacin (10µM) and tirofiban (1µg/mL) to prevent detection of proteins modified by secondary signalling mediators. Cells were lysed with ice-cold 2x Laemmli buffer (4% SDS, 10% 2-mercaptoethanol, 20% glycerol, 50mM trizma base, bromophenol blue (trace), pH 6.8) and transferred to labelled eppendorf tubes. Lysates were left on ice for 30 minutes to enable complete cell lysis. An aliquot of platelets were removed prior to lysis and were lysed separately in IP Lysis buffer (150mM NaCl,10mM Tris base, 1mM EGTA, 1mM EDTA, 1% igepal (v/v), 1mM PMSF, 2.5mM Na3VO4, 1:200 protease inhibitor cocktail (v/v), 1:200 phosphatase inhibitor cocktail (v/v) (Sigma – P0044), pH 6.8) for the purposes of determining protein concentration.

2.8.2 Protein assay for cell lysates & immunoprecipitation

Protein assays were performed using a detergent compatible protein assay kit (Bio-Rad, UK), following the manufacturer's instructions. The assay is based on the Lowry assay and follows the same principles as described above.

Serial dilutions of a BSA stock solution of known concentration (0-1.5 mg/mL) in IP lysis buffer were used to create a standard curve. 5µL of either lysate or protein standard was pipetted into a 96 well plate in triplicate, followed by the addition of the copper tartrate solution (25µL) and Folin reagent (200µL). The plate was left for 15 minutes to incubate prior to measurement of light absorption at 750nm. Protein concentration of the lysate was derived by interpolation using values from the standard curve.

2.8.3 Sodium dodecyl sulphate polyacrylamide gel electrophoresis

Cell lysates contain a complex mixture of proteins, which need to be separated out before detection can take place. Protein separation can be performed using different criteria including size, charge, solubility in order to facilitate detection. However, when a solid substrate is required, proteins separation is usually conducted by SDS-PAGE.

First described in 1970 (Laemmli, 1970), SDS-PAGE is composed of two components – a polyacrylamide gel and a salt-based running buffer. Polyacrylamide gels act as a sorting mechanism, allowing smaller proteins to migrate through the gel, whilst larger proteins remain at the top (Fig. 2.5). Gels are formed by vinyl addition chemistry- a free radical driven process - in which acrylamide monomers polymerise into long chains and are cross-linked by bis-acrylamide present in solution. The process is accelerated by the compounds ammonium persulphate (APS) and tetramethylethylenediamine (TEMED), in which TEMED catalyses the release of free radicals from APS.

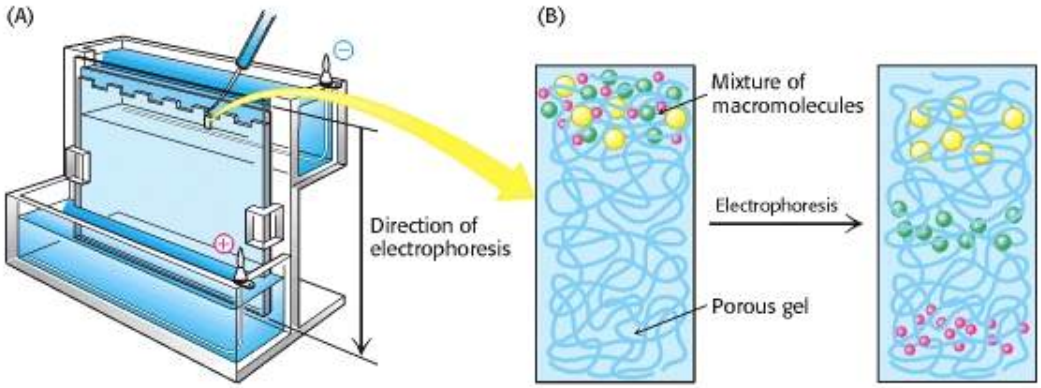


Figure 2.6– Schematic of SDS-PAGE system. Polyacrylamide gels are cast outside of the system and placed into a reservoir containing running buffer. Upon introduction of an electrical current, proteins begin to separate on the basis of size, with the smallest proteins travelling the furthest due to pores within the gel. Adapted from Berg et al. 2002.

The running buffer is essential for the migration of proteins across the gel. In order for protein mixtures to be separated, proteins are first denatured by use of Laemmli buffer which contains both SDS and β-mercaptoethanol to interfere in bond formation and the protein to lose its tertiary structure. Moreover, SDS also binds to the protein, negating the effects of the protein's intrinsic charge. The binding of SDS results in an equal charge-to-mass ratio for all proteins (one molecule SDS per two amino acids), allowing separation based on size alone.

Polyacrylamide gels (1.5mm) were prepared and composed of a resolving gel (10%) and stacking gel (3%) (Table 1) and left overnight at 4^{o}C to cool. Gels were loaded into running tanks and tanks filled with Running buffer (0.1% SDS (w/v), 25mM Tris base, 192mM glycine), allowing an electrical current to be conducted throughout the system and facilitate protein migration. Samples (5-40 µg) were loaded alongside a pre-stained protein ladder. Gels were run at 120V for 60-180 minutes, depending on protein of interest.

Table 2.1 - Reagents required to prepare two complete gels

Reagent	Resolving Gel (10%)	Stacking Gel (3%)
dH2O	6.4 mL	4.87 mL
Acrylamide	5.3 mL	0.75 mL
Buffer 1/2	4 mL (Buffer 1)	1.87 mL (Buffer 2)
APS	75 µL	75 µL
TEMED	5.3 µL	10 µL

Table 2.2 - Reagents and pH required to prepare Buffers 1 and 2 for acrylamide gels

Reagent	Buffer 1	Buffer 2
Tris Base	1.5M	0.5M
SDS	0.4% (w/v)	0.4% (w/v)
pH	8.8	6.8

2.8.4 Immunoblotting

Immunoblotting (also known as Western blotting) is a semi-quantitive technique for the detection of relative protein levels and protein modification within cells. The process requires SDS-PAGE to separate proteins, followed by transfer from the gel to a specific solid support membrane. Proteins migrate from the gel upon introduction of an electrical current and are 'caught' by a nitrocellulose or PVDF membrane in the process, capable of adhering amino acids non-specifically (Tovey and Baldo, 1989). The membrane is then exposed to a primary antibody recognising a specific target of interest. Upon binding of the primary antibody, an antibody recognising the primary antibody (secondary antibody) is added, allowing the formation of an antibody-antibody complex. The secondary antibody is directly conjugated to a reporter molecule – a fluorophore able to emit IR fluorescence, which is detected by a fluorescence-based imaging system.

Gels containing protein lysates were transferred to Bio-Rad Trans-Blot® Turbo™ Transfer packs containing a PVDF membrane and were assembled in specialist cassettes for

insertion into the Trans-Blot Turbo™ system. The system enables a current to be passed from the cathode to the anode plate, causing the migration of proteins and the adhesion to the PVDF membrane.

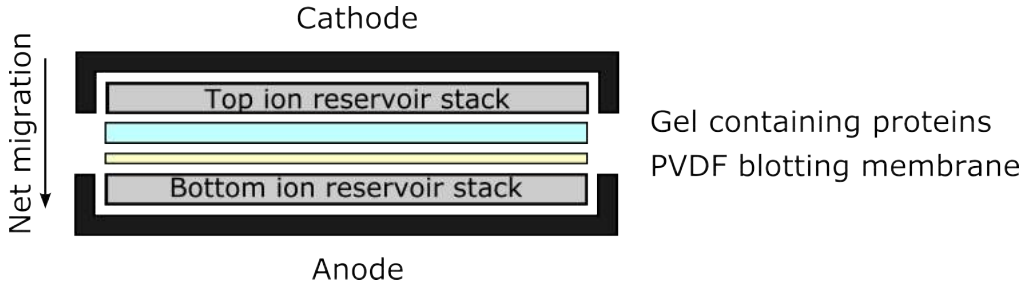


Figure 2.7 – Bio-Rad Trans-Blot® Turbo™ Transfer Pack set-up. PVDF membranes and bottom ion reservoir stacks were loaded onto the anode plate and any air spaces removed using a roller tool. The SDS-PAGE gel was loaded on top and the top ion reservoir stack placed on top. To complete the cassette, the cathode plate was locked in place and the complete cassette placed in the Trans-Blot TurboTM system. Voltage to induce protein mobility was selected from a pre-set menu according to the manufacturer's specifications.

Transferred membranes were blocked in 10% BSA/TBS-T for 1 hour to prevent non-specific antibody binding and allowed to incubate with 5mL of desired primary antibodies (described in Section 2.2) overnight at 4°C. The following morning, membranes were washed with TBS-T for 15 minutes under continual shaking and probed with fluorescently labelled secondary antibodies for 1 hour at room temperature protected from light. Secondary antibodies used to detect the protein or epitope of interest were conjugated to fluorophores containing a specific Infra-Red emitting molecule (IR Dye® 800CW anti-rabbit, anti-mouse (Li-Cor, Germany) whilst antibodies detecting a protein used for normalisation of protein levels emitted at a lower wavelength (IR Dye® 680RD anti-rabbit, anti-mouse. Both secondary antibodies were used at a 1:15,000 dilution, four subsequent washes (10 minutes, TBS-T) after secondary antibody incubation to remove any non-specific binding of secondary antibodies. Signal detection was conducted using an Odyssey CLx system (Li-Cor, Germany), allowing the simultaneous detection of both protein of interest and loading control.

2.8.5 Immunoprecipitation

In some situations, proteins could not be detected via normal immunoblotting techniques and relied on immunoprecipitation to yield a stronger signal. Unlike the preparation of

cell lysates, immunoprecipitation facilitates the isolation of a specific protein under non-reducing conditions. Immunoprecipitation utilises polysaccharide beads coated with bacterial proteins A and/or G which bind to antibody Fc regions to allow the formation of antibody-bead complexes. Antibody-bead complexes are incubated with cell lysates, binding to the protein of interest. Beads are subsequently partitioned away from residual cell lysates via centrifugation, eluted and proteins used for *in vitro* activity assays or probed via immunoblotting (Johansen and Svensson, 1998).

For immunoprecipitation, platelet samples (500µL; $7x10^8$/mL) were lysed in IP lysis buffer (1:1) and complete lysis allowed to occur for 30 minutes on ice. Protein A beads were washed with TBS-T, pelleted using a bench top centrifuge and resuspended in TBS-T (e.g. 0.2g beads + 200µL TBS-T) to form a slurry. 20µL slurry was added to chilled lysates and lysates pre-cleared by placed on a rotator for a minimum of 1 hour. By incubating the lysates with beads in the absence of antibodies, it facilitates the removal of any proteins capable of binding directly to the bead. Upon pre-clearing, lysate (200-500µg depending on protein of interest) was incubated with slurry (25µL) and antibody (1-5µg depending on protein of interest) and complexes allowed to form by gentle rotation overnight at 4^oc. The following day, beads were pelleted via centrifugation (1000*g*, 30 secs) and washed with TBS-T (75µL) three times. Upon the final wash, beads were incubated with Laemmli buffer (60µL) and boiled for 3 minutes to elute the protein-antibody complex.

2.9 Statistical analysis

Results were expressed as the mean average ± standard error of the mean (SEM). All statistical analyses were conducted using Graphpad Prism 6 (San Diego, USA), using the Student t-test or Analysis of Variance (ANOVA) functions. Significance was declared for p-values of <0.05. Percentage data was transformed using an arcsin transformation and analysed using parametric tests.

Chapter 3: Biochemical Characterisation of Oxidised LDL and the Effects on Platelet Activation

3.1 Introduction

An increased risk of thrombosis has long been associated with hyperlipidaemia (Leren, 1968; Steinberg, 2013). The modification of LDL in the vessel wall has been considered as the major factor behind this elevated risk. Since the oxidation of LDL *in vivo* is believed to be as a result of numerous different processes, including free radicals and enzymatic activity, this has translated into many different protocols being established to produce oxLDL *in vitro* and with different effects on platelet function (Berliner et al., 1990; Bielicki et al., 1996; Sigari et al., 1997; Anderson et al., 1999; Itabe et al., 2003; Boullier et al., 2006).

The oxidation of LDL *in vitro* gives rise to two major classes of oxLDL: *hydroperoxide-rich* oxLDL and *oxysterol-rich* oxLDL (Gerry et al., 2008; Levitan et al., 2010). Lipid hydroperoxides are an early marker of oxidation and are produced when LDL is oxidised under mild conditions (Levitan et al., 2010). Harsher oxidation conditions including the use of Cu^{2+} ions at 37^{o}c, yield extensively oxidised LDL which is rich in oxysterols and protein adducts, affecting the protein charge (Levitan et al., 2010). Furthermore, these differentially oxidised LDL species have been reported to have distinct effects on platelet function (Naseem et al., 1997). However, there remains a great deal of uncertainty of how these different forms of oxLDL influence platelet function.

In a physiological setting, platelets adhere to immobilised proteins such as collagen in order to cause bleeding cessation. Several studies have shown that platelets are able to bind to immobilised oxLDL (Nergiz-Unal, Lamers, et al., 2011; Wraith et al., 2013) and undergo a spreading response. Moreover, the ability for cells to bind oxLDL has mostly been attributed to scavenger receptors. Platelets possess the scavenger receptor CD36 in high abundance on both the plasma membrane and within α-granules (Berger et al., 1993) and multiple studies have shown that it able to bind oxLDL to initiate activation. In

addition, platelets also possess the receptor LOX-1 (Chen et al., 2001), another receptor reported to bind oxLDL upon platelet activation (Chan et al., 2013). The role of these receptors in binding oxLDL and triggering an activatory cellular response however, remains incompletely characterised.

3.2 Chapter aims

The experiments in this chapter were designed to produce both HP-rich and OS-rich oxidised LDL and to test the effects of these species on platelet function with a view to investigate receptor involvement. Specifically, we aimed to:

- Produce oxLDL *in vitro* and use biochemical assays to evaluate the degree of modification
- Determine of the ability of modified LDL to cause platelet activation
- To investigate the ability of oxLDL to support platelet adhesion and spreading and the role of secondary signalling mediators in this process

3.3 Biochemical characterisation of oxidised LDL

LDL contains both lipids and proteins which become modified during oxidation. In order to ascertain the degree of oxidation of the modified LDL particles generated *in vitro*, different biochemical techniques were used to measure changes to the lipid and protein components. In this study, LDL oxidation was determined using lipid hydroperoxide concentrations and the Relative Electrophoretic Mobility (REM) as markers of lipid and protein modifications respectively.

3.3.1 Effects of oxidation on Lipid hydroperoxide content

Lipid hydroperoxides (LPO) are an early marker of LDL oxidation and have been reported to be at high concentrations in LDL oxidised at 4^{o}C but absent in extensively oxidised LDL (Esterbauer et al., 1987; Esterbauer et al., 1990). In this experiment, aliquots of LDL undergoing oxidation at 4^{o}C were taken every 4 hours in order to examine the kinetics of LPO formation, with LPO formation measured via a well characterised colorimetric Iodine based assay. Lipid hydroperoxide rich ('HP-rich') oxLDL was LDL oxidised at 4^{o}C for 24 hours whilst extensively oxidised (oxysterol-rich – 'OS-rich' oxLDL) was oxidised for 24 hours at 37^{o}C.

As expected, nLDL had very low levels of LPO (3.71%±3.33) and this gradually increased with the duration of exposure to the Cu^{2+} ions (Fig. 3.1). Levels of LPO remained low for 8 hours (4 hours – 4.72%±3.29; 8 hours – 11.7%±5.94) but increased dramatically by 16 hours (79.64%±8.29). After 20 hours' oxidation, LDL was almost identical to the final HP-rich LDL used in functional studies (20 hours – 91.89%±8.76; 24 hours – 101.33%±2.46). In contrast, OS-rich oxLDL had very low detectable levels of LPO, analogous to nLDL (6.2%±5.0).

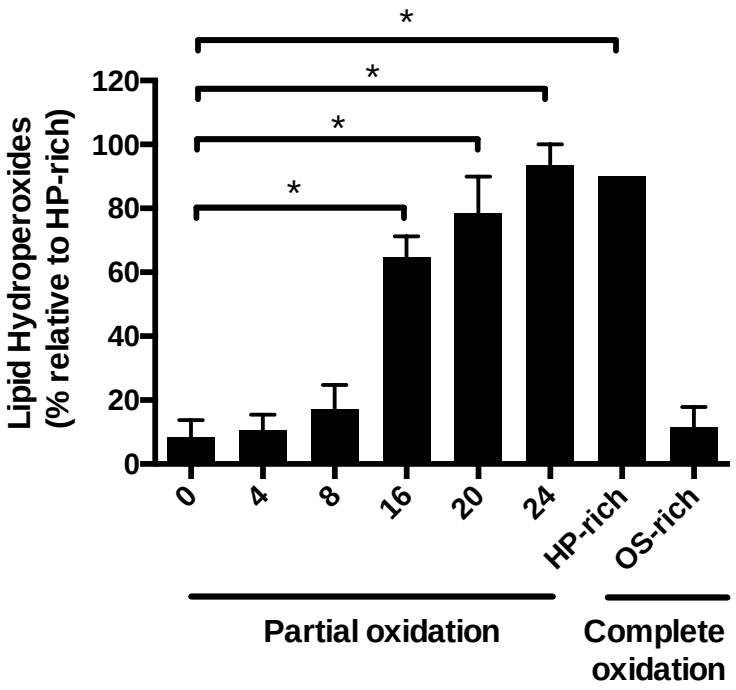


Figure 3.1 – Lipid Hydroperoxide content of LDL varies during oxidation. LDL was oxidised between 0 - 24 hours at either 4°C or 37 °C, yielding hydroperoxide (HP) rich or oxysterol (OS) rich LDL respectively. Samples oxidised at 4°C (4 – 24 hours) were taken at the indicated time points and EDTA (100µM) added to prevent further oxidation. Fully oxidised LDL (HP/OS-rich) underwent buffer exchange into phosphate buffer. Samples were measured for LPO levels using an Iodine based colorimetric assays described previously. Only OS-rich LDL was oxidised at 37 °C. Average of 3 separate experiments. * = p=>0.05 when data was arcsin transformed and ANOVA performed.

On average, nLDL contained almost undetectable levels of LPO, indicating that the isolation protocol was not causing artificial oxidation (nLDL – 4.2nmol/mg±3.5) (Fig. 3.1). HP-rich oxLDL contained significantly higher levels of LPO than nLDL (HP-rich oxLDL – 850.4nmol/mg±506.28) (p=<0.001). As expected, OS-rich oxLDL had very low levels of LPO (OS-rich oxLDL – 14.47nmol/mg±13.4) and when analysed, was not significantly different to nLDL. However, due to variation between batches, data was normalised to HP-rich oxLDL (100%).

3.3.2 Effects of oxidation on REM status

In parallel to the LPO assays, we also wanted to compare the differences in the degree of protein modification taking place between oxidation methods. Since oxidation affects the overall charge of the LDL particle, the movement of samples travelling across an agarose gel can be used as a proxy for protein modification and therefore a marker of extensive oxidation. However, since this assay only uses nLDL as a baseline, any oxidation which has taken place prior to the isolation is not detected, potentially skewing results.

Native LDL, and the subsequent buffer changes had no measurable effect on mobility (Fig. 3.2). Furthermore, LDL oxidised for short durations (<8 hours) had very little change in mobility and was consistent with LPO data (4 hours – 1.35±0.4; 8 hours – 1.49±0.4). In contrast after 16 hours, measurable protein modification had begun to occur with REM increasing to 2.34±0.62. There was only a modest increase in REM after this point (20 hours – 2.55±0.66; 24 hours -2.9±0.85; HP-rich – 2.84±0.75). As expected, OS-rich oxLDL had a greater degree of mobility and travelled furthest across the gel (OS-rich – 3.55±1.0). Despite the clear trend of increasing mobility, when analysed this narrowly failed to reach significance ($p=0.053$).

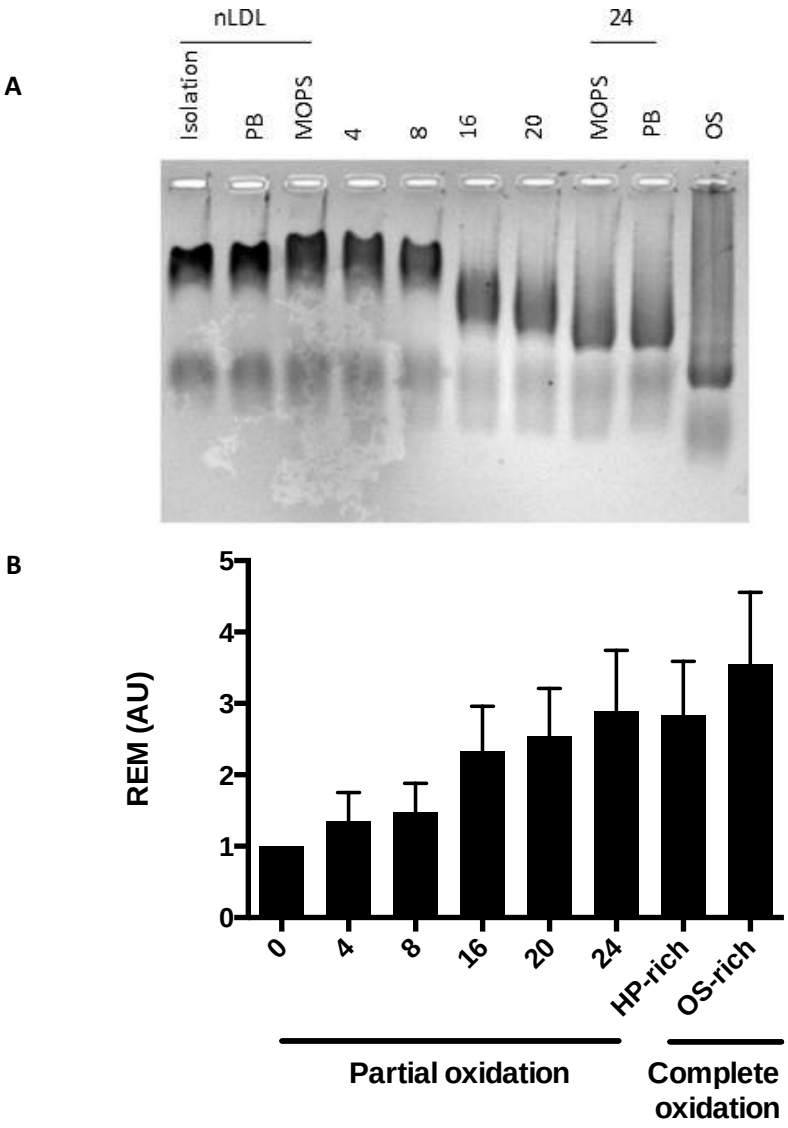


Figure 3.2 – Protein modification occurs in a time-dependent manner. Isolated LDL was incubated in a solution containing 10μM $CuSO_4$ at either 4°C (4-HP-rich) or 37 °C (OS-rich) to induce differential rates of oxidation. A – Representative image of agarose gel containing 20μg samples of LDL across a 24 hour period. Measurement is made from the well (white) to the first dark band (in millimetres) and REM value calculated. B – Measurements of relative electrophoretic mobility. PB – Buffer change into phosphate buffer, MOPS – Buffer change into MOPS buffer, HP – Hydroperoxide rich oxLDL, OS – Oxysterol rich oxLDL. Average of 3 separate experiments.

It was our intention to characterise both forms of modified LDL in the context of platelet function, having shown that the species generated via use of different oxidising conditions are biochemically distinct. However, a key publication by Chan et al. showed that they were able to identify a highly analogous form of OS-rich oxLDL, termed electronegative LDL from the plasma of STEMI patients (Chan et al., 2013). As a result, we decided to focus exclusively on OS-rich oxLDL for the rest of the project.

3.4 Platelet activation in response to oxidised LDL exposure

In order to ascertain what effects oxLDL might have on platelet function in a physiological setting, platelets from healthy donors were exposed to oxLDL in a range of different assays. Parameters which were tested included platelet activation via FACS, platelet aggregation and platelet adhesion and shape change. This was important since many of the reported effects of oxLDL on platelet function remain controversial, with factors such as oxidation methodology, donor variation and differences in experimental techniques compounding this.

3.4.1 Oxidised LDL causes expression of the α-granule marker CD62P in human platelets

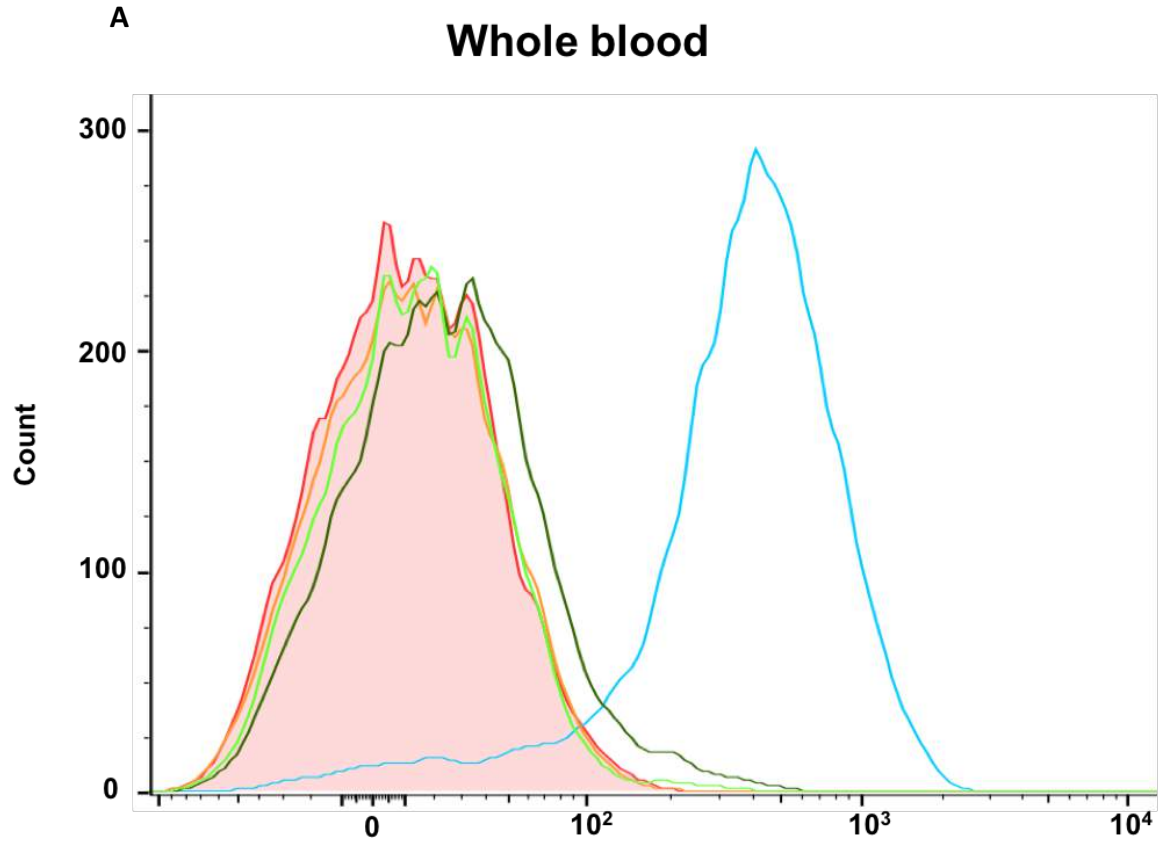
We began by determining whether oxLDL was able to activate platelets by causing degranulation and detectable expression of the alpha granule marker P-selectin (CD62P). Detection was carried out using FACS on platelets in whole blood and platelet-rich plasma and % positive cells was used as the FACS readout.

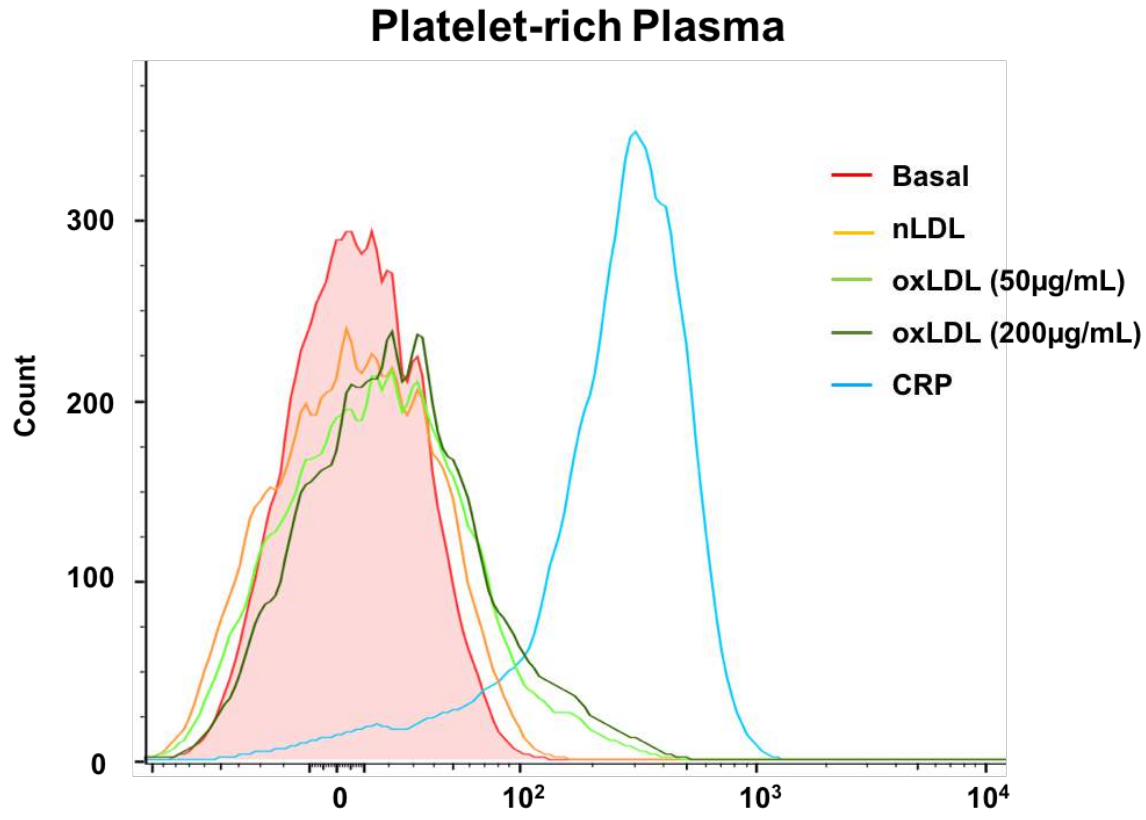
In whole blood, basal levels of P-selectin were low (Basal – 8.35%±0.58) and did not significantly increase with nLDL treatment (nLDL – 10.75%±2.34) (Fig. 3.3a). With the lower concentrations of oxLDL, levels of P-selectin stayed consistent with nLDL (10μg/mL – 10.45%±2.09; 50μg/mL – 12.98%±1.95). However, platelet pre-incubation with 200μg/mL oxLDL did cause significant increase in P-selectin expression (200μg/mL – 24.3±2.68). In order to confirm platelets were still functional, CRP was used as a positive control, causing almost all platelets to become activated and express P-selectin (CRP – 95.23%±0.97 (data not shown)).

To confirm that the platelet activation being observed was not due to effects of oxLDL on leukocytes causing the release of pro-thrombotic agents, we repeated the same experiment using PRP.

Untreated platelets from PRP showed low levels of CD62P expression (Basal – 8.23%±2.28) and similarly showed no increase in expression when stimulated with either nLDL (nLDL – 10.9%±2.29) or 10µg/mL oxLDL (10µg/mL – 11.8%±1.81) (Fig. 3.3B). In contrast to whole blood, both 50 and 200µg/mL oxLDL was able to induce significant increases in CD62P expression (50µg/mL – 22.2%±3.14; 200µg/mL – 25.4%±3.75). CRP was used as a positive control in PRP too, yielding almost 100% CD62P positive platelets (CRP – 96.35%±1.42).

A


Whole blood
Count
300
200
100
0
0
10^2
10^3
10^4


Platelet-rich Plasma
Count
300
200
100
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10^2
10^3
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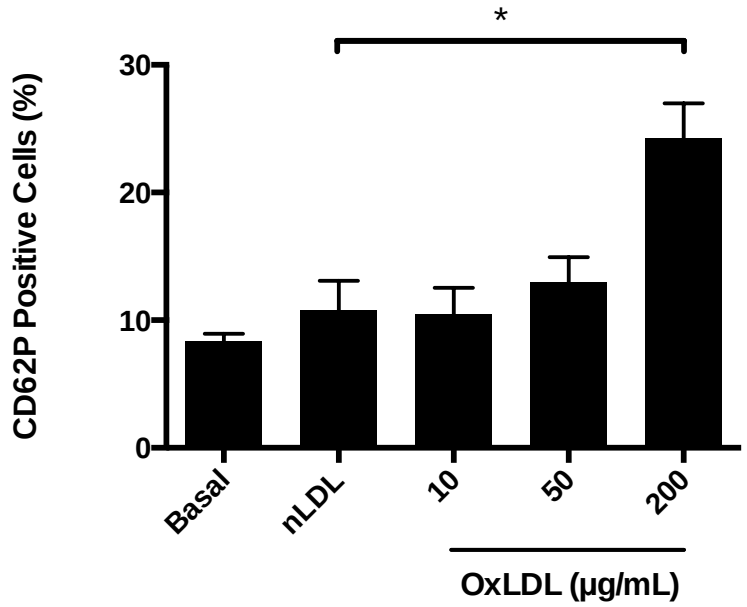
Basal
nLDL
oxLDL (50µg/mL)
oxLDL (200µg/mL)
CRP

B



C

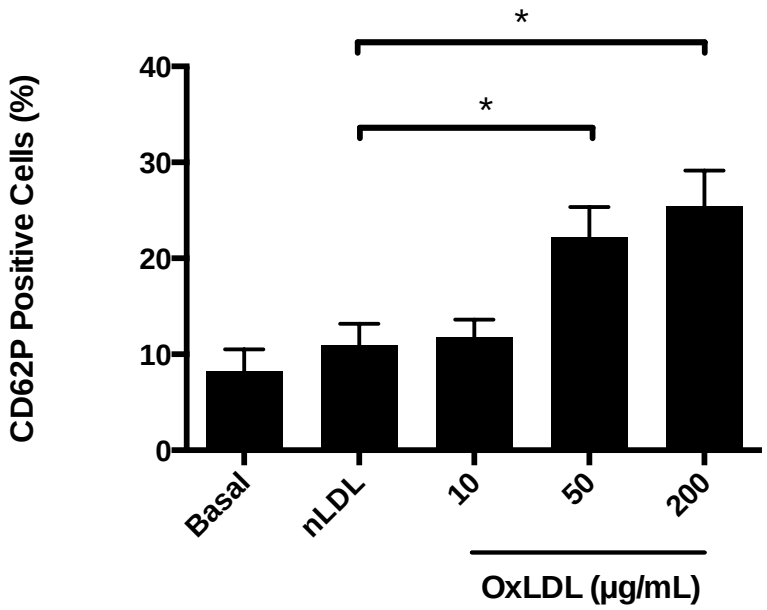


Figure 3.3 – OxLDL causes increased expression of CD62P in human platelets. A – representative flow cytometry traces of whole blood and platelet rich plasma samples. OxLDL (10µg/mL) not included for brevity. B – Analysis of platelet CD62P expression in whole blood treated with oxLDL. Blood (5µL) was incubated with an anti-CD62P antibody and treated with oxLDL at varying concentrations for 20 mins followed by fixation with formalsaline. C – Analysis of platelet CD62P expression in platelet-rich plasma. As for A, except PRP was used instead of whole blood. Average of a minimum of 4 separate experiments. For statistical analysis, percentage data was arcsin transformed and analysed via ANOVA. *= $p<0.05$

3.4.2 Oxidised LDL induces weak platelet aggregation in human platelets.

Having established that oxLDL is able to induce platelet activation in a dose dependent manner, we next wanted to determine whether oxLDL was able to induce platelet aggregation. A concentration of 50µg/mL was chosen for LDL experiments based on FACS data showing that oxLDL at this concentration was able to cause significant increases in CD62P in PRP. In addition, use of 200µg/mL oxLDL was not feasible for the type of experiment and volume required.

In order to confirm that platelets were functional at the time of testing, platelets were stimulated with collagen (Fig. 3.4). Collagen caused a robust aggregation response consistent with the literature. Native LDL showed minimal aggregation, whereas oxLDL was able to induce weak aggregation (range: 15-30%) depending on the donor (Fig. 3.4). The aggregation was not reversible and maintained for 15 minutes (longest time tested).

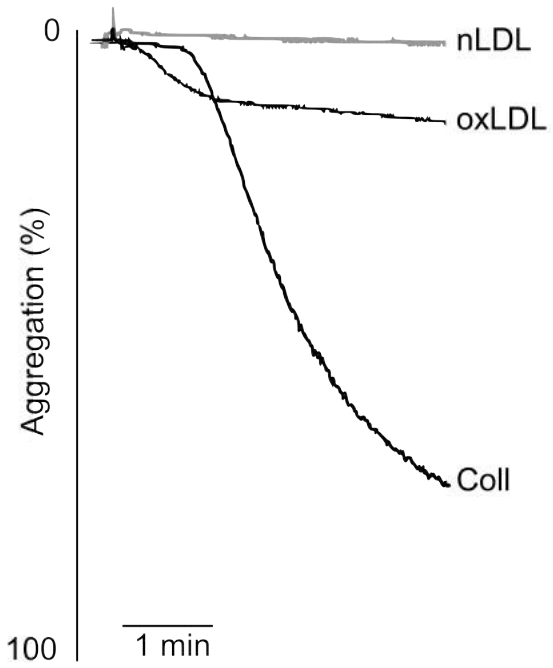


Figure 3.4 – oxLDL, but not nLDL, causes weak platelet aggregation. Platelets ($2.5x10^8$/mL) were treated with either nLDL (50µg/mL), oxLDL (50µg/mL) or collagen (10µg/mL) for 4 mins and aggregation recorded using a platelet aggregometer (Chrono-Log). Traces represent example traces from three separate experiments.

3.4.3 Oxidised LDL does not induce platelet aggregation in murine platelets.

Having shown that oxLDL is able to induce modest but consistent aggregation in human washed platelets, we next wanted to investigate whether oxLDL would be able to elicit similar levels of aggregation in murine platelets. Murine platelets deficient in specific proteins of interest serve as invaluable tools to dissect molecular signalling mechanisms and would be used later in this project.

In order to determine whether our murine platelet isolation protocol was yielding functional platelets, we began by testing platelets with thrombin (Fig. 3.5). Thrombin (0.1U/mL) induced a clear aggregation response, confirming that the protocol was yielding functionally active platelets. Next we incubated platelets with either nLDL or oxLDL (50µg/mL) but surprisingly both sets of lipoproteins failed to induce an aggregation response after 5 minutes.

3.4.4 Oxidised phospholipids induce platelet activation in murine platelets

Since murine platelets did not seem to response to human oxLDL, we began to look for alternative tools to investigate the murine response. Oxidised phospholipids were chosen as a possible alternative to oxLDL since they are present within human oxLDL (E. a. Podrez, 2002) and have been shown to have similar effects to oxLDL across multiple assays (E.A. Podrez, 2002; Magwenzi et al., 2015). Whilst a range of oxidised phospholipids have been isolated from human oxLDL and are commercially available, we selected KOdiA-PC due to high potency in macrophage studies and high levels of CD36 specificity (E. a. Podrez, 2002; E.A. Podrez, 2002). The non-oxidised form, PAPC, was used as a control and treated analogous to nLDL.

To begin with, murine platelets were treated with thrombin (0.1U/mL), triggering strong aggregation (Fig. 3.6). The native phospholipid PAPC did not induce any observable changes in murine platelets, whereas KOdiA-PC caused a robust shape change response.

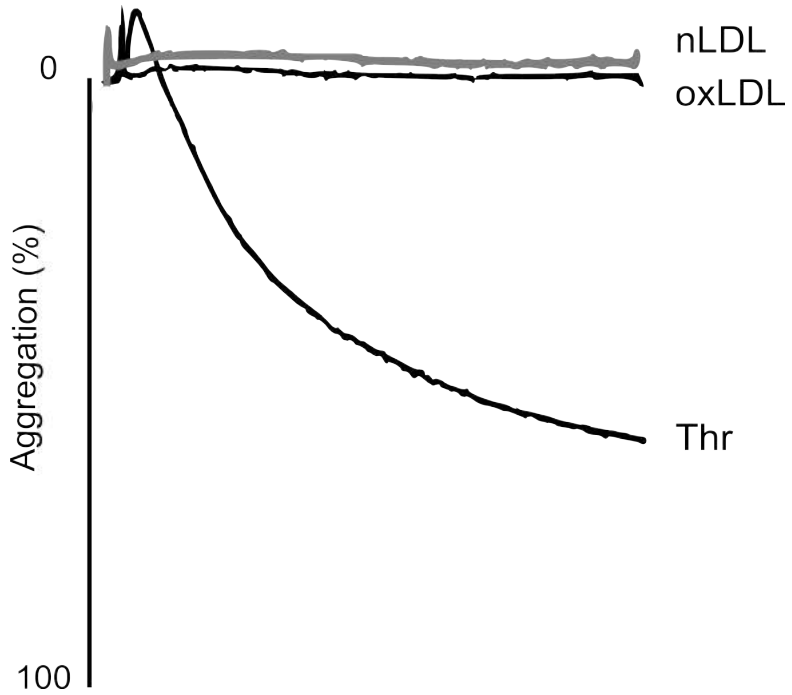


Figure 3.5 – oxLDL does not induce aggregation in murine platelets. Murine platelets ($2.5x10^8$/mL) were treated with either nLDL (50µg/mL), oxLDL (50µg/mL) or thrombin (0.1U/mL) for 5 mins and aggregation recorded using a platelet aggregometer (Chrono-Log). Traces represent example traces from three separate experiments.

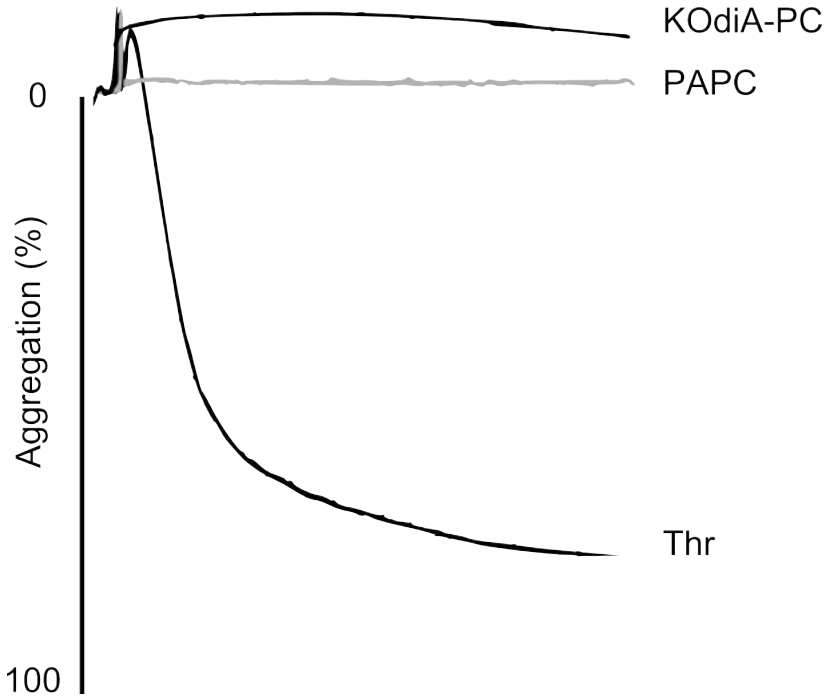


Figure 3.6 – oxidised phospholipids cause murine platelet shape change. Murine platelets ($2.5x10^8$/mL) were treated with either PAPC (5µM), KOdiA-PC (5µM) or thrombin (0.1U/mL) for 5 minutes. Aggregation was recorded using a platelet aggregometer (Chrono-Log). Traces represent example traces from three separate experiments.

3.4.5 - Oxidised LDL causes tyrosine phosphorylation and PKC activation in human platelets

Having examined the functional changes induced by oxLDL and KOdiA-PC, we next wished to examine their effects on intracellular signalling events. To begin our investigation into the intracellular signalling events triggered by oxLDL ligation, we wanted to confirm previous reports of increased phosphorylated tyrosine residues, indicative of tyrosine kinase activity and then examine whether this triggered canonical PKC activation.

Using immunoblotting, we showed that oxLDL is able to induce both tyrosine phosphorylation and PKC activation, in a time-dependent manner (Fig. 3.7A). OxLDL caused an increase in tyrosine phosphorylation at the earliest time point of 1 minute and this was sustained until the latest sampled time point of 15 minutes. Peak phosphorylation was observed at 5-15 minutes and a high dose of CRP (1µg/mL) was used as a positive control. At one minute stimulation, bands at approximate weights of 140, 90 and 70 began to become visible but were clearer at later time points. By 5 minutes, bands at approximate weights of 100, 40 and 10 were visible too. These bands were present in lysates of CRP treated platelets too, indicating that the same proteins may be activated in oxLDL signalling as collagen signalling.

Some phospho-PKC substrates were detected at very early time points (15-30 seconds), although phosphorylation was strongest at 1-5 minutes stimulation (Fig. 3.7B). Early time points clearly show PKC substrate phosphorylation of bands at approximate weights of >140 and 140 and also a possible dephosphorylation event of a protein at ~35kDa. Global pPKC substrate phosphorylation began to decrease by 15 minutes, the latest sampled time point, although was still elevated compared to basal. Taken together, this data suggests that oxLDL is capable of causing multiple changes to intracellular signalling networks which may be responsible for the functional changes observed in platelet function.

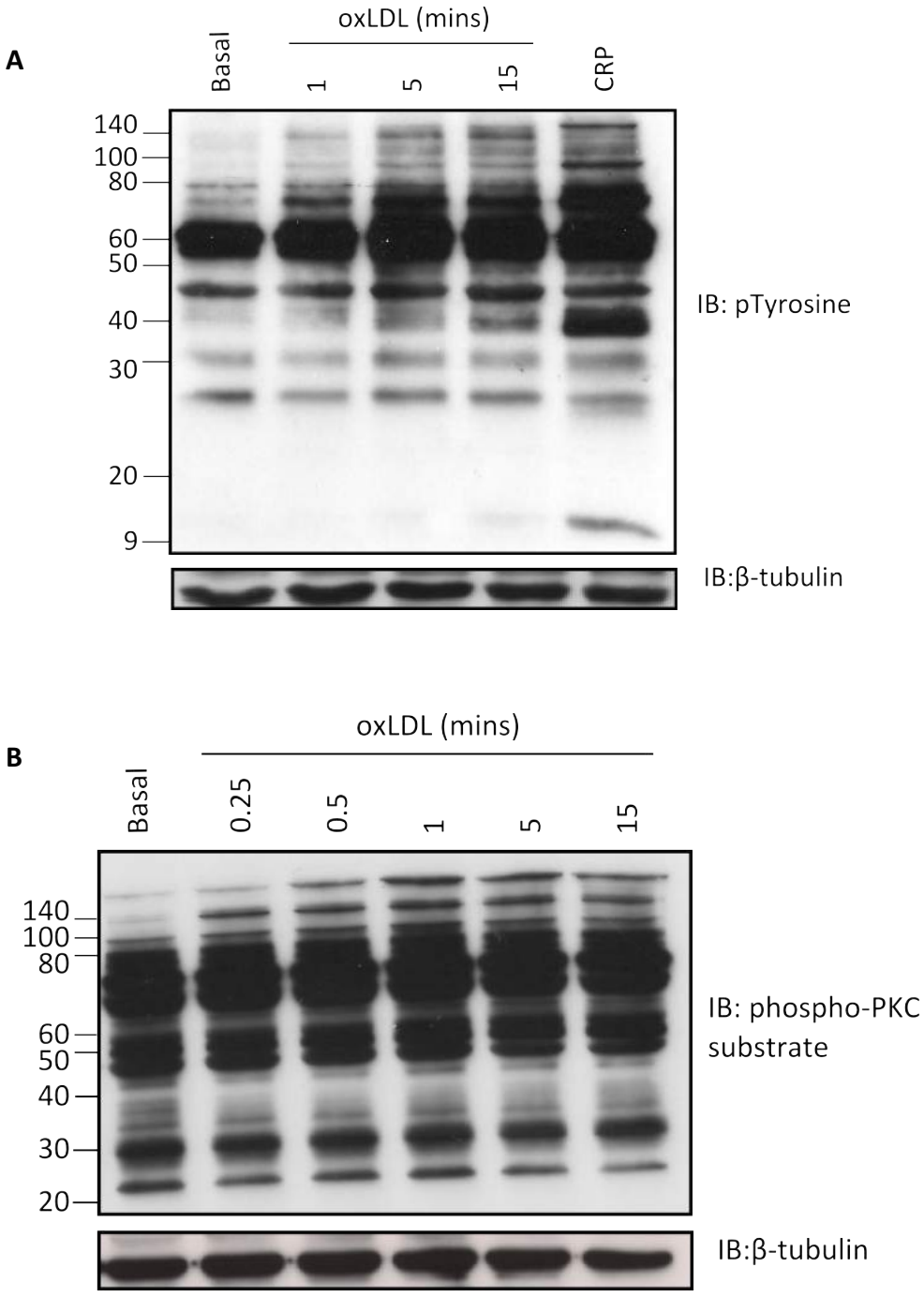


Figure 3.7 - oxLDL causes tyrosine phosphorylation and PKC activation in human platelets. Platelets ($5x10^8$/mL) were treated with apyrase (2U/mL), indomethacin (10μM), EGTA (1mM) and tirofiban (1μg/mL) for 20 minutes prior to oxLDL stimulation (50μg/mL) for the indicated time point or collagen related peptide (1μg/mL) for 1 min. Samples were lysed in Laemelli buffer, proteins separated via electrophoresis and transferred to PVDF membranes. Membranes were probed with A) anti-phosphotyrosine antibody or B) anti-phosphoPKC substrate antibody. Blots are representative of a minimum of 3 separate experiments.

3.5 OxLDL causes platelet shape change under static conditions

Platelet aggregation tested in the Born aggregometer is an excellent tool for examining the effects of strong agonists. However, the test is far removed for the physiological situation, lacking a solid support required for the initial adhesion phase. Therefore, we wanted to explore whether oxLDL could support platelet adhesion. Unlike aggregation experiments, in which platelets are continually stirred, static adhesion assays allow cells to bind and signal for extended periods of time, acting as a useful tool for investigating the receptor and signalling pathways involved.

3.5.1 Platelets adhere and spread on oxLDL

Human serum albumin, used to block untreated areas of the slide, supported very low levels of adhesion (Serum - 50±5.72) (Fig. 3.8). Immobilisation of nLDL (100µg/mL) supported some adhesion of platelets (nLDL - 142.26±28.18). A significant increase in adhesion was observed however using 100µg/mL oxLDL (100µg/mL – 277.91±21.51) when compared to nLDL *(p=0.0173)*. As a result, all subsequent experiments using oxLDL coatings were used at 100µg/mL, although some preliminary experiments were conducted using lower concentrations (data not shown). In order to determine whether our experimental technique would provide data consistent with the literature and check whether the platelets were functional, fibrinogen was used as a positive control (Fibrinogen – 408.98±50.42).

Under physiological conditions, platelets adhere to a matrix protein at the site of injury and undergo shape change, forming a spread morphology to maximise surface area coverage. This shape change represents an important part of the platelet activation process and therefore is important to measure *in vitro*. Using fibrinogen as a positive control, we showed that our platelet preparation was able to not just adhere by also spread and adopt the spread morphology (Fig. 3.9). Platelets spread on fibrinogen with a surface area of 24.9±3.1µm^2. This value was used as a benchmark to evaluate platelet spreading on oxLDL. Platelet adhesion to nLDL or oxLDL was able to induce similar levels of spreading to that of fibrinogen (nLDL –18.69±5.1µm^2; oxLDL – 18.28±5.02µm^2) and

when analysed did not show any statistical differences *(p=0.875).* However, it is important to remember that since nLDL did not support much adhesion, the cells adhering and spreading may have been either pre-activated or anomalous.

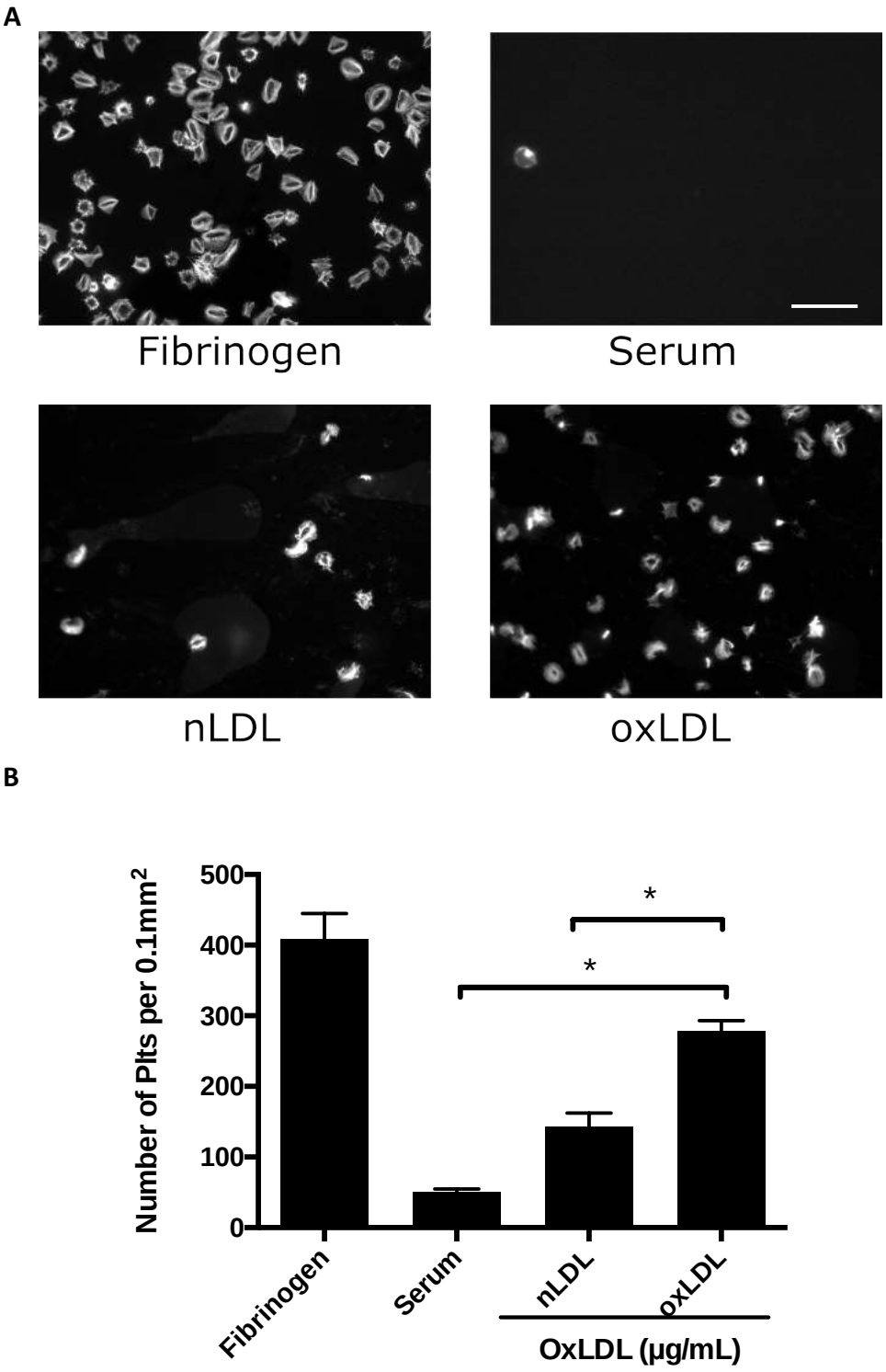


Figure 3.8 – Platelets adhere to oxLDL in a dose dependent manner. Platelets ($5x10^7$/mL) were incubated on nLDL (100µg), oxLDL (100µg/mL) or fibrinogen (100µg/mL) treated slides for 60 mins at 37°c. Adhered platelets were fixed, stained using TRITC-phalloidin (1µg/mL) for 60 mins and viewed using an inverted fluorescence microscope (Zeiss). Images were randomly acquired and quantified manually using ImageJ. Scale bar represents 100 microns. Data shown is the mean average from 3 separate experiments. *= *p=<0.05* when analysed by ANOVA.

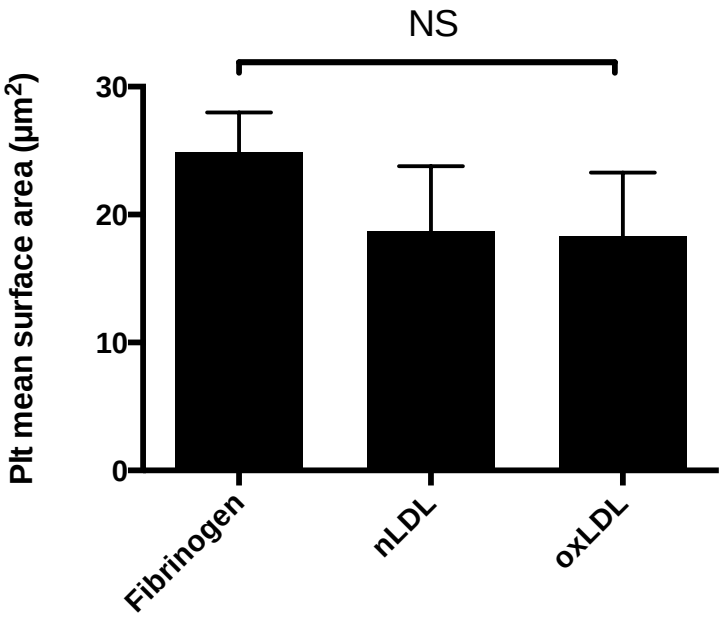


Figure 3.9 – Platelets are able to spread on oxLDL. Platelets ($5x10^7$/mL) were incubated on nLDL (100µg), oxLDL (100µg/mL) or fibrinogen (100µg/mL) treated slides for 60 mins at 37°C. Adhered platelets were fixed, stained using TRITC-phalloidin (1µg/mL) for 60 mins and viewed using an inverted fluorescence microscope (Zeiss). Images were randomly acquired and quantified manually using ImageJ. Data shown is the mean average from 3 separate experiments, with a minimum of 100 platelets analysed per treatment. *= *p=<0.05* when analysed by ANOVA.

3.5.2 Platelets adhere to oxLDL in a time-dependent manner

Having established a working dose of oxLDL capable of supporting adhesion and spreading, we next began to look at the length of time platelets were incubated for. Multiple variations of the spreading assay technique exist using shorter incubation times and we wanted to see whether this would make any difference to platelet adhesion and spreading results.

Platelets allowed to adhere and spread for 1 hour showed consistent levels of adhesion between experiments analysed throughout this thesis (60 mins – 356). However, platelet adhesion is dependent on time as shorter incubation times caused a dramatic decrease in the numbers of platelets adhering to oxLDL (5 mins – 27; 15 mins – 79; 30 mins – 91) (Fig. 3.10).

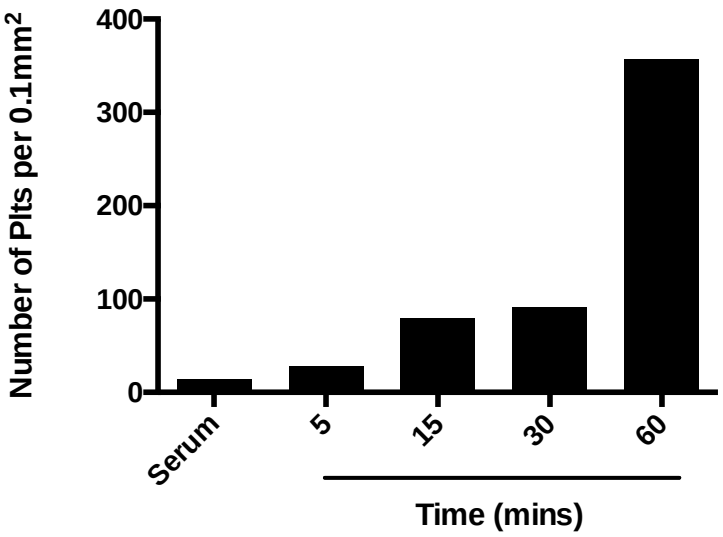


Figure 3.10 – Adherent platelet number increases with time. Platelets ($5x10^7$/mL) were incubated on oxLDL treated slides (100µg/mL) for 5-60 mins at 37^{o}c. Adhered platelets were fixed, stained using TRITC-phalloidin (1µg/mL) for 60 mins and viewed using an inverted fluorescence microscope (Zeiss). Images were acquired randomly and quantified manually using ImageJ. Data shown is from 1 experiment.

3.5.3 The role of ADP in platelet adhesion to oxLDL

Since we have shown oxLDL is able to support platelet adhesion and activation, we next wanted to investigate whether this was due to the action of the secondary mediators ADP and TxA_2 and activation of the integrin $\alpha_{IIb}\beta_3$. Furthermore, this would complement the previous functional data, showing that platelets are able to become activated (Fig. 3.3) and partially aggregate (Fig. 3.4).

Previous reports have shown that oxLDL activation of platelets is dependent on the ability of oxLDL to cause ADP secretion, leading to autocrine and paracrine activation (Nergiz-Unal, Lamers, et al., 2011; Wraith et al., 2013). Using a similar approach, we used the ADP hydrolysing enzyme, apyrase, to negate the effects of extracellular ADP and determine its effect on platelet adhesion to oxLDL (Fig. 3.11). Adhesion to oxLDL was consistent with previous findings (Untreated – 263.69±9.31) and was significantly reduced in the presence of apyrase (+ Apyrase – 130.57±35.45) *($p=<0.01$)*. Low levels of platelet adhesion to human serum was also observed (Serum – 50.8±5.73), indicative of minimal platelet activation from the preparation.

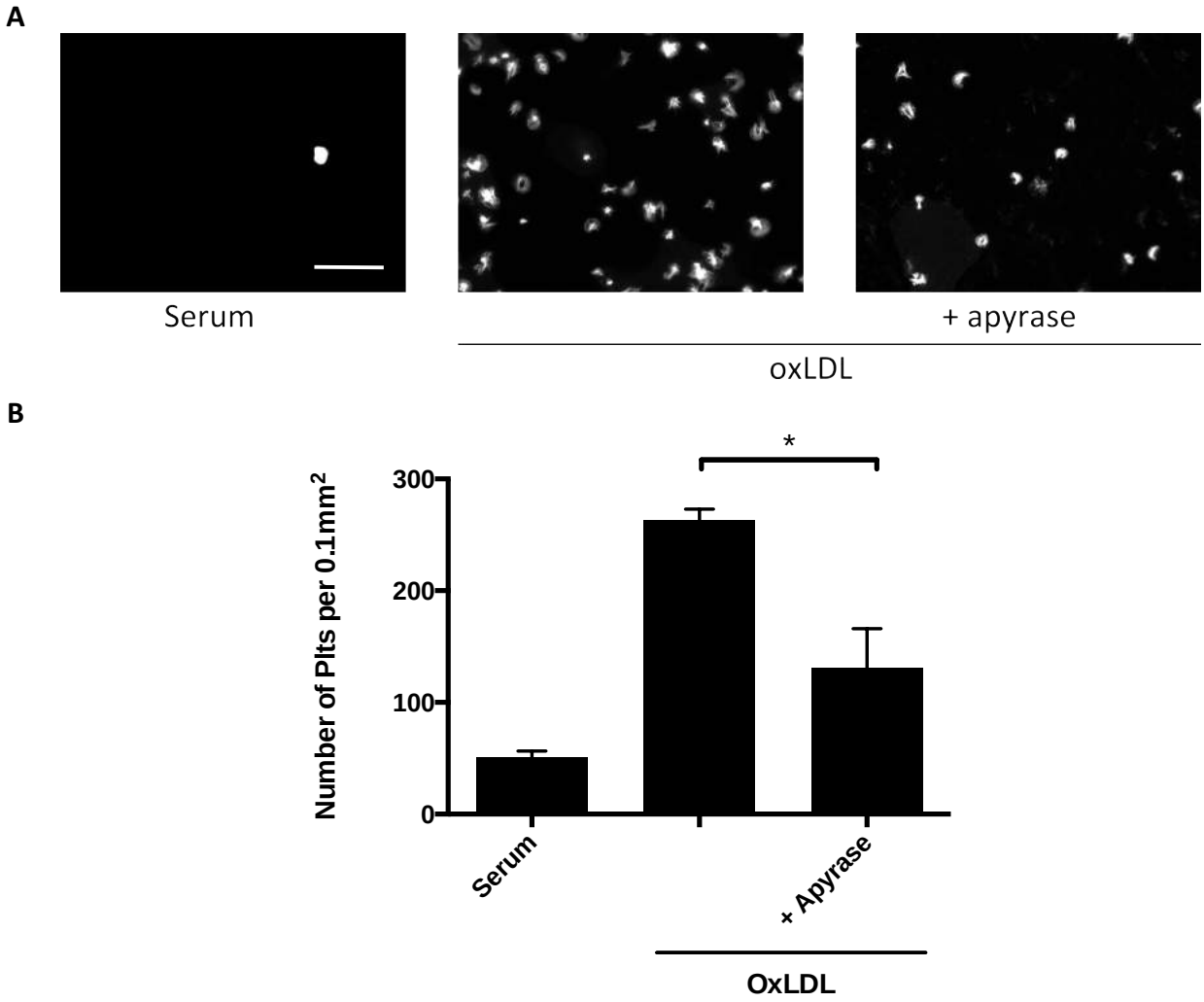


Figure 3.11 – Platelet adhesion is reduced by the presence of apyrase. Platelets ($5x10^7$/mL) were pre-incubated with apyrase (2U/mL) for 20 mins before incubation onto oxLDL treated slides (100µg/mL) for 60 mins at 37^oc. Adhered platelets were fixed, stained using TRITC-phalloidin (1µg/mL) for 60 mins and viewed using an inverted fluorescence microscope (Zeiss). Images were acquired randomly and quantified manually using ImageJ. Scale bar represents 100 microns. Data shown is the average from 3 separate experiments. *= *p=<0.05* when analysed by ANOVA.

3.5.4 The role of TxA_2 in platelet adhesion to oxLDL

Having shown that ADP is involved in adhesion of platelets to oxLDL, we next turned our attention to TxA_2. Like ADP, TxA_2 is also released from the platelet upon activation, although rather than being associated to a specific granule, it is synthesised by activation platelets via the COX enzymes. Indomethacin, a non-steroidal anti-inflammatory drug (NSAID), was used to block the production of TxA_2 by inhibiting both COX-1 and COX-2, allowing us to determine its effect in platelet recruitment (Fig. 3.12).

As in the previous figure, the treatment of platelets with indomethacin caused a significant decrease in the numbers of platelets adhering to oxLDL (Untreated – 263.69±9.31; + Indomethacin – 103.14±52.77) *(p=0.037)*. To see whether ADP and TxA_2 were acting synergistically, apyrase and indomethacin were added in combination. Levels of adhesion in the presence of both inhibitors did not change platelet adhesion levels from either apyrase or indomethacin alone (+ Apyr + Indo – 94.5±25.8) *(p=0.98)*.

3.5.4 The role of Integrin $\alpha_{IIb}\beta_3$ in platelet adhesion to oxLDL

Next, we wanted to look at the effects of secreted fibrinogen and integrin $\alpha_{IIb}\beta_3$. Fibrinogen released from intracellular stores may be involved in the recruitment of other platelets and potentiate intracellular signalling events taking place. In order to block the effects of integrin $\alpha_{IIb}\beta_{3,}$ platelets were pre-treated with a receptor antagonist: tirofiban (Fig. 3.13).

In this experiment, platelet treatment with tirofiban showed a trend of decreasing platelet adhesion (Untreated – 263.69±9.31; + tirofiban – 192.56±57.42). However, when analysed, it narrowly failed to reach statistical significance. Interestingly, incubation of platelets with all three of the used inhibitors showed very little change from apyrase or indomethacin alone, or apyrase and indomethacin in combination (+Apyr +Indo +Tiro – 108.73±23.44).

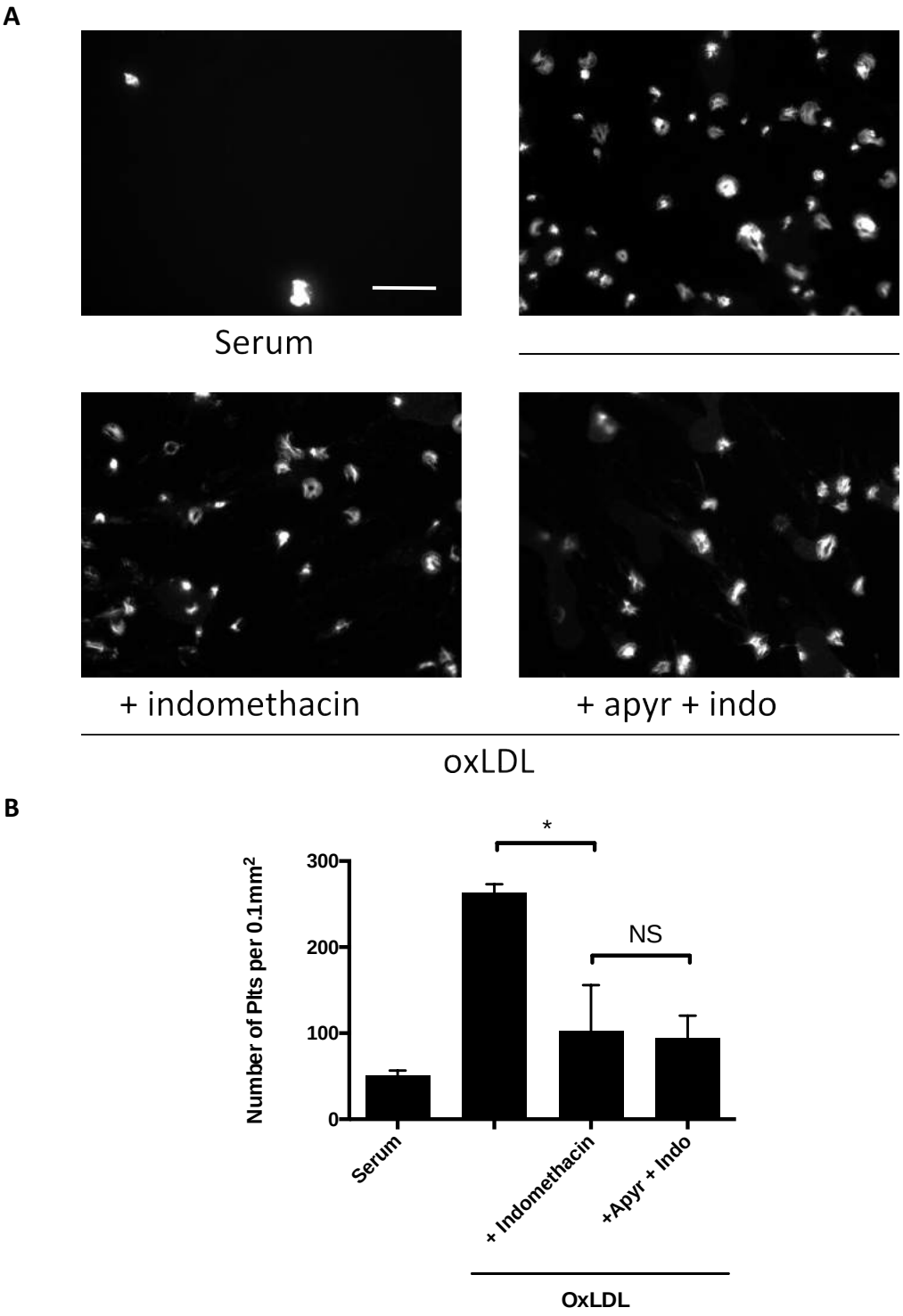


Figure 3.12 - Platelet adhesion is reduced by the presence of indomethacin. Platelets ($5x10^7$/mL) were pre-incubated with indomethacin (10µM) or apyrase and indomethacin for 20 mins before incubation onto oxLDL treated slides (100µg/mL) for 60 mins at 37°c. Adhered platelets were fixed, stained using TRITC-phalloidin (1µg/mL) for 60 mins and viewed using an inverted fluorescence microscope (Zeiss). Images were acquired randomly and quantified manually using ImageJ. Scale bar represents 100 microns. Data shown is the average from 3 separate experiments. *= *p=<0.05* when analysed by ANOVA.

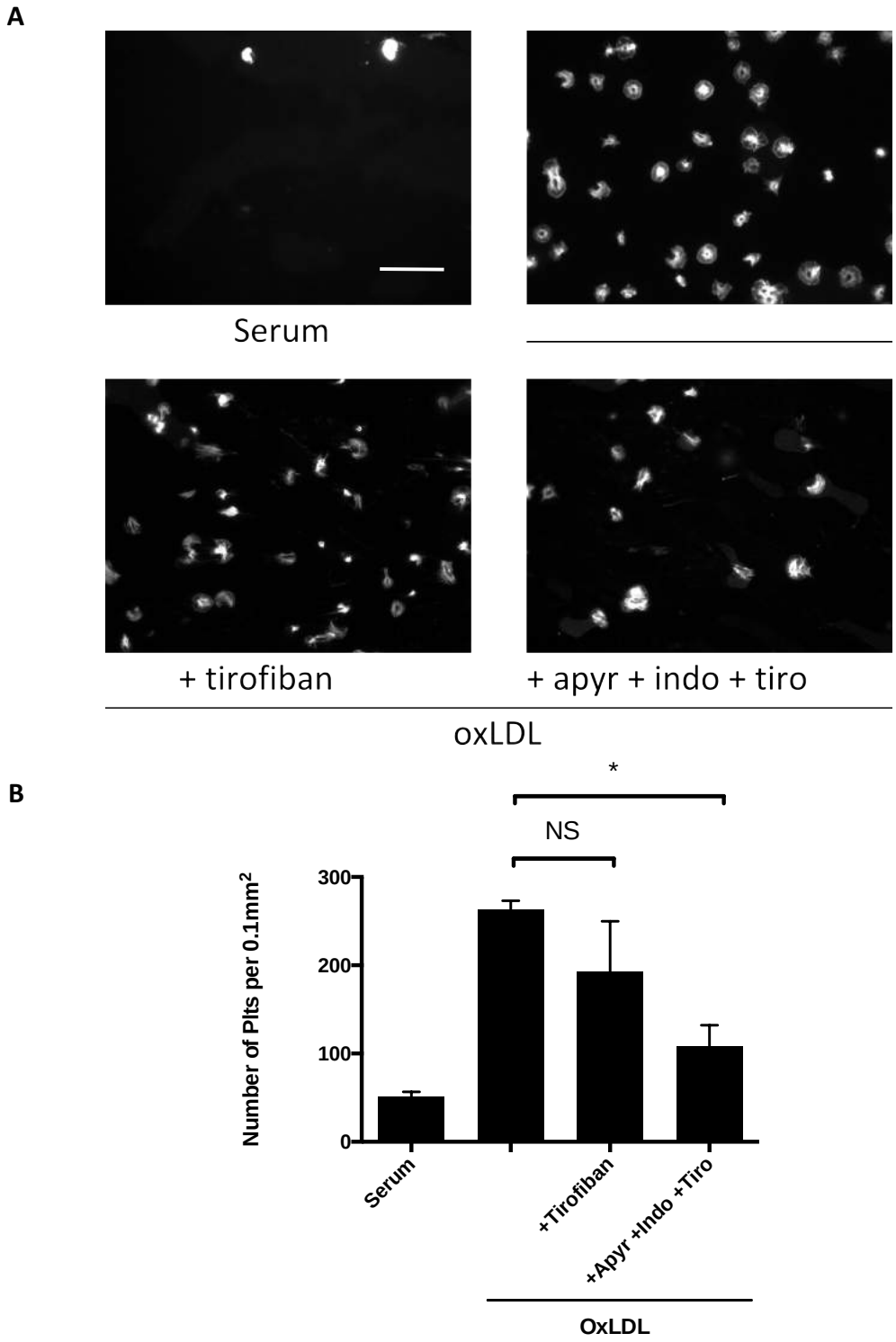


Figure 3.13 - Platelet adhesion is not reduced by the presence of tirofiban. Platelets ($5x10^7$/mL) were pre-incubated with tirofiban (1μg/mL) or apyrase, indomethacin and tirofiban in combination for 20 mins before incubation onto oxLDL treated slides (100μg/mL) for 60 mins at 37°C. Adhered platelets were fixed, stained using TRITC-phalloidin (1μg/mL) for 60 mins and viewed using an inverted fluorescence microscope (Zeiss). Images were acquired randomly and quantified manually using ImageJ. Scale bar represents 100 microns. Data shown is the average from 3 separate experiments. *= *p=<0.05* when analysed by ANOVA.

3.5.5 The effects of secondary signalling mediators on platelet spreading to oxLDL

Having established that ADP and TxA_2, and to a potentially lesser extent, $\alpha_{IIb}\beta_3$ are involved in platelet adhesion, we next began to look at whether these secondary signalling mediators were involved in platelet shape change. Consistent with previous findings, fibrinogen (100µg/mL) was able to trigger robust shape change and was used as a positive control (Fibrinogen - 28.6µm^2±4.3). In this experiment, the surface area coverage per platelet spread on oxLDL was comparable to fibrinogen (26.195µm^2±7.896). Incubation with apyrase, indomethacin or apyrase and indomethacin in combination failed to cause any measurable change in platelet spreading (+ Apyr – 29.145µm^2±5.481; + Indo – 25.499µm^2±8.642; + Apyr + Indo – 32.568µm^2±7.077). Platelet treatment with tirofiban caused a noticable decrease in adhesion (+ Tiro – 10.099µm^2±3.095) although when analysed, it failed to reach statistical significance (*p=0.29*). Interestingly, platelets treated with all three inhibitors in unison displayed a decrease in mean surface area (+ Apyr + Indo + Tiro – 20.422µm^2±4.888) compared to basal, but not as marked as tirofiban alone.

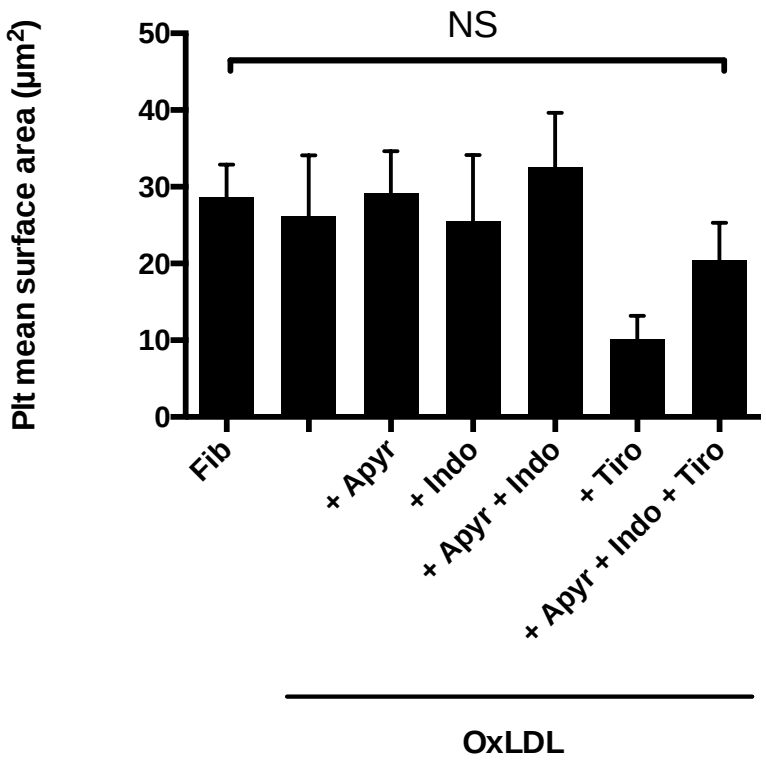


Figure 3.14 – Secondary mediators do not affect the degree of platelet spreading on oxLDL. Platelets ($5x10^7$/mL) were pre-incubated with inhibitors apyrase, indomethacin and tirofiban either alone or in combination for 20 mins before incubation onto oxLDL treated slides (100µg/mL) for 60 mins at 37°C. Adhered platelets were fixed, stained using TRITC-phalloidin (1µg/mL) for 60 mins and viewed using an inverted fluorescence microscope (Zeiss). Images were acquired randomly and quantified manually using ImageJ. Data shown is the average from 3 separate experiments with a minimum of 100 platelets analysed per treatment.

3.6 Identification and elucidation of the effects of scavenger receptors involved in platelet activation

Platelets possess multiple scavenger receptors, although CD36 is by far the most heavily implicated receptor in oxLDL mediated platelet activation. We wanted to confirm the reported findings that CD36 is involved, as well as elucidate the potential role of LOX-1 in this process.

3.6.1 Confirmation of scavenger receptors on human platelets

Having already shown that oxLDL is able to cause both tyrosine phosphorylation and PKC activation (Fig. 3.7), we wanted to begin investigating the possible mechanism and identify candidate receptors. We began by confirming the presence of specific scavenger receptors in platelet lysates and at the same time, validating the quality of the antibodies for subsequent experiments. As expected, we could clearly detect the presence of CD36 in human platelets, with band intensity increasing with increased protein lysate (Fig. 3.15). The anti-CD36 antibody H-300 was able to detect CD36 at very low protein levels, with a faint band observable at 5µg lysate.

The anti-LOX-1 antibody was unable to clearly show the presence of LOX-1 in platelets, even at the highest protein concentration of 40µg. However, the faint presence of a band suggests that the protein may be expressed at very low expression levels.

3.6.2 Confirmation of CD36 on murine platelets

Having established that the anti-CD36 antibody H-300 worked robustly in human samples, we wanted to validate the manufacturer's claims that it worked reliably in mice. In order to test antibody specificity, lysates from wild-type and $CD36^{-/-}$ mice were loaded and compared on the same membrane. In order to confirm our results, mice from both the wild-type and CD36 deficient colonies were genotyped commercially. The genotyping data obtained from an external contractor (data not shown) shows that wild-type mice possess CD36, whereas $CD36^{-/-}$ mice do not. However, immunoblotting data using H-300

did not support the genotyping data, instead showing that CD36 was absent in both strains. This is likely due to a lack of specificity for the antibody in murine tissue.

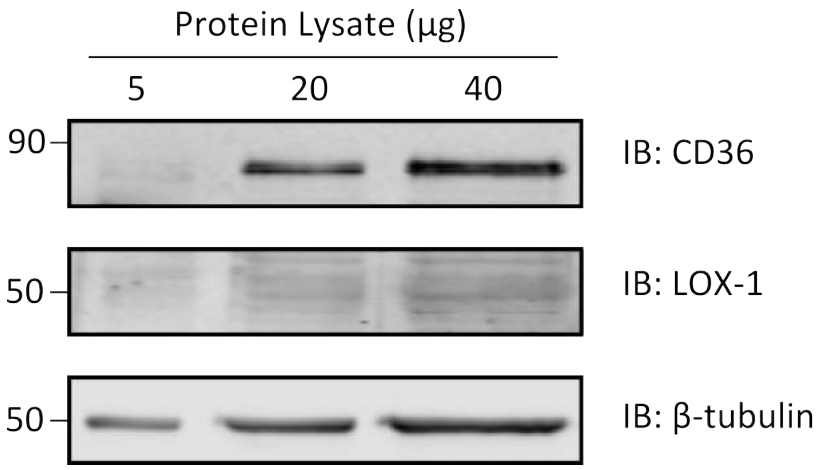


Figure 3.15 – Scavenger Receptor presence in human platelets. Platelets were lysed in Laemmli buffer and proteins separated using SDS-PAGE. Immunoblotting was used to detect the scavenger receptors CD36 and LOX-1 and β-tubulin was used as a loading control. Data shown is a representative blot of 3 separate experiments.

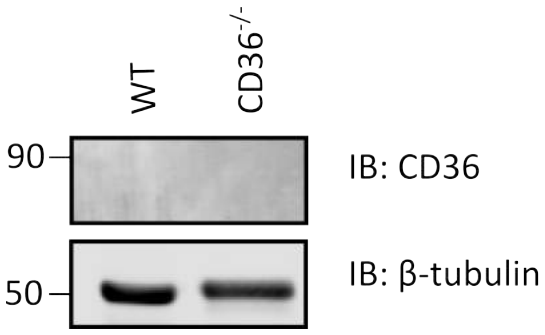


Figure 3.16 – Detection of CD36 protein in murine platelets. Wild-type and CD36 deficient murine platelets ($5x10^8$/mL) were lysed in Laemmli buffer and proteins were separated using SDS-PAGE. Immunoblotting was used to detect CD36 and β-tubulin was used as a loading control. Data shown is a representative blot of 2 separate experiments.

3.6.3 Effects of pharmaceutical blockage of CD36 and LOX-1 on platelets adhered to collagen

Having shown CD36 is present in human platelets, along with evidence of LOX-1, we wanted to investigate the effects of blocking these receptors on platelet adhesion to oxLDL. However, to begin with we wanted to determine the specificity of the inhibitors and see whether they would have an effect on processes believed to be CD36-independent – platelet adhesion to collagen (Fig. 3.17).

Platelet incubation to collagen treated slides elicited a robust adhesion and spreading response (Basal – 628.73). Platelet pre-incubation with the CD36 neutralising antibody FA6.152 caused minimal change to the numbers of adherent platelets (anti-CD36 – 605.11). Similarly, an IgG control used to determine any non-specific effects of antibody binding to platelets have very little effect (IgG – 544.91). The LOX-1 neutralising antibody caused a more noticeable decrease in platelet adhesion (anti-LOX-1 – 482.42). Minimal adhesion was observed on serum treated slides, indicating that the blocking process had worked as expected.

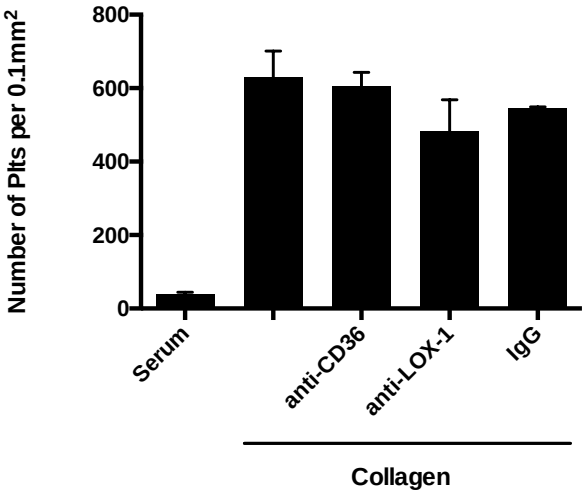


Figure 3.17 – Validation of scavenger receptor blocking antibodies on platelets adhered to collagen. Platelets ($5x10^7$/mL) were treated with an anti-CD36 antibody (FA6.152; 1µg/mL), anti-LOX-1 (Gift from University of Leeds; 1µg/mL) or an IgG control for 20 minutes prior to incubation on collagen coated slides. Adhered platelets were fixed, stained using TRITC-phalloidin and imaged using an inverted fluorescence microscope (Zeiss). Data shown is an average of two separate experiments.

3.6.4 Effects of pharmaceutical blockage of CD36 and LOX-1 on platelets adhered to oxLDL

The CD36 neutralising antibody is commonly used to block the action of CD36 across multiple cell types (Dawson et al., 1997; Nergiz-Unal, Lamers, et al., 2011; Wraith et al., 2013), whilst less evidence exists showing use of the anti-LOX-1 antibody. Previously, we sought to ensure a good degree of specificity by using the inhibitor in the presence of an agonist not related to the function of CD36 or LOX-1. We were reasonably confident in the available tools as a result of this, and next wanted to stimulate platelets on oxLDL in the presence of the neutralising antibodies to deduce the significance of these receptors in the adhesion process. We hypothesised that a decrease in platelet adhesion should be observed by blocking either of these receptors and furthermore, this would allow analysis into their relative importance.

Consistent with the previous figure, the IgG control antibody had very little effect on platelet adhesion to oxLDL (IgG – 284.42±26.12) (Fig. 3.18) when compared to basal (Basal – 309.12±35.37). However, neutralisation of CD36 and LOX-1 independently of each other caused a significant decrease in platelet adhesion (anti-CD36 – 123.46±41.59, anti-LOX-1 – 128.03±19.98) ($p=<0.001$ and $p=<0.001$ respectively).

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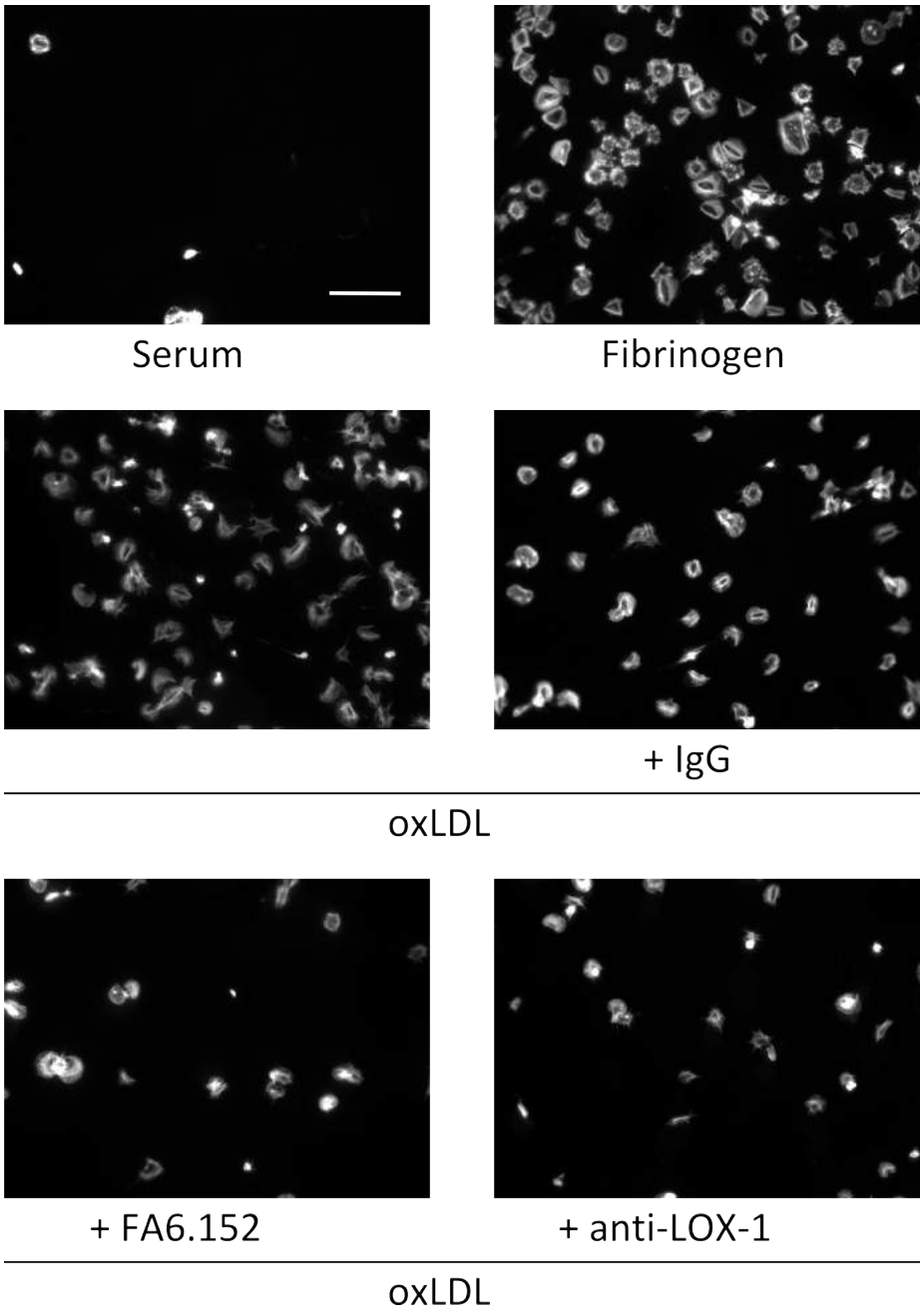

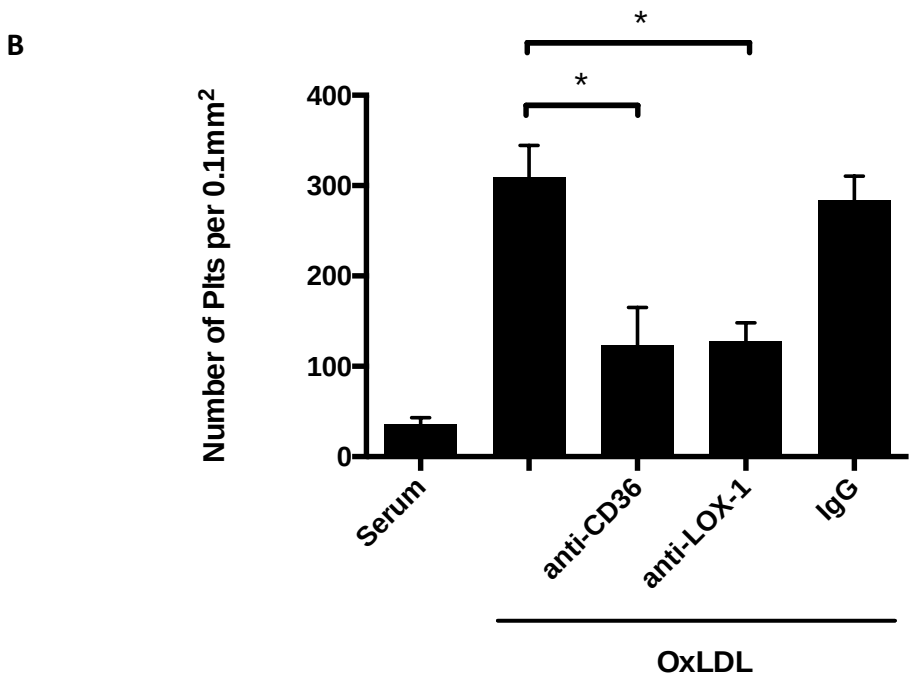

Figure 3.18 – Determining the effects of scavenger receptor inhibition on platelet adhesion to oxLDL. Platelets ($5x10^7$/mL) were treated with an anti-CD36 antibody (FA6.152; 1µg/mL), anti-LOX-1 (Gift from University of Leeds; 1µg/mL) or an IgG control for 20 minutes prior to incubation on collagen coated slides. Adhered platelets were fixed, stained using TRITC-phalloidin and imaged using an inverted fluorescence microscope (Zeiss). Scale bar represents 100 microns. Data shown is an average of five separate experiments. *= *p=<0.05* when analysed by ANOVA.

3.6.5 Genetic deletion of CD36 reduces murine platelets from adhering to oxLDL

Having convincingly shown that pharmaceutical inhibition of CD36 prevents platelet adhesion to oxLDL, we wanted to reaffirm these findings using platelets from CD36 deficient mice. Murine platelets were allowed to adhere and spread for 60 minutes on oxLDL and numbers of adhered platelets counted (Fig. 3.19). Both wild-type and CD36 deficient platelets displayed clear adhesion to fibrinogen (WT – 167.88; $CD36^{-/-}$ - 186.8), but did not spread in a fashion consistent with human platelets with no stress fibre formation (Fig. 3.18). Very few wild-type platelets were able to adhere to oxLDL (WT – 55), although even fewer $CD36^{-/-}$ platelets adhered to the same concentration of oxLDL ($CD36^{-/-}$ - 13), indicative of a weak trend. Drawing any meaningful conclusions from this data is however difficult since murine platelets do not appear to adhere well to human oxLDL and should be interpreted with caution.

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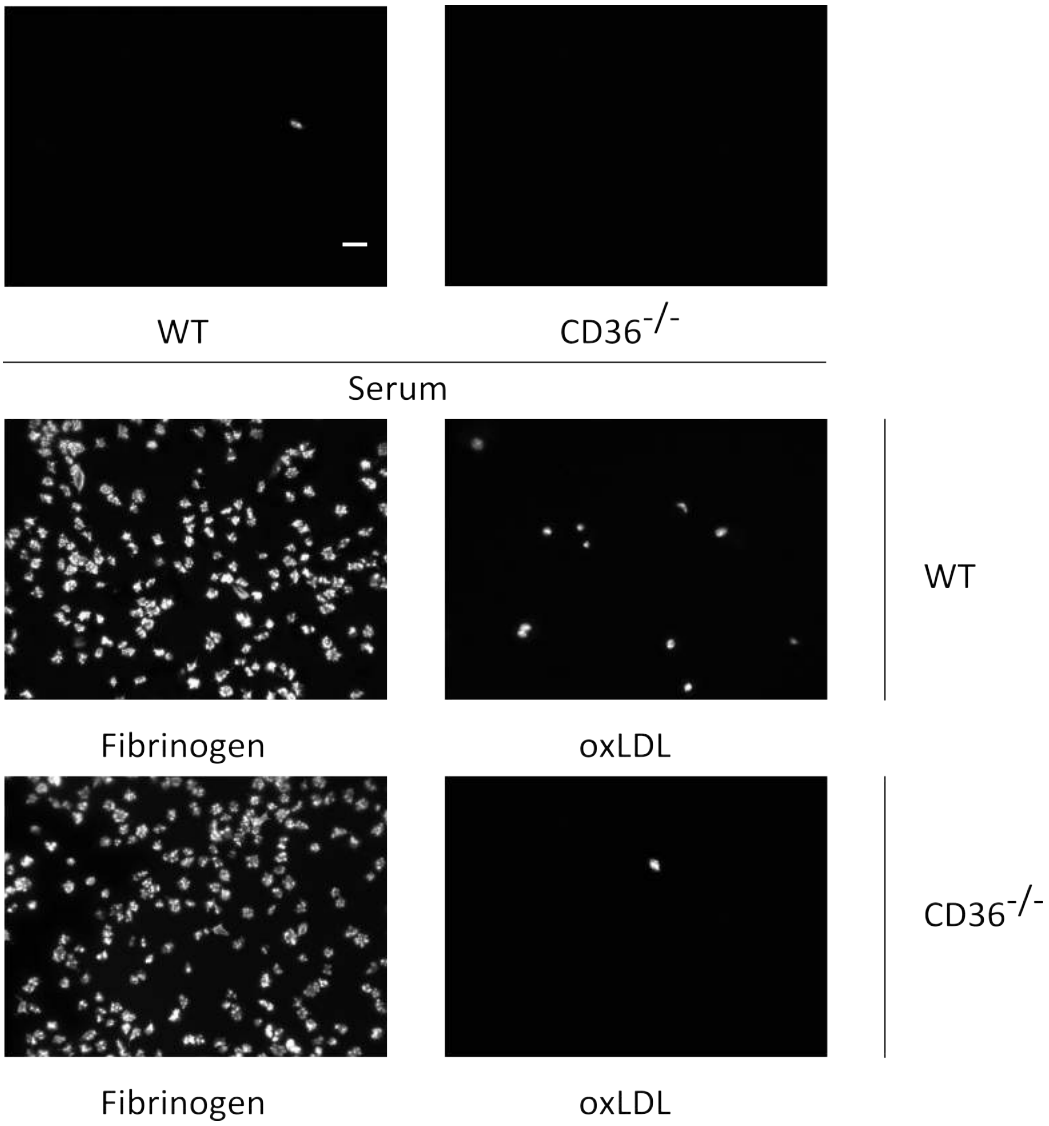

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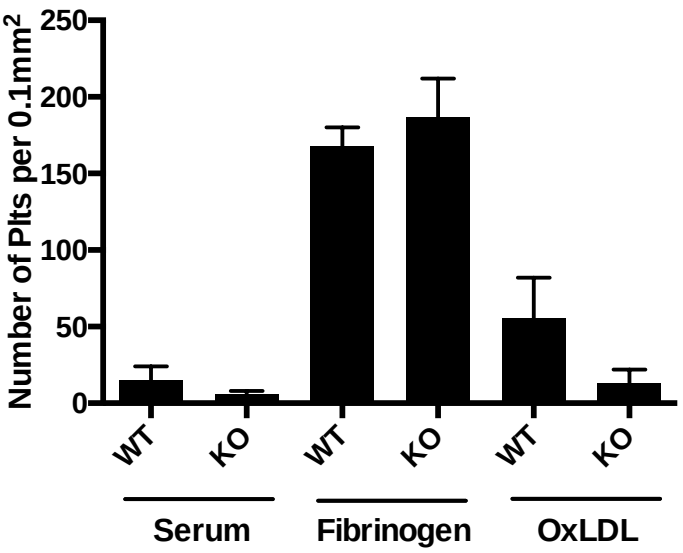


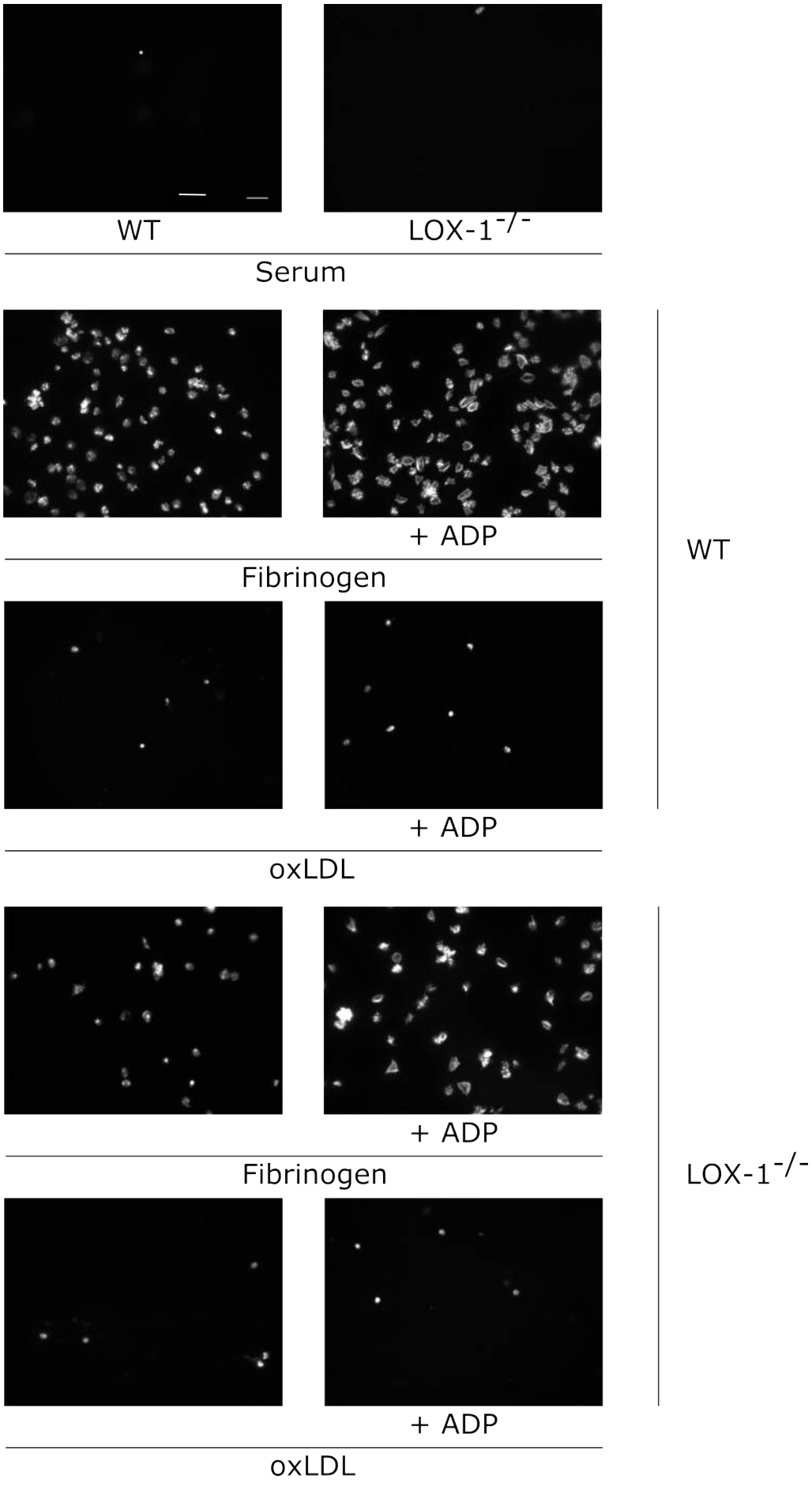
Figure 3.19 – Murine platelets do not adhere or spread well on oxLDL, although less CD36$^{-/-}$ platelets adhered compared to WT. Platelets (5x10^{7}/mL) from wild-type or CD36$^{-/-}$ mice incubated onto oxLDL treated slides (100µg/mL) for 60 mins at 37^{o}C. Adhered platelets were fixed, stained with TRITC-phalloidin (1µg/mL) for 60 mins and viewed using an inverted fluorescence microscope (Zeiss). Scale bar represents 100 microns. Images were acquired randomly and quantified manually using ImageJ. Data shown is representative of two separate experiments.

3.6.6 Genetic deletion of LOX-1 does not affect platelet adhesion to oxLDL

Since pharmaceutical inhibition of LOX-1 caused a significant decrease in platelet adhesion oxLDL, we wanted to substantiate these findings using mice deficient in the LOX-1 receptor. Since LOX-1 has been previously reported to be localised to intracellular granules (Chen et al., 2001), a low concentration of ADP (1μM) was used to cause a minor degranulation response, causing the receptor to become exposed.

We wanted to conduct an exploratory experiment using LOX-1 deficient mice to see whether a clear phenotype was apparent, but unfortunately only enough mice were available to conduct a single test. Consistent with the previous findings, wild-type platelets were able to adhere to fibrinogen but not undergo complete cytoskeleton rearrangement (Fig. 3.20). In the presence of ADP, wild-type platelets adhering to fibrinogen did display increased levels of spreading and were visually similar to human platelets spread on fibrinogen. Interestingly, LOX-$1^{-/-}$ platelets displayed less adhesion to fibrinogen than wild-type controls (WT – 378; KO – 210), but pre-treatment with ADP was able to potentiate the spreading response in both groups (WT – 492; KO – 300). However, wild-type and LOX-$1^{-/-}$ platelets displayed very low levels of adhesion to oxLDL (WT – 24; KO – 30) which did not increase in the presence of ADP (WT – 42; KO – 30).

A


WT
LOX-1-/-
Serum
+ ADP
Fibrinogen
WT
+ ADP
oxLDL
+ ADP
Fibrinogen
LOX-1-/-
+ ADP
oxLDL

B

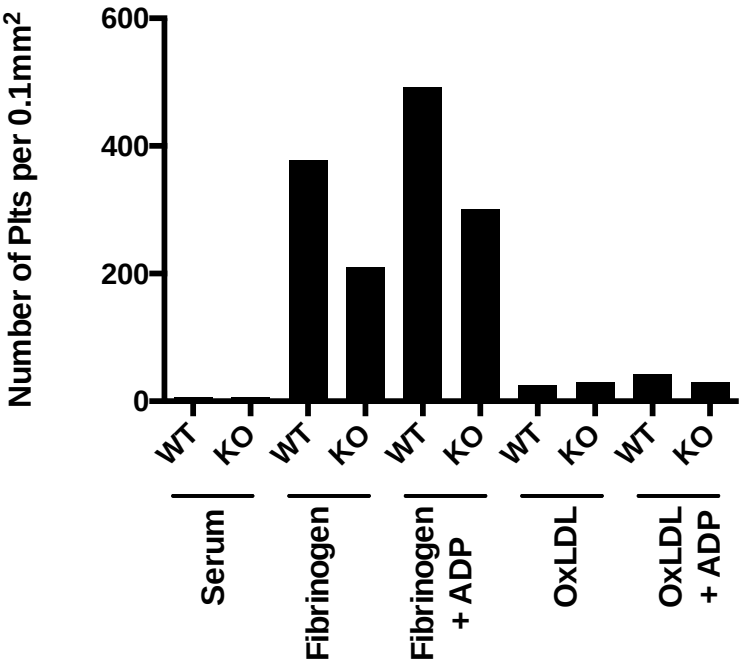


Figure 3.20 – LOX-1$^{-/-}$ platelets do not show any deficiency in adhesion to oxLDL compared to wild-type. Platelets ($5x10^7$/mL) from wild-type or LOX-1$^{-/-}$ mice incubated onto oxLDL treated slides (100µg/mL) for 60 mins at 37°C. Adhered platelets were fixed, stained with TRITC-phalloidin (1µg/mL) for 60 mins and viewed using an inverted fluorescence microscope (Zeiss). Images were acquired randomly and quantified manually using ImageJ. Scale bar represents a distance of 100 microns. Data shown is representative of a single experiment.

3.7 Chapter Discussion

Hyperlipidaemia has been linked to an increased risk of thrombosis since the 1970s (Carvalho et al., 1974). Oxidised lipoproteins have been implicated in the disease process by priming platelets *in vivo*, leading to increased reactivity and enhancing the risk of thrombotic events (AMA, 1984; Stellos et al., 2012). Whilst the ability for oxLDL to alter platelet function is becoming increasingly accepted, there remains a lack of novel drug candidates focusing on this part of the atherothrombosis process. As a result, further insight into the functional and mechanistic aspects of oxLDL ligation with platelets is required. Specifically, this section of work aimed to isolate and oxidise LDL from healthy donors and examine its effect on platelet function *in vitro*.

Biochemical characterisation of oxLDL & effects on platelet activation

Since a great deal of variability exists between LDL isolation and oxidation protocols, we began by establishing a reliable method of generating oxLDL *in vitro* for use in subsequent platelet functional studies. We generated two species of modified LDL – *hydroperoxide* rich oxLDL, obtained by oxidation using Cu^{2+} ions at 4^{o}c and *oxysterol* rich, which relies on a higher oxidising temperature (37^{o}c). The methods used in this study are adapted from a highly characterised protocol by Gerry et al., which characterises LPO and REM as well as other oxidation markers. Since there is a great deal of variation in the process of oxidising LDL between research groups, we used this protocol as a benchmark to compare our oxidation process and showed that our oxLDL preparation was uniform in approach. In our hands, LDL oxidised to yield HP-rich oxLDL was biochemically similar to that reported by Gerry. Very little differences were observed in regards to LPO (850.4 compared to approximately 800nmol/mg LDL protein) or REM (2.84AU±0.75 compared to approximately 4AU). Similarly, our preparation of extensively oxidised LDL (OS-rich) was comparable to that reported (LPO: 14.5 compared to approximately 50nmol/mg LDL protein; REM – 3.55±1.0 compared to approximately 5AU). Due to time restraints and recently published literature, it was decided that all subsequent experiments would be conducted using OS-rich oxLDL. However, it would be interesting and relevant to resume

work with the milder forms of oxLDL, also known as minimally modified LDL, since evidence exists to show that it may exert different effects compared to heavily oxidised (Naseem et al., 1997; Weidtmann et al., 1995).

Having established a reliable and consistent source of oxLDL, we wanted to begin to investigate its effect on platelet function. As preliminary work, we wanted to see whether any change in platelet function was observable when either blood or PRP was incubated with oxLDL. FACS was selected for preliminary work due to the fact it is highly sensitive and statistically rich. Interestingly, blood and PRP showed increased levels of platelet CD62P expression with oxLDL exposure. A significant increase in CD62P was observed in whole blood with 200μg/mL oxLDL, although this activatory threshold was reduced to 50μg/mL in PRP. One possible explanantion for this is the presence of other oxLDL scavenging cells in whole blood – monocytes, macrophages and dendritic cells (Berliner et al., 1990; Murphy et al., 2006), which act as a sink and increases the amounts required to activate platelets. The increase in CD62P levels gave us confidence that our oxLDL preparation was functional, even in complex environments. Moreover, this data is consistent with published literature stating that oxLDL is able to cause CD62P expression in a dose-dependent manner (Naseem et al., 1997; Podrez et al., 2007). Since the FACS studies are more physiologically relevant than experiments using washed platelets, it would be good to look at other platelet activation markers e.g. platelet-leukocyte interactions, CD40L to establish any other effects oxLDL has on platelet function. In a pathological context, oxLDL has been shown to modulate platelet function to cause platelet-leukocyte aggregates, accelerating atherogenesis and plaque rupture (Badrnya et al., 2014). It would be interesting to repeat these findings and begin to look at the mechanisms underpinning this.

Having shown that our oxLDL preparation was able to activate platelets, we decided to compare the effects of our oxLDL preparation on platelet function with previous work by using platelet aggregation. There is great disparity regarding the ability of oxLDL to induce platelet aggregation, although the bulk of studies show that oxLDL can trigger modest

aggregation (Ardlie et al., 1989; Weidtmann et al., 1995; Naseem et al., 1997; Volf et al., 1999; Korporaal et al., 2005; Wraith et al., 2013). Whilst our oxLDL preparation caused modest aggregation, it is difficult to compare between studies due to differences in oxLDL preparations and platelet counts used.

Using the same conditions, platelets were lysed and probed for two markers of platelet activation – phosphorylation of tyrosine residues and phosphorylation of PKC substrates. Both of these markers have been reported to be increased in platelets stimulated with oxLDL (Wraith et al., 2013; Magwenzi et al., 2015) and therefore we wanted to determine whether our preparation of oxLDL was equally able to trigger this. Consistent with the literature, our preparation of oxLDL was able to cause an increase in phosphorylated tyrosine/PKC substrates in a time-dependent manner. We speculate that many of the proteins shown to be phosphorylated by oxLDL are also found downstream of CRP signalling too since there was a high degree of similarity between these agonists. Published reports state that oxLDL is able to induce phosphorylation and activation of Src family kinases, Syk and PLCγ2 (Nergiz-Unal, Lamers, et al., 2011; Wraith et al., 2013; Zimman et al., 2014) and it is likely that the bands identified correlate with these proteins. Unlike tyrosine phosphorylation, which has been subject to extensive studies, changes in PKC substrate phosphorylation in platelets is less certain. Platelets possess several isoforms of PKC including PKCα, β, δ and θ and are involved in key cytoskeletal processes including shape change and granule secretion. PKC has been shown to cause the phosphorylation of many proteins in these processes such as pleckstrin, SNARE proteins complexes, SNAP-23, syntaxin 4 and Munc18c (Harper and Poole, 2010). We showed the α-granule marker P-selectin expression to be increased upon oxLDL stimulation as well as platelet spreading. Therefore, it is possible that these proteins are becoming phosphorylated and warrants further investigation.

Since the platelet aggregating capacity of oxLDL remains controversial and signalling experiments are only capable of showing a proxy of activation, we decided to investigate the activatory potential of oxLDL using a static adhesion assay. Unlike aggregation, most

reports state that oxLDL adhesion to oxDL is robust (Nergiz-Unal, Lamers, et al., 2011; Wraith et al., 2013) and this would serve as a useful tool to probe the relative importance of secreted mediators and receptors involved in the attachment process.

Platelet adhesion to oxLDL

Consistent with published literature, we managed to optimise the static adhesion assay to produce robust, measurable levels of platelet adhesion and spreading (Figs 3.7, 3.8). Furthermore, using the static adhesion assay, we corroborated previous results showing that adhesion is dependent on ADP (Nergiz-Unal, Lamers, et al., 2011) and for the first time, showed that the process is partially TxA_2 dependent. Whilst not reaching statistical significance, the data also suggested that adhesion is integrin $\alpha_{IIb}\beta_3$ dependent, in line with the work by Nergiz-Unal et al. Conversely, no significant differences in platelet spreading were observed between untreated platelets and those treated with apyrase/indomethacin/tirofiban. We hypothesised that the presence of these agents would decrease platelet spreading since the release of ADP and TxA_2 would activate complimentary signalling pathways, supporting full platelet activation. One possible explanation is that since platelet adhesion and spreading is a highly dynamic process, 60 minutes' exposure to the adhesive medium as conducted in this study could be long enough to ensure full spreading, irrespective of the ligand of choice and magnifying the effects of any small degree of oxidation with use of nLDL. One alternative approach, as used by Nergiz-Unal and colleagues, would be to record platelet spreading in real-time and see whether the presence of these inhibitors affects spreading in a time-dependent fashion.

The roles of CD36 and LOX-1 in platelet adhesion to oxLDL

Having established a robust spreading assay to measure platelet activation by oxLDL, we wanted to begin to investigate the mechanisms underpinning the activation event. Whilst several receptors including $\alpha_{IIb}\beta_3$ (Korporaal et al., 2005), LPA (Maschberger et al., 2000) and PAF receptors (Chen et al., 2009) have been implicated in the activatory response of

platelets to oxLDL, CD36 is considered to have the largest role in platelets and is expressed at very high copy numbers (Ghosh et al., 2011a). In addition, we also sought to investigate the role of LOX-1 since very little research has been conducted on this receptor in platelets, despite it playing a significant role in oxLDL ligation to endothelial cells (Sawamura et al., 1997; Kakutani et al., 2000).

We were able to detect CD36 at low protein levels of just 5µg cell lysate, suggesting that CD36 is highly expressed on platelets. This is consistent with studies investigating the copy number of CD36 on platelets, which show that it is highly expressed with up to 14,000 copies per cell (Ghosh et al., 2011a). However, no clear band was detected using the anti-LOX-1 antibody, even at the highest protein concentration. Since we concluded that the anti-LOX-1 antibody was of poor quality, we decided not to blot for LOX-1 in murine samples and surprisingly the anti-CD36 antibody could not detect the protein, despite the manufacturer's claims and supporting genotyping data.

Next we wanted to assess the role of the receptors individually on platelet adhesion using neutralising antibodies to the receptors of interest. To test this, we treated platelets with the antibodies and investigated whether platelet adhesion to collagen would be altered – a substrate not typically associated with these receptors. Minimal reduction of adhesion to collagen was observed with platelets treated with either the anti-CD36 or anti-LOX-1 antibody, indicating a reasonable degree of specificity. In contrast, the anti-CD36 or anti-LOX-1 antibody significantly reduced adhesion to oxLDL supporting the notion that these receptors are involved in oxLDL ligation *in vivo*. Published data by Heemskerk and colleagues corroborates our findings with the anti-CD36 antibody reducing platelet adhesion to oxLDL (Nergiz-Unal, Lamers, et al., 2011). However, to the best of our knowledge, this is the first attempt to use an anti-LOX-1 blocking antibody in the context of platelet adhesion to oxLDL.

To further investigate these results, we used murine platelets from animals deficient in CD36 or LOX-1 as an alternative model. Whilst having previously shown that murine

platelets do not aggregate in response to oxLDL, we hypothesised that despite this, due to the duration of incubation limited adhesion and spreading may occur.

In these experiments, murine platelets displayed a diminished adhesion response to oxLDL compared to human platelets. Whilst the reasons for this are not well known, one possible reason is that murine CD36 has an altered oxLDL binding domain, decreasing affinity to the oxidised phospholipids present (Puente Navazo et al., 1996). $CD36^{-/-}$ platelets did display decreased adhesion to oxLDL than wild-type, although levels of adhesion were very low and this may be coincidental. Subsequent experiments may benefit from using KOdiA-PC as a proxy for oxLDL. The incorporation of KOdiA-PC would allow coating of the lipid onto a glass surface and therefore we would expect it to be able to support platelet adhesion. This may provide a useful tool for the dissection of signalling pathways involved in murine platelet spreading, as well as a method of combatting oxLDL heterogeneity in human studies.

In contrast, $LOX\text{-}1^{-/-}$ platelets displayed virtually no difference to wild-type controls, although these findings were from a single experiment and therefore should be interpreted with caution. Despite the difficulties with the tools needed to investigate the role of LOX-1 signalling in platelets, this still remains an interesting concept. The ability for CD36 to cause degranulation may also result in the release of LOX-1 from granules. We hypothesise that the consequence of this would be additional oxLDL ligation and associated signalling events and moreover, the pathophysiological significance of this is an important research question.

Summary

To conclude, we have successfully produced a species of oxLDL which is able to induce platelet activation comparable to that used within the literature. Moreover, the oxLDL produced was able to initiate a range of platelet activation processes, including adhesion, secretion and aggregation. As part of this, we showed that oxLDL was able to induce intracellular signalling events and began to look at the receptors involved in this process.

The evidence from this chapter suggests that CD36 is likely to play a central role in platelet activation from oxLDL, although the tools to investigate the role of LOX-1 are not of a sufficient standard to rigorously test this hypothesis.

Later work will build on this central foundation by further investigating the role of CD36 and looking at the intracellular signalling events in greater detail.

Chapter 4: Oxidised Low-density lipoproteins affects platelet adhesion under flow

4.1 Introduction

Under physiological conditions, platelets are continually exposed to varying degrees of sheer stress that aids in binding to exposed matrix proteins at sites of injury (Kroll et al., 1996). During the last twenty years numerous methodologies have been developed in order to study the effects of sheer stress on platelet function (Westein et al., 2012). A key innovation has been the application of fluorescence microscopy and its combination with microfluidic devices to observe platelet adhesion under flow. These devices have shown the highly dynamic nature of platelet adhesion, including differences in tethering and rolling, between different protein ligands (Gutierrez et al., 2008) and have played an important role in understanding the pharmacokinetics of new anti-platelet agents (Santos-Martínez et al., 2011).

While a plethora of studies have examined platelet activation by oxLDL, very few studies have investigated their influence on platelet function under shear. A study by Korporaal et al. showed that addition of oxLDL to whole blood potentiated platelet binding to fibrinogen under both arterial and venous shear (Korporaal et al., 2007). However, in this study the authors used very high concentrations of oxLDL (1mg/mL) which is likely to be far in excess of what may be found *in vivo*. Clinical studies remain inconclusive regarding the amount of oxLDL present *in vivo*, with oxLDL plasma levels from 14.5±0.82 μg/mL in metabolic-syndrome patients (Holvoet et al., 2001), 15.0±8.1 μg/mL in CHD-risk-equivalent patients (e.g. diabetes, non-coronary atherosclerosis) and 31.1±11.9 μg/mL in high-risk coronary artery disease patients (Holvoet et al., 2003; Holvoet et al., 2004). Interestingly, the key study by Chan et al. showing the presence of electronegative LDL in vivo reported higher circulating levels of 189±210μg/mL in STEMI patients (Chan et al., 2013), indicating that further study is required.

A more recent study by the group of Heemskerk investigating the mechanisms of oxLDL pathogenicity demonstrated that, at arterial sheer, co-immobilisation of oxLDL with collagen showed an increase in platelet adhesion when compared to collagen alone (Nergiz-Unal, Lamers, et al., 2011). However, it is unclear whether this observation could be extended to other adhesive proteins or indeed whether oxLDL alone could support adhesion. An added complexity of trying to unravel the effects of oxLDL on platelet function *in vivo* is the potential influence of endothelial derived inhibitors. Blood flow helps regulate the production of NO and PGI_2 from endothelial cells lining the vessel wall (Topper et al., 1996) and aids in localising platelets to the vessel periphery, where they encounter these compounds to keep quiescent. Several reports have suggested that hyperlipidaemia can have a negative effect on the production of NO (Blair et al., 1999; Davignon, 2004) and PGI_2 (Thorin et al., 1994; Mahfouz and Kummerow, 2001). In contrast, there has been little work performed regarding hyperlipidaemia or oxLDL and its influence platelet sensitivity to NO and PGI_2. It is attractive to speculate that the pathogenic effects of oxidised lipids associated with LDL may involve modulation of both the bioavailability of NO and PGI_2 but also the sensitivity of platelets to these regulators. This could suggest that platelet hyperactivity is due, at least in part, to dysregulated inhibitory mechanisms.

4.2 Chapter aims

The aim of this section of work was to characterise the platelet adhesion response to oxLDL with a view to determining how oxLDL is able to induce platelet hyperactivity, as seen *in vivo*. Specifically, we aim to:

- Determine the effects of oxLDL and oxidised phospholipids on whole blood under physiological flow conditions
- Investigate the importance of CD36 in oxidised phospholipid mediated changes in platelet function
- Determine whether oxLDL and oxidised phospholipids affect platelet sensitivity to cyclic nucleotides

4.3 Immobilised oxLDL alone does not support adhesion under flow

4.3.1 Verification of oxLDL coating

At sites of atherosclerosis, plaques may expose their content by either rupturing or undergo erosion by blood flow (Shah, 2002) and degradation by metalloproteases (Galis and Khatri, 2002; Newby, 2005). It is generally considered that plaque rupture exposes the blood to the contents of the plaque, triggering acute occlusive thrombosis. OxLDL bound to regions of exposed vessel wall due to fibrous cap loss and erosion may present a thrombotic surface for platelets to adhere to and lead to lesion development (Shah, 2002). To test this hypothesis, it was important to determine whether oxLDL could be immobilised on glass capillary slides and be able to remain adhered whilst blood was perfused throughout the system. OxLDL was stained with the lipid dye, Nile Red (5µM, 5 mins) and allowed to adhere to the glass capillary slide overnight. To replicate blocking, PBS was flushed through the slide as albumin is known to draw the dye from cells. Emitted fluorescence was only detectable from dye contained within a hydrophobic environment and quenched in water, resulting in a high level of specificity to oxLDL.

Low levels of fluorescence were detected at 10 µg/mL, although this was still higher than untreated slides. OxLDL (100µg/mL) treated capillary slides displayed a higher level of fluorescence compared to 10 µg/mL (Fig. 4.1). Untreated slides did not show any detectable levels of fluorescence. A maximal concentration of oxLDL (3000µg/mL) treatment was used as a positive control and displayed the highest level of fluorescence. Taken together, this data indicates that oxLDL is able to adhere to the glass capillary slides and remain adhered after blocking.

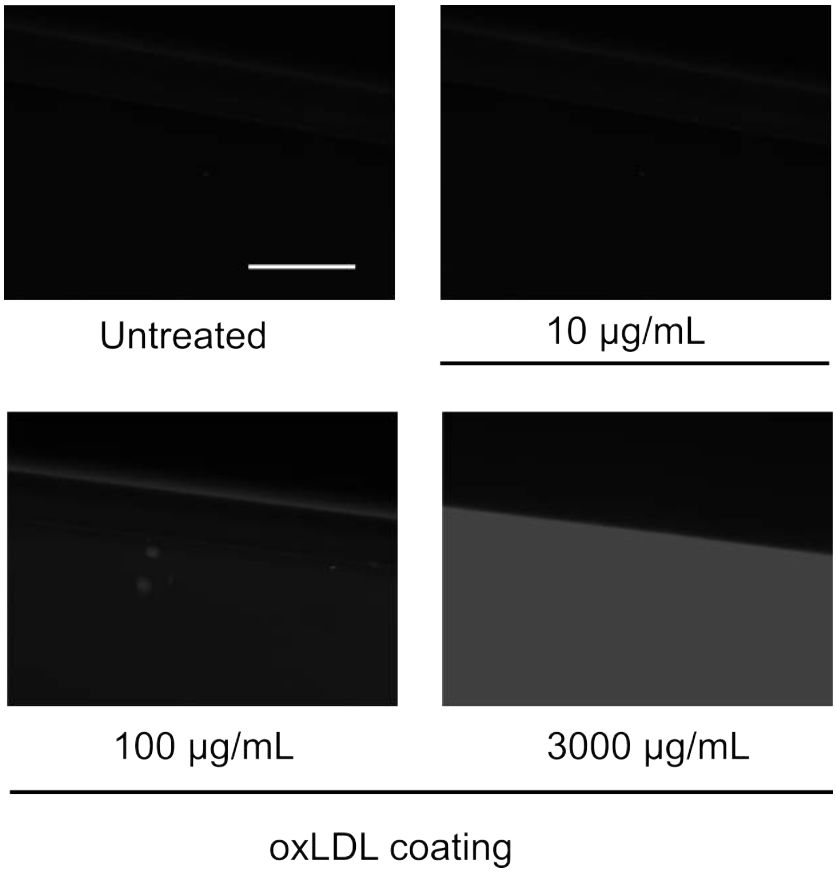


Figure 4.1 – OxLDL adheres in a dose dependent manner and is resistant to washing. OxLDL was incubated with Nile red (25µM) for 5 minutes and allowed to adhere to glass capillary slides overnight at 4^{o}C. The following day, glass capillary slides were washed using PBS and levels of fluorescence visualised using an inverted fluorescence microscope and TRITC filter. Exposure levels were kept constant throughout the experiment to compare levels of oxLDL adhesion and images taking using x60 objective. Dark regions indicate the background, whereas light regions indicate dyed oxLDL on the capillary slide. Data shown is from a single experiment. Scale bar represents 200 microns.

4.3.2 Platelet adhesion to oxLDL under arterial sheer

Having shown that oxLDL could be immobilised on glass, an investigation was undertaken to determine if platelets could adhere to the lipoproteins at differential shear rates. This is important from both a physiological context, in which adhesion may correlate with legion development (Massberg et al., 2002; Gawaz et al., 2005). From an experimental context, the results could shed light on receptor-ligand interaction strength and help elucidate signalling events analogous to the *in vivo* setting. Since atherosclerotic plaque formation occurs predominately in arterial vessels with high shear, we began by flowing platelets at this flow rate.

Under an arterial sheer rate of $1000s^{-1}$ (45 dynes), individual platelets were able to adhere to collagen and recruit additional platelets, forming micro-thrombi (Collagen – 8.33%±0.48) (Fig. 4.2). Conversely, very low levels of platelet adhesion were observed to oxLDL, independent of concentration (10μg/mL – 0.02%±0.01; 50μg/mL – 0.01%±0.01; 200μg/mL – 0.01%±0.01) although some minor rolling was observed. The concentrations 10μg/mL and 50μg/mL represent pathophysiological concentrations of oxLDL consistent with clinical findings (Holvoet et al., 2001) and previous signalling studies (Rahaman et al., 2006; Wraith et al., 2013), whereas 200μg/mL represents a super-physiological concentration. No statistical difference of platelet adhesion was observed between any of the concentrations of oxLDL and BSA-treated slides (data not shown).

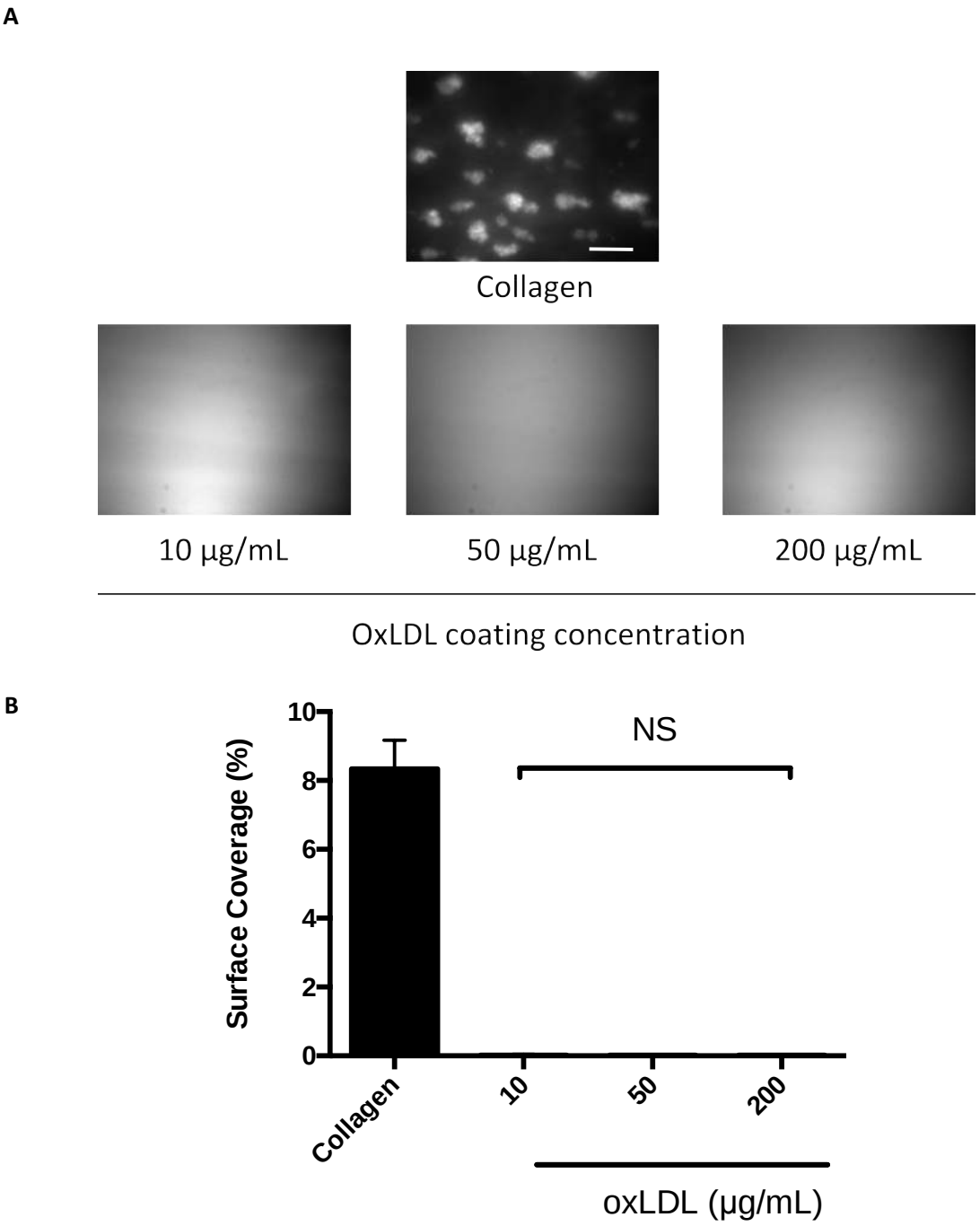


Figure 4.2 – Platelets do not adhere to oxLDL at arterial sheer. Glass capillary slides were coated with the specified concentration of oxLDL or collagen (50µg/mL) and allowed to adhere overnight at 4°C before blocking with 10% BSA for a minimum of 15 minutes. Whole blood was stained with $DiOC_6$ (0.5µM) for 10 minutes prior to flowing for 2 minutes. Platelet adhesion was visualised using an inverted fluorescence microscope (Olympus) and images captured using an XM10 CCD camera and x60 objective. Data shown is the average of three separate experiments. Scale bar represents 200 microns.

4.3.3 Platelet adhesion to oxLDL under venous sheer

Despite having shown that platelets are unable to adhere to immobilised oxLDL under arterial flow, we wanted to determine whether platelets are able to adhere under venous shear. Platelet adhesion has previously been shown to the immobilised ligand thrombospondin-1 under venous shear in a CD36 dependent manner (Kuijpers et al., 2014; de Witt et al., 2014) and therefore may be of interest.

Under venous shear, platelets adhered to collagen (Collagen – 5.83%±0.61) and formed micro-thrombi, analogous to adhesion at arterial sheer (Fig. 4.3). OxLDL was unable to support adhesion at any of the tested concentrations (10μg/mL – 0.02%±0.01; 50μg/mL – 0.02%±0.01, 200μg/mL – 0.01%±0.01) and was not significantly altered from BSA-treated control slides (data not shown). As a result, oxLDL was not used as an adhesion matrix for any subsequent experiments.

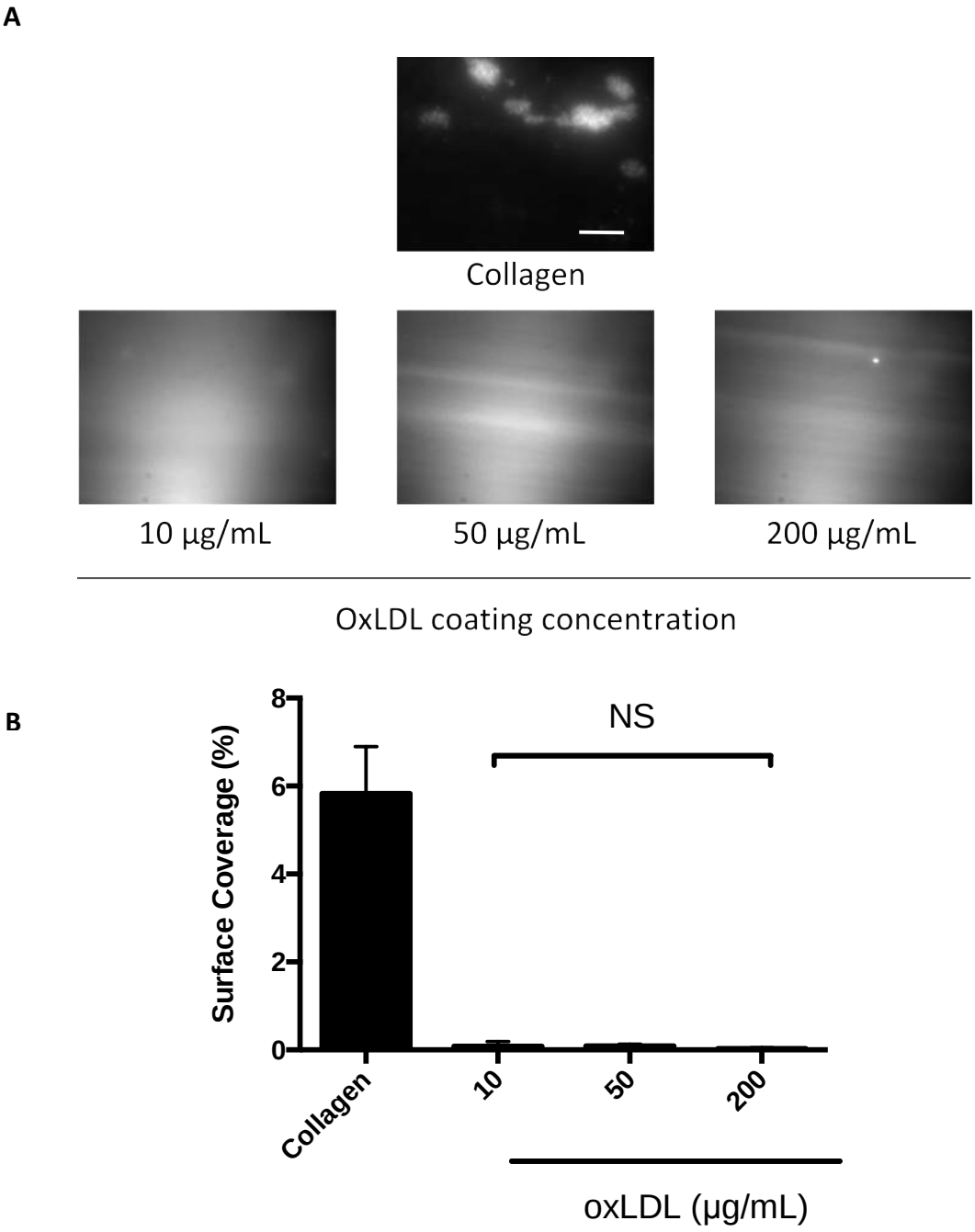


Figure 4.3 – Platelets do not adhere to oxLDL at venous sheer. Glass capillary slides were coated with the specified concentration of oxLDL and allowed to adhere overnight at 4°C before blocking with 10% BSA for a minimum of 15 minutes. Whole blood was stained with $DiOC_6$ (0.5µM) for 10 minutes prior to flowing for 2 minutes. Platelet adhesion was visualised using an inverted fluorescence microscope (Olympus) and images captured using an XM10 CCD camera and x60 objective. Data shown is the mean of three separate experiments. NS – not significant. Scale bar represents 200 microns.

4.4 The effects of oxLDL in suspension on human platelet adhesion

4.4.1 Effects of circulating oxLDL on platelet adhesion to a collagen matrix

As part of the characterisation of the effects of oxLDL on platelet function under flow, the role of oxLDL in the blood milieu and its effects on platelet adhesion to traditional agonists was examined. Collagen was chosen as a matrix protein due to the high concentrations presence at high levels at sites of vascular damage and atherosclerotic plaques. In this series of experiments, platelet adhesion to collagen was robust and consistent with the literature (Basal – 16.02%±0.95) (Fig. 4.4). No statistically significant decrease in platelet adhesion was observed with increasing oxLDL concentration (10µg/mL – 15.05%±0.96; 50µg/mL – 14.26%±1.47; 200µg/mL – 13.79%±0.12) *(p=0.49)*.

4.4.2 Effects of circulating oxLDL on platelet adhesion to a fibrinogen matrix

Having established that oxLDL treated blood displays no difference in platelet adhesion to collagen, we next wanted to investigate whether the same was true for adhesion to fibrinogen. OxLDL has been reported previously to bind to the fibrinogen receptor integrin $\alpha_{IIb}\beta_3$ (Korporaal et al., 2005) and therefore may impact on the platelet's ability to adhere to a fibrinogen matrix. Unlike binding to collagen, platelet adhesion to fibrinogen resulted in the formation of a monolayer, causing uniform coverage of the microfluidic channel (Fig. 4.5). Platelet adhesion to fibrinogen (1mg/mL) in the absence of oxLDL had a similar % coverage to collagen (Fibrinogen only – 13.55%±2.70). There was no statistical decrease in adhesion to fibrinogen in the presence of the lower concentrations of oxLDL (10µg/mL – 10.82±4.06; 50µg/mL – 7.23±2.91) *(p=0.82)*, despite a trend of decreasing adhesion. However, at the highest concentrations of oxLDL, there was a significant decrease in adhesion (200µg/mL – 5.77±1.21) *(p=0.03)*.

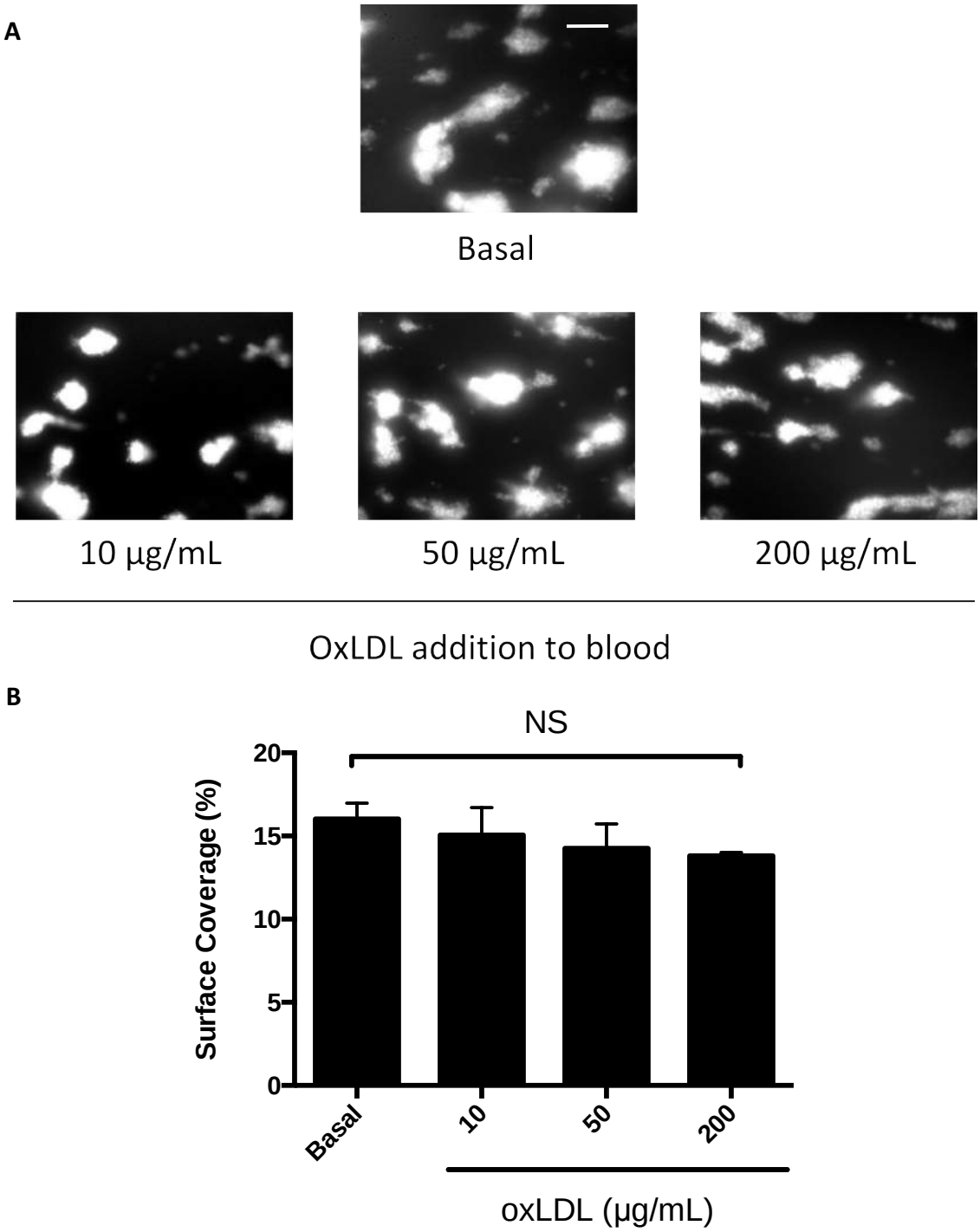


Figure 4.4 – OxLDL addition to blood has no effect on platelet adhesion to a collagen matrix. Microfluidic channels were coated in collagen (50µg/mL) overnight and blocked the following day with 10% BSA for a minimum of 15 minutes. Whole blood was stained with $DiOC_6$ (0.5µM) and treated with oxLDL (10-200µg/mL) for 15 minutes. Treated blood was flowed over collagen coated channels for 2 minutes and images taken using an inverted fluorescence microscope with x60 objective (Olympus). Data shown is the mean of three separate experiments. NS – not significant. Scale bar represents 200 microns.

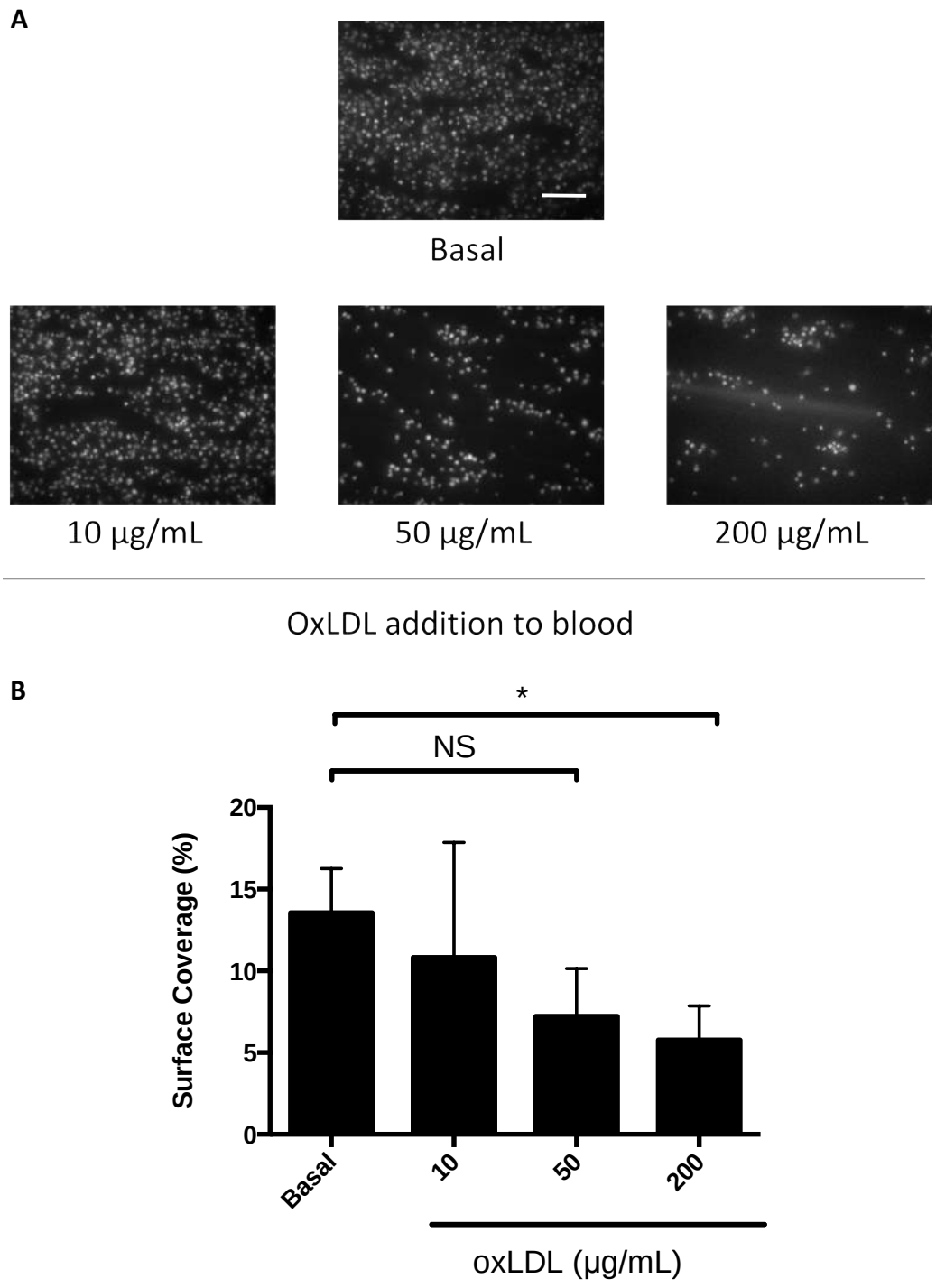


Figure 4.5 – OxLDL addition to blood causes a reduction in platelet adhesion to a fibrinogen matrix. Microfluidic channels were coated in fibrinogen (1000µg/mL) overnight and blocked the following day with 10% BSA for a minimum of 15 minutes. Whole blood was stained with $DiOC_6$ (0.5µM) and treated with oxLDL (10-200µg/mL) for 15 minutes. Treated blood was flowed over the fibrinogen coated channels for 2 minutes and images taken using an inverted fluorescence microscope with x60 objective (Olympus). Data shown is the mean of three separate experiments. NS – not significant. * = *p <0.05* when transformed and analysed using student's t-test. Scale bar represents 200 microns.

4.5 The effect of oxidised phospholipids on murine platelet adhesion

In the next series of experiments, we wished to determine the effects of oxidised LDL as a ligand for platelet CD36 and how this affected *in vitro* thrombosis. This involved the use of murine platelets deficient in CD36. Previous studies from our laboratory found that murine platelets did not respond well to human oxLDL, but do respond to oxidised phospholipids found to be present in oxLDL (E. a. Podrez, 2002; Podrez et al., 2007). KOdiA-PC is an example of an oxidised phospholipid identified from oxLDL and was used extensively in this study. Unlike oxLDL, the nature of phospholipids means that they cannot be adhered to an uncharged surface, therefore preventing us from conducting experiments looking at direct binding.

4.5.1 Effects of circulating KOdiA-PC on murine platelet adhesion to a collagen matrix

Similar to the experiments with human platelets, we began by looking at the effects of KOdiA-PC on murine platelet adhesion to collagen. The concentrations of 5µM and 25µM were selected as physiological (Podrez et al., 2007) and supermaximal concentrations respectively.

Wild-type murine platelets displayed reduced surface coverage compared to human platelets to collagen in the absence of other ligands (Collagen only - 11.27%±1.85) (Fig. 4.6). In the presence of KOdiA-PC (5µM), platelet adhesion increased (5µM - 19.7%±3.59), although when analysed it narrowly failed to meet statistical significance *(p=0.053)*. Conversely, there was a significant decrease in platelet adhesion with the higher dose of KOdiA-PC (25µM - 1.06%±0.21) *(p=<0.05)*. Interestingly, these findings differ from the effects of oxLDL binding to human platelets which show no clear change in adhesion to collagen, indicating differences in the ligands.

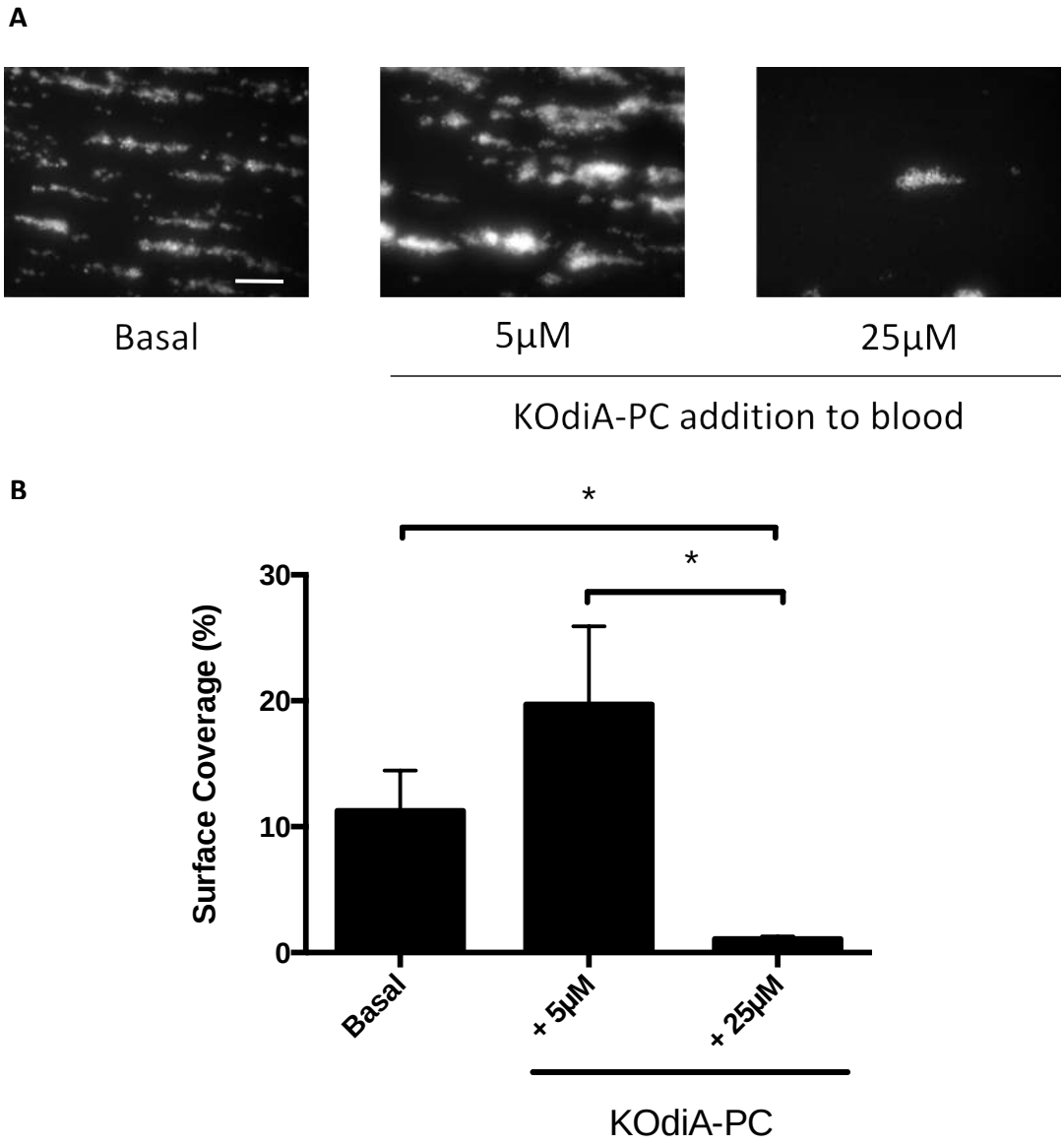


Figure 4.6 - KOdiA-PC affects murine platelet adhesion to collagen in a concentration dependent manner. Microfluidic channels were coated in collagen (50µg/mL) overnight and blocked the following day with 10% BSA for a minimum of 15 minutes. Whole blood was stained with $DiOC_6$ (0.5µM) and treated with KOdiA-PC (5-25µg/mL) for 15 minutes. Treated blood was flowed over the collagen coated channels for 2 minutes and images taken using an inverted fluorescence microscope with x60 objective (Olympus). Scale bar represents 200 microns. Data shown is the mean of three separate experiments. *=$p < 0.05$ when transformed and analysed using ANOVA.

4.5.2 Effects of circulating KOdiA-PC on murine platelet adhesion to a fibrinogen matrix

In order to determine whether KOdiA-PC was having a toxic effect at the higher dose, or affecting the ability of the platelet to bind to collagen, we next investigated whether a similar trend would be seen with platelets adhering to fibrinogen (Fig. 4.7). Like in the collagen studies, murine platelet adhesion to fibrinogen was at comparable levels to that of human platelets (Basal – 11.71%±2.28). Platelet adhesion in the presence of both 5µM and 25µM KOdiA-PC was elevated (5µM – 16.83%±2.08; 25µM – 15.74%±2.98), although these increases were less prominent and failed to reach statistical significance *(p=0.3667)*. The data from Figs. 4.5 and 4.7 show that despite the similarities of oxLDL and KOdiA-PC, they have differential effects on platelet adhesion. However, the fact that are applied to blood from different species complicates this further.

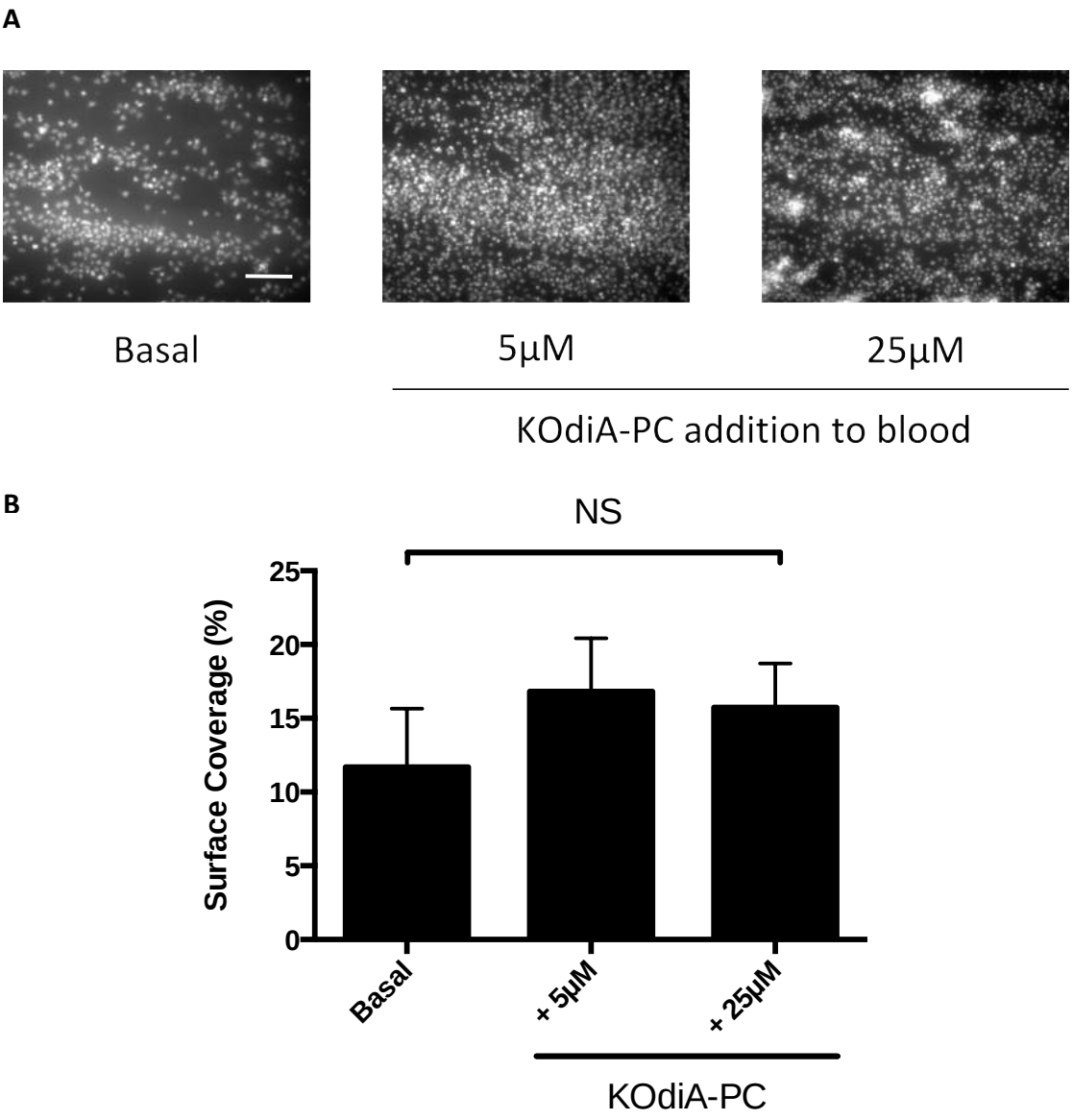


Figure 4.7 – KOdiA-PC causes an increase of murine platelet adhesion to fibrinogen. Microfluidic channels were coated in fibrinogen (1000µg/mL) overnight and blocked the following day with 10% BSA for a minimum of 15 minutes. Whole blood was stained with $DiOC_6$ (0.5µM) and treated with KOdiA-PC (5-25µg/mL) for 15 minutes. Treated blood was flowed over the fibrinogen coated channels for 2 minutes and images taken using an inverted fluorescence microscope with x60 objective (Olympus). Scale bar represents 200 microns. Data shown is the mean of three separate experiments. NS – not significant.

4.6 Genetic deletion of CD36 prevents the additive effects of KOdiA-PC on murine platelet adhesion to collagen

To confirm the findings from the previous chapter that showed that oxLDL binding to platelets is dependent on CD36. Blood from $CD36^{-/-}$ mice was obtained and flowed through collagen coated microfluidic channels. CD36 deficient platelets adhered to collagen in a manner consistent with wild-type platelets (Basal – 13.86%±1.03) (Fig. 4.8). In the presence of KOdiA-PC (5µM), $CD36^{-/-}$ platelet adhesion was not altered from basal (5µM – 14.46%±0.84) *(p=0.09)*, contrasting the effects of lose doses on wild-type platelets. At KOdiA-PC (25µM), no difference in platelet adhesion of $CD36^{-/-}$ platelets was observed when compared to untreated platelets (25µM – 14.82%±1.23) *(p=0.81)*. This contrasts strongly with the same dose on wild-type platelets, in which showed a significant decrease in adhesion, highlighting a difference between the strains and the role of CD36.

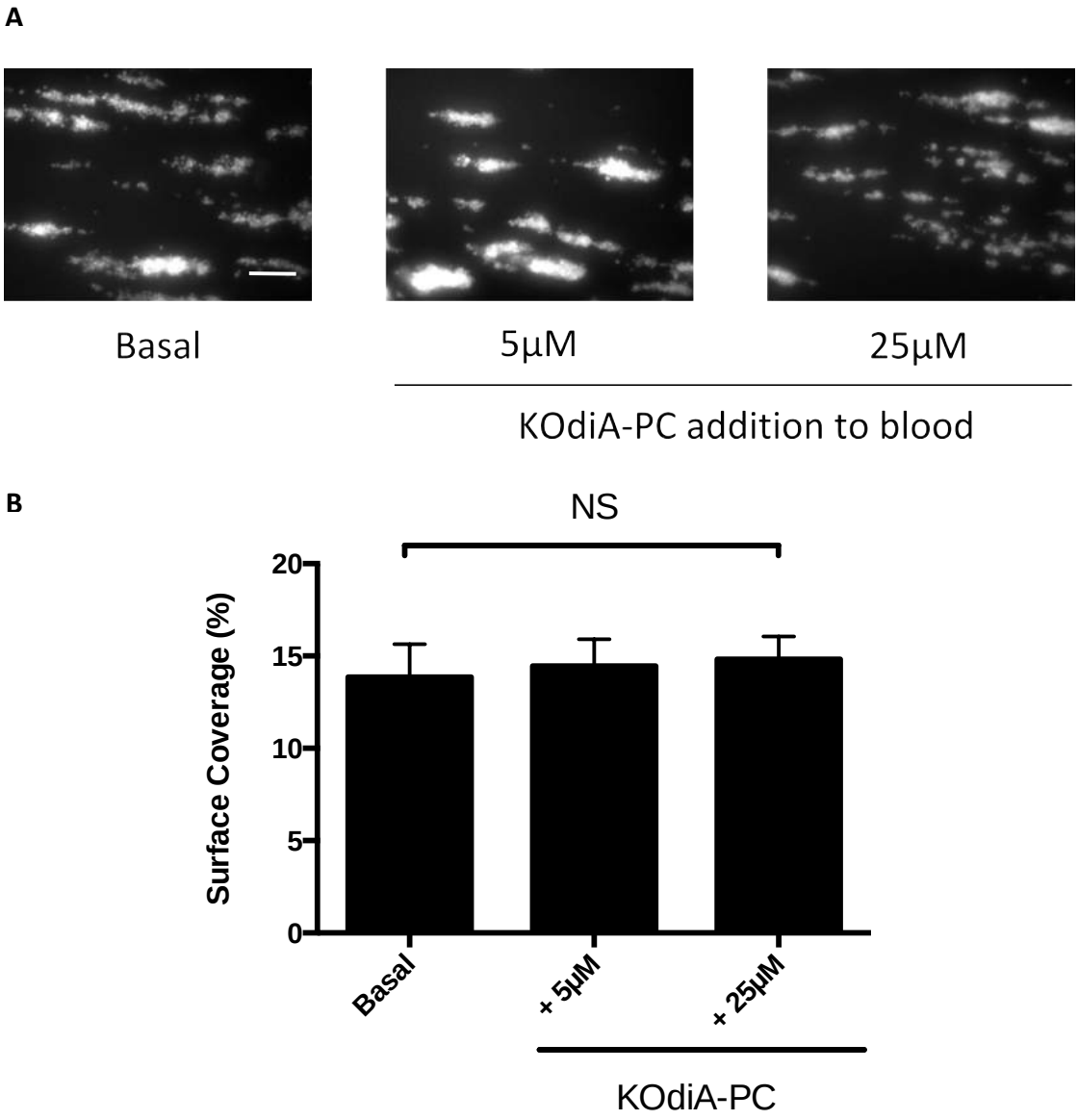


Figure 4.8 – KodiA-PC has no effect on CD36$^{-/-}$ platelet adhesion to collagen. Microfluidic channels were coated in collagen (50µg/mL) overnight and blocked the following day with 10% BSA for a minimum of 15 minutes. Whole blood was stained with $DiOC_6$ (0.5µM) and in experiments requiring additional agonists, blood was pre-treated with KOdiA-PC (5-25 nM) for 15 minutes. Blood was flowed for 2 minutes at 1000s^{-1} and images were taken using an inverted fluorescence microscope with x60 objective (Olympus). Scale bar represents 200 microns. Data shown is the mean of three separate experiments. NS – not significant.

4.7 OxLDL reduces sensitivity to cyclic nucleotides

Data in earlier part of this work, along with multiple published findings shows that oxLDL is able to cause an increase in platelet hyperactivity, leading to changes in secretion and intracellular signalling events. We hypothesised that platelet pre-treatment with oxLDL could cause an increase in adhesion to immobilised matrix proteins, although the data showed that no increase was observed to either a collagen or fibrinogen matrix. One possible explanation for this is that oxLDL may have activatory or inhibitory properties on other signalling pathways, predisposing the platelet to hyperactivity.

Platelet function *in vivo* is balanced by the endothelial derived inhibitors NO and PGI_2 and resulting signalling by proteins PKG and PKA respectively. OxLDL and hyperlipidemia has been shown to directly affect the production of these inhibitors, although very little evidence links oxLDL and direct modulation of inhibitory signalling. However, we hypothesised that platelet hyperactivity could be manifested by oxLDL altering the potential for inhibition and set out to explore that hypothesis in subsequent experiments.

4.7.1 OxLDL reduces sensitivity to cGMP in human platelets

To begin with, we wanted to investigate whether oxLDL would alter the inhibitory effects of NO and PKG signalling. Initially we intended to use the NO releasing donor S-nitrosoglutathione (GNSO) as a method of introducing NO to platelets, although it is reported that NO released from GNSO is quickly scavenged in whole blood by haemoglobin present within erythrocytes (Broniowska et al., 2013). As a result, inhibitory platelet signalling was activated using the cGMP analogue 8-cPT-cGMP. For experiments investigating the effects of oxLDL, platelets were pre-treated with oxLDL to cause hyperactivity and then followed by stimulation with 8-cPT-cGMP to determine whether the inhibitory signalling response was altered.

Under basal conditions, platelets were able to bind to collagen robustly (Basal – 9.52%±3.5) (Fig. 4.9). As expected, the presence of the cGMP analogue caused a significant decrease in platelet adhesion (cGMP – 0.9%±0.37) ($p=<0.001$). Pre-incubation

of the blood with oxLDL however, was able to reduce the inhibitory effects of cGMP and this is reflected in higher levels of platelet adhesion (oxLDL + cGMP – 5.63%±1.51) *(p=0.02)*. The vehicle control, 0.001% ethanol had no effect on platelet adhesion (data not shown).

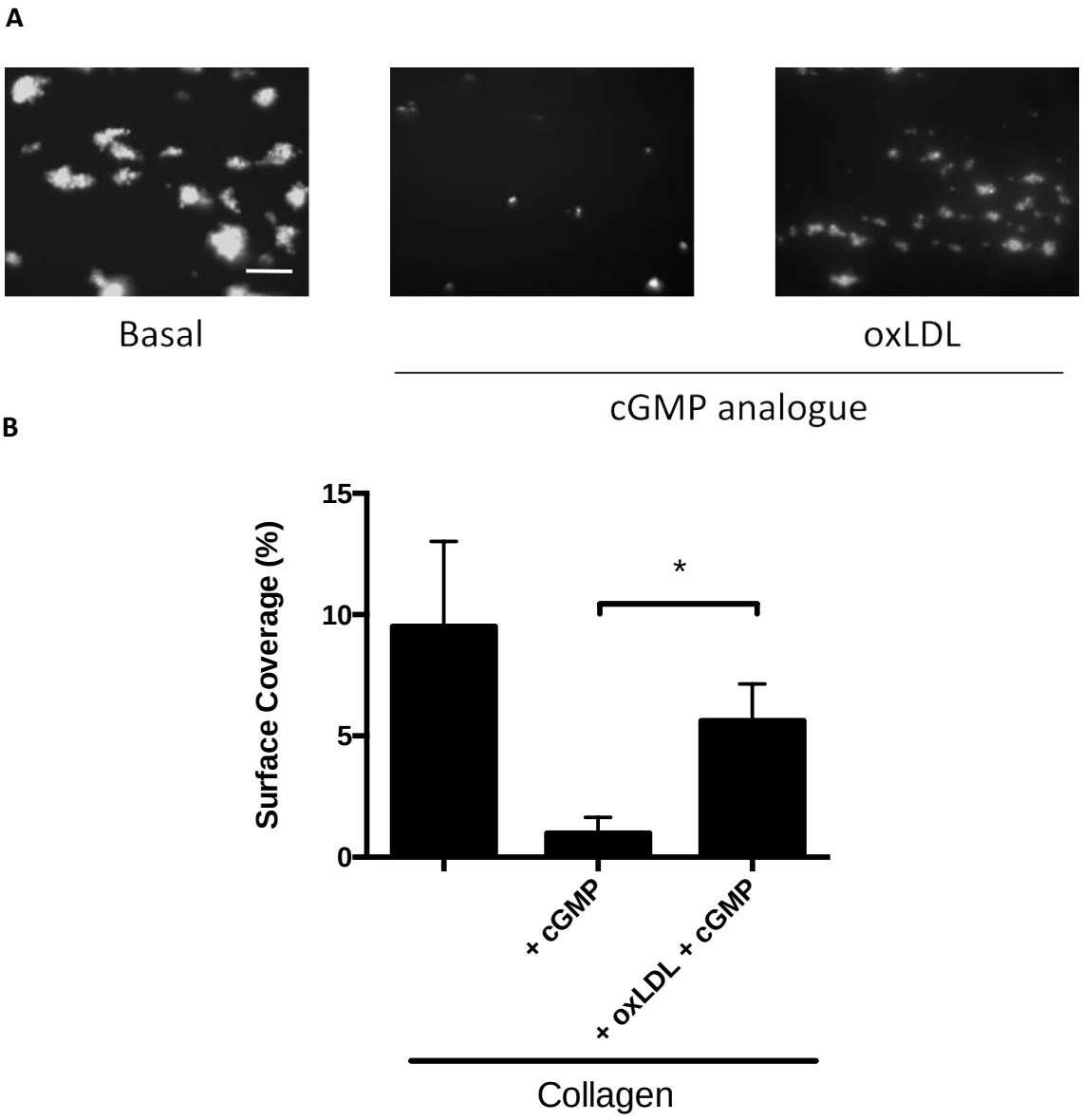


Figure 4.9 – Platelet incubation with oxLDL reduces the inhibitory effects of the cGMP analogue 8-pCPT-cGMP. Capillary slides were coated in collagen (50µg/mL) overnight and blocked the following day with 10% BSA for 1 hour. Blood was treated with oxLDL (50µg/mL) for 15 minutes and the cGMP analogue 8-cPT-cGMP (50µM) for 5 minutes prior to flowing. Due to autofluorescence of the cGMP molecule, exposure times were modified to compensate for this and allow optimum visualisation. Blood was allowed to flow for 2 minutes and the capillary tube washed with PBS to remove unbound cells. Images were taken using an inverted fluorescence microscope and x60 objective (Olympus). Data shown is the mean of three separate experiments. * = $p < 0.05$ when transformed and analysed using ANOVA.

4.7.2 OxLDL reverses platelet inhibition by prostacyclin in human platelets

Having shown that oxLDL is able to modulate the effects of the cGMP analogue, we wanted to see whether oxLDL is able to induce changes in the platelet response to PGI_2. We hypothesised that since NO and PGI_2 work by elevating levels of cyclic nucleotides within the cell, there may be some degree of similarity between the responses. Furthermore, some limited evidence exists to show that oxLDL can directly modulate cAMP levels (Korporaal et al., 2005), although not in a functional assay such as this.

Basal platelet adhesion to collagen was consistent with previous findings (Basal – 12.49±0.85) (Fig. 4.10). In our model, a low concentration of PGI_2 (10-20nM) caused a significant decrease in platelet adhesion (PGI_2 – 2.72±1.86) *(p=<0.01)*. However, in the presence of oxLDL, the effects of PGI_2 was diminished, leading to comparable levels of platelet adhesion to basal (oxLDL + PGI_2 – 10.76±3.17) *(p=0.047)*. These results indicate that oxLDL is able to affect the inhibitory processes induced by cGMP analogue and PGI_2.

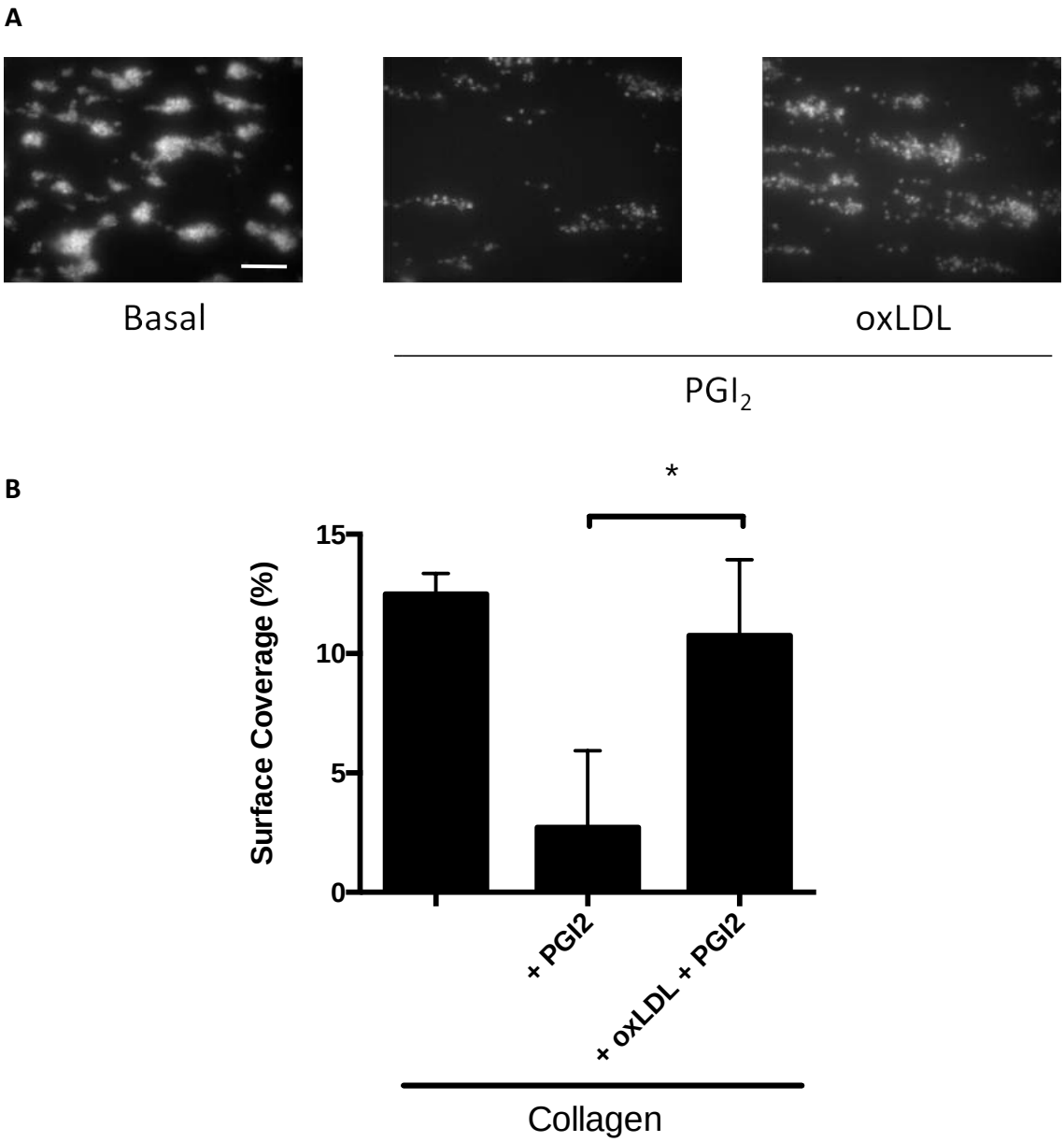


Figure 4.10 – Platelet incubation with oxLDL reduces the inhibitory effects of Prostacyclin. Microfluidic channels were coated in collagen (50µg/mL) overnight and blocked the following day with 10% BSA for a minimum of 15 minutes. Whole blood was stained with $DiOC_6$ (0.5µM) and in experiments requiring additional agonists, were treated with PGI_2 (10-20 nM) for 2 minutes and oxLDL (50 µg/mL) for 15 minutes. Treated blood was flowed for 2 minutes at $1000s^{-1}$ and images were taken using an inverted fluorescence microscope with x60 objective (Olympus). Scale bar represents 200 microns. Data shown is the mean of three separate experiments. * = $p < 0.05$ when transformed and analysed using ANOVA.

4.8 Chapter Discussion

The blood vessel is a highly complex, dynamic environment and this is exacerbated in atherosclerosis. Sheer rates can drastically affect the blood vessel integrity, first described by Virchow in the 1800s, and platelet function (Kroll et al., 1996; Cunningham and Gotlieb, 2005), becoming an important, albeit often overlooked, variable for many studies. The presence of oxLDL *in vivo* is becoming increasingly accepted, with evidence summarised well by Itabe et al. (Itabe et al., 2011). However, the location in which it exerts effects on platelets remains poorly understood. It is possible that at sites of plaque erosion, blood might be exposed to surfaces containing immobilised oxLDL, which is bound to proteoglycans present within the vessel wall (Williams and Tabas, 1995; Sneck et al., 2005). An alternative hypothesis is that oxLDL may alter platelet function in the circulation (Podrez et al., 2007), hence predisposing the blood to greater likelihood of activation. The sets of experiments presented throughout this body of work aimed to test both of these hypotheses by introducing oxLDL to platelets as an immobilised surface and in suspension. Whilst additional experimentation is required in order to deduce how platelets and oxLDL interact in vivo to cause platelet hyperactivity, data presented in this work suggests that it is circulating oxLDL which is able to modulate platelet function under shear.

The interaction between platelets and oxLDL under shear is very poorly understood. A notable study by the Heemskirk group showed that oxLDL, when coated alongside collagen, is able to exert an additive effect compared to collagen alone, potentiating platelet adhesion (Nergiz-Unal, Lamers, et al., 2011). The importance of this finding was supported by work by Stellos et al. who showed that platelets from patients with ACS not only had oxLDL bound to the surface, but this also enhanced adhesion to immobilised collagen and activated endothelial cells (Stellos et al., 2012). Studies looking at adhesion to fibrinogen are controversial, with studies showing both increased adhesion with oxLDL (Korporaal et al., 2007) and decreased (Korporaal et al., 2005), although this is further complicated by differential shear rates and very high concentrations of oxLDL. However,

to the best of our knowledge, the ability of oxLDL to induce direct platelet adhesion was uncharacterised. The aims of this section of work was to investigate the what effects oxLDL might have on platelet function under physiological shear, using a microfluidic system to allow coating of proteins of interest and the flow of blood at specified sheer rates. We hypothesised that oxLDL pre-treatment of platelets should directly result in increased platelet adhesion to matrix proteins and that immobilised oxLDL alone may be able to support limited adhesion at low shear rates.

OxLDL and human platelets

In order to recreate an environment in which blood could be flowed over immobilised oxLDL, oxLDL was adhered directly to glass capillary tubes. Since immobilisation to glass may change the protein residues present on the oxLDL particle due to the electrostatic charge (Baugh and Vogel, 2004), staining was used to confirm the oxLDL could adhere. Minimal adhesion of platelets to oxLDL was observed under either flow rate, despite the fact that oxLDL was remaining fixed to the glass (Fig. 4.1) and its ability to cause robust adhesion under static conditions. We can speculate that CD36 ligation to oxLDL alone on the platelet surface is not sufficient to tether the platelet to sites of plaque rupture, likely requiring other agonists to be present to initiate thrombus formation. Biophysical approaches show that CD36 has weak affinity for non-oxidised lipids (10-150μM) when measured via surface plasmon resonance, with even weaker affinities for oxidised lipids (Jay et al., 2015). An alternative hypothesis is that despite being adhered, immobilisation prevents exposure of the lipoprotein epitopes required for platelet receptor binding, as is the case for fibronectin (Baugh and Vogel, 2004) and fibrinogen (Sit and Marchant, 1999). Since oxLDL binds to proteoglycans in the vessel wall, an alternative strategy for future work would be to coat slides with proteoglycans and allow oxLDL to bind, potentially exposing the residues of oxLDL involved in CD36 binding *in vivo*.

Having shown that oxLDL alone cannot support platelet adhesion under shear stress when immobilised, we then looked at whether oxLDL pre incubation would lead to a direct increase in platelet adhesion when flowed over collagen and fibrinogen. This was

an important experiment to conduct since it oxLDL has been shown to ligate to platelets in disease states (Stellos et al., 2012), enhancing adhesion. In these experiments, oxLDL did not induce any additional adhesion to collagen and it would be tempting to speculate that oxLDL does not impact on adhesion to collagen and the related signalling. However, strict test conditions were used - collagen was used at a fixed concentration of 50μg/mL and this alone was able to induce strong adhesion, potentially masking subtler additive effects which might have been observed at lower concentrations. Similarly, a fixed incubation time (15 minutes) of oxLDL incubation was used throughout this work and different results may have been observed with longer and shorter durations. Further experimentation to fully characterise the effects of oxLDL exposure time and matrix concentrations would be of benefit and may yield interesting and important information. Other studies have used solubilised atherosclerotic plaque material to coat slides with (Reininger et al., 2010) and it may be interesting to stain these slides with Nile Red to see whether oxLDL in a complex matrix is affecting platelet adhesion.

In experiments where oxLDL treated blood was flowed over fibrinogen, a trend of decreasing adhesion with oxLDL dose was observed, contrasting with the previously published data by Korporaal et al. One possible explanation for this is the ability of the fibrinogen receptor integrin $\alpha_{IIb}\beta_3$ to also bind oxLDL (Koller et al., 1989; Korporaal et al., 2005) and therefore this may serve as a competitive ligand for the receptor. The ability of oxLDL to bind the integrin is also consistent with static adhesion results, in which the anti-integrin drug Tirofiban reduced adhesion (Fig. 3.13). These experiments demonstrate the activating potential of oxLDL on platelets under arterial shear conditions and shed light on a possible mechanism of increased thrombosis risk *in vivo*.

KOdiA-PC and murine platelets

For studies using murine blood, the oxidised phospholipid KOdiA-PC was used in place of human oxLDL since murine platelets displayed diminished aggregation and adhesion responses in earlier experiments (Fig. 3.6). KOdiA-PC is an oxidised phospholipid present with oxLDL (Podrez et al., 2007) and is reported to be CD36 specific (E.A. Podrez, 2002;

Gao et al., 2010). Furthermore, oxidised phospholipids, including KOdiA-PC are present in atherosclerotic plaques at high concentrations and are found in the plasma of patients with hyperlipidaemia where it correlates with the degree of platelet activation to traditional agonists (Podrez et al., 2007). KOdiA-PC serves as a valuable tool to dissect CD36 signalling in murine platelets and exploit platelets deficient in key receptors and signalling proteins.

Since phospholipids are unable to be directly immobilised to electrostatic surfaces due to charge, we immediately looked at the effects of the KOdiA-PC on platelet adhesion to collagen and fibrinogen. This would enable us to further compare the effects of KOdiA-PC and oxLDL ligands and ascertain the degree of similarity between the two. This is valuable information since KOdiA-PC is reported to have a high degree of specificity to CD36 (E. a. Podrez, 2002), whilst oxLDL is multivalent and has been shown to bind to a number of receptors, including CD36, LOX-1, SR-A and PAF receptors (Levitan et al., 2010). In contrast to oxLDL, KOdiA-PC caused a modest increase in platelet binding to collagen at the lower dose, but caused a significant decrease in adhesion at the higher dose. One possible explanation for this is that CD36 has also been previously reported as a collagen receptor (Tandon et al., 1991; Diaz-Ricart et al., 1996), although this would not explain why lower doses cause some potentiation of adhesion. An alternative hypothesis is that higher doses of KOdiA-PC prevent platelet binding to collagen, either via direct inhibition, receptor blocking or receptor shedding. The effects of oxidised phospholipids on collagen receptors and signalling is unknown.

OxLDL was shown to correlate negatively with platelet adhesion and we would expect that KOdiA-PC would also have a similar effect if it was due to competitive binding of the integrin. However, KOdiA-PC did not affect platelet adhesion to fibrinogen. Since both ligands are reported to bind CD36, although only oxLDL had an effect, it could be that it is another feature of oxLDL, such as either particle size or alternative binding epitope which causes binding to the integrin. One possible method to test this hypothesis would be to

integrate KOdiA-PC into oxLDL-sized liposomes to see whether size alone is hindering binding.

Due to the small, charged nature of KOdiA-PC, we wanted to determine whether claims of CD36 specificity were correct. Using CD36 deficient mice, we showed that regardless of concentration, KOdiA-PC exerted no observable effect. This further supports the argument that KOdiA-PC exerts its effects via CD36 and that CD36 is required for the binding of oxidised phospholipids, and by extension oxLDL to platelets.

OxLDL & Platelet Inhibition

Having established that oxLDL is able to bind to platelets, but without triggering any additional platelet recruitment, we turned our attention to whether oxLDL was instead modulating natural inhibitory pathways. *In vivo*, platelets are continually surrounded by the endogenous endothelial inhibitors PGI_2 and NO and therefore it would stand to reason that by blocking the effects of these key homeostatic compounds, the risk of thrombosis would be elevated. OxLDL has been shown previously to modulate the production of both NO (Blair et al., 1999; Davignon, 2004) and PGI_2 (Thorin et al., 1994; Mahfouz and Kummerow, 2001), affecting platelet reactivity indirectly. In a study by Korporaal et al., they showed that oxLDL was able to directly influence intracellular cAMP levels (Korporaal et al., 2005) although the ramifications of this were not investigated in much detail. Whilst the case for an altered platelet response to NO is less clear, several diseases believed to involve oxLDL including obesity, PAD and ACS all feature platelet hyporesponsiveness to NO as a symptom (Russo et al., 2010; Riba et al., 2004; Willoughby et al., 2005).

In our experiments, flow experiments in the presence of either PGI_2 or NO caused a clear reduction in adhesion, which was negated to varying degrees by pre-incubation with oxLDL. This indicates that oxLDL ligation to platelets is directly able to trigger decreased sensitivity to endogenous inhibitors and this may in turn, play a part in hyperlipidaemia

induced platelet hyperactivity. However, the mechanisms underpinning this remains unclear and warrants further investigation.

The observation that oxLDL is able to partially negate the effects of cGMP forms part of a larger body of work. In this study, oxLDL is shown to enhance production of ROS which had an inhibitory effect on PKG (Magwenzi et al., 2015). This inhibitory effect was also shown in aggregation using wild-type mice, but lost in mice deficient in the ROS generating protein ($NOX2^{-/-}$). During this work, an attempt to deduce the physiological relevance was made by investigating the effect of the cGMP analogue in mice fed a high fat diet. Platelets from hyperlipidemic mice responded in a similar fashion to human platelets treated with oxLDL, displaying poor sensitivity to cGMP (Magwenzi et al. 2015 - Appendix I). It would be of interest to take these findings and replicate the study in patients with hyperlipidemia to determine whether this is a possible mechanism of platelet hyperactivity in disease. Moreover, it would be interesting to determine whether oxLDL acts via a similar mechanism in the context of PGI_2/PKA signalling. To date, limited mechanistic data is known regarding how oxLDL is able to impact PGI_2 signalling. Whilst ROS downstream of oxLDL may play a role in inhibiting the PKA protein, an alternative hypothesis is that oxLDL ligation to the platelet surface may simply block the IP receptor, preventing signal transduction. Like cGMP, it would be interesting to see whether PGI_2 signalling is diminished in hyperlipidaemic patients but in addition, investigate whether oxLDL ligation impacts PKG or PKA signalling preferentially.

Summary

To conclude, the data presented in this chapter has broadly described the effects of oxLDL in an experimental setup more analogous to the human blood vessel with the effects of sheer. The data presented supports the notion that platelet adhesion and subsequent atherothrombosis, is unlikely to require immobilised oxLDL in the vessel and rather, it is circulating oxLDL binding to platelets which leads to hyperactivity due to modulation of cyclic nucleotide signalling. The importance of CD36 in the pathophysiology of platelet hyperactivity has been further strengthened by data shown within this body of work.

Subsequent work will investigate this further, looking at the molecular signalling pathways activated by oxLDL in an attempt to discover how this process works.

Chapter 5: The role of tyrosine kinases and phospholipase C γ2 in platelet activation and shape change in response to oxidised LDL

5.1 Introduction

The activation of platelets requires multiple changes to intracellular signalling networks. Ligation of agonists to platelet receptors initiates these signalling changes and often results in the phosphorylation and activation of a phospholipase C isoform (Blake et al., 1994; Lee et al., 1996; Ozdener et al., 2002). Known PLC activators in platelets include thrombin (Offermanns et al., 1994), collagen (Blake et al., 1994), TxA_2 (Hirata et al., 1994) and fibrinogen (Wonerow et al., 2003), whilst other agonists such as epinephrine do not (Yang et al., 2002). The PLC isoforms activated downstream of many platelet receptors are characterised, with ITAM dependent signalling requiring PLCγ2 and GPCRs coupled to Gαq requiring PLCβ (Li et al., 2010; Clemetson, 2012). However, the ability of oxLDL to cause PLC activation and the corresponding downstream effects remain poorly understood.

The human genome encodes 13 different PLC variants, which can be grouped into six isotypes: PLCβ, PLCγ, PLCδ, PLCε, PLCζ and PLCη (Cocco et al., 2015). PLC is ubiquitously expressed in a range of tissues and serves to catalyse the reaction of PIP_2 to DAG and IP_3 (Rhee et al., 1989) to elicit a range of cellular effects (Koss et al., 2014; Cocco et al., 2015). However, only PLCβ, PLCγ and PLCδ have been detected in human platelets to date (Lee et al., 1996). Whilst all isotypes perform the same function of Ca^{2+} mobilisation and PKC activation, isotypes are differentially regulated in a spatial-temporal manner and expressed in varying levels. PLCγ2 is the most abundant PLC isoform in platelets, although enforced expression of PLCγ1 can restore functionality in a PLCγ2 deficient model (Zheng, Adams, Zhi, Yu, Wen, Peter J Newman, et al., 2015). Canonically, PLC expression in platelets is as follows (in decending order): PLCγ2 > PLCβ2 > PLCβ3 > PLCβI > PLCγl > PLCδ1 > PLCβ4 as demonstrated by high performance liquid chromatography and semi-quantitative western blotting (Lee et al., 1996).

Limited evidence exists to show that oxLDL ligation can cause phosphorylation and activation of PLC. OxLDL has been shown to cause increased tyrosine phosphorylation and intracellular Ca^{2+} mobilisation (Korporaal et al., 2005; Wraith et al., 2013), with inhibition of CD36 via use of inhibitors and $CD36^{-/-}$ mice shown to prevent this from occurring (Wraith et al., 2013). Furthermore, oxLDL has also been shown to result in increased PKC substrate phosphorylation – canonically considered to be directly downstream of PLC in platelets. This evidence supports the notion of PLC activation (Magwenzi et al., 2015). However, due to the lack of specific pharmaceutical inhibitors, it remains difficult to tie the effects of oxLDL to a specific isotype. The inhibitor U73122 has been typically used as an inhibitor for PLC isoforms in multiple cell types. However, several studies have shown that U73122 also has many other targets in platelets, including PKC and Ca^{2+} channels, which might confuse findings (Pulcinelli et al., 1998; Lockhart and McNicol, 1999). Immunoprecipitation and proteomics studies have also shown a potential role for PLCγ2 in oxLDL mediated platelet activation (Wraith et al., 2013; Zimman et al., 2014), although *in vitro* data supporting the link between phosphorylation and activation of the enzyme remains lacking.

5.2 Chapter aims

The aim of this chapter was to investigate the role of PLCγ2 activation in response to oxLDL. Specifically, we wanted to:

- Determine whether PLCγ2 is phosphorylated in response to oxLDL
- Determine whether PLCγ2 activation is required for platelet activation and shape change in response to oxLDL
- Determine the significance of proteins typically believed to be upstream in the signalling pathway
- Investigate the effects of oxidised phospholipids on a PLCγ2 deficient mouse model

5.3 OxLDL causes phosphorylation of PLCγ2 in human platelets

5.3.1 OxLDL causes phosphorylation of PLCγ2 in a time-dependent manner

In order to determine whether PLCγ2 is activated by oxLDL, we began by attempting to confirm previous reports that it undergoes tyrosine phosphorylation upon stimulation (Wraith et al., 2013). We hypothesised that since phosphorylation is commonly associated with altered enzymatic activity, PLCγ2 phosphorylation would indicate the protein is active and therefore could be responsible for platelet hyperactivity.

PLCγ2 was immunoprecipitated from stimulated platelets and tyrosine phosphorylation detected using the 4G10 antibody. Minimal phosphorylation of PLCγ2 was detected under basal conditions (Fig. 5.1). Phosphorylation increased over the 60 seconds tested, in accordance with the published literature (Wraith et al., 2013). CRP-XL, a known activator of PLCγ2 (Asselin et al., 1999; Polanowska-Grabowska et al., 2003) was used as a positive control. CRP caused strong phosphorylation in comparison to oxLDL, although the concentration used was high (1μg/mL).

5.3.2 OxLDL causes phosphorylation of PLCγ2 in a dose-dependent manner

Having established that PLCγ2 phosphorylation occurs in a time-dependent manner, we next wanted to investigate the effect that oxLDL concentration might play. Consistent with the previous figure, minimal phosphorylation was observed under basal conditions, and did not rise upon stimulation with the lowest oxLDL concentration (10μg/mL) (Fig. 5.2). However, there was an increase in PLCγ2 phosphorylation with both physiological concentrations (50-100 μg/mL), with levels of phosphorylation approximately equal between the conditions. CRP was used as a positive control to aid in identification of the phosphorylated PLCγ2 band and consistently gave robust phosphorylation. As a result of these experiments, all further signalling experiments investigating PLCγ2 phosphorylation stimulated platelets with the optimised conditions of 50μg/mL oxLDL for 15-60 seconds.

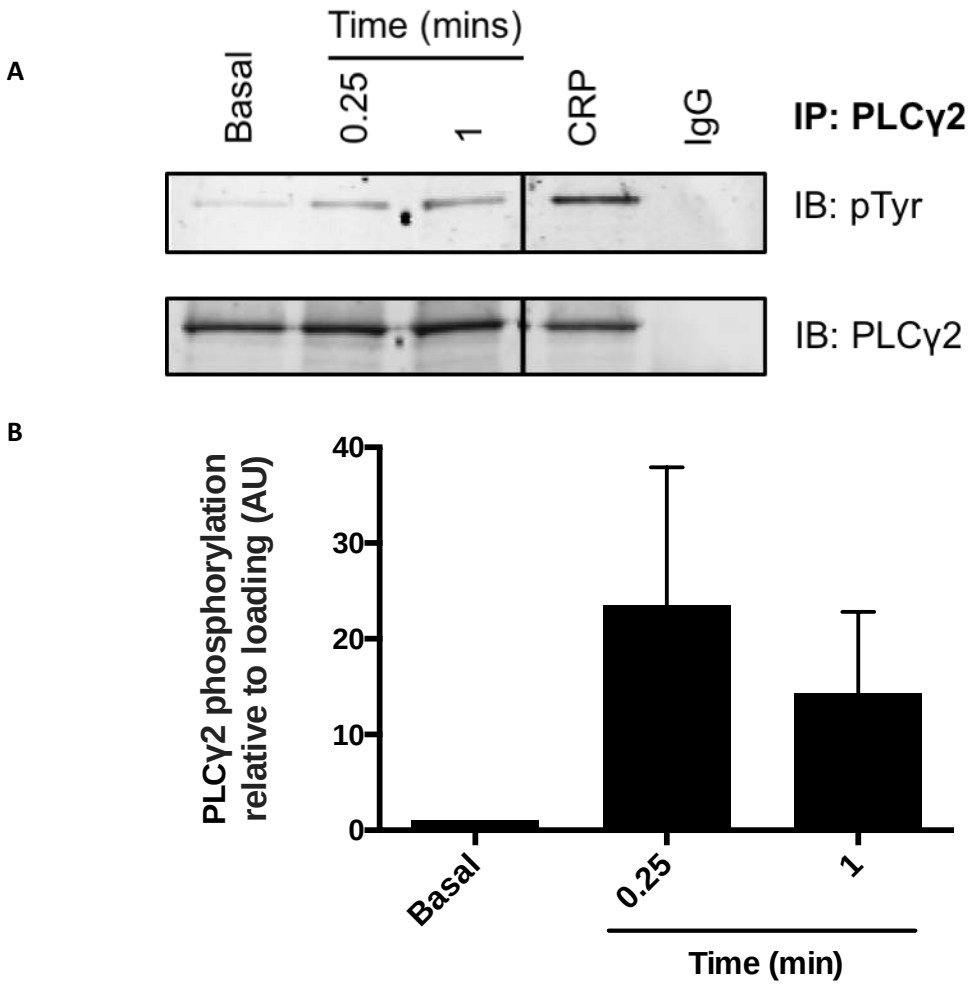


Figure 5.1 - OxLDL causes an increase in PLCγ2 phosphorylation in a time-dependent manner. Platelets ($7x10^8$/mL) were treated with CRP (1µg/mL; 1 min) or oxLDL (50µg/mL) for varying time points and lysed in IP lysis buffer. PLCγ2 was immunoprecipitated using specific antibodies and separated using SDS-PAGE. PLCγ2 tyrosine phosphorylation was measured using the antibody 4G10 and equal loading measured by use of an anti-PLCγ2 antibody. A – representative blots showing PLCγ2 tyrosine phosphorylation. Black line represents divide between two parts of the same membrane. B – mean changes in phosphorylation measured by densitometry, relative to loading controls. Data shown is an average of 4 separate experiments.

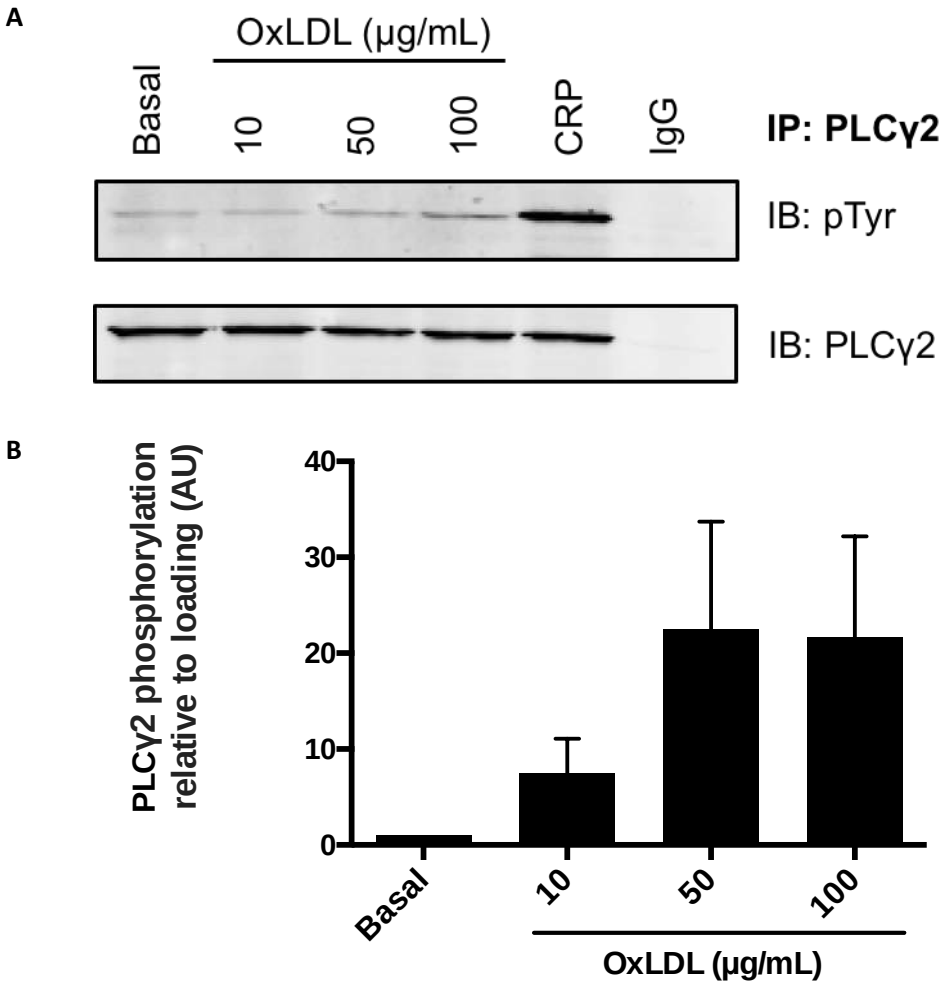


Figure 5.2 - OxLDL causes an increase in PLCγ2 phosphorylation in a dose-dependent manner. Platelets ($7x10^8$/mL) were treated with CRP (1µg/mL; 1 min) or oxLDL (10-100µg/mL) for 1 minute and lysed in IP lysis buffer. PLCγ2 was immunoprecipitated using specific antibodies and separated using SDS-PAGE. PLCγ2 tyrosine phosphorylation was measured using the antibody 4G10 and equal loading measured by use of an anti- PLCγ2 antibody. A – representative blots showing PLCγ2 tyrosine phosphorylation. B – mean changes in phosphorylation measured by densitometry, relative to loading controls. Data shown is an average of 3 separate experiments.

5.3.3 PLCγ2 phosphorylation sites involved in oxLDL and KOdiA-PC signalling

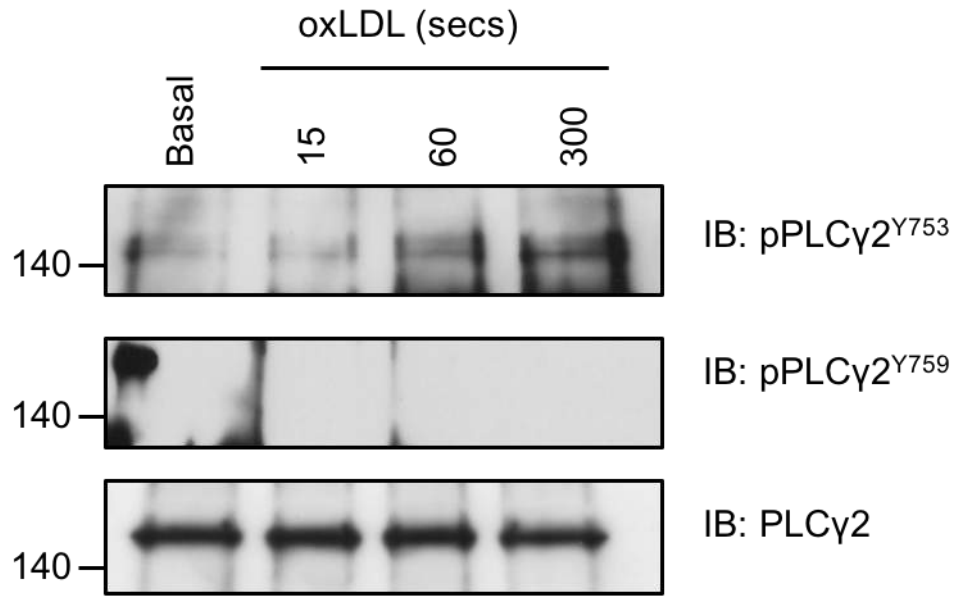
Several studies have shown that PLCγ2 can become phosphorylated downstream of multiple agonists (Blake et al., 1994; Mangin et al., 2003) and have linked PLCγ2 phosphorylation with the degree of platelet activation (Suzuki-Inoue et al., 2004). The protein PLCγ2 possesses four separate tyrosine residues which are known to be phosphorylated: Y^{743}, Y^{753}, Y^{759} and Y^{1217} (Ozdener et al., 2002; Zimman et al., 2014). However, much of the previous research has focused around Y^{753} and Y^{759} due to their roles in platelet activation downstream of classical agonists.

The GPVI agonist CRP is known to elicit strong signalling and resulting in full platelet activation, whereas vWF signalling via GPIb-V-IX exerts a weaker signal. Interestingly, these agonists were shown to cause differential phosphorylation patterns of PLCγ2. CRP has been shown to cause robust phosphorylation of Y^{753} and Y^{759}, resulting in full platelet activation (Suzuki-Inoue et al., 2004). Conversely, the same study showed that platelets treated with vWF and ristocetin displayed only weak phosphorylation of Y^{753} (Suzuki-Inoue et al., 2004). Currently, the tyrosine residues of PLCγ2 phosphorylated by oxLDL and oxidised phospholipids remain poorly understood. A mass spectroscopy study by Zimman et al. showed that the oxidised phospholipid KODA-PC is able to cause phosphorylation of PLCγ2 at Y^{759} and the novel residue Y^{1217} in a Syk-dependent manner (Zimman et al., 2014). However, the study did not explore the role of Y^{753}.

In these experiments, we attempted to determine which site(s) of PLCγ2 are phosphorylated by oxLDL. OxLDL (50μg/mL) caused a time-dependent increase in PLCγ2 phosphorylation as measured using an antibody recognising protein phosphorylation at the Y^{753} site (Fig. 5.3A). Interestingly, no detectable phosphorylation was observed using the Y^{759} antibody, a similar pattern to that previously observed to platelet stimulation with vWF and ristocetin. In murine platelets, low basal phosphorylation of PLCγ2 was observed, similar to human platelets (Fig. 5.3C). The control lipid PAPC caused a minor increase in PLCγ2 phosphorylation, whilst the lower concentration of KOdiA-PC (5μM) caused an increase. Conversely, the higher concentration of KOdiA-PC (25μM) failed to

cause an increase in phosphorylation over PAPC. CRP (1μg/mL) elicited the strongest phosphorylation response of PLCγ2, although the signal was much weaker than the same concentration used in human platelets (data not shown).

A



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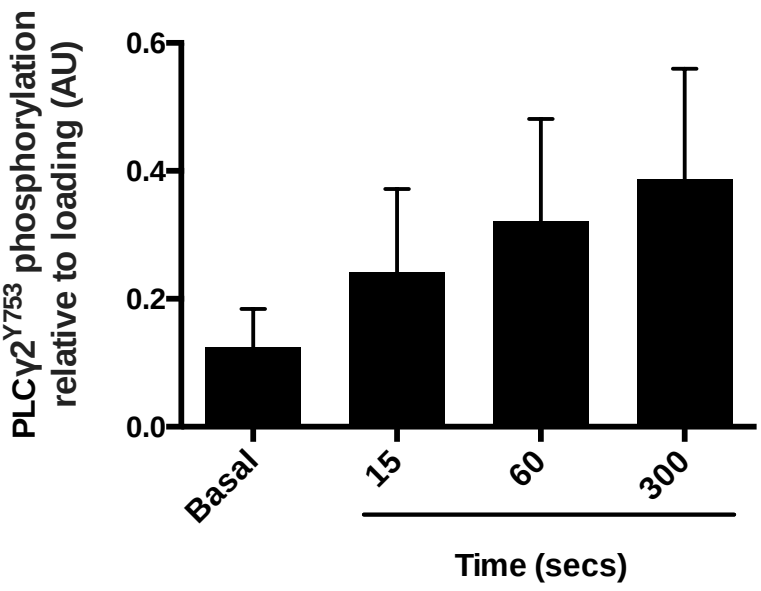


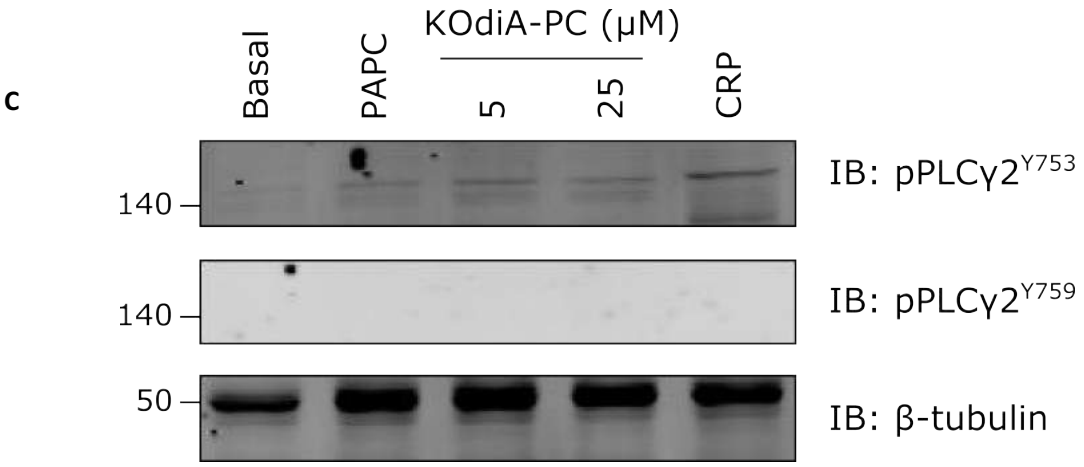


Figure 5.3 – OxLDL and KOdiA-PC causes the phosphorylation of specific tyrosine residues on PLCγ2. Platelets (human or murine) were treated with oxLDL or oxidised phospholipids and lysed in Laemmlii buffer. Lysates were separated via SDS-PAGE and PLCγ2 phosphorylation determined using antibodies raised to specific phosphorylation sites. A – Representative blot of human platelet stimulation with oxLDL showing phosphorylation of tyrosine sites Y^{753} but not Y^{759}. B - mean changes in phosphorylation measured by densitometry, relative to loading controls. C – Murine platelet stimulation with KOdiA-PC causes phosphorylation of tyrosine sites Y^{753} but not Y^{759}. Human data shown is a representative blot of 3 separate experiments whilst murine blots are from a single attempt only.

5.3.4 Inhibition of PLC prevents platelet activation in response to oxLDL

Having shown that PLCγ2 is phosphorylated by oxLDL, albeit modestly, we wanted to examine the effects of blocking the enzyme using a pharmaceutical inhibitor. U73122 has been previously used in experiments investigating collagen and vWF signalling pathways to determine the relative importance of PLC (Mangin et al., 2003; Heemskerk et al., 1997). We hypothesised that inhibition of PLC by U73122 should prevent platelet activation triggered by oxLDL; using the marker CD62P as a measure of α-granule release.

Low levels of platelet activation were detected at basal conditions (Basal – 11.8%±1.4) and this rose significantly with treatment of oxLDL, independent of dose (50μg/mL – 20.4%±2.7; 200μg/mL – 20.2%±2.8; p=0.049) (Fig. 5.4). However, in samples where PLC was inhibited, levels of CD62P did not rise upon stimulation and were decreased compared to basal (50μg/mL + U7 – 6.13%±1.38; 200μg/mL + U7 – 6.07%±1.66; p=<0.01).

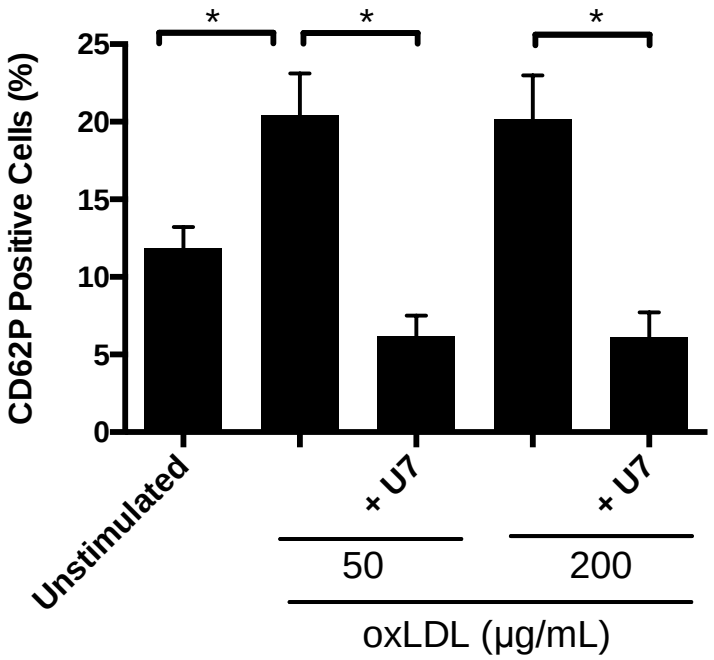
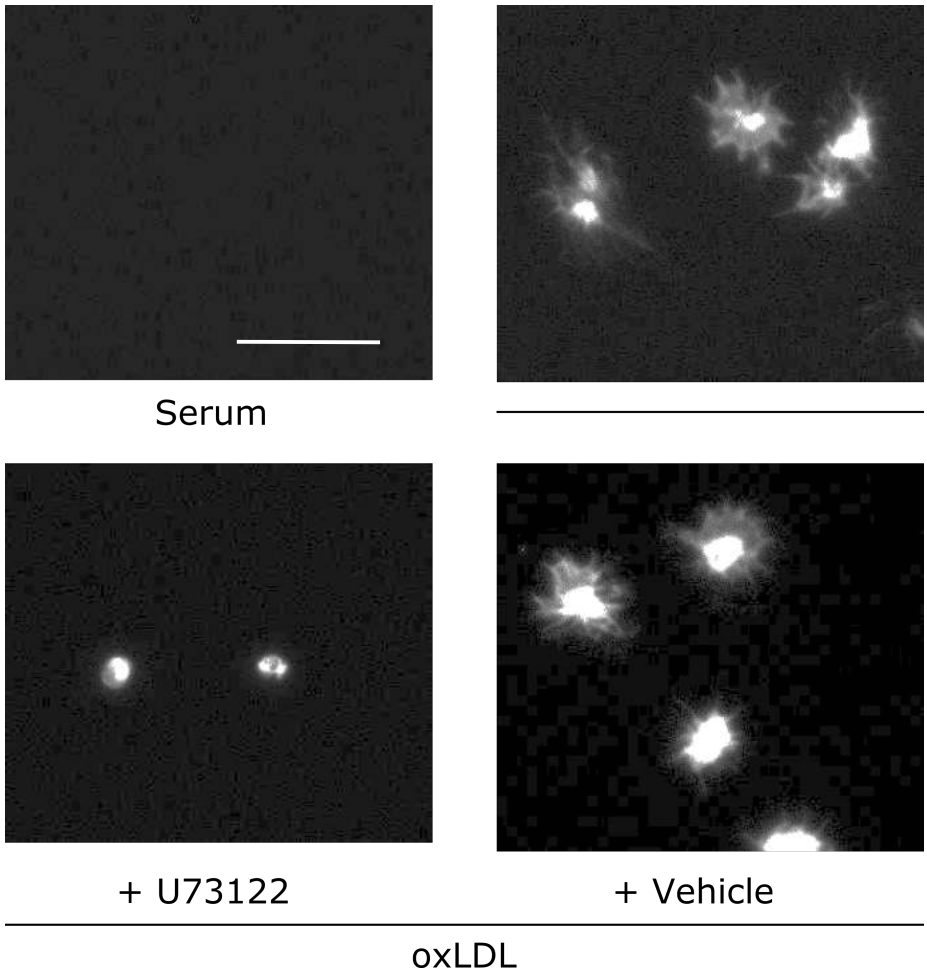


Figure 5.4 – U73122 reduces CD62P expression in oxLDL treated PRP samples. PRP was either untreated or treated with the pan-PLC inhibitor U73122 (50μM) for 20 mins. PRP (5μL) was incubated with an anti-CD62P antibody and treated with oxLDL (50-200μM) for 20 mins followed by fixation with formalsaline. Samples were run using a BD Fortessa flow cytometer and data analysed using FACSDiva software. Data shown is an average of 3 separate experiments. *=*p <0.05* when arcsin transformed and analysed using ANOVA.

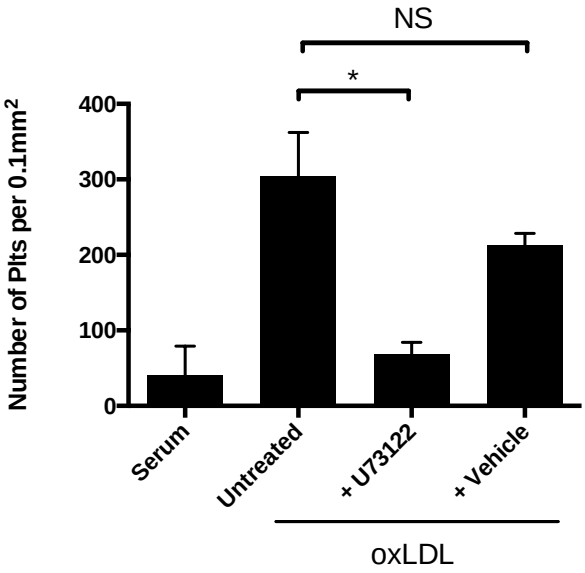
5.3.5 Inhibition of PLC prevents oxLDL mediated platelet adhesion and spreading

Having shown that inhibition of PLC prevents α-granule release, we wanted to determine whether platelet adhesion and spreading would be affected. To negate the effects of secondary signalling mediators which might activate other isoforms of PLC, platelets were pre-treated with apyrase, indomethacin, EGTA and tirofiban and as a consequence, all adhesion and spreading responses would be solely attributed to signalling initiated by oxLDL ligation. Minimal platelet adhesion was observed to glass slides coated with human serum (Serum – 40±39.) (Fig. 5.5). Despite the presence of apyrase, indomethacin, EGTA and tirofiban, platelets were still able to adhere to oxLDL (OxLDL - 304±58). Interestingly, subsequent treatment with the PLC inhibitor U73122 caused a significant reduction in platelet adhesion compared to basal (+ U73122 - 68±16.2; p=<0.01), whereas only a marginal change was seen with the vehicle control samples (+ Vehicle - 212.7±15.72). Moreover, U73122 treated platelets also displayed a significantly lower mean surface area (+ U73122 - 6.9μm^2±0.93) compared to both basal (Basal - 15.48μm^2±2.23; p=0.015) and vehicle treated platelets (+ Vehicle - 13.85μm^2±0.87). This data suggests that either PLC plays a role in platelet adhesion and spreading or U73122 is able to exhibit off-targets effects.

A


Serum
+ U73122
+ Vehicle
oxLDL

B


NS
*
Number of Plts per 0.1mm²
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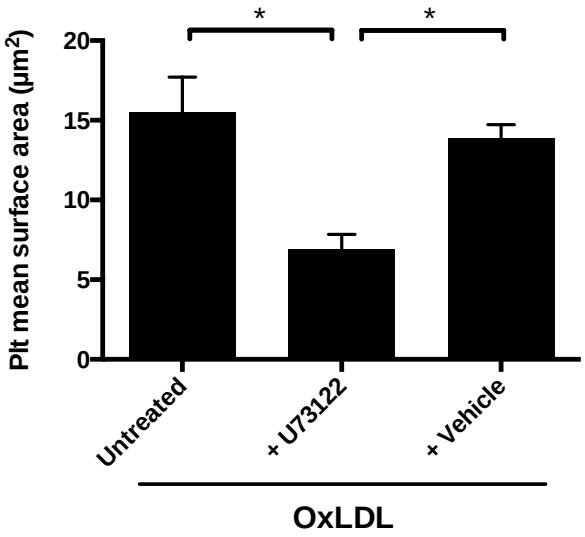


Figure 5.5 – U73122 reduces platelet adhesion and spreading to oxLDL. Platelets ($5x10^7$/mL) were pretreated with apyrase, indomethacin, EGTA and tirofiban for 5 minutes then treated with U73122 (5µM) or vehicle (DMSO) for 20 minutes prior to incubation on oxLDL coated slides. Adhered platelets were fixed, stained using TRITC-phalloidin and imaged using an inverted fluorescence microscope (Zeiss). A – Representative images taken using an inverted fluorescence microscope. B – Platelets were counted and the average number of platelets adhered per treatment condition was calculated. C – Average surface area of adhered platelets per treatment condition. Data shown is an average of three separate experiments. Scale bar represents a distance of 10 microns.

5.4 OxLDL induces signalling via a CD36 – Src – Syk pathway

So far, we have shown that oxLDL is able to cause the phosphorylation of PLCγ2 in accordance with the published literature. Moreover, inhibition of PLC using U73122 prevents multiple aspects of platelet activation, indicating that PLCγ2 is involved in the platelet activation process. Next, we wanted to investigate the signalling mechanism underpinning the phosphorylation of PLCγ2 by looking at the phosphorylation and activation of key proteins typically considered to be upstream.

5.4.1 Inhibition of CD36 prevents PLCγ2 phosphorylation by oxLDL

Previously we have shown that inhibition of CD36 prevents adhesion of platelets in response to oxLDL (Fig. 3.17) and that genetic deletion of CD36 prevents oxidised phospholipid induced platelet hyperactivity under flow (Fig. 4.8). However, since platelets are believed to possess multiple receptors able to bind these ligands, we wanted to determine whether platelet activation via CD36 was dependent on the activation of PLCγ2.

Using the PLCγ2^{Y753} specific antibody, immunoprecipitated PLCγ2 phosphorylation was assessed in the presence and absence of the anti-CD36 blocking antibody FA6.152. Minimal phosphorylation was detected under basal conditions and rose when platelets were stimulated (Fig. 5.6). In the presence of FA6.152, phosphorylation of PLCγ2 was comparable to basal levels of phosphorylation, showing a clear decrease in phosphorylation from oxLDL-treated samples. No reduction in phosphorylation was observed from platelets treated with the isotype control antibody.

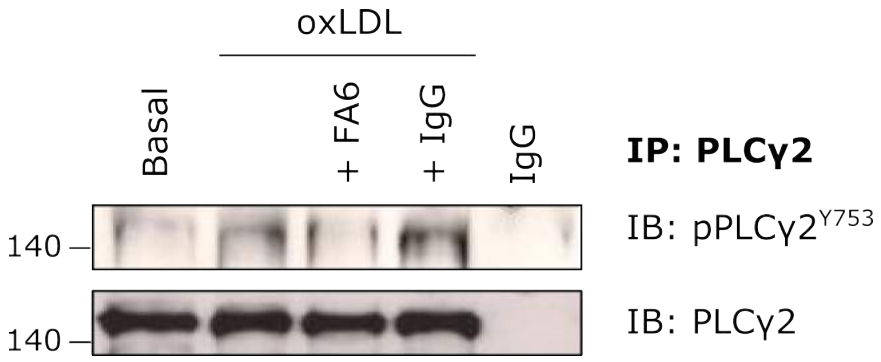


Figure 5.6 – Inhibition of CD36 prevents phosphorylation of PLCγ2 by oxLDL. Platelets ($7x10^8$/mL) were treated with apyrase, indomethacin, EGTA and tirofiban for 5 minutes followed by the anti-CD36 blocking antibody FA6.152 or isotype control for 20 minutes. Treated platelets were stimulated with oxLDL for 1 minute and lysed in IP lysis buffer. PLCγ2 was immunoprecipitated using specific antibodies and separated using SDS-PAGE. PLCγ2 tyrosine phosphorylation was measured using the site-specific antibody and equal loading measured by use of an anti- PLCγ2 antibody. Data shown represents a single experiment attempt.

5.4.2 OxLDL causes the activation of Src-family kinases and Syk

Having earlier shown that oxLDL is able to cause an increase in tyrosine phosphorylation and PKC activation (Fig. 3.10), we hypothesised that oxLDL was signalling in an ITAM-dependent manner. ITAM activation downstream of other platelet agonists typically involves the recruitment of the tyrosine kinases Src-family kinases and Syk (Watson et al., 2005; Mócsai et al., 2010) and we began by investigating whether oxLDL was able to cause phosphorylation of these proteins. Src kinases are autophosphorylated under basal conditions at the tyrosine residue 527 which causes the enzyme to fold into a 'closed' configuration (Frame, 2002). Upon agonist ligation, Src kinases become activated by a phosphorylation event at Tyr 416, freeing up the binding pocket and causing phosphorylation of the ITAM Tyr-x-x-Lys motif (Johnson et al., 1995). It is well documented that Src kinases are able to undergo autophosphorylation at Tyr 416 (Sun et al., 1998; Irtegun et al., 2013), with speculation into whether autophosphorylation allows maintenance of basal Src signalling.

Consistent with the literature, basal phosphorylation of Src kinases was observed when probed with an anti-phosphoSrc antibody recognising the Y416 motif (Fig. 5.7A). Levels of Src phosphorylation increased upon stimulation with oxLDL, but not nLDL, supporting earlier work using the anti-phosphotyrosine antibody (Fig. 3.7).

Syk is a common target of Src kinases (Mócsai et al., 2010) and has been previously shown to be involved with oxLDL signalling (Nergiz-Unal, Rademakers, et al., 2011; Wraith et al., 2013). Like Src kinases, it is also phosphorylated on tyrosine residues, specifically at Y^{352} and $Y^{525/6}$ (Suzuki-Inoue et al., 2004). Immunoprecipitation was used to isolate Syk from untreated and oxLDL treated platelets and phosphorylation status probed using an anti-phosphotyrosine antibody. Minimal tyrosine phosphorylation was observed from both untreated platelets and platelets treated with nLDL (Fig. 5.7B). Conversely, platelets treated with oxLDL displayed increased levels of Syk phosphorylation, consistent with the literature.

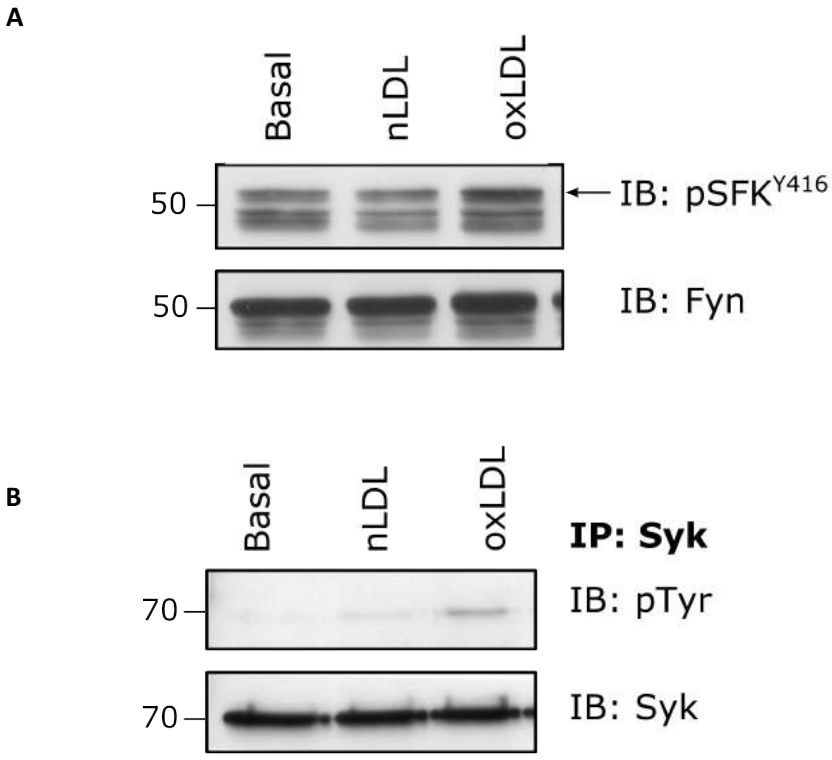


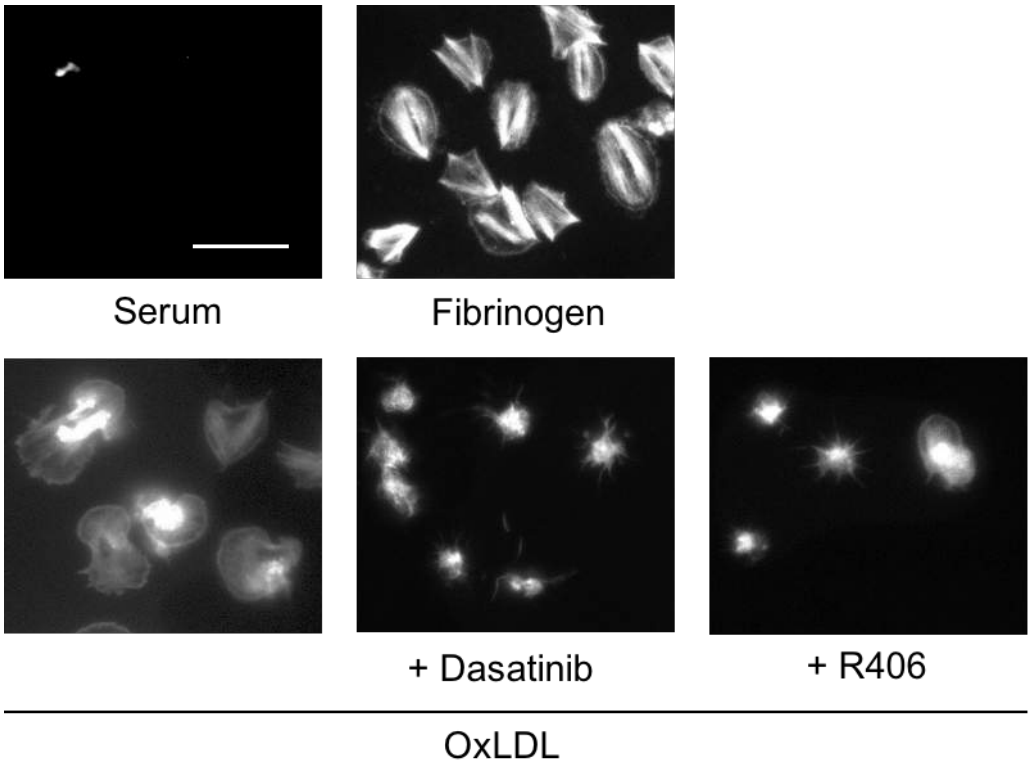
Figure 5.7 – OxLDL causes tyrosine phosphorylation of the tyrosine kinases Src family kinases and Syk. A – Platelets ($5x10^8$/mL) were treated with apyrase, indomethacin, EGTA and tirofiban for 20 minutes followed by stimulation with nLDL or oxLDL (50μg/mL) for 1 minute before lysis using Laemmli buffer. Samples were separated using SDS-PAGE and Src-family kinase phosphorylation detected using an anti-phosphoSFK^{Y416} antibody. Equal protein loading was determined using an anti-Fyn antibody. B – Platelets ($7x10^8$/mL) were treated with apyrase, indomethacin, EGTA and tirofiban for 20 minutes followed by stimulation with nLDL or oxLDL (50μg/mL) for 1 minute before lysis using IP Lysis buffer and Syk was immunoprecipitated. Samples containing immunoprecipitated Syk were loaded into gels and Syk phosphorylation probed using an anti-phosphotyrosine antibody. Images are representative blots of two separate experiments.

5.4.3 Effects of Src and Syk Inhibition on platelet adhesion and spreading to oxLDL

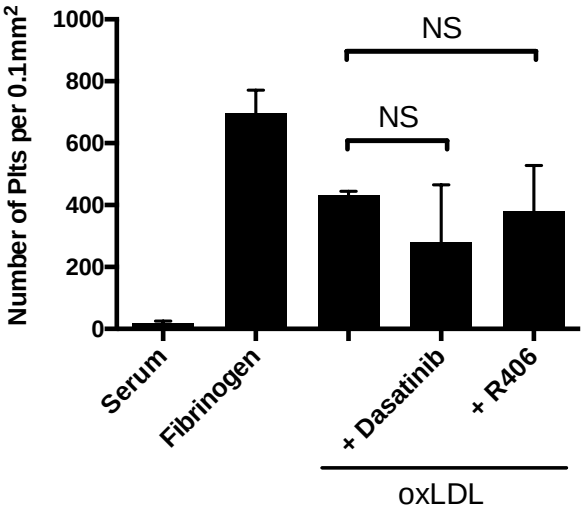
Having shown that oxLDL causes phosphorylation of SFK and Syk, we wanted to investigate what effect their inhibition would have on platelet function and tie phosphorylation and activation of these proteins together. Moreover, we hypothesised that investigation into Src kinase and Syk inhibition may provide further insight into the role of PLCγ2 since these proteins are canonically considered to be proximal to PLCγ2 in signalosome formation (Watson et al., 2005). We predicted that if Src and Syk were indeed upstream of PLCγ2, they would have very similar results on platelet adhesion and spreading to that of U73122. In addition, the involvement of Src kinase/Syk increases the likelihood that the PLC isoform responsible for spreading, as shown in Fig. 5.5 is PLCγ2 and not PLCβ since it is tyrosine kinase dependent.

As expected, minimal platelet adhesion was observed on glass slides treated with human serum (Serum – 17.5±7.09) (Fig. 5.8). Robust platelet adhesion was observed to fibrinogen (Fibrinogen – 697.3±74.18) and oxLDL treated slides (Basal - 433±11.85), consistent with previous experiments. Platelet treatment with both SFK and Syk inhibitors displayed no statistically significant effect on platelet adhesion to oxLDL (+ Dasatinib – 279.3±186.34; + R406 – 381.67±147.03). Platelets adhering to oxLDL pre-treated with dasatinib had a significantly reduced surface area (+ Dasatinib – 9.76μm^2±0.36) compared to basal (Basal – 25.85μm^2±5.16; p=0.019). Moreover, platelets treated with R406 also displayed a trend for decreased surface area (+ R406 – 13.78μm^2±1.73), although when analysed, failed to reach statistical significance (p=0.06). Taken together, the data suggests that Src and Syk inhibition does have effects on platelet adhesion and spreading on oxLDL and additional experimental repeats may have helped to confirm this statistically.

A


Serum
Fibrinogen
+ Dasatinib
+ R406
OxLDL

B


Number of Plts per 0.1mm²
1000
800
600
400
200
0
NS
NS
Serum
Fibrinogen
+ Dasatinib
+ R406
oxLDL

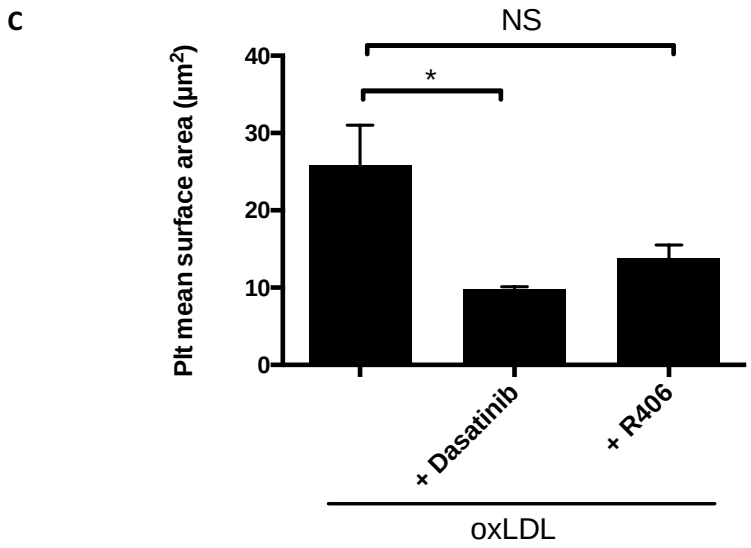


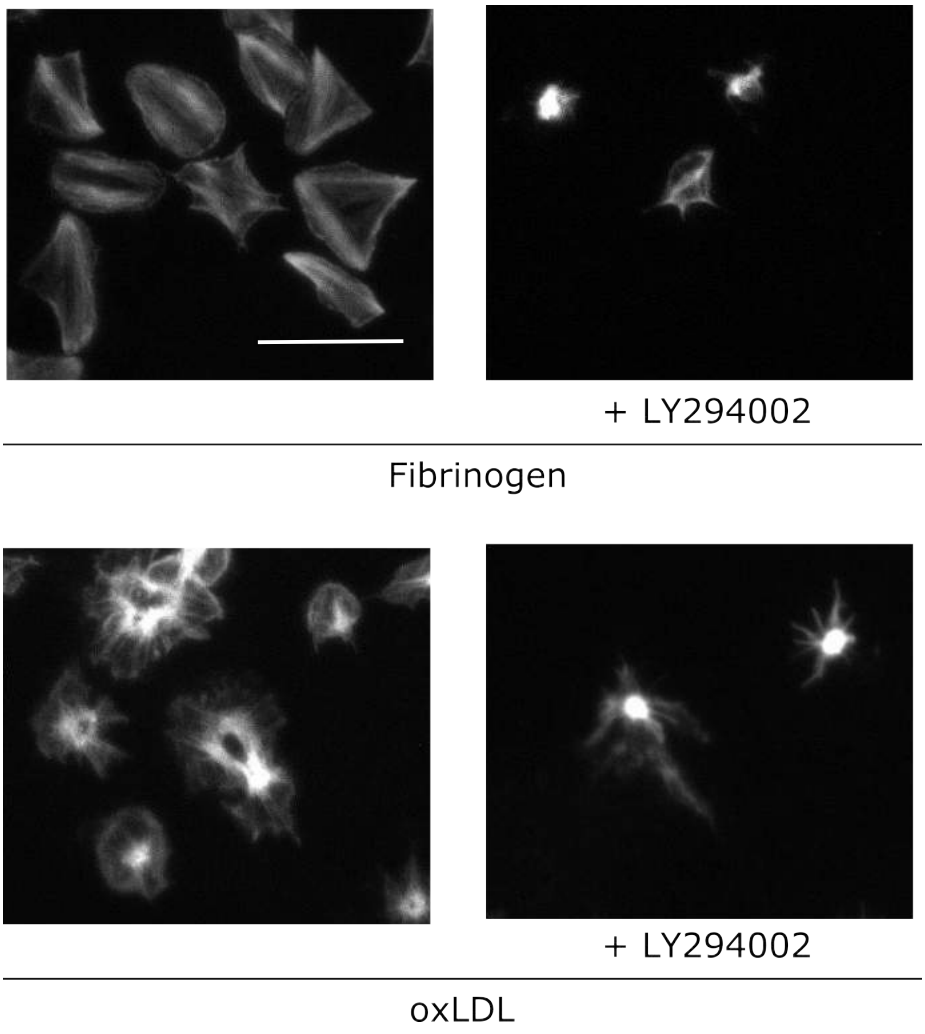
Figure 5.8 – Inhibition of Src-family kinases and Syk reduces platelet spreading, but not adhesion to oxLDL. Platelets ($5x10^7$/mL) were treated with dasatinib (10µM) or R406 (5µM) for 20 minutes prior to incubated on oxLDL treated slides (100µg/mL) for 60 mins at 37^oc. Adhered platelets were fix, stained and viewed using an inverted fluorescence microscope (Zeiss). Images were acquired randomly and quantified manually using ImageJ. A – Representative images of platelet adhesion and spreading. B – Quantification of the average number of platelets adhered. C – Quantification of average mean platelet surface area. Data shown is an average of 3 separate experiments. Scale bar represents 10 microns.

5.4.4 The role of PI3-K in platelet activation in response to oxLDL

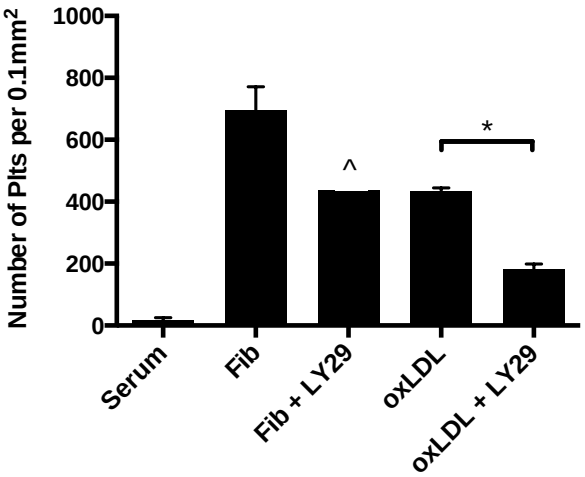
Having established that oxLDL signalling causes the phosphorylation and activation of SFK and Syk, we next wanted to look at whether the lipid kinase PI3-K was involved. This was examined because PI3-K is able to support the activation of PLCγ2 by converting PIP_2 into the secondary messenger lipid PIP_3 (Whitman et al., 1988; Auger et al., 1989) to support signalosome formation (Pasquet et al., 1999; Watson et al., 2005). Activation of PI3-K has been shown to be involved in both GPVI (Pasquet et al., 1999) and $\alpha_{IIb}\beta_3$ signalling (Yap et al., 2002), with knockouts in PI3-K isoforms shown to reduce signalling efficiency (Lian et al., 2005; Consonni et al., 2012). To investigate the role of PI3-K signalling in response to oxLDL, the inhibitor LY294002 was used.

In order to verify that the inhibitor LY294002 was working as expected, platelets were treated with the inhibitor and stimulated with fibrinogen to see whether the inhibitor would produce results consistent with the literature. A decrease in platelet adhesion was observed (Fib + LY29 – 439) when compared to untreated platelets (Fib – 756) (Fig. 5.9). Interestingly, a significant decrease in platelet adhesion was also observed when platelets were treated with LY294002 and stimulated with oxLDL, compared to oxLDL stimulation alone (OxLDL – 433±11.85; OxLDL + LY29 – 182.67±16.18; p=<0.001). However, no significant difference in surface area was detected between treated and untreated cells stimulated with oxLDL (OxLDL – 25.85μm^2±5.16; OxLDL + LY29 – 14.62μm^2±3.87). Despite this, the morphology of treated and untreated cells was different. Untreated platelets stimulated with oxLDL clearly displayed a fully spread morphology whilst those treated with the PI3-K inhibitor showed filopodia extensions only, indicating that PI3-K is likely to be involved.

A


+ LY294002
Fibrinogen
+ LY294002
oxLDL

B


Number of Plts per 0.1mm2
1000
800
600
400
200
0
^
*
Serum
Fib
Fib + LY29
oxLDL
oxLDL + LY29

C

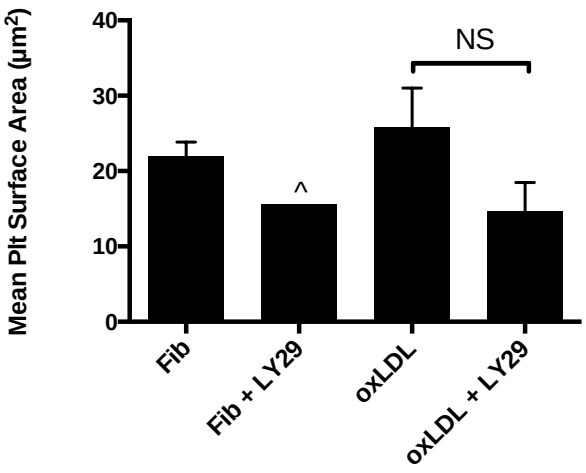


Figure 5.9 – The PI3-K inhibitor LY294002 causes a reduction in platelet adhesion to oxLDL. Platelets ($5x10^7$/mL) were treated with LY294002 (10µM) for 20 minutes prior to incubation on fibrinogen (100µg/mL) or oxLDL treated slides (100µg/mL) for 60 mins at 37°C. Adhered platelets were fix, stained and viewed using an inverted fluorescence microscope (Zeiss). Images were acquired randomly and quantified manually using ImageJ. A – Representative images of platelet adhesion and spreading. B – Quantification of the average number of platelets adhered. C – Quantification of average mean platelet surface area. ^ indicates average data from two separate experiments. Data shown is an average of 3 separate experiments. Scale bar represents 10 microns.

5.4.4 OxLDL is able to cause the phosphorylation of the PKC target Pleckstrin

We have previously shown that oxLDL stimulation of platelets is able to cause an increase in PKC activity, demonstrated via use of the anti-phospho-PKC substrate antibody (Fig. 3.10). However, whilst interesting, use of this antibody does not specify any particular substrates, nor identify a particular isoform of PKC activated in response to oxLDL. Pleckstrin is a major target of multiple PKC isoforms in platelets (Abrams et al., 1995) and its phosphorylation is frequently used as a marker of PKC activation (Lian et al., 2009). Upon platelet stimulation with thrombin, collagen or PMA (Sano et al., 1983; Gailani et al., 1990), pleckstrin is rapidly phosphorylated leading to cytoskeletal reorganisation and granule release. This has been shown by use of Plecktrin deficient mice which show abnormal exocytosis of granular contents, defective integrin activation and aggregation (Lian et al., 2009).

Using an antibody recognising Pleckstrin, Pleckstrin was immunoprecipitated and phosphorylation probed using an anti-phosphoPKC substrate antibody. Under basal conditions, minimal phosphorylation of Pleckstrin was observed (Fig. 5.11). Conversely, upon stimulation with either oxLDL or KOdiA-PC, high levels of phosphorylated Pleckstrin was detected. PMA, a direct activator of PKC was used as a positive control, although at the concentration used did not provide robust phosphorylation. It is also worth noting that the IgG sample was also able to pull down Pleckstrin from platelet lysate in equal amounts to the Pleckstrin antibody, although no phosphorylation was observed.

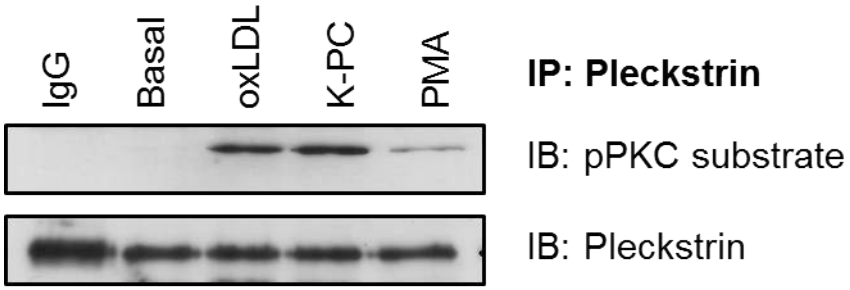


Figure 5.10 – OxLDL and KOdiA-PC are able to cause the phosphorylation of the PKC target Pleckstrin. Platelets ($7x10^8$/mL) were stimulated with oxLDL (50µg/mL), KOdiA-PC (25µM) or the positive control PMA for 1 minute before lysis using IP Lysis buffer and pleckstrin was immunoprecipitated. Samples containing immunoprecipitated pleckstrin were loaded into gels and pleckstrin phosphorylation probed using an anti-phosphoPKC substrate antibody. Images are representative blots of two separate experiments.

5.5 Characterisation of a PLCγ2 deficient murine model

5.5.1 Verification of PLCγ2$^{-/-}$ genotype

In order to begin this study, we had to source PLCγ2$^{-/-}$ mice. Embryos of PLCγ2$^{+/-}$ were a kind gift from Prof Steve Watson (University of Birmingham). The low breeding and high mortality rates of homozygous knockout mice meant that mice were bred heterozygous x heterozygous and all offspring genotyped. Mice were genotyped using specific primers and examples of wild-type, heterozygous and homozygous knockout samples are provided below (Fig. 5.12a). In order to confirm these results, we immunoblotted for the protein in platelet lysates and showed definitively that the genotyping protocol was correct and that knockout mice do not possess PLCγ2 (Fig. 5.12b).

5.5.2 PLCγ2$^{-/-}$ platelet receptor expression

Having shown that PLCγ2$^{-/-}$ platelets do not possess the protein, we wanted to ensure that any changes in platelet functional responses were due to the absence of PLCγ2 and not due to changes in receptor expression. Unstimulated platelets were incubated with fluorescently labelled antibodies recognising 5 key platelet receptors: GPVI, CD36, CD61, CD49b and CD42b in a 1:1 ratio ensuring a saturation of receptors with available antibody. No significant difference was observed in receptor expression of any of the receptors investigated, with the exception of CD61. In the case of CD61, PLCγ2$^{-/-}$ platelets displayed significantly lower fluorescence than wild-type controls, indicating that less receptor was present on the cell surface ($p=0.018$).

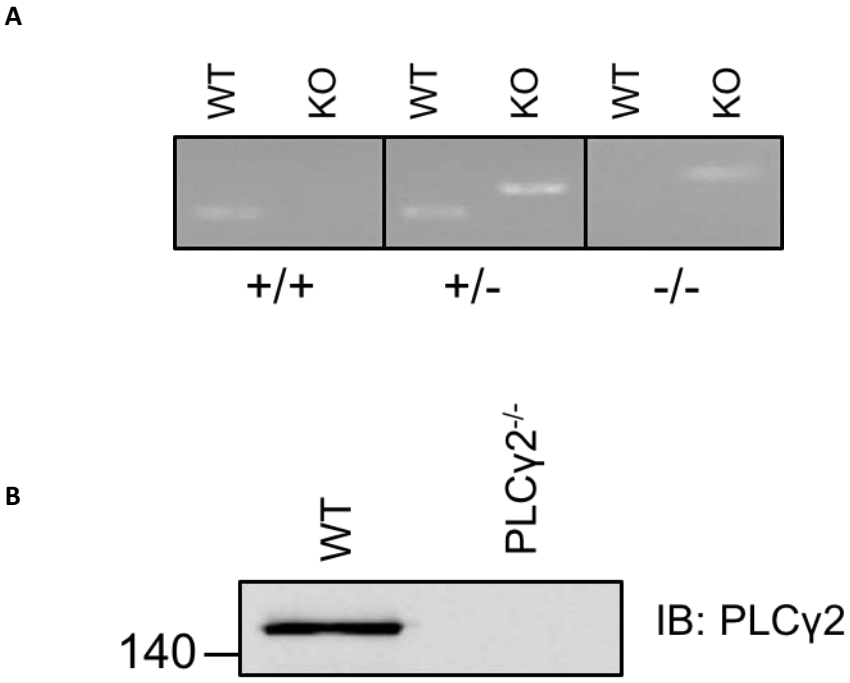


Figure 5.11 – Successful breeding of mice deficient in PLCγ2. A – Representative images of PCR bands showing differences between wild-type, heterozygous and homozygous knockout mice. B – Immunoblot of PLCγ2 in wild-type and homozygous knockout platelet samples. Platelets ($2x10^8$/mL) were used in platelet aggregation tests and subsequently lysed in 4x Lamelli buffer. 50µl of platelet lysate was separated via SDS-PAGE and PLCγ2 levels investigated using an anti-phospholipase Cγ2 antibody. Data shown is representative of one single experiment.

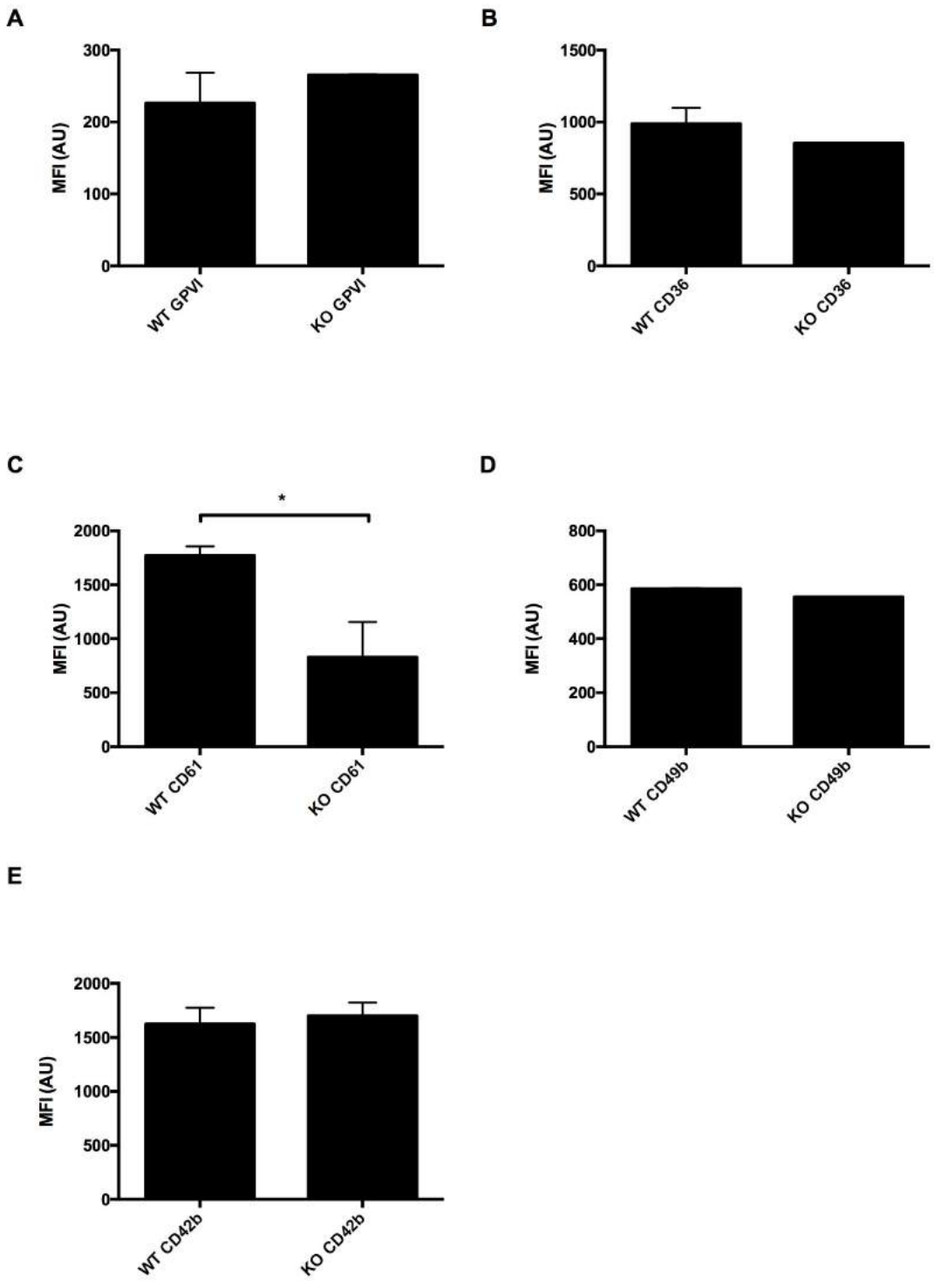


Figure 5.12 – PLCγ2$^{-/-}$ mice display similar receptor expression levels to wild-type mice, with the exception of CD61. Murine washed platelets (5µL) were incubated with 5µl of fluorescently labelled antibody recognising either GPVI, CD36, CD61, CD49b or CD42b for 5 mins prior to fixing with formalsaline for 20 mins. Median fluorescence intensity (MFI) was recorded per sample. WT – Wild-type, KO - PLCγ2 Knockout. Data represents 3 separate mice (WT) and 2 separate mice (KO).

5.5.3 PLCγ2$^{-/-}$ platelet responses to CRP

In order to investigate whether oxidised phospholipid signalling requires PLCγ2, we first wanted to test PLCγ2$^{-/-}$ platelets functionally using CRP. It is well established that collagen and CRP rely heavily on PLCγ2 in order to cause aggregation (Suzuki-Inoue et al., 2003; Nonne et al., 2005). As a result, experiments giving a large difference in aggregation should provide a clear indication of PLCγ2 deficiency, giving us greater confidence for subsequent experiments with KOdiA-PC. Consistent with the literature, platelets from both PLCγ2$^{-/-}$ mice failed to aggregate in response to CRP in contrast to wild-type controls (Fig. 5.14). Thrombin was used to test platelet viability and showed that platelets from wild-type and knockout platelets were functional and responding as expected.

5.5.3 PLCγ2$^{-/-}$ platelet responses to KOdiA-PC

Having shown that PLCγ2 deficient platelets acted as expected upon CRP stimulation, we were confident with the model and tested the effects of KOdiA-PC on PLCγ2$^{-/-}$ mice. We hypothesised that since PLCγ2 is shown to become phosphorylated by oxLDL and KOdiA-PC, as well as upstream proteins that it should become activated, triggering platelet shape change and degranulation. Interestingly, murine platelets deficient in PLCγ2 displayed similar levels of aggregation to wild-type controls (Fig. 5.15). In both experiments, platelet aggregation to KOdiA-PC was minimal (5-10%) after a minimum of 10 minutes of stirring in a Born aggregometer. In the first experiment (Fig. 5.15a), wild-type platelets were able to begin the aggregation process, whilst knockout mice displayed a clear shape change phase, but did not aggregate to a similar extent. Conversely, in the second attempt (Fig. 5.15b), very little difference was observed between the two populations, with similar levels of aggregation in both wild-type and knockout platelets.

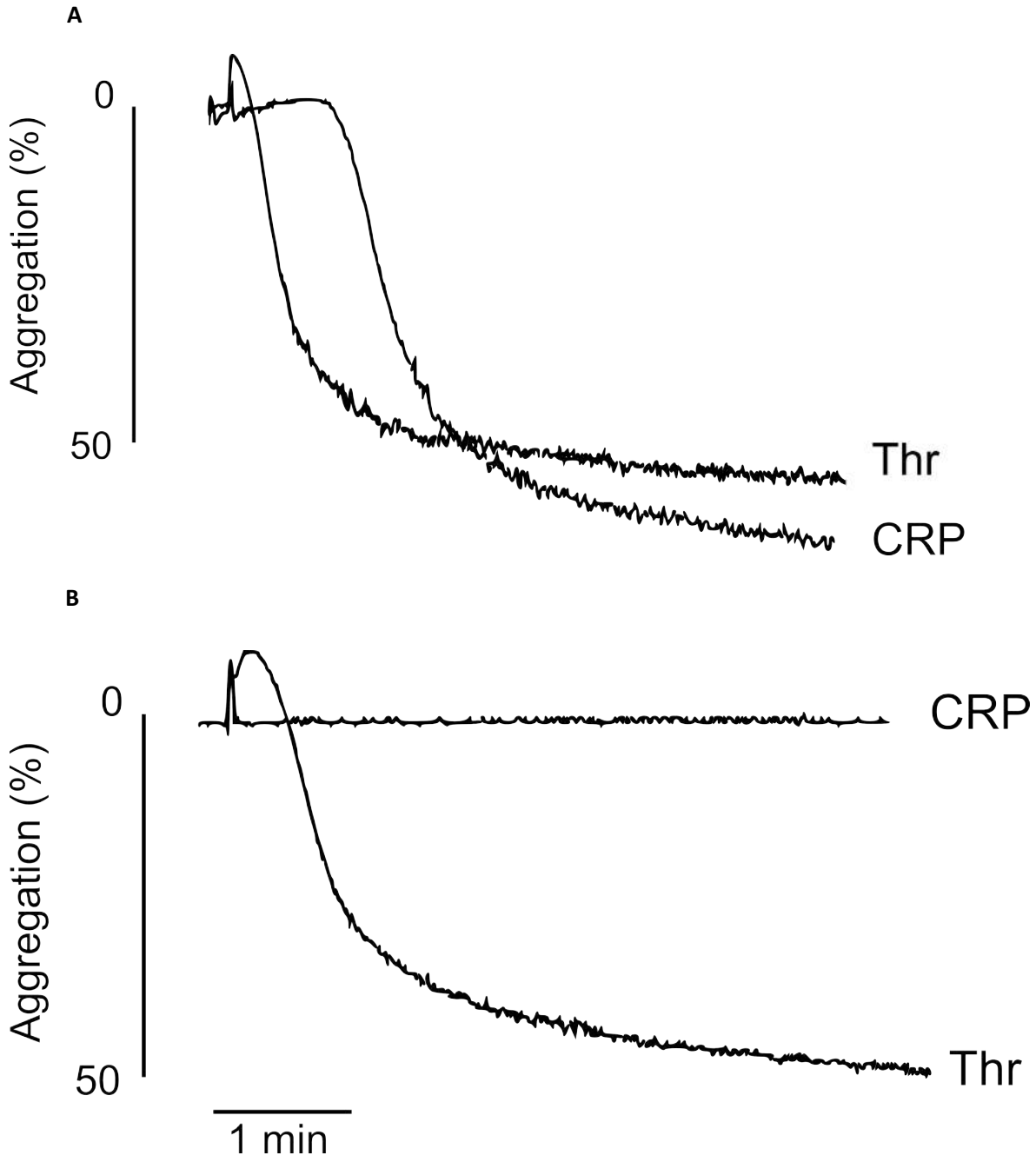


Figure 5.13 - PLCγ2 deficient platelets do not aggregate in response to CRP. A – Wild-type platelets ($2x10^8$/mL) were treated with either thrombin (0.05U/mL) or CRP (1μg/mL) and aggregation recorded for 5 minutes using an platelet light-transmission aggregometer (Chrono-Log). B – As in A, except PLCγ2$^{-/-}$ platelets were used. Data shown is representative traces from four separate experiments (Wild-type) and two separate experiments (Knockout).

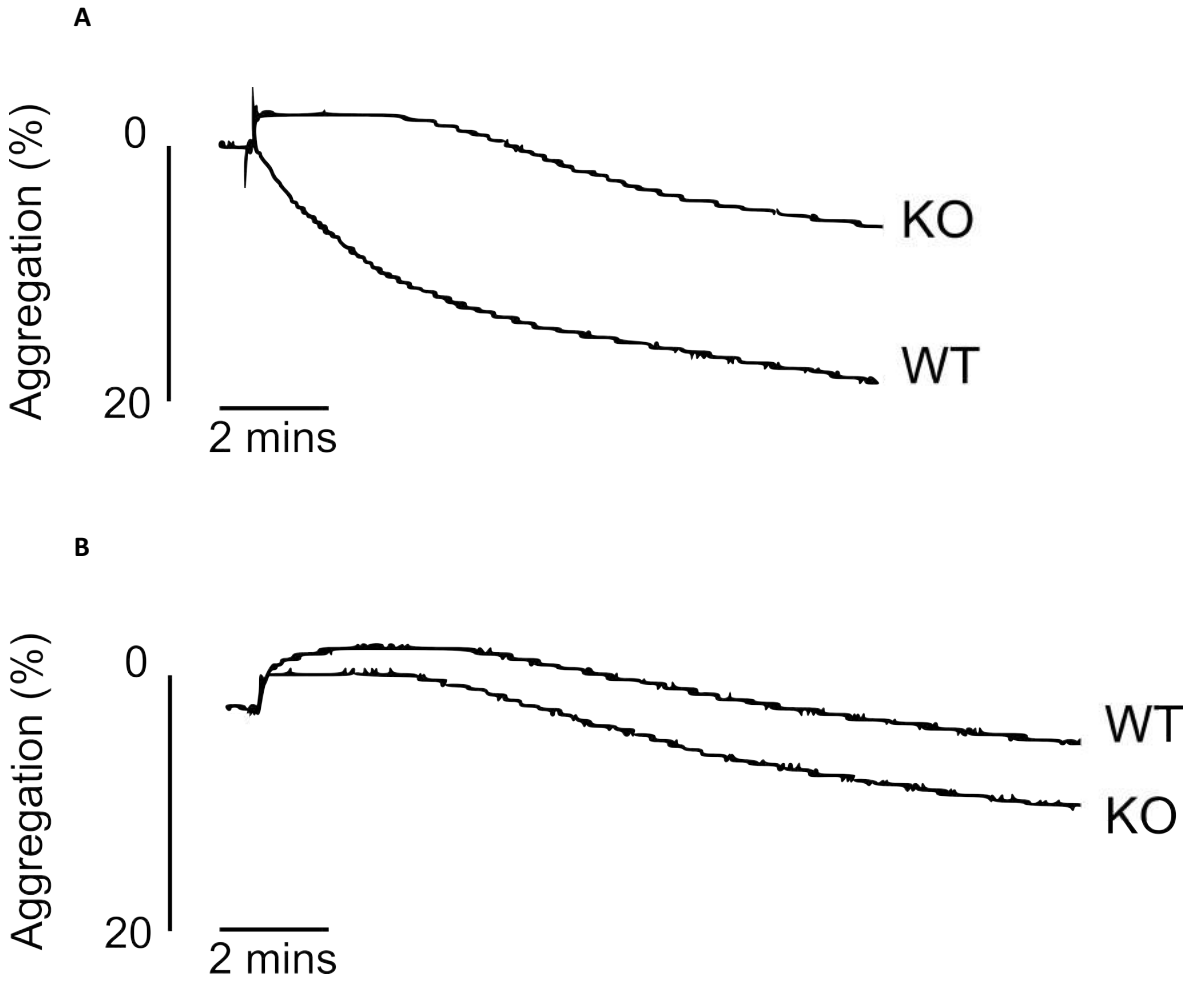


Figure 5.14 - PLCγ2 deficient platelets are able to aggregate in response to KOdiA-PC. Platelets from either wild-type or PLCγ2$^{-/-}$ mice were stimulated with KOdiA-PC (5μM) and aggregation recorded for a minimum of 10 minutes using a platelet light-transmission aggregomter (Chrono-Log). A and B denote two separate experiments.

5.6 Chapter Discussion

Phospholipases are a key effector protein in many different signalling pathways (Cocco et al., 2015). Ligands which are capable of initiating a signalling response triggering the activation of PLC cause changes in cellular function and have been reported in multiple disease states, including brain diseases (Koh, 2013), immunosuppression (Kawakami and Xiao, 2013) and cancer (Lattanzio et al., 2013). In platelets, activation of PLC isoforms by agonists including collagen, thrombin and fibrinogen causes intracellular Ca^{2+} release and increased PKC activity resulting in platelet activation (Li et al., 2010; Clemetson, 2012). However, the ability of oxLDL to trigger the activation of PLC remains incompletely understood.

Phosphorylation of PLCγ2 by oxLDL

Whilst multiple PLC isoforms exist in platelets, we focused our search initially on the role of the isoform PLCγ2. PLCγ2 is the most abundant PLC isoform present in platelets (Lee et al., 1996; Zheng, Adams, Zhi, Yu, Wen, Peter J. Newman, et al., 2015) and is known to be activated downstream of tyrosine kinase signalling pathways (Blake et al., 1994; Ozdener et al., 2002). Previously, we have shown that oxLDL is able to induce changes in phosphorylated tyrosine profiles of platelets (Fig. 3.7) and this is supported further by proteomic data suggesting that PLCγ2 can become phosphorylated in response to oxidised phospholipids (Zimman et al., 2014). Using immunoprecipitation, we managed to support previous findings that platelets do indeed possess PLCγ2 and more importantly, show that oxLDL is able induce tyrosine phosphorylation of the protein in both a time and dose dependent manner (Fig. 5.1 & 5.2) (Wraith et al., 2013). Phosphorylation was shown to become elevated after 15 seconds of stimulation and remain elevated even after 5 minutes. Due to the chronic nature of hyperlipidaemia, it would be interesting to investigate PLCγ2 phosphorylation at much later time points e.g. 1-6 hours stimulation to see whether the platelet can self-regulate this phosphorylation event via use of phosphatases. Phosphorylation of PLCγ2 was shown using two physiological concentrations of oxLDL, supporting the theory of oxLDL induced platelet activation

further. Alongside platelet stimulation with oxLDL, we also treated platelets with CRP, a known PLCγ2 activating agonist, to use as a positive control. Interestingly, PLCγ2 phosphorylation in response to oxLDL was far weaker than by CRP and perhaps is associated with the degree of platelet activation induced by the two agonists. We began to investigate the phosphorylation sites present on PLCγ2 to see whether this could play a role in determining an agonist's potency.

An early study investigating PLCγ2 phosphorylation sites in platelets showed that two major tyrosine sites govern activity – Tyrosine 753 and 759 (Suzuki-Inoue et al., 2004). CRP, considered to be the most potent ligand capable of inducing PLCγ2 activation, was shown to cause strong phosphorylation of both sites. However, the GPIb-V-IX ligand vWF causes milder aggregation in vitro and only triggered the weak phosphorylation of Y^{753}. We showed that oxLDL caused PLCγ2 phosphorylation in a manner similar to vWF, with phosphorylation observed at Y^{753} in a time-dependent manner. No phosphorylation was observed at Y^{759}, and as a result, we speculate that this may be a reason why oxLDL does not induce strong platelet activation alone. We also show limited evidence that hints of a similar phosphorylation event occurring in murine platelets treated with KOdiA-PC, although this work requires substantial further investigation. Interestingly, proteomic data by Zimman et al. showed that human platelets treated with the oxidised phospholipid KODA-PC showed phosphorylation at different sites – Y^{759} and the novel site Y^{1217} and therefore would be interesting to investigate further. Some of the discrepancies in findings between papers could in part be due to the limited specificity of the antibodies recognising phosphorylation site changes of PLCγ2 and therefore use of *in vitro* activity assays e.g. NFAT using PLCγ2 proteins mutated at the specific tyrosine sites may be of benefit.

Having shown substantial data supporting the ability of oxLDL to induce the phosphorylation of PLCγ2, albeit modest, we next wanted to investigate the functional consequences of activation. Using the pan-PLC inhibitor, platelet function in response to oxLDL was assessed using two separate assays – flow cytometric analysis of platelet P-

selectin expression and platelet adhesion and spreading. Inhibition of PLC by U73122 caused significant inhibition of platelet function, with reduced degranulation/P-selectin expression, adhesion and spreading (Fig. 5.4 & 5.5). The ability of oxLDL to induce P-selectin expression is well documented (Puccetti et al., 2005; Sener et al., 2011) and signalling studies have shown that α-granule release is largely a PKC-dependent process (Harper and Poole, 2010). As a consequence, it stands to reason that inhibition of PLCγ2, typically considered to be upstream of PKC, could prevent degranulation, with evidence in the literature supporting this with use of other upstream inhibitors including PP2 and R406 (Zimman et al., 2014).

Conversely, the effects of U73122 on platelet adhesion and spreading were ambiguous. We hypothesised that treatment of platelets should impact platelet spreading, with PLCγ2 activation known to be partially required for cytoskeletal rearrangement (Inoue et al., 2003; Wonerow et al., 2003; Wraith et al., 2013), yet not affect levels of platelet adhesion – a receptor dependent process. Whilst platelet spreading was inhibited as expected, the numbers of platelets adhering to oxLDL also decreased significantly. One possible reason for this is off-target effects of the U73122 compound, which has been shown to affect multiple other cellular targets including PKC, histamine receptors, oestrogen receptors, Ca^{2+} channels and lipoxygenases (Pulcinelli et al., 1998; Lockhart and McNicol, 1999; Wilsher et al., 2007). It is possible that U73122 may be inhibiting basally active proteins required for adhesion or blocking the effects of other secondary signalling mediators not inhibited in this set of experiments. In order to further tie down the role of PLCγ2 in oxLDL signalling and function further, use of PLC$\gamma2^{-/-}$ platelets was required.

The role of reported upstream signalling proteins

Having shown that oxLDL is able to cause phosphorylation of PLCγ2 and inhibition prevents platelet activation, we next wanted to investigate the upstream signalling proteins involved in its phosphorylation. We focused specifically on the receptor CD36, which we have previously shown to be involved in transducing signalling from oxLDL, the tyrosine kinases SFK and Syk and the lipid kinase PI3-K. Together, these proteins are

involved in the formation of a 'signalosome' in response to collagen and fibrinogen (Watson et al., 2005), which allows for the activation of PLCγ2. We also used phosphorylation of the common PKC substrate pleckstrin as a readout for PKC activity. Whilst much of this series of experiments requires further work in order to confirm the findings of the data shown throughout, it supports findings from published work (Korporaal et al., 2005; Nergiz-Unal, Lamers, et al., 2011; Wraith et al., 2013; Magwenzi et al., 2015) and provides further insight into the mechanism of oxLDL signalling.

We began by using the anti-phosphoPLCγ2^{Y753} antibody to measure changes in PLCγ2 phosphorylation in the presence and absence of the CD36 neutralising antibody FA6.152. Platelet incubation with FA6.152 caused a noticeable decrease in PLCγ2 phosphorylation when compared to both untreated platelets and platelets treated with an isotype control.

Early studies into CD36 function showed that CD36 possesses minor collagen binding ability (Tandon et al., 1989; Matsuno et al., 1996), although any possible relationship between GPVI and CD36 signalling remains uncharacterised to date. However, an early study by Daniel et al. showed that platelets from a CD36 deficient donor were able to mobilise Ca^{2+} from intracellular stores in a similar fashion to normal platelets. An in-depth signalling approach using mass spectroscopy may help answer this question by determining whether collagen stimulation causes any difference in the platelet phosphoproteome between normal and CD36 deficient donors.

Next we began to investigate the possible involvement of Src and Syk in oxLDL mediated signal transduction. Both SFK and Syk have been previously shown to become phosphorylated in platelets in response to oxLDL stimulation and we began by corroborating these findings (Wraith et al., 2013). Platelet stimulation with physiological concentrations of oxLDL caused clear increases in both SFK and Syk phosphorylation (Fig. 5.8), whilst nLDL caused very little change. Having shown that both become phosphorylated, we next wanted to investigate what effect blocking the proteins would have on platelet activation and compare it to changes seen by use of U73122. Inhibition of SFK by dasatinib displayed very similar results as those published by Wraith et al. using

the alternative SFK inhibitor PP2 (Wraith et al., 2013). Levels of platelet adhesion were comparable between treated and untreated groups, but treated platelets displayed a clear decrease in surface area (Fig. 5.9). Similarly, platelets treated with the Syk inhibitor R406 also displayed comparable levels of adhesion to untreated controls yet when analysed, there was no statistical difference in platelet spreading. However, platelets treated with R406 did display a morphological difference which is clear from visual inspection of spread platelets. It would be beneficial to tie activation of Src kinases and Syk to the phosphorylation of PLCγ2. Immunoprecipitation of PLCγ2 from dasatinib and R406-treated platelets would provide a clear link to PLCγ2 phosphorylation and therefore show a clear association between these proteins. Alternatively, PLCγ2 activity could be measured using an *in vitro* activity assay and samples of treated lysates added. Unfortunately, a lack of time prevented these experiments from being pursued.

We also sought to determine whether oxLDL stimulation of platelets required PI3-K, since this has been shown to be involved in the activation of PLCγ2 downstream of GPVI stimulation (Suzuki-Inoue et al., 2003). The PI3-K inhibitor LY294002 caused a significant reduction in platelet adhesion to oxLDL, in line with results for other platelet agonists e.g. fibrinogen (Jackson et al., 2004; Senis et al., 2005), collagen (Watanabe et al., 2003). Moreover, on visual inspection, platelets adhered to oxLDL but those treated with LY294002 displayed on average a smaller surface area than untreated cells, although when analysed, did not reach statistical significance. It is worth noting however that visually, cells stimulated on oxLDL but treated with LY294002 look morphologically different from untreated cells, displaying only filopodia extensions compared to a fully spread phenotype. It is tempting to speculate that PI3-K is required for a complete spreading response to oxLDL, although further study is required to fully elucidate this. The evidence gathered in this piece of work is encouraging and it would be of interest to determine the isoform of PI3-K that is potentially involved. One way to tackle this would be to use mice deficient in PI3-K isoforms to tie down any possible proteins and signalling pathways involved.

Data in this piece of work and others has shown that oxLDL is able to activate PKC in order to trigger shape change and degranulation. We were interested in the ability of oxLDL to activate PKC as many isoforms are activated by increased levels of Ca^{2+} and/or DAG, a result of phospholipase activation. We supported these known findings with immunoprecipitation data of the PKC substrate pleckstrin, which was shown to be highly phosphorylated by both oxLDL and KOdiA-PC. The aim of this work was to begin to establish a robust technique in order to measure PKC activity and, using pharmaceutical inhibitors, identify which isoform(s) of PKC are activated by oxLDL. Whilst this was not possible during the course of experimentation, the results provide a novel insight into an unexplored part of the oxLDL signalling pathway. Pleckstrin deficient mice have been shown to display defective exocytosis and cytoskeletal rearrangement (Lian et al., 2009), although the interplay between pleckstrin, its phosphorylation and its function within the cell is poorly understood.

Functional changes in PLCγ2 deficient mice

We attempted to support our findings by use of PLCγ2$^{-/-}$ mice and show definitively whether PLCγ2 is required for platelet activation in response to oxLDL/oxidised phospholipids. On the basis of the previous work, we hypothesised that murine platelets deficient in PLCγ2 would fail to respond to the reportedly CD36-specific ligand KOdiA-PC. Much of this work was hindered by poor breeding and survival rates of knockout mice and therefore the hypothesis could not be fully investigated during the remaining time period. Whilst not conclusive, the data suggests that PLCγ2$^{-/-}$ platelets are still able to aggregate in response to KOdiA-PC, indicating that other modes of action may be present for this ligand. One alternative theory is that the free oxidised phospholipid is able to incorporate itself into the platelet membrane, triggering small amounts of platelet activation. Incorporation of KOdiA-PC into liposomes may alter the presentation of the KOdiA-PC molecule, increasing its specificity and potentially contrast with the results presented here. Alternatively, it is possible that the free oxidised phospholipid presents itself differently to CD36 compared to oxLDL and therefore may signal differently in a

PLCγ2-independent manner. Further work to characterise the signalling response initiated by KOdiA-PC in both mice and human platelets would help answer this question.

Summary

To conclude, this piece of work has shown some convincing data, along with published literature, to show that PLCγ2 is able to become phosphorylated in response to oxLDL. It is likely that this phosphorylation event is dependent on a CD36 signalling pathway involving the proteins Src and Syk and evidence has been provided to show that PI3-K may play a role in signalosome formation. However, any functional significance of PLCγ2 activation remains only partially understood due to poor pharmaceutical inhibitors and a lack of ability to use oxLDL in murine platelets. However, the generation of PLCγ2 deficient mice raises the possibility of exciting work to further unravel the mechanism behind PLCγ2 phosphorylation and activation downstream of oxidised phospholipids.

Chapter 6: General Discussion

Despite the development of lipid-lowering agents and multiple public health campaigns, atherosclerotic cardiovascular disease remains a leading cause of mortality both in the UK and across the western world (Townsend et al., 2014). Excessive consumption of dietary fat was shown to be associated with atherosclerosis as early as 1926 (Clarkson and Newburgh, 1926), but the role of LDL and its pathogenicity upon oxidation was not discovered until the 1970s (Goldstein and Brown, 1977). Later studies described the ability for cells to oxidise LDL (Henriksen et al., 1981b) which was followed by the keystone publication by Ardlie et al. in 1989 which first described the platelet activating ability of oxLDL. Since then, many studies have been published describing different aspects of platelet activation induced by oxLDL (Zhao, Dierichs, Liu and Holling-Rauss, 1994; Zhao et al., 1995; Naseem et al., 1997; Mahfouz and Kummerow, 2000; Korporaal et al., 2005; Nergiz-Unal, Lamers, et al., 2011; Wraith et al., 2013). However, until very recently, the mechanisms underpinning these processes remained poorly characterised. The focus on the mechanisms underpinning platelet activation are of importance since current anti-platelet agents, including COX, P2Y and $\alpha_{IIb}\beta_3$ antagonists all show limited success clinically by increasing bleeding risk (Serebruany et al., 2004; Ferraris et al., 2011). Consequently, identification of target receptors or signalling proteins in atherothrombosis could have significant benefit - allowing haemostatic platelet function but not uncontrolled activation. The overarching aim of this project was to assess the ability of oxLDL to induce platelet activation, with emphasis on physiological shear conditions and to investigate scavenger receptor involvement and associated signalling.

This series of work has developed previous knowledge and understanding of CD36 and in particular, show it to be central in triggering pathophysiological signalling. Moreover, we for the first time show a detailed characterisation for the effects of oxLDL under physiological shear stress and show how it is able to potentiate platelet adhesion to both collagen and fibrinogen. This characterisation allowed us to discover that oxLDL is able to modulate platelet signalling to cyclic nucleotides and alter the inhibitory balance,

predisposing platelets to activation. This work also sheds light on the role of PLCγ2 in platelet activation, supporting and advancing previous studies.

Platelet functional changes in response to oxLDL

OxLDL has been reported to induce a multitude of platelet activation changes including shape change (Nergiz-Unal, Lamers, et al., 2011; Wraith et al., 2013), degranulation (Zhao et al., 1995; Takahashi et al., 1998), thromboxane synthesis (Mahfouz and Kummerow, 1998; Mahfouz and Kummerow, 2000) and platelet aggregation (Ardlie et al., 1989; Naseem et al., 1997). Since many of the these findings were inconsistent we began by ensuring a reliable and consistent source of oxLDL to base our experiments on and selected an oxidation protocol capable of yielding physiologically relevant modified LDL species. The oxLDL species generated was able to induce degranulation, as determined via FACS and cause weak aggregation. Consistent with the literature, we provide detailed evidence to show that oxLDL is able to induce platelet adhesion and spreading. Previous studies have shown that platelet activation to oxLDL requires secretion of ADP (Haserück et al., 2004; Korporaal et al., 2007), but scant evidence has been shown for the role TxA_2 plays in the activation process. We provide a novel insight into the role of TxA_2, showing that use of a COX inhibitor significantly reduces platelet adhesion to oxLDL and raises new questions regarding the mechanisms of synthesis and what effect this may play *in vivo*.

A large body of work within this project focused exclusively on the role of oxLDL on platelet adhesion under conditions of arterial shear. Interestingly, despite its ability to support static adhesion and induce a robust platelet spreading response, oxLDL alone was unable to support direct platelet adhesion under flow. However, where oxLDL did cause noticeable effects under flow was that pre-treatment of platelets caused significant increases in platelet adhesion to the traditional agonists collagen and fibrinogen, recreating a 'hyperactive platelet' scenario. These findings were in accordance with Nergiz-Unal et al., although were developed further by the use of fibrinogen as a matrix protein. These findings are of clinical significance because if oxLDL is in the circulation, as stated and measured in multiple studies (Holvoet et al., 2004; Chan et al., 2013), it could

support affect platelet adhesion leading to thrombus formation. At sites of atherosclerotic plaque rupture, matrix proteins are exposed allowing platelets to bind and oxLDL may 'tip the balance', leading to uncontrolled activation. In accordance with this model, and perhaps the key finding throughout this project, was the ability for oxLDL to negate the inhibitory effects of both a cGMP analogue and prostacyclin under physiological shear conditions. Little evidence has been published regarding the role of oxLDL to modulate platelet cyclic nucleotide levels. A study by Korporaal et al. showed that treatment with oxLDL caused changes in levels of intracellular cAMP, although this was not investigated in any further detail. Similarly, oxLDL was shown to negate the effect of NO treatment on thrombin-stimulated platelets, although no mechanistic data was shown (Zuliani et al., 1998). The ability of oxLDL to affect platelet signalling to endogenous inhibitors is likely to be highly deleterious *in vivo* and could potentially be directly attributed with the increase in platelet reactivity observed in obese (Santilli et al., 2012) and hyperlipidaemic patients (Aviram and Brook, 1982). The functional data presented throughout this work suggests that oxLDL acts in a dual fashion to increase platelet reactivity – by increasing the localised concentrations of platelet agonists e.g. ADP, TxA_2 and reducing sensitivity to endogenous inhibitors; potentially lowering the threshold for activation *in vivo*.

Subsequent studies following on from this work could strengthen the hypothesis of oxLDL reducing platelet sensitivity to inhibitory agents by using other platelet assays to measure disinhibition e.g. aggregometry, FACS to confirm what was shown via the *ex vivo* thrombosis assay. A separate study investigating the molecular mechanisms may be beneficial and could be used as evidence to support cyclic nucleotide signalling as a possible therapeutic target. The clinical impact of this work could be improved by flowing blood samples of hyperlipidaemic patients through the *ex vivo* thrombosis assay to see whether the effects of oxLDL *in vivo* recreate what was shown *in vitro*. It would also be of benefit to examine basal cyclic nucleotide levels of platelets from patients with hyperlipidamia and platelets treated with oxLDL as a way of comparing both techniques.

Receptor Involvement

Alongside changes in platelet function, we also invested significant resources into uncovering the mechanisms underpinning these changes. This is important for the eventual development of a therapeutic agent and provides insight into commonalities between disease processes in platelets and other cell types. CD36 is the most heavily researched receptor believed to play a central role in atherothrombosis. Pioneering work conducted by Podrez et al. showed convincingly that oxidised phospholipids are able to trigger platelet activation and oxidative stress, with genetic deletion of CD36 being highly cardioprotective (Podrez et al., 2007). Much of the attention of this work focuses on the role of CD36 in oxLDL signal transduction and largely supports the published literature (Podrez et al., 2007; Nergiz-Unal, Lamers, et al., 2011; Wraith et al., 2013; Magwenzi et al., 2015). We show convincingly that blocking CD36 via use of the antibody FA6.152 causes a reduction in platelet adhesion to oxLDL under static conditions and $CD36^{-/-}$ mice are resistant to the effects of KOdiA-PC under flow. The limitations of this work is that it relies on pharmaceutical inhibitors and mouse models, both of which may not truly reflect the situation with human platelets. One possible method to avoid this is to conduct similar experiments using platelets with low levels of CD36 present, similar to previously published work investigating the role of CD36 in collagen signalling (Daniel et al., 1994). Genetic studies of CD36 in Asian and African populations have shown that 3-8% of individuals do not possess CD36 on platelets, deemed the Nak^a phenotype (Yamamoto et al., 1990; Curtis and Aster, 1996). It would be of interest to investigate the effects of oxLDL on these populations to determine whether these platelets display the same functional and signalling changes as CD36 positive platelets. Moreover, CD36 expression varies widely amongst populations, from 200-14,000 copies (Ghosh et al., 2011b) and it would be interesting to correlate CD36 copy number to risk of oxLDL mediated hyperactivity. Nevertheless, our data does confirm a central role for CD36 in oxLDL mediated platelet activation.

One other aspect of CD36 biology not investigated in this series of work is its ability to form receptor complexes. Whilst CD36 has been shown in platelets to trigger signalling, few studies have looked at whether CD36 is signalling alone or as part of a receptor complex. Notable receptors CD36 has been previously associated with include TLR2 (Triantafilou et al., 2006), TLR4 (Stewart et al., 2010), TLR6 (Triantafilou et al., 2006; Stewart et al., 2010) and CD9 (Miao et al., 2001) and it is considered that it is this ability to act as a co-receptor which allows CD36 to bind a range of different ligands. Whilst many of these studies have been performed in leukocytes and transfected cell models, it raises the interesting question of whether CD36 requires other receptors in platelets. One possible candidate receptor that CD36 may partner with is GPVI. CD36 and GPVI share many similar signalling proteins in their respective signalling pathways (Watson et al., 2005; Nergiz-Unal, Lamers, et al., 2011; Wraith et al., 2013) and evidence suggests that they are both present in lipid rafts (Zeng et al., 2003; Quinter et al., 2007) – areas of the membrane which act as signalling hubs (Locke et al., 2002). We hypothesise that CD36 may interact with GPVI on platelets to signal and that targeting a novel, possibly platelet-specific receptor complex may serve as a drug target.

The receptor LOX-1 has also been implicated in the atherothrombosis process (Ogura et al., 2009; Chan et al., 2013). LOX-1 is believed to play an important role in endothelial cell homeostasis (Hu et al., 2007), but also binds oxLDL to induce apoptosis (Yoshimoto et al., 2011). LOX-1 has also been shown in several reports to be present on platelets (Chen et al., 2001; Marwali et al., 2007; Chan et al., 2013), although very few studies exist describing the role that it serves. One of the major hurdles for studying LOX-1 in platelets is the lack of high-quality tools. To date, no commercially available antibodies exist able to blot the protein and similarly, the blocking antibody is from another research group which makes availability difficult. Using a blocking antibody for LOX-1, we showed inhibition of LOX-1 reduced platelet adhesion to oxLDL, although this result was not repeated using LOX-1 deficient mice. On reflection, use of an *ex vivo* thrombosis assay may have provided a more detailed insight into the role of this protein and reveal any possible interplay

between it and CD36. Whilst we show limited data supporting the role of LOX-1, its expression upon platelet activation would be highly relevant to disease states. It is reported to act as a redox sensor in endothelial cells (Sakamoto et al., 2009) and be capable of signalling to activate PKC (Ogura et al., 2009; Xu et al., 2013). One possible theory is that LOX-1 may 'tip the balance', causing further activation of PKC and causing increased platelet activation and degranulation.

It is important to remember that platelets may also possess other receptors capable of binding to oxLDL. SR-A, SR-BI and CD68 have all previously been shown to bind to oxidised lipoproteins (Gillotte-Taylor et al., 2001; Korporaal et al., 2007; Daub, Siegel-Axel, et al., 2010) and may play a potentially important role in atherothrombosis. Use of mouse models deficient in these receptors would be the simplest method of assessing their relative importance since very few pharmaceutical inhibitors exist for these targets. This could be expanded further by tying down different receptors to differentially modified LDL species, as different receptors may bind different oxidised lipids and/or modified protein residues present.

Intracellular signalling events induced by oxLDL

Having established that CD36 is likely the predominant receptor involved in oxLDL signalling in platelets, we wanted to determine what signalling events were occurring upon ligation. Previous studies have shown that CD36 triggers a tyrosine kinase signalling cascade (Wraith et al., 2013) and this notion is supported by evidence showing CD36 associating with the Src kinases Fyn and Lyn (Huang et al., 1991). We confirmed that oxLDL was able to induce phosphotyrosine activity in platelet lysates and specifically looked at Src family kinases and Syk, both of which are believed to be involved in platelet signalling processes. Both SFKs and Syk were shown to be phosphorylated in response to oxLDL and inhibition of these proteins inhibited platelet spreading to an oxLDL coated matrix. These findings support the previous claims that oxLDL signalling evokes a tyrosine kinase response, although this argument could be supported further by use of mice

deficient in Src kinases and Syk to complement the work performed with pharmaceutical inhibitors.

Since we hypothesised that oxLDL ligation to platelets would result in the formation of a signalosome, we wanted to look at other proteins typically involved in this process. The lipid kinase PI3-K has been shown to be present in signalosomes formed in response to collagen and fibrinogen and acts to convert PIP_2 into PIP_3 (Bodin et al., 2003). The localised increase in PIP_3 serves multiple purposes; it causes activation of the protein kinase Akt and increase the substrate available for PLC. We showed that inhibition of PI3-K by use of the inhibitor LY294002 caused a decrease in platelet adhesion to oxLDL, indicating that it may be involved in oxLDL mediated signalosome formation. However, it would be interesting to identify which isoform of PI3-K is activated and whether activation of PI3-K downstream of oxLDL activates Akt to propagate further signalling. Particular emphasis in the latter portion of the work was placed on the ability of oxLDL to activate PLCγ2. We showed that PLCγ2 was able to become phosphorylated by oxLDL, in line with published literature (Wraith et al., 2013), but advanced current knowledge by use of a PLCγ2 deficient mouse model. We hypothesised that, having shown PLCγ2 to become phosphorylated by oxLDL, inhibition of PLCγ2 should have a marked effect on platelet activation. However, the data showed that mice deficient in PLCγ2 were still able to respond to KOdiA-PC, raising the question as to whether PLCγ2 is required for oxidised phospholipid mediated activation. It would be interesting to confirm these findings further using the PLCγ2 deficient mice in other functional assays e.g. FACS, *ex vivo* thrombosis assays. Raising sufficient numbers of mice was difficult in this study due to poor breeding, short lifespans (Corroborated by Wang et al. 2000) and high costs and this may be prohibitive for this line of enquiry in the future. Ideally, it would be of interest to place PLCγ2$^{-/-}$ mice on a high-fat diet to model clinical hyperlipidaemia, although this is not possible using the current strain. One possible way to counter this would be to use an obesity model (ApoE or LDLR$^{-/-}$) irradiated strain and transfuse with PLCγ2$^{-/-}$ bone marrow

or use of a platelet specific PLC$\gamma 2^{-/-}$ mutant. This would allow researchers to determine the effect of hyperlipidaemia without the deleterious effects of the global knockout.

The activation of PLCγ2 requires the involvement of many proteins to form a 'signalosome' (Watson et al., 2005). These additional proteins act as adaptor proteins which serve by either anchoring PLCγ2 to the plasma membrane or allowing optimal folding of the PLCγ2 protein, allowing full enzymatic activity. These proteins include, but are not limited to: LAT (Asazuma et al., 2000), SLP76 (Gross et al., 1999), BTK (Oda et al., 2000) and Rac1 (Pleines et al., 2009) and have not been investigated in platelets stimulated with oxLDL. However, these proteins have been shown to play an important role in signalling downstream of collagen/CRP (Watson et al., 2005) and it would be interesting to compare the full signalosomes of these stimulation events to determine differences between agonists.

Activation of PLCγ2 results in the mobilisation Ca^{2+} stores and activation of PKC. Whilst it is generally accepted that oxLDL is able to trigger Ca^{2+} release (Korporaal et al., 2005; Nergiz-Unal, Lamers, et al., 2011), the role of PKC remains less well characterised. A recent study by Magwenzi et al. showed that oxLDL stimulation of platelets caused an increase in phosphorylated PKC substrates, leading to the generation of ROS. However, no specific isoform of PKC was shown to be activated. We showed limited evidence that oxLDL can cause an increase in phosphorylated PKC substrates and in particular, phosphorylation of the common PKC target Pleckstrin. Phosphorylation of Pleckstrin supports data with the anti-phosphoPKC substrate antibody although does not indicate which isoform is involved. To investigate this further, it would be interesting to try a similar technique with isoform specific protein targets. However, there is a high degree of redundancy between isoforms, making this a highly challenging field of investigation. Knockout mice would likely be the most effective way of answering this question, although this is further complicated by differences in PKC isoforms present between human and mouse platelets and a high level of redundancy between isoforms (Harper and Poole, 2007; Harper and Poole, 2010).

PKC has multiple targets in platelets, including NADPH oxidase (Magwenzi et al., 2015), SNARE proteins (Lian et al., 2009) and talin (Das et al., 2014) which are all required for activation. One particular group of enzymes, phosphodiesterases, are activated by PKA, PKG and PKC and are involved in the regulation of the platelet to cyclic nucleotide signalling (Hunter et al., 2009). Platelets possess three members of this family – PDE3a, PDE2a and PDE5, although it is PDE3a which is considered to have the largest role (Schwarz et al., 2001). PDEs work by catalysing the reaction cAMP/cGMP to AMP/GMP and therefore reducing the activation of PKA or PKG respectively. It is this action which may be directly responsible for the ability of oxLDL to prevent inhibitory signalling (Law, Woodward, Naseem – Unpublished data). Conversely, research in a cancer cell line has also shown that activated Syk is able to directly inactivate PKA (Yu et al., 2013) and this may also occur in platelets. Significant further research is required in order to answer this interesting question.

Updated OxLDL - Platelet Signalling Model

Using data presented in this work and published literature, we illustrate an updated model concept for the activation of platelets by oxLDL.

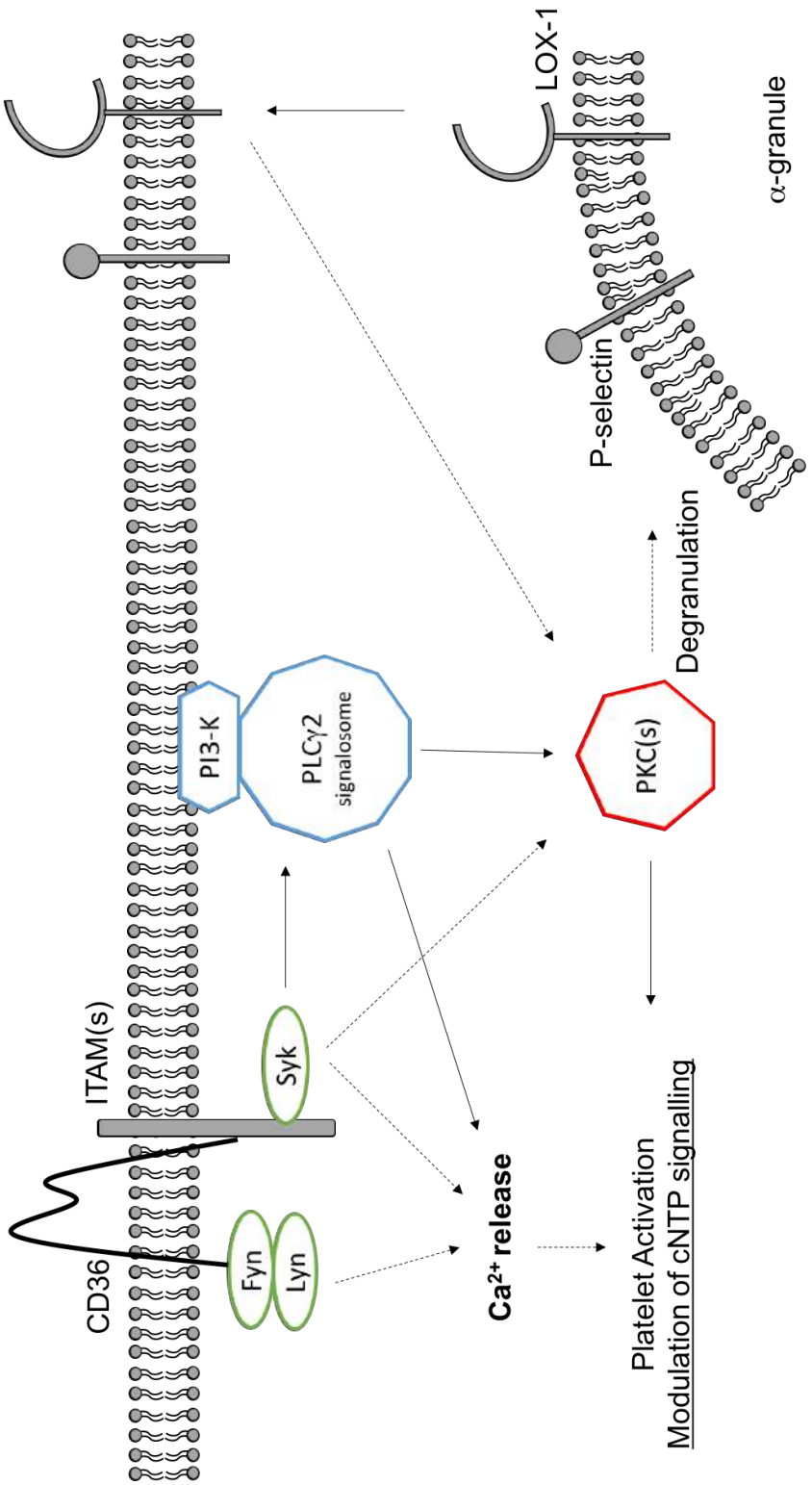


Figure 6.1 – Updated model of oxLDL signalling in human platelets. Green - tyrosine kinases, blue – lipid kinases, red – Ser/Thr kinases.

Upon oxLDL/oxidised phospholipid ligation to CD36, associated SFKs, namely Fyn and Lyn are able to cause the phosphorylation of an ITAM (Wraith & Naseem, Unpublished data) resulting in the activation of Syk. Activation of Syk is likely to cause the phosphorylation of multiple proteins, allowing the formation of a signalosome with PLCγ2 at the core. PLCγ2-independent signalling events may also occur, resulting in additional activation of PKC and/or Ca^{2+} mobilisation. The data suggests that optimal signalling is also likely to rely upon the activation of PI3-K to anchor PLCγ2 to the plasma membrane. Upon complete signalosome formation, PLCγ2 becomes activated, yielding both Ca^{2+} and DAG. The result of this is multiple aspects of platelet activation. PKC becomes active, although the specific isoform is currently unknown and therefore it could be downstream of both DAG and/or Ca^{2+}. Work from other platelet agonists has shown that PKC is integral to degranulation and there is no evidence to believe this is not in the case in oxLDL signalling. PKC could cause the release of α-granules, allowing both LOX-1 and P-selectin to be expressed. LOX-1 could then serve in a fashion analogous to P2Y/TP and support platelet activation to oxLDL by reinforcing signalling to PKC. In addition, PKC causes the activation of phosphodiesterases resulting in the modulation of cyclic nucleotide signalling. A combination of these responses could potentially be the cause of platelet hyperactivity in hyperlipidaemia.

Future Work

The completion of this body of work raises many new questions. However, questions worth investigating in detail include:

1. *What effects does mmLDL exert on platelet function? Does it signal via a different mechanism to oxLDL to induce platelet activation?*

Early experimentation showed that we were able to produce a minimally modified form of LDL via oxidised nLDL at 4^{o}C. It would be interesting to see whether this form of modified LDL is able to activate platelets via a CD36-dependent mechanism. Previously published data has shown that mmLDL is able to activate platelets via use of the lysophosphatidic acid receptor (Maschberger et al., 2000), activating Syk but not triggering Ca^{2+} mobilisation. Studies into the role of mmLDL on platelet activation would benefit from a full characterisation of platelet function. These experiments should include degranulation assays via FACS, platelet adhesion and *ex vivo* thrombosis assays in order to ascertain the potential platelet activating ability of mmLDL under physiological shear conditions. Moreover, having established whether mmLDL can sufficiently activate platelets, a series of investigative signalling experiments should be conducted to tie mmLDL to a specific receptor(s). It would be of benefit to examine the ability of mmLDL to activate murine platelets too as this may present differently to oxLDL and prevent the use of oxidised phospholipids as ligands. Signalling experiments would benefit significantly by use of a mass spectroscopy approach as conducted by Zimman et al. and this would give an insight into the signalling pathways activated.

2. *What role does LOX-1 play in platelets? Is LOX-1 integral to platelet activation in response to oxLDL?*

Despite our best efforts, we have not shown a conclusive role for LOX-1 in platelet activation. This is largely due to poor tools for blotting for the receptor, meaning that immunoprecipitation and assessment of associated signalling molecules was not possible. Investment into a suitable blotting antibody would be paramount for this work to

progress significantly. Moreover, use of the LOX-$1^{-/-}$ mouse could be used for *in vivo* studies and it would be of great interest to put LOX-$1^{-/-}$ mice onto a high-fat diet and assess platelet function. This approach has been used previously by Magwenzi et al. who compared wild-type to $NOX2^{-/-}$ mice on a high-fat diet, highlighting the role of ROS generation in response to hyperlipidaemia.

It may also be of interest to examine the role of LOX-1 under haemostatic conditions. LOX-1 has also been previously shown to bind activated platelets (Kakutani et al., 2000) via recognition of phosphatidylserine (Murphy et al., 2006) and therefore could be involved in platelet aggregation.

3. *What other proteins are involved in the oxLDL mediated 'signalosome'? How does the signalosome compare to those formed in response to GPVI and $\alpha_{IIb}\beta_3$ receptor ligation?*

Extensive investigation of the signalosome has been conducted downstream of collagen/CRP and GPVI and fibrinogen and integrin $\alpha_{IIb}\beta_3$ (Watson et al., 2005). These studies have revealed subtle differences between the signalosomes formed which may in part explain differences between the platelet activating ability of the ligands. Whilst the phosphorylation of SFK and Syk downstream of oxLDL is becoming increasingly accepted, the activation of PLCγ2 and the proteins involved with this remain unknown. An activity assay measuring the outputs of PLCγ2 e.g. IP_3 would be of direct benefit and would support the notion of phospholipase involvement. It would be of interest to stimulate platelets with oxLDL and measure levels of IP_3 within the cell and the role of PLCγ2 could be supported further by use of the PLC$\gamma2^{-/-}$ mouse. Since ITAM-dependent signalosomes tend to incorporate multiple proteins, e.g. Btk, SLP76, LAT (Boulaftali et al., 2014), it would be worthwhile investigating their involvement downstream of oxLDL. Numerous knockout mouse lines exist to investigate these questions and this could be supported with immunoblotting data.

4. *What are the mechanisms underpinning the ability of oxLDL to negate the effects of inhibitory molecules? Can this be blocked pharmaceutically to restore their effect and lower platelet activation in vivo?*

In this piece of work, we show evidence that oxLDL can cause platelet disinhibition by modulating the effects of cyclic nucleotides. However, it would be of benefit to support these findings via use of other assays, including P-selectin expression via FACS and platelet adhesion/spreading. In order to test the hypothesis that phosphodiesterase activation is responsible for platelet hyperactivity, PDEs could be blocked by pharmaceutical inhibitors and platelet function assessed. It would improve clinical significance to compare platelet populations from healthy and hyperlipidaemic patients, although many of these patients are not treatment naïve and therefore it could be potentially difficult to recruit. Since PDEs have been previously targeted in the treatment of other cardiovascular disorders (Feldman and McNamara, 2002), data may also already exist looking at the rate of atherosclerotic plaque rupture/platelet function in these groups and may provide a valuable resource to probe further.

Another approach would be to conduct a mass spectroscopy study – treat platelets with oxLDL and look at which proteins become phosphorylated in order to provide new targets to investigate.

Project Conclusions

In summary, the data presented throughout this body of work clearly shows the pathogenic action of oxLDL and the oxidised phospholipid KOdiA-PC. The data shown is in accordance with both published scientific reports and clinical findings. Moreover, we have shed light on the mechanisms behind platelet hyperactivity, specifically the role of CD36 and associated signalling pathway and supported previous studies. Crucially, the work presented in this thesis has provided novel lines of inquiry for future studies into hyperlipidaemia-induced platelet hyperactivity.

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