The Influence of Oxidised Low Density Lipoproteins on Platelet Activation

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A thesis submitted for the degree of Doctor of Philosophy in Medical Sciences

University of Hull and University of York

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Abstract

The oxidation of low density lipoproteins (LDL) may contribute to platelet hyperactivity and thrombotic events in cardiovascular diseases. Although these pathological particles are known to stimulate platelet activation, the exact signalling mechanisms involved are poorly defined. The aim of this study was to explore the mechanisms underpinning platelet activation by oxidised LDL (oxLDL). OxLDL but not native LDL (nLDL) induced a modest degree of aggregation, which was ADP dependent. However, oxLDL was able to induce shape change, one of the earliest stages of platelet activation, independently of secondary agonists. Platelet shape change requires the protein myosin, the activity of which is controlled by the phosphorylation of its regulatory light chains (MLC) at serine\(^19\). Physiological platelet agonists activating platelets through G protein coupled receptors stimulate MLC\(^{\text{Ser19}}\) phosphorylation by the activation of dual pathways that lead to a Ca\(^{2+}\) dependent activation of MLC kinase (MLCK) and the Rho kinase dependent inhibition of MLC phosphatase (MLCP). OxLDL induced platelet shape change correlated with MLC\(^{\text{Ser19}}\) phosphorylation. OxLDL also stimulated a tyrosine kinase pathway involving the proteins Syk and PLC\(\gamma\)2, which were confirmed to be involved in MLC\(^{\text{Ser19}}\) phosphorylation. Furthermore, oxLDL triggered a second pathway involving the activation of RhoA leading to the activation of Rho kinase and subsequent inhibition of MLCP. A receptor that has been continuously linked to oxLDL induced platelet activation is the scavenger receptor CD36. The expression of this scavenger receptor was found to increase upon stimulation with a range of platelet agonists. Furthermore, platelet shape change was discovered to be downstream of CD36. The findings of this report therefore elucidate two novel
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signalling pathways in response to CD36 ligation with oxLDL. Enhancements in understanding how this pathological ligand can contribute to platelet activation may lead to improvements in the treatments available for patients who suffer with hyperlipidaemia and cardiovascular diseases.
Publications

Published articles


Oral presentations

Oxidised low-density lipoproteins induce platelet activation and shape change through novel CD36 dependent signalling pathways. *British Society for Haemostasis and Thrombosis/UK Platelet Group Meeting, Bath, UK* (2012). (Runner-up of best oral presentation award)

Publications

Novel signalling pathways induced by oxidised low-density lipoproteins leading to platelet activation. *Centre for Cardiovascular and Metabolic Research Seminar, Hull, UK* (2012)


Poster presentations


**Wraith, K.S.,** Magwenzi, S., Aburima, A., Wen, Y., Leake, D. & Naseem, K.M. Oxidised low-density lipoproteins induce platelet activation and shape change through a novel
Publications


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<td>ACD</td>
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<td>Adenosine 5'-diphosphate</td>
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<td>Advanced glycation end product</td>
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<td>BSA</td>
<td>Bovine serum albumin</td>
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<td>Ca$^{2+}$</td>
<td>Calcium ion</td>
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<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
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<td>CD36</td>
<td>Cluster of differentiation 36</td>
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<td>cGMP</td>
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<td>Cu$^{2+}$</td>
<td>Copper ion</td>
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<td>CVD</td>
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<td>DAG</td>
<td>1, 2 diacylglycerol</td>
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<tr>
<td>DM</td>
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<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
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<td>Abbreviations</td>
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<td>-----------------------------------------</td>
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<tr>
<td>DTS</td>
<td>Dense tubular system</td>
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<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence</td>
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<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
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<td>Ethyleneglycoltetraacetic acid</td>
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<td>FcR γ-chain</td>
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<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
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<tr>
<td>FVα</td>
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<td>FXa</td>
<td>Activated factor X</td>
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<td>Gads</td>
<td>GRB2-related adaptor protein</td>
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<td>GAP</td>
<td>GTPase-activating protein</td>
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<tr>
<td>GDI</td>
<td>GTPase-dissociation inhibitors</td>
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<td>GDP</td>
<td>Guanosine diphosphate</td>
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<tr>
<td>GP</td>
<td>Glycoprotein</td>
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<tr>
<td>GPIb-V-IX</td>
<td>Glycoprotein Ib-V-IX receptor complex</td>
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<td>GPCR</td>
<td>G protein coupled receptor</td>
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<td>GPVI</td>
<td>Glycoprotein VI</td>
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<tr>
<td>GTP</td>
<td>Guanine 5'-triphosphate</td>
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<td>HDL</td>
<td>High density lipoproteins</td>
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<td>HDS</td>
<td>High density solution</td>
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<td>HEPES</td>
<td>N-(2-Hydroxyethyl) piperazine-N'-(2ethanesulfonic acid)</td>
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<td>HRP</td>
<td>Horseradish peroxidase</td>
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<td>IDL</td>
<td>Intermediate density lipoproteins</td>
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<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
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<tr>
<td>IP₃</td>
<td>Inositol 1,5,4-triphosphate</td>
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<tr>
<td>ITAM</td>
<td>Immunoreceptor tyrosine-based activation motif</td>
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<tr>
<td>JNK</td>
<td>c-Jun amino-terminal kinase</td>
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<tr>
<td>LAT</td>
<td>Linker for activation of T cells</td>
<td></td>
</tr>
<tr>
<td>LCFA</td>
<td>Long chain fatty acid</td>
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<tr>
<td>LDL</td>
<td>Low density lipoproteins</td>
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# Abbreviations

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<tr>
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<tr>
<td>LIMP-II</td>
<td>Lysosomal integral membrane protein-II</td>
</tr>
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<td>LPA</td>
<td>Lysophosphatidic acid</td>
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<td>MAPK</td>
<td>Mitogen activated protein kinases</td>
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<td>ML-7</td>
<td>1-(5-Iodonaphthalene-1-sulfonyl)homopiperazine</td>
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<td>MLC</td>
<td>Myosin light chain</td>
</tr>
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<td>Myosin light chain kinase</td>
</tr>
<tr>
<td>MLCP</td>
<td>Myosin light chain phosphatase</td>
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<tr>
<td>MOPS</td>
<td>3-(N-Morpholino)propanesulfonic acid</td>
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<tr>
<td>MP</td>
<td>Microparticle</td>
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<tr>
<td>nLDL</td>
<td>Native low density lipoprotein</td>
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<td>NO</td>
<td>Nitric oxide</td>
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<td>OCS</td>
<td>Open canalicular system</td>
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<td>oxLDL</td>
<td>Oxidised low density lipoproteins</td>
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<td>PAR1</td>
<td>Protease-activated receptor-1</td>
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<td>PLCβ</td>
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<td>PLCγ₂</td>
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<td>PMSF</td>
<td>Phenyl methyl sulphonyl fluoride</td>
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### Abbreviations

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<td>PP3</td>
<td>4-Amino-7-phenylpyrazol[3,4-d]pyrimidine</td>
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<td>PPP</td>
<td>Platelet poor plasma</td>
</tr>
<tr>
<td>PRP</td>
<td>Platelet rich plasma</td>
</tr>
<tr>
<td>PS</td>
<td>Phosphatidylserine</td>
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<td>PSGL-1</td>
<td>P-selectin glycoprotein ligand 1</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene fluoride</td>
</tr>
<tr>
<td>REM</td>
<td>Relative electrophoretic mobility</td>
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<tr>
<td>RGDS</td>
<td>Arginine-glycine-aspartic acid-serine</td>
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<tr>
<td>ROCK</td>
<td>Rho kinase</td>
</tr>
<tr>
<td>RhoGEF</td>
<td>Rho guanine nucleotide exchange factor</td>
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<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
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<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate-polyacrylamide gel electrophoresis</td>
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<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
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<tr>
<td>sGC</td>
<td>Soluble guanylyl cyclase</td>
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<td>SH2</td>
<td>Src homology 2</td>
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<td>SH3</td>
<td>Src homology 3</td>
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<td>SLP-76</td>
<td>Src homology 2 domain-containing leukocyte protein of 76kDa</td>
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Authors declaration

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Chapter 1

Chapter 1: General introduction

1.1 Introduction

Haemostasis is a dynamic and invaluable system involved in ensuring that the circulatory system remains in a quiescent and balanced state. In a healthy individual, this system prevents significant blood loss when the body has sustained injury. There are three central elements that contribute to this action. Firstly, the vascular wall, which, when damaged, exposes prothrombotic properties such as collagen and undergoes vasoconstriction to limit the amount of blood flow to the wounded area. Secondly, blood platelets, which act to form a primary haemostatic plug. Thirdly, the coagulation cascade, which culminates in the formation of a fibrin mesh to stabilise thrombi that are formed.

The complexity and function of a platelet is made even more remarkable on account of their tiny size. They are involved in the essential role of fastening together to form thrombi, which, along with fibrin, makes up the plug formed at the site of injury thus impeding blood loss. However, even though thrombus formation is a vital physiological response, it also heavily contributes to the pathological consequences of cardiovascular diseases (CVD). Therefore, it is vital in cardiovascular research to consider how platelets function in order to understand the role that they play in prothrombotic disorders.
Chapter 1

1.2 Platelets

When platelets encounter damage to the blood vessel wall they are rapidly activated. This process involves cytoskeletal reorganisation to facilitate shape change, granule secretion to release soluble agonists that promote activation, and integrin activation leading to platelet-platelet and platelet-vessel wall interactions. However, platelet hyperactivity is linked with many diseases including arterial thrombosis. Therefore, understanding the mechanisms by which platelets are activated both in healthy and pathological circumstances is invaluable if treatments to haemostatic dysfunction are to be identified. The roles that platelets play in haemostasis and the signalling involved in this will be discussed in further detail in the subsequent sections.

1.2.1 Platelet formation

Platelets are formed from haemostatic precursor cells called megakaryocytes. Within the bone marrow, megakaryocytes form long projections emanating from their cytoplasm. This process can occur over a period of 4-10 hours and results in the entire megakaryocyte becoming a network of tubules, which eventually separate from the nucleus. Singular proplatelet strands are released from the proplatelet mass and individual platelets are subsequently expelled from their ends into the circulation (Italiano and Hartwig, 2007). Although megakaryocytes are majorly found in the bone marrow, platelet formation has been suggested to also occur in other tissues such as the lung and the bloodstream directly (Zucker-Franklin and Philipp, 2000, Behnke and Forer, 1998). The development of a platelet is of course extremely important as it denotes their structural as well as functional attributes.
1.2.2 Platelet structure

Platelets are small cells of approximately 2-5µm in diameter but possess a highly complex ultrastructure. Under non-stimulated conditions platelets circulate in a round discoid shape but upon activation can undergo a rapid morphological transformation to pertain an irregular shape with an increased surface area, aiding in clot formation. The structure of a platelet is illustrated in Figure 1.1.
Figure 1.1 Structure of a platelet
Scanning electron microscopy image illustrating the structure and components of a platelet. Platelets contain an outer membrane consisting of the glycocalyx, phospholipid bilayer and submembrane area. Beneath this is the platelet’s cytoskeleton consisting of microtubules and actin filaments. The core of a platelet termed the cytosol contains numerous components such as α and dense granules. Adapted from (White, 2007).
1.2.2.1 Platelet membrane

The exterior surface of a platelet is composed of several layers. The outermost layer is termed the glycocalyx and consists of a thick coating found on the exterior of a number of cell types made up of polysaccharide structures (Bennett, 1962). This layer contains glycoprotein (GP) receptors that are important for platelet activation. Immediately beneath the glycocalyx is the unit membrane composed of a lipid bilayer. The lipid bilayer covers the surface of the cell as well as the open canalicular system (OCS), a series of folds within the membrane which aid in increasing surface area upon activation and is involved in the process of granule secretion (Escolar et al., 1989, White and Krumwiede, 1987). A key feature of the phospholipid membrane of platelets is the presence of lipid rafts, dynamic assemblies of sphingomyelin and cholesterol within the membrane. Lipid rafts form microdomains that are involved in focusing signalling events by clustering receptors, kinases and adaptor proteins (Lopez et al., 2005). The area directly beneath the unit membrane is the submembrane area, which remains distinct from organelles of the cytoplasm and is the site where the intracellular portions of transmembrane receptors are found (White, 2007).

1.2.2.2 Platelet cytoskeleton

The platelet cytoskeleton is a support system that maintains a round discoid shape whilst resting, but also undergoes a rapid reorganisation upon activation to facilitate platelet shape change. Firstly, this support system includes microtubules, which were identified in platelets many decades ago and are arranged in a coil formation just below the membrane and span the circumference of the cell (Behnke, 1965). They
function to support the discoid shape of the inactive platelet, however, upon
activation microtubules contract and centralise, which results in the loss of the circular
shape (White, 1968). In addition to microtubules, platelets also possess a network of
actin polymers, which make up the main cytoskeletal framework of the cell. These
actin polymers are not only the major source of cytoskeletal reorganisation during
shape change but are also involved in maintaining the separation of internal
organelles. Actin associates with actin crosslinking proteins, which interconnect actin
polymers and play a role in localising molecules such as GTPases, kinases,
phosphatases and transmembrane proteins to the plasma membrane. Therefore, the
cytoskeleton has a number of crucial roles in both shape change and localisation of
activation molecules (Hartwig, 2007).

1.2.2.3 Platelet cytosol
The platelet cytosol contains the internal organelles and numerous granules required
for platelet function. Platelets possess three main types of granules including α-
granules, dense granules and lysosomes. The most abundant are α-granules of which
there are around 50-80 per platelet and have an approximate size of 200-500nm
diameter (Blair and Flaumenhaft, 2009). There are around 10 times fewer dense
granules than α-granules and even less lysosomes. The contents of platelet granules
are summarised in Table 1.1. Briefly, α-granules contents are numerous and include
adhesion molecules, chemokines, coagulation factors, fibrinolytic molecules, growth
factors and immunological molecules. In general, the release of these factors aids in
coagulation, innate immunity and vessel wall repair. Dense granules contain small
molecules that enhance platelet activation including nucleotides such as adenosine 5'-diphosphate (ADP) and adenosine 5'-triphosphate (ATP) as well as Ca\(^{2+}\) (McNicol and Israel, 1999). The lysosomes of platelets are few but contain hydrolase enzymes, which are secreted upon activation and involved in the digestion of cellular debris (Ciferri et al., 2000). As well as granules, the platelet cytoplasm is also the location of free glycogen particles, glycogen containing glycosomes and mitochondria, which are required for energy metabolism. The dense tubular system (DTS), which is the remnants of smooth endoplasmic reticulum of the original megakaryocyte, is also critical as this is the location of stored intracellular Ca\(^{2+}\) (White, 2007).
**Table 1.1: Components of platelet granules**

Table illustrating the various contents within α-granules, dense granules and lysosomes of platelets. Taken from (McNicol and Israels, 1999)

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<thead>
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1.2.3 Platelet adhesion, activation and thrombus formation

The endothelium acts as a protective surface that prevents blood from escaping into the tissues. Platelets circulate in the blood scanning the endothelium for areas of damage. The larger cells of the blood force platelets to the periphery of the vessels where they are ideally placed to perform this function. When a blood vessel is injured a series of events are initiated involving a plethora of cells and coagulation factors to prevent significant blood loss. Platelet activation contributes substantially to the formation of a thrombus. There are a number of steps in platelet activation and it appears that many of these events occur simultaneously as opposed to one after another. Platelets, however, react quickly and efficiently to impede blood flow at the site of injury.

1.2.3.1 Platelet adhesion

Upon injury to a blood vessel, a number of extracellular matrix (ECM) proteins such as collagen and von Willebrand factor (vWF) are exposed to the flowing blood. The initial stage in the formation of a thrombus is adhesion of platelets to these ECM proteins. The process of adhesion differs depending on the type of blood vessel that injury has occurred to (Varga-Szabo et al., 2008). The sizes of blood vessels within the circulatory system differ, with blood flowing through these vessels at varying velocities. In the venous system platelets are in a low shear rate environment, that is, blood flows through these vessels at a relatively low speed and pressure. Conversely, small arteries, the microvasculature and obstructed vessels possess a high shear rate and so
blood flows through these vessels comparatively fast; therefore, platelets move past the ECM rapidly.

Under conditions of high shear stress, platelet adhesion to the damaged blood vessel is mediated through interactions with vWF. Soluble vWF found in the plasma is unable to interact with platelets as it is not in the correct confirmation. However, immobilised vWF under shear stress undergoes a conformational rearrangement from a globular to an extended shape (Siedlecki et al., 1996), which platelets can interact with. Immobilisation of vWF occurs on collagen fibres of the subendothelium, in particular, collagen types I and III which bind to the A3 domain of vWF and collagen type VI which binds to the A1 domain (Lankhof et al., 1996, Hoylaerts et al., 1997). Therefore, when collagen fibres are exposed during injury, immobilised vWF is also exposed. Platelets interact with vWF through GPIb-V-IX, which is made up of a complex of subunits GPIbα, GPIbβ, GPV and GPIX (Modderman et al., 1992) and is competent to bind vWF without prior activation. Interactions between GPIb-V-IX and vWF occur through the GPIbα subunit of the receptor and the A1 domain of vWF (Vicente et al., 1988, Sixma et al., 1991). The importance of the presence of GPIb-V-IX on platelets is apparent in the disease Bernard–Soulier syndrome, in which platelets have defective GPIb-V-IX complexes which results in macrothrombocytopenia and impaired platelet adhesion to the subendothelium (Nurden and Nurden, 2011).

Interactions between vWF and GPIb-V-IX are not stable due to the low affinity binding and the bonds between the two can break. However, initial tethering causes platelets
to slow and a “rolling” effect is seen where platelets reposition along the exposed ECM until stable adhesion can occur (Savage et al., 1996). Stable bonds can form between vWF and the integrin α_{III}β_{3} and also through several platelet receptor interactions with other ECM proteins. The main ECM constituent that platelets firmly adhere to is collagen. Platelets have at least two collagen receptors including GPVI, which is mainly involved in signalling and activation, and α_{2}β_{3}, an integrin involved in adhesion to collagen fibres (Santoro, 1986). As an integrin, α_{2}β_{3} is present on the platelet in an inactive confirmation and requires platelet activation and inside-out signalling in order to transform into a state with a high affinity for binding collagen (Jung and Moroi, 1998). Additionally, other platelet integrins involved in adhesion include α_{5}β_{1} which binds to fibronectin (Pytela et al., 1985) as well as α_{6}β_{1} which is a receptor for laminin (Sonnenberg et al., 1988).

The inside-out signalling required for early integrin activation is provided by GPVI. GPVI is non-covalently associated with an Fc receptor (FcR) γ-chain (Gibbins et al., 1997) as well as the Src kinases Fyn and Lyn, which bind to the proline rich region of the cytoplasmic tail of GPVI via their Src homology 3 (SH3) domain (Ezumi et al., 1998, Suzuki-Inoue et al., 2002). Ligation with collagen causes cross-linking of GPVI leading to the phosphorylation of the immunoreceptor tyrosine-based activation motif (ITAM) of the FcR γ-chain by the Src kinases Fyn and Lyn (Briddon and Watson, 1999) as well as recruitment of the complex into lipid rafts (Locke et al., 2002). Consequently, spleen tyrosine kinase (Syk) is recruited to the FcR γ-chain via its SH2 domain and becomes phosphorylated by Src kinases and autophosphorylated (Gibbins et al., 1996).
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The importance of the FcR γ-chain and Syk in GPVI signalling is illustrated through the use of mouse models that have had the genes to these proteins knocked-out, resulting in a loss of protein expression. These studies revealed that collagen stimulation was unable to induce tyrosine phosphorylation of Syk or phospholipase Cγ2 (PLCγ2) in FcR γ-chain deficient mice. Phosphorylation of PLCγ2 was also absent in mice deficient in Syk however FcR γ-chain phosphorylation was maintained, demonstrating that Syk is downstream of FcR γ-chain but upstream of PLCγ2. Furthermore, there was a reduction in aggregation and secretion responses observed in both knock-out animal models but importantly platelet responses to thrombin, which signals through GPCR, were maintained (Poole et al., 1997).

Following the activation of Syk, a downstream signalling cascade occurs involving the formation of a signalosome. Central to this signalosome are the adaptor proteins LAT, SLP-76 and Gads involved in organising effector proteins to allow signal transduction. Deletion of SLP-76 or LAT abolishes or strongly impairs platelet responses to GPVI signalling respectively, revealing their importance in this pathway (Judd et al., 2002). A key effector protein in GPVI signalling is PLCγ2, as illustrated above, phosphorylation of this protein is downstream of FcR γ-chain, Syk, SLP-76 and LAT. Absence of PLCγ2 results in a substantial reduction of platelet responses to GPVI agonists including reduced aggregation, P-selectin expression and fibrinogen binding (Suzuki-Inoue et al., 2003). Both Syk and PLCγ2 can become phosphorylated at several sites including tyrosine525, tyrosine526 and tyrosine352 on Syk and tyrosine753 and tyrosine759 on PLCγ2, with GPVI stimulation inducing phosphorylation at all of these sites (Suzuki-Inoue et
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al., 2004). Activation of PLCγ2 results in the formation of inositol 1,5,4-triphosphate (IP$_3$) and 1, 2 diacylglycerol (DAG), which are involved in the release of Ca$^{2+}$ from intracellular stores and the activation of PKC respectively. These are required by other aspects of platelet activation

1.2.3.2 Platelet shape change

Upon activation, platelets undergo a cytoskeletal reorganisation in order to change their shape and cover a larger surface area at the site of damage. Initial shape change involves the platelet adjusting from a discoid shape to a spherical one. This is followed by the formation of “finger-like” projections, or filopodia, and subsequently platelets flatten and spread over the affected surface (Figure 1.2). Mechanistically, shape change occurs through the polymerisation of actin filaments, which distorts the shape of the platelet resulting in filopodia (Hartwig, 2007). The mechanical force required for shape change is thought to be provided by the interactions of actin with the protein myosin IIA. The structure of myosin includes two globular head domains, which contain both ATP and actin binding sites. The head regions are each attached to an essential light chain, which stabilises this structure, as well as a regulatory light chain (MLC), which regulates the activity of myosin through phosphorylation at serine$^{19}$. Finally, this is followed by a long coiled-coil domain (Vicente-Manzanares et al., 2009) (Figure 1.3).
Figure 1.2: Platelet shape change
Scanning electron microscope images of non-stimulated and activated platelets. (A) The non-stimulated platelet possesses a round discoid shape with the surface having a convoluted appearance. (B) Projections emanate from the platelets central body during early shape change subsequent to activation. (C) Platelet spreading on an adherent surface increases the platelet surface area. Taken from (White, 2007).
Figure 1.3: Structure of non-muscle myosin IIA

Schematic diagram of the structure of non-muscle myosin IIA. Myosin contains two globular head domains, two essential light chains (ELC), two regulatory light chains (RLC) and two heavy chains. Taken from (Vicente-Manzanares et al., 2009).
It has been established for a number of years that MLC can become phosphorylated in platelets (Adelstein et al., 1973). Phosphorylation of MLC is associated with stimulation by platelet agonists and leads to an increase in Mg$^{2+}$ATPase activity and interaction with the platelet cytoskeleton. This suggested that MLC phosphorylation and subsequent myosin activation is required for actin-myosin associations and cytoskeletal rearrangement (Adelstein and Conti, 1975, Sellers et al., 1981, Fox and Phillips, 1982). Subsequently, phosphorylation of MLC was linked to the functional response of platelet shape change by a study that demonstrated the phosphorylation of MLC to marginally precede the shape change response when platelets were stimulated with several agonists (Daniel et al., 1984).

The phosphorylation of MLC is determined by the activity of two enzymes, the Ca$^{2+}$-calmodulin dependent myosin light chain kinase (MLCK) and the RhoA/Rho kinase dependent myosin light chain phosphatase (MLCP) (Bauer et al., 1999) (Figure 1.4). Major platelet agonists such as collagen, thrombin, ADP and Thromboxane A$_2$ (TXA$_2$) raise intracellular Ca$^{2+}$, which activates MLCK leading to the phosphorylation of MLC (Hathaway and Adelstein, 1979). To ensure this phosphorylation is maintained, the activity of MLCP can be simultaneously inhibited by G-protein coupled receptors (GPCR) linked to Go$_{12/13}$. Stimulation of these receptors leads to the activation of RhoA and subsequent activation Rho kinase (Klages et al., 1999). RhoA proteins are small GTPases which are found inactive when bound to guanine diphosphate (GDP) but become activated upon guanine triphosphate (GTP) binding. The activity of RhoA is controlled by several regulators including: Rho-guanine nucleotide exchange factors.
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(RhoGEFs), which induce the disassociation of GDP and subsequent association of GTP; GTPase-activating proteins (GAPs), which increase the GTPase activity, therefore inducing an inactivation of the protein; and GTPase-dissociation inhibitors (GDIs) which prevent the binding of GTP (Wettschureck and Offermanns, 2002). Once RhoA has activated Rho kinase, this protein then proceeds to inhibit MLCP, which occurs through the phosphorylation of two key sites, at thronine\textsuperscript{853} and threonine\textsuperscript{696} of its regulatory subunit designated MYPT1 (Kimura et al., 1996, Nakai et al., 1997, Feng et al., 1999). Rho kinase has also been implicated in the direct phosphorylation of MLC (Amano et al., 1996).

As GPVI and P2Y receptors are not directly linked to G\textsubscript{a}12/13, stimulation by collagen and ADP induce MLC phosphorylation predominantly though the Ca\textsuperscript{2+} pathway due to the activation of PLC\gamma2 and PLC\beta. On the other hand, thrombin and TXA\textsubscript{2} receptor stimulation, which are linked with G\textsubscript{a}12/13, results in both Ca\textsuperscript{2+} and Rho kinase pathways (Bauer et al., 1999). This illustrates how diverse platelet signalling induced by various agonists can lead to a similar functional response.
Figure 1.4: Signalling induced by G-protein coupled receptors leading to myosin light chain phosphorylation

Diagrammatic representation of the dual signalling pathways initiated to induce MLC phosphorylation. GPCRs associated with $G_{\alpha_q}$ initiate a rise in intracellular $Ca^{2+}$ resulting in the activation of MLCK and phosphorylation of MLC. Simultaneously, $G_{\alpha_{12/13}}$ receptor stimulation results in the activation of RhoA which goes on to activate Rho kinase (ROCK) leading to inhibition of MLCP and preventing the phosphatase from dephosphorylating MLC. Adapted from (Wettschureck and Offermanns, 2002).
1.2.3.3 Platelet secretion and secondary activation

Once platelets are primarily adhered to and activated by collagen in the ECM, secondary platelet activation occurs from stimulation by a number of agonists such as ADP, adrenalin, TXA\textsubscript{2} and thrombin. Platelets secrete soluble agonists, such as ADP, through the release of their granule contents by the conventional method of exocytosis, which relies on cytoskeletal reorganisation in order for platelet granules to come into close proximity and fuse with the OCS of the plasma membrane. The molecular mechanisms underlying platelet granule secretion involve a group of proteins called soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptors (SNAREs), which regulate granule fusion with the membrane. Interactions occur between SNAREs that are located on both the vesicle and plasma membranes, which brings these two structures into close proximity with one another (Flaumenhaft, 2003). Secretion of soluble agonists is critical for the recruitment of further platelets to the site of injury.

ADP released from dense granules has two purinergic receptors on the platelet's surface, P2Y\textsubscript{1} and P2Y\textsubscript{12}. P2Y\textsubscript{1} acts through G\alpha\textsubscript{q} and stimulates PLC\beta leading to IP\textsubscript{3} production and intracellular Ca\textsuperscript{2+} mobilisation as well as DAG production and PKC activation. Platelets deficient in P2Y\textsubscript{1} from genetically modified mice are unable to aggregate in response to ADP and low doses of collagen and lose the ability to change shape. P2Y\textsubscript{1} deficient mice also have an increase in tail bleeding time compared to wild type control mice (Fabre et al., 1999). P2Y\textsubscript{12} is coupled to G\alpha\textsubscript{i}, which is involved in the inhibition of adenylate cyclase (AC) and thereby decreases cyclic adenosine 5'-
monophosphate (cAMP) levels. This prevents platelets from being fully inhibited and allows for greater activation (Daniel et al., 1998, Jin et al., 1998). Utilisation of pharmacological inhibitors revealed that both of these receptors are required for full platelet aggregation induced by ADP (Jin and Kunapuli, 1998).

Adrenalin is a weak platelet agonist that binds to the $\alpha_{2A}$ adrenergic receptor, which is coupled to the $G_\alpha_z$ protein and, similar to $G_\alpha_i$ stimulation, reduces cAMP levels (Wong et al., 1992). Therefore, adrenalin also lessens the inhibition that platelets are constitutively under, enabling other platelet agonists to induce activation. Mice lacking the $G_\alpha_z$ protein have weakened responses to adrenalin and are unable to inhibit AC activity (Yang et al., 2000).

$\text{TXA}_2$ is produced through intracellular $\text{Ca}^{2+}$ mobilisation by platelet agonists, which activates cytoplasmic phospholipase $A_2$ (PLA$_2$). This cleaves phosphatidylcholine (PC) in the platelet membrane, liberating arachidonic acid, which is subsequently converted to $\text{TXA}_2$ by cyclooxygenase-1 (COX-1) and thromboxane synthase. Once produced, $\text{TXA}_2$ diffuses outside the platelet where it binds to its receptors TPA and TPB. The $\text{TXA}_2$ receptors are coupled to both $G_\alpha_q$ and $G_\alpha_{12/13}$ therefore also evoking PLCB activation and $\text{Ca}^{2+}$ mobilisation as well as initiating the RhoA pathway (Knezevic et al., 1993, Djellas et al., 1999).

Thrombin, a strong platelet agonist, is generated through the complex events of the coagulation cascade. It is cleaved from its inactive form prothrombin by
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prothrombinase, a complex involving activated factor X (FXa), activated factor V (FVa) and Ca\(^{2+}\) on the phosphatidylserine (PS) containing surface of the platelet membrane. Under resting conditions, platelets do not express PS on the outer surface of the membrane, however, upon activation this phospholipid becomes exposed to allow the formation of the prothrombinase complex (Wolberg, 2007, Riddel et al., 2007). Once converted to its active form, thrombin is involved in key responses required for thrombus formation including converting fibrinogen to fibrin in the ultimate step of the coagulation cascade to produce a fibrin mesh that strengthens clot formation. Thrombin also acts on platelets through the GPCRs protease-activated receptor-1 (PAR1) and protease-activated receptor-4 (PAR4) in humans (Kahn et al., 1998, Kahn et al., 1999). It activates platelets by cleaving the N-terminus of these receptors, which reveals a new sequence that acts as a tethered ligand to activate the receptor (Vu et al., 1991). Both PAR1 and PAR4 are associated with the G proteins G\(_{\alpha_q}\) and G\(_{\alpha_{12/13}}\) (Offermanns et al., 1994). Activation of G\(_{\alpha_q}\) results in stimulation of PLC\(\beta\), promoting IP\(_3\) production to raise intracellular Ca\(^{2+}\) levels as well as DAG production to activate PKC. Indeed, platelets lacking the G\(_{\alpha_q}\) subunit present with an inability to aggregate, stimulate IP\(_3\) production or raise intracellular Ca\(^{2+}\) levels to a number of platelet agonist as well as having a substantially increased tail bleeding time compared to wild type control mice (Offermanns et al., 1997). Stimulation of the G\(_{\alpha_{12/13}}\) subunit on the other hand initiates the RhoA/Rho kinase pathway culminating in the reorganisation of the actin cytoskeleton as mentioned above (Klages et al., 1999). Absence of G\(_{\alpha_{13}}\) in particular, results in a loss of RhoA activation and MLC phosphorylation as well as a loss of shape change and reduction in aggregation in response to thrombin and the
TXA$_2$ analogue U46619. Mice lacking G$\alpha_{12}$ and G$\alpha_{13}$ or G$\alpha_{13}$ alone also displayed increased bleeding times \textit{in vivo}, demonstrating the importance of this subunit (Moers et al., 2003). Intracellular signalling pathways initiated by the various agonists discussed here, and others, are illustrated in Figure 1.5.
Signalling pathways initiated in platelets by various agonists link together to induce maximal and efficient activation. GPCR signalling results in activation of the RhoA pathway, inhibition of the AC pathway and activation of PLCβ resulting in an increase in intracellular Ca\(^{2+}\) and PKC activation. GPVI, GPIb-V-IX and integrin signalling results in PLCγ2 activation leading to an increase in intracellular Ca\(^{2+}\) and PKC activation. Taken from (Varga-Szabo et al., 2008).
1.2.3.4 Platelet aggregation

The capture of free flowing platelets from the blood by platelets in the monolayer, leading to the formation of a thrombus, is a process termed platelet aggregation. Important to aggregation are a group of surface receptors called integrins, particularly $\alpha_{\text{IIb}}\beta_3$. Signalling events from GPVI and GPCRs transform these receptors from low affinity conformations in resting platelets to high affinity conformation in activated platelets. $\alpha_{\text{IIb}}\beta_3$ is a heterodimeric receptor composed of $\alpha_{\text{IIb}}$ and $\beta_3$ subunits each made up of a large extracellular domain, a short transmembrane region and an intracellular portion. The C-terminals of the subunits reside intracellularly while the N-terminals are extracellular (Shattil et al., 1998). Under non-stimulated conditions, integrins are in a "bent" conformation, resulting in a low affinity state for their ligands. However, stimulation of platelets results in intracellular signalling cascades which lead to the activation of integrins, a process known as inside-out signalling. Once bound to their ligand, integrins can then induce a signalling pathway resulting in further platelet activation, a process known as outside-in signalling (Shattil et al., 1998). All ligands of integrins possess an arginine, glycine, aspartic acid (RGD) sequence required for interactions to occur. $\alpha_{\text{IIb}}\beta_3$ can bind to many different ligands such as vWF, fibronectin and TSP-1, however, the main ligand which plays an essential role in platelet aggregation is fibrinogen. Platelets with active $\alpha_{\text{IIb}}\beta_3$ integrins aggregate by binding divalent plasma fibrinogen and multivalent vWF, which bridges associations between platelets in thrombi.
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1.2.4 Regulation of platelet function

While understandings of the processes that drive platelet activation have improved dramatically, it is important to recognise that these activating pathways must be regulated to maintain a quiescent state whilst platelets circulate in the blood stream. The most significant inhibitors of platelets are endothelial derived nitric oxide (NO) and prostacyclin (PGI$_2$) (Palmer et al., 1987, Weksler et al., 1977). The pathways initiated by these platelet inhibitors are illustrated in Figure 1.6.

NO is a free radical gaseous messenger produced by an enzymatic reaction of nitric oxide synthase (NOS) with the amino acid L-arginine. NO acts by entering cells and stimulating soluble guanylyl cyclase (sGC), which converts GTP into the second messenger cyclic guanosine monophosphate (cGMP) (Mellion et al., 1981). The main role of cGMP involves the activation of protein kinase G (PKG), which phosphorylates a number of proteins associated with the inhibition of platelet function. Mice deficient in PKG have been studied to elucidate its importance and findings have shown the life span of these mice to be dramatically reduced (Feil et al., 2003). Investigations have found that cGMP analogues cannot inhibit aggregation or induce the phosphorylation of vasodilator-stimulated phosphoprotein (VASP) in mouse platelets lacking PKG (Massberg et al., 1999).

PGI$_2$ is a lipid messenger synthesised from prostaglandin H$_2$ (PGH$_2$) by PGI$_2$ synthase subsequent to numerous enzyme mediated reactions. Inhibition of platelets by PGI$_2$ is initiated by its interaction with the G-protein coupled receptor IP. This receptor is
associated with a G-protein containing a Gαs subunit that activates AC, which converts ATP to cAMP (Best et al., 1977). This cyclic nucleotide is then able to activate protein kinase A (PKA). Mouse models have been used to clarify the significance of PGI₂ signalling in the regulation of platelet function by deletion of the IP receptor, which, upon damage to the endothelium, resulted in obstructive thrombi that lead to fatality in knockout mice compared to the formation of mural thrombi and survival of wild type mice (Murata et al., 1997).

Both cAMP and cGMP pathways inhibit platelet activity through a number of mechanisms including the inhibition of intracellular calcium elevation, cytoskeletal reorganisation, granule secretion and integrin activation (Schwarz et al., 2001). A major protein phosphorylated by PKA and PKG is VASP (Halbrugge et al., 1990), which contains three phosphorylation sites: serine₁⁵⁷, serine₂³⁹ and threonine²⁷⁸. The extent to which each site is phosphorylated depends on the kinase that acts upon the protein (Butt et al., 1994). PKG has a higher affinity for serine²³⁹ whereas PKA favours phosphorylation of serine₁⁵⁷. However, these kinases are not limited to these phosphorylation sites and some overlapping does occur. It is believed that phosphorylation of VASP can lead to the inhibition of the integrin α₁Iibβ₃ as well as the suppression of actin polymerisation and filament bundling (Horstrup et al., 1994, Harbeck et al., 2000), thus preventing fibrinogen binding and aggregation. It was discovered that cAMP and cGMP mediated platelet inhibition was reduced and binding of platelets to fibrinogen increased in VASP deficient mice, demonstrating its vital role (Aszodi et al., 1999). Other targets of the cAMP and cGMP pathways include the IP₃
receptor, which becomes phosphorylated in response to elevated cAMP and cGMP, preventing the Ca\(^{2+}\) releasing action of IP\(_3\) from intracellular stores (Cavallini et al., 1996); as well as the GPIbβ subunit of the GPIb-V-IX complex, which becomes phosphorylated and may be involved in the inhibition of actin polymerisation in response to elevated cAMP (Fox and Berndt, 1989).

The formation of cGMP and cAMP and thus the activation of PKG and PKA are regulated by a group of enzymes termed phosphodiesterases (PDE). These enzymes hydrolyse cyclic nucleotides into inactive forms, preventing levels from rising limitlessly. Several forms of PDEs have been identified in platelets including PDE2, PDE3 and PDE5 (Ito et al., 1996). PDE2 and PDE5 are stimulated by cGMP whereas PDE3 is inhibited by cGMP but stimulated by PKA. PDE2 and PDE3 are involved in hydrolysing both cGMP and cAMP whereas PDE5 preferentially acts upon cGMP (Schwarz et al., 2001).
Figure 1.6: Inhibitory signalling pathways in platelets
Schematic diagram illustrating the major inhibitory signalling pathways in platelets. NO diffuses through the cell membrane and interacts with sGC resulting in the formation of cGMP, which goes on to activate PKG. PGI₂ interacts with the membrane IP receptor, which associates with the G protein Gαs. This G protein subsequently activates AC leading to the production of cAMP and activation of PKA. The resultant effect of both pathways is the inhibition of platelet activation to prevent redundant thrombus formation.
1.3 Cardiovascular diseases and atherosclerosis

Cardiovascular diseases (CVD) are a group of disorders that encompass a number of conditions related to the circulatory system such as hypertension, coronary heart disease and hyperlipidaemia. The World Health Organisation (WHO) reported that 17.3 million people from around the world died as a result of CVD in 2008, although it is thought to be most prominent in industrialised nations primarily due to lifestyle.

Atherosclerosis is the major pathology that contributes to the development of CVD, with its key characteristic being the formation of atherosclerotic plaques in blood vessel walls. A healthy blood vessel wall is composed of several layers: the outer tunica adventitia, made up of fibrous connective tissue; the tunica media, consisting of smooth muscle; and the inner most layer, the tunica intima, composed of connective tissue and numerous collagen fibres. A single layer of endothelial cells coats the inner surface of a blood vessel. The endothelium not only acts as a physical barrier between the blood and the underlying tissue but also plays a multitude of roles in regulating haemostasis, blood flow, blood pressure, immunity and the movement of mediators between the blood and tissue. One such class of particles that pass through this physiological barrier and also play a major role in the development of atherosclerosis are low density lipoproteins (LDL).

1.3.1 Atherosclerotic plaque development

There are several stages in the formation of an atherosclerotic plaque (Figure 1.7). One of the earliest steps involves LDL passing through the endothelium and
accumulating within the subendothelial space. This has been proposed to be the crucial step in the initiation of atherogenesis by the response-to-retention hypothesis (Williams and Tabas, 1995). Entrapment of LDL within the subendothelial space results from interactions between LDL and proteoglycans found within the intima. Mechanistically, this occurs through ionic binding between the positive charges on the apolipoprotein B-100 (apoB-100) portion of LDL and the negatively charged glycosaminoglycans of the proteoglycans (Iverius, 1972). Once LDL is deposited within the subendothelial space, it undergoes modification resulting in the formation of a pathological mediator. Modified LDL can alter the function of numerous surrounding cells including endothelial cells, fibroblasts and underlying smooth muscle cells. These cells are thought to release chemokines, which attracts monocytes to infiltrate into the subendothelial space. Once here, monocytes undergo differentiation into macrophages, which then take up these modified lipoproteins in an unregulated manner, resulting in the formation of foam cells. Examples of modification that can occur to LDL that enhance uptake by macrophages include acetylation (Goldstein et al., 1979) and aggregation (Khoo et al., 1988). Perhaps the most important form of modification that occurs to the LDL, which contributes to the development of atherosclerosis, is oxidation. Evidence of the presence of oxidised LDL (oxLDL) within atherosclerotic plaques was determined several decades ago (Yla-Herttuala et al., 1989). More recently, ELISA assays utilising an antibody that recognises oxidised PC in oxLDL demonstrated that plasma levels of this modified lipoprotein in CVD patients are significantly higher (0.18 ± 0.01ng/µg apoB-100) than in healthy control subjects (0.13 ± 0.01ng/µg apoB-100) and levels in carotid plaques are also substantially higher (11.9
± 1.7ng/µg apoB-100) than in healthy intima (1.86 ± 0.59ng/µg apoB-100) (Nishi et al., 2002). Finally, the release of numerous cytokines by activated macrophages leads to the proliferation and infiltration of vascular smooth muscle cells, which synthesize extracellular matrix proteins leading to the formation of a fibrous cap covering the foam cell infested core.

Interestingly, atheromas appear to arise at specific sites of the vasculature rather than being homogenously distributed throughout. A number of studies have shown that lesion formation is largely prominent at locations where blood flow is most turbulent (Zand et al., 1999, Prado et al., 2008), for example, branching points or bends in vessels.

Rupture of an atherosclerotic plaque can lead to thrombosis and clinical events such as myocardial infarction. The underlying mechanisms that contribute to these thrombotic developments are still not fully understood, however, the rupture of atherosclerotic plaques exposes platelets in the blood to oxLDL. The work presented here will therefore investigate the influence that oxLDL has on platelet function to further understand how these cells become activated in the context of atherosclerosis.
Figure 1.7: Atherosclerotic plaque development
Illustration representing the many stages of atherosclerotic plaque development. LDL molecules become trapped within the subendothelial space of a blood vessel wall where they subsequently become oxidised and endocytosed by macrophages to form foam cells. An atherosclerotic plaque then forms, guarded by a fibrous cap of connective tissue. Instability of a plaque results in its rupture leading to its contents seeping into the blood vessel lumen. Adapted from (Toth, 2009).
1.4 Low density lipoproteins

Lipoproteins are a group of molecules involved in carrying lipophilic substances around in the aqueous environment of the blood. They function to transport their hydrophobic content, such as lipids and lipid soluble vitamins, to and from tissues of the body. There are several classes of lipoproteins which are characterised by their density. These include chylomicrons (CM), very low density lipoproteins (VLDL), intermediate density lipoproteins (IDL), LDL, and high density lipoproteins (HDL). The density of a lipoprotein depends on the ratio of lipid to protein content and their size is inversely correlated with their density. Although lipoproteins play an essential role within the body, LDL is thought to be the most clinically dangerous form, as elevated levels are associated with increased risk of CVD.

1.4.1 Structure and function of low density lipoproteins

LDL are small spherical particles, approximately 22nm in diameter. They are classified as having a density of between 1.019-1.063g/ml and are formed as a product of metabolism. As VLDL pass through the circulation, the triglyceride rich core is hydrolysed by lipoprotein lipase on the surface of the endothelium. This leads to the formation of lipoproteins that are denser, resulting in IDL and subsequently LDL (Schneider, 2008).

LDL are a heterogeneous species made up of a multitude of components and have a complex structure (Figure 1.8) that enables them to perform their function of transporting lipids around the circulatory system. The outer shell of LDL is majorly
comprised of a monolayer of phospholipids, primarily PC and also small amounts of sphingomyelin and lysophosphatidylcholine. The LDL particle also contains, in less abundant amounts, phosphatidylethanolamine, diacylglycerol, ceramide and phosphatidylinositol (Hevonoja et al., 2000). Evidence suggests that the orientation of the phospholipids of LDL is similar to that of phospholipid bilayers and comprises the hydrophilic head regions facing the outer external environment and the hydrophobic fatty acid chains facing the inner core, thus allowing the molecule to circulate in an aqueous environment (Yeagle et al., 1977, Fenske et al., 1990, Hevonoja et al., 2000). Additionally, the outer shell contains approximately two-thirds of the particles unesterified cholesterol content (Lund-Katz and Phillips, 1986).

The protein constituent of lipoproteins varies depending on their density and includes apolipoproteins A, B, C and E. LDL contains one molecule of apoB-100, which is one of the largest monomeric proteins known. Apo-B100 contains 4536 amino acid residues (Yang et al., 1986) and has a molecular weight of approximately 549kDa (Kane et al., 1980). In order for LDL to be endocytosed, it must bind to receptors expressed on the surface of cells. Interactions between LDL and its receptor occur through a region known as site B on apoB-100 which consists of amino acids 3359-3369 (Law and Scott, 1990, Boren et al., 1998).

The inner core of an LDL molecule contains the reason for its existence. Here the lipoprotein carries its cargo of hydrophobic molecules around the blood stream. The main lipids transported by LDL are cholesterol esters, of which there are around 1600
per LDL particle. To a lesser extent than cholesterol, LDL also carries triglycerides, around 170 per LDL particle. Small amounts of unesterified cholesterol can also be found within the core. Importantly an LDL particle also contains a number of antioxidants to prevent its components for becoming oxidatively modified. These include such antioxidants as α-tocopherol, the most abundant, as well as γ-tocopherol and β-carotene (Esterbauer et al., 1990, Hevonoja et al., 2000).

The major function of LDL is to transport cholesterol to the peripheral tissues. Cholesterol is required for: maintaining cell membrane stability and fluidity; the production of a number of hormones including the sex hormones oestrogen and testosterone; the production of bile in the liver; and is a component of the myelin sheath of neurons. LDL enters cells through a process of receptor mediated endocytosis (Goldstein and Brown, 1974). LDL receptors are found on cell membranes in clusters within small clathrin coated pits. Once bound, the LDL-receptor complex undergoes endocytosis, where it becomes encompassed within a small vesicle. These vesicles diffuse through the cell in order for LDL to reach its destination, the lysosomes (Anderson et al., 1977, Pearse, 1976, Anderson et al., 1976). The decreased pH within lysosomes leads to dissociation of LDL from the receptor. The LDL particle is then digested by the lysosome and cholesterol esters are subsequently hydrolysed by lysosomal acid lipase (Goldstein et al., 1975a, Goldstein et al., 1975b). The LDL receptor however is recycled back to the plasma membrane where it can once again engage in the transportation of LDL into the cell (Basu et al., 1981). Importantly, to prevent cholesterol overloading in cells, LDL receptor production is regulated by
transcription factors called sterol regulatory element binding proteins (SREBPs). Cholesterol within the cell blocks these transcription factors from entering the nucleus and therefore prevents LDL receptor synthesis. However, in cholesterol depleted cells, SREBPs are able to reach the nucleus, allowing the synthesis of LDL receptors to occur (Goldstein and Brown, 2009).
Figure 1.8: Structure of low density lipoprotein

Diagrammatic representation of a molecule of LDL composed of an outer shell of phospholipids, unesterified cholesterol and a molecule of apoB-100. The inner core of the particle consists of esterified and unesterified cholesterol, triglycerides and antioxidants.
1.4.2 Oxidation of low density lipoproteins

The oxidative modification of LDL is an irreversible process that leads to alterations in the lipoproteins structure and functions. The phospholipid monolayer of the LDL particle is most susceptible to oxidation as it is this surface that first comes into contact with components that can cause alterations, such as metal ions and oxidative enzymes. Therefore, the primary stage of LDL modification involves the oxidation of the phospholipid content in a process known as lipid peroxidation. To understand how the phospholipids of LDL undergo oxidation it is first important to understand their structure, an example of which is illustrated in Figure 1.9. There are numerous types of phospholipids which are classified into two groups called phosphoglycerides and sphingophospholipids. Phosphoglycerides consist of a glycerol backbone, which adjoins two non-polar, hydrophobic hydrocarbon chains at the C-1 and C-2 positions. A polar phosphate containing head group is located at the C-3 position of the glycerol molecule. Sphingophospholipids are similar in structure but contain a sphingosine backbone rather than glycerol adjoined to a phosphate containing group and a long chain fatty acid (Hanahan, 1997).
Schematic diagram representing the structure of a phospholipid. These molecules are composed of a head region containing a phosphate group, a glycerol back bone and two fatty acid tails. Adapted from (Germann and Stanfield, 2005).
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The unsaturated fatty acid chains of the phospholipid are particularly vulnerable to modification under circumstances of oxidative stress (Figure 1.10). The carbon-carbon double bonds of the fatty acids are typically in a cis configuration, which reduces carbon-hydrogen bond energies. In the correct oxidative environment, this arrangement allows for the abstraction of a hydrogen atom, adjacent to a carbon-carbon double bond, from the hydrocarbon chain by free radical attack. Abstraction results in the formation of a carbonyl radical and the molecular rearrangement of the hydrocarbon chain to a conjugated diene. The resultant carbonyl radical is highly reactive and thus, in an aerobic environment, undergoes oxidation leading to the formation of a peroxyl radical. Finally, the addition of a hydrogen atom to the peroxyl radical forms a lipid hydroperoxide. This stage of lipid peroxidation is known as the propagation stage as the hydrogen atom that forms the hydroperoxide can be taken from an adjacent hydrocarbon chain, leading to the peroxidation of another fatty acid and thus ensuing in a chain reaction (Halliwell and Gutteridge, 1989, Reis and Spickett, 2012).
Lipid peroxidation occurs to unsaturated fatty acid chains by abstraction of a hydrogen atom through free radical attack. This results in the formation of a carbonyl radical and molecular rearrangement of the chain to form a conjugated diene. Oxidation results in the formation of a peroxyl radical and addition of a hydrogen atom to this leads in the formation of a lipid hydroperoxide. Adapted from (Jialal and Devaraj, 1996).

**Figure 1.10: Lipid peroxidation of an unsaturated fatty acid chain**

Lipid peroxidation occurs to unsaturated fatty acid chains by abstraction of a hydrogen atom through free radical attack. This results in the formation of a carbonyl radical and molecular rearrangement of the chain to form a conjugated diene. Oxidation results in the formation of a peroxyl radical and addition of a hydrogen atom to this leads in the formation of a lipid hydroperoxide. Adapted from (Jialal and Devaraj, 1996).
In addition to the oxidation of phospholipids in the outer shell of LDL, prolonged oxidation induces the modification of the inner core of cholesterol. In its free form, the structure of cholesterol includes a four ring arrangement attached to a hydrocarbon side chain. The esterified form of cholesterol results from a fatty acid binding to this structure. Therefore, the oxidation of cholesterol within LDL may involve the modification of the sterol, i.e. the ring structure/side chain, as well as the fatty acid chain. Similar to phospholipids, modification of the fatty acid chain of cholesterol esters results in the development of lipid hydroperoxides. Modification of the sterol, however, leads to the formation of oxysterols, the most important involved in atherosclerosis being 7-ketocholesterol, 27-hydroxycholesterol and 7β-hydroxycholesterol (Leonarduzzi et al., 2007). Although there is inconsistency in the exact amounts found within lesions, perhaps due to varying analytical techniques, these oxysterols are consistently present at increased levels in both rabbit and human plaques (Upston et al., 2001, Iuliano et al., 2003, Vaya et al., 2001). Indeed the extent of plaque severity appears to correlate with an increase in the amount of 27-hydroxycholesterol and 7-ketocholesterol found (Upston et al., 2002).

Continued oxidation of LDL results in the breakdown of carbon-carbon bonds of lipid hydroperoxides, derived from both phospholipids and cholesterol esters. This leads to the formation of aldehydes such as malondialdehyde and 4-hydroxynonenal, which are found to be present in atherosclerotic plaques (Palinski et al., 1989). In particular to esterified cholesterol, a major aldehyde product formed during oxidation that is abundant in atherosclerotic lesions is 9-oxononanoyl cholesterol, which derives from
cholesteryl linoleate, a key component of LDL (Karten et al., 1998). The formation of aldehyde products culminates in the irreversible modification of the protein component of LDL. The aldehyde products form a variety of adducts with positively charged amino acids of the apolipoprotein through the formation of a Schiff base. This results in an increased net negative charge of the protein and a loss of recognition from the LDL receptor.

There are a number of mechanisms that may be responsible for the oxidative modification of LDL in vivo, which are divided into enzymatic and non-enzymatic processes. The non-enzymatic process of modification involves potentially free transition metal ions such as iron and copper, which have been found at elevated levels within atherosclerotic lesions (Stadler et al., 2004) and are involved in catalysing lipid peroxidation thus initiating the reactions culminating in LDL modification. Enzymatic modification of LDL includes enzymes such as lipoxygenase, which directly oxygenates polyunsaturated fatty acids, and myeloperoxidase, which catalyses the formation of hypocholrus acid leading to the formation of acetylated LDL (Yoshida and Kisugi, 2010). For many years, oxidation of LDL in vitro has involved its incubation with cells in the presence of metal ions such as iron or copper or by incubation with these metals alone. Interestingly, incubation with cells alone is unable to modify LDL, illustrating the importance of transition metal ions in initiating oxidative reactions (Steinbrecher et al., 1984, Heinecke et al., 1984). Additionally, incubation with metal ions, in particular copper, results in the formation of many products present in atherosclerotic plaques, such as lipid hydroperoxides and 7-ketocholesterol, suggesting
its reliability as a method to investigate the effects of oxLDL within experimental circumstances (Gerry et al., 2008).

1.4.3 Cellular effects induced by oxidised low density lipoproteins

OxLDL induces a number of proatherogenic properties in a range of cell types found within the regions of atheromas. A major example of this is the increased uptake of oxLDL by macrophages leading to the formation of lipid laden foam cells (Henriksen et al., 1981), a process that largely contributes to the development of atherosclerotic plaques. Endothelial cells incubated with oxLDL are stimulated to release higher levels of chemotactic factor for monocytes and adhere to a greater extent to these cells (Berliner et al., 1990). Aortic smooth muscle cells have been shown to migrate further (Autio et al., 1990) and increase collagen synthesis (Jimi et al., 1995) in the presence of oxLDL, which aids in the formation of a fibrous covering over the plaque area. Therefore, many of the cellular defects observed in atherosclerosis appear to be driven by the presence of oxLDL. Importantly, a vital property of oxLDL that may be significant in contributing to thrombotic events in atherosclerosis is the ability of this modified lipoprotein to induce the activation of platelets.

1.4.4 Platelet activating properties of oxidised low density lipoproteins

The platelet activating properties of oxLDL have been studied for many years, since enhanced activity of platelets in patients with hyperlipoproteinaemia in response to the platelet agonists collagen, ADP and adrenalin was first observed (Carvalho et al., 1974). The resuspension of healthy platelets into plasma from patients with familial
hypercholesterolemia resulted in a greater response to physiological platelet agonists (Aviram and Brook, 1982). Early studies on isolated LDL found it to induce platelet aggregation and enhance agonist induced aggregation (Aviram and Brook, 1983, Hassall et al., 1983). This response, however, may have been due to partial oxidation of the lipoprotein during isolation before much was known regarding its oxidative modification. Subsequently, over the years, numerous studies have found oxLDL to induce a small degree of aggregation and degranulation of platelets, depending on the extent of oxidation (Ardlie et al., 1989, Zhao et al., 1994, Naseem et al., 1997). One of the earliest stages of platelet activation, platelet shape change, was observed to occur upon stimulation with oxLDL and was found to be Rho kinase dependent as the Rho kinase inhibitor Y27632 ablated this effect (Retzer et al., 2000). Therefore, oxLDL appears to have the ability to induce many stages of platelet activation, which may result in thrombosis at sites in the body where levels are high.

1.4.5 Scavenger receptors

The many proatherogenic responses to oxLDL appear to be mediated on numerous cell types by the same group of receptors designated scavenger receptors. These are a group of proteins that are structurally unrelated and divided into 8 subfamilies, classes A-H. Scavenger receptors are involved in the recognition of pathological ligands, in particular, modified forms of LDL. They were first identified by Goldstein and colleagues, who found macrophages to bind and take up acetylated LDL at a much greater rate than nLDL by a separate mechanism to the LDL receptor (Goldstein et al., 1979). In particular to platelets, two scavenger receptors have been implicated in
mediating the activating responses to oxLDL. These include scavenger receptor A (SR-A) (Korporaal et al., 2007) and CD36 (Chen et al., 2008). CD36 is in the class B category of scavenger receptors, in which there are three members including scavenger receptor BI (SR-BI) and lysosomal integral membrane protein-II (LIMP-II) as well as CD36 and are all thought to derive from the same ancestral gene (Moore and Freeman, 2006). Importantly, genetic deletion of CD36 has been found to protect hyperlipidaemic mice from a prothrombotic phenotype, suggesting this receptor as a key regulator of platelet dysfunction in diseases with increased circulating lipids (Podrez et al., 2007). Therefore, CD36 functions are a vital aspect for research as a potential new drug target within the setting of hyperlipidaemia to prevent thrombotic events.
1.5 Cluster of differentiation 36

Cluster of differentiation 36 (CD36), also known as glycoprotein IV (GPIV), was first recognised by the monoclonal antibody OKM5, which identified an 88KDa antigen present on monocytes and platelets (Talle et al., 1983). Subsequent investigations revealed this antigen to be a receptor for TSP on platelets, as OKM5 blocked TSP interactions with thrombin activated platelets (Asch et al., 1987). Since these early observations, CD36 has been found to be expressed on a number of different cell types and to have an array of ligands, resulting in its participation in a plethora of functions which will be discussed in the subsequent sections.

1.5.1 CD36 expression

CD36 was first found to be expressed on platelets and monocytes in 1983 (Talle et al., 1983). Subsequently, its expression has been described on a number of cell types including: endothelial cells of the microvasculature but not on large vessels (Knowles et al., 1984, Swerlick et al., 1992); adipocytes, where it is involved in long chain fatty acid (LCFA) transportation (Abumrad et al., 1993); and both skeletal and smooth muscle cells (Bonen et al., 1999, Ricciarelli et al., 2000).

There have been numerous reports on the levels of CD36 expressed on platelets. Early work demonstrated CD36 to be the fourth major glycoprotein on these cells (Okumura and Jamieson, 1976). Furthermore, a recent review stated that a single platelet can express between 10,000-25,000 copies of this receptor (Nergiz-Unal et al., 2011b). However, flow cytometric analysis of platelets from 500 patients
characterised CD36 expression levels as 7876±1924 molecules of CD36 per platelet, with a range of 2000-18,000 molecules (Ghosh et al., 2011).

On platelets, CD36 is located on the phospholipid membrane within lipid rafts (Dorahy et al., 1996b) as well as within α-granule membranes (Berger et al., 1993). Interestingly, CD36 expression on the platelet surface increases upon stimulation with the platelet agonists thrombin, ADP, collagen, adrenalin and the TXA$_2$ analogue U46619. This is likely to be the result of degranulation upon platelet activation resulting in a redistribution of CD36 from α-granules to the plasma membrane (Michelson et al., 1994).

There are some individuals that do not express CD36. The Nak$^a$ alloantigen was first found to be carried on CD36 over 20 years ago (Tomiyama et al., 1990). Nak$^a$ deficiency is most common in Asian populations, where 3-11% of people lack the antigen. Investigations of Nak$^a$ deficient healthy blood donors revealed that CD36 could not be detected on the platelets from these individuals, suggesting that Nak$^a$ deficiency correlates with CD36 deficiency (Yamamoto et al., 1990). CD36 deficiency is an inherited disorder which can be categorised into type I, where CD36 is not expressed on any tissues, or type II, where only platelets lack the glycoprotein. Patients who do not express this scavenger receptor do not present with bleeding disorders, they do however appear to have an increased risk for hyperlipidaemia, impaired glucose metabolism due to insulin resistance and mild hypertension (Yamashita et al., 2007). It is difficult to determine if these phenotypes occur due to
CD36 deficiency or through other risk factors such as life style choices. It does, however, appear that CD36 deficiency itself is not life threatening but it can nevertheless bring about critical conditions.

1.5.2 Structure of CD36

The gene that encodes CD36 is located on chromosome 7q11.2 (Fernandez-Ruiz et al., 1993). The protein consists of 472 amino acids, which results in a molecular mass of 78-88kDa depending on the extent of post-translational glycosylation (Rac et al., 2007). Its structure, illustrated in Figure 1.11, is composed of two short intracellular segments at the amino and carboxyl termini, two transmembrane domains and a large extracellular loop. The intracellular amino terminal portion of CD36 consists of just 6 amino acids, residues 1-6, and the carboxyl terminal section contains 12 amino acids, residues 461-472 (Rac et al., 2007). Both amino and carboxyl termini are post-translationaly palmitoylated at cysteine residues 3, 7, 464 and 466, confirming their intracellular residence (Tao et al., 1996). The two transmembrane domains pass through the plasma membrane between amino acids 7-28 and 439-460 (Rac et al., 2007). The extracellular domain contains a proline rich region between amino acids 242-333 and a hydrophobic region between amino acids 184-204, which is thought to possibly interact with the plasma membrane (Collot-Teixeira et al., 2007). The extracellular domain also possesses 10 glycosylation sites at asparagines 79, 102, 134, 163, 205, 220, 235, 247, 321 and 417 (Hoosdally et al., 2009).
The extracellular region of CD36 is the site of binding for numerous ligands. An important functional domain located on CD36 between amino acids 155 and 183 was first identified in 1995 using a range of monoclonal antibodies (Daviet et al., 1995). Shortly after, this functional domain was recognized as the binding site for oxLDL (Puente Navazo et al., 1996) and to some extent advanced glycation end products (AGEs) (Ohgami et al., 2001). Additionally, the sequence of amino acids between 93 and 120, now designated the CD36 LIMP II Emp structural homology (CLESH) domain, has been shown to be the binding site for TSP-1 (Frieda et al., 1995). Another binding region proposed to exist on the extracellular domain is between amino acids 127 and 279, thought to interact with fatty acids (Baillie et al., 1996). Finally, *Plasmodium falciparum* infected erythrocytes are believed to interact with a binding site between amino acids 139-184, with particular emphasis on the regions between amino acids 146-164 and 145-171 (Baruch et al., 1999). Therefore, it can be seen that there are numerous functional sites within the large extracellular loop of CD36 that contribute to its diverse array of functions.
The scavenger receptor possesses two intracellular regions at the amino and carboxyl termini, two transmembrane domains and a large extracellular loop containing a hydrophobic region and a proline rich region. Adapted from (Collot-Teixeira et al., 2007).

Figure 1.11: Diagrammatic representation of CD36
The scavenger receptor possesses two intracellular regions at the amino and carboxyl termini, two transmembrane domains and a large extracellular loop containing a hydrophobic region and a proline rich region. Adapted from (Collot-Teixeira et al., 2007).
1.5.3 Functions of CD36

The functional consequences of CD36 stimulation is highly dependent on the cell type on which it is expressed and the ligand that binds to it. CD36 has a variety of physiological ligands such as TSP-1 and collagen but also a number of pathological ligands including oxLDL, AGEs and endothelial derived microparticles (MP). Collagen was one of the earliest physiological ligands found to associate with CD36. Indeed, the main role played by CD36 on platelets was first thought to involve adhesion to collagen (Tandon et al., 1989), more specifically collagen type V (Kehrel et al., 1993). However, later experiments revealed that the role CD36 plays in collagen adhesion is somewhat redundant as platelets deficient in CD36 are able to adhere to the ECM (Saelman et al., 1994), aggregate (Yamamoto et al., 1992) and induce collagen signal transduction to comparable levels of platelets with CD36 (Daniel et al., 1994).

TSP-1 is found within α-granules of platelets and is released upon activation. Early discoveries found TSP-1 to enhance thrombin and ADP stimulated platelet aggregation, with the involvement of CD36 as the receptor mediating it’s interaction with platelets (Tuszynski et al., 1988). More recent studies discovered that TSP-1 reverses the inhibitory effects of NO by preventing sGC mediated formation of cGMP as well as preventing VASP^{Ser239} phosphorylation by directly inhibiting PKG. Using modified CD36 and CD47 binding peptides, it was determined that these results were dependent upon TSP-1 binding to either CD36 or CD47 (Isenberg et al., 2008). PGE_{1} inhibition of platelet aggregation is also reversed in the presence of TSP-1, which is accompanied with a reduction in VASP^{Ser157} phosphorylation. These responses were found to be
CD36 mediated. Furthermore, inhibition of adhesion to fibrinogen under physiological flow conditions by PGE\textsubscript{1} was reversed in the presence of TSP-1, illustrating that under conditions replicating flowing blood in an injured artery, TSP-1 prevents PGE\textsubscript{1} from inhibiting platelet adhesion and thus promotes thrombus formation at the site of injury (Roberts et al., 2010). Therefore, TSP-1-CD36 interactions may enhance thrombus formation by reducing the effects of physiological inhibitors constitutently present in the circulation.

AGEs are the resulting molecules of the Maillard reaction, accordingly named after the scientist who first investigated the reaction in the early part of the 20\textsuperscript{th} century (Maillard, 1912). This reaction involves the non-enzymatic interaction between a reducing sugar and a lipid or protein, also known as glycation. Initial investigations by Hangaishi and colleagues found that glycated albumin potentiated both ADP and U46619 induced platelet aggregation in human platelet rich plasma (PRP) whereas non-glycated albumin had no effect (Hangaishi et al., 1998). Further investigations by other groups have found that glycated albumin: induces PS exposure on platelets surfaces (Wang et al., 2007); enhances serotonin induced aggregation, with plasma levels of AGEs in diabetic subjects positively correlating with the extent of aggregation induced by serotonin (Hasegawa et al., 2002); and enhances thrombin receptor agonist peptide (TRAP) and collagen induced aggregation as well as flow induced activation (Rubenstein and Yin, 2009). Additionally, AGEs isolated from food and human diabetic serum have been found to induce P-selectin expression as a marker of platelet activation (Gawlowski et al., 2009). A more recent study by the Silverstein group
showed that glycated albumin specifically binds to platelets through CD36 and that this interaction enhances ADP induced aggregation. They also revealed that AGE levels were greater in plasma of diabetic mice both with and without CD36 but AGE incorporation into thrombi and the prothrombotic phenotype observed in diabetic mice was protected by CD36 deficiency (Zhu et al., 2012). Therefore, AGEs appear to both induce platelet activation as well as enhance activation induced by other agonists.

MPs are tiny fragments that are released from cells such as leukocytes, platelets and endothelial cells upon activation or apoptosis. Endothelial derived MPs have been found to associate with platelets via interactions between platelet CD36 and PS on MPs. Incubation of human platelets with either endothelial derived MPs, monocyte derived MPs, platelet derived MPs or human blood derived MPs resulted in increased aggregation responses to ADP, however, this was not observed in individuals that did not express CD36. Additionally, endothelial derived MPs were shown to accumulate with CD36 in thrombi of wild type but not CD36 deficient mice (Ghosh et al., 2008).

1.5.3.1 Functional consequences of oxidised low density lipoproteins binding to CD36

In 1993 oxLDL was found to ligate CD36 (Endemann et al., 1993). It is believed that specific oxidised phospholipids derived from oxLDL, known as oxPC\textsubscript{CD36}, bind directly to CD36. The characteristic required for CD36 binding involves structurally related regions on the unsaturated fatty acid in the \textit{sn}-2 position of PC (Podrez et al., 2002b). Confirmation of oxPC\textsubscript{CD36} as a ligand for CD36 came from experiments using
macrophages, which revealed that these ligands could bind and be endocytosed by wild type macrophages but could not bind to macrophages deficient in CD36. Furthermore, investigations of normal and atherosclerotic rabbit aortas in the same study confirmed these oxidised phospholipid ligands to be present at higher levels in atherosclerotic plaques (Podrez et al., 2002a). The binding site for oxLDL on CD36 is between amino acids 155-183, however, more recent investigations have shown that of particular importance to oxLDL and oxPC \textsubscript{CD36} interactions are the positively charged amino acids lysine\textsuperscript{164} and lysine\textsuperscript{166} (Kar et al., 2008). Conversely, other investigations using oxLDL generated through modification by hypochlorite, which alters only the protein constituent of the molecule, have found this type of oxLDL to induce platelet aggregation as well as P-selectin and CD40L surface expression on platelets in a CD36 dependent manner (Volf et al., 2000, Assinger et al., 2010). This suggests apoB-100 to interact with CD36. Therefore, a combination of modified lipid and protein components of oxLDL may play a role in its binding to CD36.

Studies using knockout mice deficient in both CD36 and SR-A illustrated that these are the key receptors involved in the formation of foam cells (Kunjathoor et al., 2002). The role that CD36 plays in CVD is further supported by observations from apoE deficient mice that are predisposed to hyperlipidaemia. ApoE knockout mice, that were also CD36 deficient, were found to have a 76% reduction in atherosclerotic lesion area compared to mice that were deficient in apoE alone (Febbraio et al., 2000). This suggests that CD36 not only plays a prominent role in foam cell formation but also in the pathological formation of atherosclerosis \textit{in vivo}. The functions of CD36 on
macrophages are not, however, limited to foam cell formation. Interestingly, a study investigating macrophage migration used both *in vitro* and *in vivo* techniques to demonstrate that macrophage migration was inhibited by oxLDL, which was prevented in the absence of CD36. This suggests that CD36 may be involved in regulating macrophage migration in an oxLDL dependent manner resulting in the trapping of macrophages in the arterial intima and thus promoting atherosclerosis (Park et al., 2009).

1.5.3.2 The influence of oxidised low density lipoproteins interactions with CD36 on platelet activity

Since early investigations that illustrated the platelet activating properties of oxLDL (Ardlie et al., 1989), more recent studies have revealed CD36 to be the major receptor involved in carrying out this function. As oxLDL only stimulates a weak platelet aggregation response, investigators have since used P-selectin expression on platelets as a marker for activation and noted that this response to oxLDL occurs through binding of the modified lipoprotein to CD36 (Takahashi et al., 1998). Platelets also adhere to and spread on immobilized oxLDL in a CD36 dependent manner (Nergiz-Unal et al., 2011a).

A pioneering study carried out by Podrez and colleagues illustrated the major role that oxLDL binding to CD36 has in the development of CVD. This group used both wild type and CD36 deficient hyperlipidaemic mice. They found that CD36 deficiency significantly protects mice from a prothrombotic phenotype observed within
hyperlipidaemia. Using aggregation assays they showed that hyperlipidaemic mouse platelets aggregated to a greater extent in response to ADP than wild type. However, when wild type platelets were resuspended into hyperlipidaemic plasma, aggregation was greater than when in their own plasma. When hyperlipidaemic platelets were resuspended into wild type plasma their activity was diminished. Critically, there was no difference in the extent of aggregation in CD36 deficient platelets when they were resuspended into either plasma. This suggested that a constituent of the hyperlipidaemic plasma was promoting a prothrombotic phenotype. Further investigations illustrated that oxPC$_{CD36}$ were at high levels in the hyperlipidaemic plasma and that these molecules, as well as oxLDL, bound to platelets and induced $\alpha_{IIb}\beta_3$ activation and P-selectin expression in a CD36 dependent manner (Podrez et al., 2007).

Although oxLDL is known to activate platelets, some studies suggest that highly modified forms, as well as inducing slight aggregation, can impede aggregation and fibrinogen binding induced by other agonists (Aviram, 1989, Naseem et al., 1997, Korporaal et al., 2005). This was thought to be due to binding of oxLDL to CD36 blocking interactions between fibrinogen and $\alpha_{IIb}\beta_3$ (Korporaal et al., 2005) as CD36 has been previously found to associate with this integrin (Dorahy et al., 1996a, Miao et al., 2001).
1.5.4 CD36 signalling in platelets

Even though it is now well established that oxLDL activates platelets through CD36, the signalling pathways are still poorly defined. A major and consistent finding between studies, however, is that CD36 associates with Src kinases. Platelets possess a range of Src family kinases including Src, Fyn, Lyn, Yes, Fgr, Lck and Hck. Specifically, CD36 has been found to be associated with Fyn, Lyn and Yes in platelets (Huang et al., 1991). Previous reports have been contradictory as to how Src kinases associate with CD36. It was originally proposed that there is an association between CD36 and Src kinases through interactions with the carboxyl terminal of the cytoplasmic domain of CD36 due to the similarity of its sequence with CD4 and CD8 receptors on T lymphocytes that interact through a Cys-X-X-Cys region of Lck (Shattil and Brugge, 1991). In addition, a fairly recent study has used CD36/GST fusion proteins to show a direct association between the carboxyl terminal of CD36 and Lyn in macrophages (Rahaman et al., 2006). However, it has also been reported that interactions between CD36 and Lyn are lipid mediated (Thorne et al., 2006). Despite the uncertainty as to how CD36 and Src kinases associate, several studies confirm Src kinase involvement in CD36 signalling, due to pharmacological inhibition of Src kinases preventing: macrophage foam cell formation; the platelet activating effects of oxLDL; and the dis-inhibitory effects of TSP-1 in platelets (Rahaman et al., 2006, Chen et al., 2008, Nergiz-Unal et al., 2011a, Roberts et al., 2010).

Other proteins that have been implicated in being associated with CD36, which are involved in platelet activation by other receptors, include the FcR y-chain and the
tyrosine kinase Syk. Firstly, FcR γ-chain involvement in CD36 signalling was investigated using a peptide of the C-terminal domain of TSP-1. It was found that this peptide induced phosphorylation of FcR γ-chain, Syk, PLCγ2 and SLP-76 in platelets. Additionally, tyrosine phosphorylation was blocked by the Src kinase inhibitor PP1, implicating a number of potential proteins possibly involved in CD36 signalling (Tulasne et al., 2001). Furthermore, Syk has also been found to associate with CD36 in cultured human dermal microvascular endothelial cells (Kazerounian et al., 2011) and becomes phosphorylated in platelets when they adhere to immobilised oxLDL (Nergiz-Unal et al., 2011a). The data therefore strongly indicates that Syk tyrosine kinase plays a substantial role in CD36 mediated signalling.

The group of guanine nucleotide exchange factors called Vav proteins have recently been extensively investigated for their role in the CD36 signalling pathway in a number of cell types. There are three Vav family members, however, only Vav1 and Vav3 are expressed in platelets. The exact role of Vav proteins in platelets is not well defined, however thrombin and collagen both induce the tyrosine phosphorylation of the proteins (Cichowski et al., 1996). Additionally, genetic deletion of either Vav1 or Vav3 in mice does not considerably affect collagen induced aggregation, however, genetic deletion of both isoforms substantially reduced both collagen and collagen related peptide (CRP) induced aggregation. This therefore suggests that Vav1 and Vav3 together, but not alone, are involved in platelet activation (Pearce et al., 2004). Interestingly, Vav1 and Vav3 both become phosphorylated in platelets stimulated with oxLDL, with phosphorylation of Vav1 occurring much quicker than Vav3. This response
to oxLDL is again Src kinase mediated and genetic deletion of Vav proteins appears to protect hyperlipidaemic mice against a prothrombotic phenotype normally observed in wild type mice (Chen et al., 2011). Additionally, Vav1, Vav2 and Vav3 are phosphorylated in response to oxLDL in macrophages in a CD36 dependent manner and genetic deletion of Vav1 and Vav3 significantly reduces CD36 dependent oxLDL uptake and macrophage foam cell formation (Rahaman et al., 2011a).

The mitogen activated protein kinases (MAPK) p38 and JNK are present in platelets and, although little is known about their exact role, these proteins are thought to be involved in platelet activation by several agonists (Adam et al., 2008). With regards to CD36 signalling, p38 has been found to become phosphorylated by oxLDL in both a CD36 and SR-A dependent manner in platelets (Korporaal et al., 2007). Additionally, JNK has also been shown to become phosphorylated in response to oxLDL in both platelets and macrophages and in response to TSP-1 in platelets. Pharmacological inhibition of JNK prevents oxLDL from inducing platelet activation and macrophage foam cell formation as well as TSP-1 induced dis-inhibition of PGE$_1$ in platelets, all of which were shown to occur through CD36 (Chen et al., 2008, Rahaman et al., 2006, Roberts et al., 2010).
Chapter 1

1.6 Aims of the study

It has been well established for many years now that oxLDL plays a prominent role in the development of atherosclerosis and CVD. Additionally, it is also known that oxLDL interacts with and initiates the activation of platelets, therefore, contributing to the prothrombotic state of CVD. Only recently has it been identified that the scavenger receptor CD36 plays a critical role in the activation of platelets by oxLDL and the signalling pathways involved in this are only now becoming apparent. As the activation of platelets by oxLDL may be a potential target for new pharmacological developments, it is important to understand exactly how the stimulatory mechanisms of these pathological ligands work. Therefore the aims of this study are to identify how oxLDL activates platelets through the scavenger receptor CD36. This will be achieved by:

- Producing an oxidised form of LDL and identifying the platelet activating properties that it possesses.
- Identifying the scavenger receptor CD36 on platelets using flow cytometry and evaluating whether its expression can alter in response to platelet agonists.
- Investigating the signalling mechanisms that are involved in oxLDL induced platelet activation, with particular emphasis on the pathways leading to platelet shape change.
Chapter 2

Chapter 2: Materials and methods

2.1 Chemicals and reagents

Acrylamide was purchased from Bio-Rad (Hemel Hempstead, UK). 1,2-Bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetrakis(acetoxyethyl ester) (BAPTA-AM), Ethylenediaminetetraacetic acid (EDTA), JNK inhibitor I, 1-(5-iodonaphthalene-1-sulfonyl)homopiperazine (ML-7), AG 1879, 4-Amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (PP2), 4-Amino-7-phenylpyrazol[3,4-d]pyrimidine (PP3), RGDS, wortmannin and (R)-(+-trans-N-(4-Pyridyl)-4-(1-aminoethyl)-cyclohexanecarboxamide (Y27632) were from Calbiochem (Nottingham, UK). R406 was from Selleckchem (Suffolk, UK). 1-[6-[[17β]-3-Methoxyestra-1,3,5(10)-tri-en-17-yl]amino]hexyl]-1H-pyrrrole-2,5-dione (U73122) was from Tocris (Bristol, UK). PAR1 receptor agonist (SFLLRN) and PAR4 receptor agonist (AYPGKF) were from Cambridge Bioscience (Cambridge, UK). The CD36 blocking antibody FA6.152 was purchased from Santa Cruz (Wembley, UK). CRP-XL was from the Department of Biochemistry, University of Cambridge (Cambridge, UK). Collagen was from Axis Shield (Dundee, UK). All other chemicals were ordered from Sigma (Poole, UK).

2.2 Antibodies

Anti-CD36 antibody was from Novus (Cambridge, UK). Anti-phospho-MLCSer19, anti-phospho-MYPT1Thr853 and anti-phospho-Src familyTyr416 antibodies were from cell signalling (Hitchen, UK). Anti-phospho-MYPT1Thr696, anti-PLCy2, anti-Syk and anti-Vav1 antibodies were from Santa Cruz (Wembley, UK). Anti-RhoA antibody was from
Cytoskeleton (Cambridge, UK). Anti-phospho-tyrosine, anti-β-tubulin, normal mouse IgG isotype control and normal rabbit IgG isotype control antibodies were from Upstate (Watford, UK). Fluorescein isothiocyanate (FITC) conjugated mouse anti-human CD36 antibody was from Biolegend (Cambridge, UK). FITC conjugated rabbit anti-human fibrinogen antibody was from Dako (Ely, UK). FITC conjugated mouse anti-human CD42b, phycoerythrin (PE) conjugated mouse anti-human CD62p, FITC conjugated mouse IgG isotype control and PE conjugated mouse IgG isotype control antibodies were from BD Biosciences (Oxford, UK).

2.3 Methodology for the isolation and oxidation of low density lipoproteins

LDL was used from healthy human volunteers to ascertain the effects that its oxidation has on platelet function. Isolation was performed using sequential density ultracentrifugation and oxidation was carried out using a dialysis procedure. Confirmation of oxidation was assessed by calculation of relative electrophoretic mobility (REM) and lipid hydroperoxide levels. LDL was provided as a generous gift from Professor David Leake (University of Reading).

2.3.1 Isolation of low density lipoproteins

LDL was isolated using a sequential density ultracentrifugation method previously described (Wilkins and Leake, 1994) (Figure 2.1). Firstly, blood was drawn from fasted, healthy volunteers into sterile EDTA (150mM) to prevent clotting and inhibit oxidation of LDL. Blood was then centrifuged at 1500g for 30 minutes at 4°C to separate plasma from blood cells. Centrifugation was carried out at low temperatures to help prevent
oxidation during the isolation process. Plasma was removed and adjusted to a density of 1.019g/ml by addition of a high density solution (HDS) (2.97M KBr, 2.62M NaCl, 297μM EDTA, pH7.4). Plasma was then dialysed for 1-2 hours against a solution of 1.019g/ml density, made from the addition of 87.54ml HDS to 2000ml low density solution (LDS) (150mM NaCl, 297μM EDTA), at 4°C. Subsequently, the plasma was centrifuged at 115,000g for 18 hours at 4°C followed by retrieval of the bottom most layer. This was then adjusted to a density of 1.063g/ml with HDS and dialysed against a solution of 1.063g/ml density, made from the addition of 451ml HDS to 2000ml LDS, for 1-2 hours at 4°C. Plasma was again centrifuged at 115,000g for 18 hours at 4°C followed by removal of the upper most layer, which contained the LDL fraction. Dialysis was then performed against a solution of 1.063g/ml density for 1-2 hours at 4°C followed by centrifugation at 115,000g for 18 hours. LDL was then removed and dialysed against a phosphate buffer (140mM NaCl, 8.1mM Na₂HPO₄, 1.9mM NaH₂PO₄, 100μM EDTA, pH 7.4) overnight at 4°C to remove excess amounts of salts and EDTA. LDL was filtered to sterilise and finally stored at 4°C. Protein was measured using the modified Lowry assay (Markwell et al., 1981).
Chapter 2

Diagram illustrating the various stages of LDL isolation, in which plasma is separated from the blood and subsequently subjected to several dialysis and density ultracentrifugation steps to produce a pure isolation of the lipoprotein.

Figure 2.1: Isolation of low density lipoproteins

Diagram illustrating the various stages of LDL isolation, in which plasma is separated from the blood and subsequently subjected to several dialysis and density ultracentrifugation steps to produce a pure isolation of the lipoprotein.
2.3.2 Oxidation of low density lipoproteins

The oxidation of LDL in this study was achieved by exposing nLDL to Cu$^{2+}$ ions. This relies on the ability of transition metal ions to exist in multiple states, allowing them to cause fission of any existing peroxide bonds. This creates a chain reaction leading to the formation of multiple oxidation products. Experimentally, the method was based on that described by Gerry et al (Gerry et al., 2008), which utilises a series of dialysis steps. Native LDL (nLDL) was dialysed for 4.5 hours at 4°C against 3 changes of phosphate buffer (140mM NaCl, 8.1mM Na$_2$HPO$_4$, 1.9mM NaH$_2$PO$_4$, pH 7.4) to remove any residual EDTA from the LDL preparation. The nLDL was then dialysed against 2 changes of MOPS buffer (150mM NaCl, 10mM MOPS, pH 7.4) containing washed Chelex-100 (0.1%, w/v) for 24 hours at 4°C to remove traces of metal ions that may already be present. After this, nLDL was diluted to 2mg/ml with MOPS buffer containing CuSO$_4$ (10µM) and dialysed against 2 changes of the same buffer for 24 hours at 4°C to produce hydroperoxide rich oxLDL or at 37°C to produce oxysterol rich oxLDL. To terminate oxidation, EDTA (1mM) was added and the oxLDL was further dialysed for 24 hours against 2 changes of phosphate buffer containing EDTA (100µM) to ensure a final concentration of 100µM EDTA was reached. Finally, oxLDL was passed through a 0.2µm filter to sterilise and stored at 4°C. Protein concentration was determined using the Bio-Rad DC protein assay kit (Section 2.6.2). All dialysis was performed under stirred conditions using snakeskin dialysis tubing with a 10,000 M, cut-off. 1L of buffer was used per 10mg of LDL protein.
2.3.3 Agarose gel electrophoresis

During the oxidative modification of LDL, the net negative charge of apoB-100 can increase, leading to greater migration on agarose gels. Therefore, measurement of the REM of LDL is a useful technique to evaluate oxidation. Measurements were performed by running both the oxidised and non-oxidised forms of LDL on agarose gels as has been performed previously (Naseem et al., 1997). Agarose gels (1%) were cast by dissolving agarose in TAE buffer (40mM Tris base, 0.1% glacial acetic acid (v/v), 1mM EDTA, pH8). Both nLDL and oxLDL (20µg) were loaded onto gels and were run at 100V for 1 hour in TAE buffer. Gels were then stained using a Coomassie Blue Staining system to visualise the protein. This involved incubation of the gel in staining solution (0.025% coomassie brilliant blue (w/v), 40% methanol (v/v), 7% glacial acetic acid (v/v)) for 2 hours followed by incubation in destaining solution-I (40% methanol (v/v), 7% acetic acid (v/v)) for 1 hour. Gels were finally incubated in destaining solution-II (5% methanol (v/v), 7% acetic acid (v/v)) overnight. REM was calculated by measuring the distance that the nLDL protein and oxLDL protein migrated from the loading well through the gel and dividing these measurements by the distance that the nLDL protein migrated.

2.3.4 Lipid hydroperoxide assay

The extent of lipid hydroperoxides present in both nLDL and oxLDL was assessed using a colorimetric assay in which lipid hydroperoxides present within the LDL preparations convert iodide to iodine, which can be measured photometrically at 365nm (el-Saadani et al., 1989). Hydrogen peroxide was used to produce a standard curve. Samples of
nLDL and oxLDL (25µg) in 250µl were added to eppendorfs in triplicate, followed by the addition of 1ml of colour reagent (163mM KH$_2$PO$_4$, 37mM K$_2$HPO$_4$, 120mM KI, 0.2% Triton X-100 (w/v), 0.15mM sodium azide, 0.01% benzalkonium chloride (w/v), 10µM ammonium molybdate, pH6). Samples were then mixed and incubated in the dark for 1 hour. After this time readings were taken at 365nm and concentrations of lipid hydroperoxides were calculated from the standard curve and expressed as nmol/mg LDL protein.

2.4 Methodology for the isolation of human blood platelets

It was necessary to isolate platelets from healthy human volunteers in order to assess how pathological ligands, such as oxLDL, can interact and affect the function of these cells directly, without other contributing factors being present. This is the first step in elucidating the ways in which platelets function in vitro to further our knowledge of how these cells work in vivo.

2.4.1 Volunteers

Blood was obtained from healthy adult volunteers who had given their informed consent and had confirmed that they had not taken any medication that may interfere with platelet function, such as aspirin, within the 14 days prior to blood donation. Ethical approval was granted by the Postgraduate Medical Institute (Hull York Medical School, Hull, UK). Blood was drawn from the median cubital vein in the cubital fossa using a 21g needle. The first 3ml of blood was discarded to eliminate platelets activated artificially. Following this, blood was collected into 20ml syringes containing
either acid citrate dextrose (ACD; 113.8mM D-glucose, 29.9mM Tri-sodium citrate, 72.6mM NaCl, 2.9mM citric acid, pH6.4) 1:5 (v/v) for washed platelet studies or into tri-sodium citrate (109mM) 1:9 (v/v) for the use of whole blood or PRP. Syringes were gently mixed and blood was then placed into 50ml falcon tubes for centrifugation.

2.4.2 Isolation of platelets from whole blood

Platelets were isolated from human blood using a reduced pH method, in which platelets are kept in an environment with a pH of 6.5 to prevent activation and aggregation during the isolation procedure (Flatow and Freireich, 1966). Blood was centrifuged at 200g for 20 minutes at room temperature, which separated the blood into three separate layers. This included the bottom most layer of red blood cells, a thin middle layer of leukocytes (buffy coat) and the upper layer of PRP. The PRP layer was gently aspirated, with careful attention not to disrupt the bottom two layers of cells, and transferred into a clean 15ml falcon tube. Citric acid (300mM) was then added to the PRP at a ratio of 1:50 (v/v) to reduce the pH to 6.5 and prevent activation in subsequent centrifugation steps. In some cases indomethacin (10µM) and/or apyrase (0.5U/ml) were also added to the PRP to prevent thromboxane production and ADP stimulation respectively. PRP was then centrifuged at 800g for 12 minutes at room temperature. Platelet poor plasma (PPP) was discarded and the platelet pellet obtained was gently resuspended into wash buffer (36mM citric acid, 10mM EDTA, 5mM D-glucose, 5mM KCl, 90mM NaCl, pH6.5). This was once more centrifuged at 800g for 12 minutes at room temperature to wash platelets and ensure no plasma proteins would be present within the platelet preparation. Finally, platelets were
resuspended into 1-2ml of modified Tyrode's buffer (150mM NaCl, 5mM HEPES, 0.55mM NaH$_2$PO$_4$, 7mM NaHCO$_3$, 2.7mM KCl, 0.5mM MgCl$_2$, 5.6mM D-glucose, pH7.4) for counting, before adjustment to the required platelet count.

2.4.3 Platelet Count

Platelet numbers within the suspension of isolated washed platelets were quantified using a haemocytometer. Firstly, a sample of the platelet suspension was diluted in ammonium oxalate (1%, w/v) 1:100 (v/v) to lyse any contaminating erythrocytes present. This sample was then mixed, applied to a double sided haemocytometer and left to settle for 10 minutes. Platelets were counted using a light microscope (Leitz) to ascertain the final count within the isolated suspension.

The haemocytometer is designed for cell counting and consists of two counting grids etched into a glass block. The area of each counting grid is 3mmx3mm which is divided into 9 squares, each having an area of 1mm$^2$. These are further divided into 25 squares each having an area of 0.04mm$^2$. When a coverslip is placed over the haemocytometer, the depth within the counting chamber is 0.1mm. Therefore, the volume in one of the 25 counting squares is 0.004mm$^3$. The number of platelets was counted in 10 of the 25 counting squares, 5 squares from each side of the haemocytometer, as indicated in Figure 2.2. Therefore, the number of platelets counted was in a total volume of 0.04mm$^3$. To apply this to the number of platelets per ml in the final isolated platelet suspension, calculations were performed to correct for the volume measured in the haemocytometer and the dilution into ammonium
oxalate. As the volume counted in the haemocytometer was 0.04mm$^3$ (equal to 0.04µl), this would have to be multiplied by 25,000 to equal 1ml, therefore the number of platelets counted (n) will also have to be multiplied 25,000. Additionally, as the sample of the platelets was diluted 1:100 into ammonium oxalate, to correct for this dilution n must also be multiplied by 100. Therefore, to calculate the number of platelets per ml from the number of platelets in 10 of the counting squares, the following equation was performed:

$$\text{Platelets/ml} = n \times 25000 \times 100$$
Platelets were counted in the squares indicated by red crosses on each side of the haemocytometer.

**Figure 2.2: Illustration of a haemocytometer used to count platelets**

Platelets were counted in the squares indicated by red crosses on each side of the haemocytometer.
2.5 Methodology for the assessment of platelet functional responses

To evaluate the functional responses of platelets to various agonists and inhibitors, several functional assays were employed. These included light transmission aggregometry, to measure the extent of aggregation induced by numerous agonists, and flow cytometry, to measure receptor expression and markers of activation on the platelet surface.

2.5.1 Light transmission aggregometry

Platelet aggregation, a vital technique used in many platelet laboratories, is a turbidimetric assay that involves the measurement of a transmitted light beam through a stirred suspension of platelets by a photometer (Born, 1962) (Figure 2.3A). It is assumed that when using small stirred volumes, resting platelets are uniformly distributed in suspension. This forms an optically dense medium which is refractory to the passage of light. Therefore, a non-stimulated platelet suspension is representative of 0% aggregation whereas a platelet free solution (modified Tyrode's buffer only or PPP if washed platelets or PRP were used respectively) represents 100% aggregation. When platelets are stimulated, aggregation results in spaces appearing between the aggregates and therefore light transmission increases. By comparing this with the platelet free solution, the amount of light transmitted through the suspension correlates with the extent of aggregation. As can be seen in Figure 2.3B, a classical platelet aggregation response consists of an initial stage where light transmission is briefly reduced, indicating platelet shape change. This is followed by two stages of aggregation, including the primary phase which is reversible, followed by the
secondary phase which is irreversible and results from the release of secondary mediators from platelets.

Platelet aggregation was measured in an optical aggregometer (Chrono-log). Washed platelets or PRP (250µl) were incubated in the aggregometer under non-stirring conditions for 2 minutes to allow platelets to equilibrate to 37°C. Platelets were then stimulated with the required agonist under stirred conditions (1000rpm) and aggregation responses were monitored for 4 minutes. In experiments to measure shape change only, washed platelets were preincubated with Ethyleneglycoltetraacetic acid (EGTA) (1mM), apyrase (2U/ml) and indomethacin (10µM) to prevent integrin activation, ADP signalling or TXA2 production respectively. EGTA is a chelating agent with a high affinity for Ca2+, which is required for integrin activation, and so prevents integrin signalling. Apyrase is an enzyme that hydrolyses ATP/ADP to AMP and therefore breaks down any ADP present within the platelet preparation, thus preventing ADP receptor stimulation. Indomethacin is an inhibitor of cyclooxygenase, an enzyme involved in the production of TXA2, and therefore prevents TXA2 receptor stimulation. The use of these agents allows the action of any added agonist to be studied in isolation.
Figure 2.3: Light transmission aggregometry

(A) Schematic diagram of the measurement of platelet aggregation. (B) Representative platelet aggregation trace when platelets have been stimulated with thrombin (0.1U/ml).
2.5.2 Flow Cytometry

The method of flow cytometry involves the measurement of various characteristics on individual cells within a fluid suspension (Figure 2.4). Cells are hydrodynamically focussed to create a single stream. This results from sheath fluid being pumped through a laminar flow chamber. The sample is injected into the centre if this flow system, resulting in the single stream of cells that pass separately through several laser beams at the interrogation point. As the cells move through the lasers, light is scattered in different directions and any fluorescent antibodies present are excited, producing signals that are subsequently collected by different lenses and detectors. The two major characteristics measured by the scattering of light from the laser beams are the size of the cell, measured by forward scattered light, and cell granularity, measured by side scattered light. Fluorescently labelled antibodies can be incubated with cells prior to analysis to examine the presence of specific antigens present on a cells surface.
Diagram illustrating the fluidics system of a flow cytometer in which individual cells become focused and pass through several laser beams, resulting in the scattering of light. This is subsequently detected, converted into electronic signals and displayed onto a computer screen.

**Figure 2.4: Flow cytometry**
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2.5.2.1 Sample preparation

Samples were prepared for flow cytometric analysis as previously described (Goodall and Appleby, 2004). Modified Tyrode’s buffer (50µl), FITC or PE conjugated antibodies (2-5µl) and the agonist to be tested (5µl) were added to the appropriate sample tubes. Tubes were prepared no longer than 30 minutes prior to venepuncture to prevent deterioration of fluorescent signals. Whole blood or PRP (5µl) were next added to each tube and mixed by gentle flicking. Samples were left at room temperature to incubate for 20 minutes, unless stated otherwise, followed by fixation with formyl saline (0.2%, v/v) (0.5ml). Finally, samples were left for a minimum of 10 minutes before analysis was carried out.

2.5.2.2 Flow cytometric analysis of samples

Samples were analysed on either a BD FACS Aria™II or BD FACSCalibur (BD Biosciences). For each experiment performed, isotype control antibodies were prepared to first determine background fluorescence. The platelet bitmap was next identified using a FITC-conjugated mouse anti-human CD42b antibody. CD42b detects GPIb, a subunit of GPIb/V/IX found in the blood only on platelets. Therefore, only cells expressing fluorescence for this antibody were examined. Experimental samples were then run in ascending order of agonist concentration. 10,000 events were recorded for each sample.
2.5.2.3 Compensation

In experiments that required more than one fluorescently labelled antibody to be incubated in the same sample tube, compensation was performed manually with BD compensation beads. Figure 2.5A and Figure 2.5B show the fluorescent signals for the BD compensation beads before compensation was carried out. FITC labelled beads showed fluorescence, above $10^1$, through both FL1 (FITC) (Figure 2.5Ai) and FL2 (PE) (Figure 2.5Aii) channels whereas PE fluorescence could only be detected above $10^1$ through the FL2 channel (Figure 2.5Bii), with very little detected through the FL1 channel (Figure 2.5Bi). After compensation was performed, FITC fluorescence could only be detected through the FL1 channel (Figure 2.5C) and PE fluorescence was unaltered (Figure 2.5D). To confirm that compensation was appropriate for the platelet samples, FITC-conjugated CD42b expression was measured before (Figure 2.5E) and after (Figure 2.5F) compensation. There was very little fluorescence observed in the FL2 channel with the FITC labelled CD42b antibody before compensation (Figure 2.5Eii), however, this was abolished after compensation had been performed (Figure 2.5Fii).
Samples containing either (A and C) FITC labelled BD compensation beads, (B and D) PE labelled BD compensation beads or (E and F) PRP incubated with FITC labelled CD42b antibody (5µl, 125ng) were prepared. Analysis was then performed on a BD FACSCalibur both before (A,B and E) and after (C,D and F) compensation. Histograms represent fluorescence in either the (i) FL1 (FITC) channel or (ii) FL2 (PE) channel.

Figure 2.5: Compensation of fluorescently labelled antibodies
2.6 Methodology for examination of platelet signalling events

Investigating signalling events within platelets is vital for contributing to the understanding of how these cells work. In the present study, a combination of electrophoresis, immunoblotting and immunoprecipitation were used to examine the post translational modification of platelet proteins under a variety of experimental conditions.

2.6.1 Sample Preparation

Samples for the examination of platelet signalling events were made using washed platelets. In all cases, platelets were incubated with EGTA (1mM), apyrase (2U/ml) and indomethacin (10µM) to prevent integrin activation, ADP signalling and TXA$_2$ production respectively. Platelets were warmed to 37°C followed by the addition of any necessary inhibitors being tested. Platelets were then stimulated with agonists under stirred conditions (1000rpm). Reactions were terminated by the addition of 2x Laemmli buffer (4% SDS (w/v), 10% 2-mercaptoethanol (v/v), 20% glycerol (v/v), 50mM Tris base, trace bromophenol blue, pH6.8) which results in cell lysis, making proteins accessible for analysis (Laemmli, 1970).

2.6.2 Measurement of protein concentrations

Protein concentrations of samples were determined using the Bio-Rad DC protein assay kit based on the Lowry assay (Lowry et al., 1951). This method is a colorimetric assay in which proteins react with a copper tartrate solution in an alkaline environment. Subsequently, the copper treated proteins reduce Folin reagent, by
removal of 1-3 oxygen atoms, leading to its change in colour from yellow to blue. The intensity of blue colour correlates with the amount of protein present within the sample and is quantified at an absorbance of 750nm.

Protein standards were made with bovine serum albumin (BSA) at concentrations of 0, 0.3, 0.6, 0.9, 1.2 and 1.5mg/ml in immunoprecipitation lysis buffer (150mM NaCl, 10mM Tris base, 1mM EGTA, 1mM EDTA, 1% igepal (v/v)). A sample of washed platelets was lysed in the same buffer at a ratio of 1:1 (v/v). Protein standards and platelet samples (5µl) were added in triplicate to a 96 well plate followed by addition of the copper tartrate solution (25µl) and Folin reagent (200µl). Sample plates were gently mixed and incubated at room temperature for 15 minutes. Plates were then read at an absorbance of 750nm and protein concentration of the platelet sample was calculated from a standard curve.

2.6.3 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

Electrophoresis allows for the separation of charged macromolecules in an electrical field. When applied to a porous matrix, such as a gel, molecules can be separated based on their size and charges. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) uses a combination of SDS and polyacrylamide gels to separate proteins according to their molecular masses by electrophoretic migration. Polyacrylamide gels are made from acrylamide molecules that polymerise into long linear chains, which are cross-linked by N,N-methylenebis-acrylamide (Bis). This polymerisation is accelerated by the presence of free radicals. Hence, ammonium
persulfate (APS) is added when casting gels as it decomposes to release \( \text{SO}_4^- \) ions. 
N,N,N\(^1\),N\(^1\)-tetramethylethylenediamine (TEMED) is also included to catalyse the decay of APS. The percentage of acrylamide used in these solutions determines the pore size and therefore the relative separation of the proteins within the mixture. Electrophoretic mobility is influenced by protein shape, charge density and molecular weight. In order to separate proteins solely by molecular weight, SDS is added to the protein mixture (Shapiro et al., 1967). SDS is an anionic detergent that binds and denatures proteins leaving them with a similar, rod-shaped tertiary structure. Furthermore, it confers equal negative charge per unit protein mass (1.4g SDS per 1g protein). In the presence of a reducing agent, such as 2-mercaptoethanol, disulphide bonds are broken and proteins become fully denatured. Proteins treated this way are then separated by the disparity of their migration through a polyacrylamide gel under an electric field. The gel may then be immersed in a protein stain to indicate their relative positions.

Gels were run in a discontinuous buffer system (Laemmli, 1970) which employs the use of different buffer ions within the gel compared to the buffer within the electrode reservoirs as well as a varying pH between the stacking gel (pH6.8) and the resolving gel (pH 8.8). The stacking gel lies above the resolving gel and has large pores in order to converge or "stack" proteins into a concentrated zone before entering the resolving gel, which has smaller pores and separates proteins on account of their size. When a current is applied, proteins migrate toward an anode and separation occurs.
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2.6.3.1 Procedure used for SDS-PAGE of platelet proteins

In this study both 10-18% gradient and 10% polyacrylamide gels of 1.5mm thickness were used. Resolving gels were made with the appropriate reagents (Table 2.1 and Table 2.2) using a gradient mixer and peristaltic pump. APS and TEMED were added to the gel mixer immediately prior to pouring the gel. Gels were then left to set for approximately 1 hour at room temperature. Once set, 3% stacking gels (Table 2.3) were layered over the top of resolving gels, well forming combs (1.5mm) were inserted and gels were left to set for a further 15 minutes. Gels were placed into an electrophoresis tank containing running buffer (0.1% SDS (w/v), 25mM Tris base, 192mM glycine) before samples (15-30µg) and a biotinylated protein ladder were loaded. The gels were run at 120V for 1.5-2.5 hours depending on the size of the protein of interest.
Table 2.1: Reagents required to prepare one 10-18% gradient polyacrylamide resolving gel

<table>
<thead>
<tr>
<th>Reagent</th>
<th>10%</th>
<th>18%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water</td>
<td>1.418ml</td>
<td>0.708ml</td>
</tr>
<tr>
<td>Acrylamide</td>
<td>1.182ml</td>
<td>1.961ml</td>
</tr>
<tr>
<td>Buffer 1</td>
<td>0.886ml</td>
<td>0.886ml</td>
</tr>
<tr>
<td>APS (10%) (w/v)</td>
<td>18μl</td>
<td>18μl</td>
</tr>
<tr>
<td>TEMED</td>
<td>2μl</td>
<td>2μl</td>
</tr>
</tbody>
</table>

Table 2.2: Reagents required to prepare two 10% polyacrylamide resolving gels

<table>
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<tr>
<th>Reagent</th>
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</tr>
</thead>
<tbody>
<tr>
<td>Distilled water</td>
<td>6.4ml</td>
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<tr>
<td>Acrylamide</td>
<td>5.3ml</td>
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<tr>
<td>Buffer 1</td>
<td>4ml</td>
</tr>
<tr>
<td>APS (10%) (w/v)</td>
<td>75μl</td>
</tr>
<tr>
<td>TEMED</td>
<td>5.3μl</td>
</tr>
</tbody>
</table>

Table 2.3: Reagents required to prepare two 3% polyacrylamide stacking gels

<table>
<thead>
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<th>Reagent</th>
<th>3%</th>
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</thead>
<tbody>
<tr>
<td>Distilled water</td>
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<tr>
<td>Acrylamide</td>
<td>0.75ml</td>
</tr>
<tr>
<td>Buffer 2</td>
<td>1.87ml</td>
</tr>
<tr>
<td>APS (10%) (w/v)</td>
<td>75μl</td>
</tr>
<tr>
<td>TEMED</td>
<td>10μl</td>
</tr>
</tbody>
</table>

Table 2.4: Reagents required for preparing buffer 1 and buffer 2

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Buffer 1 (pH8.8)</th>
<th>Buffer 2 (pH6.8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris Base</td>
<td>1.5M</td>
<td>0.5M</td>
</tr>
<tr>
<td>SDS</td>
<td>0.4% (w/v)</td>
<td>0.4% (w/v)</td>
</tr>
</tbody>
</table>
2.6.4 Immunoblotting

To identify specific proteins separated from the complex mixture found in lysates, immunoblotting was used. Here, proteins are transferred from a gel to an adhesive matrix such as nitrocellulose or polyvinylidene fluoride (PVDF) membranes under an electric field. Once transferred, the membranes are probed with primary antibodies raised against specific target proteins. A secondary antibody raised against the primary antibody species is then added. Secondary antibodies are commonly conjugated to horseradish peroxidase (HRP), which allows for the detection of their position on the membrane following exposure to photographic film by enhanced chemiluminescence (ECL).

2.6.4.1 Procedure used for immunoblotting of platelet proteins

In the present study, two procedures were used to transfer proteins to a PVDF membrane. In the first method, gels were placed into a cassette containing multiple layers consisting of: foam pads, blotting paper, polyacrylamide gel, PVDF membrane, blotting paper and foam pads. Care was taken to ensure no air bubbles were present in these layers. Cassettes were then placed into a Bio-Rad transfer tank, covered with transfer buffer (25mM Tris base, 192mM glycine, 20% methanol (v/v)) and placed into an ice box to keep cool. Transfer was carried out at 100V for 2.5 hours. The second method for the transfer of proteins involved the use of the Bio-Rad Trans-Blot Turbo Transfer system in which gels were placed into premade transfer packs containing a PVDF membrane and transferred using the turbo blot system for 10 minutes.
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Following transfer, membranes were blocked in either 10% (w/v) BSA/Tris-buffered saline-tween (TBS-T) (150mM NaCl, 20mM Tris base, 0.1% tween-20 (v/v)) or 5% (w/v) milk/TBS-T, depending on the antibody to be used, for 30 minutes. This step prevented non-specific binding of the primary antibody to the membranes. Membranes were subsequently incubated with the appropriate primary antibody, diluted in either 2% (w/v) BSA/TBS-T or 2% (w/v) milk/TBS-T, overnight at 4°C. After removal of the primary antibody solution, membranes were washed in TBS-T twice for 10 minutes followed by incubation with the appropriate HRP conjugated secondary antibody in TBS-T (1:10,000 (v/v)) for one hour at room temperature. Table 2.5 illustrates the dilutions for the various primary antibodies and the suitable secondary antibodies used. Membranes were simultaneously incubated with a HRP conjugated anti-biotin antibody (1:2000 (v/v)) to detect the biotinylated protein ladder. Following incubation with the secondary antibody, membranes were washed for 1.5 hours at 15 minute intervals in TBS-T before developing using ECL, a system that employs a chemical reaction to produce the emission of light. Visualisation of the bands of interest relies on HRP, which is conjugated to the secondary antibody, and its ability to catalyse the oxidation of luminol, from the ECLI solution, by hydrogen peroxide, from the ECLII solution. This results in the emission of light, which is captured by a photographic film. Both ECLI (250mM luminol, 90mM p-coumaric acid, 100mM Tris base) and ECLII (100mM Tris base, 30% H$_2$O$_2$ (v/v)) solutions were added to membranes at a ratio of 1:1 (v/v) and incubated in the dark for 90 seconds. Membranes were placed into an exposure cassette and exposed to...
chemiluminescence film in a dark room. Films were then developed using Kodak developer solution, followed by washing and fixation in Kodak fixer solution.

To assess that samples had been equally loaded onto gels, membranes were stripped using Restore™ PLUS western blot stripping buffer (Thermo Scientific, UK) for 30 minutes at room temperature. This was followed by two 15 minute washes in TBS-T and a 30 minute incubation in 10% (w/v) BSA/TBS-T. Membranes were then incubated overnight with anti-β-tubulin antibody at 4°C and subsequently developed as above. All incubations and washing steps were performed with gentle agitation. Densitometry to assess the intensity of bands was performed using Image J software. Figure 2.6 illustrates the steps performed in immunoblotting.
Table 2.5: Dilutions of primary antibodies and appropriate secondary antibodies

Primary antibodies were diluted as stated in either 2% BSA/TBS-T or 2% milk/TBS-T solutions and detected with the referred secondary antibody.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Dilution</th>
<th>Secondary antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-β-tubulin</td>
<td>1:1000 in BSA</td>
<td>Mouse</td>
</tr>
<tr>
<td>Anti-CD36</td>
<td>1:1000 in BSA</td>
<td>Rabbit</td>
</tr>
<tr>
<td>Anti-phospho-MLC&lt;sup&gt;Ser19&lt;/sup&gt;</td>
<td>1:1000 in BSA</td>
<td>Rabbit</td>
</tr>
<tr>
<td>Anti-phospho-MYPT1&lt;sup&gt;Thr696&lt;/sup&gt;</td>
<td>1:500 in milk</td>
<td>Rabbit</td>
</tr>
<tr>
<td>Anti-phospho-MYPT1&lt;sup&gt;Thr853&lt;/sup&gt;</td>
<td>1:250 in milk</td>
<td>Rabbit</td>
</tr>
<tr>
<td>Anti-phospho-Src family&lt;sup&gt;Tyr416&lt;/sup&gt;</td>
<td>1:1000 in BSA</td>
<td>Rabbit</td>
</tr>
<tr>
<td>Anti-phospho-tyrosine</td>
<td>1:1000 in BSA</td>
<td>Mouse</td>
</tr>
<tr>
<td>Anti-PLCγ2</td>
<td>1:1000 in BSA</td>
<td>Rabbit</td>
</tr>
<tr>
<td>Anti-RhoA</td>
<td>1:1000 in BSA</td>
<td>Mouse</td>
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<tr>
<td>Anti-Syk</td>
<td>1:1000 in BSA</td>
<td>Mouse</td>
</tr>
<tr>
<td>Anti-Vav1</td>
<td>1:1000 in BSA</td>
<td>Rabbit</td>
</tr>
</tbody>
</table>
In the procedure of immunoblotting, proteins are first separated by SDS-PAGE followed by transfer to PVDF membranes. These are then blocked to prevent non-specific binding and subsequently probed with the appropriate primary antibody. A secondary antibody is then applied and detected using ECL.

Figure 2.6: Principles of immunoblotting

In the procedure of immunoblotting, proteins are first separated by SDS-PAGE followed by transfer to PVDF membranes. These are then blocked to prevent non-specific binding and subsequently probed with the appropriate primary antibody. A secondary antibody is then applied and detected using ECL.
2.6.5 Immunoprecipitation

The method of immunoprecipitation can be performed to isolate a protein of interest from a cell lysate, allowing the protein to be examined separately to assess its phosphorylation state as well as its interactions with other proteins. Antibodies that are selective for the protein of interest are firstly immobilised on an insoluble matrix such as agarose or sepharose beads conjugated to protein A or G. Conjugation of the antibody to the protein A/G bead occurs through interactions of the heavy chains of the Fc region of the antibody with protein A/G. Antibody/bead complexes are then incubated with cell lysates to allow the protein of interest to form interactions and precipitate from the mixture of proteins in the lysate. The protein complex is then separated from the rest of the cell lysate by centrifugation steps which results in the immune complex being sedimented into a pellet. After the supernatant is discarded, the antibody-antigen complex is liberated from the beads by boiling in Laemmlili buffer. Therefore, the resultant sample will remain with only the protein of interest and any physically associated proteins. The isolated protein may be studied further using SDS-PAGE and immunoblotting. The procedures used in immunoprecipitation are illustrated in Figure 2.7.

2.6.5.1 Procedure used for immunoprecipitation of platelet proteins

Protein A sepharose beads (200µl) were first washed to remove storage ethanol. This was carried out by pelleting beads using centrifugation and discarding the supernatant. The beads were washed twice in TBS-T (75µl), mixed and centrifuged. Beads were then resuspended into 50% TBS-T (w/v). Beads (25µl) were incubated with the primary
antibody at 4°C with gentle rotation for a minimum of 3 hours. Incubation was performed in the presence of BSA (1mg/ml) dissolved in modified Tyrode's buffer and immunoprecipitation lysis buffer at a ratio of 1:1 (v/v). This was to allow incubation to occur in a larger volume to enhance mixing of beads and antibody as well as to block non-specific binding sites on beads with BSA.

Washed platelet samples were prepared as in Section 2.6.1 except reactions were terminated by the addition of ice cold immunoprecipitation lysis buffer (150mM NaCl, 10mM Tris base, 1mM EGTA, 1mM EDTA, 1% igepal (v/v), 1mM PMSF, 2.5mM Na$_3$VO$_4$, 1:200 protease inhibitor cocktail (v/v), 1:200 phosphatase inhibitor cocktail (v/v)). Platelet lysates were incubated on ice for 30 minutes followed by pre-clearing with non-antibody bound protein A beads for a minimum of 1 hour. Preclearing was performed to account for any non-specific binding that may occur between proteins and beads. The platelet lysates (300-500µg protein) were then incubated with antibody/bead complexes overnight at 4°C with gentle rotation.

Immune complexes were pelleted by centrifugation and supernatants were removed and stored at -20°C. Beads were then washed once in immunoprecipitation lysis buffer (25µl), twice in TBS-T (25µl) and finally mixed with Laemmli buffer (65µl). Samples were boiled for 3 minutes to break interactions between beads, antibodies and antigens. Samples were finally centrifuged to pellet unwanted beads and the supernatants were transferred to clean eppendorfs and stored at -20°C until needed.
Samples were then run on SDS-PAGE and immunoblotted as in Sections 2.6.3 and 2.6.4.
Figure 2.7: Immunoprecipitation

The immunoprecipitation technique used involved the immobilisation of an appropriate antibody onto protein A sepharose beads followed by incubation with cell lysates. Centrifugation next isolated the bead/antibody/protein complex and elusion resulted in a sample containing the protein of interest.
2.6.6 RhoA activity assay

The activity of RhoA was assessed using an assay kit purchased from Cytoskeleton (Cambridge, UK). The kit utilises the properties of the Rho binding domain (RBD) of the Rho effector protein rhotekin, which binds with high affinity to GTP bound Rho. The rhotekin-RBD is expressed as a GST fusion protein bound to glutathione-sepharose beads. Therefore, similar to the immunoprecipitation technique, incubation of cell lysates with these beads results in active RhoA only binding to beads to allow for its subsequent isolation.

Washed platelet lysates were prepared as in Section 2.6.1 except reactions were terminated with ice cold lysis buffer (1:1 (v/v)) supplied with the kit. Samples were then immediately snap frozen in liquid nitrogen. Once all samples had been prepared, they were removed from liquid nitrogen with forceps, thawed at room temperature and immediately kept on ice. A portion of each sample (50µl) was taken and mixed with Laemmli buffer (1:1 (v/v)) to assess for total RhoA in each sample. Platelet lysates (300µg protein) were then incubated with rhotekin-RBD beads (30µg) for 1.5 hours at 4°C with gentle rotation. Samples were then centrifuged to pellet RhoA/bead complexes and the supernatant removed and stored at -20°C. Beads were then washed once in wash buffer (500µl) supplied with the kit, pelleted by centrifugation and resuspended in Laemmli buffer (30µl). Samples were subsequently stored at -20°C until needed. Samples were boiled for 3 minutes before use and run on SDS-PAGE and immunoblotted as in Sections 2.6.3 and 2.6.4.
Chapter 2

2.7 Statistical analysis

Data are expressed as mean ± SEM. Statistical comparisons of data were carried out using Students t-test and one-way ANOVA. Statistical tests were performed using Microsoft Excel 2007 and IBM SPSS Statistics 19. Statistical significance was considered for values of $p<0.05$. 
Chapter 3

Chapter 3: Oxidised low density lipoproteins induce platelet aggregation

3.1 Introduction

LDL can become oxidatively modified within the subendothelial space of blood vessel walls, which is thought to contribute to the development of atherosclerosis and thrombotic events (Lusis, 2000). In order to more fully understand the potential effects of oxLDL on atherothrombosis, it is important to characterise its effects on the cells that are known to form the basis of atherosclerotic plaque associated thrombi. In the first section of this work, a simple but important confirmation was sought to establish that the oxidative modification of LDL caused these particles to interact with platelets in a manner that resulted in activation and that the native form of LDL had no effect, since there have been inconsistent reports over the years as to whether nLDL influences platelet function. Early work that was carried out investigating the effects of nLDL on platelets found these particles to induce platelet shape change and spontaneous aggregation (Hassall et al., 1983). However, it is now likely that these observations were the result of LDL being oxidised during the isolation process. Therefore, it was imperative to distinguish between the nLDL and oxLDL used in this study. OxLDL appears to affect platelet aggregation in a variety of ways. Investigators have found oxLDL to both induce platelet aggregation but also to inhibit other agonist induced aggregation (Korporaal et al., 2005). Crucially, it appears that the extent of oxidation is a key feature to distinguish the role that oxLDL may play (Naseem et al., 1997).
The aims of this chapter include:

- Evaluating the extent of oxidation of the LDL species produced in this project.
- Investigating the platelet aggregating properties of oxLDL.
- Assessing whether platelet aggregation induced by oxLDL is secondary messenger mediated.
3.2 Measurement of the oxidation of low density lipoproteins

Oxidation of LDL was carried out as previously described (Gerry et al., 2008). This method involved oxidation at 37°C for 24 hours to produce a species that was rich in oxysterols, the modified cholesterol constituent. To confirm the oxidation of LDL, two techniques were employed: the use of agarose gels to measure REM and a lipid hydroperoxide assay.

3.2.1 Measurement of the electrophoretic mobility of oxidised low density lipoproteins

The oxidation of LDL results in the modification of the positively charged amino acids of apoB-100, with the consequence of an increased overall negative charge. Increasing concentrations of nLDL and oxLDL were separated by electrophoresis on agarose gels (1%). This was done to determine the appropriate quantity of protein needed to enable suitable detection and to calculate REM. As demonstrated by Figure 3.1, oxLDL prepared for this study migrated toward the anode to a much greater extent than nLDL. Based on this experiment, 20µg of protein was chosen to be used for the measurement of REM for subsequent batches of LDL as this amount of protein could be confidently visualised. The REM measured for nLDL in this study was 1.0±0 and the REM for oxLDL was 3.9±0.2, indicating a significant increase (p<0.001) in REM following oxidation. The findings here were similar to levels found by other groups that have used this method of oxidation (Gerry et al., 2008).
(A) Varying doses of nLDL and oxLDL (10-30µg) were loaded onto agarose gels and run at 100V for 1 hour. (B) REM was calculated for both nLDL (20µg) and oxLDL (20µg) from 7 of the LDL preparations used. *** indicates p<0.005.

Figure 3.1: Measurement of the relative electrophoretic mobility of non-oxidised and oxidised low density lipoproteins
3.2.2 Measurement of lipid hydroperoxides in oxidised low density lipoproteins

The levels of lipid hydroperoxides within both the nLDL and oxLDL species were examined. Consistent with a number of published studies, small levels of lipid hydroperoxides were found to be present in nLDL preparations (Gerry et al., 2008, Naseem et al., 1997, el-Saadani et al., 1989), but as Figure 3.2A illustrates, the oxidation of LDL by Cu$^{2+}$ led to a significant increase. The lipid hydroperoxide levels within nLDL were found to be 9.9±7.2 nmol/mg LDL protein compared to 74.0±11.5 nmol/mg LDL protein for oxLDL (p=0.03). Since the nLDL was used for several experiments over a number of weeks, it was important to ensure that the preparations did not undergo significant oxidation while being stored. To assess that the nLDL was stable and that it would remain unoxidised during the experimental period in which it was used, levels of lipid hydroperoxides were measured over a 5 week period following isolation. Figure 3.2B confirms that very little oxidation products were present in nLDL within the first 4 weeks after isolation from the blood. Lipid hydroperoxide oxidation products only began to form after 5 weeks post-isolation in which lipid hydroperoxide levels were still relatively low (24.36 nmol/mg LDL protein). Therefore, nLDL was used in experiments for up to a 4 week period post-isolation.
Figure 3.2: Measurement of lipid hydroperoxides within non-oxidised and oxidised low density lipoproteins

(A) Lipid hydroperoxide levels were measured in nLDL and oxLDL from 3 separate LDL preparations. (B) Lipid hydroperoxide levels were measured in a batch of nLDL over a 5 week period following isolation from the blood. * indicates p<0.05-0.01.
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3.3 Oxidised low density lipoproteins induce platelet aggregation

In the first instance, the ability of oxLDL to induce platelet aggregation was established. These experiments were to clarify the extent of platelet aggregation that could be induced by oxLDL and also to determine whether aggregation was mediated by secondary signalling. However, before the pro-aggregatory properties of oxLDL were assessed, platelet responses were first tested in response to the two physiological agonists collagen and thrombin, to ensure that the platelet preparations were functionally competent. The two different agonists were used to examine sensitivity to both tyrosine kinase and GPCR linked activation mechanisms.

3.3.1 Platelet aggregation induced by the physiological agonists collagen and thrombin

The addition of collagen (0.1-10µg/ml) induced a concentration dependent increase in aggregation. At high doses, collagen (10µg/ml) induced 78.0±6.5% aggregation (Figure 3.3A). Similarly, stimulation of platelets with the GPCR agonist thrombin also caused a concentration dependent increase in aggregation with maximal effects of 89.0±2.7% aggregation observed with 0.1U/ml (Figure 3.3B).
Washed platelets (2.5x10^8/ml) were stimulated with varying doses of (A) collagen (0.1-10µg/ml) or (B) thrombin (0.01-0.1U/ml) and aggregation responses were recorded for 4 minutes. (Ai) and (Bi) Representative traces and (Aii) and (Bii) quantitative analysis of 3 separate experiments.

**Figure 3.3: Platelet aggregation responses to collagen and thrombin**

Washed platelets (2.5x10^8/ml) were stimulated with varying doses of (A) collagen (0.1-10µg/ml) or (B) thrombin (0.01-0.1U/ml) and aggregation responses were recorded for 4 minutes. (Ai) and (Bi) Representative traces and (Aii) and (Bii) quantitative analysis of 3 separate experiments.
3.3.2 Platelet aggregation in response to oxidised low density lipoproteins

Having established that platelet preparations were functionally sensitive to agonists, the effects of oxLDL were examined. nLDL did not induce detectable platelet aggregation under any of the conditions tested. In contrast, oxLDL did induce aggregation, although the extent was highly variable between individual platelet donors. A dose of 50µg/ml oxLDL induced a range of aggregation responses from 8-44%. This range was observed in aggregation experiments from 15 platelet donors across 5 individual batches of oxLDL. Figure 3.4A illustrates typical aggregation traces provoked by nLDL and oxLDL. OxLDL (50µg/ml) appears to stimulate a weak platelet aggregation response when compared to collagen (10µg/ml), whereas nLDL (50µg/ml) does not induce any aggregation. Additionally, aggregation responses to a series of oxLDL and nLDL concentrations were compared (Figure 3.4B). The responses stimulated by oxLDL were significantly higher than the corresponding dose of nLDL (10µg/ml p=0.04, 50µg/ml p=0.02 and 100µg/ml p<0.01). Aggregation responses induced by oxLDL appeared to be maximal at a concentration of 50µg/ml.

In addition to the highly oxidised form of LDL used in these experiments, a more minimally oxidised form of LDL was also tested for its aggregation inducing properties. Hydroperoxide rich LDL (hpLDL) contains predominantly lipid hydroperoxides with little oxysterol formation or protein modification (Gerry et al., 2008). This form of modified LDL also induced platelet aggregation, although not to the same extent as oxLDL. For comparison, hpLDL (50µg/ml) stimulated 13.0±1.7% aggregation and oxLDL (50µg/ml) 29.7±2.4% aggregation (p=0.03) (Figure 3.5).
Figure 3.4: Platelet aggregation induced by oxidised low density lipoproteins

(A) Washed platelets (2.5x10^8/ml) were stimulated with nLDL (50µg/ml), oxLDL (50µg/ml) or collagen (10µg/ml) and aggregation responses were recorded for 4 minutes. Shown are representative traces of 6 experiments. (B) Washed platelets (2.5x10^8/ml) were stimulated with varying doses (10-100µg/ml) of nLDL and oxLDL and aggregation was recorded for 4 minutes. Data is analysed from 3 separate experiments. * indicates p<0.05-0.01 and ** p<0.01-0.005
Washed platelets (2.5x10^8/ml) were stimulated with either nLDL (50µg/ml), hpLDL (50µg/ml) or oxLDL (50µg/ml) and aggregation responses were recorded for 4 minutes. Data is expressed from 3 separate experiments. * indicates p<0.05-0.01, ** p<0.01-0.005 and *** p<0.005.

**Figure 3.5: Platelet aggregation induced by hydroperoxide rich low density lipoproteins and oxidised low density lipoproteins**

Washed platelets (2.5x10^8/ml) were stimulated with either nLDL (50µg/ml), hpLDL (50µg/ml) or oxLDL (50µg/ml) and aggregation responses were recorded for 4 minutes. Data is expressed from 3 separate experiments. * indicates p<0.05-0.01, ** p<0.01-0.005 and *** p<0.005.
3.3.3 The effects of hydroperoxide rich low density lipoprotein on thrombin induced aggregation

Having found that oxidatively modified LDL induce only small amounts of aggregation, their influence on aggregation induced by other agonists was tested. Only hpLDL was examined in this experiment and its effects were observed on the physiological agonist thrombin. Figure 3.6A demonstrates that hpLDL alone induced a small extent of aggregation at both concentrations tested (10µg/ml - 15.7±5.2% and 50µg/ml - 23.0±7.6%). A low dose of thrombin (0.005U/ml) alone stimulated a mid-range of aggregation (36.7±16.8%). HpLDL and thrombin added together also provoked a mid-range aggregation response (10µg/ml hpLDL + thrombin - 49.7±16.3% and 50µg/ml hpLDL + thrombin - 45.7±15.9%). The difference in aggregation responses induced by thrombin alone compared to thrombin in combination with hpLDL was not statistically significant across the range of experiments tested (Figure 3.6B). Aggregation did however appear to occur at a faster rate when hpLDL was present with thrombin compared to thrombin alone.
Washed platelets ($2.5 \times 10^8 / ml$) were stimulated with either thrombin (0.005U/ml), hpLDL (10µg/ml) or hpLDL (50µg/ml) alone or a combination of thrombin (0.005U/ml) and hpLDL (10µg/ml) or thrombin (0.005U/ml) and hpLDL (50µg/ml) and aggregation traces were recorded for 4 minutes. (A) Representative traces and (B) quantitative analysis of 3 independent experiments.

Figure 3.6: Aggregation responses to hydroperoxide rich low density lipoproteins and thrombin

Washed platelets ($2.5 \times 10^8 / ml$) were stimulated with either thrombin (0.005U/ml), hpLDL (10µg/ml) or hpLDL (50µg/ml) alone or a combination of thrombin (0.005U/ml) and hpLDL (10µg/ml) or thrombin (0.005U/ml) and hpLDL (50µg/ml) and aggregation traces were recorded for 4 minutes. (A) Representative traces and (B) quantitative analysis of 3 independent experiments.
3.3.4 Oxidised low density lipoprotein induced aggregation is integrin dependent

To confirm that aggregation responses induced by both oxLDL and hpLDL were indeed due to platelet aggregation and integrin activation and not due to the agglutination of platelets, further experiments were performed. Here, platelets were tested in the presence of the reagents EGTA and RGDS. EGTA is a chelating agent with a high affinity for Ca\(^{2+}\); without this cation, integrins are unable to acquire their activated confirmation and aggregation is therefore blocked. RGDS is a synthetic peptide composed of the amino acids arginine, glycine, aspartic acid and serine, which corresponds with the sequence of amino acids on the A\(\alpha\) chain of fibrinogen that binds to the integrin \(\alpha_{IIb}\beta_3\). This peptide blocks fibrinogen from interacting with the receptor through competitive inhibition and, therefore, prevents aggregation (Gartner and Bennett, 1985). To firstly confirm their effectiveness, these reagents were used in combination with collagen. Both EGTA (1mM) and RGDS (1mM) blocked collagen (5µg/ml) induced aggregation, illustrating their ability to inhibit integrin activity (Figure 3.7A). The differences in aggregation responses induced in the presence and absence of EGTA and RGDS by hpLDL (50µg/ml) were not found to be significant (Figure 3.7D). Similarly, Only EGTA was found to cause a significant decrease in aggregation responses (p=0.03) induced by oxLDL (50µg/ml) (Figure 3.7E). This suggests that aggregation induced by oxidatively modified LDL is integrin dependent although perhaps some agglutination may occur.
Washed platelets \((2.5 \times 10^8/\text{ml})\) were incubated in the presence or absence of either EGTA (1mM) or RGDS (1mM) for 5 minutes followed by stimulation with (A) collagen (5µg/ml), (B) hpLDL (50µg/ml) or (C) oxLDL (50µg/ml) and aggregation traces were recorded for 4 minutes. (A) Traces of an individual experiment. (B) and (C) are representative traces and (D) and (E) are quantitative analysis of 4 independent experiments. * indicates p<0.05-0.01.

**Figure 3.7:** Oxidised low density lipoprotein and hydroperoxide rich low density lipoprotein induced platelet aggregation is integrin dependent
3.3.5 Oxidised low density lipoprotein induced aggregation is ADP dependent

Platelets release ADP from their dense granules and generate TXA$_2$ in response to physiological platelet agonists, both of which enhance aggregation responses. Therefore, it was of interest to establish whether oxLDL induced aggregation was dependent on these secondary messengers. To investigate this, platelets were treated with apyrase and indomethacin prior to stimulation with oxLDL. Apyrase is an enzyme that hydrolyses ADP and thus prevents this nucleotide from interacting with its receptors, whereas indomethacin is an inhibitor of the cyclooxygenase enzyme and consequently prevents TXA$_2$ production. To first of all establish that these reagents were effectual, aggregation was observed in response to collagen (Figure 3.8). Indomethacin significantly reduced aggregation responses to collagen (p<0.001). Apyrase was not as effective, however, a substantial reduction was still observed, but the extent of inhibition was much more variable. Consistent with previous studies, both reagents incubated together further reduced the extent of collagen induced aggregation (p<0.001) (Atkinson et al., 2001).

Interestingly, indomethacin did not significantly inhibit oxLDL induced aggregation, although a very slight delay was observed. Conversely, apyrase almost abolished oxLDL induced aggregation (p=0.02) and complete inhibition was seen in the presence of both reagents (p=0.02) (Figure 3.9). This strongly suggests that oxLDL stimulates the secretion of ADP from dense granules, which in turn provokes platelet aggregation.
To finally confirm that aggregation induced by oxLDL was due to the modified lipoprotein and not one of the reagents found in the buffer that it was diluted in during the oxidation procedure, platelets were also stimulated with a sample of this MOPS buffer (150mM NaCl, 10mM MOPS, pH7.4) alone. As can be seen in Figure 3.10 oxLDL does again induce a small extent of aggregation whereas no effect was observed with the MOPS buffer alone.
Washed platelets (2.5x10^8/ml) were incubated with either apyrase (2U/ml), indomethacin (10µM) or a combination of both for 20 minutes followed by stimulation with collagen (5µg/ml) and aggregation responses were recorded for 4 minutes. (A) Representative traces and (B) quantitative analysis of 3 independent experiments. *** indicates p<0.005.

Figure 3.8: Enhancement of collagen induced aggregation by ADP and thromboxane A₂
Washed platelets (2.5x10^8/ml) were incubated with either apyrase (2U/ml), indomethacin (10µM) or a combination of both for 20 minutes followed by stimulation with oxLDL (50µg/ml) and aggregation responses were recorded for 4 minutes. (A) Representative traces and (B) quantitative analysis of 3 independent experiments. * indicates p<0.05-0.01.

**Figure 3.9: Oxidised low density lipoprotein induced aggregation is ADP dependent**
Washed platelets \((2.5 \times 10^8/\text{ml})\) were stimulated with either oxLDL (50µg/ml) or the equivalent volume of MOPS buffer and aggregation was recorded for 4 minutes. Traces are representative of 2 separate experiments.

**Figure 3.10: MOPS buffer used to dilute oxidised low density lipoproteins does not affect platelet aggregation**

Washed platelets \((2.5 \times 10^8/\text{ml})\) were stimulated with either oxLDL (50µg/ml) or the equivalent volume of MOPS buffer and aggregation was recorded for 4 minutes. Traces are representative of 2 separate experiments.
3.4 Discussion

The major aim of the initial section of this work was to establish that oxLDL prepared in this laboratory was able to activate platelets and to confirm the often contradictory literature in the field. The main finding was that oxLDL can induce the aggregation of human platelets and it appears that this response is mediated primarily through the release of the secondary messenger ADP. These data indicate that the oxLDL used in this project is indeed a potential pathological activator of platelets and is a useful tool for studying atherosclerotic derived ligands on platelet function.

3.4.1 Oxidised low density lipoproteins possess altered apolipoprotein B-100 molecules and lipid hydroperoxides

The oxidation of LDL is a complex process and results in a multitude of different products including lipid hydroperoxides, oxysterols such as 7-ketocholesterol, aldehyde products such as malondialdehyde and apoB-100 modification (el-Saadani et al., 1989, Jialal et al., 1991, Gerry et al., 2008). There are a number of assays that can be performed to assess the oxidation state of LDL. These include the measurement of thiobarbituric acid reactive substances (TBARS), conjugated dienes, lipid hydroperoxides, oxysterols and modifications of protein. The TBARS assay involves the reaction of malondialdehyde, formed during oxidation, with thiobarbituric acid (TBA), which can subsequently be spectrophotometrically quantified at an absorbance of 532nm and has been used extensively in the past (Slater and Sawyer, 1971). However, these reactions are not specific and other products such as sugars and amino acids can also react with TBA (Jialal and Devaraj, 1996). Consequently, this assay can be
unreliable and was therefore not used in this study. Other assays that can be used to measure oxidation of LDL, which were not employed in this project, include conjugated diene formation (Esterbauer et al., 1989), which determines the rearrangement of double bonds within the fatty acid chains of phospholipids and cholesterol esters, and measurement of oxysterols by high performance liquid chromatography (HPLC) (Kritharides et al., 1993). It would be of interest in future work to perform these assays to further assess the oxidation state of the oxLDL used.

In order to confirm the oxidative modification of LDL in this study, agarose gel electrophoresis was used to measure the REM of oxLDL. The alteration of the apoB-100 component of LDL results in an increase in the overall negative charge. This occurs due to a reaction between aldehydes formed from the breakdown of lipid hydroperoxides and the positively charged lysine and arginine residues as well as the histidine and proline residues, which may also be converted to negatively charged aspartate and glutamine (Jialal and Devaraj, 1996). Therefore, the measurement of REM can be used to establish the full oxidation of LDL that involves both lipid and protein modification. The data obtained here illustrate that the oxidation protocol used caused the increased mobility of oxLDL compared to nLDL, verifying full oxidation. A further test was also performed to validate the oxidation state of the LDL used, which involved the measurement of lipid hydroperoxides within the LDL preparations. OxLDL contained greater levels of lipid hydroperoxides than nLDL, again clarifying the oxidation state of the oxLDL used.
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The protocol applied to oxidise LDL in this project was adapted from a highly optimised technique (Gerry et al., 2008). The study from this group characterised in detail the different oxidation products formed during this procedure. Importantly, REM reported by the group was approximately 5, similar to observations in this study (3.9±0.2). Lipid hydroperoxide levels were approximately 50nmol/mg LDL protein, which is again consistent with observations in this report (74.0±11.5 nmol/mg LDL protein). The study by Gerry and colleagues described high levels of 7-ketocholesterol and a decrease in nonesterified and esterified cholesterol content. As the LDL used in this project was modified using an identical procedure and because the REM and lipid hydroperoxide levels were comparable, it can be suggested that the oxLDL used in this project may also be rich in oxysterols. Therefore, from the oxidation assays performed and consistency with the literature, it can be confidently proposed that the oxLDL used in subsequent experiments was of a highly oxidised nature.

3.4.2 Platelet aggregation induced by oxidised low density lipoproteins

The first response to oxLDL tested in this project was platelet aggregation. Two forms of modified LDL were investigated in this sense: hpLDL, which was rich in lipid hydroperoxides and so minimally modified; and oxLDL, which was rich in oxysterols and, therefore, highly oxidised. Both forms of LDL induced a small extent of platelet aggregation and when tested in comparison on the same platelet donor, oxLDL stimulated a greater aggregation response than hpLDL. Platelet aggregation induced by multiple forms of oxLDL used over a range of studies is highly variable. Early work on oxLDL found the modified lipoprotein to induce platelet aggregation (Ardlie et al.,
which is consistent with the findings in this report. In support of this, a more recent study has also found oxLDL to induce a small extent of aggregation and that this response was greater in the more highly oxidised species of LDL (Korporaal et al., 2005). Conversely, other investigators have found only minimally modified LDL to induce platelet aggregation by itself and that highly oxidised LDL did not induce aggregation at all, even though the oxLDL in these studies were produced using CuSO₄, as did this project (Weidtmann et al., 1995, Naseem et al., 1997). Therefore, the platelet aggregating properties of oxLDL appear to be varied, which was also illustrated in this report by the inconsistency of aggregation responses induced by the oxLDL used here. This may be explained by the heterogeneous nature of LDL isolated from different donors, which when oxidised would create an array of structurally diverse products. LDL have been categorised into four major classes termed LDL-I to IV from small, dense LDL to larger, less dense particles. The components of LDL can range from approximately 18-23% phospholipids, 3-7% triglycerides, 40-46% esterified cholesterol, 7-10% unesterified cholesterol and 18-29% protein (Berneis and Krauss, 2002). A study investigating 10 LDL donors showed that there was a substantial range in the extent of oxidation products from the individual batches including differences in protein modification by measuring REM, lipid peroxidation using the TBARS assay and oxysterol levels (Jialal et al., 1991). Therefore, for example, a donor with greater levels of cholesterol may result in an oxidised form of LDL with higher levels of oxysterols. Depending on the exact oxidative products within LDL that induce platelet activation, if levels of this vary significantly between batches, this may result in varying degrees of activation. Additionally, the protocols used for oxidising LDL are inconsistent within
the literature with methods for oxidising including exposure to CuSO$_4$, FeSO$_4$ and air being used (Weidtmann et al., 1995, Korporaal et al., 2005, Naseem et al., 1997). Different procedures may create a variety of species that are able to interact with platelets in diverse ways. Previous demonstrations have shown that oxPC$_{CD36}$ in LDL bind to and induce the activation of platelets (Podrez et al., 2007) and so preparations containing high levels of this component may react more readily with platelets. The protocol used in this study to induce the oxidation of LDL involved incubation with Cu$^{2+}$, a method that has previously been shown to produce oxPC$_{CD36}$ species in LDL (Podrez et al., 2002b), making it an appropriate procedure to examine the platelet activating properties of oxLDL. Therefore, the heterogeneous nature of oxLDL may illuminate an explanation for variability of aggregation responses observed both here and within the literature.

It is postulated that oxLDL may act as ligands that sensitise platelets to more physiological agonists and thereby increase activation. The hpLDL used in this project was found to slightly enhance thrombin induced aggregation, however, this was not found to be significant. Other research groups have established minimally oxidised LDL to enhance platelet aggregation, but highly oxidised LDL to inhibit aggregation in response to ADP in washed platelets and PRP (Naseem et al., 1997). Inhibition of aggregation by oxLDL has also been seen in response to other agonists (TRAP) (Korporaal et al., 2005). Some researchers have found oxLDL to inhibit collagen induced aggregation in PRP, which was attributed to an increase in cAMP levels and VASP$^{Ser157}$ phosphorylation (Chou et al., 2006). Interestingly, oxLDL has also been
found to enhance aggregation to ADP and collagen in whole blood but not in PRP or washed platelets (Tornvall et al., 1999). It is difficult to make comparisons between all of these findings as different agonists and LDL preparations were used in each study, however, they do highlight the heterogeneous nature of oxidatively modified LDL. It would be appealing to include as future work for this project, a more detailed study into the effects oxLDL can have on other agonists. Within the body, especially at sites of atherosclerotic plaque rupture, other platelet agonists may also be present in the vicinity of oxLDL. Therefore, it would be beneficial to compare the effects that oxLDL, which have been oxidised to different extents, have on a variety of platelet agonists.

It was important in this work to distinguish the mechanism in which oxLDL induces platelet aggregation. Findings showed that oxLDL and hpLDL induced aggregation was inhibited when integrin activity was blocked by the reagents EGTA and RGDS, however, this was not found to be significant for RGDS. This lack in significance nevertheless may be explained by the high variability in aggregation responses induced by oxLDL alone. As EGTA significantly inhibited oxLDL induced aggregation, therefore indicating integrin activation, this was the first sign of evidence in this study that oxLDL does activate platelets. To further dissect the mechanisms of oxLDL induced aggregation, platelets were also treated with apyrase and indomethacin, to block ADP and TXA\textsubscript{2} signalling respectively. Interestingly, the majority of aggregation induced by oxLDL occurred from ADP signalling, as apyrase blocked aggregation whereas indomethacin had little effect. This indicates that oxLDL induces dense granule secretion of platelets, which has major implications when considering atherosclerotic lesions.
oxLDL does not cause a large aggregation response, if platelets were to come into contact with oxLDL, for example in the region of a ruptured atherosclerotic plaque, ADP would be secreted, which may recruit and activate nearby platelets. This may result in partially activated circulating platelets, consequently leading to thrombosis. Early work on oxLDL induced aggregation, consistent with these experiments, also found indomethacin to have little inhibitory effect on oxLDL induced platelet aggregation (Ardlie et al., 1989). However, Weidtmann and colleagues attributed aggregation induced by minimally modified LDL to be completely TXA$_2$ dependent, with aspirin inhibiting aggregation (Weidtmann et al., 1995). However, this LDL was only minimally modified compared to the highly modified LDL tested in this project, again suggesting that the heterogeneous nature and the level of oxidation of LDL may produce different signalling responses in platelets.

To summarise, this chapter has provided evidence that the oxLDL used in this project is of a highly oxidised nature and induces a mild aggregation response in platelets. Additionally, this aggregation response can be attributed, at least partially, to integrin activation and also to ADP secretion. These two lines of evidence strongly suggest that oxLDL activates platelets, which can be highly problematic when considering situations within the body where platelets may come into contact with oxLDL, such as in atherosclerosis.
Chapter 4: The expression of CD36 on platelets

4.1 Introduction

In light of the findings in the previous chapter, illustrating that oxLDL can induce platelet activation and aggregation, the next line of questioning involved the assessment of the receptor on platelets that may facilitate oxLDL signalling. Therefore, investigations in this chapter concerned the examination of the scavenger receptor CD36. CD36 was first identified on platelets several decades ago (Talle et al., 1983) and is highly expressed on these cells, which are thought to have around 10,000-25,000 copy numbers per cell (Nergiz-Unal et al., 2011b) as well as more residing intracellularly in the membranes of α-granules (Berger et al., 1993). However, the physiological role of this receptor on haemostatic cells is not well understood. Early reports suggested that CD36 plays an activating role on platelets, particularly in response to both collagen (Tandon et al., 1989) and TSP-1 (Tuszynski et al., 1988). Furthermore, CD36 has also been established as a receptor for oxLDL (Endemann et al., 1993). Indeed, recent findings suggest that some of the platelet activating properties of oxLDL occur through its interactions with CD36 (Chen et al., 2008). Within a more pathophysiological setting in mice, CD36 was shown to be the critical receptor for inducing a prothrombotic state within hyperlipidaemia, due to its interactions with oxLDL species (Podrez et al., 2007). Therefore, this evidence suggests that CD36 may play an important role in platelet function, particularly in response to ligation with oxLDL. In the first instance, the expression of CD36 on platelets was evaluated using two separate techniques, immunoblotting and flow cytometry. This was important to ascertain as high levels of CD36 in conjunction with a hyperlipidaemic state may
predispose patients to increased platelet activation and an escalated prothrombotic condition.

The aims of this chapter include:

- Identifying CD36 on platelets.
- Assessing whether CD36 expression alters upon stimulation with platelet agonists.
- Evaluating the specificity of FA6.152 as a CD36 blocking antibody.
4.2 Assessment of CD36 expression on platelets using immunoblotting

To begin with, CD36 expression on platelets was confirmed using immunoblotting. Platelet whole cell lysate (10-50µg protein) were separated using SDS-PAGE and immunoblotted with an antibody specific to CD36. Figure 4.1A illustrates that CD36 is present in platelets and shows that this protein can be visualised with as little as 10µg of whole cell lysate protein. To further confirm this finding, CD36 was immunoprecipitated from non-stimulated whole cell lysates followed by immunoblotting with the CD36 antibody. A band was detected in the CD36 immunoprecipitated sample at the approximate reported molecular weight of CD36. In contrast, no band was detected in the IgG control antibody sample (Figure 4.1B).
Figure 4.1: CD36 expression in platelets

(A) Non-stimulated washed platelet samples (7x10^8/ml) were lysed followed by separation of proteins using SDS-PAGE and immunoblotting for CD36. Membranes were then stripped and re-probed for β-tubulin. Shown is a western blot from a single experiment. (B) Non-stimulated washed platelet samples (7x10^8/ml) were lysed followed by immunoprecipitation of CD36. Samples were subsequently separated by SDS-PAGE and immunoblotted for CD36. Shown is a representative western blot from 3 independent experiments.
4.3 Optimisation of the method used to assess surface antigens on platelets using flow cytometry

Immunoblotting and immunoprecipitation experiments confirmed the presence of CD36 in platelet whole cell lysates, however, these experiments cannot identify the location of receptors in cells. Therefore, to further investigate the expression of CD36 on the platelet's surface, the technique of flow cytometry was utilised. By examining non-permeabilised cells using flow cytometry, the extracellular surface expression of CD36 can be established. However, to begin with, the method to identify platelets and detect surface antigens in both whole blood and PRP samples was optimised.

4.3.1 Detection of platelets with the CD42b antibody

The platelet population in PRP and whole blood was identified by incubation of platelets with a CD42b antibody that recognises the GPIb subunit of the GPIb/V/IX receptor. Firstly, a titration of the antibody was performed. PRP was incubated with increasing volumes (1µl, 5µl and 10µl; equivalent to 25ng, 125ng and 250ng) of the CD42b antibody and the fluorescence produced by each sample was measured. Figure 4.2 demonstrates that the fluorescent signal detected was greater with 5µl of the CD42b antibody (mean fluorescence intensity (MFI) = 3164) than with 1µl (MFI = 991), whereas little difference was observed between 5µl and 10µl (MFI = 4130). Therefore, 5µl of the CD42b antibody was used to gate the platelet population in subsequent experiments in conjunction with forward scatter and side scatter properties.
Figure 4.2: Titration of the CD42b antibody

PRP was incubated with either (A) 1µl (25ng), (B) 5µl (125ng) or (C) 10µl (250ng) of the CD42b antibody for 20 minutes followed by fixation. Samples were then analysed on a BD FACS Aria™II. Histograms are from a single experiment.
4.3.2 Evaluation of the stability of flow cytometry samples over time

The stability of flow cytometer samples was next evaluated in order to determine the time period in which the samples should be made and subsequently analysed. PRP was incubated with the CD42b antibody and this sample was examined every 30 minutes for a period of 3 hours. Figure 4.3 shows that the dot plots, representing the platelet population, and the histograms, illustrating the CD42b antibody fluorescence, are relatively stable over the course of a 3 hour period. However, close inspection reveals that the side scatter properties of the platelets slightly decline when comparing analysis immediately post-fixation (Figure 4.3Ai) with analysis 3 hours after fixation (Figure 4.3Di). This suggests a decrease in granularity, possibly due to the secretion of granule content. Interestingly, forward scatter properties, indicating the size of platelets, do not alter over time. Additionally, there is also a slight decrease observed in CD42b antibody fluorescence when comparing Figure 4.3Aii to Figure 4.3Dii, suggesting decay in the antibody signal over time. Therefore, subsequent samples were always analysed as soon as possible after fixation, within a maximum period of 2 hours, to allow for optimal fluorescence signal to be detected.
Figure 4.3: Analysis of sample stability over time

PRP was incubated with the CD42b antibody (5µl, 125ng) for 20 minutes followed by fixation. Samples were then analysed on a BD FACS Aria™ II immediately after fixation and every 30 minutes thereafter. (Ai-Di) Dot plots of the platelet population. (Aii-Dii) Histograms of CD42b antibody fluorescence. (A) Analysis immediately post-fixation (22 minutes). (B) Analysis 60 minutes post-fixation. (C) Analysis 120 minutes post-fixation. (D) Analysis 180 minutes post-fixation. Data is from a single experiment performed.
4.3.3 Identification of the platelet population in whole blood and platelet rich plasma

After optimising the conditions for analysing platelet samples with the flow cytometer, whole blood and PRP samples were next analysed to ensure the correct identification of the platelet population. Figure 4.4Ai illustrates a sample of whole blood, where two distinct populations can be identified. The smaller, less dense population (highlighted in blue) was analysed for its CD42b fluorescence. More than 95% of cells in this population were positive for CD42b expression (Figure 4.4Aiii), indicating that these cells are platelets. Furthermore, when analysing PRP samples, only one population of cells was observed (Figure 4.4Bi), due to their isolation by centrifugation. However, to confirm that these cells were platelets, CD42b expression was analysed and again more than 95% of cells were shown to be platelets (Figure 4.4Biii). These samples were performed in every flow cytometer experiment carried out to ensure that only platelets were analysed.
Chapter 4

Figure 4.4: Analysis of platelet populations in whole blood and platelet rich plasma
(A) Whole blood and (B) PRP were incubated with either IgG control antibody (5µl, 250ng) or CD42b antibody (5µl, 125ng) for 20 minutes followed by fixation. Samples were then analysed on the BD FACS Aria™ II. Shown are the dot plots of the (Ai) whole blood sample and (Bi) PRP sample. (Aii) and (Bii) Histogram of the IgG control antibody fluorescence. (Aiii) and (Biii) Histogram of the CD42b antibody fluorescence. Images are representative from all flow cytometer experiments performed.
4.4 Surface antigen expression on non-stimulated and stimulated platelets

The analysis of CD36 expression on platelets by flow cytometry was required to confirm previous immunoblotting experiments (Figure 4.1) and also to examine if the surface expression of CD36 changed under stimulated and non-stimulated conditions. However, before this was performed, the conditions required for platelet activation were established. A CD62p antibody, to detect P-selectin expression and an antibody that recognises fibrinogen, to detect fibrinogen binding to platelet surfaces, were used. All experiments performed in this chapter were under conditions where cells were not permeabilised. Therefore, any antigen detected was from expression on the surface of platelets.

4.4.1 Platelet activation by ADP induces P-selectin expression and fibrinogen binding on platelets in whole blood

Samples of whole blood were incubated with increasing concentrations of ADP (0.1-10µM) in combination with a CD62p antibody (5µl, equivalent to 31ng) that recognises P-selectin and has been used under similar conditions previously (Riba et al., 2004). As expected, when stimulated with ADP, platelet P-selectin expression increased in a concentration dependent manner (Figure 4.5). For example, the percentage of platelets that expressed P-selectin increased from 2.3±0.2% in non-stimulated samples to 33.9±1.6% in platelets stimulated with ADP (10µM) (p=0.001) (Figure 4.5F). The MFI for CD62p expression was also calculated as a relative increase from basal levels. There was a significant increase in MFI upon stimulation with ADP (1µM, p<0.001) and ADP (10µM, p<0.001) (Figure 4.5G).
Fibrinogen binding was also assessed in whole blood samples in response to ADP stimulation (0.1-10µM) (Figure 4.6). For the fibrinogen antibody used in these experiments, there was no reliable isotype control antibody. Therefore, a sample containing the fibrinogen antibody plus EDTA (6mM), was used to assess non-specific binding. The presence of EDTA inhibits integrin activity and therefore prevents fibrinogen binding. This technique was taken from a protocol previously developed (Goodall and Appleby, 2004). Whole blood was incubated with the fibrinogen antibody (5µl, equivalent to 6µg) under similar conditions that have been used previously (Riba et al., 2004). ADP stimulation induced a concentration dependent increase in fibrinogen binding. The percentage of platelets positive for fibrinogen in non-stimulated samples was 8.8±4.1% and in platelets stimulated with ADP (10µM) was 93.7±0.6% (p<0.001) (Figure 4.6F). An increasing trend was also seen when analysing the MFI of the fibrinogen antibody relative to basal levels (Figure 4.6G). However, this was not found to be significant at any of the concentrations tested, possibly due to an anomalous result obtained in a non-stimulated sample in one of the experiments performed.
Whole blood was incubated with either (A) an isotype control antibody (5µl, 250ng) or (B-E) a CD62p antibody (5µl, 31ng). Samples were simultaneously incubated with either (A and B) no ADP (C) 0.1µM ADP, (D) 1µM ADP or (E) 10µM ADP for 20 minutes followed by fixation. Samples were then analysed on a BD FACS Aria™II. Shown are (A-E) representative histograms and (F and G) quantitative analysis of 3 independent experiments. * indicates p<0.05-0.01 and *** p<0.005.

**Figure 4.5: ADP induces P-selectin expression on platelets in whole blood**

Whole blood was incubated with either (A) an isotype control antibody (5µl, 250ng) or (B-E) a CD62p antibody (5µl, 31ng). Samples were simultaneously incubated with either (A and B) no ADP (C) 0.1µM ADP, (D) 1µM ADP or (E) 10µM ADP for 20 minutes followed by fixation. Samples were then analysed on a BD FACS Aria™II. Shown are (A-E) representative histograms and (F and G) quantitative analysis of 3 independent experiments. * indicates p<0.05-0.01 and *** p<0.005.
Whole blood was incubated in the presence (A) or absence (B-E) of EDTA (6mM) with a fibrinogen antibody (5µl, 6μg). Samples were simultaneously incubated with either (A and B) no ADP (C) 0.1µM ADP, (D) 1µM ADP or (E) 10µM ADP for 20 minutes followed by fixation. Samples were then analysed on a BD FACS Aria™II. (A-E) Representative histograms and (F and G) quantitative analysis of 3 independent experiments. *** indicates p<0.005.

**Figure 4.6: ADP induces fibrinogen binding on platelets in whole blood**
4.4.2 CD36 expression levels on platelets in whole blood

Having established the reliability of the flow cytometry technique used to measure platelet activation with P-selectin and fibrinogen binding as markers, the expression of CD36 was investigated under both non-stimulated and stimulated conditions. To begin with, an antibody titration was performed to identify the appropriate amount of antibody needed to identify CD36 on platelets. Figure 4.7 reveals little difference in fluorescence intensity at any of the amounts of antibody tested. The MFI of CD36 expression was 1539 at 1µl (equivalent to 50ng) of antibody, 1743 at 2µl (equivalent to 100ng) of antibody, 1784 at 5µl (equivalent to 250ng) of antibody and 1791 at 10µl (equivalent to 500ng) of antibody. Therefore, in subsequent experiments, 2µl of antibody was used to detect CD36 expression on platelets.

When the levels of CD36 expressed on the platelets surface were examined it was found that 98.0±0.8% of platelets stained positive for CD36 under basal conditions. Stimulation of platelets with ADP did not alter the percentage of cells positive for CD36 (Figure 4.8F). When the data was examined as MFI, it was found that ADP did not stimulate a significant increase in the amount of CD36 on the cell surface (Figure 4.8G).
Whole blood was incubated with either (A) IgG control antibody (5µl, 250ng) or (B) 1µl (50ng), (C) 2µl (100ng) (D) 5µl (250ng) or (E) 10µl (500ng) CD36 antibody for 20 minutes followed by fixation. Samples were then analysed on a BD FACS Aria™II. Histograms are from a single experiment.

Figure 4.7: Titration of the CD36 antibody

Whole blood was incubated with either (A) IgG control antibody (5µl, 250ng) or (B) 1µl (50ng), (C) 2µl (100ng) (D) 5µl (250ng) or (E) 10µl (500ng) CD36 antibody for 20 minutes followed by fixation. Samples were then analysed on a BD FACS Aria™II. Histograms are from a single experiment.
Whole blood was incubated with either (A) an isotype control antibody (2µl, 100ng) or (B-E) a CD36 antibody (2µl, 100ng). Samples were simultaneously incubated with either (A and B) no ADP, (C) 0.1µM ADP, (D) 1µM ADP or (E) 10µM ADP for 20 minutes followed by fixation. Samples were then analysed on a BD FACS Aria™II. Shown are (A-E) representative histograms and (F and G) quantitative analysis of 4 independent experiments.

Figure 4.8: CD36 expression on platelets in whole blood
4.4.3 Multiple platelet agonists increase the surface expression of CD36

CD36 expression in response to a variety of platelet agonists was next investigated. These studies were performed in PRP to investigate platelet responses in isolation from other cell types. For these experiments, a combination of a FITC-conjugated CD36 antibody and a PE-conjugated CD62p antibody, were incubated with platelets in the same sample tube, which allowed for simultaneous measurements of α-granule secretion and CD36 expression. Due to the presence of two fluorescently labelled antibodies in the same tube, compensation was first performed (as in section 2.5.5.3) to ensure that the fluorescent signal from the FITC labelled antibody did not interfere with the fluorescent signal of the PE labelled antibody.

4.4.3.1 CD36 and P-selectin expression increase in response to platelet agonists

It was important to establish if increases in expression of CD36 occurred downstream of a specific pathway or was a general response to platelet activation. To test this, the following agonists were used: ADP, which stimulates P2Y_1 and P2Y_12 receptors; a PAR1 receptor agonist (SFLLRN); a PAR4 receptor agonist (AYPGKF); and CRP, which stimulates GPVI. ADP induced a dose dependent increase in the percentage of cells that were positive for P-selectin, with a significant increase observed at ADP (1µM, p<0.001) and ADP (10µM, p<0.001). No difference was observed in the percentage of cells positive for CD36 at any of the concentrations tested. The MFI for P-selectin expression, relative to basal levels, also increased upon ADP stimulation, with significance observed at ADP (0.1µM, p<0.005), ADP (1µM, p<0.005) and ADP (10µM, p<0.005) (Figure 4.9Aii). Furthermore, a small increase in the MFI for CD36 expression,
when measured relative to basal levels, was observed which was significant at ADP (0.1µM, p=0.04), ADP (1µM, p=0.001) and ADP (10µM, p<0.005) (Figure 4.9Ai). Stimulation with the PAR1 agonist induced a significant increase in the percentage of cells positive for P-selectin expression at a concentration of 10µM (p<0.001) but had no effect on the percentage of cells positive for CD36. MFI for both P-selectin and CD36 expression was increased by the PAR1 agonist (Figure 4.9Bi and Bii). Similar results were obtained for the PAR4 receptor agonist, where the percentage of cells positive for P-selectin significantly increased at PAR4 agonist (10µM, p<0.001) and PAR4 agonist (100µM, p<0.001) but no difference was seen in the percentage of cells positive for CD36. MFI did, however, increase for both P-selectin and CD36 expression using PAR4 agonist (10µM, p<0.01 for P-selectin and p<0.005 for CD36) and PAR4 agonist (100µM, p=0.02 for P-selectin and p=0.001 for CD36) (Figure 4.9Ci and Cii).

Finally, CRP appeared to be the strongest of the agonists tested, with all concentrations inducing a large increase in the percentage of cells positive for P-selectin expression - CRP (0.1µg/ml, <0.001), CRP (1µg/ml, p<0.001) and CRP (10µg/ml, p<0.001). An increase in the MFI for both P-selectin and CD36 was again observed at concentrations of CRP (0.1µg/ml, p=0.03 for P-selectin and p=0.001 for CD36), CRP (1µg/ml, p=0.04 for P-selectin and p<0.005 for CD36) and CRP (10µg/ml, p=0.04 for P-selectin and p<0.01 for CD36) (Figure 4.9Di and Dii). Therefore, the number of platelets that express CD36 does not alter in response to agonist stimulation, however, the amount of CD36 expression does increase in correlation with α-granule secretion of P-selectin.
**Figure 4.9: CD36 expression on platelets increases dose dependently in response to a variety of agonists**

PRP was incubated with either (A) ADP (0.1-10µM), (B) PAR1 receptor agonist (0.1-10µM), (C) PAR4 receptor agonist (1-100µM) or (D) CRP (0.1-10µg/ml) in combination with a PE-conjugated P-selectin antibody (5µl, 31ng) and a FITC-conjugated CD36 antibody (2µl, 100ng) for 20 minutes followed by fixation. Samples were then analysed on a BD FACSCalibur. Shown are results for (i) CD36 expression and (ii) P-selectin expression. Data are from 3 separate experiments performed. * indicates p<0.05-0.01, ** p<0.01-0.005 and *** p<0.005.
4.4.3.2 Measurement of CD36 and P-selectin expression over time

Investigations were next performed to assess whether, the incubation time that platelets were stimulated with the various agonists for, could affect P-selectin and CD36 expression. Therefore, PRP was incubated with either ADP (1µM), the PAR1 receptor agonist (1µM), the PAR4 receptor agonist (10µM) or CRP (1µg/ml) for 5, 10 or 20 minutes (Figure 4.10). A significant increase from basal levels in the percentage of cells positive for P-selectin expression was observed for ADP, the PAR4 receptor agonist and CRP at all of the time point tested (Figure 4.10A). Conversely, when considering the MFI for P-selectin expression, there was only a significant increase from basal levels with CRP stimulation at 5 minutes. ADP became significant at 10 minutes stimulation whereas the PAR4 receptor agonist did not become significant until 20 minutes stimulation (Figure 4.10B). The MFI for PAR1 receptor agonist stimulation did not reach significance at any of the time points tested. Therefore, even though the amount of cells that express P-selectin increases rapidly upon stimulation, the amount of P-selectin expressed appears to take a longer time to rise.

There was, again, no significant increase observed in the percentage of cells positive for CD36 expression at any of the time points tested (Figure 4.10C). The MFI for CD36 expression increased much more rapidly than P-selectin expression, with ADP, PAR4 receptor agonist and CRP stimulation for 5 minutes inducing a significant increase. The PAR1 receptor agonist also induced a significant increase in MFI of CD36 after just 10 minutes of stimulation (Figure 4.10D). This again illustrates that the amount of CD36 expressed on platelets increases with agonist stimulation.
PRP was incubated with either ADP (1µM), PAR1 receptor agonist (1µM), PAR4 receptor agonist (10µM) or CRP (1µg/ml) in combination with a PE-conjugated P-selectin antibody (5µl, 31ng) and a FITC-conjugated CD36 antibody (2µl, 100ng) for 5, 10 or 20 minutes. This was followed by fixation and analysis on a BD FACSCalibur. (A) Percentage positive cells and (B) mean fluorescence of P-selectin expression. (C) Percentage positive cells and (D) mean fluorescence of CD36 expression. Data are from 3 separate experiments performed. * indicates p<0.05-0.01, ** p<0.01-0.005 and *** p<0.005.
4.4.3.3 Platelet agonists that increase CD36 expression also induce platelet aggregation

In addition to measuring P-selectin expression as a marker of platelet activation when stimulating platelets with various agonists, aggregation was also used as a second confirmatory technique. Similar to flow cytometry experiments, PRP was also used in aggregation and was stimulated with ADP (0.1-10µM), the PAR1 receptor agonist (0.1-10µM), the PAR4 receptor agonist (1-100µM) and CRP (0.1-10µg/ml). Interestingly, aggregation responses to the various concentrations of agonists were much weaker compared to measurements of P-selectin expression. ADP did not induce aggregation at low concentrations (0.1µM and 1µM) whereas 10µM of ADP induced a mid-range of aggregation (41%) that was reversible (Figure 4.11A). The PAR1 receptor agonist also did not induce aggregation at the concentrations 0.1µM and 1µM but stimulated full aggregation at a concentration of 10µM (Figure 4.11B). Similarly, the PAR4 receptor agonist did not induce aggregation at the lowest concentrations tested (1µM and 10µM) but also stimulated full aggregation at the highest concentration tested (100µM) (Figure 4.11C). Finally, CRP did not induce aggregation at 0.1µg/ml but did induce full aggregation at 1µg/ml and 10µg/ml (Figure 4.11D). These results indicate that flow cytometric analysis of P-selectin expression is a much more sensitive technique in assessing platelet activation compared to aggregation.
Figure 4.11: Agonist induced aggregation in PRP

PRP was stimulated with either (A) ADP (0.1-10µM), (B) PAR1 receptor agonist (0.1-10µM), (C) PAR4 receptor agonist (1-100µM) or (D) CRP (0.1-10µg/ml) and aggregation was recorded for 4 minutes. Traces are from an individual experiment.
4.5 CD36 as a receptor for oxidised low density lipoproteins on platelets

A key tool used in this study to investigate whether CD36 is a receptor on platelets responsible for the platelet activating properties of oxLDL was the CD36 blocking antibody FA6.152. FA6.152 was first produced in 1986 (Edelman et al., 1986) and was later found to bind to CD36 (Kieffer et al., 1989). More specifically, binding was shown to occur to the sequence between amino acids 155-183 (Daviet et al., 1995), which is the binding site for oxLDL. This makes it ideal to block oxLDL binding with CD36 in order to determine the significance of these interactions. However, before this blocking antibody was used in experiments, its specificity for CD36 was first examined.

4.5.1 Confirmation of FA6.152 specificity for CD36

Investigations were first performed to confirm that FA6.152 does indeed bind specifically to CD36. Samples of PRP were incubated with either FA6.152 (1µg/ml), IgG control antibody (1µg/ml) or the equivalent volume of modified Tyrode’s buffer for 15 minutes at 37°C. These conditions have been previously used to block CD36 signalling in our laboratory (Roberts et al., 2010). Samples were then incubated with a FITC-conjugated CD36 antibody or a FITC-conjugated CD42b antibody and analysed by flow cytometry. The rationale for these experiments was that if FA6.152 does indeed block CD36, then interactions between platelets and the FITC-conjugated CD36 antibody should be prevented. FA6.152 should not, however, block interactions between platelets and the CD42b antibody. Figure 4.12B demonstrates high levels of CD36 in non-stimulated samples (MFI = 1814). The MFI of CD36 is, however, substantially
reduced (176) in the presence of FA6.152, indicating that this blocking antibody is preventing the FITC-conjugated CD36 antibody from binding to platelets (Figure 4.12D). Additionally, the IgG control antibody had no effect on the MFI (1813) of the CD36 antibody (Figure 4.12F). Importantly, neither FA6.152 nor the IgG control antibody had a major effect on the binding of the CD42b antibody, with a MFI of 259 observed with modified Tyrode’s buffer, 213 with FA6.152 and 245 with the IgG control antibody (Figure 4.12C, E and G). This indicates that FA6.152 does not affect the CD42b antibody from binding to GPIb and, therefore, is specific for CD36.
PRP was incubated with either (A, B and C) modified Tyrode’s buffer, (D and E) FA6.152 (1µg/ml) or (F and G) an IgG control antibody (1µg/ml) for 15 minutes at 37°C. Samples were then incubated with either (A) FITC-conjugated isotype control antibody (2µl, 100ng), (B, D and F) FITC-conjugated CD36 antibody (2µl, 100ng) or (C, E and G) FITC-conjugated CD42b antibody (2µl, 50ng) for 20 minutes followed by fixation. Samples were then analysed on a BD FACS Aria™II. Histograms are from an independent experiment.

Figure 4.12: Confirmation that the CD36 blocking antibody FA6.152 binds to CD36
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4.6 Discussion

The aim of this section of work was to investigate the scavenger receptor CD36, in order to understand whether this receptor plays a role in oxLDL induced platelet activation. However, it was also important to understand the factors affecting its expression. To achieve this, a flow cytometry protocol was developed and optimised for use in this laboratory. This was followed by investigating the use of FA6.152 as a CD36 blocking antibody.

4.6.1 CD36 is present in platelets and is expressed on the surface of these cells

Immunoblotting was first used to examine CD36 expression in platelets. Both immunoblotting of whole cell lysates and immunoprecipitation experiments confirmed the presence of CD36 in platelets. This is consistent with previous findings that provide evidence for CD36 as a protein within platelets (Talle et al., 1983). There was a slight discrepancy in the molecular weights observed when immunoblotting CD36 from whole cell lysates compared to immunoprecipitation, although all bands detected were still in the correct molecular weight region of 78-88kDa. This may be due to the varying extent of glycosylation on the scavenger receptor protein (Rac et al., 2007).

Whilst immunoblotting confirmed the presence of CD36, it did not demonstrate where in the platelet CD36 was expressed. Therefore, a flow cytometry protocol was used, with antibodies that were not cell permeable, therefore indicating that the expression of CD36 is on the surface of platelets. Flow cytometric analysis of both whole blood and PRP revealed the expression of CD36 on the surface of platelets. This technique
was also used to assess the expression of other platelet surface antigens upon stimulation with agonists. It was found that stimulation of whole blood with ADP induced both the expression of P-selectin and the binding of fibrinogen on platelet surfaces, similar to levels previously observed (Janes et al., 1994). Interestingly, the amount of P-selectin expression detected appeared to be greater in samples of stimulated PRP compared to samples of stimulated whole blood. This may be due to other cells being present within the whole blood samples, whereas in PRP there are only platelets. Therefore, more interactions may occur between the agonist and platelets in PRP. Additionally, despite basal P-selectin levels being low in PRP samples, centrifugation to obtain the PRP may have partially activated the platelets and, therefore, made them more sensitive to agonist stimulation. Measurements of P-selectin and fibrinogen binding, however, were a good indicator that platelets had been activated when measuring CD36 expression in response to agonists.

The surface expression of CD36 increased in PRP when platelets were stimulated with several agonists. Interestingly, the number of platelets that expressed CD36 did not alter upon stimulation. This is because the vast majority of platelets express CD36 under basal conditions, in contrast to P-selectin expression and fibrinogen binding, where non-stimulated platelets do not possess these surface antigens and so stimulation causes an increase in the percentage of cells positive for them. However, the amount of CD36, as quantified by the MFI for the FITC-conjugated CD36 antibody, did increase upon platelet stimulation. This was true of agonists that stimulate a range of platelet receptors, including: ADP, which stimulates P2Y₁ and P2Y₁₂ receptors; a
PAR1 receptor agonist; a PAR4 receptor agonist; and CRP, which stimulates GPVI. Therefore, this suggests, that increased surface expression of CD36 occurs from a general platelet activation response and is not specific to one receptor signalling pathway. These results are consistent with previous findings that showed CD36 expression to increase upon stimulation with thrombin, U46619, ADP, collagen and adrenaline (Michelson et al., 1994). Due to the correlation between increased CD36 expression and P-selectin expression, it could be suggested that CD36, which is found in the membranes of α-granules (Berger et al., 1993), is secreted upon activation, resulting in further expression on the platelets surface. This effect may be detrimental in the condition of hyperlipidaemia, which is known to involve platelet hyperactivity (Carvalho et al., 1974), as more CD36 expression may result in an increase in oxLDL binding leading to augmented platelet activity.

In light of the findings that CD36 expression increases during platelet activation, it would be beneficial in future work to discover the pathway that leads to this feature. As mentioned above, stimulation with a number of agonists results in the increased expression of CD36, suggesting a common pathway involved. Two mutual events that are involved in secretion, and result from stimulation by a number of agonists, include an increase in intracellular Ca\(^{2+}\) and the activation of PKC (Walker and Watson, 1993). Additionally, PKC\(\alpha\) knockout mice have more recently been found to have decreased α-granule secretion in response to agonists (Konopatskaya et al., 2009). Therefore, it would be of interest to use pharmacological reagents to block these intracellular responses to observe whether CD36 expression increases upon agonist stimulation.
In addition to the future work that could be carried out investigating CD36 expression, interactions of CD36 with intracellular proteins would also be of great interest to investigate. A valuable aspect to the technique of immunoprecipitation is that, once the protein of interest has been isolated from the whole cell lysate, experiments can be performed to discover whether other proteins associate with it. Therefore, it would be beneficial to carry out co-immunoprecipitation studies in order to clarify other platelet proteins that can associate with CD36. To begin with, this could involve the immunoprecipitation of CD36 followed by western blotting and probing with a phospho-tyrosine antibody, which would result in a general indication of the tyrosine kinase related proteins that associate with CD36. This experiment could be performed both with and without platelet stimulation with oxLDL, to determine whether proteins associate with this scavenger receptor upon ligation. It is of interest to look at tyrosine kinase related proteins, as Src kinases have been known to associate with CD36 in platelets for many years (Huang et al., 1991). Additionally, proteins such as Syk, Vav and phosphatidylinositol 3-kinase (PI3K), have also more recently been found to associate with CD36 in other cell types (Kazerounian et al., 2011). Therefore, increasing our knowledge of the types of signalling molecules that associate with this scavenger receptor, may aid in enhancing our understanding of how this receptor works, especially when stimulated with pathological ligands.

As well as using flow cytometry to examine the expression levels of CD36 on platelets, experiments were also performed in this study to determine the specificity of FA6.152 as a blocking antibody for CD36. FA6.152 prevented a FITC-conjugated CD36 antibody
from binding to platelets, but had no effect on a FITC-conjugated CD42b antibody, therefore suggesting that FA6.152 binds to and blocks CD36 without having an effect on other platelet receptors. This makes it a key tool in investigating CD36 interactions with oxLDL.

To summarise, the expression of CD36 on platelets has been confirmed in these studies by two separate techniques, immunoblotting and flow cytometry. The surface expression of CD36 was also found to increase upon activation by several platelet agonists, possibly due to its secretion from α-granules.
Chapter 5

Chapter 5: Oxidised low density lipoproteins induce platelet shape change through a tyrosine kinase mediated pathway

5.1 Introduction

Shape change is the earliest physiological response to platelet activation and involves a dynamic reorganisation of the cytoskeletal framework. This response acts to enlarge the surface area of these cells, allowing interactions to occur with nearby platelets and the exposed ECM. The cytoskeletal protein myosin IIA is critical to platelet shape since it is proposed to interact with actin polymers to disrupt the quiescent discoid form.

The activity of myosin, and to some degree platelet shape change, is controlled by the phosphorylation of its regulatory light chains at serine\(^{19}\) (MLC\(^{\text{Ser19}}\)) (Daniel et al., 1984). The light chains are phosphorylated by MLCK but can also be dephosphorylated by MLCP. Therefore, in order for platelet shape change to occur, physiological platelet agonists initiate signalling pathways leading to a Ca\(^{2+}\) dependent activation of MLCK and a RhoA/Rho kinase dependent inhibition of MLCP (Paul et al., 1999). From experiments performed in chapter 3, it has been established that oxLDL can induce platelet aggregation, although this response was found to be variable. However, despite the variation in the degree of aggregation in response to oxLDL, the lipoproteins ability to induce platelet shape change was consistent. Therefore, investigations were performed to assess whether oxLDL could induce platelet shape change independently of secondary mediators; and to elucidate the signalling pathways involved in this response.
Since CD36 is a platelet receptor for oxLDL (Endemann et al., 1993), investigations focussed on the potential role of this receptor in platelet shape change. Additionally, CD36 has been known to associate with Src kinases for many years (Huang et al., 1991) and a growing body of evidence suggests that a tyrosine kinase pathway is involved in CD36 signalling. More specifically, Src kinases have been found to play a prominent role in oxLDL induced platelet activation (Chen et al., 2008); Syk has been shown to become phosphorylated in response to platelet adhesion to oxLDL (Nergiz-Unal et al., 2011a); and the RhoGEF Vav has been found to become tyrosine phosphorylated in response to oxLDL in platelets (Chen et al., 2011). Therefore, the role of a tyrosine kinase mediated signalling pathway leading to platelet shape change was assessed.

The aims of this chapter include:

- The determination of whether oxLDL stimulates platelet shape change independently of secondary signalling mediators.
- The assessment of whether CD36 is involved in oxLDL induced platelet shape change.
- The investigation of a tyrosine kinase mediated signalling pathway leading to oxLDL induced platelet shape change.
5.2 Oxidised low density lipoproteins induce platelet shape change

In order to assess the ability that oxLDL has to induce platelet shape change, independently of integrin activation and the secondary mediators ADP and TXA₂, platelets were stimulated under non-aggregatory conditions. Experiments were performed in the presence of EGTA (1mM), apyrase (2U/ml) and indomethacin (10µM) to prevent integrin activation, ADP stimulation and TXA₂ production, respectively. Shape change responses were recorded in an optical aggregometer. In the first instance, shape change in response to thrombin, which is known to induce this response through stimulation of GPCR, was examined. Figure 5.1A shows that increasing doses of thrombin stimulated platelets to change shape. Next tested was the ability of oxLDL (10-200µg/ml) to induce the shape change response. OxLDL at 10-200µg/ml was able to induce shape change (Figure 5.1B). In contrast, the same concentrations of nLDL did not provoke any shape change (Figure 5.1C). This indicates that it is only the oxidised form of the lipoprotein that has the ability to induce platelet shape change and that this effect is independent of integrin activation, ADP or TXA₂.
Washed platelets (2.5x10^8/ml) were treated with EGTA (1mM), apyrase (2U/ml) and indomethacin (10µM) for at least 20 minutes prior to shape change experiments. Platelets were stimulated with either (A) thrombin (0.01-0.1U/ml), (B) oxLDL (10-200µg/ml) or (C) nLDL (10-200µg/ml) and shape change responses were recorded for 1 minute. (A) Shape change traces from a single experiment performed and (B and C) representative shape change traces from 4 independent experiments.

**Figure 5.1: Oxidised low density lipoproteins induce platelet shape change**

Washed platelets (2.5x10^8/ml) were treated with EGTA (1mM), apyrase (2U/ml) and indomethacin (10µM) for at least 20 minutes prior to shape change experiments. Platelets were stimulated with either (A) thrombin (0.01-0.1U/ml), (B) oxLDL (10-200µg/ml) or (C) nLDL (10-200µg/ml) and shape change responses were recorded for 1 minute. (A) Shape change traces from a single experiment performed and (B and C) representative shape change traces from 4 independent experiments.
5.3 Oxidised low density lipoproteins induce platelet shape change through the scavenger receptor CD36

Having established that oxLDL can independently induce platelet shape change, next to be investigated was the possible involvement of CD36 in this functional response. To achieve this, the CD36 blocking antibody FA6.152 was used. OxLDL (50µg/ml) induced a robust shape change but this effect was inhibited by the presence of FA6.152 (1µg/ml). In contrast, the IgG control antibody (1µg/ml) had no effect on oxLDL induced shape change (Figure 5.2A). Importantly, neither FA6.152 nor the IgG control antibody had an effect on platelet shape change provoked by thrombin (Figure 5.2B). When platelets were incubated with FA6.152 or the IgG control antibody alone, in the presence of EGTA (1mM), apyrase (2U/ml) and indomethacin (10µM), no shape change was observed (Figure 5.2C). This evidence supports the possibility that oxLDL ligation of CD36 can drive shape change independently of other agonists.
Washed platelets \((2.5 \times 10^8/\text{ml})\) were treated with EGTA (1mM), apyrase (2U/ml) and indomethacin (10µM) for a minimum of 20 minutes prior to shape change experiments. (A) Platelets were incubated in the absence or presence of either FA6.152 (1µg/ml) or IgG control antibody (1µg/ml) for 15 minutes at 37°C followed by stimulation with oxLDL (50µg/ml) and shape change responses were recorded for 1 minute. (B) As in (A) except platelets were stimulated with thrombin (0.05U/ml) instead of oxLDL. (C) Platelets were stimulated with either FA6.152 (1µg/ml) or IgG control antibody (1µg/ml) and shape change responses were recorded for 1 minute. Shape change traces are representatives of 4 separate experiments performed.

**Figure 5.2: Oxidised low density lipoprotein induced platelet shape change is CD36 dependent**

Washed platelets \((2.5 \times 10^8/\text{ml})\) were treated with EGTA (1mM), apyrase (2U/ml) and indomethacin (10µM) for a minimum of 20 minutes prior to shape change experiments. (A) Platelets were incubated in the absence or presence of either FA6.152 (1µg/ml) or IgG control antibody (1µg/ml) for 15 minutes at 37°C followed by stimulation with oxLDL (50µg/ml) and shape change responses were recorded for 1 minute. (B) As in (A) except platelets were stimulated with thrombin (0.05U/ml) instead of oxLDL. (C) Platelets were stimulated with either FA6.152 (1µg/ml) or IgG control antibody (1µg/ml) and shape change responses were recorded for 1 minute. Shape change traces are representatives of 4 separate experiments performed.
Chapter 5

5.4 The phosphorylation of platelet myosin light chains on serine$^{19}$

The previous results demonstrate that oxLDL provokes a shape change response in platelets, potentially through CD36. Since platelet shape change is associated with the phosphorylation of myosin (Daniel et al., 1984), the ability of oxLDL to induce this post-translational modification was tested.

5.4.1 G protein coupled receptor stimulation results in myosin light chain phosphorylation

Stimulation of platelets with thrombin (0.01-0.1U/ml) provoked a concentration dependent increase in MLC$^{\text{Ser19}}$ phosphorylation (Figure 5.3A). Phosphorylation of myosin in response to thrombin was rapid and occurred within 15 seconds of stimulation, before beginning to decline at 5 minutes stimulation (longest time tested) (Figure 5.3B). These results confirm that MLC$^{\text{Ser19}}$ phosphorylation occurs following GPCR stimulation and is a rapid response, in keeping with the early onset of platelet shape change.
Washed platelets (3x10^8/ml) were treated with EGTA (1mM), apyrase (2U/ml) and indomethacin (10µM) for a minimum of 20 minutes before experiments were performed. Platelets were treated with (A) thrombin (0.01-0.1U/ml) for 15 seconds or (B) thrombin (0.05U/ml) for 15-300 seconds followed by lysis. Samples were then subjected to SDS-PAGE and immunoblotted for phospho-MLC^{Ser19} followed by stripping and re-probing for β-tubulin. Immunoblots are from an individual experiment performed.

**Figure 5.3: Thrombin induced myosin light chain phosphorylation**

Washed platelets (3x10^8/ml) were treated with EGTA (1mM), apyrase (2U/ml) and indomethacin (10µM) for a minimum of 20 minutes before experiments were performed. Platelets were treated with (A) thrombin (0.01-0.1U/ml) for 15 seconds or (B) thrombin (0.05U/ml) for 15-300 seconds followed by lysis. Samples were then subjected to SDS-PAGE and immunoblotted for phospho-MLC^{Ser19} followed by stripping and re-probing for β-tubulin. Immunoblots are from an individual experiment performed.
5.4.2 Oxidised low density lipoproteins stimulate myosin light chain phosphorylation

Having established the conditions to examine MLC\textsuperscript{Ser19} phosphorylation in response to thrombin, identical procedures were used in the presence of EGTA (1mM), apyrase (2U/ml) and indomethacin (10µM) to examine the effects of oxLDL. OxLDL (10 - 200µg/ml) stimulated a concentration dependent increase in MLC\textsuperscript{Ser19} phosphorylation, with maximal phosphorylation reached at 100µg/ml (Figure 5.4A). A time course of oxLDL stimulation revealed that this pathological ligand induced detectable changes in MLC\textsuperscript{Ser19} phosphorylation as early as 15 seconds (Figure 5.3B). This phosphorylation induced by oxLDL also declined over time, with levels almost reaching basal after 5 minutes (Figure 5.4B). A concentration of 50µg/ml oxLDL has been used previously to investigate the effects of this lipoprotein on platelets (Chen et al., 2008). Therefore, as this dose induced substantial platelet shape change and MLC\textsuperscript{Ser19} phosphorylation, and due to the rapid onset of platelet shape change, subsequent experiments were performed with a standard concentration of 50µg/ml oxLDL and a standard stimulation time of 15 seconds.

To confirm that it is the oxidised form of the lipoprotein that stimulates MLC\textsuperscript{Ser19} phosphorylation, experiments were also performed with nLDL. Figure 5.5 illustrates that nLDL does not induce MLC\textsuperscript{Ser19} phosphorylation at any of the concentrations or time points tested. Importantly, thrombin was used as a positive control in these experiments and did stimulate MLC\textsuperscript{Ser19} phosphorylation.
Chapter 5

Aii

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MLC<sup>Ser19</sup> Phosphorylation Relative to β-tubulin

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Time (Seconds)

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MLC<sup>Ser19</sup> Phosphorylation Relative to β-tubulin

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Washed platelets (3x10^8/ml) were treated with EGTA (1mM), apyrase (2U/ml) and indomethacin (10µM) for a minimum of 20 minutes before experiments were performed. Platelets were stimulated with (A) oxLDL (10-200µg/ml) for 15 seconds or (B) oxLDL (50µg/ml) for 15-300 seconds. Samples were then lysed followed by separation by SDS-PAGE and immunoblotted for phospho-MLC^{Ser19}. Membranes were then stripped and re-probed for β-tubulin. (Ai and Bi) Representative immunoblots and (Aii and Bii) quantitative analysis of 3 individual experiments performed.
Washed platelets ($3 \times 10^8$/ml) were treated with EGTA (1mM), apyrase (2U/ml) and indomethacin (10µM) for a minimum of 20 minutes before experiments were performed. Platelets were stimulated with (A) nLDL (10-200µg/ml) for 15 seconds or (B) nLDL (50µg/ml) for 15-300 seconds. Samples were then lysed followed by separation by SDS-PAGE and immunoblotted for phospho-MLC$^{\text{Ser19}}$. Membranes were then stripped and re-probed for β-tubulin. Shown are representative immunoblots of 3 individual experiments performed.

Figure 5.5: Native low density lipoproteins do not induce myosin light chain phosphorylation

Washed platelets ($3 \times 10^8$/ml) were treated with EGTA (1mM), apyrase (2U/ml) and indomethacin (10µM) for a minimum of 20 minutes before experiments were performed. Platelets were stimulated with (A) nLDL (10-200µg/ml) for 15 seconds or (B) nLDL (50µg/ml) for 15-300 seconds. Samples were then lysed followed by separation by SDS-PAGE and immunoblotted for phospho-MLC$^{\text{Ser19}}$. Membranes were then stripped and re-probed for β-tubulin. Shown are representative immunoblots of 3 individual experiments performed.
5.4.3 Phosphorylation of myosin light chains by oxidised low density lipoproteins requires the scavenger receptor CD36

Experimental data in section 5.3 demonstrated that oxLDL provoked platelet shape change required CD36, and so investigations were performed to determine whether signalling through this scavenger receptor resulted in MLC\textsuperscript{Ser19} phosphorylation. OxLDL (50µg/ml) induced the phosphorylation of MLC\textsuperscript{Ser19} after only 15 seconds of stimulation. However, the phosphorylation response was abolished in the presence of FA6.152 (p=0.02), whereas the IgG control antibody had no effect (p=0.38) (Figure 5.6). Critically, FA6.152 did not induce MLC\textsuperscript{Ser19} phosphorylation by itself. These data suggest that oxLDL induces MLC\textsuperscript{Ser19} phosphorylation, and platelet shape change, through a mechanism that involves an interaction with CD36.
Washed platelets (3x10^8/ml) were treated with EGTA (1mM), apyrase (2U/ml) and indomethacin (10µM) for a minimum of 20 minutes before experiments were performed. Platelets were pre-incubated at 37°C for 15 minutes in the absence or presence of either FA6.152 (1µg/ml) or IgG control antibody (1µg/ml). Platelets were then stimulated with oxLDL (50µg/ml) for 15 seconds and then lysed. Samples were next separated by SDS-PAGE and immunblotted for phospho-MLC^{Ser19} followed by stripping and re-probing for β-tubulin. (A) Representative blot and (B) quantitative analysis of 3 independent experiments performed. End sample containing FA6.152 alone was performed in only one experiment. * indicates p<0.05-0.01.

Figure 5.6: Oxidised low density lipoproteins stimulate myosin light chain phosphorylation through CD36
5.5 Oxidised low density lipoprotein induced myosin light chain phosphorylation involves a tyrosine kinase signalling pathway

The preceding findings demonstrate that oxLDL can stimulate platelet shape change and MLC$^{\text{Ser19}}$ phosphorylation through a CD36 dependent signalling pathway. As Src kinases are known to associate with CD36 (Huang et al., 1991) and have been shown to be involved in oxLDL/CD36 induced platelet activation (Chen et al., 2008), experiments were performed to establish the involvement of Src kinases in the shape change pathway.

5.5.1 Oxidised low density lipoproteins stimulate the activatory phosphorylation of Src kinases

Phosphorylation of Src kinases on the tyrosine$^{416}$ residue correlates with their activation (Reuter et al., 1990). OxLDL (50µg/ml) induced a significant increase in the phosphorylation of tyrosine$^{416}$ on Src family kinases ($p=0.03$). In contrast, nLDL had no effect beyond that observed under basal conditions ($p=0.28$) (Figure 5.7). This suggests that oxLDL, potentially through ligation of CD36, induces the activation of Src family kinases.
Washed platelets (5x10⁸/ml) were treated with EGTA (1mM), apyrase (2U/ml) and indomethacin (10µM) for a minimum of 20 minutes before experiments were performed. Platelets were stimulated with either oxLDL (50µg/ml) or nLDL (50µg/ml) for 15 seconds followed by lysis. Samples were then separated by SDS-PAGE, immunoblotted for phospho-Src<sup>Y416</sup>, stripped and re-probed for β-tubulin. (A) Representative immunoblot and (B) quantitative analysis of 4 separate experiments performed. * indicates p<0.05-0.01.

**Figure 5.7: Oxidised low density lipoproteins stimulate the activatory phosphorylation of Src kinases**

Washed platelets (5x10⁸/ml) were treated with EGTA (1mM), apyrase (2U/ml) and indomethacin (10µM) for a minimum of 20 minutes before experiments were performed. Platelets were stimulated with either oxLDL (50µg/ml) or nLDL (50µg/ml) for 15 seconds followed by lysis. Samples were then separated by SDS-PAGE, immunoblotted for phospho-Src<sup>Y416</sup>, stripped and re-probed for β-tubulin. (A) Representative immunoblot and (B) quantitative analysis of 4 separate experiments performed. * indicates p<0.05-0.01.
5.5.2 Inhibition of Src kinases prevents oxidised low density lipoprotein induced platelet shape change and myosin light chain phosphorylation

Evidence from others suggests that Src kinases associate with CD36 and investigations from this work suggest that oxLDL stimulation induces their activation. Therefore, the Src kinase inhibitor PP2 was used to discover if suppression of Src kinase activity can block shape change triggered by oxLDL. Initially, PP2 was tested in aggregation experiments with collagen. Src kinases are associated with GPVI and are essential for its signalling (Briddon and Watson, 1999), therefore, PP2 should block collagen induced aggregation. The concentration of PP2 (20µM) used in these experiments has been used previously (Spalton et al., 2009). Figure 5.8A demonstrates that PP2 completely abolishes collagen induced aggregation, whereas PP3, its inactive analogue, has very little effect, suggesting that the inhibition of Src kinases is critical for aggregation induced by collagen.

Platelets were next treated with PP2 (20µM) in the presence of EGTA (1mM), apyrase (2U/ml) and indomethacin (10µM), to produce non-aggregatory conditions in order to observe platelet shape change. Incubation with PP2 substantially reduced the shape change response provoked by oxLDL (Figure 5.8B), while PP3 had no effect. These data suggest that inhibition of Src kinases prevents oxLDL from stimulating platelet shape change.

Having demonstrated that Src kinases are important for shape change induced by oxLDL, their role in the phosphorylation of MLC\textsuperscript{Ser19} was evaluated. Consistent with
previous experiments, oxLDL induced a robust phosphorylation of $\text{MLC}^{\text{Ser}19}$. However, in the presence of PP2, the increased phosphorylation of $\text{MLC}^{\text{Ser}19}$ in response to oxLDL was abolished ($p=0.01$). Importantly, PP3 had no effect ($p=0.27$) (Figure 5.8C). Therefore, Src kinases must lie upstream of $\text{MLC}^{\text{Ser}19}$ phosphorylation in response to stimulation by oxLDL.
Chapter 5

A

Collagen

\[ \text{Aggregation (\%)} \]

1 Minute

PP2

PP3

B

oxLDL

1 Minute

PP2

PP3

Ci

oxLDL

PP2

PP3

Phospho-MLC<sup>α19</sup>

β-tubulin

Gli

oxLDL

PP2

PP3

MLC<sup>α19</sup>

Phosphorylation Relative to β-tubulin

*
Figure 5.8: Oxidised low density lipoprotein induced platelet shape change and myosin light chain phosphorylation is Src kinase dependent

(A) Washed platelets (2.5x10^8/ml) were pre-incubated in the absence or presence of either PP2 (20µM) or PP3 (20µM) at 37°C for 20 minutes followed by stimulation with collagen (5µg/ml) and aggregation responses were recorded for 3 minutes. (B) Washed platelets (2.5x10^8/ml) and (C) washed platelets (3x10^8/ml) were treated with EGTA (1mM), apyrase (2U/ml) and indomethacin (10µM) for a minimum of 20 minutes before experiments were performed. Platelets were then incubated in the absence or presence of either PP2 (20µM) or PP3 (20µM) at 37°C for 20 minutes followed by (B) stimulation with oxLDL (50µg/ml) and shape change responses recorded for 1 minute and (C) stimulation with oxLDL (50µg/ml) for 15 seconds followed by lysis. Samples were then subjected to SDS-PAGE and immunoblotted for phospho-MLC^{Ser19}, stripped and re-probed for β-tubulin. (A) Representative traces of samples performed for each experiment that used PP2. (B) Representative traces of 3 independent experiments performed. (C) Representative immunoblot and (Cii) quantitative analysis of 4 independent experiments performed. * indicates p<0.05-0.01.
5.5.3 Oxidised low density lipoprotein stimulation results in the Src kinase dependent tyrosine phosphorylation of a number of platelet proteins

Since the tyrosine kinase Src was involved in the signalling pathway resulting in platelet shape change, immunoblotting was used to assess whether oxLDL could induce the tyrosine phosphorylation of proteins in platelets. For this, a general tyrosine phosphorylation antibody was utilised, which has been previously employed to assess tyrosine phosphorylation of protein in whole cell lysates (Briddon and Watson, 1999). OxLDL stimulation triggered a concentration dependent increase in tyrosine phosphorylation of a broad range of proteins with the most prominent bands observed at molecular weights of approximately 25kDa, 30kDa, 45kDa, 70kDa, 90kDa and 140kDa (Figure 5.9A). This occurred at the early time point of 15 seconds, which is in keeping with the platelet shape change response. However, unlike MLC\textsuperscript{Ser19} phosphorylation, tyrosine phosphorylation levels remain constant over a period of 5 minutes (Figure 5.9B). In contrast, nLDL did not induce tyrosine phosphorylation beyond that observed under basal conditions. These data suggest that tyrosine phosphorylation events are stimulated by oxLDL and may be involved in platelet shape change as well as other, more long lasting, platelet activation responses.

Tyrosine phosphorylation of platelet proteins in response to oxLDL were also assessed in the presence of the Src kinase inhibitor. These experiments illustrate that tyrosine phosphorylation induced by oxLDL is completely blocked in the presence of PP2, while PP3 has no effect (Figure 5.9C). This implies that all tyrosine phosphorylation events
stimulated by oxLDL are downstream of Src kinases, which potentially may be the most upstream element of the CD36 signalling pathway.
Washed platelets (5x10^8/ml) were treated with EGTA (1mM), apyrase (2U/ml) and indomethacin (10µM) for a minimum of 20 minutes prior to experiments. Platelets were stimulated with (A) oxLDL (10-200µg/ml) or nLDL (50µg/ml) for 15 seconds, (B) oxLDL (50µg/ml) for 15-300 seconds or (C) incubated in the absence or presence of either PP2 (20µM) or PP3 (20µM) at 37°C for 20 minutes followed by stimulation with oxLDL (50µg/ml) for 15 seconds. Samples were then lysed and proteins separated by SDS-PAGE followed by immunoblotting for phospho-tyrosine. Membranes were then stripped and re-probed for β-tubulin. Immunoblots are representative of 3 separate experiments performed.
5.5.4 Oxidised low density lipoproteins stimulate the Src kinase dependent phosphorylation of Syk and PLCγ2

Data presented in section 5.5.3 demonstrates that oxLDL induces the tyrosine phosphorylation of a number of proteins. However, the antibody used in these experiments can only detect general tyrosine phosphorylation events and does not verify which proteins in particular become phosphorylated. However, the molecular weights of the bands observed provide an indication of which proteins may be phosphorylated in response to oxLDL. The phosphorylated bands detected at approximately 70kDa and 140kDa may correspond with Syk and PLCγ2, respectively (Clark and Brugge, 1996). To confirm this, immunoprecipitation experiments were performed to isolate these proteins and assess their phosphorylation state. Immunoprecipitation of Syk from platelet lysates demonstrated that it became phosphorylated upon platelet stimulation with oxLDL (p=0.01) (Figure 5.10). This was found to be Src kinase dependent as PP2 significantly reduced the level of phosphorylation (p=0.001), whereas PP3 had no effect (p=0.2). A JNK inhibitor (10µM) was also applied to platelets, as previous reports have found this protein to be involved in CD36 signalling provoked by oxLDL (Chen et al., 2008). However, the JNK inhibitor, previously used at this concentration (Roberts et al., 2010), had no effect on Syk phosphorylation under these experimental conditions, suggesting that this protein is not found upstream of Syk activation by oxLDL. The activation of PLCγ2 was also assessed using immunoprecipitation. Figure 5.11 illustrates that oxLDL stimulates the tyrosine phosphorylation of PLCγ2 (p<0.005) and that this is also Src kinase dependent, as PP2 significantly reduces phosphorylation (p<0.001) whereas PP3 has no effect.
(p=0.07). Therefore, it can be proposed from this data that oxLDL stimulates the phosphorylation and activation of both Syk and PLCγ2 downstream of Src kinases.
Washed platelets ($7 \times 10^8$ /ml) were treated with EGTA (1mM), apyrase (2U/ml) and indomethacin (10µM) for a minimum of 20 minutes prior to experiments. Platelets were incubated in the absence or presence of either JNK inhibitor1 (10µM), PP2 (20µM) or PP3 (20µM) at 37°C for 20 minutes followed by stimulation with oxLDL (50µg/ml) for 15 seconds. Platelets were then lysed and Syk was immunoprecipitated. Samples were then separated by SDS-PAGE and immunoblotted for phospho-tyrosine followed by stripping and re-probing for Syk. (A) Representative immunoblot and (B) quantitative analysis of 3 separate experiments performed. * indicates p<0.05-0.01 and *** p<0.005.

Figure 5.10: Oxidised low density lipoproteins induce the tyrosine phosphorylation of Syk
Washed platelets (7x10^8/ml) were treated with EGTA (1mM), apyrase (2U/ml) and indomethacin (10µM) for a minimum of 20 minutes prior to experiments. Platelets were incubated in the absence and presence of either PP2 (20µM) or PP3 (20µM) at 37°C for 20 minutes followed by stimulation with oxLDL (50µg/ml) for 15 seconds. Platelets were then lysed and PLCγ2 was immunoprecipitated. Samples were then separated by SDS-PAGE and immunoblotted for phospho-tyrosine followed by stripping and re-probing for PLCγ2. (A) Representative immunoblot and (B) quantitative analysis of 3 individual experiments performed. *** indicates p<0.005.

Figure 5.11: Oxidised low density lipoproteins induce the tyrosine phosphorylation of PLCγ2
5.5.5 Syk and PLCγ2 play a role in oxidised low density lipoprotein induced myosin light chain phosphorylation

Having confirmed that oxLDL stimulates the phosphorylation of Syk and PLCγ2 in platelets, the involvement of these proteins in shape change was next investigated. Syk and PLCγ2 are known to be involved in producing a Ca\(^{2+}\) response in platelets downstream of other receptors (Rivera et al., 2009). Therefore, it was predicted that stimulation of these proteins by oxLDL may result in a rise in intracellular Ca\(^{2+}\) leading to the activation of MLCK. To test this hypothesis, the Syk inhibitor piceatannol (20µM) and the PLC inhibitor U73122 (5µM) were utilised. The PI3K inhibitor, wortmannin (100nM), and the JNK inhibitor (10µM) were also examined. Previous experiments have been performed in platelets, with similar concentrations, of piceatannol (Law et al., 1999), U73122 (Riba et al., 2008b), wortmannin (Riba et al., 2008a) and the JNK inhibitor (Roberts et al., 2010).

To confirm that the inhibitors were effective under the conditions used, they were tested in aggregation in response to collagen. Figure 5.12 demonstrates that piceatannol, U73122 and wortmannin all substantially reduce collagen stimulated aggregation. The JNK inhibitor did not have an effect on collagen induced aggregation.

Platelets were next treated with the above inhibitors, followed by stimulation with oxLDL and MLC\(^{\text{Ser19}}\) phosphorylation was assessed. Piceatannol, U73122 and the JNK inhibitor each significantly reduced oxLDL stimulated MLC\(^{\text{Ser19}}\) phosphorylation (piceatannol - p<0.01, U73122 - p<0.01 and JNK inhibitor p<0.005). Inhibition of PI3K
by wortmannin did reduce phosphorylation levels, however, this was not found to be significant (p=0.11) (Figure 5.13). Therefore, it can be proposed that the activation of Syk and PLCγ2 by oxLDL results in their contribution to the CD36 signalling pathway leading to MLC^{Ser19} phosphorylation and platelet shape change.
Washed platelets (2.5x10^8/ml) were incubated in the absence and presence of either piceatannol (20µM), U73122 (5µM) or wortmannin (100nM) at 37°C for 20 minutes followed by stimulation with collagen (5µg/ml). Aggregation traces were recorded for 3 minutes. Shown are representative traces of samples performed for each experiment that used piceatannol, U73122 and wortmannin.

Figure 5.12: Syk, PLC and PI3K inhibitors reduce collagen stimulated aggregation

Washed platelets (2.5x10^8/ml) were incubated in the absence and presence of either piceatannol (20µM), U73122 (5µM) or wortmannin (100nM) at 37°C for 20 minutes followed by stimulation with collagen (5µg/ml). Aggregation traces were recorded for 3 minutes. Shown are representative traces of samples performed for each experiment that used piceatannol, U73122 and wortmannin.
Washed platelets (3x10^8/ml) were treated with EGTA (1mM), apyrase (2U/ml) and indomethacin (10µM) for a minimum of 20 minutes before experiments were performed. Platelets were pre-incubated with either piceatannol (20µM), U73122 (5µM), wortmannin (100nM) or JNK inhibitor 1 (10µM) at 37°C for 20 minutes followed by stimulation with oxLDL (50µg/ml) for 15 seconds. Platelets were then lysed and samples separated by SDS-PAGE followed by immunoblotting for phospho-MLC^{Ser19}. Membranes were then stripped and re-probed for β-tubulin. (A) Representative immunoblot and (B) quantitative analysis of 3 independent experiments performed. ** indicates p<0.01-0.005 and *** p<0.005.

**Figure 5.13: Syk, PLC and JNK are involved in oxidised low density lipoprotein stimulated myosin light chain phosphorylation**

Washed platelets (3x10^8/ml) were treated with EGTA (1mM), apyrase (2U/ml) and indomethacin (10µM) for a minimum of 20 minutes before experiments were performed. Platelets were pre-incubated with either piceatannol (20µM), U73122 (5µM), wortmannin (100nM) or JNK inhibitor 1 (10µM) at 37°C for 20 minutes followed by stimulation with oxLDL (50µg/ml) for 15 seconds. Platelets were then lysed and samples separated by SDS-PAGE followed by immunoblotting for phospho-MLC^{Ser19}. Membranes were then stripped and re-probed for β-tubulin. (A) Representative immunoblot and (B) quantitative analysis of 3 independent experiments performed. ** indicates p<0.01-0.005 and *** p<0.005.
5.5.6 Confirmation of Syk involvement in oxidised low density lipoprotein induced platelet shape change

To confirm the participation of Syk in the oxLDL/CD36 signalling pathway leading to MLC\textsuperscript{Ser19} phosphorylation, a second Syk inhibitor was employed. R406 has been previously used with platelets to examine the function of Syk in these cells (Spalton et al., 2009). However, as this inhibitor has not been used in this laboratory before, a dose and time response to R406 was first performed in aggregation experiments with collagen to find an effective concentration of the inhibitor. Figure 5.14A illustrates that a range of concentrations of R406 (0.1-10µM) reduce aggregation, although 1µM appeared to be the lowest dose to cause full inhibition with 5 minutes incubation time. R406 (1µM) abolished aggregation at all of the time points tested (1-10 minutes) (Figure 5.14B). A standard concentration of 1µM and an incubation time of 5 minutes were chosen to be used in subsequent experiments.

Immunoblotting was performed to examine MLC\textsuperscript{Ser19} phosphorylation induced by oxLDL in the presence of piceatannol and R406. Both Syk inhibitors reduced the extent of phosphorylation induced by oxLDL (Figure 5.15). This result strengthens the evidence for Syk involvement in the CD36 signalling pathway in platelets that leads to shape change.
Washed platelets ($2.5 \times 10^8$/ml) were incubated with (A) R406 (0.1-10µM) for 5 minutes or (B) R406 (1µM) for 1-10 minutes followed by stimulation with collagen (5µg/ml). Aggregation traces were recorded for 4 minutes. Traces are from a single experiment performed.

Figure 5.14: R406 inhibits collagen induced aggregation
Washed platelets (3x10^8/ml) were treated with EGTA (1mM), apyrase (2U/ml) and indomethacin (10µM) for a minimum of 20 minutes before experiments were performed. Platelets were incubated at 37°C in the absence and presence of piceatannol (20µM) for 20 minutes or R406 (1µM) for 5 minutes followed by stimulation with oxLDL (50µg/ml) for 15 seconds. Platelets were then lysed, separated using SDS-PAGE and immunoblotted for phospho-MLC^{Ser19}. Membranes were stripped and re-probed for β-tubulin. Immunoblot is from a single experiment performed.

**Figure 5.15: Piceatannol and R406 inhibit oxidised low density lipoprotein induced myosin light chain phosphorylation**

Washed platelets (3x10^8/ml) were treated with EGTA (1mM), apyrase (2U/ml) and indomethacin (10µM) for a minimum of 20 minutes before experiments were performed. Platelets were incubated at 37°C in the absence and presence of piceatannol (20µM) for 20 minutes or R406 (1µM) for 5 minutes followed by stimulation with oxLDL (50µg/ml) for 15 seconds. Platelets were then lysed, separated using SDS-PAGE and immunoblotted for phospho-MLC^{Ser19}. Membranes were stripped and re-probed for β-tubulin. Immunoblot is from a single experiment performed.
5.5.7 Myosin light chain kinase phosphorylates myosin light chains in response to oxidised low density lipoproteins

The previous experiments demonstrate the involvement of Src, Syk and PLCγ2 in oxLDL induced MLC\textsuperscript{Ser19} phosphorylation. This sequence of events leads to a Ca\textsuperscript{2+} response in platelets downstream of other receptors (Rivera et al., 2009). If a Ca\textsuperscript{2+} response is provoked by oxLDL, this may lead to the activation of MLCK. Therefore, to determine if MLCK is indeed activated by oxLDL, leading to MLC\textsuperscript{Ser19} phosphorylation, platelets were treated with the MLCK inhibitor ML-7. This MLCK inhibitor has been previously applied to platelets at the concentration used in these experiments (Roberts et al., 2009). Figure 5.16 illustrates that ML-7 (5µM) significantly reduces MLC\textsuperscript{Ser19} phosphorylation stimulated by oxLDL (p=0.001). These data indicate that CD36 signalling, generated by oxLDL, results in a tyrosine kinase pathway and MLCK activation leading to MLC\textsuperscript{Ser19} phosphorylation and platelet shape change.
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Washed platelets (3x10^8/ml) were treated with EGTA (1mM), apyrase (2U/ml) and indomethacin (10µM) for a minimum of 20 minutes prior to experiments. Platelets were incubated in the absence and presence of ML-7 (5µM) at 37°C for 20 minutes followed by stimulation with oxLDL (50µg/ml) for 15 seconds. Platelets were then lysed and samples were separated by SDS-PAGE followed by immunoblotting for phospho-MLC^{Ser19}. Membranes were stripped and re-probed for β-tubulin. (A) Representative immunoblot and (B) quantitative analysis of 5 independent experiments performed. *** indicates p<0.005.

Figure 5.16: Oxidised low density lipoprotein stimulated myosin light chain phosphorylation requires myosin light chain kinase

Washed platelets (3x10^8/ml) were treated with EGTA (1mM), apyrase (2U/ml) and indomethacin (10µM) for a minimum of 20 minutes prior to experiments. Platelets were incubated in the absence and presence of ML-7 (5µM) at 37°C for 20 minutes followed by stimulation with oxLDL (50µg/ml) for 15 seconds. Platelets were then lysed and samples were separated by SDS-PAGE followed by immunoblotting for phospho-MLC^{Ser19}. Membranes were stripped and re-probed for β-tubulin. (A) Representative immunoblot and (B) quantitative analysis of 5 independent experiments performed. *** indicates p<0.005.
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5.6 Discussion

The data presented in this chapter has demonstrated that the pathological ligand oxLDL can stimulate one of the earliest stages in platelet activation, the shape change response. This effect was shown to be specific to the oxidised form of the lipoprotein. The experimental results suggest that the signalling pathway facilitating this response was stimulated through the scavenger receptor CD36 and required a tyrosine kinase mediated pathway.

5.6.1 Oxidised low density lipoproteins stimulate platelet shape change

The first line of evidence that established platelet shape change could be stimulated by oxLDL was aggregation experiments performed under non-aggregatory conditions. Samples contained the reagents: EGTA, to prevent integrin activation and outside-in signalling; apyrase, to breakdown any secreted ADP, thus avoiding its interactions with P2Y receptors; and indomethacin, to inhibit TXA₂ production, preventing stimulation of the TXA₂ Gαq and Gα₁₂/₁₃ coupled receptors. By blocking these factors it was established that oxLDL induced primary signalling events that led to the activation of tyrosine kinase dependent pathways, which in turn resulted in shape change. Therefore, as investigations were carried out in the presence of these reagents, it can be concluded that shape change and MLC\textsuperscript{Ser19} phosphorylation stimulated by oxLDL is a consequence of the interactions between the oxidised lipoprotein with platelets and independent of secondary mediators. Interestingly, two previous studies have found a mildly oxidised form of LDL to also induce platelet shape change (Retzer et al., 2000,
Maschberger et al., 2000), suggesting that both high and low levels of oxidation can produce species capable of stimulating the early stages of platelet activation.

The receptor responsible for oxLDL induced platelet shape change was also investigated in this chapter. The major tool to approach this was the use of the CD36 blocking antibody FA6.152 and therefore it was important to confirm that FA6.152 alone did not influence platelet shape change. Three pieces of experimental data were presented to give confidence that the antibody was an appropriate tool, (i) FA6.152 did not induce platelet shape change when added to platelets, (ii) FA6.152 did not affect thrombin induced platelet shape change and (iii) FA6.152 did not trigger MLC\textsuperscript{Ser19} phosphorylation on its own. Therefore, the CD36 blocking antibody was determined to be acceptable for use to assess the contribution of this scavenger receptor in the shape change signalling pathway.

FA6.152 reduced both oxLDL induced platelet shape change and MLC\textsuperscript{Ser19} phosphorylation, strongly suggesting CD36 as a receptor for this signalling response. However, platelets possess multiple receptors that can potentially interact with oxLDL and so; it would be beneficial in future work to investigate these in more detail. For example, SR-A has been implicated as a receptor that can contribute to the platelet activating properties of oxLDL (Korporaal et al., 2007). Therefore, future work could involve the use of SR-A inhibitors such as fucoidan and polyinosinic acid (Dhaliwal and Steinbrecher, 1999). Previous reports have suggested the lysophosphatidic acid (LPA) receptor to be responsible for shape change induced by oxLDL (Maschberger et al.,
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However, this study used minimally modified LDL, therefore, suggesting that LDL oxidised to varying extents may interact with different receptors on platelets, but lead to similar responses. To strengthen the findings that CD36 is the receptor for oxLDL induced platelet shape change, it would be beneficial to use CD36 deficient mice. However, for unknown reasons, human oxLDL did not stimulate murine platelets therefore preventing this line of investigation from being performed in this work. Nevertheless, the findings in this chapter strengthen previous conclusions implicating CD36 as the critical receptor for oxLDL interactions with platelets leading to activation and a prothrombotic phenotype (Chen et al., 2008, Podrez et al., 2007).

5.6.2 Oxidised low density lipoprotein induced platelet shape change involves a tyrosine kinase mediated pathway

Investigations into the contribution of Src kinases to the signalling pathway that leads to platelet shape change stimulated by oxLDL were crucial due to the well established participation of Src kinases in CD36 signalling in platelets as well as other cell types. The results from this chapter demonstrate oxLDL to stimulate the rapid activatory phosphorylation of Src kinases at tyrosine\(^{416}\) and that platelet shape change, MLC\(^{\text{Ser}19}\) and tyrosine phosphorylation were all abolished in the presence of the Src kinase inhibitor PP2. Src kinases were first found to associate with CD36 in 1991 (Huang et al., 1991). Since this finding, these proteins have been shown to be involved in: a CD36 signalling pathway leading to platelet activation (Chen et al., 2008); CD36 dependent macrophage foam cell formation (Rahaman et al., 2006); and TSP-1 induced CD36 signalling in platelets (Roberts et al., 2010). This suggests that Src kinases are crucial to
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CD36 signalling and also strengthens the finding that CD36 is the receptor for oxLDL induced platelet shape change. An important discovery that this work has brought to light is that the activation of Src kinases by oxLDL occurs rapidly at 15 seconds stimulation, compared with longer time points that have been previously observed (Chen et al., 2008), indicating a rapid signal transduction mechanism. It would be appealing in future work to establish exactly which Src kinase that associates with CD36, Fyn, Lyn, Yes, or a combination, participate in the shape change response and whether different Src kinases become activated at various time points.

The observations in this report are the first of rapid receptor mediated tyrosine phosphorylation events in platelets stimulated by oxLDL. This pathological lipoprotein stimulated the tyrosine phosphorylation of at least 6 different proteins in platelets over a time course of 15-300 seconds. As this response was shown to be mediated by Src kinases, comparisons can be made with other platelet receptors such as GPVI, GPIb-V-IX and α_{IIb}β_{3}. Each of these receptors are associated with Src kinases that mediate signalling (Briddon and Watson, 1999, Falati et al., 1999, Obergfell et al., 2002). GPVI, GPIb-V-IX and α_{IIb}β_{3} also require the contribution of an ITAM to mediate further signal transduction (Poole et al., 1997, Wu et al., 2001, Boylan et al., 2008), although α_{IIb}β_{3} signalling has been suggested to be ITAM independent (Gao et al., 1997), indicating that CD36 signalling may also be coupled with ITAM activity. To date, CD36 has not been linked with ITAM mediated signalling and therefore further investigations are required to determine whether this may be the case. However, like other tyrosine kinase linked receptor signalling, CD36 is associated with Src kinases.
that are primarily responsible for the downstream signalling pathways provoked by oxLDL ligation.

The identities of the tyrosine phosphorylated proteins were investigated by immunoprecipitation. These experiments showed that both Syk and PLCγ2 become tyrosine phosphorylated in platelets in response to only 15 seconds stimulation with oxLDL. These findings are in agreement with the literature, in which others have also found Syk to become phosphorylated with stimulation of mildly oxidised LDL (Maschberger et al., 2000) and upon platelet adhesion to oxLDL (Nergiz-Unal et al., 2011a). In addition, PLCγ2 has been shown to become phosphorylated in response to oxLDL in macrophages (Rahaman et al., 2011b). Therefore, these proteins appear to also be crucial in CD36 signalling. Furthermore, these events reveal additional resemblances with GPVI, GPIb-V-IX and αIIbβ3 signalling, in which Syk and PLCγ2 phosphorylation are initiated (Poole et al., 1997, Wu et al., 2001, Wonerow et al., 2003). Consequently, a number of similarities are beginning to appear between the signalling pathways of the scavenger receptor CD36 and other tyrosine kinase mediated platelet receptors.

Syk and PLCγ2 phosphorylation were shown to be downstream of Src kinases. Although PLCγ2 is known to be downstream of Syk upon stimulation with other platelet receptors (Poole et al., 1997), it would have been beneficial to also apply the Syk inhibitors piceatannol and R406 to confirm that the phosphorylation of PLCγ2 is indeed downstream of Syk in response to oxLDL. In addition, it would be of interest in
future work to determine which particular sites on Syk and PLCγ2 are phosphorylated. Syk can become autophosphorylated at tyrosine\textsuperscript{525} and tyrosine\textsuperscript{526} as well as phosphorylation occurring at tyrosine\textsuperscript{352}, whereas PLCγ2 can become phosphorylated at tyrosine\textsuperscript{753} and tyrosine\textsuperscript{759}. Furthermore, different agonists can induce varying extents of phosphorylation at each of these sites at distinctive time points (Suzuki-Inoue et al., 2004). Therefore, examination of which sites that each protein becomes phosphorylated and the incubation times needed for this would be of interest to observe.

Inhibition of JNK was found to reduce MLC\textsuperscript{Ser19} phosphorylation, although this protein was interestingly noted not to be upstream of Syk phosphorylation. Previous studies have established JNK to be involved in platelet activation by oxLDL and that activation of JNK is Src kinase dependent (Chen et al., 2008). This suggests that JNK may be either involved in a separate pathway to that of Syk or that it may be downstream of this protein. Further investigations are required to fully establish the role that JNK has in oxLDL induced platelet shape change. Additionally, Syk and PLC inhibitors were also discovered to reduce oxLDL stimulated MLC\textsuperscript{Ser19} phosphorylation, suggesting their involvement in the shape change signalling pathway. An important response to Syk and PLCγ2 stimulation in platelets is an increase in intracellular Ca\textsuperscript{2+} levels, which can lead to a number of platelet activation responses. The activation of MLCK requires a Ca\textsuperscript{2+}/calmodulin response (Hathaway and Adelstein, 1979) and the involvement of MLCK in oxLDL induced MLC\textsuperscript{Ser19} phosphorylation was confirmed by the use of the MLCK inhibitor ML-7, which reduced MLC\textsuperscript{Ser19} phosphorylation. Therefore, it could be
speculated that stimulation of platelets with oxLDL through CD36 leads to the Src kinase dependent activation of Syk and PLCγ2, resulting in a rise in intracellular Ca$^{2+}$ and MLCK activation. These events then culminate in the phosphorylation of MLC$^{\text{Ser19}}$ and platelet shape change (Figure 5.17).

It could also be hypothesised that as oxLDL stimulates platelets to change shape, this may aid in the secretion of ADP, which is necessary for oxLDL induced platelet aggregation, as shown in chapter 3. This illustrates how minimal stimulation induced by CD36 signalling, leading to platelet shape change, can result in a larger thrombotic event occurring, if ADP is secreted as a consequence.

Interestingly, the reagents used in this chapter to inhibit Syk, PLC, JNK and MLCK do not appear to completely abolish oxLDL stimulated MLC$^{\text{Ser19}}$ phosphorylation. This suggests the possible involvement of another pathway contributing to the shape change response. In addition to the Ca$^{2+}$ dependent phosphorylation of MLC$^{\text{Ser19}}$, a RhoA/Rho kinase pathway also plays a prominent role, especially as a result of Go$^{\text{12/13}}$ coupled receptor stimulation (Klages et al., 1999). Previous work examining the effects of oxLDL on platelet shape change have found these modified lipoproteins to induce a Rho kinase dependent signalling pathway (Retzer et al., 2000). Therefore, this led to the prediction that, along with a tyrosine kinase mediated pathway, oxLDL also stimulates a RhoA/Rho kinase pathway. This will be next considered in the subsequent chapter.
Schematic representation of the CD36 signalling pathway stimulated by oxLDL, in which Src kinase dependent activation of Syk and PLCγ2 leads to an increase in intracellular Ca\(^{2+}\). This increase results in the activation of MLCK, MLC\(^{\text{Ser19}}\) phosphorylation and platelet shape change.

Figure 5.17: A proposed model of oxidised low density lipoprotein induced platelet shape change through a CD36/tyrosine kinase signalling pathway

Schematic representation of the CD36 signalling pathway stimulated by oxLDL, in which Src kinase dependent activation of Syk and PLCγ2 leads to an increase in intracellular Ca\(^{2+}\) resulting in the activation of MLCK, MLC\(^{\text{Ser19}}\) phosphorylation and platelet shape change.
Chapter 6: Oxidised low density lipoproteins stimulate a RhoA/Rho kinase signalling pathway that contributes to platelet shape change

6.1 Introduction

Platelet shape change and the phosphorylation of $\text{MLC}^{\text{Ser19}}$, in response to platelet stimulation by physiological agonists, require the dual regulation of MLCK and MLCP. MLCK is activated through $\text{Ca}^{2+}$/calmodulin stimulation, facilitating phosphorylation of $\text{MLC}^{\text{Ser19}}$ (Hathaway and Adelstein, 1979). To ensure a net increase in $\text{MLC}^{\text{Ser19}}$ phosphorylation during platelet activation MLCP must be regulated. The pathway normally involved in this response is stimulated by $\text{G} \alpha_{12/13}$ receptors (Klages et al., 1999). Ligation of receptors coupled to $\text{G} \alpha_{12/13}$ brings about the activation of the GTPase RhoA by RhoGEFs, which induce the disassociation of GDP and the association of GTP, transforming RhoA into its activated state. RhoA then proceeds to activate Rho kinase, which in turn induces the inhibitory phosphorylation of MLCP at two key sites, threonine$^{853}$ and threonine$^{696}$ (Nakai et al., 1997, Feng et al., 1999). The consequence of this pathway is that MLCP is unable to dephosphorylate $\text{MLC}^{\text{Ser19}}$, allowing platelet shape change to occur.

Data from the previous chapter demonstrated that oxLDL ligation of CD36 induced the activation of MLCK leading to $\text{MLC}^{\text{Ser19}}$ phosphorylation. Therefore, this chapter was designed to elucidate whether oxLDL also stimulates the RhoA/Rho kinase pathway, in order to inhibit MLCP and enhance $\text{MLC}^{\text{Ser19}}$ phosphorylation, leading to the shape change response.
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The aims of this chapter include:

- Assessing the contribution of the Rho kinase pathway to oxLDL stimulated platelet shape change.
- Determining whether oxLDL can induce the activation of RhoA.
- Investigating the inhibitory phosphorylation state of MLCP provoked by oxLDL.
6.2 Oxidised low density lipoproteins induce both Ca\(^{2+}\) and Rho kinase pathways leading to platelet shape change

In light of the findings of the previous chapter, in which inhibition of Syk, PLC and MLCK only partially reduced MLC\(^{\text{Ser19}}\) phosphorylation stimulated by oxLDL, investigations into the possible involvement of a second pathway were next performed. This was first considered by use of two reagents: the intracellular Ca\(^{2+}\) chelator BAPTA-AM, to confirm the contribution of intracellular Ca\(^{2+}\) signalling; and the Rho kinase inhibitor Y27632, to reveal the involvement of the RhoA/Rho kinase pathway.

6.2.1 Characterisation of the effects of BAPTA-AM and Y27632 on myosin light chain phosphorylation

In the first instance, the effects of the Rho kinase inhibitor Y27632 and the intracellular Ca\(^{2+}\) chelator BAPTA-AM on MLC\(^{\text{Ser19}}\) phosphorylation were established. When used independently, these reagents both induced a dose-dependent inhibition of MLC\(^{\text{Ser19}}\) phosphorylation stimulated by thrombin (Figure 6.1). However, neither inhibitor was able to completely abolish MLC\(^{\text{Ser19}}\) phosphorylation. Concentrations of 20µM BAPTA-AM and 10µM Y27632 were chosen to be used in subsequent experiments as these doses substantially reduced MLC\(^{\text{Ser19}}\) phosphorylation and have also been previously used in the literature with platelets (Oberprieler et al., 2007, Getz et al., 2010).
Washed platelets (3x10^8/ml) were treated with EGTA (1mM), apyrase (2U/ml) and indomethacin (10µM) for a minimum of 20 minutes before experiments were performed. (A) Platelets were incubated in the absence or presence of BAPTA-AM (1-50µM) at 37°C for 20 minutes followed by stimulation with thrombin (0.05U/ml) for 15 seconds. (B) Platelets were incubated in the absence or presence of Y27632 (1-50µM) at 37°C for 20 minutes followed by stimulation with thrombin (0.05U/ml) for 60 seconds. Samples were then lysed, separated by SDS-PAGE and immunoblotted for phospho-MLC^{Ser19}. Membranes were stripped and re-probed for β-tubulin. Immunoblots are from a single experiment performed.

Figure 6.1: Inhibition of thrombin stimulated myosin light chain phosphorylation by BAPTA-AM and Y27632
6.2.2 Oxidised low density lipoprotein induced myosin light chain phosphorylation proceeds through both Ca$^{2+}$ and Rho kinase pathways

Platelets were treated with BAPTA-AM, Y27632 or a combination of both, followed by oxLDL, to determine whether stimulation of MLC$^{\text{Ser19}}$ phosphorylation by this pathological ligand occurred through Ca$^{2+}$ and Rho kinase pathways. Treatment with both reagents individually, reduced significantly but did not abolish MLC$^{\text{Ser19}}$ phosphorylation triggered by oxLDL (Y27632 - p<0.01, BAPTA-AM - p=0.04) (Figure 6.2). When platelets were pre-incubated with a combination of the inhibitors, MLC$^{\text{Ser19}}$ phosphorylation was maintained at basal levels (p<0.001). Importantly, dimethyl sulphoxide (DMSO) was used as a vehicle control and did not have any effect on MLC$^{\text{Ser19}}$ phosphorylation (p=0.18). This data suggests that CD36 signalling, stimulated by oxLDL, can induce two pathways, which lead to a rise in intracellular Ca$^{2+}$ and Rho kinase activation. Both of these pathways may contribute to MLC$^{\text{Ser19}}$ phosphorylation and platelet shape change.

It was suggested in the previous chapter that Syk and PLCγ2 activation by oxLDL were contributing factors that lead to increases in intracellular Ca$^{2+}$. Therefore, to examine this hypothesis, platelets were treated with the Syk inhibitor piceatannol, the PLC inhibitor U73122, the Rho kinase inhibitor Y27632 or a combination. Each of these reagents significantly reduced MLC$^{\text{Ser19}}$ phosphorylation alone (Y27632 - p<0.01, piceatannol - p=0.005, U73122 - p=0.03). However, when piceatannol or U73122 were used in combination with Y27632, MLC$^{\text{Ser19}}$ phosphorylation was inhibited to basal levels (piceatannol + Y27632 - p<0.001, U73122 + Y27632 - p<0.001) (Figure 6.3). This
suggests that, similar to the dual inhibition of Ca\textsuperscript{2+} and Rho kinase, inhibition of either Syk and Rho kinase or PLC and Rho kinase, prevents oxLDL from stimulating MLC\textsuperscript{Ser19} phosphorylation. Therefore, these data taken together imply that oxLDL are indeed provoking two separate signalling pathways leading to MLC\textsuperscript{Ser19} phosphorylation. One of these involves a tyrosine kinase dependent pathway that may increase intracellular Ca\textsuperscript{2+} whereas a second involves a Rho kinase pathway.
Washed platelets (3x10^8/ml) were treated with EGTA (1mM), apyrase (2U/ml) and indomethacin (10µM) for a minimum of 20 minutes before experiments were performed. Platelets were incubated in the absence and presence of Y27632 (10µM), BAPTA-AM (20µM), a combination of these or DMSO (0.2%) at 37°C for 20 minutes followed by stimulation with oxLDL (50µg/ml) for 15 seconds. Samples were then lysed, separated by SDS-PAGE, immunoblotted for phospho-MLC^{Ser19}, stripped and re-probed for β-tubulin. (A) Representative immunoblot and (B) quantitative analysis of 4 independent experiments performed. * indicates p<0.05-0.01, ** p<0.01-0.005, *** p<0.005.

Figure 6.2: Oxidised low density lipoprotein stimulated myosin light chain phosphorylation is both Ca^{2+} and Rho kinase dependent
Washed platelets (3x10⁸/ml) were treated with EGTA (1mM), apyrase (2U/ml) and indomethacin (10µM) for a minimum of 20 minutes before experiments were performed. Platelets were incubated in the absence and presence of either Y27632 (10µM), piceatannol (20µM), U73122 (5µM), a combination of Y27632 and piceatannol or a combination of Y27632 and U73122 at 37°C for 20 minutes. Platelets were then stimulated with oxLDL (50µg/ml) for 15 seconds and lysed. Samples were separated by SDS-PAGE and immunoblotted for phospho-MLC<sup>Ser19</sup> followed by stripping and re-probing for β-tubulin. (A) Representative immunoblot and (B) quantitative analysis of 3 separate experiments performed. * indicates p<0.05-0.01, ** p<0.01-0.005 and *** p<0.005.
6.3 Assessment of RhoA activity upon stimulation with oxidised low density lipoproteins

After demonstrating the potential involvement of a Rho kinase pathway leading to oxLDL induced MLC\textsuperscript{Ser19} phosphorylation, the participation of its upstream activator, RhoA was next considered. This was assessed using a RhoA pull-down assay, in which GTP-bound RhoA was isolated from platelet lysates followed by immunoblotting to determine the conditions under which RhoA can become activated.

6.3.1 Oxidised low density lipoproteins stimulate RhoA activation in a Src kinase dependent manner

The RhoA activity assay revealed that stimulation of platelets with oxLDL significantly increased the amount of GTP-bound RhoA present within the cell (p=0.03) (Figure 6.4). However, this was not to the extent that was seen in thrombin stimulated platelets. Importantly, nLDL did not induce the activation of RhoA, indicating that the oxidative modification of the lipoprotein is required to stimulate this signalling pathway. As Src kinases lie upstream of the tyrosine kinase pathway evaluated in the previous chapter, the contribution of these proteins in stimulating the RhoA/Rho kinase pathway was also assessed. OxLDL stimulated RhoA activation was significantly reduced in the presence of the Src kinase inhibitor PP2 (p=0.02), whereas PP3 had no effect (p=0.30) (Figure 6.5). These results were also mirrored in the phosphorylation state of MLC\textsuperscript{Ser19}. The Rho kinase inhibitor was also tested in the RhoA assay and, as predicted, had no effect on RhoA activation but reduced MLC\textsuperscript{Ser19} phosphorylation. This confirms that Rho kinase is indeed downstream of RhoA but upstream of MLC.
Washed platelets (5x10⁸/ml) were treated with EGTA (1mM), apyrase (2U/ml) and indomethacin (10µM) for a minimum of 20 minutes prior to experiments being performed. Platelets were stimulated with either oxLDL (50µg/ml), nLDL (50µg/ml) or thrombin (0.05U/ml) for 15 seconds followed by lysis. Active RhoA was then isolated using rhotekin-RBD bound glutathione-sepharose beads. Samples were next separated using SDS-PAGE and immunoblotted for RhoA. (A) Representative immunoblot and (B) quantitative analysis of 4 individual experiments performed. * indicates p<0.05-0.01 and ** p<0.01-0.005.
Washed platelets ($5 \times 10^8$/ml) were treated with EGTA (1mM), apyrase (2U/ml) and indomethacin (10µM) for a minimum of 20 minutes prior to experiments being performed. Platelets were incubated in the absence and presence of either PP2 (20µM), PP3 (20µM) or Y27632 (10µM) at 37°C for 20 minutes followed by stimulation with oxLDL (50µg/ml) for 15 seconds. Cells were then lysed and active RhoA was isolated using rhotekin-RBD bound glutathione-sepharose beads. Samples were next separated using SDS-PAGE and immunoblotted for RhoA. (A) Representative immunoblot and (B) quantitative analysis of 3 independent experiments performed. End sample containing Y27632 was performed in a single experiment. * indicates p<0.05-0.01.

Figure 6.5: RhoA activation stimulated by oxidised low density lipoproteins is Src kinase dependent

Washed platelets ($5 \times 10^8$/ml) were treated with EGTA (1mM), apyrase (2U/ml) and indomethacin (10µM) for a minimum of 20 minutes prior to experiments being performed. Platelets were incubated in the absence and presence of either PP2 (20µM), PP3 (20µM) or Y27632 (10µM) at 37°C for 20 minutes followed by stimulation with oxLDL (50µg/ml) for 15 seconds. Cells were then lysed and active RhoA was isolated using rhotekin-RBD bound glutathione-sepharose beads. Samples were next separated using SDS-PAGE and immunoblotted for RhoA. (A) Representative immunoblot and (B) quantitative analysis of 3 independent experiments performed. End sample containing Y27632 was performed in a single experiment. * indicates p<0.05-0.01.
6.3.2 Oxidised low density lipoproteins stimulate the tyrosine phosphorylation of the Rho guanine nucleotide exchange factor Vav1

Having demonstrated that oxLDL can stimulate the conversion of RhoA into its active GTP bound state; investigations were next performed to ascertain how this may transpire. It has been recently found that oxLDL can induce the phosphorylation of Vav1 and Vav3 in platelets (Chen et al., 2011). Vav proteins are RhoGEFs, which are involved in the disassociation of GDP and the association of GTP in RhoGTPases (Adams et al., 1992, Gulbins et al., 1993). As Chen and colleagues observed Vav3 to become phosphorylated at a late time point of 30 minutes, attention was focused onto Vav1. Phosphorylation of Vav1 was assessed using immunoprecipitation followed by Immunoblotting for phosphotyrosine. In preliminary experiments it was found that Vav1 could be detected with 3µg of antibody (Figure 6.6A), therefore, this quantity was used in subsequent experiments.

Tyrosine phosphorylation of Vav1 could be detected in samples in which platelets had been stimulated with oxLDL for only 15 seconds (Figure 6.6B), which corresponds with the early signalling events already observed to be downstream of oxLDL. In addition, nLDL did not provoke any phosphorylation of Vav1, indicating the importance of oxidative modification for this signalling to occur. The Src kinase inhibitor PP2 was also tested in these experiments and found to significantly impede Vav1 phosphorylation stimulated by oxLDL (p=0.02), whereas PP3 had no effect (p=0.18). This data implies that, similar to the previous observations of oxLDL/CD36 signalling, Vav1 phosphorylation is downstream of Src kinases.
Washed platelets (7x10^8/ml) were treated with EGTA (1mM), apyrase (2U/ml) and indomethacin (10µM) for a minimum of 20 minutes before experiments were performed. (A) Non-stimulated platelet lysates were incubated with Vav1 antibody (1-5µg) or IgG control (1µg). Immunoprecipitated Vav1 and non-stimulated whole cell lysate (WCL) were subjected to SDS-PAGE followed by immunoblotting for Vav1. Shown is an immunoblot from an individual experiment performed. (B) Platelets were incubated in the absence or presence of either PP2 (20µM) or PP3 (20µM) at 37°C for 20 minutes followed by stimulation with either oxLDL (50µg/ml) or nLDL (50µg/ml). Platelets were then lysed and Vav1 immunoprecipitated. Samples were next separated by SDS-PAGE, immunoblotted for phospho-tyrosine, stripped and re-probed for Vav1. (Bi) Representative immunoblot and (Bii) quantitative analysis of 3 separate experiments performed. * indicates p<0.05-0.01, ** p<0.01-0.005 and *** p<0.005.
6.4 Activation of the RhoA/Rho kinase pathway by oxidised low density lipoproteins results in the inhibitory phosphorylation of myosin light chain phosphatase

Activation of the RhoA/Rho kinase pathway can result in the inhibitory phosphorylation of MLCP at the sites threonine\(^{853}\) and threonine\(^{696}\) of its regulatory subunit MYPT1 (Feng et al., 1999, Lincoln, 2007). In platelets, this prevents the phosphatase from dephosphorylating MLC, allowing shape change to occur. Therefore, as oxLDL has been shown to stimulate the RhoA/Rho kinase pathway, immunoblotting experiments were performed to determine whether this pathological species could also stimulate the inhibitory phosphorylation of MLCP.

6.4.1 Oxidised but not native low density lipoproteins can stimulate the inhibitory phosphorylation of myosin light chain phosphatase

Stimulation of platelets with oxLDL significantly increased the phosphorylation of both MYPT1\(^{Thr853}\) (p=0.02) and MYPT1\(^{Thr696}\) (p=0.02) above basal levels (Figure 6.7). In contrast, there was no difference observed in the phosphorylation state between non-stimulated and nLDL stimulated samples at both MYPT1\(^{Thr853}\) (p=0.14) and MYPT1\(^{Thr696}\) (p=0.48). Importantly, thrombin, as a positive control, also stimulated the inhibitory phosphorylation at both MYPT1\(^{Thr853}\) and MYPT1\(^{Thr696}\). This suggests that oxLDL stimulation of platelets results in the inhibitory phosphorylation of MLCP, which may contribute to increasing the phosphorylation of MLC\(^{Ser19}\) and platelet shape change.
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A

oxLDL  nLDL  Thrombin

Phospho-MYPT1\textsuperscript{Thr853}

140

140

50

Phospho-MYPT1\textsuperscript{Thr696}

\(\beta\)-tubulin

B

\begin{align*}
\text{MYPT1}\textsuperscript{Thr853} & \quad \text{Phosphorylation} \\
& \quad \text{Relative to } \beta\text{-tubulin}
\end{align*}

\begin{align*}
\text{oxLDL} & \quad \text{nLDL} \quad \text{Thrombin}
\end{align*}

C

\begin{align*}
\text{MYPT1}\textsuperscript{Thr696} & \quad \text{Phosphorylation} \\
& \quad \text{Relative to } \beta\text{-tubulin}
\end{align*}

\begin{align*}
\text{oxLDL} & \quad \text{nLDL} \quad \text{Thrombin}
\end{align*}
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Figure 6.7: Oxidised low density lipoproteins induce the inhibitory phosphorylation of myosin light chain phosphatase

Washed platelets (5x10^8/ml) were treated with EGTA (1mM), apyrase (2U/ml) and indomethacin (10µM) for a minimum of 20 minutes prior to experiments. Platelets were stimulated with either oxLDL (50µg/ml), nLDL (50µg/ml) or thrombin (0.05U/ml) for 15 seconds followed by lysis. Samples were then separated by SDS-PAGE and immunoblotted for phospho-MYPT1^{Thr853}. Membranes were stripped and re-probed for phospho-MYPT1^{Thr696} followed by stripping and re-probing for β-tubulin. (A) Representative immunoblot and (B and C) quantitative analysis of 3 independent experiments performed. * indicates p<0.05-0.01.
6.4.2 CD36 mediates oxidised low density lipoprotein stimulated myosin light chain phosphatase phosphorylation

Although observations in the previous chapter have indicated CD36 as the receptor in which oxLDL stimulates MLC$^{\text{Ser19}}$ phosphorylation and platelet shape change, investigations were performed to confirm this as the receptor that leads to the activation of the RhoA/Rho kinase pathway triggered by oxLDL. The CD36 blocking antibody FA6.152 was again utilised for this purpose. Prevention of interactions between oxLDL and CD36, with FA6.152, significantly inhibited phosphorylation of MYPT1$^{\text{Thr853}}$ ($p<0.001$) and MYPT1$^{\text{Thr696}}$ ($p=0.001$) by oxLDL (Figure 6.8). A small decline in phosphorylation levels was also observed with the IgG control antibody, however, this was not found to be significant for either MYPT1$^{\text{Thr853}}$ ($p=0.19$) or MYPT1$^{\text{Thr696}}$ ($p=0.14$). This indicates that CD36 signalling can indeed stimulate the RhoA/Rho kinase pathway, which leads to the downstream inhibitory phosphorylation of MLCP.
Figure 6.8: Myosin light chain phosphatase phosphorylation induced by oxidised low density lipoproteins is CD36 dependent

Washed platelets (5x10^8/ml) were treated with EGTA (1mM), apyrase (2U/ml) and indomethacin (10µM) for a minimum of 20 minutes before experiments were performed. Platelets were incubated in the absence and presence of either FA6.152 (1µg/ml) or IgG control antibody (1µg/ml) at 37°C for 15 minutes followed by stimulation with oxLDL (50µg/ml) for 15 seconds. Samples were then lysed, separated by SDS-PAGE and immunoblotted for phospho-MYPT1 Thr853. Membranes were stripped and re-probed for phospho-MYPT1 Thr696 followed by stripping and re-probing for β-tubulin. (A) Representative immunoblot and (B and C) quantitative analysis of 3 independent experiments performed. *** indicates p<0.005.
6.4.3 Oxidised low density lipoprotein induced myosin light chain phosphatase phosphorylation is Rho kinase dependent but Ca\(^{2+}\) independent

To strengthen observations that oxLDL stimulates a RhoA/Rho kinase pathway that leads to the inhibition of MLCP, phosphorylation was assessed in the presence of the Rho kinase inhibitor Y27632. The intracellular Ca\(^{2+}\) chelator BAPTA-AM was also employed to demonstrate that Ca\(^{2+}\) signalling stimulated by oxLDL leads to a separate pathway, distinct from the Rho kinase pathway. The Rho kinase inhibitor Y27632 abolished oxLDL induced MYPT1\(^{Thr696}\) phosphorylation to basal levels (p<0.01), whereas chelation of intracellular Ca\(^{2+}\) by BAPTA-AM had no effect (p=0.15) (Figure 6.9A). This suggests that oxLDL does indeed induce the Rho kinase dependent phosphorylation of MLCP at this residue, which is independent of Ca\(^{2+}\) signalling.

Interestingly, more ambiguous results were obtained when examining MYPT1\(^{Thr696}\) phosphorylation. Basal phosphorylation levels at this residue were high and, therefore, oxLDL alone was unable to induce a significant increase in phosphorylation. Here, the Rho kinase inhibitor only impeded phosphorylation in one of the experiments performed, whereas BAPTA-AM did not have an effect on phosphorylation in any (Figure 6.9B). It can therefore be proposed that MYPT1\(^{Thr696}\) phosphorylation stimulated by oxLDL is more variable and less reliant on Rho kinase than that of MYPT1\(^{Thr853}\).
Washed platelets (5x10^8/ml) were treated with EGTA (1mM) apyrase (2U/ml) and indomethacin (10µM) for a minimum of 20 minutes before experiments were performed. Platelets were incubated in the absence and presence of either Y27632 (10µM) or BAPTA-AM (20µM) at 37°C for 20 minutes followed by stimulation with oxLDL (50µg/ml) for 15 seconds. Samples were then lysed and separated by SDS-PAGE in duplicate followed by immunoblotting for either (A) phospho-MYPT1^Thr853 or (B) phospho-MYPT1^Thr696. Membranes were then stripped and re-probed for β-tubulin. (Ai and Bi) Representative immuno blots and (Aii and Bii) quantitative analysis of 3 separate experiments performed. ** indicates p<0.01-0.005.

**Figure 6.9: Oxidised low density lipoproteins stimulate the Rho kinase dependent phosphorylation of myosin light chain phosphatase at the threonine^853 residue**

Washed platelets (5x10^8/ml) were treated with EGTA (1mM) apyrase (2U/ml) and indomethacin (10µM) for a minimum of 20 minutes before experiments were performed. Platelets were incubated in the absence and presence of either Y27632 (10µM) or BAPTA-AM (20µM) at 37°C for 20 minutes followed by stimulation with oxLDL (50µg/ml) for 15 seconds. Samples were then lysed and separated by SDS-PAGE in duplicate followed by immunoblotting for either (A) phospho-MYPT1^Thr853 or (B) phospho-MYPT1^Thr696. Membranes were then stripped and re-probed for β-tubulin. (Ai and Bi) Representative immuno blots and (Aii and Bii) quantitative analysis of 3 separate experiments performed. ** indicates p<0.01-0.005.
6.4.4 Myosin light chain phosphatase phosphorylation stimulated by oxidised low density lipoproteins is dependent on Syk and PLC

To consider whether there may be cross-over between the tyrosine kinase and Rho kinase pathways stimulated by oxLDL, MLCP phosphorylation was examined in the presence of the Syk inhibitor piceatannol and the PLC inhibitor U73122. As the previous experiment illustrated MYPT1\(^{Thr853}\) to be more consistently phosphorylated downstream of Rho kinase, only this residue was examined. Intriguingly, both reagents significantly reduced MYPT1\(^{Thr853}\) phosphorylation back to basal levels (piceatannol - \(p<0.01\), U73122 - \(p=0.005\)) (Figure 6.10), indicating that both Syk and PLC contribute to the phosphorylation of MLCP at this residue. However, Figure 6.9A revealed that MYPT1\(^{Thr853}\) phosphorylation is not downstream of Ca\(^{2+}\). Therefore, this data taken together suggests the involvement of another factor.

To ensure that the above finding was indeed due to the inhibition of Syk and PLC, and not a non-specific effect of the reagent, an experiment was also performed with thrombin. This agonist induces MLCP phosphorylation downstream of G\(_{\alpha_{12/13}}\) receptors; therefore, neither inhibitor should have an effect. Figure 6.11 illustrates piceatannol and U73122 to again reduce oxLDL stimulated MYPT1\(^{Thr853}\) phosphorylation, however, piceatannol did not affect thrombin stimulated phosphorylation and U73122 had only a minor effect.
Washed platelets (5x10^8/ml) were treated with EGTA (1mM), apyrase (2U/ml) and indomethacin (10µM) for a minimum of 20 minutes before experiments were performed. Platelets were incubated in the absence and presence of either piceatannol (20µM) or U73122 (5µM) at 37°C for 20 minutes followed by stimulation with oxLDL (50µg/ml) for 15 seconds. Samples were then lysed, separated by SDS-PAGE and immunoblotted for phospho-MYPT1^Thr853. Membranes were next stripped and re-probed for β-tubulin. (A) Representative immunoblot and (B) quantitative analysis of 3 independent experiments performed. ** indicates p<0.01-0.005 and *** p<0.005.

Figure 6.10: Syk and PLC contribute to myosin light chain phosphatase inhibition by oxidised low density lipoproteins
Washed platelets (5x10⁸/ml) were treated with EGTA (1mM), apyrase (2U/ml) and indomethacin (10µM) for a minimum of 20 minutes before experiments were performed. Platelets were incubated in the absence and presence of either piceatannol (20µM) or U73122 (5µM) at 37°C for 20 minutes followed by stimulation with either thrombin (0.05U/ml) or oxLDL (50µg/ml) for 15 seconds. Samples were then lysed, separated by SDS-PAGE and immunoblotted for phospho-MYPT1⁷⁸⁵³. Membranes were next stripped and re-probed for β-tubulin. Immunoblot is from an individual experiment performed.

Figure 6.11: Syk and PLC contribution to myosin light chain phosphatase phosphorylation is specific to oxidised low density lipoprotein but not to thrombin stimulation

Washed platelets (5x10⁸/ml) were treated with EGTA (1mM), apyrase (2U/ml) and indomethacin (10µM) for a minimum of 20 minutes before experiments were performed. Platelets were incubated in the absence and presence of either piceatannol (20µM) or U73122 (5µM) at 37°C for 20 minutes followed by stimulation with either thrombin (0.05U/ml) or oxLDL (50µg/ml) for 15 seconds. Samples were then lysed, separated by SDS-PAGE and immunoblotted for phospho-MYPT1⁷⁸⁵³. Membranes were next stripped and re-probed for β-tubulin. Immunoblot is from an individual experiment performed.
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6.5 Discussion

The results in this chapter have revealed, for the first time, that the ligation of oxLDL with platelets through CD36 can lead to the RhoA/Rho kinase signalling pathway resulting in the potential inhibition of MLCP. These findings shed new light on the diverse signalling pathways that can be stimulated by CD36 resulting in platelet activation events, in particular platelet shape change.

6.5.1 Oxidised low density lipoproteins stimulate two distinct signalling pathways leading to platelet shape change

The previous chapter reported a tyrosine kinase dependent signalling pathway, triggered through CD36 by oxLDL stimulation, that lead to the phosphorylation of MLC$^{\text{Ser19}}$ and platelet shape change. However, inhibition of this pathway with various reagents only partially diminished oxLDL induced MLC$^{\text{Ser19}}$ phosphorylation. Therefore, investigations were performed to uncover other contributing pathways. The most logical signalling event to first focus on was the Rho kinase pathway, as this is known to contribute significantly to MLC$^{\text{Ser19}}$ phosphorylation and platelet shape change by physiological agonists such as thrombin and TXA$_2$ (Bauer et al., 1999). The use of the Rho kinase inhibitor Y27632 confirmed the contribution of this pathway to oxLDL induced MLC$^{\text{Ser19}}$ phosphorylation. The data here demonstrated that this inhibitor partially blocked the ability of both thrombin and oxLDL to induce MLC$^{\text{Ser19}}$ phosphorylation. These findings are consistent with a previous report that demonstrated minimally oxLDL can stimulate platelet shape change in a Rho kinase dependent manner (Retzer et al., 2000). Interestingly, this group did not believe
Ca\(^{2+}\)/MLCK to play a role in shape change as no detectable Ca\(^{2+}\) response was observed upon stimulation with minimally oxLDL. As the work in this project found the chelation of intracellular Ca\(^{2+}\) to diminish responses to oxLDL, and others have found oxLDL to raise intracellular Ca\(^{2+}\) in response to oxLDL (Nergiz-Unal et al., 2011a), this suggests that a more highly modified form of LDL does induce a Ca\(^{2+}\) response. This highlights a major dissimilarity between minimally oxLDL and fully oxLDL signalling. A possible explanation for the diverse responses to varying oxidation states of LDL may be the receptors responsible for the transduction of intracellular signalling responses as previous reports suggest CD36 to be a major receptor for oxLDL (Chen et al., 2008) whereas the LPA receptor may be stimulated by mildly oxLDL (Maschberger et al., 2000). Additionally, a milder oxidised form of LDL may simply induce a weaker activation response in platelets resulting in no observable intracellular Ca\(^{2+}\) elevations.

Therefore, it would be of interest in future work to measure Ca\(^{2+}\) in platelets upon stimulation with LDL oxidised to varying extents to determine the specific properties of the modified lipoprotein that produce different responses. This could also be performed in combination with various receptor blockers to identify which receptors are stimulated in response to the many forms of the modified lipoprotein.

There were two important lines of evidence presented in this report to support the contribution of two distinct signalling pathways leading to oxLDL stimulated platelet shape change. Firstly, either inhibition of Rho kinase or chelation of intracellular Ca\(^{2+}\) only partially reduced oxLDL induced MLC\(^{\text{Ser19}}\) phosphorylation, while in combination they abolished it. Secondly, inhibition of Syk or PLC also incompletely diminished
MLC$^{\text{Ser19}}$ phosphorylation, but when activation of either protein was blocked in combination with Rho kinase, phosphorylation was again abolished. This suggests that Syk, PLC and Ca$^{2+}$ each contribute to one pathway, which when inhibited does not completely impede MLC$^{\text{Ser19}}$ phosphorylation due to the involvement of the Rho kinase pathway. Similarly, Rho kinase must contribute to a separate pathway as inhibition of this protein again partially reduces MLC$^{\text{Ser19}}$ phosphorylation, leaving the Syk/PLC/Ca$^{2+}$ pathway to stimulate slight activation. When inhibited together, full ablation of MLC$^{\text{Ser19}}$ phosphorylation suggests that there is no additional pathway that contributes to this event. To further strengthen these findings, it would be beneficial to perform experiments on mice deficient in the proteins Syk, PLCγ2 and RhoA to categorically confirm their involvement in the two distinct pathways. As mentioned earlier, human LDL did not appear to stimulate murine platelets, making this method of experimentation difficult to perform. However, a possible line of future investigations could be to isolate murine LDL to determine its effects on knockout platelets.

### 6.5.2 Oxidised low density lipoproteins activate RhoA with possible contribution from the Rho guanine nucleotide exchange factor Vav1

It has been shown here for the first time that oxLDL can convert RhoA from its inactive GDP to its active GTP bound form in platelets. This was proven to be unique to the oxidised class of the lipoprotein as nLDL did not have an effect. There is currently little evidence linking CD36 to the RhoA pathway. However, a recent study has found RhoA to be associated with this scavenger receptor in human dermal microvascular endothelial cells (Kazerounian et al., 2011). Interestingly, RhoA activation by oxLDL
was established to be dependent on Src kinases. An earlier study has also found that inhibition of Src kinases can reduce RhoA activation in Chinese hamster ovary cells downstream of integrin αIIbβ3 (Salsmann et al., 2005), indicating that there are Src kinase dependent mechanisms for activating RhoA, although conventional platelet agonists that stimulate RhoA activation through Gα12/13 receptors do so independently of Src kinases (Klages et al., 1999). There is no evidence linking CD36 to G proteins in platelets and the addition of apyrase and indomethacin prevented the stimulation of ADP and TXA2 GPCR respectively. Therefore, the findings in this report suggest that the RhoA pathway can be stimulated downstream of receptors in platelets that are not coupled to G proteins. Additionally, this also illustrates that CD36 signalling can promote a variety of pathways through the contribution of Src kinases.

To establish a link between CD36/Src kinases and the RhoA pathway, the RhoGEF Vav1 was considered. It has been recently found that Vav1 becomes phosphorylated, in a Src kinase dependent manner, in platelets upon stimulation with oxLDL (Chen et al., 2011). This report has shown that Vav1 is phosphorylated after as little as 15 seconds stimulation with oxLDL, which is in keeping with the early signalling events required for platelet shape change. Furthermore, Vav has also been found to be associated with CD36 in human dermal microvascular endothelial cells (Kazerounian et al., 2011). A growing body of evidence is beginning to strongly link Vav with CD36 signalling in a number of cell types such as platelets (Chen et al., 2011), macrophages (Rahaman et al., 2011a) and monocytes (Wilkinson et al., 2006). Moreover, Vav proteins have been linked with the activation of RhoA in other cell types (Gakidis et al., 2004, Gao et al.,
2005, Bhavsar et al., 2009), although Vav does show preference for the RhoGTPase Rac in platelets (Aslan and McCarty, 2012). Therefore, Vav1 may be the protein responsible for the conversion of RhoA from a GDP to a GTP bound form. Further work is required to confirm the ability of Vav1 to activate RhoA downstream of oxLDL/CD36. Additionally, the protein p115RhoGEF is linked with RhoA activation downstream of Go13 receptors in platelets (Huang et al., 2007). Therefore, it would also be of interest in future work to investigate the whether this protein is involved in the CD36 signalling pathway leading to RhoA activation.

6.5.3 Oxidised low density lipoproteins stimulate the inhibitory phosphorylation of myosin light chain phosphatase

In addition to presenting data that reveals oxLDL can induce the activation of RhoA and Rho kinase, this report has also demonstrated the subsequent downstream phosphorylation of MLCP. This event was shown to be inhibited in the presence of the CD36 blocking antibody, confirming that the RhoA/Rho kinase pathway is indeed downstream of CD36. Early work has demonstrated that mildly oxLDL can also reduce the activity of MLCP in human endothelial cells, illustrating in this case comparisons between signalling events stimulated by both mildly and highly oxLDL (Essler et al., 1999). To confirm that the phosphorylation induced by oxLDL does correlate with the inhibition of MLCP, it would be beneficial to measure the activity of this phosphatase in platelets that have been stimulated with oxLDL at similar time points that have been tested here. Interestingly, the inhibitory phosphorylation of MLCP induced by oxLDL appeared to be mainly downstream of Rho kinase at the residue threonine853. This is
in keeping with previous suggestions that Rho kinase phosphorylates \( \text{MYPT1}^{\text{Thr853}} \) more efficiently than \( \text{MYPT1}^{\text{Thr696}} \) (Lincoln, 2007).

To summarise, the work from this chapter, in combination with the previous chapter, has elucidated two signalling pathways, illustrated in Figure 6.12. Both pathways are initiated by interactions of oxLDL with CD36 leading to Src kinase activation. Divergence at this point is observed resulting in a Syk/PLC\( \gamma_2 \) dependent increase in intracellular \( \text{Ca}^{2+} \) and MLCK activation. Simultaneously, RhoA activation leads to the activation of Rho kinase and subsequent inhibition of MLCP. Although questions remain as to the involvement of the RhoGEF Vav1, the culminating effects of both pathways are the phosphorylation of MLC\(^{\text{Ser19}}\) and platelet shape change.
Diagrammatic representation of the CD36 signalling pathways stimulated by oxLDL in platelets. Ligation of CD36 with oxLDL results in the Src kinase dependent activation of Syk and PLCγ2 leading to an increase in intracellular Ca\textsuperscript{2+} and MLCK activation. Additionally, RhoA is activated leading to the activation of Rho kinase and inhibition of MLCP. This results in MLC phosphorylation and platelet shape change.

**Figure 6.12:** A proposed model of oxidised low density lipoprotein induced platelet shape change through both tyrosine kinase and Rho kinase mediated pathways
Chapter 7: General discussion

Subjects with heterozygous familial hypercholesterolaemia have an approximate two-fold increase of plasma LDL concentrations and are known to be at risk of premature myocardial infarctions below the age of 30 years. Even more intriguing is the homozygous form of familial hypercholesterolaemia, in which circulating levels of LDL can be six to ten-folds higher than in healthy individuals, with patients suffering from myocardial infarctions in early childhood. Complications observed in homozygous familial hypercholesterolaemia at such a young age, suggest a direct correlation between elevated LDL levels and atherosclerotic events (Goldstein and Brown, 2009).

While the elevated risk of CVD is associated with plasma dyslipidaemia, understandings of the cellular events underpinning CVD continue to evolve. The oxidation of plasma LDL produces a cytotoxic species, which can induce dysregulation of the surrounding cells. These events are proposed to play a major role in atherogenesis. Platelet hyperactivity has been associated with elevated circulating LDL levels, such that are found in hyperlipidaemia, for almost forty years (Carvalho et al., 1974), but again the precise mechanism of how hyperlipidaemia activates platelets remains poorly defined. One factor that has emerged is the potential for oxLDL to act as a platelet activator. This has been confirmed by various in vitro studies demonstrating the platelet activating properties of oxLDL (Ardlie et al., 1989, Naseem et al., 1997, Retzer et al., 2000, Volf et al., 2000, Korparaal et al., 2005, Chen et al., 2008, Nergiz-Unal et al., 2011a). Since oxLDL represents a pathological ligand that could contribute to unwanted platelet aggregation found in disease, it is of great
importance to delineate the mechanisms by which oxLDL can stimulate platelet activation.

The current project has established a number of findings that contribute to the understanding of how oxLDL can activate platelets. OxLDL was found to induce weak platelet aggregation that is entirely dependent on ADP stimulation. This implies that, although oxLDL is not a strong platelet agonist, it is able to induce dense granule secretion, leading to the release of soluble agonists. Since ADP plays a key role in the recruitment of circulating platelets, this could suggest that at sites where platelets are exposed to oxLDL, such as ruptured atherosclerotic plaques, these particles could help increase the potential for thrombotic events. A second major finding of this report is that oxLDL can consistently stimulate the early activation response of platelet shape change, which may contribute to the secretion of dense granule content. The signalling involved in this response was shown to be dependent on two pathways - a tyrosine kinase mediated pathway and a Rho kinase mediated pathway. This report is the first line of evidence to demonstrate that these two signalling pathways can be stimulated by oxLDL. Finally, a principle finding of this work is the ability of the scavenger receptor CD36 to stimulate the intracellular signalling induced by oxLDL. Ligation of this receptor by oxLDL was found to be crucial in initiating the shape change response in platelets and was linked to Src kinase mediated activation of several signalling events. Interestingly, although the expression level of this scavenger receptor is already high under basal conditions, experiments in this project demonstrated that stimulation with platelet agonists generates an even greater
amount of the protein on the cell surface. The role of increased expression of CD36 on platelets is unclear, although based on the findings here it could contribute further to thrombotic complications in the presence of oxLDL.

The results in this project are consistent with previous studies that have found oxLDL to also induce platelet shape change (Retzer et al., 2000, Maschberger et al., 2000). However, this initial work has been extended significantly by the discovery of the prominent role that CD36 appears to play in this response. A previous study examining the role of CD36 as a receptor for oxLDL driving platelet activation, suggested a number of proteins to be involved in the signalling pathway induced by this scavenger receptor. These included both Src kinases and the MAP kinase JNK (Chen et al., 2008). However, this study did not identify the cascade in which these proteins become activated and how exactly they contribute to platelet activation. Similarly, CD36 contributes to oxLDL stimulated signalling in macrophages, which promote atherosclerosis through their phenotypic transformation into foam cells. A group from the same institute also investigated CD36 signalling in response to oxLDL in macrophages. Src kinases and JNK were too established to be involved in CD36 dependent signalling in these cells (Rahaman et al., 2006). These studies support the findings in this report that demonstrate Src kinases to be major contributors in oxLDL/CD36 signalling and the possible contribution of JNK. However, nothing has been shown as to how the activation of Src, or JNK, actually drives platelet activation. The data from the current project begin to dissect this mechanism by demonstrating that the CD36-Src signalling pathway is associated with the phosphorylation of a
number of proteins including Syk, PLCγ2, Vav1, MLC and MLCP. Furthermore, the phosphorylation of these proteins is associated with a functional response that occurs as a consequence.

As mentioned earlier, to definitively tie down the signalling receptors and proteins involved in oxLDL induced platelet activation, it would be desirable to perform future work in mice that are deficient of proteins such as Syk, PLCγ2, RhoA and CD36. However, human oxLDL does not appear to interact with murine platelets in the same way that it does with human platelets, resulting in an inability to observe shape change and MLC<sup>Ser19</sup> phosphorylation in murine platelets in response to oxLDL in this study. A possible line of investigation that could be performed, which has been used by previous studies to counteract this predicament, is the use of apoE deficient mice, which are predisposed to hyperlipidaemia, that are crossed with genetically deficient mice of the protein of interest, for example CD36 and Vav (Podrez et al., 2007, Chen et al., 2011). These studies suggest that the prothrombotic phenotype presented in apoE deficient mice is protected against upon deletion of CD36 and Vav, implicating the involvement of these proteins in oxLDL signalling in platelets. There is one study in the literature that does use human oxLDL with murine platelets (Korporaal et al., 2007). This study found that both nLDL and oxLDL induced that phosphorylation of the MAPK p38 but that oxLDL responses were reduced in CD36 and SRA deficient platelets. The concentration of LDL used in the study was 1mg/ml, which is much greater than that used in this report. The oxidation method used included incubation with FeSO<sub>4</sub> at 20°C for 24-72 hours to produce a moderate to high level of oxidation. This is similar to the
oxidation procedure used in this project as both forms were oxidised using transition metal ions. However, it is problematical to compare the extent of oxidation in the Koporaal study with this project as measurements were made of conjugated dienes as opposed to REM and lipid peroxides. Additionally, actually conjugated diene levels were not reported and only percentage of oxidation was confirmed. Therefore, differing properties of the oxLDL may have varying effects on murine platelets. However, as this study stands alone in the literature in stimulating murine platelets with human oxLDL, this raises the question as to why more researchers have not performed experiments with oxLDL on genetically modified mice.

The physiological implications of platelet activation induced by oxLDL may differ depending on the location in which this pathological ligand can act upon platelets. Atherosclerotic plaques have considerably higher levels of oxLDL compared to healthy intima, suggesting these sites to be prime locations for oxLDL interactions with platelets (Nishi et al., 2002). However, as intact plaques are separated from the blood by a layer of endothelial cells, contact can only occur upon plaque rupture. Therefore, thrombus formation during this late stage of plaque development may be attributed to oxLDL released from the ruptured plaque coming into contact with circulating platelets. However, there is also evidence that oxLDL is elevated in the plasma of CVD patients compared to healthy control subjects, although these levels are much lower than those within plaques (Nishi et al., 2002). High levels of oxLDL are not thought to exist within the circulation due to the presence of antioxidants and its clearance from the blood by the liver (Van Berkel et al., 1991). However, the presence of some oxLDL
in the plasma of CVD patients could suggest that oxLDL may also interact with circulating platelets to some degree, perhaps priming these cells to activation. Therefore, it cannot be categorically confirmed where platelets come into contact with oxLDL, however, it appears most likely that these cells come into contact with oxLDL in CVD, with highest levels being present upon rupture of an atherosclerotic plaque.

OxLDL is a heterogeneous species, which is likely to contain a mixture of components that become oxidised to varying degrees. Mildly oxidised LDL, widely defined as LDL with only phospholipid modification is distinct from highly oxidised LDL, which can consist of modified cholesterol and protein constituents. Mildly oxidised forms of LDL can stimulate events such as platelet shape change and tyrosine phosphorylation of Syk, which is similar to findings in this report (Retzer et al., 2000, Maschberger et al., 2000). A controversial difference between these two studies is that one found the mildly oxLDL not to induce a rise in intracellular Ca\(^{2+}\) (Retzer et al., 2000) whereas the other found this lipoprotein did induce Ca\(^{2+}\) elevations, thought mostly to occur from an influx across the plasma membrane with little mobilisation from intracellular stores (Maschberger et al., 2000). An additional study has shown that nLDL does not provoke a Ca\(^{2+}\) response but that oxidation of LDL from 1-15% produces a species that induces slight Ca\(^{2+}\) mobilisation in platelets in a Ca\(^{2+}\) free buffer and this response increased as the oxidation state became greater (Korporaal et al., 2005). With regard to highly oxidised forms of LDL, this pathological ligand can induce platelet aggregation, with higher oxidation states inducing greater aggregation responses (Naseem et al., 1997, Korporaal et al., 2005). Highly oxLDL can also induce P-selectin expression (Chen et al.,
2008) and can support platelet adhesion, spreading and induce rises in intracellular Ca\textsuperscript{2+} when immobilised (Nergiz-Unal et al., 2011a). Additionally, a study using hypochlorite modified LDL, in which only the protein constituent was modified, found this lipoprotein to induce platelet aggregation and dense granule secretion (Volf et al., 2000). Therefore, there are some similarities and some differences between the platelet activating properties of mildly and highly oxLDL and it may be likely that these different LDL species interact with a number of receptors on platelet surfaces. For example, more highly oxidised forms of LDL can interact with scavenger receptors such as CD36 and SR-A (Chen et al., 2008, Korporaal et al., 2007), whereas mildly oxidised forms may induce their effects through the LPA receptor (Maschberger et al., 2000). Therefore, it is possible to suggest that while distinct receptors may bind the different forms of LDL, common signalling may facilitate physiological responses.

A possible connection between the signalling pathways stimulated by both mildly and highly oxidised LDL could be the presence of an ITAM bearing protein(s). These activation motifs, found on immunoreceptors or proteins associated with immunoreceptors, become phosphorylated by Src kinases and this leads to the activation of the tyrosine kinase Syk. Subsequently, a signalling cascade is initiated leading to the activation of a number of signalling molecules such as SLP-76, LAT, PLC\textgamma2 and Vav (Kasirer-Friede et al., 2007). The sequence of events downstream of immunoreceptors contains striking similarities with the tyrosine kinase pathway discovered to be downstream of CD36 in this report. Although analysis of the sequence of CD36 demonstrates that it does not contain an ITAM, it may be possible
that it is coupled to an ITAM bearing protein. For example, the collagen receptor GPVI is associated with the FcR γ-chain, which contains an ITAM and stimulates downstream activating events (Gibbins et al., 1997). Therefore, like this, CD36 associated Src kinases may phosphorylate an ITAM of a coupled receptor, leading to Syk activation and the downstream signalling cascade observed. ITAM signalling is not limited to platelets and occurs in nearly all hematopoietic cells with similar Src kinase mediated signalling pathways (Kasirer-Friede et al., 2007, Lowell, 2011). As other CD36 signalling events such as macrophage foam cell formation are also linked to Src kinases, other cell types may also require ITAM mediated signalling downstream of CD36. This suggests a new line of investigation to be explored in different cell types. The identification of the ITAM protein involved is a major area of future research.

Considering a larger perspective, the work reported in this project is of extreme importance when contemplating CVDs that are brought about by the occurrence of elevated LDL levels in hyperlipidaemia. Indeed, of great significance is a study that was performed with CD36 deficient mice, in which animals were genetically modified to become predisposed to hyperlipidaemia. The mice that contained elevated cholesterol levels in the presence of CD36 were susceptible to a prothrombotic phenotype whereas animals with high cholesterol levels in the absence of CD36 were protected from this (Podrez et al., 2007). This study highlights the importance of CD36, in an in vivo model, as a contributing receptor to CVD. Therefore, the work in this project further enhances these findings by piecing together the signalling cascade downstream of CD36 following ligation with oxLDL. As genetic deletion of CD36 can
protect against a prothrombotic phenotype in mice, it may be possible that this receptor could be a potentially new drug target. Pharmacological blockade of CD36 in individuals with hyperlipidaemia may therefore be beneficial to prevent thrombotic complications from occurring.

**Future Work**

The work in this project has elucidated two CD36 signalling pathways in platelets stimulated by oxLDL. However, to further advance understandings of how this receptor signals and how this results in platelet activation, additional investigations are required. Therefore, future work could include the following:

- The important role that Src kinases play in CD36 signalling has been highlighted in this report and within the literature (Chen et al., 2008). However, the exact mechanism by which these proteins interact with this scavenger receptor, leading to signalling, is poorly understood. Therefore, investigations could be performed to assess exactly how Src kinases associate with CD36 in platelets. There have been suggestions that associations are direct between Src kinases and the carboxyl terminal of CD36 (Rahaman et al., 2006), whereas others have reported associations to be lipid mediated (Thorne et al., 2006). Therefore, experiments could be performed with GST-linked peptides relating to both the amino and carboxyl termini of CD36 to reveal if Src kinases, and which particular Src kinases, associate with either of these portions. Additionally, GST-linked peptides for each terminal containing slight mutations could also be
used to determine the particular amino acid sequence that is necessary for these associations.

- As suggested above, the tyrosine kinase pathway stimulated by oxLDL corresponds closely with the events that are downstream of ITAM containing receptors. Therefore, investigations could be performed to assess the possible contribution of an ITAM in CD36 signalling. As this scavenger receptor is not known to possess this motif itself, associations may occur with another receptor that does. Two possible candidates for this, which contain ITAMs in platelets, are the FcR γ-chain and the FcγRIIa. Therefore, to begin with, western blotting experiments could be performed to determine whether oxLDL stimulation of platelets results in the tyrosine phosphorylation of either of these receptors. Additionally, immunoprecipitation experiments could also be performed to clarify whether CD36 associates with either of these receptors.

- The findings from this report imply that Ca\(^{2+}\) responses may be stimulated by an oxLDL induced tyrosine kinase signalling pathway. An additional activation response in platelets downstream of Syk/PLC\(\gamma\)2 is the stimulation of PKC. Therefore, to determine whether this protein is also activated by oxLDL, western blotting experiments could be performed to examine the phosphorylation state of PKC substrates subsequent to platelet stimulation with oxLDL. Additionally, PKC inhibitors could also be employed to analyse the
effects of this enzyme on downstream activation markers such as $\text{MLC}^{\text{Ser19}}$ phosphorylation.

Conclusions

The experiments in this project have demonstrated that stimulation of platelets with the pathological ligand oxLDL can lead to the functional responses of shape change and dense granule secretion. In particular reference to platelet shape change, the signalling cascade that leads to this response requires both tyrosine kinase and Rho kinase pathways, mediated through the scavenger receptor CD36. Enhancing our knowledge of how this scavenger receptor signals in platelets upon stimulation with oxLDL will increase our understanding of how these cells become unnecessarily activated in CVD. This will hopefully lead to the development of new drug targets in order to prevent thrombotic events in diseases such as hyperlipidaemia.
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Chapter 8


Oxidized low-density lipoproteins induce rapid platelet activation and shape change through tyrosine kinase and Rho kinase –signaling pathways

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PLATELETS AND THROMBOPOIESIS

Oxidized low-density lipoproteins induce rapid platelet activation and shape change through tyrosine kinase and Rho kinase–signaling pathways

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Key Points

- Oxidized LDL stimulates rapid change in platelet shape through ligation of CD36.
- Ligation of CD36 by oxidized LDL simultaneously activates tyrosine and Rho kinase–dependent signaling pathways.

Oxidized low-density lipoproteins (oxLDL) generated in the hyperlipidemic state may contribute to unregulated platelet activation during thrombosis. Although the ability of oxLDL to activate platelets is established, the underlying signaling mechanisms remain obscure. We show that oxLDL stimulate platelet activation through phosphorylation of the regulatory light chains of the contractile protein myosin IIa (MLC). oxLDL, but not native LDL, induced shape change, spreading, and phosphorylation of MLC (serine 19) through a pathway that was ablated under conditions that blocked CD36 ligation or inhibited Src kinases, suggesting a tyrosine kinase–dependent mechanism. Consistent with this, oxLDL induced tyrosine phosphorylation of a number of proteins including Syk and phospholipase Cγ2. Inhibition of Syk, Ca2+ mobilization, and MLC kinase (MLCK) only partially inhibited MLC phosphorylation, suggesting the presence of a second pathway. oxLDL activated RhoA and RhoA kinase (ROCK) to induce inhibitory phosphorylation of MLC phosphatase (MLCP). Moreover, inhibition of Src kinases prevented the activation of RhoA and ROCK, indicating that oxLDL regulates contractile signaling through a tyrosine kinase–dependent pathway that induces MLC phosphorylation through the dual activation of MLCK and inhibition of MLCP. These data reveal new signaling events downstream of CD36 that are critical in promoting platelet aggregation by oxLDL. (Blood. 2013;122(4):580-589)

Introduction

Blood platelets play an important role in atherothrombosis, the pathologic condition that underpins myocardial infarction and stroke. Hyperlipidemia is a key risk factor for atherothrombosis and is associated with a number of cellular changes including activation of endothelial cells, proliferation of vascular smooth muscle cells, formation of lipid-laden foam cells, and platelet hyperactivity.1,2 The generation of oxidized low-density lipoproteins (oxLDL), a heterogeneous group of modified particles, is intimately linked to hyperlipidemia and is proposed to induce many of the cellular changes observed in atherogenesis. Numerous studies have demonstrated that oxLDL activates blood platelets and potentiates the effect of physiological agonists.3,5 The mechanisms, including receptors and signaling enzymes, underlying these activatory effects are still unclear. To date a number of platelet receptors including integrin αIIbβ3,6 platelet-activating factor receptor,7 scavenger receptor A (SR-A),8 and the class B scavenger receptor CD369 have been identified. Binding of oxLDL to CD36 results in platelet activation through Src kinase and mitogen-activated protein kinase–dependent pathways,8 although signaling through extracellular signal–regulated kinase and focal adhesion-associated kinase may also play a role.8,10 Importantly, CD36 is key to both platelet hyperactivity and accelerated thrombosis in murine models of hyperlipidemia, effects that are mediated via oxidized lipids associated with oxLDL.11 Thus, CD36 plays a prominent role in platelet activation in disease, although the signaling mechanism triggering platelet activity through CD36 remains elusive.

Platelet shape change is the earliest physiological response after activation and is driven by a dynamic remodeling of the actin cytoskeleton. The phosphorylation of regulatory myosin light chains (MLC) on serine 19 of myosin IIA instigates ATPase activity that facilitates myosin interaction with actin filaments.12 This actin–myosin interaction triggers contraction of the actin cytoskeleton required for shape change and granule secretion.13 The phosphorylation state of MLC is determined by the activities of 2 critical regulatory enzymes: MLC kinase (MLCK) and MLC phosphatase (MLCP). MLCK phosphorylates myosin at serine 19 (Ser19), whereas MLCP dephosphorylates this same residue. Platelet agonists stimulate coordinated signaling events that lead to simultaneous activation of MLCK and inhibition of MLCP,14,15 which collectively promote maximal MLC phosphorylation. In human and murine platelets, elevated intracellular Ca2+ in response to numerous platelet agonists activates MLCK through a calmodulin-dependent mechanism,16,17 resulting in the phosphorylation of MLC, shape change, and secretion.16–20 Signaling downstream of G-protein–coupled receptors inhibits MLCP through RhoA-activated Rho kinase (ROCK),
which phosphorylates and inhibits MLCP.14 Here we describe novel signaling events where ligation of CD36 by oxLDL triggers 2 Src kinase–dependent pathways, a Src/Syk-dependent and Src/ROCK–dependent pathway, which stimulate platelet shape change through activation of MLCK and inhibition of MLCP, respectively.

Materials and methods

Materials

These studies were approved by the Hull York Medical School Ethics Committee and were conducted in accordance with the Declaration of Helsinki. c-Jun N-terminal kinase (JNK) inhibitor 1, pyrolopyrimidine 2 and 3 (PP2, PP3), Y27632, 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid-acetoxymethyl ester (BAPTA-AM), and ML-7 were obtained from Calbiochem (Nottingham, United Kingdom). R406 was obtained from Selleckchem (Suffolk, United Kingdom). Sulfosuccinimidy l oleate (SSO), antibodies to CD36 (FA6.152), Syk, and phospholipase C γ2 (PLCγ2) were obtained from Santa Cruz (Wembley, United Kingdom). Phospho-MLC Ser19 (Biosource) antibody, phospho-MYPT (myosin phosphatase targeting subunit) 1 (Thr553) antibody, and phospho-Src-tyr416 antibodies came from Cell Signaling (Hitchen, United Kingdom). Antibodies to β-tubulin, phosphotyrosine (4G10), and IgG control came from Upstate (Watford, United Kingdom). Collagen was obtained from Axis Shield (Dundee, United Kingdom). The RhoA pull-down assay was obtained from Cytoskeleton (Cambridge, United Kingdom). All other chemicals came from Sigma (Poole, United Kingdom).

Platelet preparation

Human blood was taken from drug-free volunteers by clean venepuncture using acid citrate dextrose (29.9 mM of sodium citrate, 113.8 mM of glucose, 72.6 mM of sodium chloride, and 2.9 mM of citric acid (pH 6.4) as anticoagulant. Platelet-rich plasma was obtained by centrifugation of whole blood at 200 g at 20°C for 20 minutes. Platelet-rich plasma was treated with citric acid (0.3 mM) and indomethacin (10 μM) and was centrifuged at 800 g for 12 minutes. The platelet pellet was then suspended in wash buffer (36 mM citric acid (0.3 mM) and indomethacin (10 μM)) for 20 minutes before addition of oxLDL. For immunoprecipitated proteins, washed platelets (7×10^6 platelets/mL) were lysed with ice-cold lysis buffer.26 Syk or PLCγ2 were then precipitated as described previously.26 Proteins were separated by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and were transferred to polyvinylidene difluoride membranes. Membranes were blocked for 60 minutes with 10% bovine serum albumin (or 5% milk for MLCP experiments), dissolved in Tris-buffered-saline-Tween (0.1%), then incubated for 1 hour with anti-phospho-MLC Ser19, anti-phospho-Src-tyr416, anti-phospho-MYPT-1 (Thr553), anti-MLCP (Biosource) antibody, and phospho-Src-tyr416 antibodies came from Cell Signaling (Hitchen, United Kingdom). Antibodies to β-tubulin, phosphotyrosine (4G10), and IgG control came from Upstate (Watford, United Kingdom). Collagen was obtained from Axis Shield (Dundee, United Kingdom). The RhoA pull-down assay was obtained from Cytoskeleton (Cambridge, United Kingdom). All other chemicals came from Sigma (Poole, United Kingdom).

Platelet aggregation and shape change

Washed platelets (2.5×10^9 platelets/mL) were treated with oxLDL or native LDL (nLDL; 10-200 μg/mL), and aggregation was recorded under constant stirring conditions (1000 rpm) for 4 minutes using a Chronolog aggregation module–dual channel light aggregometer. To monitor shape change, platelets were preincubated with apyrase (2 U/mL), indomethacin (10 μM), and EGTA (1 mM), before addition of LDL.

Platelet-spreading analysis by microscopic analysis

Glass microscope slides were coated with nLDL or oxLDL (50 μg/mL) for 12 hours at 4°C. Washed platelets (5×10^7) were adhered for 30 minutes at 37°C in the presence of troglitazone (2 μM).21 In some cases, platelets were incubated with the CD36-blocking agent SSO (75 μM), FA6.152 (1 μg/mL), IgG (1 μg/mL), or PP2 or PP3 (20 μM) for 20 minutes at 37°C before adhesion. Adherent platelets were stained for 1 hour with isothiocyanate-phalloidin and were viewed with an IX71 fluorescence microscope using a XM10 CCD camera (Olympus, Japan). Images were captured with ×60 magnification and were analyzed with IMAGEJ software (National Institutes of Health, Bethesda, MD).

LDL preparation and oxidation

LDL was prepared from fresh human plasma by sequential density ultracentrifugation,22 and protein concentration was determined by a modified Lowry assay.23 LDL was oxidized in the presence of CuSO4 (10 μM) at 37°C for 24 hours, before being dialyzed against phosphate buffer (140 mM of NaCl, 8.1 mM of Na2HPO4, 1.9 mM of NaH2PO4, pH 7.4) containing EDTA (100 μM) extensively to remove copper ions.24 The extent of oxidation was determined by measurement of lipid hydroperoxides (LPOs) and relative electrophoretic mobility on agarose gels. For measurement of LPOs, LDLs (100 μg/mL) were mixed with color reagent (163 mM of K2HPO4, 37 mM of K2HPO4, 120 mM of KI, 2g/L of Triton X-100, 0.15 mM of sodium azide, 0.1g/L of alkylbenzylidimethylammonium chloride, and 0.01 mM of (NH4)2MoO4), incubated in the dark for 60 minutes and absorbance read at 365 nm. LPO levels of nLDL were 9.7 ± 2.2 nmol/L/mg protein and of oxLDL were 74.0 ± 11.5 nmol/L/mg protein (P < .05 compared with nLDL).25 Samples of agarose gels (1%) were loaded with 20 μg of both nLDL and oxLDL and were run at 100V for 60 minutes. Gels were then stained with Commassie blue stain, and relative electrophoretic mobility was calculated for nLDL (1) and oxLDL (3.9 ± 0.2; P < .05 compared with nLDL).

Immunoprecipitation and immunoblotting

For signaling studies, suspended platelets (3-5×10^9 platelets/mL) were incubated with apyrase (2 U/mL), indomethacin (10 μM), and EGTA (1 mM) to prevent secondary signaling events. Platelets were then treated with nLDL or oxLDL (0-200 μg/mL; for 0.25-5 minutes) before termination with Laemmli buffer. In some cases, platelets were incubated with SSO (50 μM), CD36-blocking antibody FA6.152 (1 μg/mL) or IgG control for 15 minutes, or ML-7 (5 μM), BAPTA-AM (20 μM), Y27632 (10 μM), PP2 (20 μM), PP3 (20 μM), R406 (1 μM), fucoidan (5 μg/mL) or JNK inhibitor 1 (10 μM) for 20 minutes before addition of oxLDL. For immunoprecipitated proteins, washed platelets (7×10^6 platelets/mL) were stimulated with nLDL or oxLDL for 15 seconds in the presence or absence of the stated inhibitors. Platelets were lysed with ice-cold lysis buffer.26 Syk or PLCγ2 were then precipitated as described previously.26 Proteins were separated by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and were transferred to polyvinylidene difluoride membranes. Membranes were blocked for 60 minutes with 10% bovine serum albumin (or 5% milk for MLCP experiments), dissolved in Tris-buffered-saline-Tween (0.1%), then incubated with anti-phospho-MLC Ser19 (1:1000), anti-phospho-Src-tyr416 (1:1000), anti-phosphoMLC-Thr19 (1:250), anti-phospho-tyr (4G10, 1:1000), anti-Syk (1:1000), anti-PLCγ2 (1:1000), or an anti β-tubulin antibody (1:1000). Immunoblots were processed as described previously.27

RhoA activity assay

Activity of RhoA in platelets was assessed using a guanosine triphosphate (GTP)-RhoA pull-down assay. Briefly, washed platelets (5×10^7 platelets/mL) were incubated with apyrase (2 U/mL), indomethacin (10 μM), and EGTA (1 mM) before treatment with nLDL or oxLDL (50 μg/mL) for 15 seconds in the presence or absence of PP2 or PP3 (20 μM). Platelets were lysed with ice-cold lysis buffer and immediately snap frozen in liquid nitrogen. Lysates were thawed and incubated with rhoetkin-RBD beads to pull down activated RhoA (GTP-bound). Proteins were separated by SDS-PAGE and were transferred to polyvinylidene difluoride membranes as described above.

Statistical analysis

Results are expressed as means ± SEM and were analyzed using the Student t test. The results were considered significant when P values were < .05.

Results

OxLDL induce platelet shape change and phosphorylation of MLC

OxLDL, but not nLDL, induced a small degree of aggregation in washed human platelets (20 ± 3% at 50 μg/mL) (Figure 1A). OxLDL-induced aggregation was variable among platelet donors, ranging from 8% to 29% (oxLDL – 50 μg/mL) but consistently

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Figure 1. OxLDL induces platelet aggregation, platelet shape change and MLC phosphorylation. (A) Platelets (2.5 \times 10^8/mL) were stimulated with nLDL (50 μg/mL), oxLDL (50 μg/mL), or collagen (10 μg/mL) for 4 minutes under stirring conditions. Shown are representative aggregation traces of 3 separate experiments. (B) Samples were processed as in (A), except that platelets were preincubated with apyrase (2 U/mL), indomethacin (10 μM), and EGTA (1 mM) followed by stimulation with either oxLDL or nLDL (0-200 μg/mL). Traces were recorded for 2 minutes. Shown are representative traces of 3 separate experiments. (C) Platelets (3 \times 10^8/mL) were stimulated with varying concentrations of oxLDL (10-200 μg/mL) or thrombin (0.05 U/mL) for 15 seconds followed by lysis, separation by SDS-PAGE, immunoblot for phospho-MLC Ser19, and reprobing for β-tubulin. (Ci) Representative blots. (Cii) Densitometric analysis of 3 independent experiments. (D) Platelets were incubated with 50 μg/mL of oxLDL for various time points (15-300 seconds) or thrombin (0.05 U/mL) for 15 seconds before lysis. Samples were then processed as in (C). (Di) Representative blots. (Dii) Densitometric analysis of 3 independent experiments. (E) Samples were processed as in (C), except that platelets were stimulated with nLDL. (Ei) Samples were processed as in (D), except that platelets were stimulated with nLDL. All immunoblots are representatives of 3 separate experiments and were carried out in the presence of apyrase (2 U/mL), indomethacin (10 μM), and EGTA (1 mM). Data are expressed as mean ± SEM.
caused shape change. The ability of oxLDL to induce shape change was maintained under conditions that inhibited the effects of ADP, TxA2, and integrin signaling, confirming that the effect was independent of other agonists (Figure 1B).

A critical event driving platelet shape change is phosphorylation of MLC\textsuperscript{Ser19}.\textsuperscript{28-30} Under the same conditions that prevented secondary signaling, oxLDL (10-200 \textmu g/mL) induced a time- and concentration-dependent increase in phospho-MLC\textsuperscript{Ser19}. Increased phosphorylation of MLC was evident at concentrations as low as 10 \textmu g/mL, with maximal effects observed with 100 \textmu g/mL (Figure 1C). The phosphorylation was rapid with 50 \textmu g/mL, inducing maximal effects at 15 seconds before declining back to near-basal levels after 5 minutes (longest time tested) (Figure 1D). In contrast, nLDL failed to stimulate MLC phosphorylation at any of the times or concentrations tested (Figure 1E). Because of the rapid onset of platelet shape change, we used standard conditions of oxLDL (50 \textmu g/mL) for 15 seconds to evaluate the effects of oxLDL on platelet signaling in further experiments.

Platelet shape change and MLC phosphorylation induced by oxLDL require CD36 and occur through both Ca\^{2+}-dependent and Rho kinase–dependent pathways

MLC phosphorylation is regulated by a Ca\^{2+}–pathway that stimulates MLCK and a Rho kinase (ROCK)–dependent pathway that phosphorolyses and inhibits MLCP.\textsuperscript{28} To further dissect the pathway initiated by oxLDL leading to cytoskeletal reorganization, we evaluated these 2 pathways using ML7 to inhibit MLCK, BAPTA-AM (20 \textmu M) to chelate intracellular Ca\^{2+}, and the ROCK inhibitor Y27632 (10 \textmu M). ML-7 (5 \textmu M) significantly reduced, but did not abolish, phospho-MLC\textsuperscript{Ser19} induced by oxLDL (Figure 2A), suggesting that Ca\^{2+}-dependent MLCK was responsible for phosphorylation of MLC, but that other pathways could be involved. When BAPTA-AM–treated platelets were stimulated with oxLDL (50 \textmu g/mL), it led to a significant, but incomplete, inhibition of MLC phosphorylation (\textit{P} < .05) (Figure 2B). Similarly, incubation of platelets with Y27632 before stimulation with oxLDL also significantly reduced MLC phosphorylation (\textit{P} < .01) without abolishing the response. However, a combination of these inhibitors ablated phospho-MLC (\textit{P} = .0005) (Figure 2B), suggesting that both Ca\^{2+}-dependent and ROCK-dependent pathways were important for oxLDL-induced MLC phosphorylation.

CD36 is highly expressed on platelets\textsuperscript{31} and has emerged as a potentially key receptor for oxLDL on platelets.\textsuperscript{3} We explored the possibility that oxLDL-induced shape change and associated signaling responses lay downstream of CD36. This experiment is necessary because oxLDL is proposed to bind to multiple receptors on the platelet surface.\textsuperscript{6-8} The expression of CD36 on platelets was confirmed by immunoblotting and flow cytometry using specific antibodies (not shown). The role of CD36 was evaluated using the CD36-blocking antibody FA6.152 (1 \textmu g/mL) and the CD36 inhibitor SSO (50 \textmu M).\textsuperscript{32} Platelet shape change induced by oxLDL (50 \textmu g/mL) in the absence of secondary signaling was abolished by FA6.152 but was unaffected by a control IgG (Figure 3A). Neither of these antibodies influenced shape change initiated by thrombin, which stimulates platelets through G\textsubscript{q} and G\textsubscript{12/13}. Similarly, SSO completely blocked oxLDL-induced shape change but had no effect on that induced by thrombin (Figure 3B).

To further explore the role of CD36 in shape change, we found that adhering platelets to immobilized oxLDL (50 \textmu g/mL), but not nLDL, resulted in spreading. Importantly, blocking of CD36 with either SSO or FA6.152 ablated adhesion and spreading (Figure 3C). Consistent with a role for CD36 in oxLDL-induced shape change and spreading, blocking CD36 with FA6.152 or SSO prevented oxLDL-induced phosphorylation of MLC (\textit{P} = .02) in platelets (Figure 3D). In contrast, fucoidan, which has been used as an inhibitor of SR-A,\textsuperscript{8} had only minor effects that were not significant (Figure 3D).

A tyrosine kinase–signaling pathway mediates phosphorylation of MLC in response to oxLDL

In platelets, Src-family kinases are constitutively associated with CD36\textsuperscript{33} and lie upstream of signaling pathways diverging from the
Figure 3. OxLDL signals through CD36 to induce platelet shape change. (A) Platelets (2.5 × 10^6/mL) were treated with FA6.152 (1 μg/mL) or control IgG (1 μg/mL) for 15 minutes in the presence of apyrase (2 U/mL), indomethacin (10 μM), and EGTA (1 mM) followed by stimulation with oxLDL (50 μg/mL) or thrombin (0.05 U/mL). Traces were recorded for 2 minutes. Shown are representative traces of 7 independent experiments. (B) Samples were processed as in (A), except that platelets were treated with SSO (50 μg/mL) or control IgG (1 μg/mL), or control IgG (1 μg/mL), or control IgG (1 μg/mL), for 30 minutes and were viewed by fluorescence microscopy. Representative images of 3 independent experiments were taken under 60× magnification. Bar = 20 μm. (C) Platelets (3 × 10^6/mL) were treated with FA6.152 (1 μg/mL) or control IgG (1 μg/mL), SSO (50 μg/mL), or fucoidan (5 μg/mL) for 15 minutes in the presence of apyrase (2 U/mL), indomethacin (10 μM), and EGTA (1 mM) followed by stimulation with oxLDL (50 μg/mL) for 15 seconds. Platelets were lysed and separated by SDS-PAGE and immunoblotted for phospho-MLC (Ser19) followed by re-probing for β-tubulin. (Di) Representative blots. (Di) Densitometric analysis of 3 independent experiments. Data are presented as mean ± SEM. *P < .05.

OXLDL stimulates a RhoA/ROCK pathway to regulate MLCP

Because MLC phosphorylation in response to oxLDL was prevented in part by the ROCK inhibitor Y27632, we examined the potential regulation of MLCP via RhoA/ROCK. To confirm that oxLDL activated the ROCK-signaling pathways, we examined its upstream activator RhoA. Using a RhoA-GTP pull-down assay, we demonstrate that oxLDL (50 μg/mL) induces activation of RhoA. In contrast, nLDL had no effect on the GTP loading of RhoA (Figure 6A). This finding is consistent with early studies indicating that oxLDL activates ROCK, although the mechanisms remained unclear.33 The proposed downstream target of RhoA/ROCK is MLCP, which is dually phosphorylated at Thr-696/Thr-853, with resultant inhibition of phosphatase activity.14,36 OxLDL, but not nLDL,
induced inhibitory phosphorylation of thr853 (Figure 6B) and thr696 (not shown). oxLDL-mediated phosphorylation was blocked by Y27632 but was unaffected by chelation of intracellular Ca2+ by BAPTA-AM (Figure 6C). Moreover, oxLDL-stimulated phospho-MYPT1thr853 was ablated by FA6.152 and SSO but not by fucoidan. Together, these data suggest that oxLDL ligation of the scavenger receptor activates ROCK signaling to phosphorylate MYPT1 (Figure 6D). We used the Src kinase inhibitor to explore whether the CD36-Src kinase pathway was responsible for the activation of RhoA. oxLDL induced robust activation of RhoA, which was abolished by PP2 (P = .02) but not by PP3 (Figure 6E).

Interestingly, the inhibition of Syk had no effect on the activation of RhoA induced by oxLDL (not shown). Thus, oxLDL stimulates a CD36-Src kinase–dependent activation of RhoA, leading to ROCK activation with resultant phosphorylation and inhibition of MLCP (Figure 6E). Finally, we confirmed that both Src/Syk and ROCK pathways were required for maximal phosphorylation of MLC. Treatment of platelets with the Syk inhibitor R406 and Y27362 alone attenuated, but did not fully block, MLC phosphorylation (Figure 6F). However, when R406 was used in combination with Y27362, the ability of oxLDL to drive the phosphorylation of MLC was abolished (Figure 6F).

Figure 4. OxLDL induces the activation of Src kinases, which leads to platelet shape change and the phosphorylation of MLC. (A) Platelets (2.5 × 10⁸/mL) were treated with PP2 (20 μM) or PP3 (20 μM) for 20 minutes, followed by stimulation with oxLDL (50 μg/mL). Traces were recorded for 2 minutes. Shown are representative traces of 6 independent experiments. (B) Platelets (5 × 10⁷/mL) were adhered to nLDL or oxLDL (50 μg/mL) slides in the presence or absence of PP2 or PP3 (20 μM) for 30 minutes and were viewed by fluorescence microscopy. Representative images of 3 independent experiments were taken under ×60 magnification. Bar = 20 μm. (C) Platelets (5 × 10⁷/mL) were stimulated with either oxLDL (50 μg/mL) or nLDL (50 μg/mL) for 15 seconds. Samples were then lysed, separated by SDS-PAGE, and immunoblotted for phospho-SrcY416 followed by reprobing for β-tubulin. (C)i Representative blots. (C)ii Densitometric analysis of 4 independent experiments. *P < .05. (D) Platelets (3 × 10⁶/mL were treated with either PP2 (20 μM) or PP3 (20 μM) for 20 minutes, followed by stimulation with oxLDL (50 μg/mL) for 15 seconds. Samples were then processed as in (C) and were immunoblotted for phospho-MLCSer199 followed by reprobing for β-tubulin. (Di) Representative blots. (D)ii Densitometric analysis of 3 independent experiments. **P < .05. (E) Platelets (5 × 10⁷/mL) were incubated with 50 μg/mL of oxLDL for various time points (15-300 seconds) before lysis. Representative blots of 4 independent experiments are shown. (E)i Platelets (5 × 10⁷/mL) were treated with either PP2 (20 μM) or PP3 (20 μM) for 20 minutes, followed by stimulation with oxLDL (50 μg/mL) for 15 seconds. Samples were then processed as in (B) and were immunoblotted for phosphotyrosine, followed by reprobing for β-tubulin. All experiments were carried out in the presence of apyrase (2 U/mL), indomethacin (10 μM), and EGTA (1 mM), and are representative of 3 independent experiments.
Discussion

Modified lipoproteins such as oxLDL act as potential pathological ligands that drive platelet activation in disease states. In our present study, we describe new signaling pathways by which oxLDL activates blood platelets. Our data demonstrate for the first time that oxLDL simultaneously activates 2 pathways that lead to the phosphorylation of MLC on Ser19 required for platelet shape change. The ligation of CD36 by oxLDL leads to the sequential activation of Src kinases and Syk, initiating a signaling cascade that leads to tyrosine phosphorylation and activation of PLCγ2. OxLDL-induced signaling events requiring CD36-Src also activates a RhoA/ROCK-dependent pathway that leads to the phosphorylation and inhibition of MLCP. We show that both of these pathways are required for maximal phosphorylation of MLC and platelet activation in response to oxLDL (Figure 7).

Focusing on human platelets, our data demonstrate that oxLDL, but not nLDL, induces shape change under conditions that prevent secondary signaling, indicating that the morphologic changes were in direct response to the modified lipoproteins. Consistent with numerous physiological agonists such as thrombin, collagen, and TxA₂, oxLDL is able to induce rapid and concentration-dependent phosphorylation of MLC. The ability of oxLDL to induce shape change, spreading, and MLCP phosphorylation was abolished in the presence of 2 distinct CD36-blocking agents but not a putative blocker of SR-A. This observation is important because oxLDL can signal through both SR-A and CD36. Recently, several laboratory studies have suggested that CD36 may be a key receptor for oxLDL that drives both platelet activation and thrombosis, and in that regard, our data place a central importance of CD36 in the early stages of platelet activation by oxLDL. It is suggested that Src kinases play a key role in transducing signals on ligation of CD36, although the downstream targets remain to be fully established.

Our data provide new insights into the potential roles of CD36-associated Src kinases, which seem to play a critical role in facilitating platelet shape change. Consistent with other studies, we found that oxLDL induced phosphorylation of Src family kinases, which was prevented by blocking CD36. Work by Chen et al suggest that Fyn and Lyn are potential members of the family, which is activated by oxLDL. However, phosphorylation of Fyn and Lyn takes up to 15 minutes, but we observed Src family kinase phosphorylation within 15 seconds of exposure. The reason for this finding is unclear, but multiple Src kinases are associated with CD36 in platelets including Fyn, Lyn, and Yes, and it is possible that the kinetics for phosphorylation of these family members are dependent on the conditions. Regardless of which member of the family is responsible, it is clear that platelet shape change, spreading, and MLCP phosphorylation seemed to be dependent on Src kinases, suggesting a key role for a tyrosine kinase–dependent signaling pathway. In endothelial cells, Syk has been shown to be associated with CD36, consistent with that observation, oxLDL induced rapid tyrosine phosphorylation of a number of platelet proteins, including Syk and PLCγ2. These events resemble those related to signaling events downstream of GPIb-IX-V and integrin αIIbβ3, which also lead to activation of Syk. It is possible that activation of Syk and PLCγ2 may drive the Ca²⁺ mobilization required for platelet activation and spreading. Indeed, Nergiz-Unal et al recently demonstrated that immobilized oxLDL can stimulate CD36-dependent mobilization of Ca²⁺. Our work confirms the importance of CD36 and Src but extends these findings significantly to demonstrate that the shape change and spreading are rapid (not requiring extensive interactions with immobilized ligands) and are mediated by phosphorylation of MLCP. Unfortunately, we found that our oxLDL did not induce tyrosine phosphorylation in murine platelets (K.S.W. and K.M.N., unpublished data), suggesting that oxLDL may signal differently in human and murine platelets, but also precluding the use of Syk and PLCγ2–deficient murine platelets. However, the central role of Syk is supported by data demonstrating that inhibition of the kinase by R406 reduces phosphorylation of MLCP and abolishes platelet spreading induced by oxLDL. Interestingly, inhibition of PLCγ2 using the nonspecific inhibitor U73122 also reduced MLCP phosphorylation in response to oxLDL (K.S.W. and K.M.N., unpublished data). This finding could suggest that oxLDL may increase intracellular Ca²⁺ through activation of PLCγ2, which is required for the activation of MLCK and is consistent with the ability of ML-7, the MLCK inhibitor, to reduce phosphorylation of MLCP in response to oxLDL (Figure 7).
A second critical finding of our current study is that, for the first time, we identify RhoA/ROCK as a target of CD36 signaling. The induction of shape change by physiological agonists involves at least 2 pathways: a Ca2+-dependent and a RhoA/ROCK–dependent pathway, with agonists activating platelets via Gq/G12/13 activating both pathways.28 To the best of our knowledge, oxLDL do not signal through Gq/G12/13–coupled receptors. Therefore, our data suggest a novel CD36-signaling target, which may have implications in other cell types. Certainly, the recent observation that RhoA is associated with CD36 in HUVECs supports findings that RhoA activation may occur downstream of CD36 in platelets.38 Using a number of pharmacologic inhibitors to isolate tyrosine kinase, Ca2+, and ROCK pathways, we demonstrate that oxLDL-stimulated MLC phosphorylation was partially independent of Ca2+ because it was still evident

Figure 6. OxLDL, but not nLDL, induces the activation of RhoA and the inhibitory phosphorylation of MLCP. (A) Platelets (5 × 10⁸/mL) were stimulated with oxLDL (50 µg/mL), nLDL (50 µg/mL), or thrombin (0.05 U/mL) for 15 seconds followed by lysis. Samples with then subjected to a RhoA activation assay, separated by SDS-PAGE and immunoblotted for RhoA. (Ai) Representative blots. (Aii) Densitometric analysis of 4 independent experiments. *P < .05. (B) Platelets (5 × 10⁸/mL) were stimulated with oxLDL (50 µg/mL), nLDL (50 µg/mL), or thrombin (0.05 U/mL) for 15 seconds followed by lysis, separation by SDS-PAGE, immunoblot for phospho-MYPT1Thr853, and reblotting for β-tubulin. (Bi) Representative blots. (Bii) Densitometric analysis of 4 independent experiments. *P < .05. (C) Platelets (5 × 10⁸/mL) were treated with Y27632 (10 µM) or BAPTA-AM (20 µM) for 20 minutes, followed by stimulation with oxLDL (50 µg/mL) for 15 seconds. Samples were then processed as in (B). (Ci) Representative blots. (Cii) Densitometric analysis of 4 independent experiments. *P < .05. (D) Platelets (5 × 10⁸/mL) were treated with FA6.152 (1 µg/mL), SSO (50 µM), or fucoidan (5 µg/mL) for 20 minutes followed by stimulation with oxLDL (50 µg/mL) for 15 seconds and were processed as in (B). (Di) Representative blots. (Dii) Densitometric analysis of 3 independent experiments. *P < .05. (E) Platelets (5 × 10⁸/mL) were treated with either PP2 (20 µM) or PP3 (20 µM) for 20 minutes, followed by stimulation with oxLDL (50 µg/mL) for 15 seconds. Samples were then subjected to a RhoA activation assay and were processed as in (A). (Ei) Representative blots. (Eii) Densitometric analysis of 3 independent experiments. *P < .05. (F) Platelets (3 × 10⁸/mL) were preincubated with Y27632 (10 µM), R406 (1 µM), or a combination of Y27632 and R406 for 20 minutes followed by stimulation with oxLDL (50 µg/mL) for 15 seconds and lysis. Samples were then separated by SDS-PAGE and were immunoblotted for phospho-MLCSer19, followed by reprobing for β-tubulin. (Fi) Representative blots. (Fii) Densitometric analysis of 3 independent experiments. *P < .05. Data are presented as mean ± SEM. Experiments were carried out in the presence of apyrase (2 U/mL), indomethacin (10 µM), and EGTA (1 mM).
under conditions that prevented mobilization of intracellular Ca^{2+} and signaling through Syk. The remaining MLC phosphorylation under these conditions was prevented by the Rho kinase inhibitor Y27632, suggesting that oxLDL activated dual pathways to drive MLC phosphorylation.

We present 4 pieces of evidence to support activation of a RhoA/ROCK pathway. First, treatment of platelets with oxLDL led to the conversion of RhoA to its GTP-loaded form. Second, the activation of RhoA by oxLDL was prevented by blocking the activity of Src kinases, which lie downstream of CD36. Third, oxLDL induces inhibitory phosphorylation of MLCP-thr696/853 on the MYPT subunit. Fourth, the inhibitory phosphorylation by oxLDL was lost in the absence of both CD36 and ROCK activity, suggesting that oxLDL activated dual pathways to drive MLC phosphorylation (Figures 6 and 7). Importantly, our data demonstrate that beyond ligation of CD36 and activation of Src kinases, multiple signaling pathways emerge that lead to the activation of RhoA (data not shown). The activation of JNK-1 is also linked to the CD36/Src kinase-signaling hub but, again, is divergent from the Syk/PLCγ2 and ROCK pathways because it takes up to 15 minutes to become activated compared with the rapid activation of Syk and RhoA. It is clear that future studies are required to link the multiple signaling events downstream of CD36 to specific aspects of platelet function to enhance our understanding of platelet activation induced by oxLDL and hyperlipidemia.

In conclusion, we have described for the first time that oxLDL induce coordinated targeting of the phosphorylation status of MLC. Our data demonstrate that beyond ligation of CD36 and activation of Src kinases, multiple signaling pathways emerge that lead to the activation of MLCK and inhibition of MLCP. These data further our understanding of the potential mechanism by which oxLDL activate platelets contributing to the unwanted and unregulated platelet activation that occurs in hyperlipidemia and atherothrombosis.

**Authorship**

Contribution: K.S.W. performed experiments and analyzed and interpreted the data; S.M. and A.A. performed experiments and analyzed the data; Y.W. performed experiments; D.L. provided essential material and contributed to the writing of the manuscript; and K.M.N. designed research, analyzed and interpreted the data, and wrote the manuscript.

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