universität wien

DISSERTATION

Titel der Dissertation

Platelet function under systemic oxidative stress

The role of native and modified lipoproteins

angestrebter akademischer Grad

Doktorin der Naturwissenschaften (Dr. rer.nat.)

Verfasserin: Alice Assinger
Matrikel-Nummer: 0008660
Dissertationsgebiet (lt. Studienblatt): Ernährungswissenschaften
Betreuer: o.Univ.-Prof. Dr. Ibrahim Elmadfa

Wien, am 11. November 2008

Preface

This thesis is based upon studies conducted during April 2006 to September 2008 at the Institute of Physiology (Centre of Physiology and Pathophysiology) at the Medical University of Vienna.

Alice Assinger,   Oktober 2008

Contents

Abbreviations:

ADP: adenosine diphosphate

Apo A: apolipoprotein A

Apo B: apolipoprotein B

Apo E: apolipoprotein E

ASA: acetylsalicylic acid

CD: cluster of differentiation

CD40L: CD40 ligand

Cox: cyclooxygenase

CuOxHDL: copper oxidized high density proteins

CuOxLDL: copper oxidized low density proteins

GFP: gel filtered platelets

EDTA: ethylene diamine tetraacetic acid

eNOS: endothelial nitric oxide synthase

FACS: fluorescence-activated cell sorting

FITC: fluorescein-5-isothiocyanat

HDL: high density lipoproteins

HOCl: hypochlorous acid

HSA: human serum albumin

hypOxHDL: hypochlorite oxidized high density lipoproteins

hypOxLDL: hypochlorite oxidized low density lipoproteins

IL-1β: interleukin-1β

LDL: low density lipoproteins

L-NMMA: N-monomethyl-L-arginine

LOX-1: Lectin-like oxidized LDL receptor

mHSA: maleylated human serum albumin

NO: nitric oxide

PBS: phosphate buffered saline

PE: phycoerythrin

PF-4: platelet factor 4

PGE$_1$: prostaglandine E$_1$

PRP: platelet rich plasma

RANTES: regulated upon activation normal T-cell expressed and secreted

REM: relative electrophoretic mobility

RNA: ribonucleic acid

VASP: vasodilator stimulated phosphoprotein

VLDL: very low density lipoproteins

vWf: von Willebrand factor

1   Introduction

Platelets and their reactivity state play an important role in the development of atherosclerotic disease, which represents the major source of morbidity and mortality in the Western world.

The aim of this work was to investigate the effects of native lipoproteins and lipoproteins under conditions of oxidative stress on human platelets.

Since this work brings many different fields together, an introduction of all players involved is necessary.

Therefore, the introduction starts by giving an insight in the development and clinical manifestations of atherosclerosis, followed by discussing the role of lipoproteins and the impact of oxidative stress, particularly with regard to atherosclerotic events.

Thereafter, the role of platelets, which are important factors in haemostasis, inflammation and atherosclerosis, is outlined.

For a better understanding of platelet lipoprotein interaction, it is necessary to mention details of the regulatory mechanisms of platelet function, to get an understanding of signal transduction events involved in platelet adhesion and aggregation. Moreover, inhibitory mechanisms of platelet function are discussed. Subsequently, the current knowledge regarding the interaction of human platelets with native and modified lipoproteins is reviewed. The introduction concludes with summarizing the aim of this work.

1.1 Development and clinical manifestation of atherosclerosis

Atherosclerosis is a pathological process that underlies most cardiovascular diseases, which represent the leading cause of death worldwide.

The apparent beginning of an atherosclerotic event is characterised by accumulation of cholesterol deposits in macrophages or smooth muscle cells of large and medium sized arteries, which leads to so called foam cells, noticeable as fatty streaks.

Extensive studies over the last decades investigated the mechanisms responsible for initiating atherosclerosis. Whereas atherogenesis has traditionally been viewed as a response to an vascular injury (response-to-injury hypothesis)[1] or a simple retention of lipids within the vessel wall (response-to-retention hypothesis)[2], more recent data emphasize the importance of oxidative stress in this process by highlighting the requirement of lipoprotein oxidation (oxidative modification hypothesis)[3].

The initial cause for the development of atherosclerosis is still discussed controversially, but it is for sure a highly complicated, active process and essentially hallmarked by an inflammatory reaction, in which, codetermined by oxidative stress, lipoproteins and platelets play a leading role.

The progressive course of this disease is characterised by changes in the subendothelial region, where certain cell types proliferate, more cholesterol accumulates and the vessel wall lumen is gradually impinged. These changes also lead to activation of the immune system and migration of inflammatory cells, which create (due to respiratory burst) a pro-oxidative environment and endothelial dysfunction, central factors for further atherogenesis.

Atherosclerosis without flow-limiting thrombosis is a slowly progressing disease, which often lasts for decades without clinical manifestations. The usual mechanism responsible for a sudden transition from a stable disease to a symptomatic life threatening condition is the denudation and erosion of the endothelial surface or plaque disruption, which leads to exposure of deep arterial wall components to flowing blood, followed by thrombosis and compromised oxygen supply to target organs[4]. Reduced blood flow can result in the loss of heart or brain functions, known as heart attack or stroke.

1.2 Lipoproteins and oxidative stress

Cholesterol, apart from being important in vitamin D and steroid synthesis as well as membrane viscosity, is considered a pivotal risk factor for the development of atherosclerotic disease. It derives either from dietary origin or de novo synthesis. Due to its insolubility in blood, cholesterol, like all other lipophilic substances, has to be transported through the circulatory system in the form of transport molecules, known as lipoproteins.

Lipoprotein particles are composed of a surface monolayer of phospholipids, which renders the particle soluble in water, a core of lipids, including cholesterol, and surface apolipoprotein molecules that allow cells to recognize and take up the particle. Lipoproteins are usually classified by their density: high density lipoproteins (HDL), low density lipoproteins (LDL) and very low density lipoproteins (VLDL). These lipoproteins vary in their ratio of protein to lipid and in their particular apolipoproteins and lipids which they contain and hence their function essentially differs. The major apolipoprotein of LDL is Apo B-100, which is essential for the receptor mediated cellular uptake of LDL. In HDL the most abundant apolipoprotein is Apo A-I, but HDL also comprise a number of other apolipoproteins, like Apo A-II, Apo C, Apo D and Apo E, which play an important role in activating lipases and lecithin-cholesterol acyltransferase (LCAT). Since this work emphasis on LDL and HDL, only these two lipoproteins are discussed in detail.

1.2.1 LDL and its atherogenic effects

LDL is responsible for cholesterol transport from the liver to peripheral tissues, where cells take up LDL by receptor-mediated endocytosis. The surface expression of the LDL receptor, which recognizes and binds apolipoprotein B-100, is strictly regulated by concentration of intracellular cholesterol on a transcriptional level. High intracellular cholesterol concentrations lead to a decreased release of sterol regulatory element binding protein (SREBP), which is responsible for LDL receptor transcription[5]. This negative feedback prevents

cells from excessive cholesterol uptake. Therefore, internalization of cholesterol by this route can not result in foam cell formation.

Native LDL have to undergo oxidative modification in order to be taken up at an enhanced level. Due to these modifications, LDL lose their specifity for the classical LDL receptor and are recognized by scavenger receptors, which are a group of receptors that recognize oxidised or acetylated lipoproteins and do not underlie a negative feedback regulation. Therefore, intracellular lipid accumulation by cellular uptake of lipoproteins is feasible by this pathway. Scavenger receptors are found on the surface of different types of cells, among them macrophages and smooth muscle cells. Due to scavenger receptors these cells take up oxidised LDL, transforming them into foam cells, with huge cytoplasmic lipid droplets[6].

The existence of such an alternative pathway for cellular uptake of LDL is supported by the fact that macrophages completely lacking LDL receptors can still become foam cells[7].

Although there are other candidate lipoprotein modifications that can enhance LDL uptake by macrophages in vitro (including self-aggregation of lipoproteins, immune-complex formation and complex-formation with proteoglycans[8]), most interest to date has focused on the oxidation of LDL.

1.2.2 The protective role of HDL

Unlike LDL cholesterol, that represents a major cardiovascular risk factor, HDL cholesterol levels are proven to inversely correlate with the risk for atherosclerosis[9].

The classical function of HDL is its participation on removal of cholesterol from peripheral tissues followed by cholesterol transport to the liver for excretion, known as reverse cholesterol transport[10].

Moreover, HDL have the capacity to increase endothelial nitric oxide synthase expression and activity as well as prostacyclin release and to decrease endothelial cell apoptosis, proliferation and migration[11]. In addition, HDL enhance anticoagulant activities of protein S and activated protein C [12].

HDL also possess antioxidative properties, which is mainly mediated by Apo A-I, but also other apolipoproteins have been demonstrated to bear antioxidative capacity[13]. By protection of other lipoproteins from oxidation, HDL themselves can undergo oxidative modification, which alters its function[14].

1.2.3 Oxidative stress within blood vessel and its impact on lipoproteins

Oxidative stress is described as an imbalance between oxidants and antioxidants in favour of the former, potentially leading to damage of biomolecules such as DNA, lipids or proteins[15].

Oxidative stress increases with individual risk factors of atherosclerosis such as obesity, hypertension, hyperlipidemia, diabetes and smoking[16].

The fact that lipoprotein oxidation occurs only in a prooxidative environment, makes oxidative stress an important factor in the initiation of atherosclerosis.

The in vivo mechanism of the initiation and progression of lipoprotein oxidation is investigated intensively, but presently still unclear. Relevant information come from immunohistochemical and biochemical analysis of the atherosclerotic lesions and lipoproteins within, showing that nonenzymatic and enzymatic mechanisms may contribute to LDL oxidation[17]. Among them, ceruloplasmin, 15-lipoxygenase and myeloperoxidase (MPO) are discussed to be involved in oxidation of lipoproteins.

Ceruloplasmin, the major copper carrying protein in blood, exhibits a copper-dependent oxidase activity, associated with possible oxidation of ferrous iron into ferric iron and is suggested to provoke LDL oxidation by its redoxactive metal ions. But since specific markers of metal ion-catalyzed protein damage are not elevated in early and intermediate lesions, it appears rather unlikely that ceruloplasmin or metal ions significantly contribute to LDL oxidation in vivo[18].

15-lipoxygenase, an enzyme that participates in arachidonic acid and linoleic acid metabolism has been suggested to be involved in LDL modification in vivo[19]. Several results promote that 15-lipoxygenase is proatherogenic: it has been shown that fibroblasts overexpressing 15-lipoxygenase are able to transform native LDL into minimally modified LDL. Moreover, oxidative

modification of LDL that is achieved by coincubation of LDL and cultured endothelial cells or monocytes could be shown to, be mediated by 15-lipoxygenase[20].

Recent studies in a mouse model have shown that MPO, a haem enzyme secreted by human phagocytes upon activation, is present in atherosclerotic tissue, co-localizing with macrophages[21]. The enzyme is a potent catalyst of LDL oxidation in vitro and it generates products that are detectable in atherosclerotic plaque. Moreover hypochlorite/hypochlorous acid (referred to as HOCl within this work), the major strong oxidant generated by MPO, has been implicated in the in vivo oxidation of LDL[22]. In vitro studies have shown that hypochlorite oxidised LDL (hypOxLDL) are able to cause foam cell formation, enhance leukocyte oxidant and cytokine production, degranulation, migration and adherence of endothelial cells and increase vascular permeability[17]. Moreover our group has demonstrated an important role of hypOxLDL in platelet activation[23].

Taken together, these findings suggest that myeloperoxidase plays an important role in LDL oxidation occuring in vivo. The fact that oxidised LDL have not only be found in atherosclerotic lesions but also in plasma itself[24] provides a solid basis for the in vivo relevance of investigations dealing with platelet interaction with oxidised LDL.

While these studies only concern LDL oxidation, there is also evidence for the presence of oxidised HDL in vivo – both are present in atherosclerotic lesions as well as in plasma[25].

Apo A-I in HDL recovered from atherosclerotic lesions displays significant oxidative modifications[26] and lipids isolated from HDL and LDL found in atherosclerotic lesions have been reported to be oxidised to a comparable extent, increasing with severity of disease[27, 28].

Upon oxidative modification of HDL, these lipoproteins not only lose important protective functions, but also acquire severe pro-inflammatory and pro-thrombotic properties.

In detail, it could be shown that oxidatively modified HDL interfere with reverse cholesterol transport, activate mitogen-activated protein kinase and upregulate

the expression of cyclooxygenase-2 (Cox-2), plasminogen activator inhibitor-1 and matrix-degrading proteases in endothelial cells[29].

Additionally, it was reported that oxidised HDL stimulate the delivery of intracellular cholesterol to the cell surface, where it becomes available for removal by other, non-oxidised, HDL particles[14].

1.3   Platelets – important factors in haemostasis, immune response, inflammation and atherosclerosis

1.3.1 Primary haemostasis

Platelets, which are produced in the bone marrow by megakaryocytes, represent anucleate cells that lack genomic DNA, but contain megakaryocyte-derived messenger RNA and the translational machinery, needed for protein synthesis.

After leaving their site of origin, platelets circulate in the blood for about 10 days. There platelets mediate the process of primary haemostasis, a process by which a barrier against blood loss is created in the case of injury. The contact of platelets with (mostly) collagen fibrils leads to signal transduction, which results in platelet adhesion, followed by activation and aggregation of platelets.

In the absence of injury, the endothelium prevents haemostasis by providing a physical barrier and by secreting nitric oxide (NO) and prostaglandin (PGI), which counteract platelet activation.

1.3.2 Platelets in immune response

Upon activation, platelets secrete several antimicrobial peptides like "**r**egulated upon **a**ctivation **n**ormal **T**-cell **e**xpressed and **s**ecreted" (RANTES), platelet factor 4 (PF-4), thymocin-β and its derivates, which can deaden microbes by forming pores in the microbial membrane, causing depolarisation and breakdown of the membrane[30, 31]. RANTES and PF-4 also act as

chemoattractants for monocytes and promote their differentiation into macrophages. They also induce expression of E-selectin by endothelial cells[32]. Moreover, PF-4 may directly facilitate atherosclerosis by inhibiting LDL catabolism and enhancing uptake of oxidised LDL by macrophages[33, 34]. Since PF-4 deposition correlates with lesion severity in atherosclerosis, persistent platelet activation may contribute to the evolution of atherosclerotic lesions[35].

Apart from secreting factors important for immune response, platelets also directly interact with cells of immune response via platelet surface receptor P-selectin, derived from the α-granule membrane, which, upon activation, is expressed on the platelet surface.

P-selectin binding to P-selectin glycoprotein ligand-1 (PSGP-1), on lymphocytes, enhances adhesion of lymphocytes to endothelial tissue.

Platelet-monocyte binding favours transmigration of monocytes to sites of inflammation[36, 37], which also leads to translocation of platelets into extravascular tissue, where they can further interact with leucocytes[38].

1.3.3 Platelets in inflammation

Inflammation is a complex biological response to harmful stimuli like pathogens, injury or irritants. Due to activation of endothelial cells and macrophages, platelets indirectly favour inflammatory reactions. Platelets are also able to participate directly in inflammation by the release of soluble agents, like Interleukin-1β (IL-1β), which is synthesised upon platelet activation and can induce endothelial cells to express genes that mediate the adhesion of leukocytes. Moreover endothelial cells, activated by IL-1β, release chemokines and up-regulate molecules that promote adhesion of neutrophils and monocytes to the endothelium[39].

By no doubt the most important inflammatory mediator released by stimulated platelets is the trimeric transmembrane protein CD40 Ligand (CD40L, CD154), structurally related to cytokine tumour necrosis factor α (TNFα). CD40L is stored in the cytoplasma of resting platelets and rapidly appears on the cell surface after platelet activation. In a period of minutes to hours (depending on the activator) CD40L undergoes cleavage from the platelet surface and a functional

soluble fragment is generated[40]. CD40L can trigger specific immune response through dentritic cell maturation, T cell activation and isotype switching of immunoglobulin from IgM to IgG[41]. Moreover, CD40L can induce inflammatory responses in the endothelium, such as the release of interleukin-8 (IL-8) and monocyte chemoattractant protein 1 (MCP-1)[42], as well as secretion of chemokines and adhesion molecules for leukocyte recruitment[43].

Recently "homologous to lymphotoxin; exhibits inducible expression and competes with HSV glycoprotein D for herpes virus entry mediator"; a receptor expressed on T cells (LIGHT, TNFSF 14), which also belongs to the TNF superfamily, was identified on the platelet surface upon activation. LIGHT leads to pro-inflammatory and pro-thrombotic responses of vascular endothelial cells comparable to CD40L, by enhancing platelet adhesion to endothelium and endothelial activation via NFkB. In vitro studies with recombinant, soluble LIGHT showed upregulation of inflammatory markers like ICAM-1, tissue factor (TF) and IL-8 on the endothelium[44].

Elevated serum levels of CD40L and LIGHT indicate an acute risk for coronary events[45, 46]. Since platelets are the main source of CD40L and an important source for LIGHT, their crucial role of in the pathogenesis of atherosclerosis is undoubted.

1.3.4 The role of platelets in the initiation of atherosclerosis

One of the key events in the initiation of atherosclerosis is the monocyte arrest on vascular endothelium and recruitment of blood monocytes into the arterial wall. Via MCP-1 secretion platelets play an important role in this process and they are also involved in macrophage differentiation via RANTES and PF-4. Platelet adhesion to the intact endothelium provokes leukocyte recruitment and, through multistep adhesive and signalling events, leads to infiltration of inflammatory cells into the blood vessel[47]. Moreover, adhesion of platelets to the endothelial surface generates signals recruiting monocytes to the site of inflammation[48]. These processes represent a vicious cycle and the principle of cause and effect is hard to dissect. Due to their activation, platelets, macrophages, leukocytes and endothelial cells produce potent inflammatory

and mitogenic substances which alter adhesive and proteolytic properties of the endothelium, leading to recruitment of further inflammatory cells[49] and as a consequence to a pro-oxidative microenvironment.

1.4 Regulation of platelet function

1.4.1 Platelet function

As mentioned before, the classical role of human platelets is to seal injured vessels, by adhesion and subsequent aggregation. Platelet adhesion is mediated by binding of von Willebrand factor (vWF) to glycoprotein (GP) Ib-IX-V and by direct binding of integrin α2β1 (GPIa/IIa) to collagen fibrils.

Platelet adhesion to the vessel wall leads to platelet activation, which causes shape change of platelets and activation of integrin αIIbβ3 (GPIIb/IIIa), which then binds fibrinogen and vWF. Upon activation, platelets synthesize and release thromboxane A_2 (TxA_2) and platelet activating factor (PAF), which are potent platelet aggregating agonists and vasoconstrictors. Moreover, platelets immediately respond by exocytosis of α-granules and dense bodies, which leads to the release of adhesion molecules, coagulation factors, cytokines, growth factors and antimicrobial compounds, by which platelets interact with other cells and recruit further platelets. Besides the release of soluble compounds, platelet degranulation also leads to the expression of new membrane proteins (like GPIb, GPIIb/IIIa, P-selectin and granulophysin) on the surface of activated platelets.

Platelet aggregation is mediated by fibrinogen, which links adjacent platelets by activated GPIIb/IIIa, leading to platelet clustering. This primary, reversible platelet plug must then be stabilized by the formation of fibrin.

1.4.2 Inhibition of platelet function

Since the activation of platelets has strong impact on different cell types and the consequences of inadvertent platelet activation (e.g. thrombosis and

atherosclerosis) can be lethal, platelets have to be prevented from unintentional activation.

Therefore, one of the physiological functions of endothelial cells is to downregulate platelet function by releasing nitric oxide (NO) and prostacyclin (PGI$_2$) Under physiological conditions endothelium constantly releases small amounts of NO and PGI$_2$, which prevent platelet activation[50]. Not only the endothelium, but also platelets themselves are able to limit size and growth of thrombus via production of NO.

1.4.2.1 Nitric oxide (NO)

The potent platelet inhibitor and vasodilator NO was first discovered in endothelial cells in 1980[51]. Biosynthesis of NO is carried out by a family of enzymes called nitric oxide synthases (NOS), which produce NO either in a constitutive or an inducible manner (endothelial NOS (eNOS), inducible NOS (iNOS), neuronal NOS (nNOS) and mitochondrial NOS (mtNOS)). Membrane-associated eNOS is expressed by platelets and endothelial cells[52] and catalyzes, in the presence of several cofactors (calmodulin/Ca^{++}, NADPH, tetrahydrobiopterin(BH$_4$), FAD and FMN), the multi-electron oxidation reaction of L-arginine with oxygen, forming L-citrulline and releasing NO[53]. The activity of eNOS is regulated by intracellular Ca^{++} concentration, (de)phosphorylation at various tyrosine, serine and threonine residues as well as the association or dissociation of eNOS interacting proteins[54]. In the absence of L-arginine or BH$_4$ or in the presence of eNOS inhibitors N-monomethyl-L-arginine (L-NMMA) or L-N^6-Nitroargenine methyl ester (L-NAME), eNOS can undergo a process called "uncoupling", whereby superoxide is produced instead of NO which immediately reacts with NO to form peroxynitrite, that represents a powerful oxidant[55].

Besides its vasodilatory effects, endothelial derived NO has also antithrombotic effects, leading to inhibition of activation, adhesion and aggregation of platelets[51, 56]. Moreover, platelet derived NO, which is released during rest and activation, is important for modulation of platelet function itself. Therefore a large amount of NO is released shortly after activation to prevent further aggregation[57].

NO binds soluble guanylyl cyclase (sGC), thereby increasing its activity and leading to an increase of intracellular cyclic guanosine 5′-monophosphate (cGMP). This affects multiple signalling pathways, including protein kinases and receptor proteins[58]. Furthermore, increased cGMP levels lead to a decrease of intracellular Ca^{++} flux by cGMP-dependent protein kinase G (PKG) by inhibiting Ca^{++} entry and Ca^{++} release from the dense tubular system[59].

The decrease in Ca^{++} levels inhibits the conformational change in GPIIb/IIIa required for activation and thus a decrease of platelet association with fibrinogen. Moreover GPIIb/IIIa affinity for fibrinogen is lowered by cGMP dependent phosphorylation of vasodilator-stimulated phosphoprotein (VASP) on serine 157[60].

1.4.2.2 Prostacyclin

Prostacyclin (PGI_2), a derivative of the (semi)essential unsaturated fatty acid arachidonic acid, was discovered in 1976 as potent vasodilator, antithrombotic and antiplatelet agent with a very limited lifetime under physiologial conditions[61]. Prostacyclin synthesis by endothelial cells is regulated by two mechanisms, which involve either endogenous precursors or endoperoxides (like TxA_2) derived from activated platelets[62]. The rate limiting enzyme in PGI_2 production is cyclooxygenase (Cox), which exists in two isoforms[63]. HDL could be shown to increase Cox-2 expression in vascular smooth muscle cells[64].

PGI_2 is stabilized by serum albumin, which enhances receptor binding and activity, while a possible stabilizing role of HDL could not be proven[65].

The platelet prostacyclin receptor, a G protein-coupled receptor, is located in the plasma membrane and binds the cyclopentane ring of both PGI_2 and prostaglandins PGE_1 and PGE_2.

Via the platelet prostacyclin receptor, PGI_2 inhibits platelet activation, limits thrombus size and prevents platelet and leukocyte adhesion to endothelial cells. PGI_2 binding to its receptor induces a signal cascade, which leads via adenylate cyclase (AC) to an increase of intracellular cAMP levels[66].

As a consequence of increased cAMP production protein kinase A (PKA) becomes activated, which then phosphorylates several key proteins, like myosin

light chain kinase (MLCK), inositol 1,4,5-triphosphate receptor and the vasodilator stimulated phosphoprotein (VASP).

This leads to inhibition of MLCK and Rho-kinase, which subsequently inhibits granule secretion and activation of GPIIb/IIIa and PKC. Moreover, increase of intracellular Ca^{++} levels is inhibited[67]. The induction of these inhibitory mechanisms enables PGI_2 not only to inhibit platelet aggregation, but also facilitates disaggregation of existing platelet aggregates.

1.4.3 Interaction of platelet and lipoproteins

Lipoproteins have traditionally been viewed as simple carriers of cholesterol, phospholipids and triglycerides, but increasing evidence indicates that lipoproteins are also able to induce intracellular signalling pathways in different target cells.

The interaction of platelets with lipoproteins has been under investigation for many years, since epidemiological studies revealed a correlation between lipid profiles and hyperaggregability of platelets[68]. Platelets of hypercholesterolemic patients show enhanced activation in vivo, suggesting that LDL enhances platelet responsiveness. HDL seem to oppose these activating properties, whereas the effects of chylomicrons, VLDL or IDL on platelet function are discussed controversially[69]. These changes on platelet reactivity are either results of cholesterol-phospholipid uptake by platelet membranes during platelet formation or are based on signal transduction induced by direct contact with lipoproteins[70]. While older literature favours lipid uptake during platelet formation, nowadays direct interaction of lipoproteins and circulating platelets is proposed.

1.4.3.1 Potential lipoprotein receptor candidates

It is presently unclear which component of the lipoprotein, lipid or protein, is responsible for its influence on platelet function. Some work argues that receptor independent, lipid interactions are responsible for platelets response, whereas others regard protein moiety, for example apolipoproteins, like Apo

B-100 (in LDL and VLDL) and Apo A or Apo E (in HDL), as the responsible factors for binding and signal transduction.

The exact nature of receptors for native and/or modified lipoproteins on human platelets is presently unclear. The following section gives an overview of receptors currently discussed to be involved in lipoprotein platelet interaction.

CD36 (FAT, GPIV) belongs to the scavenger receptor family class B and has been known for decades, but its role in lipid uptake and its important role in atherosclerosis was not recognized until 15 years ago, when macrophage derived CD36 was proven to bind moderately oxidised LDL (reviewed by MOORE et al.[71]). Moreover, CD36 was discovered to bind native HDL, LDL and VLDL, as well as thrombospondin-1, collagen, fatty acids and pathogen derived ligands in mammalian cells[72, 73].

Via CD36, macrophages internalize fatty acids which activate peroxisome proliferator-activated receptor γ (PPARγ) and this process stimulates further expression of CD36[74, 75], leading to a positive feedback loop. This supports the importance of this receptor in foam cell formation and atherosclerosis.

CD36, which is expressed on the platelet surface, is discussed intensively as one, if not the only, receptor for oxidised LDL on resting platelets. It has been suggested that platelet activation induced by oxidised LDL or by glycerophospholipids formed during oxidation is mediated by CD36, which then triggers activation of GPIIb/IIIa and P-selectin surface expression[76]. In contrast, others discuss a combined action of CD36 and scavenger receptor class A (SR-A)[77].

Our group could show that human platelets specifically bind oxidised LDL, but not acetylated LDL[78]. As acetylated LDL represent the classical ligand of class A scavenger receptors, these results rule out the existence of SR-A on human platelets.

In contrast to CD36, lectin-like oxidised LDL receptor (LOX-1) appears on the platelet surface only upon platelet activation. In resting platelets, LOX-1 is stored in α-granules. LOX-1 was originally identified as a receptor for oxidised

LDL in aortic endothelial cells. Since it is only expressed in activated platelets, LOX-1 does not seem to play a major role in circulating resting platelets[79].

Class B scavenger receptor B1 (hSR-B1/CLA-1) has been shown to interact with both native and modified LDL as well as HDL in transfected cells and therefore might contribute to lipid metabolism and atherogenesis[80].

On human platelets and megakaryocytes, one group was able to detect CLA-1. This group also showed that the level of CLA-1 expression on human platelets inversely correlates with cholesterol ester content of platelets (from patients with atherosclerotic disease) and extent of platelet aggregation[81]. In this regard, it is noteworthy that the expression of CLA-1 on human platelets could not be confirmed by others[82] nor by our group. Therefore, there is doubt if CLA-1 is really expressed on human platelets.

LDL receptor-related protein 8 (LRP8), a splicing variant of apoER2, which belongs to the LDL receptor (LDLR) gene family, was identified on human platelets and has been proven to bind Apo E particles and thereby HDL[83]. Nevertheless, it is still unknown, if LRP8 is the only platelet receptor for native HDL.

The receptor responsible for LDL interaction with human platelets is discussed controversially. Since platelets do not possess the classical Apo B receptor and the expression of CLA-1 is doubted, there must be another way responsible for interaction. Up to today, it remains unclear, if LDL directly interact with platelets, since different studies revealed controversial outcome. While GPIIb/IIIa, CD36 and LDL receptor-related protein 8 (LRP8) are discussed intensively as receptor candidates (reviewed by KOLLER et al.[84]), others believe that platelet LDL interaction is carried out by a receptor independent mechanism[85].

1.4.3.2 Platelet interaction with HDL

It has been demonstrated that HDL are able to directly influence platelet reactivity, since they mediate a dose dependent inhibition of platelet activation induced by various agonists[86]. Moreover an inverse correlation between HDL abundance and P-selectin positive platelets could be found in humans[11].

Various reasons are responsible for these positive effects. On the one hand blocking of saturable binding sites by HDL leads to less binding of other agonists that bind to the same receptors. On the other hand HDL mediate the production of the atheroprotective signalling molecule nitric oxide by upregulating endothelial NO synthase (eNOS) and promote prostacyclin synthesis, both of which mediate inhibitory effects on platelets. The antithrombotic properties of HDL may also be related to their ability to attenuate the expression of tissue factor and selectins in endothelial cells and platelets and subsequenty decrease thrombin generation[11]. Also downregulation of TxA_2 plays an important role within this scenario, whereat in general HDL_2 subclass is reported to be more effective than HDL_3[87]. This might relate to the Apo E content, which differs between the subclasses, being significantly higher in HDL_2. Moreover, purified ApoE (in phospholipid vesicles) could be proven to stimulate eNOS in platelets and thereby upregulate cGMP[88].

Also in endothelial cells HDL was observed to play a role in eNOS activation and to stabilize eNOS localisation, thereby counteracting its depletion by oxidised LDL[89]. Apo A-I is the principally responsible - although solitary not sufficient - apolipoprotein for this atheroprotective feature of HDL[90, 91].

Since the regulation of eNOS activity demands complex signal transduction pathways, it is not astonishing that a variety of experiments in endothelial cells indicate multiple signalling events that are activated by HDL, among them eNOS phosphorylation of Ser1179, Scr activation and activation of MAP and Act kinases[92]. Moreover HDL could be shown to upregulate eNOS enzyme abundance by extending its half life[93].

1.4.3.3 Platelet interaction with LDL

LDL interaction with platelets has been under investigation for many years, revealing several signalling pathways induced in vitro.

Since it seems difficult to obtain completely unmodified LDL, it is hard to distinguish between effects of native or minimally modified LDL. Therefore, especially older data have to be accepted under reserve.

It could be shown that the presence of LDL itself increases binding of fibrinogen by platelets[94]. Moreover, native LDL are suggested to stimulate TxA_2 formation, activation of PKC, which leads to an increase in diacylgylcerol (DAG) and inositol 1,4,5-triphosphate ($InsP_3$), Ca^{++} release and inhibition of Na^+/H^+ antiporter and subsequent intracellular acidification (reviewed by KOLLER et al.[84]). Also, phosphorylation of p38 mitogen-activated protein kinase (p38MAPK) and p125 focal adhesion kinase (p125FAK), which induces further activation, is reported to be induced by LDL. Since LDL also act on platelet-endothelial cell adhesion molecule (PECAM-1), these lipoproteins might also be able to downregulate p38MAPK activation and thereby counteract platelet aggregation[95]. Due to the ambiguous effects of LDL, the interpretation of their actions remains still unclear.

1.4.3.4 Platelet interaction with oxidised lipoproteins

Oxidative modification of lipoproteins is associated with the generation of new bioactive compounds within the lipoproteins. Whereat copper modification is proven to have an influence on lipid moiety of lipoproteins, the formation of lipid peroxides could be ruled out for modification of LDL by hypochlorite[96, 97]. It could be shown that the relative electrophoretic mobility (REM), a measure of protein modification, corresponded to the hypochlorite concentration used for modification and that thiobarbituric acid reactive substances (TBARS), an index for lipid peroxidation, were not formed[97]. Hence, changes in protein moiety are regarded to be responsible for platelet-stimulating effects of hypochlorite oxidised LDL.

Comparison between platelet aggregation induced by CuOxLDL and hypOxLDL, identified hypOxLDL as the stronger platelet agonist[97].

LDL, which were modified with physiological concentrations of hypochlorite, were reported to mediate increasing effects on agonist-induced platelet aggregation[96].

Since the effects of hypOxLDL could be completely inhibited by mHSA, a potent inhibitor of binding of oxidised lipoproteins to all scavenger receptors, influence

of lipoproteins on platelet function seems to be transmitted via binding of lipoproteins to specific platelet receptors[98].

Moreover, hypOxLDL could be shown to induce P-selectin surface expression and induction of p38MAPK phosphorylation[77, 99]. Other results showed, that in contrast to CuOxLDL, hypOxLDL lead to stimulation of Ca-ATPase in isolated platelet membranes and a decrease of intracellular Ca^{++}[100].

Less is known about the impact of oxidatively modified HDL on platelets. Up to now only few investigations of platelet interactions with oxidised HDL exist, which reveal contradictory results. No precise specifications of oxidised HDL binding to human platelets or of effects of oxidised HDL on platelet function have been made so far.

It has been reported that upon oxidation of HDL with copper, these lipoproteins trigger spontaneous platelet aggregation[101]. Moreover, copper oxidised HDL promote platelet activation and intracellular Ca^{++} flux in washed platelets (but not in platelet rich plasma)[102]. The effects of oxidised HDL on platelet aggregation are not attributable to increased production of TxA_2, since Cox inhibitors show little effect. Therefore, changes in membrane fluidity are suggested as the underlying mechanism[101].

By contrast, copper oxidised HDL_3 were also shown to inhibit thrombin-induced platelet aggregation and fibrinogen binding to platelets via decreased production of DAG and $InsP_3$ to the same extent as unmodified HDL_3[103].

In terms of HOCl modification, oxidised HDL_3 have been reported to have no impact on platelet aggregation[96] but stimulate Ca-ATPase activity in isolated platelet membranes, which results in a decrease of intracellular Ca^{++} in functional platelets[100].

Taken together, platelet activating properties of oxidised LDL are well established, although receptors and signal transduction pathways are not clarified yet. In contrast to oxidised LDL, the effects of oxidised HDL are discussed controversially.

1.5 Aims of this study

In light of the central importance of platelets to the atherosclerotic process it was the aim of this work to investigate and characterise the impact of LDL and HDL – both in their native state as well as after oxidative modification – on several aspects of platelet function. By means of the performed experiments, the effects of these lipoproteins on platelet aggregation, degranulation, GPIIb/IIIa activation, VASP phosphorylation, CD40L expression and calcium flux should be determined. Moreover, binding studies should reveal if lipoproteins show specific and saturable binding to human platelets and potential receptor candidates investigated for their role in this proposed binding process and their influence on lipoprotein-mediated effects on platelet function. In light of the redox-sensitive nature of platelet function and as there are only very limited data concerning the impact of oxidatively modified HDL on platelets, it was the main aim of this work to also ascertain the effects of hypochlorite oxidised HDL on platelet function.

2   Material and methods

2.1   Material

2.1.1 Laboratory equipment

Aggregometer: 490-4D 4 channel aggregometer (Chrono-Log)

Centrifuge: RT6000 and RT6000D with a H1000B Rotor (Sorvall)

         Allegra X12R (Beckman Coulter)

Flow Cytometer: FACS Calibur analytic flow cytometer with a two laser system:

         Argon-488nm and 635nm Diode (Becton Dickinson)

Gel electrophoresis: equipment type Minnie HE 33 (Hoefer)

Microplate Reader: Anthos HT III (Anthos Labtec Instruments)

Microplate Spectrofluorometer: Spectra Max Gemini XS (Molecular Devices)

Spectrophotometer: U-3200 (Hitachi)

Ultracentrifuge: OTD Combi with a T-885 rotor (Sorvall)

        Optima LXP Series with a 50.2 Ti rotor (Beckman Coulter)

2.1.2 Buffers

Phosphate buffered saline (PBS):

       potassium phosphate (1.5mM)

       potassium chloride (2.7mM)

       sodium chloride (137mM)

       sodium phosphate (8.3mM)

Hoefer buffer:

       diethylbarbituric acid (50mM)

       sodium acetate (70mM)

       pH: 8.2

Fixing solution (gel electrophoresis):

      methanol (500ml)

      glacial acetic acid (20ml)

      distilled water (480ml)

Lowry solutions:

      A1: copper sulphate (40mM)

      A2: potassium sodium tartrat (70mM)

      B: 50ml 2% (w/v) sodium carbonate +1ml A

        (A: 500µl A1+ 500µl A2, freshly mixed)

      C: Folin reagent + H_2O (1:1)

      D: NaOH (1N)

Tyrode-HEPES buffer (with glucose and albumin):

      sodium chloride (140mM)

      potassium chloride (3mM)

      magnesium chloride (1mM)

      sodium hydrogen carbonate (16.62mM)

      HEPES (10mM)

      (D-glucose (5.5mM))

      (human serum albumin (0.5%))

For platelet isolation by gel filtration and all functional platelet experiments buffer was set to a pH of 7.35. For platelet washing it was set to pH 6.2. Buffer without glucose and albumin was stored at 4°C and used within 2 month. Buffer with glucose and albumin was used immediately or frozen at -20°C and never refrozen.

Borate buffer with EDTA:

      boric acid (0.1M)

      EDTA (100µM)

      pH: 7.2

<u>Borate buffer with sodium chloride</u>

> boric acid (0.1M)
>
> sodium chloride (0.05M)
>
> pH: 7.2

2.1.3 Chemicals and solutions

Platelet agonists were always freshly prepared from ADP (Sigma-Aldrich), thrombin (Sigma-Aldrich) and collagen (Nycomed) stock solutions.

ADP and thrombin stock solutions were deeply frozen stored in small portions (100µl) (-80°C) and never refrozen. Collagen stock solution was always prepared on the same day.

PGE_1 (Sigma Aldrich) was dissolved in pure ethanol at 10mM and stored at -80°C. Further dilutions were performed in PBS and always freshly prepared immediately before use.

For cell fixation, 10% formaldehyde stock solution in 0.9% sodium chloride was used.

Cell permeabilization was carried out with 0.2% triton (triton x-100, Sigma Aldrich) solution.

For lipoprotein oxidation, sodium hypochlorite solution (NaOCl) from Sigma Aldrich was used. Its concentration was determined spectrophotometrically before use (ε_{290}=350 L mol^{-1}cm^{-1}).

Reduction of oxidised lipoproteins was performed with 200mM methionine stock solution (Sigma Aldrich).

For platelet gel filtration sepharose 4B (GE Healthcare) was used.

Gelfiltration of lipoproteins was performed with Econo-Pac 10DG polyacrylamide chromatography columns (Bio-Rad).

Lipoproteins were filtered using Rotilabo PVDF syringe membrane with a pore diameter of 0.45 µm (Roth).

For concentrating lipoprotein solutions an ultrafree-15 unit with a molecular weight cut-off of 100 000 (Millipore) was used.

2.1.4 Antibodies and fluorescence marker

The following antibodies were obtained from Becton Dickinson:

monoclonal anti CD62 (cat # 348107, PE conjugated)

monoclonal anti CD63 (cat # 556020, FITC conjugated)

monoclonal anti PAC-1 (cat # 340507, FITC conjugated)

monoclonal anti CD40L (cat # 555699, FITC conjugated)

polyclonal anti-mouse IgG antibody (cat # 349031, FITC conjugated)

Antibodies and fluorescence markers obtained from Invitrogen/Molecular
   Probes:

polyclonal anti-goat IgG (cat # 45624A , Alexa 488 conjugated)

Fluo-4 and Fura-Red (cat # 28B1-2, cat # 28B2-3, acetoxymethylester-
   derivatives, diluted in DMSO to a final concentration of 1µg/µl)

Alexa Fluor 633 (cat # 34571A, carboxylic acid, succinimidyl ester)

Further antibodies:

polyclonal anti Apo B (cat # 600111101, Rockland)

polyclonal anti Apo A-I (cat # 0510013255, Chemicon International)

polyclonal anti CD36 (cat # K1606, Santa Cruz)

monoclonal anti CD36, FA6. 152 antibody (cat # ab17044, Immunotech)

monoclonal anti CD32, AT10 antibody (azide free, cat # MCA1075XZ,
   Serotec)

monoclonal anti-phospho-VASP (cat # 0153S0202, pSer 239,
   clone 22E11, nanoTools)

2.2   Methods

2.2.1 Platelet isolation

Venous blood from healthy donors, who declared to be free of any medication for at least one week, was drawn into anticoagulation tubes filled with 3.8% sodium citrate (used in a 1:9 volume ratio). Platelet-rich plasma (PRP) was obtained by centrifugation immediately after taking the sample (125g; 20 minutes; Beckman Coulter).

2.2.1.1 Gel filtration technique

To obtain gel filtered platelets, a sepharose 4B filled column was prepared, by cutting off the tip of a serological, plastic pipette (20ml) with hot wire and stuffing the cone end with a piece of nylon to hold back liquid sepharose. Thereafter the pipette was filled with sepharose and washed with Tyrode-HEPES buffer containing glucose and albumin (pH: 7.35). Then 3ml of PRP were loaded and 500µl fractions collected in test tubes. Columns were always freshly prepared and only used once. The platelet fraction can be identified by a change from clear fractions (buffer) to a milky clouding (platelets), which, after about 6 fractions, start getting a yellowish tint, indicating the occurrence of plasma. Only pure platelet fractions were used for platelet studies.

2.2.1.2 Washing technique

To obtain washed platelets, 10ml PRP were incubated with PGI_2 at a final concentration of 1µM and centrifuged for 10minutes at 2000g. The supernatant was discarded and the pellet resuspended in 10ml Tyrode-HEPES buffer (pH: 6.2) with PGI_2 (f.c.: 1µM) and centrifuged again (10minutes; 2000g). The pellet was resuspended with Tyrode-HEPES buffer (pH: 7.35) in a volume of 1ml. Washed platelets were used between half an hour and two hours after isolation, so that platelet function was no longer inhibited by PGI_2 activity.

2.2.2 Patelet count

2.2.2.1 Counting chamber method:

For determination of platelet count, a 25-, a 50- and a 100-fold dilution of gel filtered platelets was prepared from each donor. Dilution was performed in 1% ammonium oxalate, to lyse all erythrocytes left in the filtrate. A Bürker and Türk counting chamber was filled with 10µl of the platelet dilution. Thereafter the chamber was incubated in a damp cloth for at least 15 minutes, allowing platelets to settle. Afterwards platelets were counted under the 40X objective with the help of the specific counting lines of the Bürker and Türk counting chamber. Platelets in 5 squares were counted and the number of platelets was calculated according to the following formula:

$$\text{Platelets per } \mu l = \text{counted platelets} * \text{dilution factor} * 40$$

2.2.2.2 Photometrical method:

For photometric determination of platelet count, a 50-, a 100-, a 500- and a 1000-fold dilution of platelets from each donor was prepared. Plastic cuvettes (10 mm optical path) were filled with 1ml of the platelet dilution and placed in a photometer. The photometric measurements of the platelet dilutions were performed at 800 nm. The instrument was set for zero with Tyrode-HEPES buffer. Stated numbers of platelet count are always means of all 4 dilutions.
The standard formula for platelet count calculation by Walkowiak[104] was used:

$$N\,(10^8/\text{ml}) = \left(\frac{6.23}{2.016 * w * k * E/800} - 3.09 \right) * R$$

N............... estimated platelet count
R............... sample dilution
w............... used wavelength
E .... extinction of the sample
k............... geometrical factor equal 1 for 10 mm optical path

2.2.3 Platelet aggregation

2.2.3.1 Aggregometer

Platelet aggregation was carried out in a 4-channel aggregometer (Chrono-Log), measuring light transmission. 420µl of gel filtrated platelets were pipetted into a coated glass cuvettes with stirring bars and placed into the instrument, where they were preincubated at 37°C for 5 min without stirring. Then stirring (1000 rpm) was started and the zero level as well as 100% transmission was adjusted. Potential antagonists (nHDL, FA6.152 or mHSA in volumes of 5-40µl) were added. To avoid artificial results due to possible spontaneous platelet aggregation, experiments proceeded only if no changes in light transmission occurred during the first minute. Thereafter the aggregation process was triggered by the addition of platelet agonists (ADP, hypOxLDL, hypOxHDL in volumes between 5µl and 40µl) and effects observed for 12 min. Total volume of each probe always amounted 500µl, therefore volumes of controls were adjusted, to grant the same amount of platelets in all probes.

2.2.3.2 Microplate reader

Platelet aggregation was also monitored in a microplate reader, which was able to maintain temperature and continuously shake (forth and back) the 96 well microplate between readings. For the aggregation process temperature was set at 37°C and readings - performed at a wavelength of 405nm - were repeated every 20 sec for about 12 minutes.

In detail, 50µl platelets were added to each well. According to the design of the experiments, some probes had to be preincubated with antagonists (10µl nHDL, 5µl mHSA, 1µl FA6.152); therefore the volume of the other probes was adjusted to grant the same platelet density in all probes. Just before the probes were placed into the microplate reader, platelet agonists (or buffer for controls), in 5µl volume, were added using a multichannel pipette, and reading was started. The sample surfaces in every well had to be free from any air-bubbles in order to get well reproducible and smooth aggregation curves[105]. Controls for 100%

transmission (buffer) and zero level (GFP) were observed during the whole period of measurements. The last control allowed observing the degree of basal aggregation of platelets under the chosen experimental conditions. Moreover a positive control for maximal aggregation (induced by 50μM ADP) was always carried out as well. The collected data were finally converted to aggregation curves and evaluated by Microsoft Excel and Sigma Plot.

2.2.4 Lipoprotein isolation

Freshly drawn venous blood from healthy donors was drawn into anticoagulation tubes filled with 3.8% sodium citrate used in 1:9 volume ratio. Platelet-poor plasma was obtained by centrifugation (1300g; 20 minutes; 4°C). Plasma was used immediately or stored at -20°C. To avoid inadvertent oxidation of lipoproteins, all manipulations were performed at 4°C.

2.2.4.1 Isolation of VLDL

By addition of potassium bromide (KBr) the fresh or freshly defrozen plasma was brought to a density of 1.019g/ml which corresponds to a 2.85% KBr solution.

Since the density of plasma corresponds to 1.1% KBr solution, only the balance of 1.75% KBr solution is necessary. The amount of KBr needed can therefore be calculated with the following formula:

$$g_{(KBr)} = \frac{g_{(plasma)} * 1.75}{98.25}$$

After adding the calculated amount of KBr, the plasma was filled into centrifugation tubes in 15ml portions and overlaid with 5ml 2.85% KBr solution. The plasma was then centrifuged for 16 hours at 35 000g at a temperature of 4°C. Thereafter VLDL floated above the KBr layer and were carefully taken off with a syringe, while the centrifugation tubes were constantly kept on ice to reduce the risk of unwanted oxidative consequences.

2.2.4.2 Isolation of LDL:

By adding KBr, the remaining plasma was brought to a density of 1.063g/ml, which corresponds to 8.6% KBr solution. The amount of KBr needed can therefore be calculated with the following formula:

$$g_{(KBr)} = \frac{g_{(plasma)} * 5.75}{94.25}$$

Each centrifugation tube was filled with 15ml plasma and overlaid with 5ml 8.6% KBr solution. For isolation of LDL, centrifugation for 20 hours at 35 000g at a temperature of 4°C was performed. Thereafter LDL, which floated on top of the KBr layer, could be taken off and were filtered through a PVDF membrane with a pore size of 0.45μm. LDL and remaining plasma were always kept on ice or stored at 4°C.

2.2.4.3 Isolation of HDL$_2$:

By adding KBr, the remaining plasma was brought to a density of 1.125g/ml, which corresponds to 16.1% KBr solution. The amount of KBr needed can therefore be calculated with the following formula:

$$g_{(KBr)} = \frac{g_{(plasma)} * 7.5}{92.5}$$

After addition of KBr, each centrifugation tube was filled with 15ml plasma and overlaid with 5ml 16.1 % KBr solution. For isolation of HDL$_2$ centrifugation was performed for 48 hours at 35 000g and a temperature of 4°C. After centrifugation, HDL$_2$ floated on top of the KBr layer and were taken off and filtered through a PVDF membrane with a pore size of 0.45μm and stored at 4°C.

2.2.4.4 Isolation of HDL$_3$:

By adding KBr, the remaining plasma was brought to a density of 1.21g/ml, which corresponds to 25.2% KBr solution. The amount of KBr needed can therefore be calculated with the following formula:

$$g_{(KBr)} = \frac{g_{(plasma)} * 9.1}{90.9}$$

Each centrifugation tube was filled with 15ml plasma and overlaid with 5ml 25.2% KBr solution. Isolation of HDL$_3$ was performed by centrifugation for 48 hours at 35 000g and a temperature of 4°C. After centrifugation HDL$_3$ floated on top of the KBr layer and were taken off and filtered through a PVDF membrane with pore size of 0.45µm and stored at 4°C.

The protein concentration of LDL, HDL$_2$ and HDL$_3$ was determined and lipoproteins stored at 4°C and used within three weeks.

2.2.5 Lipoprotein modification

2.2.5.1 Modification by hypochlorite

Hypochlorite modification of HDL and LDL was performed according to the protocol by Arnhold et al[106]. 1ml of native lipoprotein was rebuffered into borate buffer with EDTA by size exclusion chromatography. Thereafter HOCl/NaOCl in 5µl portions was added to the lipoprotein, while rapidly and gently mixing. All manipulations were performed on ice and probes were allowed two minutes of incubation time after addition of each 5µl portion of HOCl/NaOCl. For maximal oxidation, hypochlorite was added until lipoproteins were bleached completely, which usually corresponds to a 300-fold molar excess of hypochlorite over HDL and a 400-fold molar excess of hypochlorite over LDL. For minor degrees of modifications, lipoproteins were incubated with half or quarter the amount of hypochlorite used for maximal modification. The molar excess of hypochlorite used for the preparation of oxidised LDL (hypOxLDL) was usually 400 or 300 for oxidised HDL (hypOxHDL). If another degree of modification was used, the molar excess of hypochlorite over LDL or HDL is indicated in square brackets. After treatment with HOCl/NaOCl, the possibly remaining oxidant was removed

by size exclusion chromatography and concomitant transfer to isotonic borate buffer. Therefore, 1ml of oxidised lipoprotein was loaded on an Econo-Pac column calibrated with isotonic borate buffer. Upon migration of the lipoprotein, borat buffer was loaded and, since the dead volume of the column amounts to 3ml, the third millilitre, which contained the lipoproteins, was collected.

HypOxLDL were always prepared on the day before use, since LDL of some donors showed autofluorescence during the first couple of hours after oxidation. HypOxHDL were always prepared immediately before use.

2.2.5.2 Copper oxidised lipoproteins

LDL oxidation by copper ions was performed according to a commonly used protocol. After gelflitration of LDL into borate buffer, lipoproteins were diluted to a concentration of 200µg/ml and incubated with copper sulphate at a final concentration of 5µM at 37°C for 24 hours. Thereafter incubation was stopped and the lipoproteins were concentrated to approximately 2 mg/ml. Subsequently, copper oxidised LDL (CuOxLDL) were transferred in isotonic borate buffer by gelfiltration as described for hypochlorite-modified lipoproteins. CuOxLDL were always stored on ice and used within a week.

2.2.6 Lipoprotein analysis

2.2.6.1 Protein determination according to Lowry

Lipoprotein concentrations are expressed in terms of their total protein content, which was analysed according to a protocol by Lowry[107].

For each probe two different dilutions of lipoproteins in PBS were prepared (1:20 and 1:50). 250µl of each dilution (or 250µl buffer as control) were added to 125µl 1N sodium hydroxide and incubated for 30 minutes at room temperature.

Thereafter 1.25ml of Lowry solution B were added. After 10 minutes of incubation, probes were mixed rapidly with 125µl of 50% Folin reagent in H_2O and incubated for another 30 minutes. The absorbance of each sample was

determined at 750nm and protein concentration was calculated by the following formula:

$$c_{protein} = (E_{sample} - E_{buffer}) * 303 * d$$

$c_{protein}$ .... protein concentration in µg/ml
E_{sample} .... extinction of the sample
E_{buffer} .... extinction of the buffer
D .... dilution factor

2.2.6.2 Relative electrophoretic mobility

Electrophoretic mobility of native and modified lipoproteins was assessed by electrophoresis[108] with 0.8% agarose gel in Hoefer buffer. Native and oxidised lipoproteins were adjusted for their protein concentration (1000µg/ml) by dilution with PBS. Thereafter 15µl of the lipoproteins were mixed with 1µl bromide phenol blue to increase the visibility of the probes, and some saccharose crystals, to weight down the probes to facilitate loading. All 16µl of the probes were loaded and electrophoresis performed for 3 hours at 40V and 4°C. Afterwards the gel was incubated overnight in fixing solution, which made protein bands visible.

2.2.6.3 Determination of free amino groups

Determination of free amino groups was carried out with fluorescamin (540µM in acetone) according to a protocol by Bohlen[109]. 1ml native or modified lipoproteins were diluted with PBS to a protein concentration of 500µg/ml. 20µl of lipoproteins were added to 730µl borate buffer (pH 8.5). Thereafter, 250µl fluorescamin was added and probes mixed immediately. After 30 minutes of incubation fluorescent readings at a wavelength of 390nm/475nm (excitation/emission) were performed.

Free amino groups of the corresponding native lipoproteins were set as 100% and decrease in free amino groups by oxidation of the same charge of lipoproteins was calculated in relative percent.

2.2.6.4 Chloramine content:

Chloramines were quantified according to a protocol by Riddles[110] using 2,'2 dinitro-5,5' dithio-bibenzoic acid (DTNB), which hydrolysis to TNB^{2-} in alkali. N-Cl derivatives oxidize the sulfhydryl group of TNB^{2-} to its disulphide (DTNB), which can then be detected photometrically.

DTNB was dissolved in 0.1M NaOH. After 5 minutes of exposure, it was centrifuged and TNB^{2-}, the supernatant, taken off. 50µl TNB^{2-} were diluted in 950µl PBS and photometrically measured at a wavelength of 412nm. Thereafter 20µl native or modified lipoproteins (which were always adjusted for their protein content) were added and changes in extinction before and after addition of lipoproteins determined. The difference between the two extinctions corresponds to the amount of TNB^{2-} oxidised by the chloramines of the lipoprotein. Since two TNB^{2-} are oxidised by one Cl^+, the obtained value has to be divided by two. To determine chloramines in HDL, the following formula was used:

$$\text{Chloramines }_{(per\ HDL)}: \frac{(E_{pre} - E_{post})\,/\,2}{13\,600 \,*\, 1000 \,*\, 1000} \,*\, R$$

E_{pre} .... extinction measured before addition of lipoprotein
E_{post} .... extinction after adding HDL
R .... dilution factor of HDL

2.2.7 Flow cytometric analysis of surface- and intracellular markers of platelet activation

For all functional platelet studies by flow cytometry, probes were prepared in wells of 96 well plates. Each probe for flow cytometry was carried out in duplicates and means were calculated. Concentrations given in the results section represent always final concentrations.

2.2.7.1 Surface expression of P-selectin and granulophysin

Surface exposure of P-selectin or granulophysin was determined by flow cytometry. Wells of a 96-well plate were filled with 30µl of washed or gel filtered platelets. According to the investigation question, some probes were preincubated with 2-10µl nHDL, 3µl mHSA (f.c.: 50µg/ml) 1µl FA6.152 (f.c.: 3µg/ml) or 1µl polyclonal CD36 antibody (f.c.: 3µg/ml) for 10 minutes, whereat all probes had to be brought to the same volume again by adding PBS, so that each probe had the same platelet density.

Thereafter 4-8µl of platelet agonists (see pipetting scheme in Table 1) were added and incubated for 5 minutes (for classical agonists) and 10 minutes (for modified lipoproteins). If no or less agonist was added to one probe, volume was balanced by addition of PBS so that the final volume of all probes amounted 48µl. Platelets were then fixed by adding 5µl of 10% formaldehyde and incubated for 15 minutes. Then the 96 well plate was centrifuged at 1700g for 10 minutes. The supernatant was discarded, the pellet resuspended in 30µl PBS and 2µl of antibody directed against CD62P (P-selectin) or CD63 (granulophysin) were added.

Incubation was performed for one hour at room temperature and probes were kept in a dark place. Thereafter probes were diluted in 470µl PBS and analysed immediately. PE marked CD62 was detected in FL2 and FITC labelled CD63 detected in FL1. 10 000 events, gated for platelets according their size, were measured and analysed using BD CellQuest Pro and Microsoft Excel.

substance	concentration	volume added	final concentration
ADP	500µM	4µl	50µM
ADP	50µM	4µl	5µM
ADP	25µM	4µl	2.5µM
ADP	10µM	4µl	1µM
thrombin	2000U/ml	4µl	200U/ml
thrombin	400U/ml	4µl	40U/ml
thrombin	200U/ml	4µl	20U/ml
thrombin	20U/ml	4µl	2U/ml
collagen	1000µg/ml	4µl	100µg/ml
collagen	500µg/ml	4µl	50µg/ml
collagen	200µg/ml	4µl	20µg/ml
collagen	13.3µg/ml	4µl	1.33µg/ml
hypoxHDL	1000µg/ml-1300µg/ml	1µl-10µl	25µg/ml-32µg/ml -250µg/ml-325µg/ml
hypoxLDL	1000µg/ml-1300µg/ml	1µl-10µl	25µg/ml-32µg/ml -250µg/ml-325µg/ml

Table 1: pipetting scheme for platelet agonists

2.2.7.2 CD40L surface expression

CD40L surface expression was detected according to a protocol by Inwald[111]. 10µl of whole blood, PRP or gel filtered platelets were incubated for 2 to 10 minutes with 2µl ADP (f.c.:50µM), 2µl thrombin (f.c.:200U/ml), 2µl collagen (f.c.:100µg/ml), 2µl hypOxHDL (f.c.:100µg/ml) or 2µl hypOxLDL (f.c.:100µg/ml). For some studies, platelets were preincubated with 2µl mHSA (f.c.:100µg/ml) were added. Incubations were performed in a volume of 20µl. For investigations with fixed platelets, platelets were incubated with 2µl of 10% formaldehyde for 15 minutes before the antibody was added.

For studies on unfixed platelets 1µl of anti CD40L was added immediately. Incubation was performed for 30 minutes at room temperature in the dark. Thereafter unfixed platelets were fixed with 2µl of 10% formaldehyde and incubated for 5 minutes or analyzed immediately.

2.2.7.3 Detection of activated glycoprotein IIb/IIIa

Activated glycoprotein IIb/IIIa (GPIIb/IIIa) was determined by PAC-1 antibody. 10µl of gel filtered platelets were incubated with 2µl ADP (f.c.:50µM), 2µl thrombin (f.c.:200U/ml), 2µl collagen (f.c.:100µg/ml), 2µl hypOxHDL (f.c.:100µg/ml) or 2µl hypOxLDL (f.c.:100µg/ml).

Incubations were performed in a volume of 20µl and 1µl of PAC-1 antibody was added to each probe. Incubation was performed in the dark for 30 minutes. Thereafter probes were fixed with 2µl of 10% formaldehyde and analysed immediately.

2.2.7.4 Quantification of VASP phosphorylation

To determine the impact of lipoproteins on VASP phosphorylation, 30µl of gel filtered platelets were incubated with 100µg/ml to 300µg/ml nHDL, hypOxHDL or hypOxLDL, which amounted to a volume between 2µl and 10µl, whereat volume was adjusted to 40µl in all probes by PBS. After an incubation time of 10 minutes, 10µl of different concentrations of PGE_1 (ranging between 1nM to 100µM) were added to the probes. Platelets were incubated for exactly two minutes, afterwards cells were fixed by adding 5µl of 10% formaldehyde. To detect intracellular Ser 239 VASP phosphorylation, cells were permeabilized with 80µl of 0.2% triton. After 10 minutes cells were centrifuged (1700g for 10min) and the pellet was resuspended in 50µl PBS. 5µl of anti-phospho-VASP antibody were added to each probe and platelets incubated for one hour in the dark.

Unbound antibody was eliminated by centrifugation (1700g, 10min) and resuspension of platelets in 40µl PBS. Subsequently, 4µl of secondary FITC labelled anti-mouse antibody were added. After 20 minutes of incubation, flow cytometric measurement was performed and signals were acquired in FL1.

2.2.7.5 Measurement of cytosolic free calcium

Free cytosolic calcium in platelets was measured by time-dependent flow cytometry. 300µl of platelet-rich plasma were incubated in the dark with 1µl Fluo4 and 2µl Fura-Red in the dark, at room temperature for 30 minutes. Afterwards, platelets were isolated by gel-filtration as described for platelet isolation. 20µl of gel filtered platelets were diluted in 500µl PBS in a FACS tube and put into the flow cytometer. After acquisition of basal calcium levels for one minute, the probe was removed, platelet agonists (between 5 and 10µl) were

added and put back into the cytometer immediately, so that data acquisition was continued as soon as practicable. Fluo-4 signals were acquired in FL1 and Fura Red in FL3 using BD Cell Quest Pro Software. Fluorescence ratio was calculated for each cell using WEASEL 2.3 Software[112].

2.2.8 Binding studies

2.2.8.1 Lipoprotein labelling with Alexa 633

Direct labelling of lipoproteins with Alexa 633 was performed with copper oxidised LDL (CuOxLDL) or native HDL, which were diluted in borate buffer (with 0.9%NaCl) to a final concentration of 1mg/ml. For labelling 500µl of the lipoprotein were incubated with 10µl Alexa 633 (solved in DMSO; f. c.: 10mg/ml) for one hour at room temperature in the dark and lipoproteins were shaken from time to time. After incubation, excess Alexa 633 dye was removed by size exclusion chromatography. Therefore the Alexa 633 lipoprotein mix was loaded on an Econ Pac column, equilibrated with isotonic borate buffer, and the flow-through was collected in 500µl fractions in test tubes. Alexa 633 lipoproteins could be detected easily as a bright blue fraction. Alexa 633 marked CuOxLDL or nHDL were used within one week. To avoid possible cellular uptake of lipoproteins in subsequent experiments, gel filtered platelets were pre-chilled for at least 30 minutes and kept on ice throughout the experimental procedure.

50µl of gel filtered platelets were put into eppendorf tubes, and lipoproteins in final concentrations between 5 and 325µg/ml (corresponding to a volume between 0.5µl and 20µl) were added. For displacement studies cells were coincubated with a 10- to 25-fold excess of unlabelled lipoproteins, 7µl mHSA (f.c.: 50µg/ml) or 1µl FA6.152 (f.c.: 3µg/ml) according to the experimental protocol.

Since the maximum volume amounted 70µl, all probes which had less volume were filled up to ensure the same cell count per volume for all experiments.

Incubation time ranged between two and three hours and was performed at 4°C. Thereafter cells were fixed with 7µl 10% formaldehyde, diluted with 420µl PBS and analyzed immediately by flow cytometry.

2.2.8.2 Indirect labelling of lipoproteins with antibodies against Apo A or Apo B

For indirect labelling of lipoproteins, hypOxLDL or hypOxHDL in final concentrations between 2.5µg/ml and 300µg/ml (ranging in volume from 0.5µl to 20µl) were added to 30µl of pre-chilled platelets (incubation for 30 minutes on ice) in an eppendorf tube and kept on ice.

To show displacement, probes were coincubated with mHSA (5µl which corresponds to a final concentration of 50µg/ml), FA6.152 (1µl which corresponds to a final concentration of 4µg/ml) or a different class of lipoproteins (between 5µl and 10µl, final concentrations between 100µg/ml and 300µg/ml).

The maximal volume of all probes amounted 50µl, therefore all probes where brought to a final volume of 50µl by adding PBS.

After two hours of incubation on ice, platelets were fixed with 5µl 10% formaldehyde, centrifuged (2000g, 90 seconds) and resuspended in 50µl PBS. HypOxLDL were incubated with 1µl antibody against Apo B and hypOxHDL with 1µl antibody against Apo A. After another hour of incubation, unbound antibody was removed by centrifugation (2000g, 90 seconds) and subsequent resuspension of platelets in 30µl PBS. Subsequently, platelets were incubated with 0.2µl of Alexa Fluor 488 labelled antibody against goat IgG, which binds anti Apo A and anti Apo B antibodies.

After an incubation time of 30 minutes, probes were transferred into FACS tubes filled with 470µl PBS and analyzed immediately. Apo A or Apo B positive platelets were detected in FL1 using BD Cell Quest Pro Software.

2.2.9 Statistical evaluation and graphics

Results are presented as mean values plus standard deviation. Platelet parameters were subjected to KS test for confirming normal distribution and to subsequent t-tests for unpaired samples using SPSS 16.0 and Microsoft Excel software. An error probability of less than 0.05 was considered as statistically significant.

Graphics of the calculated data were drawn with Sigma Plot 10.0. FACS figures were obtained directly from BD CellQuest Pro Software and edited with Adobe Photoshop CS2.

2.2.10 Calculation of results from flow cytometric binding studies

No quantitative data are available for the fluorescence yield of the bound fluorescently labelled ligand. Therefore, the factor of proportionality α was included in the equations.

The measured fluorescence includes specific and non-specific binding portions obeying the following equations used for fitting the binding curves:

$$F_{cal} = \alpha * B + \alpha * k * L_t = \text{ specific binding (with B given in equation 2)}$$
$$+ \text{ non-specific binding}$$

(1)

$$B = \frac{B_{max} + L_t + Kd}{2} - \sqrt{\left(\frac{B_{max} + L_t + Kd}{2}\right)^2 - B_{max} * L_t}$$

(2)

F_{cal} .... calculated fluorescence
α............ factor correlating fluorescence yield and concentration of labelled ligand
B .... bound ligand
k............ constant for non-specific binding
L_t .... ligand concentration (bound + free)
B_{max} ...... maximal bound ligand
Kd......... dissociation constant

3   Results

3.1   Establishing platelet-associated techniques

3.1.1 Platelet count

Determination of exact platelet count is necessary for most types of experiments to assure precision, reproducibility and comparability of results. Therefore, platelets are normally counted under a microscope in a Bürker and Türk counting chamber. Since this method is time intensive, less time consuming photometric measurement of platelet count by the method of Walkowiak[113] was implemented and evaluated for its precision and comparability with manual microscopical count. To do so, the number of platelets from ten different donors was determined by both techniques. Each probe was analysed in triplicate, thereafter means were calculated and the results compared between the two different methods. The mean platelet count obtained by microscopy was calculated to be 361 582 platelets/µl (± 55 400), while photometrical determination led to a number of 375 179 platelets/µl (± 64 325) (see Figure 1). No significant difference between results obtained by these two methods can be observed (p>0.05) and therefore photometrical platelet count is used in all experiments.
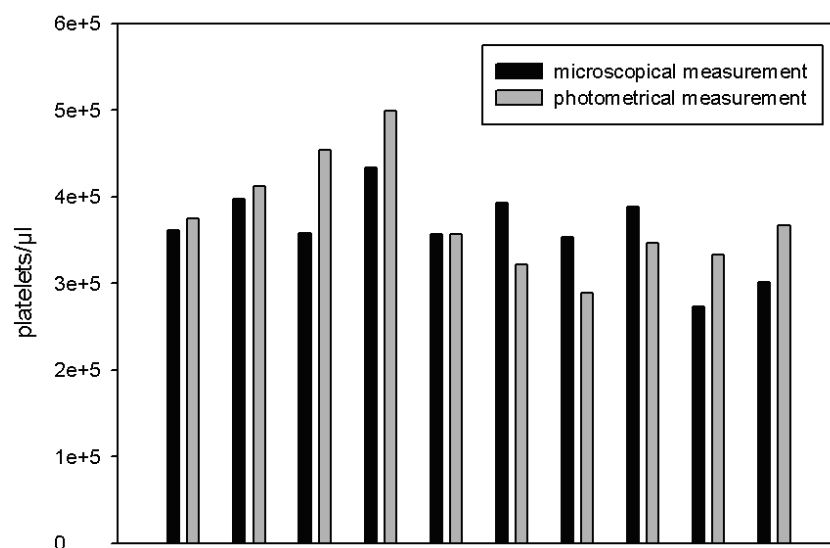
Figure 1: Platelet count: microscopical versus photometric measurement
   Platelets from 10 different donors; means of three dilutions per donor shown for each
   method

3.1.2 Studies on platelet isolation

3.1.2.1 Quality aspects of platelets isolated by different techniques

Isolated platelets can be obtained either by washing procedures (basing on sedimentation and resuspension steps) or by gel filtration. Platelets which are isolated by gel filtration always show similar platelet count as estimated in whole blood of the same donor, whereas washing method of platelets allows adjustment of platelet number per volume, since pelleted platelets are obtained that can be resuspended in a volume of choice. While preparation time is comparable between the two methods, washing method is less cost-intensive. Nevertheless, gel filtration has a big advantage over washing method, since no addition of platelet antagonists is necessary during the platelet isolation procedure. This provides a big merit, since gel filtered platelets therefore represent the in vivo state more accurate than washed platelets.

To evaluate the two isolation techniques in terms of basal platelet activation and platelet responsiveness to agonists, washed platelets (WP) and gel filtrated platelets (GFP) were compared to platelets in platelet rich plasma (PRP) for their basal surface expression of P-selectin (an indicator for platelet activation status) and the surface expression of P-selectin upon activation with 50μM ADP. As depicted in Figure 2, washed platelets show a slightly higher basic activation than GFP and PRP. Nevertheless, the amount of this increase in surface expression of P-selectin was not significant. After addition of 50μM ADP, increase of P-selectin was significantly lower in washed platelets than in gel filtered platelets and platelet rich plasma ($p<0.05$), which indicates that platelets isolated by washing method are no longer fully responsive to ADP.

In terms of functionality, estimated by surface expression of P-selectin in response to submaximal concentrations of ADP, washed platelets also show a significant different response to this agonist than gel filtered platelets ($p<0.05$). Surface expression of P-selectin induced by ADP concentrations between 1μM and 5μM is significantly decreased in WP compared to GFP (shown in Figure **3**). Taken together, platelets isolated by gel filtration can be regarded as fully resting and fully functional and were therefore used for functional studies performed in this work.
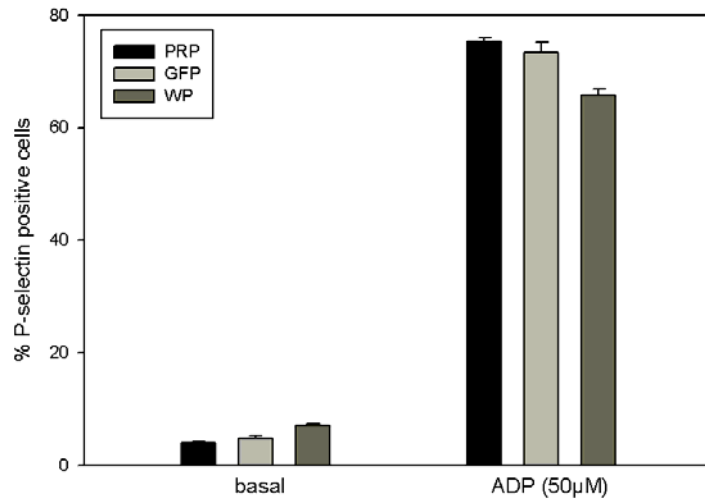
Figure 2: Surface expression of P-selectin following platelet isolation
    Results of flow cytometric analysis of surface P-selectin expression in platelet rich plasma (PRP), gel filtered platelets (GFP) and washed platelets (WP) before and after addition of ADP (50µM); mean and standard deviations of results from 10 experiments
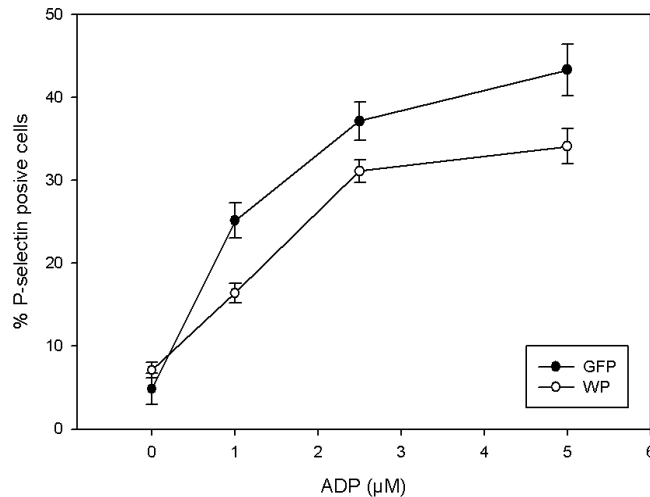

Figure 3: Influence of platelet isolation technique on surface expression of P-selectin
    Results of flow cytometric analysis of surface P-selectin in gel filtered platelets (GFP) and washed platelets (WP) after addition of submaximal concentrations of ADP; means and standard deviations of results from 5 experiments

3.1.2.2 Relevance of human serum albumin during platelet isolation

After establishing gel filtration as the superior technique for platelet isolation, attempts to optimize and economise this technique were made.

Human serum albumin (HSA), a common ingredient in isolation buffer, is time- and cost intensive in production, especially in the case of pure HSA that is used in our laboratory. To economise the costs of experiments, platelets were isolated in tyrode HEPES buffer containing different amounts of HSA as well as in buffer without HSA and the effects of buffer HSA concentration on platelet reactivity were evaluated.

Tyrode HEPES buffer was used containing 0.5% HSA, 0.25% HSA or 0% HSA.

A buffer HSA concentration of 0.25% or buffer lacking HSA led to a significant reduction in platelet count compared to PRP ($p < 0.05$). At a final concentration of 0.25% HSA in isolation buffer, platelet count was reduced to 69% ($\pm$ 6.4%) of platelet count observed in PRP, whereas HSA free buffer led to a loss of 78% ($\pm$ 8.7%) of platelets. Platelet count of gel filtered platelets, obtained with isolation buffer containing 0.5% HSA and platelet count in PRP was almost identical, which demonstrates the importance of the presence of HSA during platelet isolation.

As the striking differences in platelet count might be a consequence of platelet activation occurring within the isolation column, basal platelet activation and platelet reactivity after isolation in the presence of different concentrations of HAS was determined. To do so, surface expression of P-selectin was measured immediately after collection of platelets and after incubation with ADP (50µM). Surprisingly, no significant difference, neither in basal activation state nor in platelet reactivity could be detected between the different platelet preparations isolated with different HSA concentrations in tyrode buffer (shown in Figure 4). As platelet activation and platelet adhesion occurring in the isolation column would be supposed to influence neighbouring platelets (what would be considered even more likely as eluted platelets are obviously in a fully functional state), the reasons for the observed differences in platelet count remain curious. In summary it can be stated that HSA seems to mediate a beneficial impact in the course of platelet isolation by gel filtration and should therefore be present

throughout the isolation procedure. As a consequence, isolation buffer containing 0.5% HSA, was used for the experiments performed within this work.
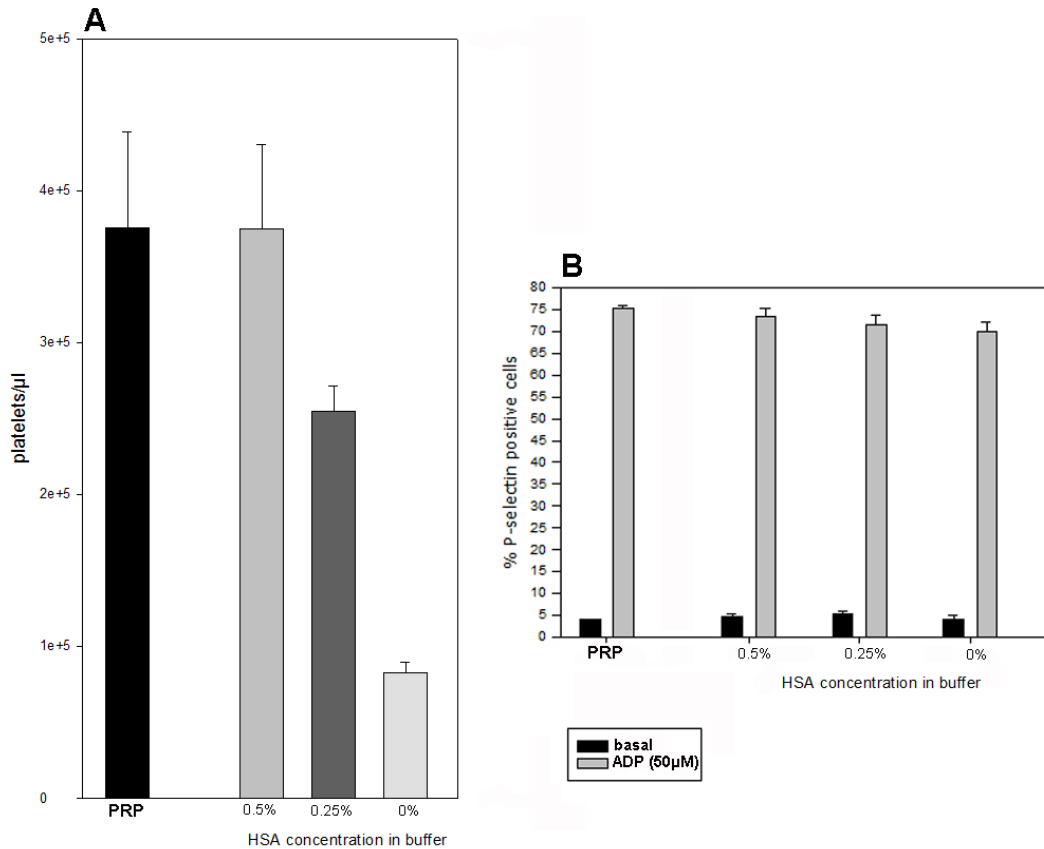

Figure 4: Influence of HSA concentration in isolation buffer on platelet count, basal platelet activation and platelet reactivity

> **A: Influence of HSA concentration in isolation buffer on platelet count**
> PRP: 375 513 platelets/µl (± 63 545)
> 0.5% HSA: 375 179 platelets/µl (± 55 400)
> 0.25% HSA: 255 122 platelets/µl (± 16 328)
> 0% HSA: 82 539 platelets/µl (± 7 180)
> Means and standard deviations of results from 10 experiments
>
> **B: Influence of HSA concentration in isolation buffer on platelet P-selectin expression**
> Results of flow cytometric analysis; means and standard deviations of results from 10 experiments

3.1.3 Submaximal platelet activation by ADP, thrombin and collagen

For several functional platelet studies (especially when determining the impact of protective agents or synergistic actions of weak agonists) it is important that platelet activation is induced to only a submaximal extent. Therefore, submaximal concentrations of classical platelet agonists (ADP, thrombin and collagen) were ascertained.

Platelets from ten different donors were tested for their sensitivity towards the mentioned agonists, in order to determine the appropriate concentrations for submaximal platelet activation.

As shown in Figure 5A, platelets start to show surface expression of P-selectin (indicating degranulation of α-granules as a consequence of platelet activation) at a concentration of 1.25µM ADP. Full platelet activation is induced by ADP concentrations of 15µM and above.

Therefore, ADP concentrations between 1.25µM and 15µM were used in experiments where submaximal platelet activation was needed. Results of similar experiments obtained with thrombin are shown in Figure 5B: no significant platelet activation can be observed at a concentration of 0.05U/ml thrombin, while activation is almost fully induced at thrombin concentrations of 20U/ml. Hence, thrombin concentrations between 0.05U/ml and 20U/ml were employed. Submaximal activation by collagen occurs above a concentration of 1µg/ml and below 20µg/ml collagen (depicted in Figure 5C).
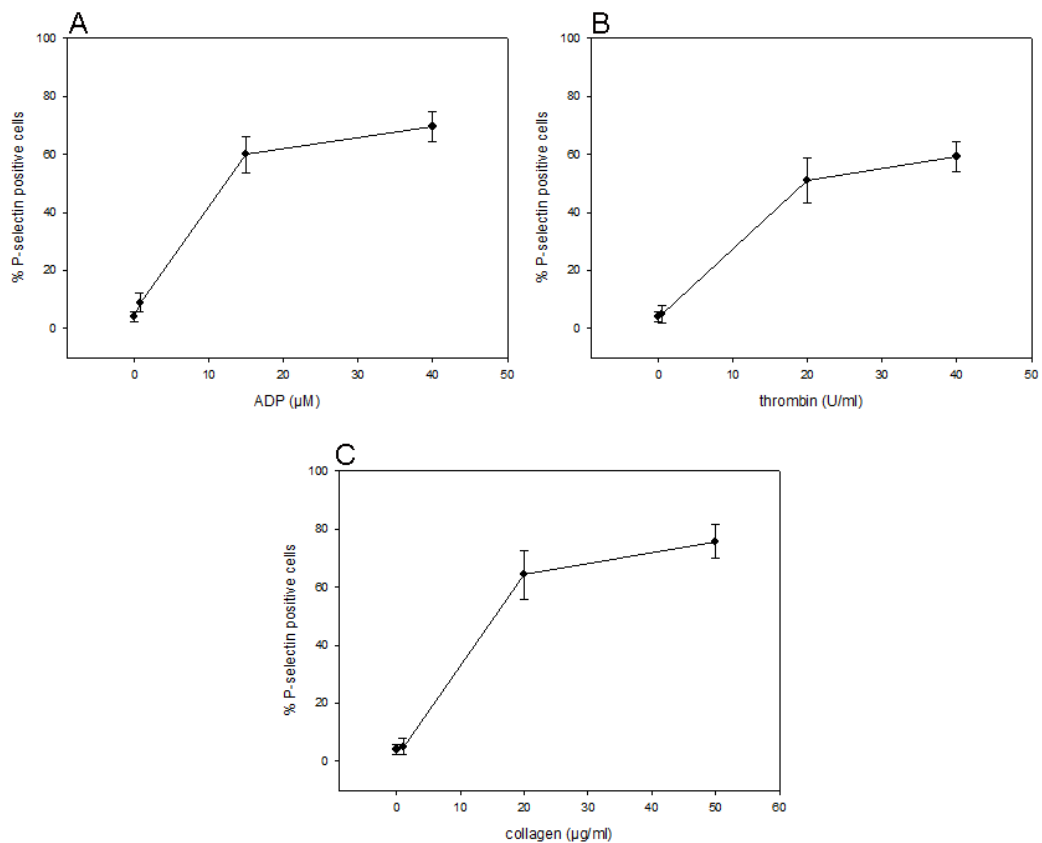
Figure 5: Surface expression of P-selectin induced by different agonists
Results of flow cytometric analysis of gel filtered platelets
A: ADP (0-40μM)
B: thrombin (0-40U/ml)
C: collagen (0-50μg/ml)
Means and standard deviations of results from 10 experiments

3.1.4 Platelet aggregation studies

Optical platelet aggregometry is still accepted as the "gold standard" assay for platelet function studies in response to agonists. The basis of measurement is the decrease in optical density which occurs in suspension as platelets aggregate. This change recorded as a function of time is represented graphically as a platelet aggregation curve.

Optical platelet aggregometry is usually performed in an aggregometer, in which platelet suspension is constantly stirred and kept at a temperature of 37°C. It records a dynamic measure of light transmission induced by platelet aggregation. Alternatively platelet aggregation can be measured using a

microplate reader, which also detects changes in light transmission, but instead of constantly stirring the platelet suspension, the whole plate is shaken. The big advantage of the microplate reader technique is its higher throughput rate and the smaller amount of platelets needed. Since platelets are treated differently in an aggregometer and a microplate reader, the two methods had to be evaluated for the comparability of their resulting aggregation curves. Therefore the effects of different concentrations of ADP (10µM to 50µM) on platelets were compared between the two methods. As shown in Figure 6, no significant difference in aggregation course could be detected between the two methods. Not only the intensity of platelet aggregation but also the time pattern show very good correlation between the two methods.
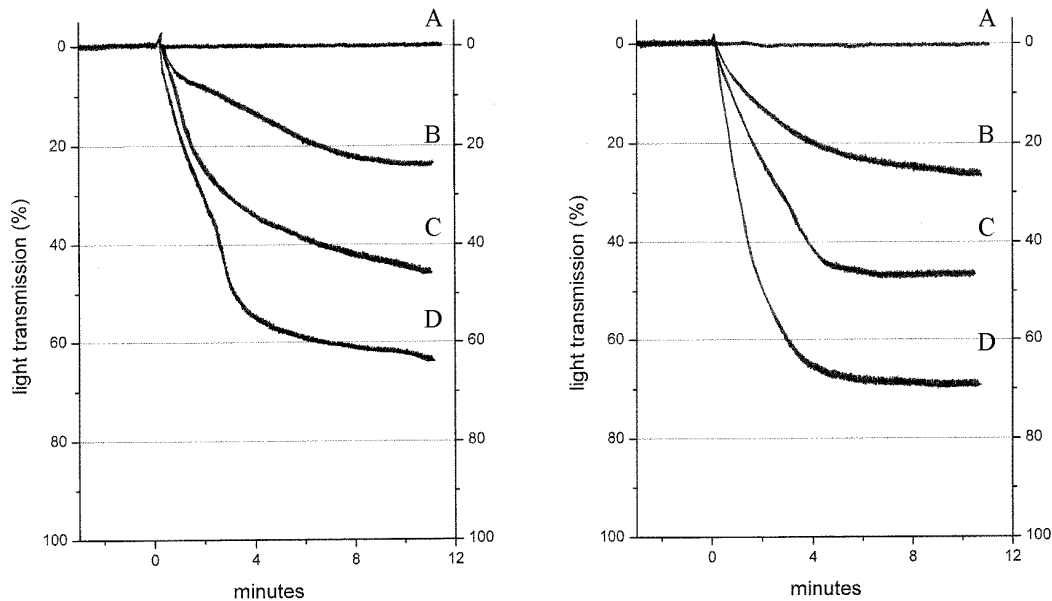
Figure 6: Platelet aggregation: aggregometer versus microplate reader
Aggregation curves, showing aggregation response of GFP after addition of agonists
left side: classical aggregometer; **right side:** microplate reader
A: control
B: ADP (final concentration 10µM)
C: ADP (final concentration 20µM)
D: ADP (final concentration 50µM)

3.1.5 Quantification of CD40L on platelets

3.1.5.1 CD40L staining in whole blood, PRP and GFP

CD40L, an important platelet-derived mediator of immune response, is expressed on the cell surface upon platelet activation. Since CD40L antibody had not been used in our laboratory so far, detection of surface expression of CD40L under basal and activated conditions was compared between whole blood, platelet rich plasma and gel filtered platelets.

First, basal surface expression of CD40L was compared between unfixed platelets in whole blood, PRP and GFP. Results of these experiments are depicted in Figure 7A, showing the lowest CD40L level in whole blood and the highest in GFP. Upon activation with ADP (50μM), significant increase of CD40L could be detected in platelets in all three environments, whereas GFP again showed the highest and whole blood the lowest signal. In Figure 7B the calculated relative increase after activation by ADP is shown. Basal CD40L expression was set for 100% and the relative increase of each fraction was calculated, showing no statistical difference between the three environments (p>0.05). Since the observed differences between resting and activated platelets in CD40L surface expression are statistically not significant, GFP were used for the following studies.
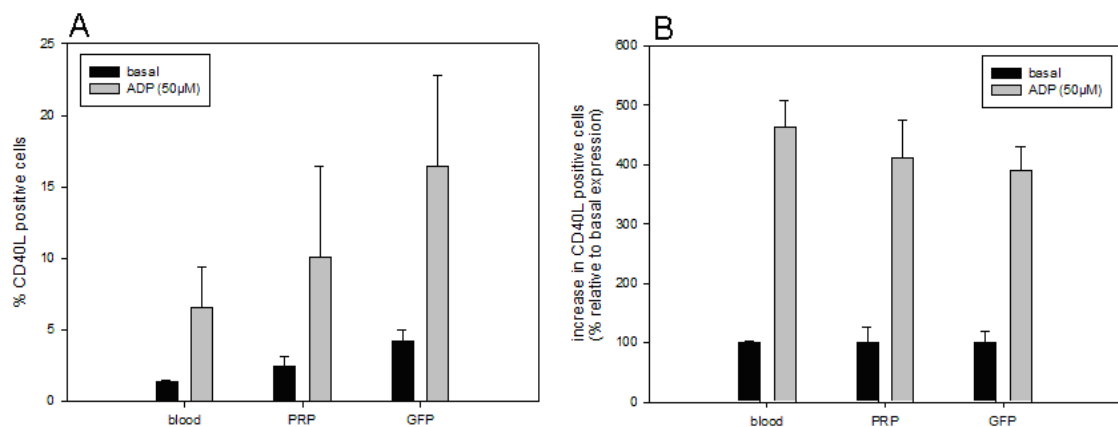
Figure 7: Surface expression of CD40L of unfixed platelets in blood, PRP and gel filtered platelets
A: CD40L positive platelets before and after addition of ADP (50μM)
B: relative increase in the number of CD40L positive platelets after addition of ADP (50μM)
Means and standard derivation of results from 6 experiments

3.1.5.2 Platelet fixation by formaldehyde abolishes CD40L antibody binding

To optimize the method used for CD40L surface staining, unfixed and fixed platelets were compared. Cell fixation facilitates probe handling by assuring constant conditions, but some antibodies show far lower affinities to their binding sites after fixation. Therefore binding of CD40L antibody to fixed (1% formaldehyde) and unfixed platelets was compared.

Platelets, freshly obtained by gel filtration, were either left untreated or fixed with formaldehyde (1%) for 15 minutes. Thereafter fixed and unfixed platelets were incubated with FITC labelled CD40L antibody. As shown in Figure 8, the fluorescence signal for CD40L positive cells was almost identical between fixed and unfixed platelets, although standard deviation was much higher in fixed cells. To evaluate CD40L increase induced upon activation, platelets were incubated with ADP (50µM) prior to fixation. As shown in Figure 8, an increase in fluorescence signal could only be detected in the unfixed fraction. Therefore, unfixed platelets were used for CD40L-related experiments performed within this work.
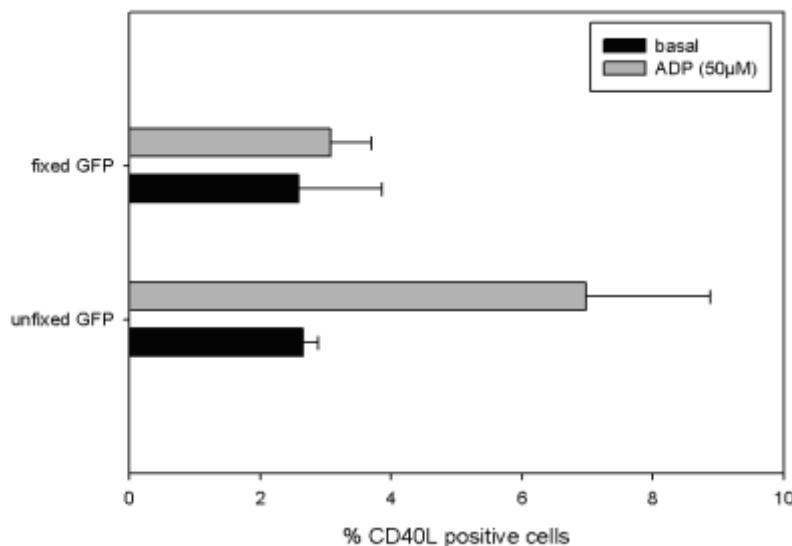
Figure 8: Influence of platelet fixation with formaldehyde on anti CD40L binding
Results from flow cytometric analysis of fixed and unfixed gel filtrated platelets ± ADP (50µM); Means and standard deviations of results from 6 experiments

3.2 Characterisation of modified lipoproteins

3.2.1 Relative electrophoretic mobility

To characterise (both chemical and oxidative) lipoprotein modification, relative electrophoretic mobility (REM) of native and modified lipoproteins was compared. Lipoproteins have a negatively charged surface and migrate to the anode in agarose gel electrophoresis under non denaturing conditions. Oxidation, by modification of amino acids, renders lipoproteins more negatively charged and accordingly their electrophoretic mobility increases. A measurable index of this is the REM, which is the ratio of migration distance of native to that of modified lipoproteins[114]. REM provides a very reliable way to quantify protein modification in the course of lipoprotein oxidation. Since platelet lipoprotein interactions seem to be mediated by apolipoprotein dependent binding, evaluation of modifications of the protein moiety is very important. REM was used to evaluate the extent of lipoprotein modification induced by different molar excess of oxidant hypochlorite and for studies regarding the comparability of lipoprotein modification by copper or hypochlorite. Although the in vivo relevance of copper oxidised lipoproteins is contested, this modification was used in binding studies with directly fluorescence labelled lipoproteins, since hypOxLDL bear bleaching properties and destroy the employed fluorophor. As shown in Figure 9, CuOxLDL and hypOxLDL (oxidised by a 300-fold molar excess of hypochlorite over LDL) were comparable in their REM and therefore used in comparative studies.
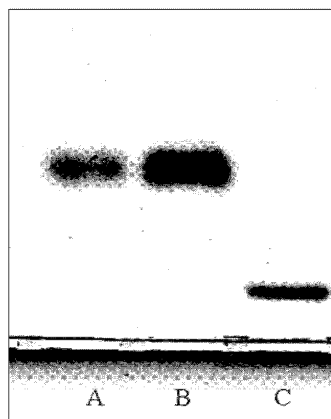

Figure 9: Typical electrophoretic mobility of LDL
  A: hypOxLDL
  B: CuOxLDL
  C: native LDL
  Results of one typical experiment shown

In similar studies with high density lipoproteins, REM was mainly used to quantify the extent of modification. It could be shown that the relative electrophoretic mobility of HDL rose with severity of modification, whereas treatment of hypOxHDL with methionine (that has been reported to result in the elimination of protein-associated chloramines) resulted in a decrease of REM (shown in Table 2). These findings are in accordance with the results of further analysis of changes in the protein moiety of modified HDL.

3.2.2 Protein analytics

For further analysis and characterization of the protein moiety of oxidised HDL, the influence of oxidation by hypochlorite on the formation of chloramines and the number of free amino groups was determined.

As shown in Table 2, the number of free free amino groups declined depending on the severity of lipoprotein modification: while a 75-fold molar excess of hypochlorite reduced free amino groups to 50% (± 21), a 300-fold molar excess of hypochlorite over HDL led to a reduction to 15% (± 5). Free amino groups were partly restored upon treatment of hypOxHDL with methionine (increase from 15% (± 5) to 55% (± 7)).

In addition, the influence of hypochlorite modification of HDL on chloramine formation was determined. Number of chloramines within HDL increased dose-dependently on hypochlorite modification and treatment of oxidised lipoproteins with methionine showed strong reduction of chloramine number, with a decrease from 62.6 (± 13.7) to 9.8 (± 5.4) chloramines per HDL.

	nHDL	hypOxHDL[75]	hypOxHDL[150]	hypOxHDL[300]	methionine -hypOxHDL[300]
free amino groups (%)	100	50.6 ± 21.2	30.4 ± 17	15.4 ± 5.1	55.4 ± 7.3
REM	1	1.2 ± 0.1	1.5 ± 0.1	1.7 ± 0.2	1.3 ± 0.1
Chloramines per HDL	0	24.2 ± 4.4	43.3 ± 7.4	62.6 ± 13.7	9.8 ± 5.4

Table 2: Physico-chemical properties of HDL
Values in squared brackets indicate the molar excess of hypochlorite over HDL; means and standard deviations of results from 8 experiments

3.3   Influence of native HDL on platelet reactivity

3.3.1 Native HDL are able to impair ADP-induced platelet aggregation

It has been demonstrated by several groups that HDL are able to influence platelet reactivity in that they are able to interfere with platelet activation induced by various agonists[86]. To support and extend the current knowledge on the action of native HDL on human platelets, the influence of HDL on platelet reactivity was investigated, starting with studies regarding the influence of HDL on platelet aggregation. Therefore gel filtered platelets were preincubated with nHDL and their response to ADP in concentrations which led to submaximal (5µM ADP) and maximal (50µM ADP) platelet aggregation was compared to effects seen after addition of ADP in the absence of HDL.

As shown in Figure 10, nHDL could be proven to attenuate aggregatory effects of ADP on platelets. Aggregation induced by submaximal concentrations of ADP (5µM) was partly prevented by preincubation of platelets with nHDL. If platelet aggregation was induced to a maximum extent (f.c.: ADP: 50µM), nHDL showed no influence on intensity of aggregation.
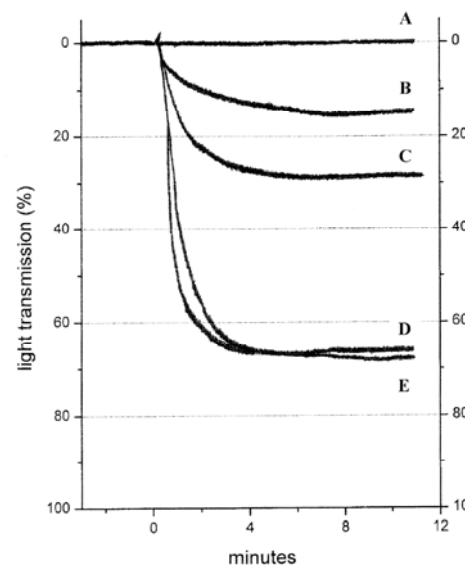
Figure 10: Influence of nHDL on platelet aggregation induced by ADP
Aggregation curves, showing aggregation response of platelets after addition of:
A: nHDL (260µg/ml)
B: nHDL (260µg/ml) + ADP (final concentration 5µM)
C: ADP (final concentration 5µM)
D: nHDL (260µg/ml) + ADP (final concentration 50µM)
E: ADP (final concentration 50µM)

3.3.2 Protective role of native HDL on ADP-induced surface expression of P-selectin

Since aggregation studies revealed a protective influence of nHDL on platelet aggregation induced by ADP, further analyses were performed to determine the influence of HDL on the surface expression of several proteins specific for platelet activation. First, studies regarding a potential attenuation of surface expression of P-selectin by HDL were performed. Platelets were preincubated with nHDL, thereafter different amounts of ADP were added and the impact of ADP on surface expression of P-selectin was compared with results from identical experiments without nHDL. As shown in Figure 11C, surface expression of P-selectin induced by ADP (1µM to 5µM) was significantly reduced upon preincubation of platelets with native HDL. Again, at higher ADP concentrations nHDL showed no protective impact on platelet activation (data not shown). Figure 11A and Figure 11B depict FACS dot plots of a typical experiment, which shows the impressive protective impact of nHDL on P-selectin expression after addition of ADP.
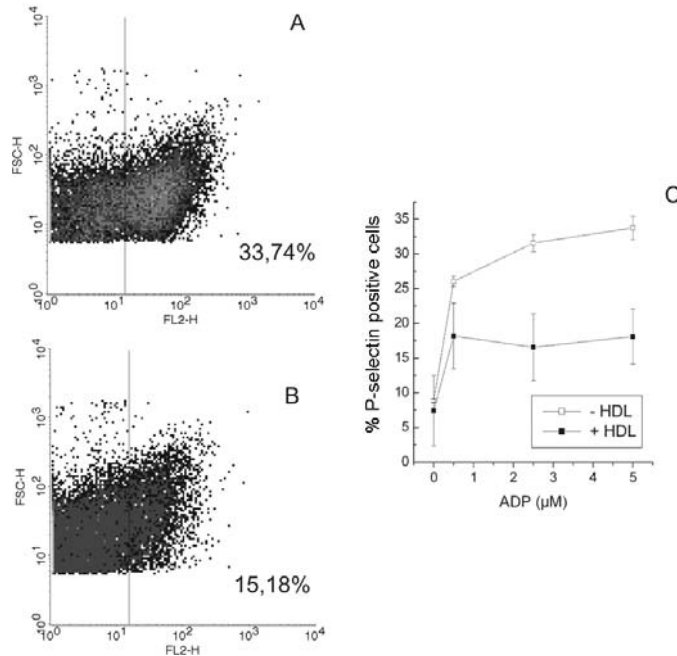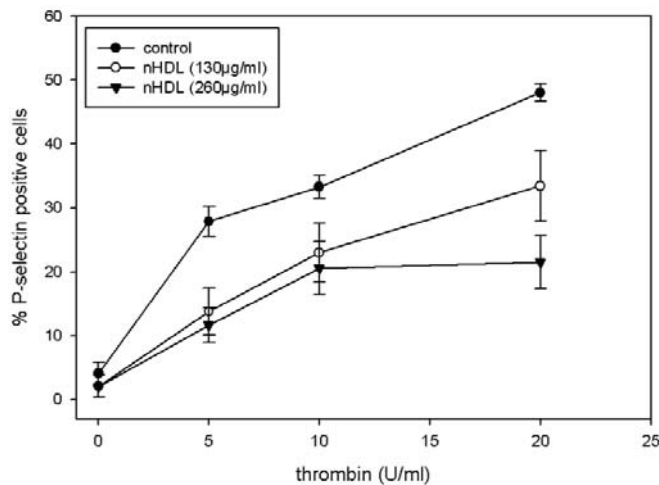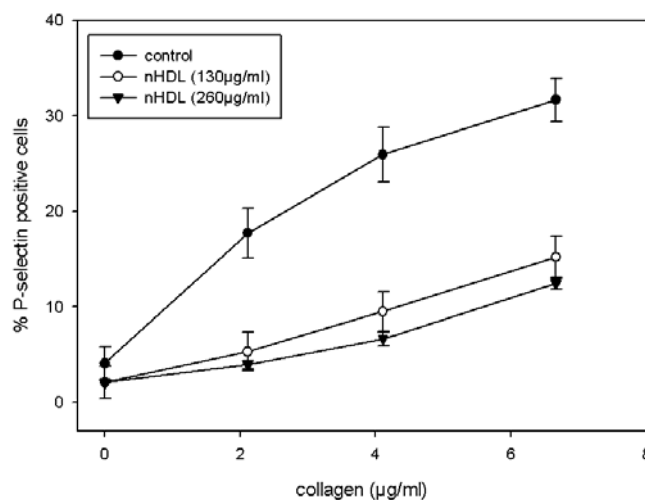

Figure 11: Influence of nHDL on ADP-induced surface expression of P-selectin
Results of flow cytometric experiments with platelets stained for P-selectin (PE), detected in FL2; percentage of P-selectin positive cells id indicated in the lower right
A: dot plot of a typical experiment: ADP (2.5µM)
B: dot plot of a typical experiment: nHDL (260µg/ml) + ADP (2.5µM)
C: means and standard deviation of results from 16 experiments

3.3.3 Protective role of native HDL on thrombin- and collagen- induced surface expression of P-selectin

To evaluate if the protective influence of nHDL on platelet activation was limited to platelet activation by ADP, identical experiments were carried out with platelet agonists thrombin and collagen. Consistent with results of studies on attenuation of ADP-induced surface expression of P-selectin, nHDL were able to significantly impair α-granule release by both agonists, which is shown in Figure 12 and Figure 13.

Figure 12: Influence of nHDL on thrombin-induced surface expression of P-selectin
Results of flow cytometric experiments with platelets; nHDL from 4 different donors; means and standard deviations of results from 9 experiments

Figure 13: Influence of nHDL on collagen-induced surface expression of P-selectin
Results of flow cytometric experiments with platelets; nHDL from 4 different donors; means and standard deviations of results from 9 experiments

3.3.4 Influence of native HDL on ADP-induced surface expression of granulophysin

Granulophysin represents another granule-derived protein that is expressed on the platelet surface only after degranulation. In contrast to P-selectin, granulophysin derives from dense granules and hence, an increase in granulophysin is associated with dense granule release. To further evaluate the role of nHDL on platelet function, the impact of preincubation of platelets with nHDL on surface expression of granulophysin was quantified in platelets that were then activated with submaximal concentrations of ADP.

Gel filtered platelets were either incubated with nHDL or left untreated. Thereafter submaximal concentrations of ADP (1.25M to 25µM) were added and both platelet fractions compared for their surface expression of granulophysin. As shown in Figure 14, ADP dose dependently increased surface expression of granulophysin. After preincubation of platelets with native HDL, granulophysin expression was significantly lowered (p<0.05). Therefore it can be concluded that nHDL are not only able to impair ADP-induced α- granule release but also reduce dense granule release induced by this agonist.
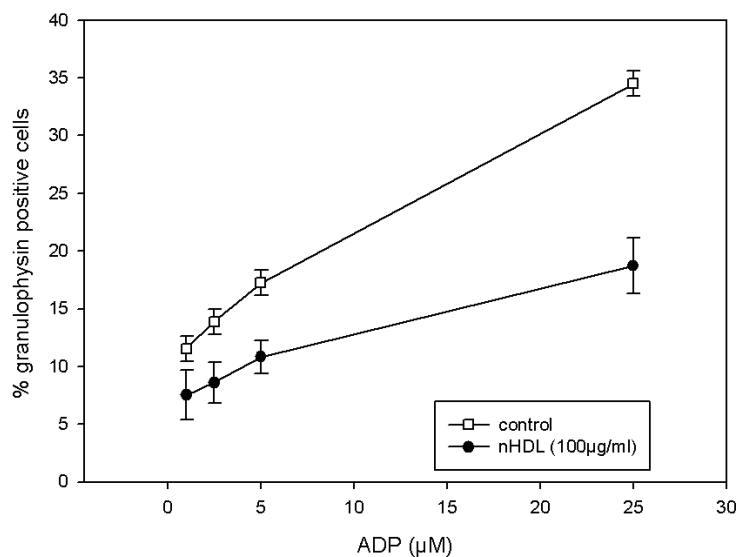

Figure 14: Influence of nHDL on ADP-induced surface expression of granulophysin
Results of flow cytometric experiments with platelets; nHDL from 3 different donors; means and standard deviations of results from 12 experiments

3.3.5 Influence of native HDL on VASP phosphorylation

Determination of intracellular VASP phosphorylation represents a quite novel test that is usually used to determine the sensitivity of platelets towards different agents which counteract platelet activation (NO, PGE_1, PGI_2). The more VASP protein is phosphorylated, the weaker the capability of human platelets to become activated. Therefore, determination of VASP phosphorylation might also provide additional insights into the protective effects of lipoproteins on platelets. By now, there are no data available on (potential) effects of nHDL on VASP phosphorylation.

Since solitary nHDL failed to have an impact on serine 239 VASP phosphorylation (data not shown), submaximal concentrations of PGE_1 were included in these experiments to evaluate potential summative effects of HDL and PGE_1.

Following preincubation of platelets with nHDL (130µg/ml or 260µg/ml), different concentrations of PGE_1 (0.6nM-2.5nM) were added and effects on VASP phosphorylation were quantified and compared with results obtained with platelets under the same experimental conditions, but in the absence of nHDL.

As shown in Figure 15, both employed concentrations of nHDL led to an increase of intracellular VASP phosphorylation (compared to control), whereat the higher nHDL concentration led to higher phosphorylation response.

Although these results tend to demonstrate VASP phosphorylation by nHDL, no significant difference in VASP phosphorylation between platelets with nHDL and controls could be determined ($p > 0.05$). In light of the high standard deviations resulting from these experiments, sample size might be too small to obtain statistically significant results.

At higher concentrations of PGE_1, no influence of nHDL on VASP phosphorylation could be observed.

Notably, there was a remarkable variation in the platelet response to different PGE_1 concentrations between different donors in terms of VASP phosphorylation. Some experiments were not able to show effects of minor concentrations of PGE_1 on VASP phosphorylation and consequently no effects of nHDL on PGE_1-induced VASP phosphorylation occurred. Moreover, not all

charges of nHDL were able to cause an increase in VASP phosphorylation. The number of experiments was too low to analyse gender or life style (smoking, etc.) specific effects. But further experiments, which take these factors into account, are planned.

So far it can be stated that nHDL from most donors show a inhibitory effect on platelets by increasing PGE$_1$-induced VASP phosphorylation, whereat the reasons for non-responsiveness of platelets and the ineffectiveness of nHDL from some donors, require further investigations.
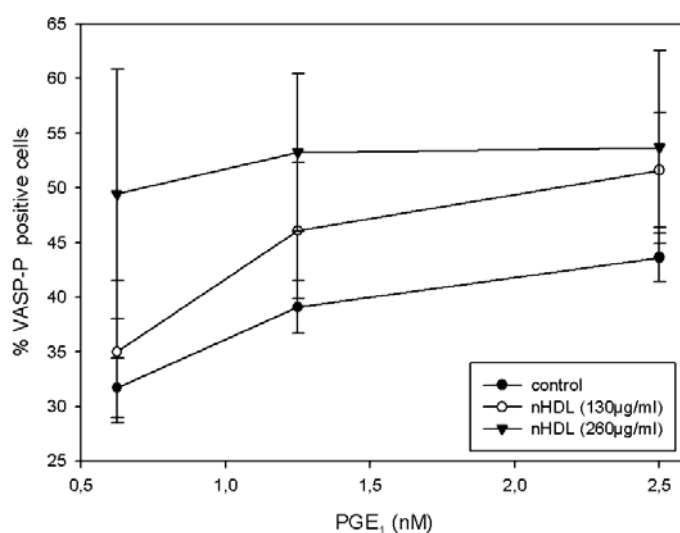
Figure 15: Influence of nHDL on VASP phosphorylation induced by PGE$_1$
> Results of flow cytometric experiments with platelets ± nHDL (130μg/ml or 260μg/ml); VASP-P stands for Ser 239 VASP phosphorylation; nHDL from 3 different donors; means and standard deviations of results from 8 experiments

3.3.6 Differences in VASP phosphorylating effects between HDL$_2$ and HDL$_3$

Since HDL subclasses HDL$_2$ and HDL$_3$ differ in their Apo E content, these lipoprotein subclasses might also differ in their potential to induce VASP phosphorylation, as increased levels of cGMP lead to VASP phosphorylation and Apo E has been reported to cause upregulation of cGMP in human platelets[88].

Differences in the influence of HDL subclasses on serine 239 VASP phosphorylation were determined by comparing the additive effects of PGE_1 (1nM-10nM) and both lipoprotein subclasses.

As shown in Figure 16, coincubation of platelets with both HDL subclasses induced an increase of intracellular VASP phosphorylation. Surprisingly, HDL_3, which contains less Apo E, seems to have a stronger influence on VASP phosphorylation than HDL_2, although differences were statistically not significant ($p > 0.05$).

At higher concentrations of PGE_1 (or without PGE_1) neither HDL_2 nor HDL_3 are able to induce VASP phosphorylation (data not shown).

Taken together, it can be stated that both subclasses show a tendency to favour VASP phosphorylation induced by PGE_1, whereat the effects obviously can not be attributed to the Apo E content of the lipoproteins.
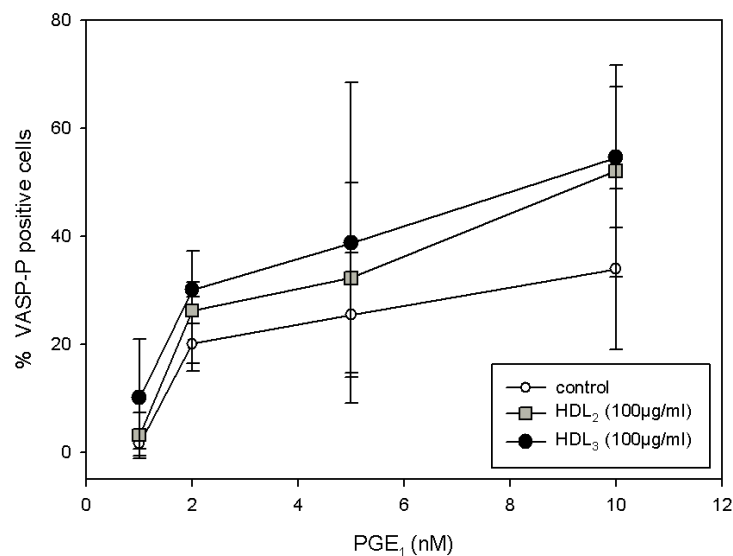
Figure 16: Influence of nHDL-subclasses on VASP phosphorylation in the presence of PGE_1

Results of flow cytometric experiments with platelets ± HDL_2 (100µg/ml) or HDL_3 (100µg/ml); VASP-P stands for Ser 239 VASP phosphorylation; nHDL from 4 different donors; means and standard deviations of results from 12 experiments

3.3.7 Binding of native HDL to human platelets

To answer the question if nHDL specifically bind to human platelets, two different methods were used: binding of lipoproteins to platelets was investigated by direct labelling of nHDL with Alexa Fluor 633 dye and by indirect labelling of platelet bound nHDL with an antibody directed against Apo A-I.

To make the obtained results comparable between the different experimental conditions, identical platelet counts used within these experiments are essential – therefore, platelet count was adjusted and 3.15×10^8 platelets/ml were employed in all experiments.

Specific binding is no directly measurable quantity, since only total binding can be determined directly, which consists of specific and non-specific binding (and, eventually uptake of ligand).

Specific binding implicates binding to one or more class(es) of specific receptor(s), whereat affinity to other binding sites is marginal. Nevertheless, each ligand also shows some extent of unspecific binding to biological or artificial material. Therefore, unspecific binding has to be evaluated and subducted. This is usually done by adding unlabelled ligand in high (100- to 1000-fold) molar excess. Under these conditions, since all specific binding sites are occupied with the unlabelled ligand, remaining binding of the labelled ligand will represent its binding to unspecific sites.

An additional characteristic of specific binding is that binding shows saturable binding kinetics, as only a definite number of specific receptors for binding are available. As a consequence, at increasing concentrations of ligand, the observed increase in bound ligand will reach a plateau, whereas unspecific binding is not saturable.

3.3.7.1 Binding of Alexa Fluor 633 labelled native HDL to human platelets

The first attempts to investigate if nHDL show specific binding to human platelets were made with nHDL that had been labelled with Alexa Fluor 633 dye. Binding studies performed with labelled ligands are more direct and reduce error-proneness besides less laborious study procedures compared with other techniques. Nevertheless, conjugation of HDL with the fluorophor and

incubation in the course of lipoprotein-labelling might cause distinct modification of the protein moiety, leading to changes in receptor specifity of the labelled lipoproteins.

Binding of HDL was investigated by adding increasing concentrations of Alexa 633 labelled nHDL to gel filtered platelets, followed by flow cytometrical detection of platelet-associated fluorescence. To distinguish between unspecific and specific binding, platelets were additionally incubated with an excess of unlabelled nHDL from the same donor.

Figure 17A shows binding of Alexa 633 labelled nHDL to human platelets. Fluorescence signals were successfully reduced by coincubation with nHDL (250µg/ml).
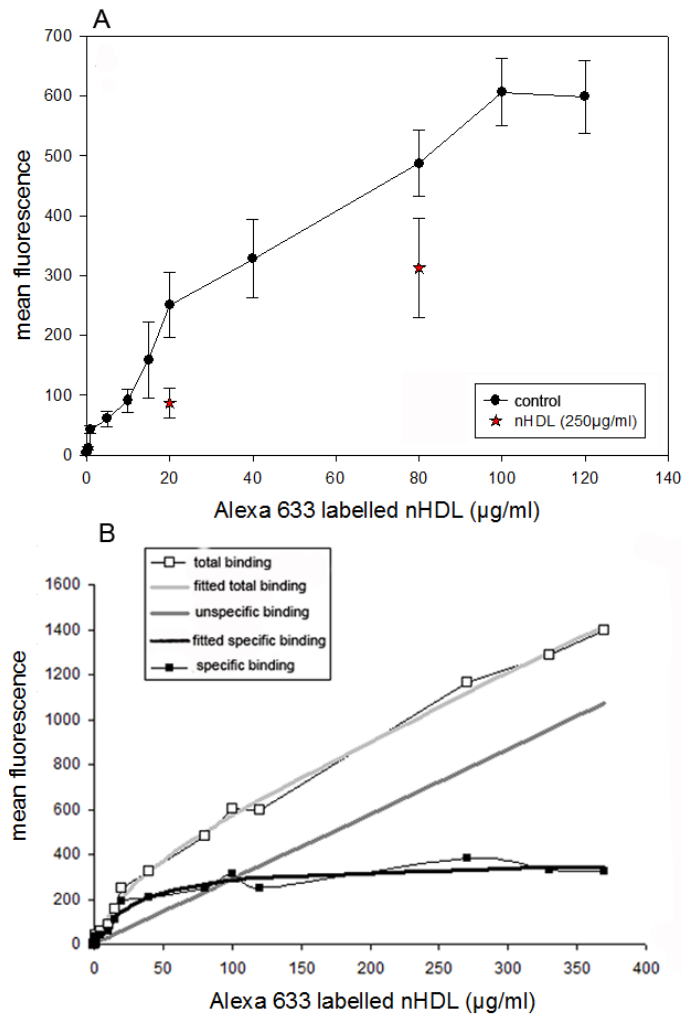
Figure 17: Total binding of Alexa Fluor 633 dyed nHDL to gel filtered platelets
    A: means and standard deviation of results from 8 experiments; nHDL from 2 different donors
    B: calculated total binding of data shown in **A**

Unfortunately, experimental conditions did not allow to exceed a 12.5-fold molar excess of unlabelled nHDL. Therefore, only incomplete displacement of labelled ligand by the competitor could be achieved what prevents quantitative insight into non-specific binding. Therefore, unlabelled nHDL were mathematically treated like labelled nHDL and their (supposed) fluorescence extrapolated (shown in Figure 17B). These data were fitted according to equation 1, leading to estimates for Kd (31.2µg/ml or 3.12×10^{-10}M, Bmax (0.209µg/ml) and k ($1.63*10^{-3}$), respectively. Due to the insufficient number of data points, however, these results are not very reliable. Therefore, another method to determine nHDL binding to platelets was implemented

3.3.7.2 Native HDL binding studies by indirect labelling of Apo A-I

Another way to determine specific binding of nHDL to platelets is to label platelet bound nHDL with an antibody against Apo A-I, which can then be detected by flow cytometry. This method ensures that lipoproteins are not modified in the course of the labelling procedure, but does not allow displacement by the same class of lipoprotein. Preliminary studies revealed that mHSA (which was originally reported to block binding of oxidised LDL to scavenger receptors) is able to compete with native HDL for binding to the platelet surface – therefore, mHSA was used for displacement of of nHDL.

Therefore gel filtered platelets were incubated with different concentrations of nHDL (in the presence or absence of mHSA) and analyzed for Apo A-I positive cells.

In Figure 18A binding of nHDL to platelets is shown together with reduction of nHDL binding upon coincubation with mHSA.

Unlike in experiments with fluorescence-labelled nHDL (see 3.3.7.1), binding of nHDL determined by labelled anti Apo A-I does not reveal linear (i.e. non-saturable) binding to any significant degree. One possible explanation might be that non-specific binding observed in experiments with directly labelled nHDL is mediated by distinct modification of the lipoprotein particle introduced by the modification process (either the fluorescence label itself, or, more likely, some additional oxidativr modification in the lipid or protein moiety). Accordingly, the

effects of maleylated human serum albumin require careful interpretation. In fact, mHSA successfully competes for binding of nHDL to human platelets. This effect is illustrated in Figure 18. Moreover, the calculated binding was fitted to equation 3, which allows calculation of the effects of an inhibitor (I) competing with ligand (L) for the same class of binding sites (shown in Figure 2B).

$$B = \frac{B_{max} + L_t + Kd \ * (1 + I / Ki\ )}{2} - \sqrt{\left(\frac{B_{max} + L_t + Kd \ * (1 + I / Ki)}{2}\right)^2 - B_{max} * L_t} \qquad (3)$$

B............bound ligand
B_{max}.......maximal bound ligand
L_t...........ligand concentration (bound + free)
Kd ....dissociation constant
I.............inhibitor
Ki ....dissociation constant of the inhibitor

Fitting the experimental data to this function, however, reveals that true competition obviously is not observed, since the total binding capacity for nHDL is reduced to about half the value in the absence of mHSA, whereas the binding affinity remains virtually unaffected.

This behaviour reflects non-competitive inhibition rather than competition for identical sites. One explanation could be that nHDL and mHSA bind to non-overlapping sites on the same protein on the platelet surface with mutual exclusion of binding of either of the two ligands after binding of the respective other one. From Figure 18 the dissociation constant for the mHSA-platelet receptor is calculated to be 2.10^{-5}M. The reported decrease in nHDL-binding in the presence of mHSA might, however, also reflect some sort of "quenching". Quenching effects might be generated by rearrangement in the platelet surface (e.g. receptor translocation into the platelet interior, either crosslinking of receptors or dissociation of receptor complexes into their components, or even degradation of receptor proteins), or a loss in cell count, which, however, can be ruled out in this case.

From the binding isotherms with and without mHSA an average dissociation constant for nHDL-binding was calculated (21.9µg/ml). With 2.19 x 10^{-7}M the

calculated Kd is only a third of the dissociation constant estimated by other groups[115]. Notably HDL show a higher affinity to platelets than oxidised LDL (calculated in chapter 3.6.10.2).
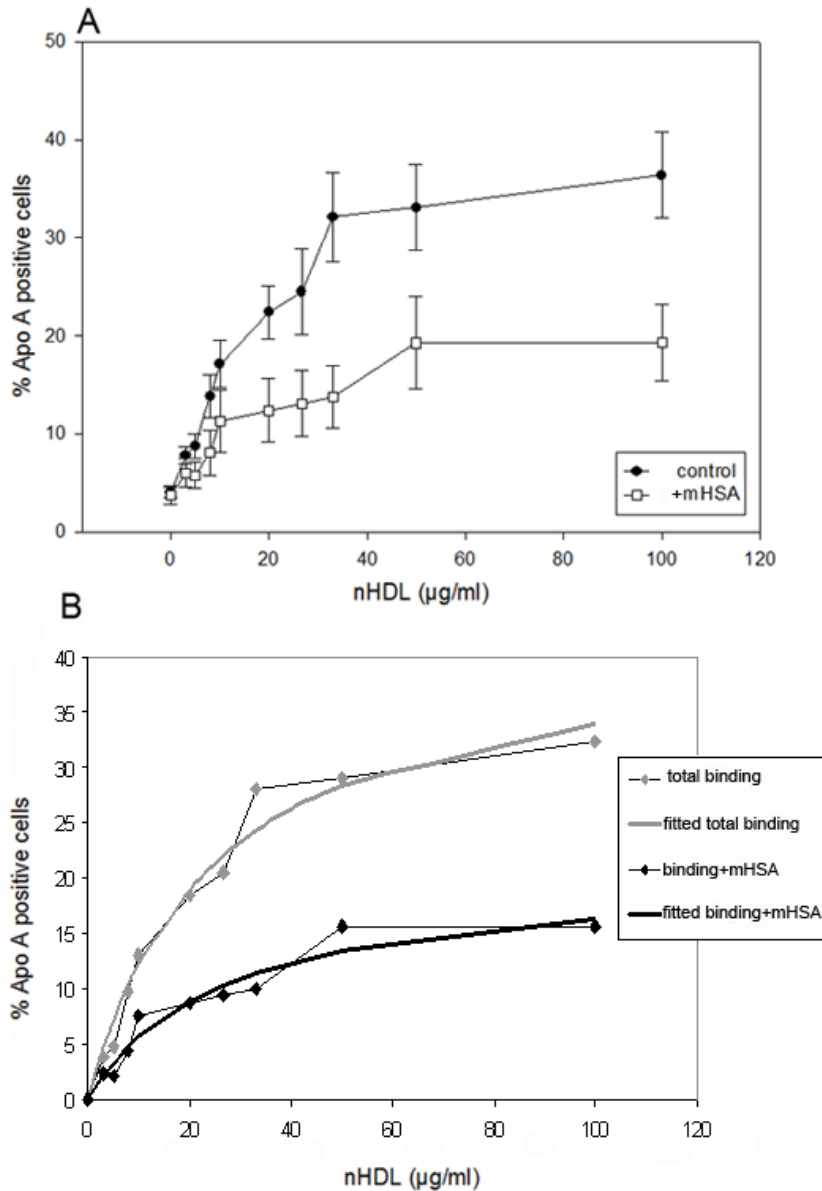

Figure 18: nHDL: total binding to platelets determined by Apo A-I detection and displacement

A: platelet-bound nHDL were detected by anti Apo A-I and analyzed by flow cytometry; displacement was performed with mHSA (500µg/ml); nHDL from 4 different donors, means of results from 12 experiments;

B: calculation of binding curves, assuming that mHSA blocks nHDL binding sites on platelets

3.4   Interaction of platelets and hypochlorite-modified HDL

3.4.1 hypOxHDL enhance platelet activation induced by ADP, collagen and thrombin

After showing the protective impact of native HDL on platelet aggregation and platelet activation, the effects of HDL after oxidative modification were investigated.

In contrast to oxidative modification of LDL, the effects of hypOxHDL on platelet function are discussed controversially and far less data exist.

To determine if hypochlorite modification alters the effects of HDL on platelet function, native HDL and hypOxHDL were compared for their effects on ADP-, thrombin- and collagen-induced platelet activation.

Gel filtered platelets were either left untreated or preincubated with nHDL or hypOxHDL. Thereafter platelets were treated with classical platelet agonists (ADP, collagen, and thrombin) in different concentrations and surface expression of P-selectin was quantified by flow cytometry.

As shown in Figure 19, hypOxHDL significantly amplify platelet degranulation induced by ADP, whereas nHDL from the same donor show protective effects on ADP-induced platelet activation.

Experiments carried out with submaximal concentrations of thrombin (Figure **20**) and collagen (Figure 21), show similar results: in both cases the addition of hypOxLDL lead to a strong increase of surface P-selectin, while nHDL are once again proven to have a protective impact on thrombin- and collagen-induced α-granule release.

Since synergistic effects of hypOxHDL with these agonists lead to such striking results in which dose dependency of the classical agonists seems to play a minor role, the question ariose, if hypOxHDL are able to trigger platelet activation in the absence of other agonists.
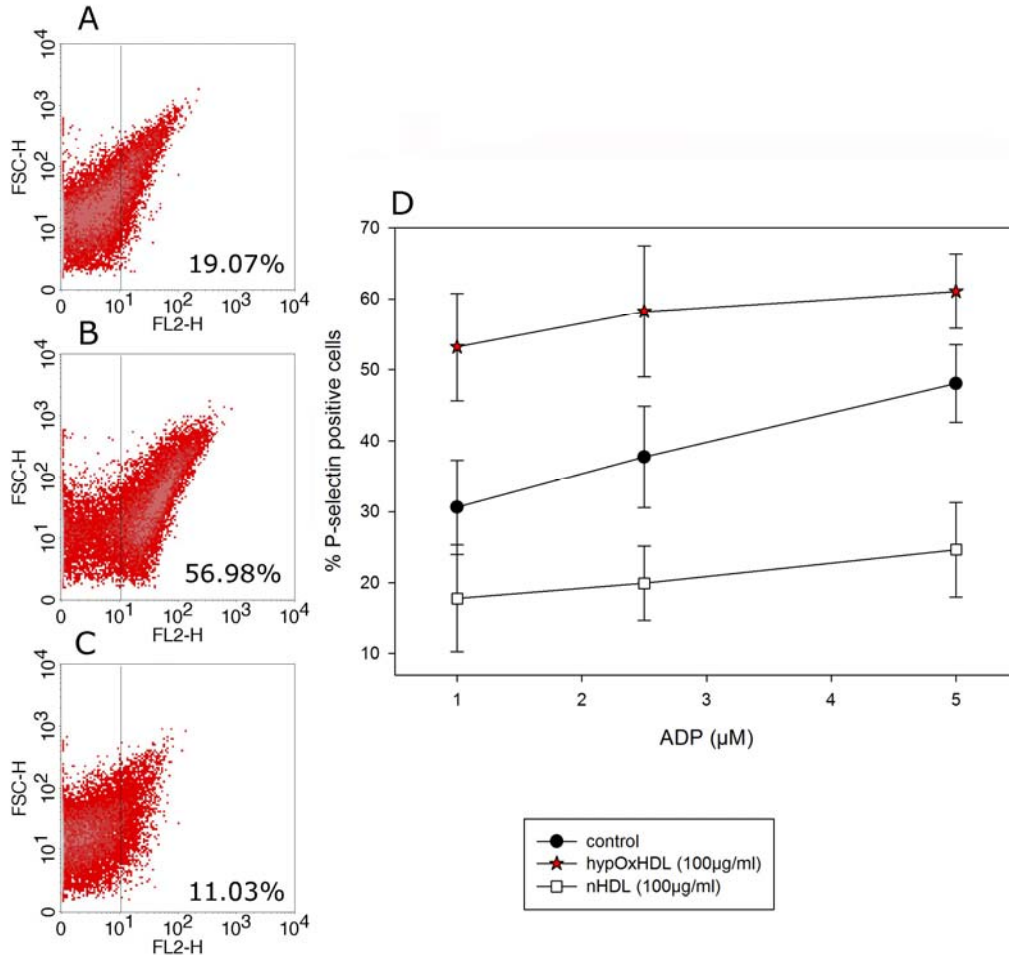
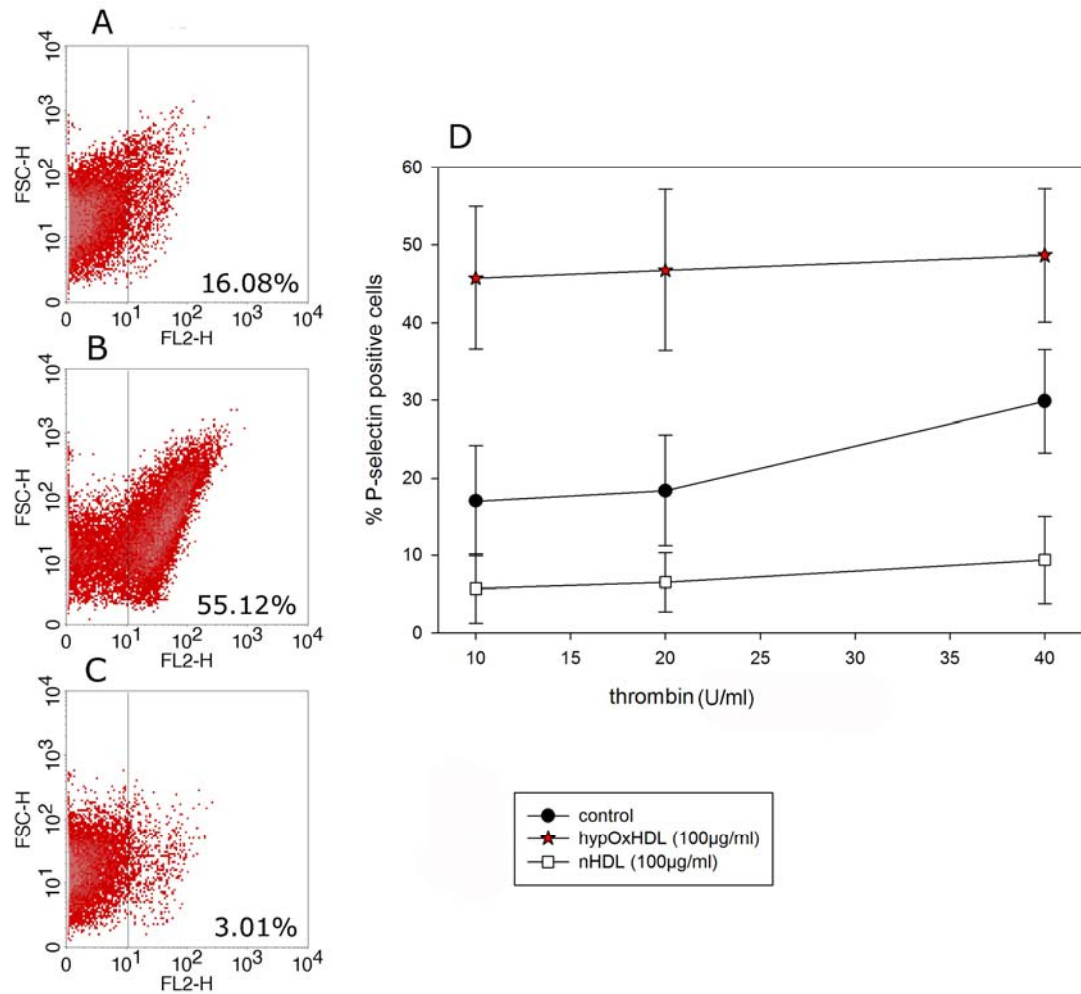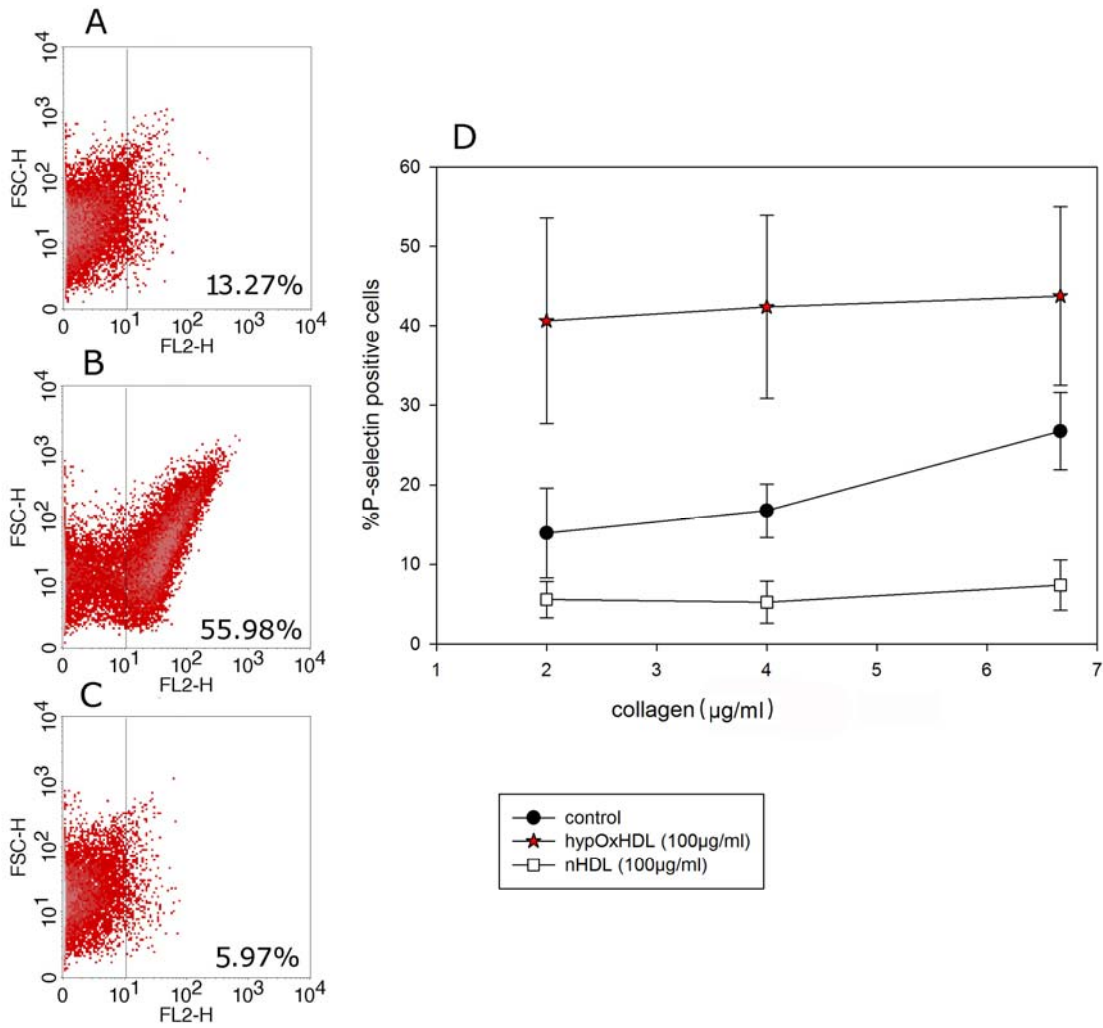**Figure 19: Influence of nHDL and hypOxHDL on ADP-induced surface expression of P-selectin on human platelets**

A, B and C show FACS dot plots of gel filtered platelets, stained for surface expression of P-selectin (PE), detected in FL2; percentage of P-selectin positive cells are indicated in the lower right of each figure

A: ADP (1µM)

B: ADP (1µM) + hypOxHDL (100µg/ml)

C: ADP (1µM) + nHDL (100µg/ml)

D: means and standard deviations of results from 10 experiments

Figure 20: Influence of nHDL and hypOxHDL on thrombin-induced surface expression of P-selectin on human platelets

A, B and C show FACS dot plots of gel filtered platelets, stained for surface expression of P-selectin (PE), detected in FL2; percentage of P-selectin positive cells are indicated in the lower right of each figure

A: thrombin (10U/ml)

B: thrombin (10U/ml) + hypOxHDL (100μg/ml)

C: thrombin (10U/ml) + nHDL (100μg/ml)

D: means and standard deviations of results from 10 experiments

Figure 21: Influence of nHDL and hypOxHDL on collagen-induced surface expression of P-selectin on human platelets

A, B and C show FACS dot plots of gel filtered platelets, stained for surface expression of P-selectin (PE), detected in FL2; percentage of P-selectin positive cells are indicated in the lower right of each figure

A: collagen (2μg/ml)

B: collagen (2μg/ml) + hypOxHDL (100μg/ml)

C: collagen (2μg/ml) + nHDL (100μg/ml)

D: means and standard deviations of results from 10 experiments

3.4.2 hypOxHDL induce surface expression of P-selectin

Since hypOxHDL show synergistic effects with classical agonists, the question arose, if hypOxHDL by themselves are able to activate human platelets.

Therefore gel filtered platelets were incubated with 260µg/ml of HDL, which had been modified with a 75-fold, a 150-fold or a 300-fold molar excess of hypochlorite. Moreover, the impact of hypOxHDL[300], treated with methionine (methionine-hypOxHDL), on surface expression of P-selectin was determined.

Figure 22 shows results of a typical experiment, which reveales a modification dependent induction of P-selectin expression by hypOxHDL. Compared with these results, a decrease of α-granule release could be observed if hypOxHDL had been treated with methionine before they were added to the platelets.
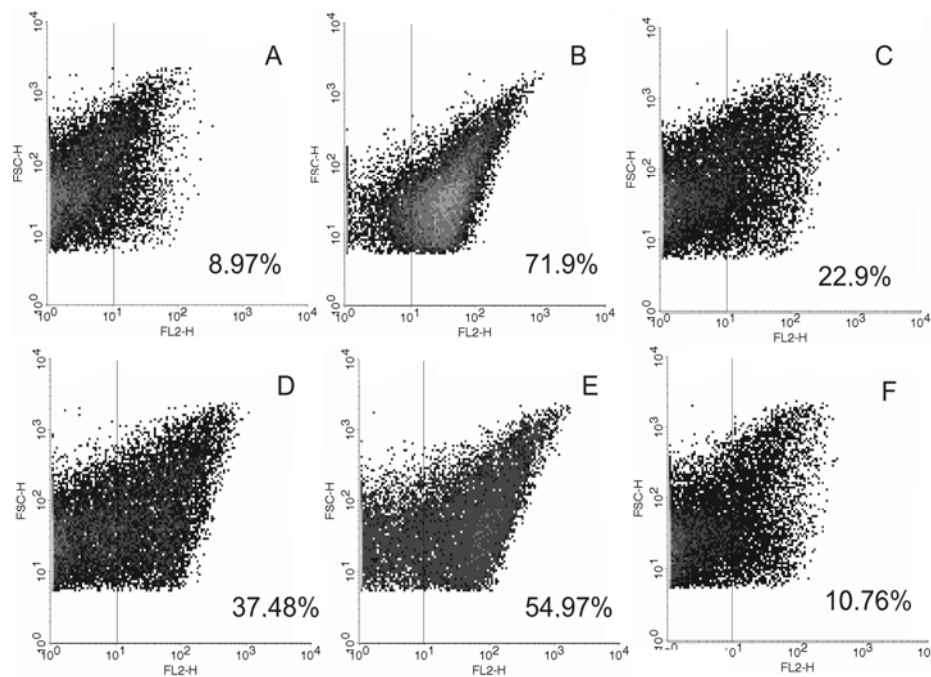
Figure 22: Surface expression of P-selectin induced by hypOxHDL
Dot plots, showing surface expression of P-selectin (PE), detected in FL2, on gel filtered platelets after stimulation with hypOxHDL with different degrees of modification
A: basal
B: ADP (50µM)
C: hypOxHDL[75] (260 µg/ml)
D: hypOxHDL[150] (260 µg/ml)
E: hypOxHDL[300] (260 µg/ml)
F: methionine-hypOxHDL[300] (260 µg/ml)
Values in squared brackets indicate molecular excess of hypochlorite over HDL; percentages in the lower right indicate percentage of P-selectin positive cells

In addition to results of a typical experiment shown in Figure 22, Figure 23 depicts means and standard deviations out of 18 experiments, showing significant differences in P-selectin expression between basal platelet activation and activation after treatment with hypOxHDL (75-fold, 150-fold and 300-fold excess of hypochlorite over HDL), but no significant difference between basal platelet activation and activation after addition of methionine-treated hypOxHDL.
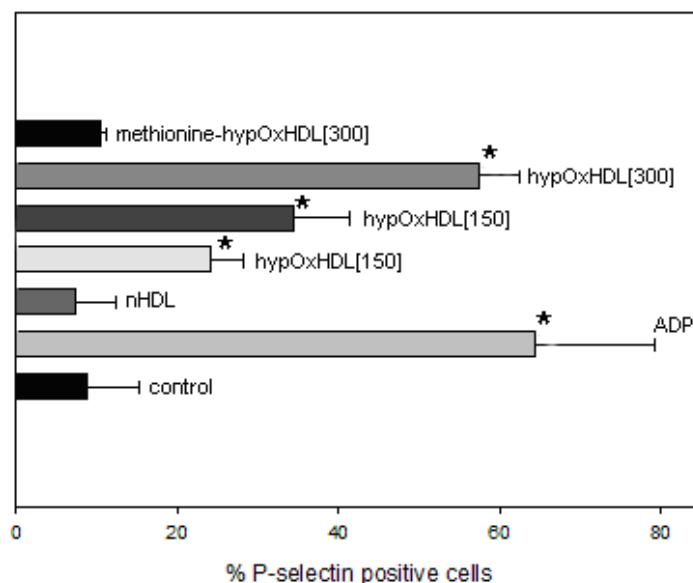

Figure 23: P-selectin surface expression induced by hypOxHDL modified to a different degree
   * indicates statistically significant difference to controls ($p<0.05$); values in squared brackets indicate molecular excess of hypochlorite over HDL; means and standard deviations of results from 18 experiments

3.4.3 hypOxHDL are able to trigger platelet aggregation

To further determine the effects of hypOxHDL on platelet function, their effects on platelet aggregation were analyzed.

Therefore, HDL, modified with different molar excess of hypochlorite over HDL, were added in different concentrations to resting platelets, in order to determine the severity of HDL modification and the concentration of hypOxHDL needed to eventually trigger platelet aggregation. This is of special interest to evaluate potential in vivo relevance of these new findings.

Figure 24 shows the effects of hypOxHDL on platelet aggregation, revealing that hypOxHDL are able to trigger platelet aggregation in the absence of other

agonists. As shown in Figure 24A, the intensity of platelet aggregation triggered by hypOxHDL directly correlates with the molar excess of hypochlorite used for HDL modification. While nHDL did not trigger platelet aggregation, HDL modified with a 75-fold molar excess of hypochlorite over HDL, provoked platelet aggregation to a submaximal extent.
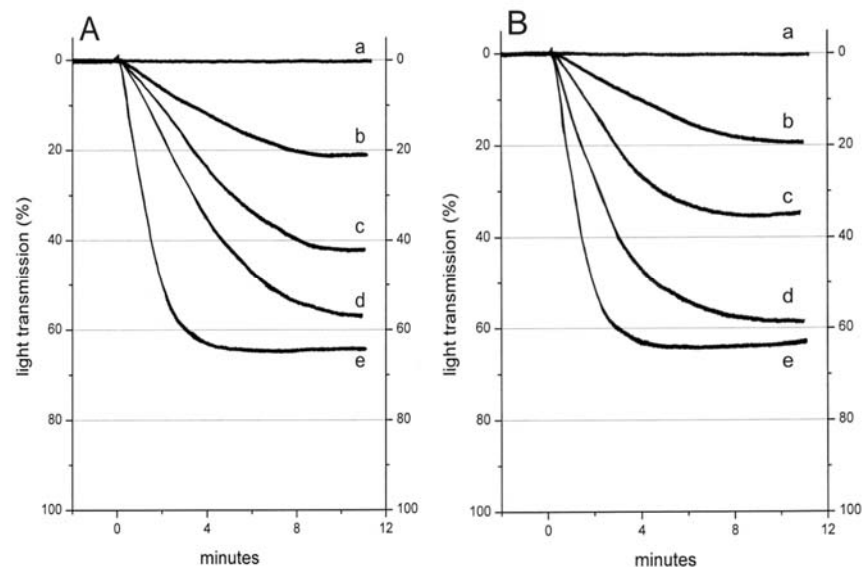

Figure 24: Influence of hypOxHDL on platelet aggregation
Aggregation curves, showing aggregation response of GFP
A: Impact of different degrees of hypOxHDL modification
a: nHDL added to a final concentration of 260μg/ml
b: hypOxHDL[75] added to a final concentration of 260μg/ml
c: hypOxHDL[150] added to a final concentration of 260μg/ml
d: hypOxHDL[300] added to a final concentration of 260μg/ml
e: ADP added to a final concentration of 50μM
B: Impact of different concentrations of hypOxHDL
a: buffer
b: hypOxHDL[300] added to a final concentration of 26μg/ml
c: hypOxHDL[300] added to a final concentration of 130μg/ml
d: hypOxHDL[300] added to a final concentration of 260μg/ml
e: ADP added to a final concentration of 50μM
Values in squared brackets indicate molar excess of hypochlorite over HDL

In Figure 24B, dose dependency of hypOxHDL[300] induced platelet aggregation is shown: even small concentrations of hypOxHDL (26μg/ml) are able to trigger platelet aggregation. At final concentrations of 260μg/ml hypOxHDL[300], the extent of the observed aggregation is comparable with results obtained with 50μM ADP. Moreover, platelet aggregation is totally

inhibited upon pre-treatment of hypOxHDL[300] with methionine (aggregation curve virtually identical to buffer shown in Figure 24B; authentic traces for methionine-hypOxHDL[300] are shown in Figure 26).

3.4.4 Influence of native HDL on hypOxHDL-induced platelet activation and aggregation

Since a protective effect of nHDL on platelet activation and aggregation induced by classical agonists could be shown, the question arose if nHDL also have a protective influence on hypOxHDL-induced platelet activation.

Therefore gel filtered platelets were incubated with different concentrations of nHDL and compared to platelets in buffer for their activation response upon addition of hypOxHDL.

Figure 25 depicts the influence of nHDL on surface expression of P-selectin induced by hypOxHDL.
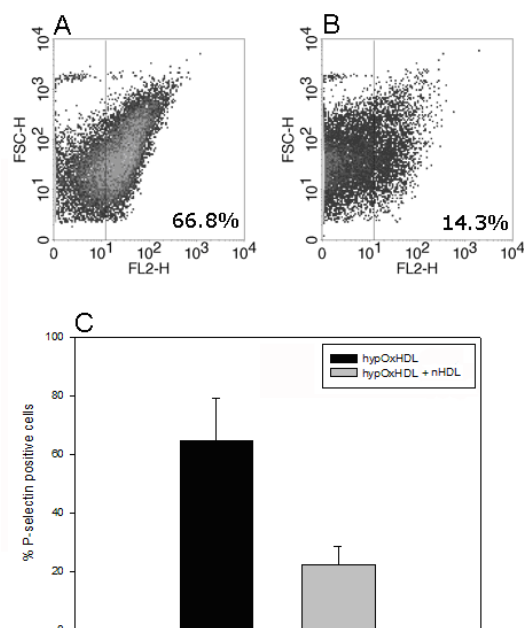
Figure 25: Influence of nHDL on hypOxHDL-induced surface expression of P-selectin
A-B: FACS dot plots of platelets stained for P-selectin (PE), detected in FL2; values in the lower right indicate percentage of P-selectin positive cells
A: hypOxHDL (100μg/ml)
B: nHDL (200μg/ml) + hypOxHDL (100μg/ml)
C: means and standard deviations of results from 10 experiments; nHDL (200g/ml) from 4 different donors were used

Figure 25A and Figure 25B show dot plots of a typical flow cytometric experiment and Figure 25C shows means and standard deviations of 10 experiments. Statistically significant differences in hypOxHDL-induced surface expression of P-selectin exist between platelets after preincubation with nHDL and controls (p<0.05).

As shown in Figure 26, nHDL mediate also a protective effect on hypOxHDL-induced platelet aggregation. The higher the nHDL concentration, the weaker is the aggregation response induced by hypOxHDL. Moreover, it could be shown that preincubation of hypOxHDL with methionine abolishes the ability of hypOxHDL to trigger platelet aggregation (also depicted in Figure 26).

Whether the protective effects of nHDL on hypOxHDL-induced platelet activation originate from competing with hypOxHDL for binding to the same receptor or if these effects are based on intracellular signalling induced by nHDL remains to be determined.
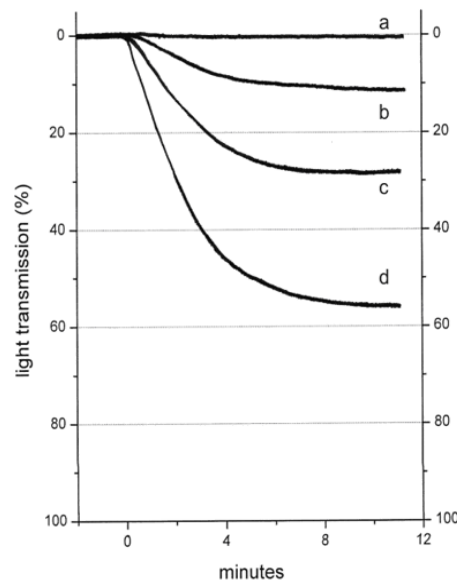
Figure 26: Influence of methionine pre-treatment and nHDL on hypOxHDL-induced platelet aggregation

Aggregation curves, showing aggregation response of platelets after addition of:
a: methionine-hypOxHDL[300] (260μg/ml)
b: nHDL (540μg/ml) + hypOxHDL[300] (260μg/ml)
c: nHDL (260μg/ml) + hypOxHDL[300] (260μg/ml)
d: hypOxHDL[300] (260μg/ml)

3.4.5 The role of scavenger receptor CD36 in hypOxHDL-induced platelet activation and aggregation

To investigate the role of scavenger receptors in hypOxHDL-induced platelet activation, platelets were incubated with maleylated human serum albumin (mHSA) - an antagonist of oxidised LDL binding to scavenger receptors - or with FA6.152, an antibody that is directed against the oxidised LDL-binding domain of CD36.

Figure 27 shows that hypOxHDL (100µg/ml) is able to induce GPIIb/IIIa activation. Upon coincubation with mHSA, GPIIb/IIIa activation by hypOxHDL is reduced to an almost basal level, which might indicate a role of specific binding of hypOxHDL to platelet receptors as a prerequisite for its stimulating effects.
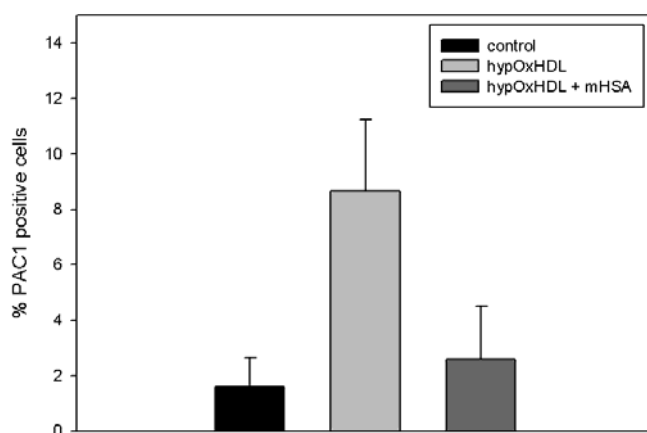
Figure 27: Influence of mHSA on hypOxHDL-induced GPIIb/IIIa activation (PAC-1)
    Results of flow cytometric experiments with platelets ± mHSA (50µg/ml);
    hypOxHDL (100µg/ml) from 5 different donors; means and standard deviations of
    results from 12 experiments

The binding domain domain of mHSA on scavenger receptors obviously plays a major role in mediating hypOxHDL-induced surface expression of P-selectin (depicted in Figure 28). Besides effects of mHSA, Figure 28 also shows results of experiments regarding hypOxHDL-induced surface expression of P-selectin in the presence nHDL and FA6.152, which reveal that surface expression of P-selectin induced by hypOxHDL can be significantly impaired by coincubation with FA6.152 or nHDL. FA6.152 is a stronger inhibitor of hypOxHDL-induced platelet activation than nHDL or mHSA. This suggests that the binding domain

of oxidised LDL on CD36 also plays an important role for platelet interaction with hypOxHDL. It has to be mentioned, that since the Fc portion of FA6.152 is able to trigger platelet aggregation, platelets had to be preincubated with antibody AT10, which is capable of blocking the Fcγ receptor.
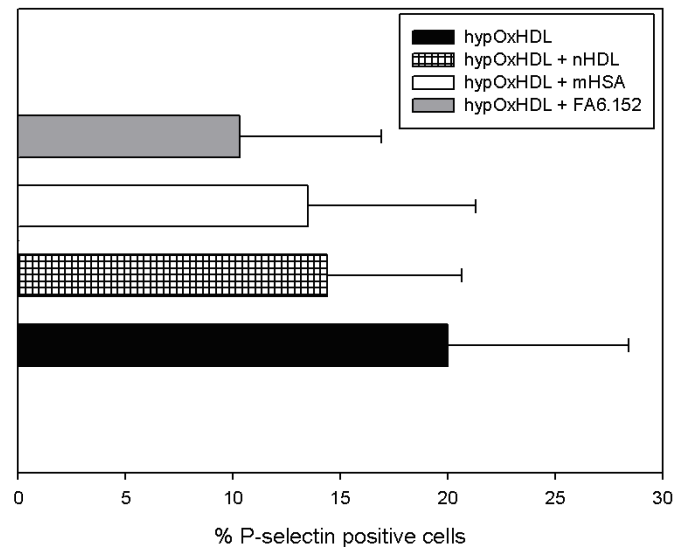

Figure 28: Influence of mHSA, FA6.152 and nHDL on hypOxHDL-induced expression of P-selectin
FA6.152 (3μg/ml) together with AT10 (1μg/ml); mHSA (50μg/ml); hypOxHDL (50μg/ml) and nHDL (200μg/ml) from 5 different donors; means and standard deviations of results from 12 experiments

In Figure 29 the influence of FA6.152 on hypOxHDL-induced platelet aggregation is depicted. Also in these experiments, preincubation of platelets with AT10 was necessary. To exclude unspecific inhibitory or activating effects of the added antibodies, ADP-induced aggregation in the presence and absence of AT10 and FA6.152 was compared, revealing no difference in the aggregation behaviour of platelets (shown in Figure 29). Taken together, platelet aggregation induced by hypOxHDL can be totally inhibited by blocking an epitope on CD36 responsible for binding of oxidised LDL, indicating that interaction of hypOxHDL with CD36 is necessary for platelet response.
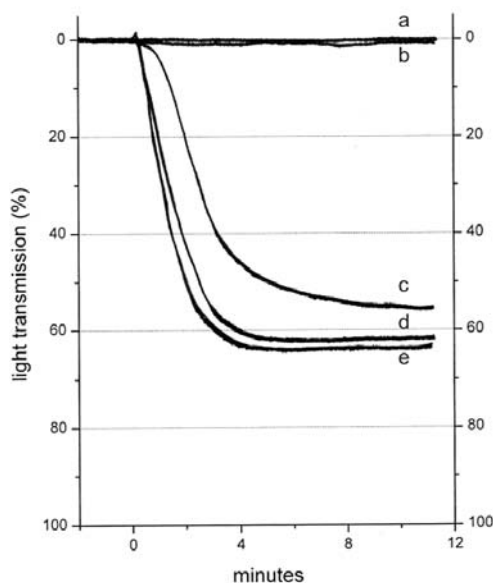
Figure 29: Influence FA6.152 antibody on hypOxHDL-induced platelet aggregation

Aggregation curves, showing aggregation response of platelets after addition of:
a: buffer
b: AT10 (1µg/ml) + FA6.152 (1µg/ml) + hypOxHDL (260µg/ml)
c: hypOxHDL (260µg/ml)
d: AT10 (1µg/ml) + FA6.152 (1µg/ml) + ADP (50µM)
e: ADP (50µM)

3.4.6 Influence of hypOxHDL on intraplatelet calcium

The measurement of intracellular free calcium levels is an elegant way to study the response of platelets to different agonists. In contrast to other flow cytometric methods, analysis is performed with unfixed and vital cells – therefore, the response of platelets to different agonists can be observed over time and changes are visualized immediately.

As depicted in Figure 30, addition of hypOxHDL to human platelets leads to an immediate calcium influx, with kinetics comparable to that obtained with ADP. In accordance with results obtained from aggregation experiments, antibody FA6.152 prevents calcium influx provoked by hypOxHDL, but does not affect calcium influx in response to ADP. As a consequence of the fact that platelet suspension was not stirred in this type of experiments, it was possible to omit AT10 in these experiments, since FA6.152 alone had no impact on intracellular free calcium levels.
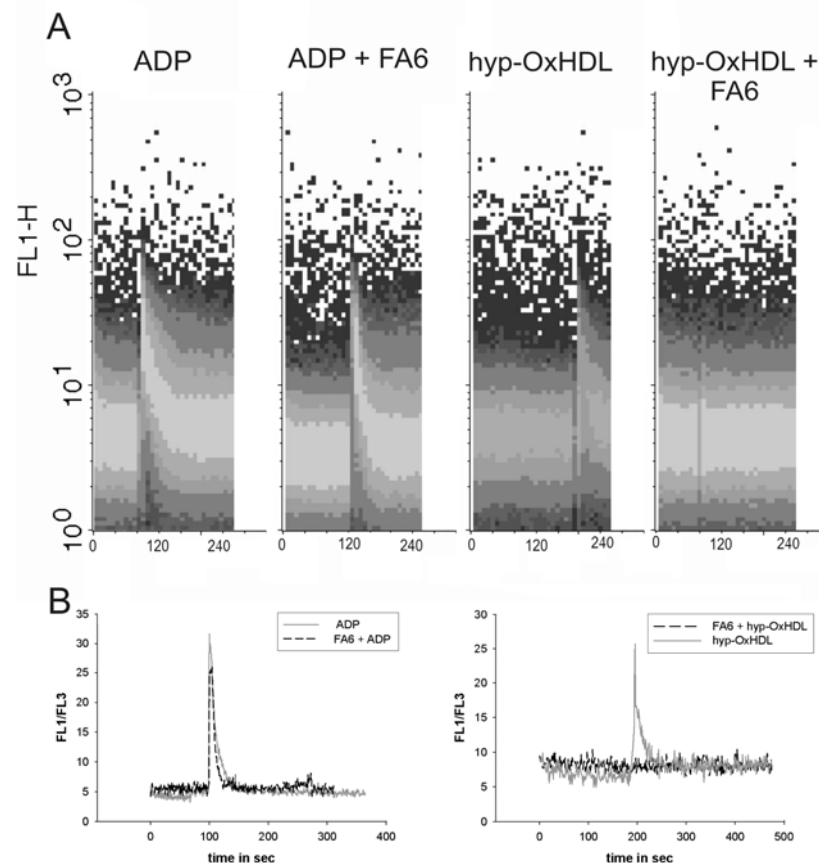
Figure 30: Effects of hypOxHDL on intraplatelet calcium
    A: Fluo-4 fluorescence (detected in FL1) after addition of indicated agonists (ADP (50μM) or hypOxHDL (260μg/ml)) to GFP and GFP incubated with FA6.152 (4μg/ml)
    B: influence of FA6.152 (4μg/ml) on sliding means (Fluo-4/Fura Red; detected in FL1/FL3) of calcium influx either induced by ADP (50μM) or hypOxHDL (260μg/ml)

3.4.7 Impact of hypOxHDL on VASP phosphorylation

As mentioned before, determination of VASP phosphorylation in platelets provides a new way to quantitate the sensitivity of platelets towards antagonists of platelet activation. Since platelet activating effects of hypOxHDL could be proven, the question arose if hypOxHDL are also able to counteract PGE_1 action on human platelets. Therefore the influence of hypOxHDL on PGE_1-induced serine 239 VASP phosphorylation was investigated.

Gel filtered platelets were either preincubated with hypOxHDL or left untreated. Then submaximal concentrations of PGE_1 were added (or both substances

were coincubated, whereat this difference in the experimental procedure was proven to lead to no difference in the outcome).

Figure 31 shows FACS dot plots of a single experiment, which reveal that hypOxHDL is able to reduce PGE$_1$-induced Ser 239 VASP phosphorylation.
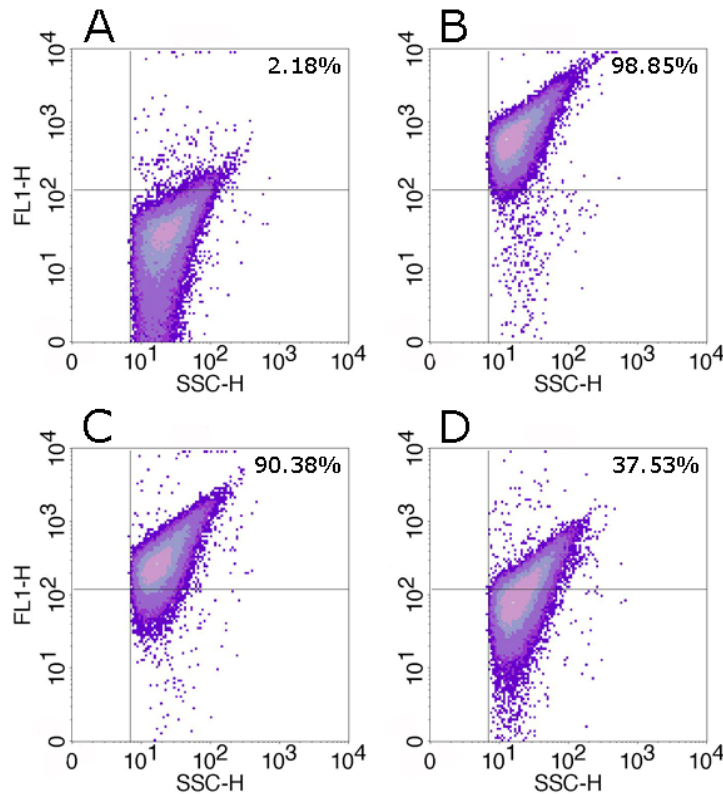

Figure 31: Influence of hypOxHDL on PGE$_1$-induced VASP phosphorylation (1)
Results of flow cytometric experiments with platelets stained for Ser 239 VASP phosphorylation (FITC), detected in FL1; values in the upper right indicate percentage of VASP positive cells
A: basal
B: PGE$_1$ (10µM) fully induced VASP phosphorylation
C: PGE$_1$ (0.1µM)
D: hypOxHDL (100µg/ml) + PGE$_1$ (0.1µM)

Unfortunately, outcome of individual experiments varied, so that after calculating of means and standard deviations of all experiments, only a tendency but no significant difference could be determined ($p > 0.05$).

In Figure 32 means and standard deviations of 24 experiments are shown. Differences in effectiveness of hypOxHDL, to counteract PGE$_1$ induced VASP phosphorylation were not related to gender or known health status of the

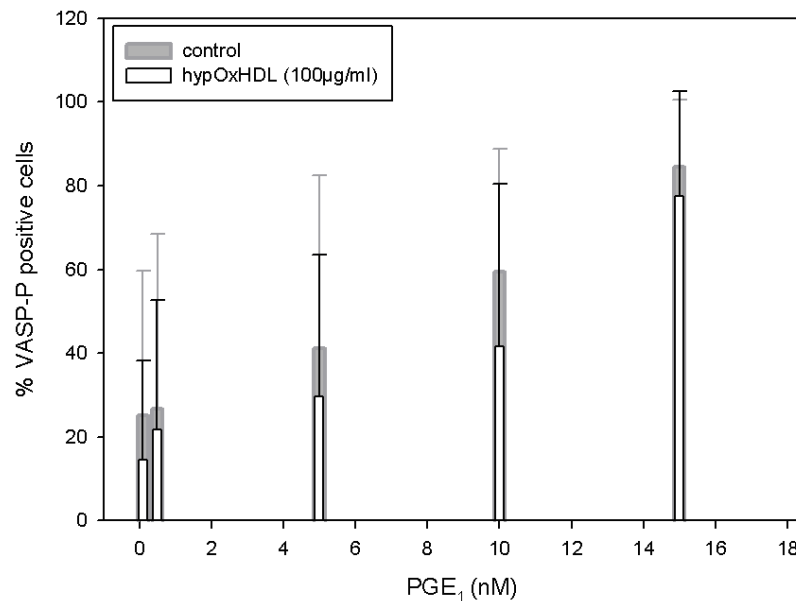donors. Lifestyle habits, like smoking or other sources of oxidative stress were not determined.

Figure 32: Influence of hypOxHDL on PGE$_1$-induced VASP phosphorylation (2)
Results of flow cytometric experiments with platelets; VASP-P stands for Ser 239 VASP phosphorylation; PGE$_1$ (0.2nM-15nM); hypOxHDL (100µg/ml) from 6 donors; means and standard deviations of results from 24 experiments

The variability in the outcome of these experiments led to the idea of blocking NO production. The rationale behind this strategy is that, as mentioned before, a large amount of NO is released by platelets shortly after their activation to prevent further aggregation. Since hypOxHDL are proven to activate platelets, this might lead to NO production and thereby to VASP phosphorylation.

To exclude any influence of NO, its synthesis was inhibited by the arginine analogue L-NMMA.

Surprisingly, preincubation of platelets with L-NMMA failed to show the proposed impact on the obtained results: As shown in Figure 33A and Figure 33B, the PGE$_1$ counteracting action of hypOxHDL is reduced in the presence of L-NMMA. The extent of the effects of hypOxHDL on PGE$_1$-induced VASP phosphorylation did not increase upon preincubation with L-NMMA. Quite on the contrary, even reverse effects could be observed. Therefore the hypothesis that NO, released shortly after platelet activation (by hypOxHDL) is responsible for the high standard deviations and low effects on PGE$_1$-induced VASP

phosphorylation can not be uphold. In Figure 33C means and standard deviations of results with and without L-NMMA are compared for all three concentrations of PGE_1.
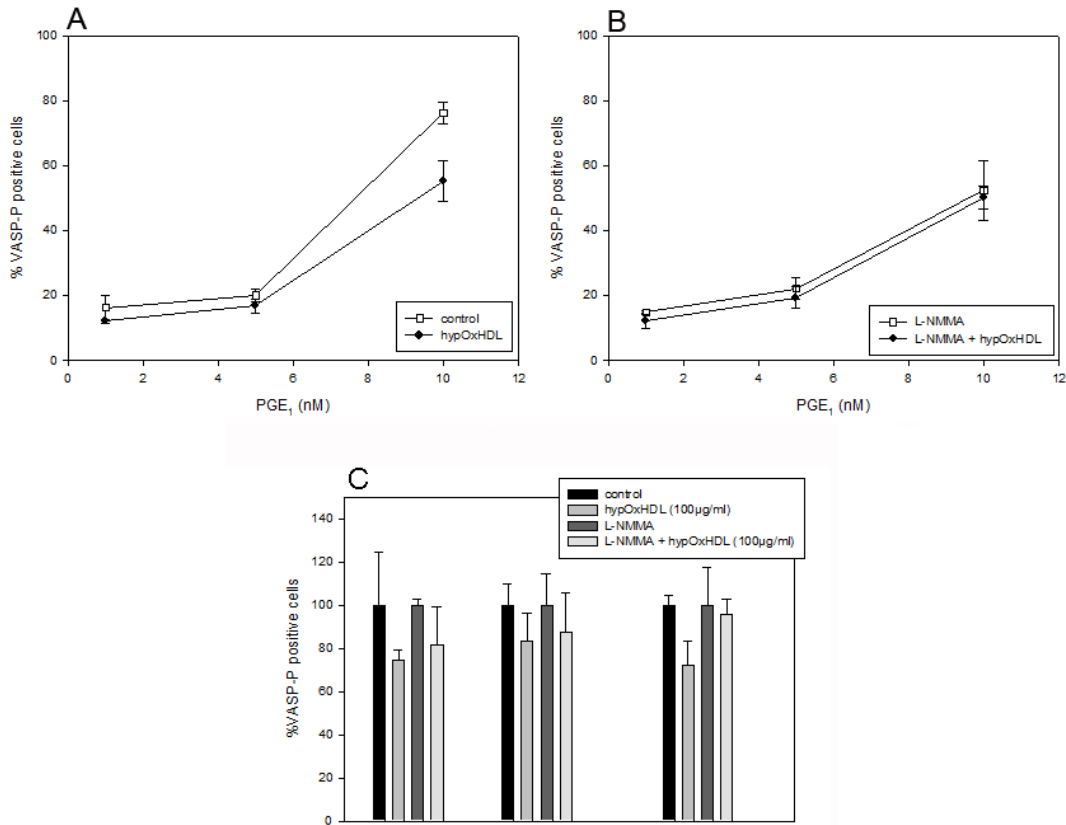

Figure 33: Impact of inhibiting NO synthesis on hypOxHDL-induced reduction of PGE_1-induced VASP phosphorylation
Results of flow cytometric experiments with platelets; VASP-P stands for Ser 239 VASP phosphorylation
A,B: PGE_1 (1nM-10nM) ± hypOxHDL (100µg/ml)
A: platelets
B: platelets preincubated with L-NMMA (50µM)
C: means and standard deviations, preincubation of platelets ± L-NMMA (50µM); hypOxHDL from 4 donors; means and standard deviations of results of 8 experiments

3.4.8 Effects of inhibiting distinct platelet activation pathways on hypOxHDL-induced platelet aggregation

To get an insight into signal transduction pathways involved in hypOxHDL-induced platelet activation, platelets were treated with different inhibitors (quinacrine, ethylene diamine tetraacetic acid (EDTA) and acetylsalicylic acid (ASA)) in order to knock out distinct pathways.

Quinacrine blocks arachidonate release from human platelets and interferes with platelet activation and aggregation by inhibiting phosphatidylinositol-specific phospholipase C and cyclic-GMP phosphodiesterase[116]. Quinacrine also inhibits phospholipase A_2, which is important for the production of lysophosphatidic acid and therefore essential for thrombin-induced platelet aggregation[117].

EDTA, a potent chelator of Ca^{++}, reduces ionized Ca^{++} levels, which leads to inhibition of platelet aggregation. Extracellular Ca^{++} is required for various signalling events as well as the Ca^{++} dependent stabilisation of GPIIb/IIIa[118].

ASA leads to an inhibition of TxA_2 synthesis, resulting in inhibition of platelet aggregation via inhibition of Cox1[119], which is an enzyme required for production of TxA_2, a stimulator of platelet aggregation.

To evaluate the effects of these different inhibitors on hypOxHDL-induced platelet aggregation, it was necessary to treat these - partly inhibited - platelets with classical agonists as a positive control and for comparability of results.

As depicted in Figure 34, aggregation curves of the differently inhibited platelets varied between the different agonists.

EDTA was shown to be the most potent, ASA the weakest inhibitor of ADP-induced platelet aggregation (shown in Figure 34A). No dose dependency of ADP could be observed for the inhibitory action of quinacrine, EDTA or ASA.

In Figure 34B the impact of the three inhibitors on thrombin-induced platelet aggregation is depicted. In these experiments inhibitory effects of ASA and quinacrine depended on the dosage of agonists, whereas the inhibitory effect of EDTA was dose independent. Depending on thrombin concentration, either EDTA, or in lower concentrations quinacrine was the most potent inhibitor of thrombin-induced platelet aggregation.

Figure 34C demonstrates the effects of the inhibitors on collagen-induced platelet aggregation. All three inhibitors reduced collagen-induced platelet activation dose dependently. EDTA was shown to anticipate agonist-induced platelet aggregation more severely than the other inhibitors.

In Figure 34D, finally, the results of hypOxHDL-induced aggregation in the presence of inhibitors are shown. All three inhibitors are able to block platelet

aggregation induced by hypOxHDL. No dose dependent effects could be observed nor a significant difference of the effects between the three inhibitors. This leads to the assumption that all inhibited pathways are involved in hypOxHDL-induced platelet activation.
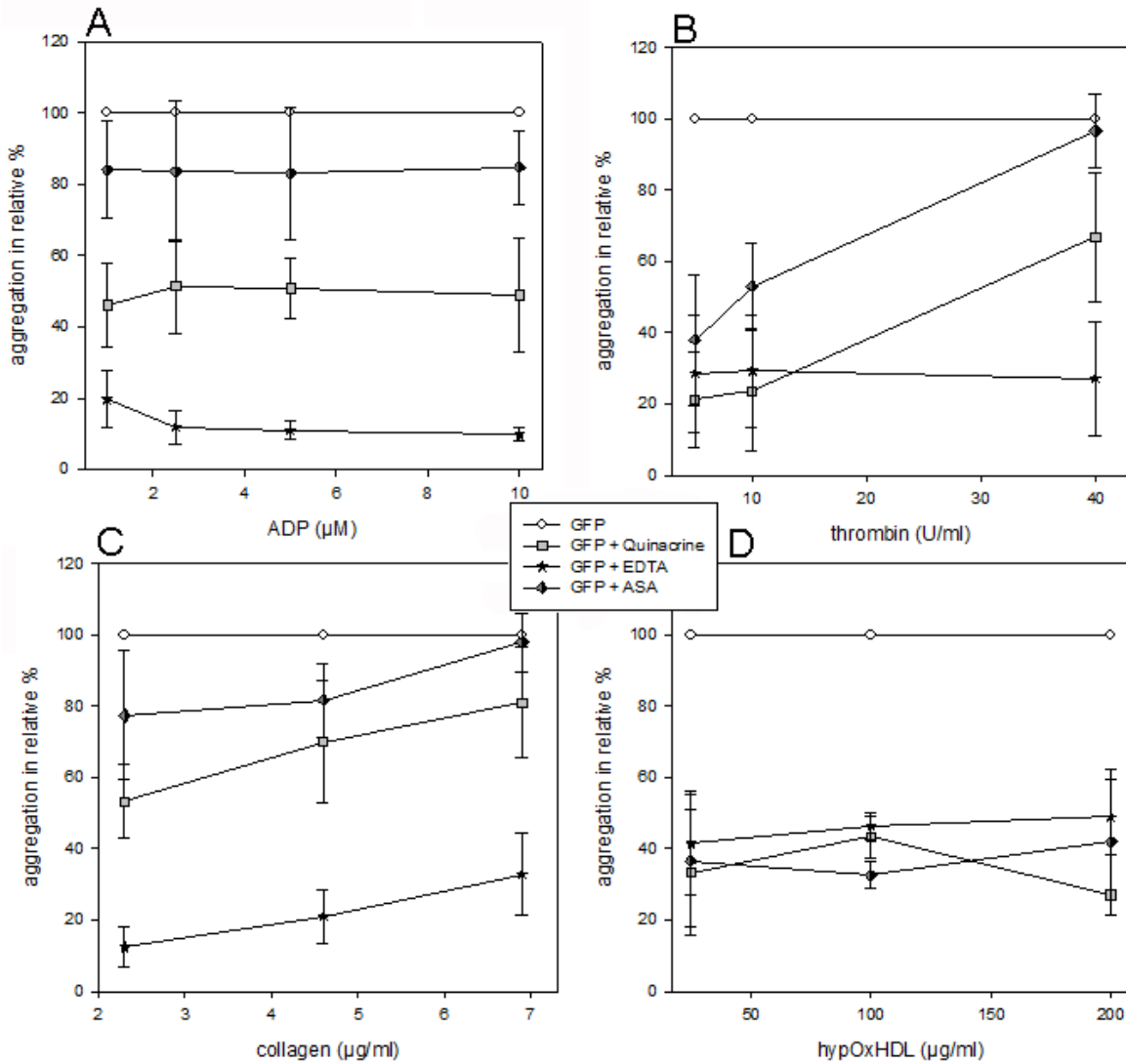

Figure 34: Effects of inhibiting distinct platelet activation pathways on platelet aggregation induced by different agonists

Aggregation in the absence of inhibitors was set 100% and attenuation by quinacrine (20µM), EDTA (1mM) or ASA (1mM)
calculated as relative % of these 100%
A: ADP (1µM-10µM)
B: thrombin (5U/ml-40U/ml)
C: collagen (2.3µg/ml-6.7µg/ml)
D: hypOxHDL (20µg/ml-200µg/ml) from 3 different donors;
Means and standard deviations of results of 12 experiments

3.4.9 Binding of hypOxHDL to human platelets

3.4.9.1 hypOxHDL binding studies by Apo A-I detection

Since Apo A-I is the main apolipoprotein of HDL, detection of this apoprotein should consequently be utilisable to investigate hypOxHDL binding to human platelets. To determine characteristics of receptors involved in hypOxHDL binding to platelets, mHSA, polyclonal CD36 antibody and monoclonal antibody FA6.152 were evaluated for their ability to interfere with binding of hypOxHDL to human platelets. In other cell types, CD36 has been shown to bind (copper) oxidised HDL, therefore polyclonal CD36 antibody was used to determine the role of this receptor in platelets. To test if oxidised LDL and oxidised HDL might bind to the same domain of CD36, monoclonal antibody FA6.152, directed against the binding domain of oxidised LDL on CD36, was used.

Gel filtered platelets (with or without the indicated potential binding antagonists) were incubated with hypOxHDL (20µg/ml–200µg/ml) and analyzed for Apo A-I positive cells by flow cytometry.

As shown in Figure 35A, a dose dependent increase in Apo A-I positive cells after addition of increasing concentrations of hypOxHDL could be detected (total binding). Displacement by mHSA, CD36 antibody and FA6.152 was successful in all concentrations of hypOxHDL. Relative percent of inhibition of hypOxHDL binding to platelets is depicted in Figure 35B. FA6.152 turned out to be the strongest competitor, indicating the important role of the binding domain for oxidised LDL on CD36 for hypOxHDL binding to human platelets. Specific binding was calculated by means of data obtained with platelets coincubated with hypOxHDL and FA6.152. Binding curves were mathematically adjusted. Unfortunately, the quantity of data was insufficient to allow reliable calculation of the dissociation constant.
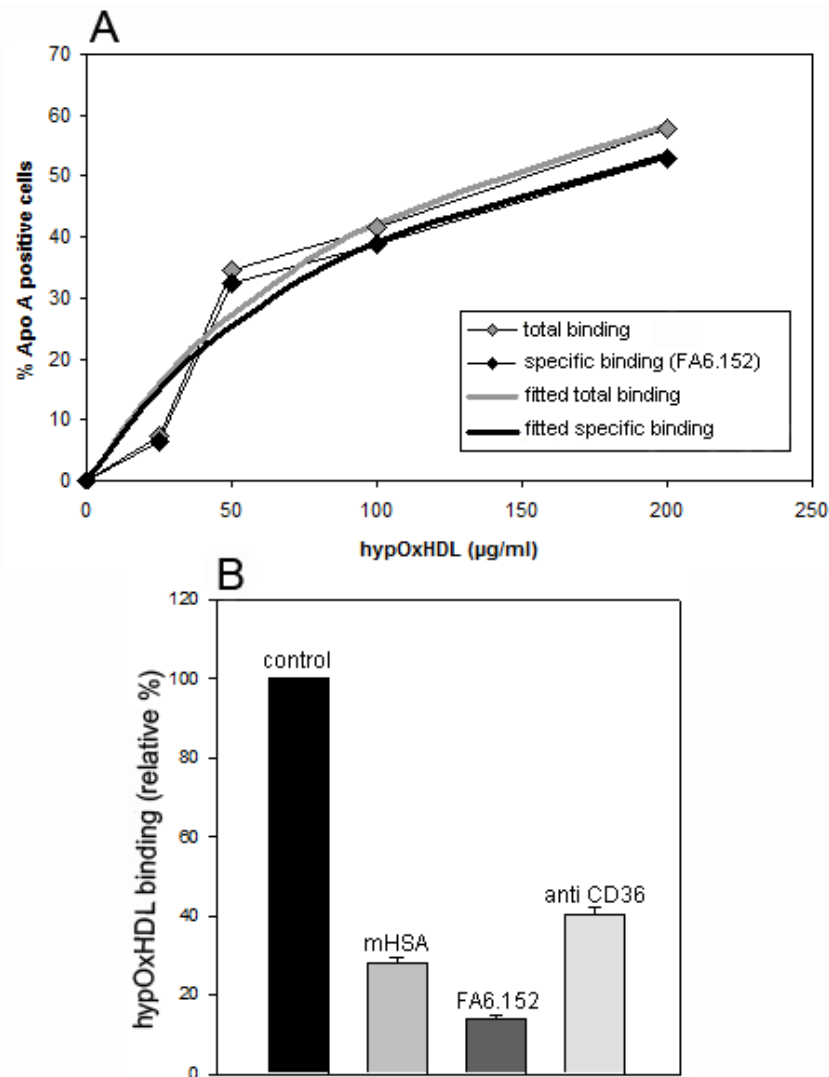
Figure 35: hypOxHDL: total binding to platelets determined by Apo A-I detection and displacement

   A: measured and fitted total and specific binding of hypOxHDL; specific binding was calculated by displacement with FA6.152

   B: displacement of hypOxHDL by mHSA (500µg/ml), FA6.152 (3µg/ml) and anti CD36 (=polyclonal CD36 antibody (3µg/ml)); hypOxHDL from 4 different donors; means and standard deviations of results from 8 experiments;

3.5   Interaction of platelets and native LDL

3.5.1   Native LDL impair agonist-induced surface expression of P-selectin

To distinguish between lipoprotein class specific and modification dependent effects, the effects of native LDL and oxidatively modified LDL on platelet activation were investigated. The impact of native LDL on platelet function is discussed controversially. While some authors stated protective effects, others were able to show activating effects of native LDL on human platelets[84]. Since it is difficult to obtain completely unmodified LDL, these controversial results might arise from problems in isolation or storage of lipoproteins. LDL used for this work were isolated with highest precautions in order to obtain lipoproteins as native as possible. To evaluate if native LDL are able to interfere with platelet activation, platelets were activated by submaximal amounts of classical agonists and analyzed for changes in surface expression of P-selectin due to the presence of native LDL. Hence, freshly obtained gel filtered platelets, preincubated with native LDL or left untreated, were exposed to submaximal concentrations of ADP and thrombin. Results of these experiments are depicted in Figure 36 and show that native LDL are able to impair ADP- (Figure 36A) and thrombin- (Figure 36B) induced surface expression of P-selectin in a dose dependent way.
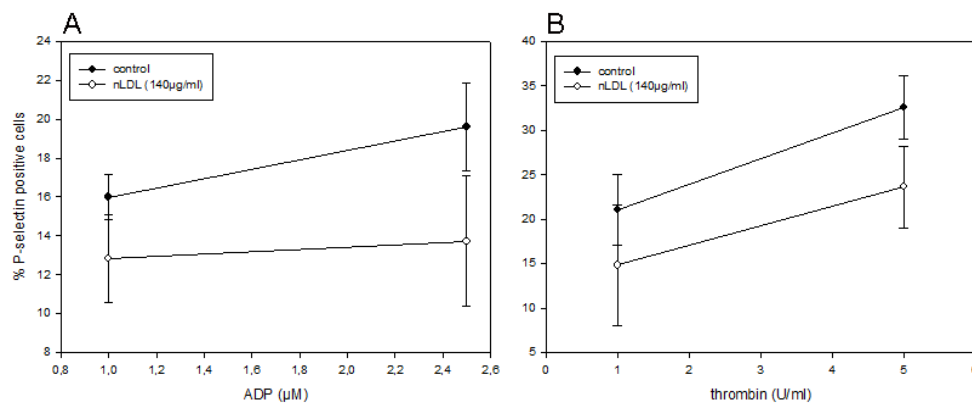

Figure 36: Effects of nLDL on P-selectin expression induced by ADP or thrombin
Results of flow cytometric experiments with platelets; LDL from 4 different donors;
A: ADP
B: thrombin
Means and standard deviations of results from 8 experiments

3.5.2 Native LDL enhance PGE$_1$-induced VASP phosphorylation

A potential inhibitory impact of native LDL on platelet activation was further investigated by experiments regarding determination of platelet serine 239 VASP phosphorylation. Since no effects on VASP phosphorylation could be observed upon addition of native LDL to platelets (data not shown), potential synergistic effects with submaximal concentrations of PGE$_1$ were investigated. Gel filtered platelets were preincubated with native LDL (140µg/ml) and subsequently, PGE$_1$ (2nM-10nM) was added and platelets analyzed for intracellular VASP phosphorylation. Means and standard deviations of results from 8 experiments are depicted in Figure 37: in the presence of native LDL, platelets show an increase in VASP phosphorylation compared to controls, although the difference was only significant at a concentration of 10nM PGE$_1$ (p<0.05). Comparable to studies with native HDL, native LDL from some donors failed to increase intracellular VASP phosphorylation.

In some experiments, platelets did not respond to minor concentrations of PGE$_1$ and therefore the obtained results had to be excluded from evaluation. Since the number of experiments was not sufficient to further correlate the lack of protective effects with increased risk factors (e.g.: smoking) of platelet donors, no statement on the influence of life style habits on protective effects of native LDL on platelet function can be made at the moment.
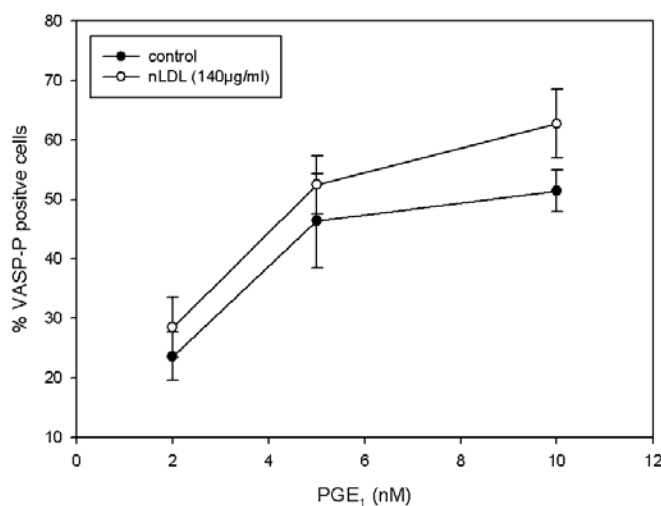

Figure 37: Effects of nLDL on PGE$_1$-induced VASP phosphorylation
　　Results of flow cytometric experiments with platelets ± nLDL (140µg/ml); VASP-P stands for Ser 239 VASP phosphorylation; PGE$_1$ (2nM-10nM); LDL from 4 different donors; means and standard deviations of results from 8 experiments

3.6   Interaction of platelets and oxidised LDL

It has already been shown by our group that LDL, modified with hypochlorite, are able to activate human platelets[23]. Nevertheless, investigations of platelet interaction with hypOxLDL performed in this work started with repeating aggregation experiments to confirm published findings, followed by additional experiments regarding platelet activation by hypOxLDL. In this regard, effects of coincubation time of platelets and hypOxLDL as well as dose dependency of hypOxLDL-induced surface expression of P-selectin have not been investigated so far. Moreover, the impact of hypOxLDL on platelet CD40L surface expression, GPIIb/IIIa activation and VASP phosphorylation was investigated for the first time.

3.6.1   hypOxLDL trigger platelet aggregation

According to results of prior studies, LDL, treated with a 400-fold molecular excess of hypochlorite were able to independently trigger platelet aggregation, whereas native LDL from the same donor showed no effect. To prove that scavenger receptors are involved in platelet activating signal transduction induced by hypOxLDL, gel filtered platelets were pretreated with mHSA and compared to platelets in buffer avoid of mHSA. Such blocking of scavenger receptors by mHSA led to a strong attenuation of hypOxLDL-induced platelet aggregation (shown in Figure 38).
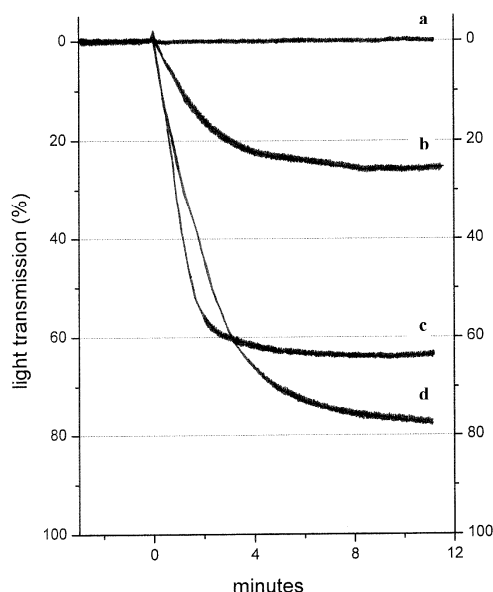
Figure 38: Effects of hypOxLDL on platelet aggregation
Aggregation curves, showing aggregation response of platelets after addition of:
a: nLDL (100µg/ml)
b: mHSA(50µg/ml) + hypOxLDL (100µg/ml)
c: ADP (50µM)
d: hypOxLDL (100µg/ml)

3.6.2 hypOxLDL induce surface expression of P-selectin

The finding that hypOxLDL are able to induce surface expression of P-selectin has already been shown[76]. Hence, to determination of the amount of hypOxLDL needed to induce α-granule release and the time pattern of hypOxLDL-mediated platelet activation is of great interest.

3.6.2.1 Influence of hypOxLDL concentration on surface expression of P-selectin

To evaluate the in vivo relevance of platelet activation by hypOxLDL it is also important to know the concentration needed for platelet activation. Therefore, gel filtered platelets were incubated with different amounts of hypOxLDL and the impact of rising hypOxLDL concentrations on surface expression of P-selectin was determined.

As shown in Figure 39, hypOxLDL are able to stimulate surface expression of P-selectin in a dose dependent mode. An increase of surface expression of

P-selectin could be seen at hypOxLDL concentrations as low as 5µg/ml, reaching peak activation at a concentration of 75µg/ml. There seems to be no significant difference in P-selectin expression between concentrations of 75µg/ml and 300µg/ml.
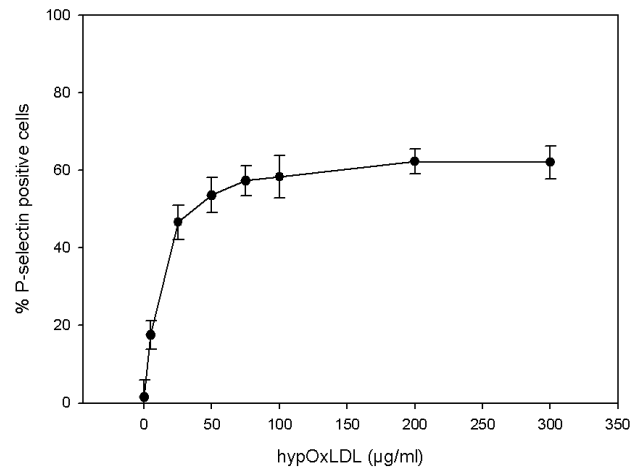

Figure 39: Concentration-dependent effects of hypOxLDL on P-selectin expression
Results of flow cytometric experiments with platelets; hypOxLDL from 3 donors; means and standard deviations of results from 6 experiments

3.6.2.2 Impact of incubation time of platelets with hypOxLDL on surface expression of P-selectin

To determine the time course of hypOxLDL induced platelet activation, platelets were incubated with 100µg/ml hypOxLDL for 1 to 15 minutes at room temperature and the impact of incubation time on surface expression of P-selectin was evaluated.

As depicted in Figure 40, it took about 8 minutes of coincubation of platelets with hypOxLDL to reach their maximal P-selectin surface expression. Between 8 and 15 minutes, no significant further increase could be detected. This observation was quite surprising, since platelet aggregation is triggered immediately after addition of hypOxLDL and full aggregation can be observed only 4 to 6 minutes after addition of 100µg/ml hypOxLDL (see Figure 38). The fact that platelet aggregation is performed at 37°C, whereat platelets in experiments regarding surface expression of P-selectin are incubated at room temperature might explain this phenomenon. Moreover, in aggregometrical

studies platelets are stirred or shaken, which facilitates cell contacts and accelerates platelet activation.
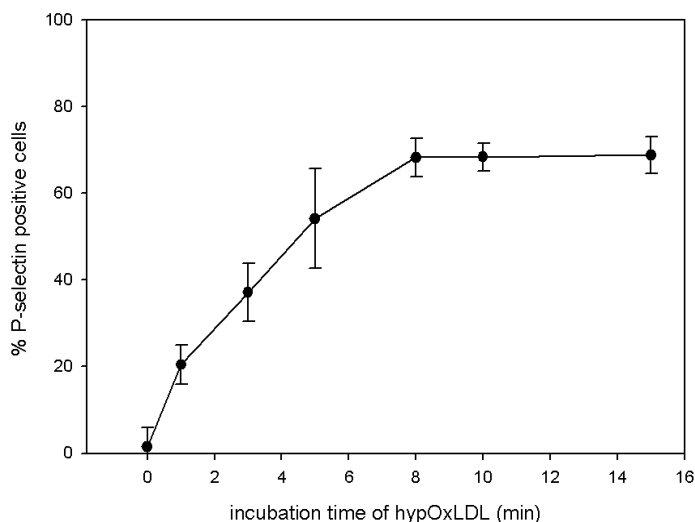

Figure 40: Time-dependent effects of hypOxLDL on surface expression of P-selectin
Results of flow cytometric experiments with platelets; hypOxLDL (100g/ml) from 2 different donors; means and standard deviations of results from 6 experiments

3.6.3 Synergistic effects of hypOxLDL and other agonists of platelet activation

Synergistic effects of hypOxLDL with ADP and thrombin were investigated to evaluate if submaximal concentrations of hypOxLDL synergistically increase surface expression of P-selectin induced by classical agonists and if, in the presence of hypOxLDL, less amount of other agonists is needed to induce platelet activation.

Therefore, freshly obtained gel filtered platelets were incubated with 20µg/ml hypOxLDL, which led to submaximal activation (38.6% P-selectin positive cells, shown in Figure 41C). Thereafter, ADP or thrombin in different concentrations was added to platelets that had been preincubated with or without hypOxLDL.

As shown in Figure 41, hypOxLDL show synergistic effects with both agonists. In the presence of hypOxLDL, less ADP or thrombin is needed to trigger full platelet activation.
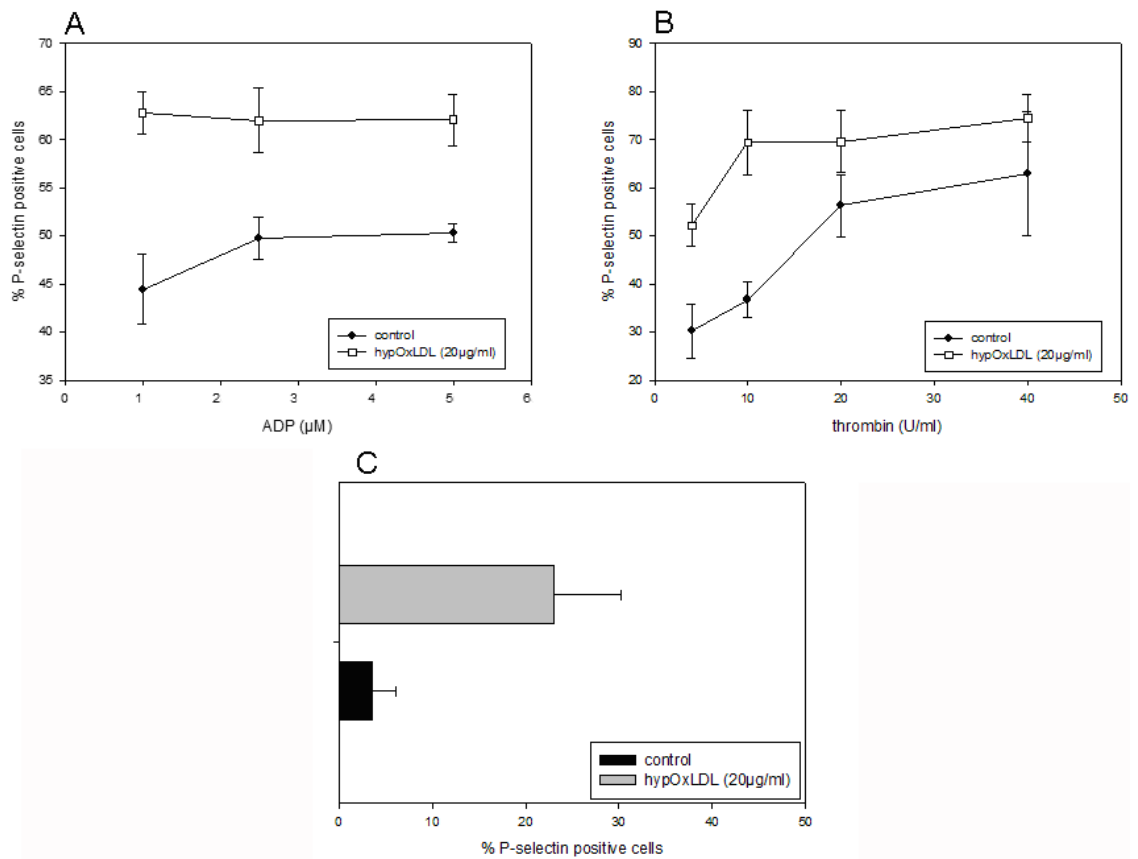
Figure 41: Effects of hypOxLDL on surface expression of P-selectin
    A: ADP (1µM-5µM) ± hypOxLDL (20µg/ml)
    B: thrombin (5U/ml-40U/ml) ± hypOxLDL (20µg/ml)
    C: hypOxLDL (20µg/ml)
    Results of flow cytometric experiments with platelets; means and standard deviations
    of results from 6 experiments

3.6.4 hypOxLDL induce surface expression of CD40L on platelets

In contrast to P-selectin and granulophysin, which are stored in granules, CD40L is reported to be stored in the cytosol of human platelets. Platelet activation leads to a translocation of CD40L to the platelet surface. Due to its important role in immune response, determination of CD40L surface expression by hypOxLDL is of special interest to further characterise the atherogenic role of oxidised LDL.

To investigate the impact of hypOxLDL on CD40L expression of human platelets, platelets were incubated with ADP, thrombin and hypOxLDL at agonist

concentrations which induced full platelet aggregation. Thereafter, platelets were stained for CD40L surface expression and analyzed by flow cytometry.

As depicted in Figure 42, thrombin, ADP and hypOxLDL lead to a significant increase in CD40L surface expression, whereat hypOxLDL show the highest impact on CD40L expression. Strategies to block binding of hypOxLDL to platelets by mHSA reveal that hypOxLDL-induced CD40L surface expression is significantly inhibited by coincubation with mHSA ($p<0.05$, also shown in Figure **42**).
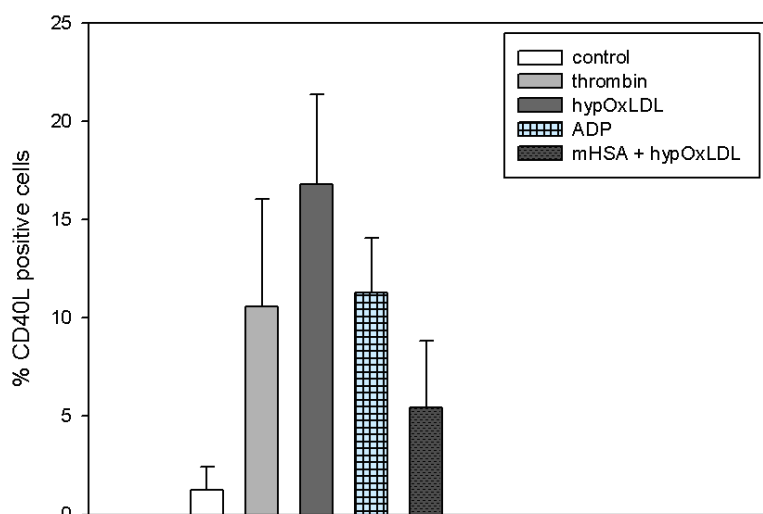

Figure 42: Effects of hypOxLDL on CD40L expression
    Results of flow cytometric experiments with platelets; thrombin (40U/ml), ADP (50μM) and hypOxLDL (100μg/ml) and mHSA (50μg/ml) + hypOxLDL (100μg/ml)
    LDL from 4 different donors; means and standard deviation of results from 11 experiments

3.6.5 Influence of hypOxLDL on activation state of GPIIb/IIIa

Antibody PAC-1 recognizes an epitope on the GPIIb/IIIa complex, which is only present on activated platelets and hence it can be used to determine platelet activation.

To investigate the impact of hypOxLDL on GPIIb/IIIa activation, resting platelets were incubated with hypOxLDL (100μg/ml) and analyzed for binding of antibody PAC-1. To further determine the binding effects of hypOxLDL, scavenger receptor binding of oxidised lipoproteins was blocked by coincubation with mHSA, and effects of mHSA on hypOxLDL-induced GPIIb/IIIa activation were evaluated.

As shown in Figure 43, hypOxLDL is able to induce GPIIb/IIIa activation. This stimulating effect of hypOxLDL can be successfully inhibited by preincubation of platelets with mHSA (also shown in Figure 43).
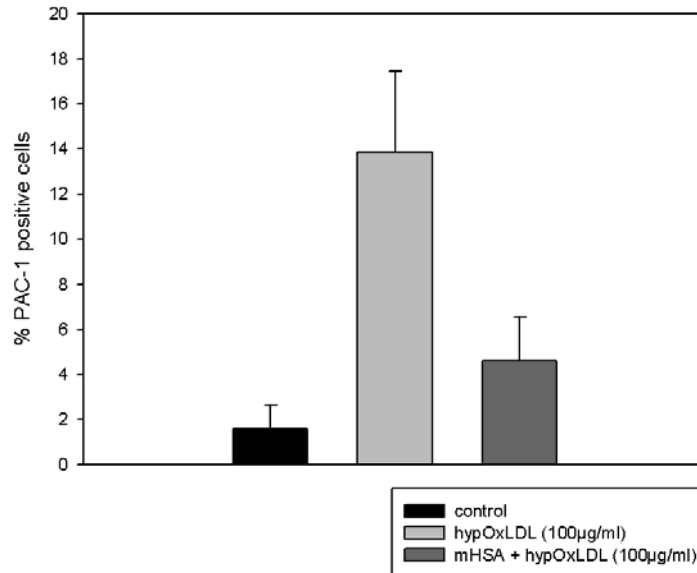

Figure 43: Influence of hypOxLDL on GPIIb/IIIa activation
Results of flow cytometric experiments with platelets stained for PAC-1 (activated GPIIb/IIIa); mHSA (50µg/ml); hypOxLDL (100µg/ml) from 4 different donors; means and standard deviation of results of 12 experiments

3.6.6 Attenuation of hypOxLDL-induced platelet aggregation by HDL

Since nHDL show a protective influence on ADP-, collagen- thrombin- and hypOxHDL-induced platelet aggregation, the question arose if nHDL are also able to attenuate platelet aggregation induced by hypOxLDL.

As shown in Figure 44, nHDL are able to impair platelet aggregation induced by different concentrations of hypOxLDL.

It can not be excluded that this effect of nHDL on hypOxLDL-induced platelet aggregation is not (only) attributable to inhibitory pathways induced by nHDL, but (also) might be due to the fact that nHDL and hypOxLDL compete for binding to the same receptor(s).
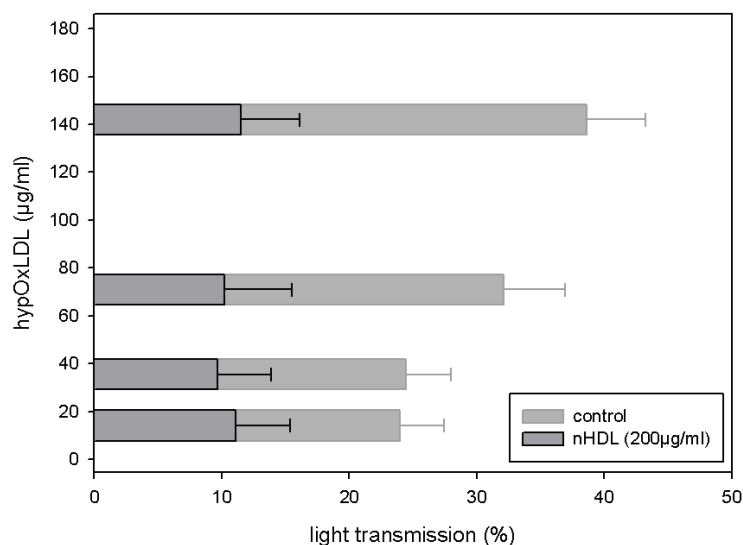
Figure 44: Influence of nHDL on hypOxLDL-induced platelet aggregation
Results of aggregometric experiments: platelets ± nHDL (200µg/ml) were compared in their aggregation response to hypOxLDL (18µg/ml to 145µg/ml); LDL from 4 different donors; means and standard deviation of results from 11 experiments

3.6.7 Influence of HDL$_2$, HDL$_3$ and antibody FA6.152 on hypOxLDL-induced surface expression of P-selectin

To further evaluate the effects of nHDL on hypOxLDL-induced platelet activation, differences between the HDL subclasses HDL$_2$ and HDL$_3$ were analyzed with regard to their influence on hypOxLDL-induced surface expression of P-selectin. Moreover, the influence of FA6.152 on hypOxLDL-induced α-granule release was investigated. FA6.152 is an antibody, which is directed against the binding domain for oxidised LDL on CD36 (amino acids Gln155 to Lys183).

Gel filtered platelets were preincubated with FA6.152, HDL$_2$ or HDL$_3$ and the influence of these preincubations on hypOxLDL (100µg/ml) induced surface expression of P-selectin was measured. Since the presence of antibody FA6.152 in stirred platelet suspensions leads to platelet aggregation[120] unfixed platelets were always preincubated with AT10 before incubation with antibody FA6.152.

As shown in Figure 45, blocking of OxLDL-binding site on CD36 by FA6.152 results in a very strong inhibition of platelet activation by hypOxLDL.

In identical experiments, HDL_2 and HDL_3 also decrease the number of P-selectin positive cells – furthermore, these two lipoprotein classes significantly differ in their impact on hypOxLDL-induced platelet activation. HDL_2, which contains more Apo E, seems to bear stronger platelet inhibiting capacities than HDL_3. This is in line with results of experiments on ADP induced platelet aggregation by other groups[87], which characterised the Apo E content as the responsible factor for the inhibitory action of HDL on (this type of) platelet aggregation.
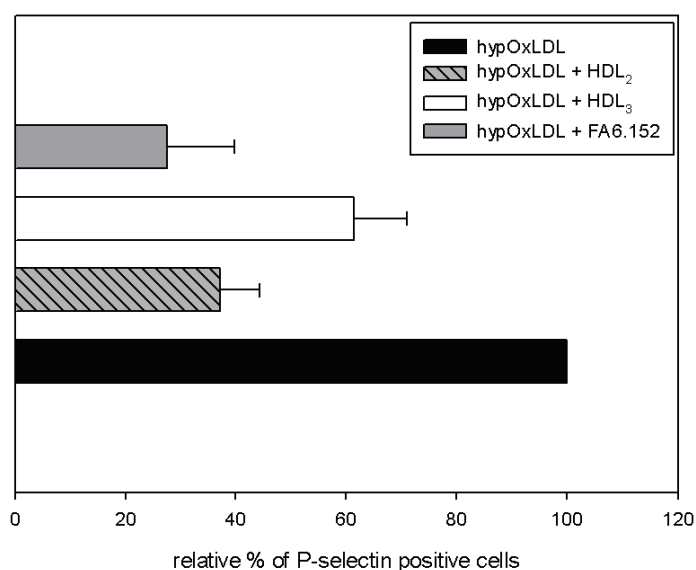

Figure 45: Influence of HDL_2, HDL_3 and antibody FA6.152 on hypOxLDL-induced surface expression of P-selectin
Out of 4 experiments - hypOxLDL (100µg/ml) induced P-selectin expression was set 100% and relative attenuation by HDL_2 (200µg/ml), HDL_3 (200µg/ml) and FA6.152 (4µg/ml + AT10 (2µg/ml)) was calculated

3.6.8 Effects of antibody FA6.152 on hypOxLDL-induced surface expression of P-selectin in pre-activated platelets

Besides CD36, another receptor candidate for oxidatively modified LDL (LOX-1) is reported to be present in human platelets. Since surface expression of LOX-1 is only induced upon platelet activation[79], platelets would be supposed to be pre-activated in order to be able to test a potential role of LOX-1 on hypOxLDL-induced platelet activation.Therefore, the ability of antibody FA6.152 to impair

surface expression of P-selectin induced by hypOxLDL was investigated in ADP-stimulated platelets (in presence and absence of FA6.152).

As shown in Figure 46, blocking of OxLDL binding site on CD36 leads to a significant reduction of P-selectin surface expression ($p<0.05$), revealing, once again, the importance of this scavenger receptor for intracellular signal transduction triggered by hypOxLDL. Compared to resting platelets shown in Figure 45, pre-activated platelets (depicted in Figure 46) do not show a reduced ability of FA6.152 to inhibit hypOxLDL-induced platelet activation. Antibody FA6.152 (for reasons outlined above in combination with antibody AT10) itself did not show any influence on data obtained with ADP (see Figure 46).
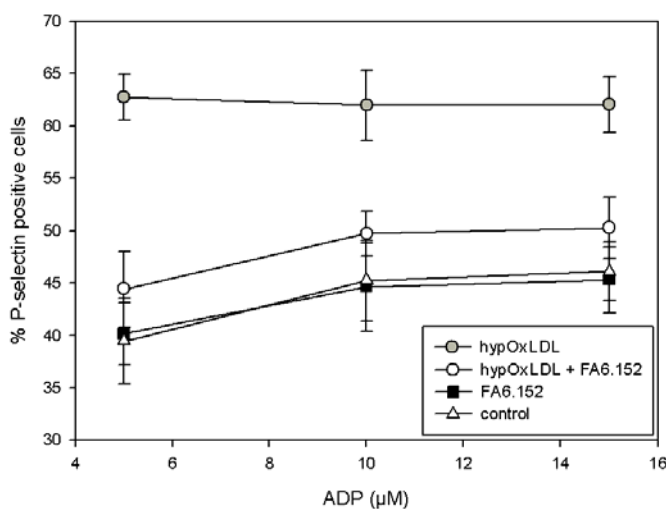

Figure 46: Effects of antibody FA6.152 on hypOxLDL-induced P-selectin expression in pre-activated platelets
Results of flow cytometric experiments with platelets; hypOxLDL (100µg/ml) from 4 different donors; all experiments with FA6.152 (4µg/ml) were performed in the presence of AT10 (2µg/ml); means and standard deviations of results from 8 experiments

Since FA6.152 leads to such a strong reduction of degranulation induced by hypOxLDL, the significance for LOX-1 in hypOxLDL-induced platelet activation has to be doubted.

3.6.9 Impact of hypOxLDL on VASP phosphorylation

The impact of oxidised LDL on VASP phosphorylation in human platelets has not been investigated so far. In light of platelet-stimulating effects of hypOxLDL, experiments concerning the impact of hypOxLDL on VASP phosphorylation open a new gateway to determine if hypOxLDL are also able to counteract PGE_1 action on human platelets.

Therefore, hypOxLDL, were tested for their ability to modulate intracellular VASP phosphorylation in the presence of different concentrations of PGE_1.

In Figure 47 results of 12 experiments on the influence of hypOxLDL on serine 239 VASP phosphorylation induced by submaximal concentrations of PGE_1 are shown. In submaximal concentrations of PGE_1 significant differences between untreated platelets and platelets coincubated with hypOxLDL were obtained, whereas at high concentrations of PGE_1 hypOxLDL failed to have an impact on VASP phosphorylation.
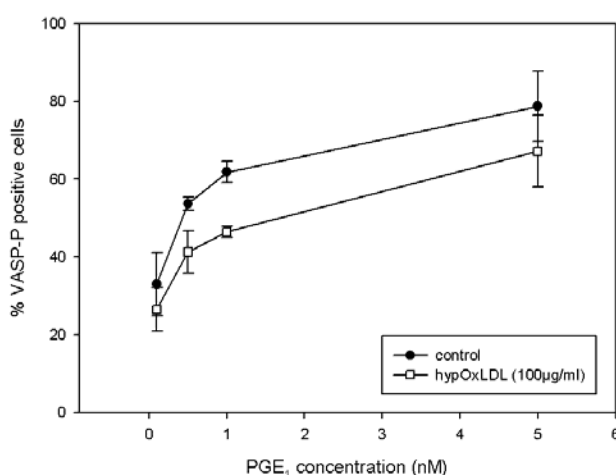

Figure 47: Influence of hypOxLDL on PGE_1-induced Ser 239 VASP phosphorylation
Results of flow cytometric experiments with platelets; VASP-P stands for Ser 239 VASP phosphorylation; PGE_1 (0.1-5nM); hypOxLDL from 3 different donors; means and standard deviations of results from 8 experiments

In accordance with results obtained from experiments with hypOxHDL. Again, platelet donors varied in their response to PGE_1. Therefore results of experiments in which platelets did not respond to submaximal concentrations of PGE_1 were excluded from evaluation

3.6.9.1 Impact of modification degree of hypOxLDL on PGE$_1$-induced VASP phosphorylation

To evaluate the in vivo relevance of attenuation of VASP phosphorylation by hypOxLDL it is important to determine the degree of modification necessary for the observed effects. As native LDL were shown to cause an increase of PGE$_1$-induced VASP phosphorylation, these investigations are of special interest.

To characterise the degree of modification necessary to reduce VASP phosphorylation, native LDL from 3 different donors were modified with 100- to 400-fold molar excess of hypochlorite and the impact of these oxidatively modified LDL on VASP phosphorylation was analyzed.

Figure 48 shows the influence of different degrees of LDL modification by hypochlorite on PGE$_1$–induced VASP phosphorylation.
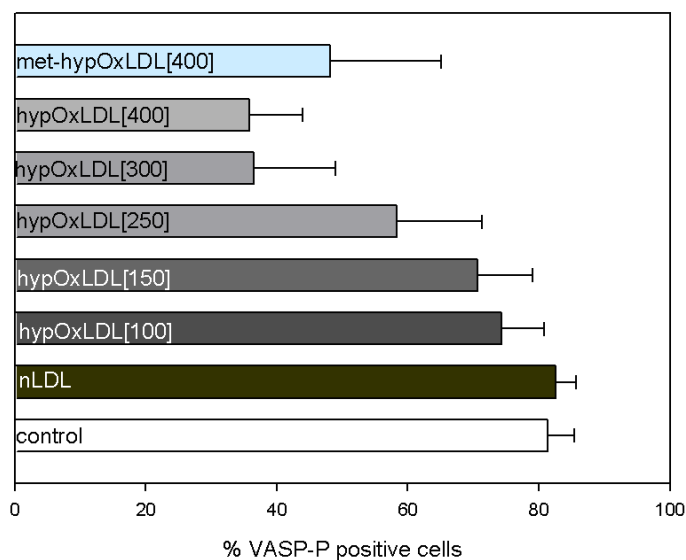

Figure 48: Effects of different degrees of hypOxLDL modification on PGE$_1$-induced VASP phosphorylation
Values in squared brackets indicate molar excess of hypochlorite over LDL; VASP-P stands for Ser 239 VASP phosphorylation; LDL from 3 different donors; means and standard deviations of results from 12 experiments

Unfortunately standard deviations were quite high, so that no statistically significant difference could be detected. It can be stated that, upon modification with a 100-fold molar excess of hypochlorite over LDL, hypOxLDL reduce PGE$_1$–induced VASP phosphorylation.

Methionine treated hypOxLDL[400], lead to a minor decrease of PGE_1–induced VASP phosphorylation than untreated hypOxLDL[400], but failed to restore the degree of VASP phosphorylation to values obtained with nLDL.

3.6.9.2 Inhibition of hypochlorite oxidised LDL effects on PGE_1-induced VASP phosphorylation

As shown in Figure 44-46, nHDL and antibody FA6.152 are able to interfere with platelet activating effects of hypOxLDL. Consequently, experiments were performed to determine if HDL and FA6.152 are also able to interfere with the effects of hypOxLDL on VASP phosphorylation. In addition, hexarelin was included in these experiments, as hexarelin has been reported to bind to CD36 at a binding domain that overlaps with the binding domain for oxidised LDL[121]. In Figure 49 FACS dot plots of one experiment are shown.
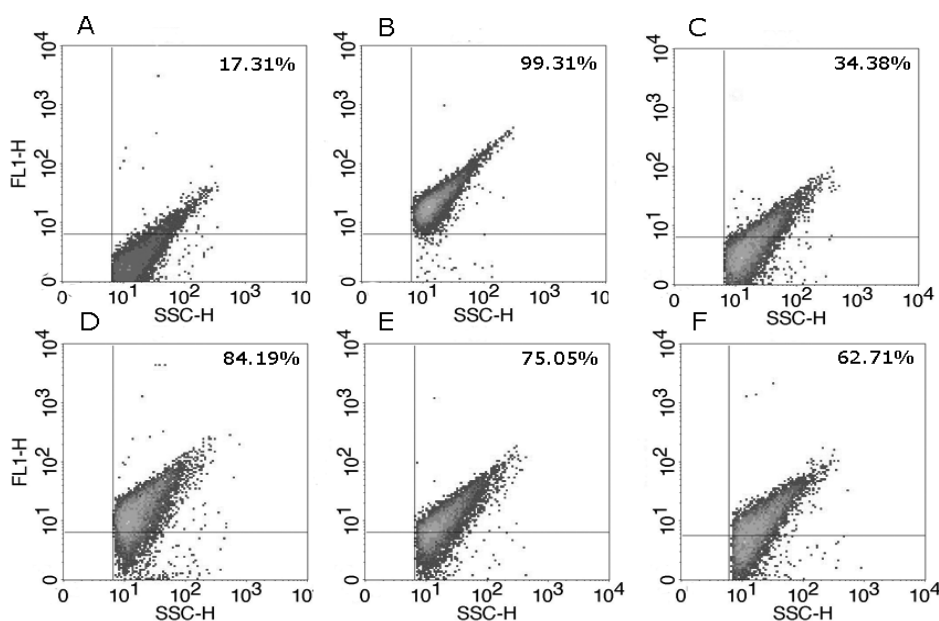

Figure 49: Effects of hypOxLDL on Ser 239 VASP phosphorylation
FACS dot plot of platelets, stained for serine 239 VASP phosphorylation (FL1); percentage of VASP phosphorylation positive cells are indicated in the upper right of each figure
A: control
B: PGE_1 (0.5μM)
C: hypOxLDL (100μg/ml) + PGE_1 (0.5μM)
D: AT10 (1μg/ml) + FA6.152 (4μg/ml) + hypOxLDL (100μg/ml) + PGE_1 (0.5μM)
E: nHDL (260μg/ml) + hypOxLDL (100μg/ml) + PGE_1 (0.5μM)
F: hexarelin (10μg/ml) + hypOxLDL (100μg/ml) + PGE_1 (0.5μM)

PGE$_1$, in a concentration of 0.5µM, is able to trigger Ser 239 VASP phosphorylation in almost all platelets (99.31%).

Upon coincubation with hypOxLDL only a third of the cells were VASP phosphorylation positive (34.38%). In the presence of FA6.152, VASP phosphorylation was restored to a significant part (84.19%).

Also coincubation with nHDL led to protection of VASP phosphorylation (75.05%). Moreover, hexarelin was proven to have a protective impact (62.71%).

3.6.10 Binding of oxidised LDL to human platelets
3.6.10.1 Binding of Alexa 633-labelled copper-oxidised LDL

To investigate if oxidised LDL specifically bind to human platelets, oxidised LDL were directly labelled with Alexa Fluor 633 dye. Because hypochlorite bleaches all fluorophors, LDL had to be oxidised with copper (CuOxLDL).

In Figure 50A binding of Alexa 633-labelled CuOxLDL to platelet and displacement with unlabelled CuOxLDL, mHSA and FA6.152 is shown.

Since the molar excess of CuOxLDL was too low to block all saturable binding sites, binding was calculated assuming that unlabelled and labelled lipoproteins bind in an identical way to platelets and the fluorescence signal was extrapolated. Figure 50B shows total binding calculated by extrapolation. This procedure obviously leads to unreasonably high "binding".

The only way to explain this phenomenon is to assume that unlabelled and labelled lipoproteins actually do not bind in identical manner. Therefore, a protocol avoiding fluorescence-labelling of OxLDL was developed (cf. 1.1.3.2.).
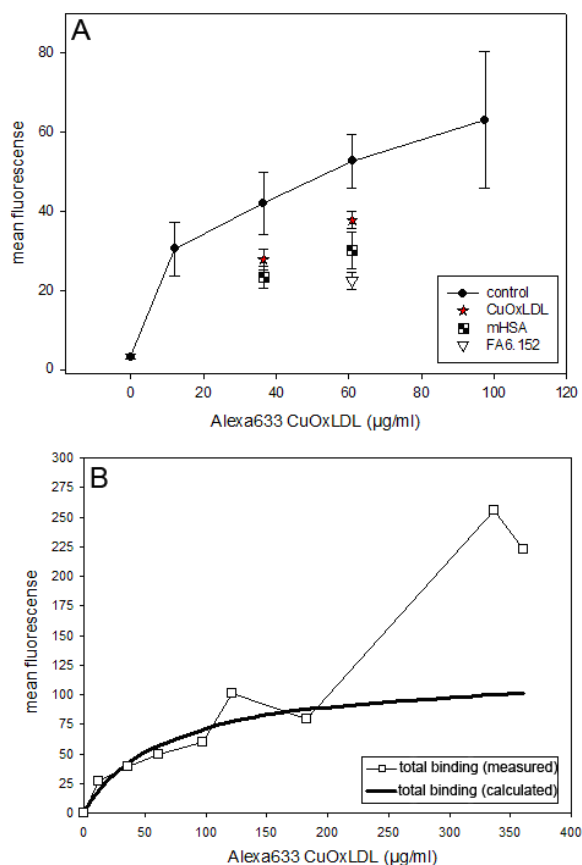
Figure 50: Binding of Alexa 633 CuOxLDL to platelets
CuOxLDL (300µg/ml), mHSA (50µg/ml) and FA6.152 (3µg/ml)
A: means and standard deviations of 8 experiments
B: calculated binding of the data depicted in **A**

3.6.10.2 hypOxLDL binding studies by Apo B detection

Besides the mentioned disadvantages of direct labelling, it is not the ideal tool to show binding anyway, since only CuOxLDL but not hypOxLDL can be investigated. Therefore a second protocol using anti Apo B antibody was implemented. Platelets were incubated with different concentrations of hypOxLDL and potential competitors of hypOxLDL binding to human platelets (nHDL, hypOxHDL, mHSA, polyclonal anti CD36 and FA6.152).

Figure 51A shows the measured and fitted binding curves of hypOxLDL to platelets by Apo B detection. Since displacement with the same class of lipoproteins is not possible with this technique, polyclonal CD36 antibody in excess was employed to calculate specific binding of hypOxLDL, which is also depicted in Figure 51A. The dissociation constant was calculated to be

10.3 x 10^{-8}M, which is in accordance with data obtained for copper oxidised LDL (9.6 x 10^{-8}M)[122].

In Figure 51B displacement of hypOxLDL by other potential competitors is depicted. The most efficient displacement occurred in the presence of mHSA. Also nHDL and hypOxHDL seem to compete for binding with hypOxLDL. Polyclonal CD36 antibody and monoclonal CD36 antibody FA6.152, had a severe impact on hypOxLDL binding to platelets, but less than the before mentioned competitors. Since mHSA is a significantly stronger competitor for hypOxLDL binding to platelets than antibodies against CD36, the question arises if binding sites different to CD36 exist, which can be blocked by mHSA. Interestingly hypOxHDL, which seems to bind only to CD36 also blocks hypOxLDL binding more efficient than both CD36 specific antibodies.
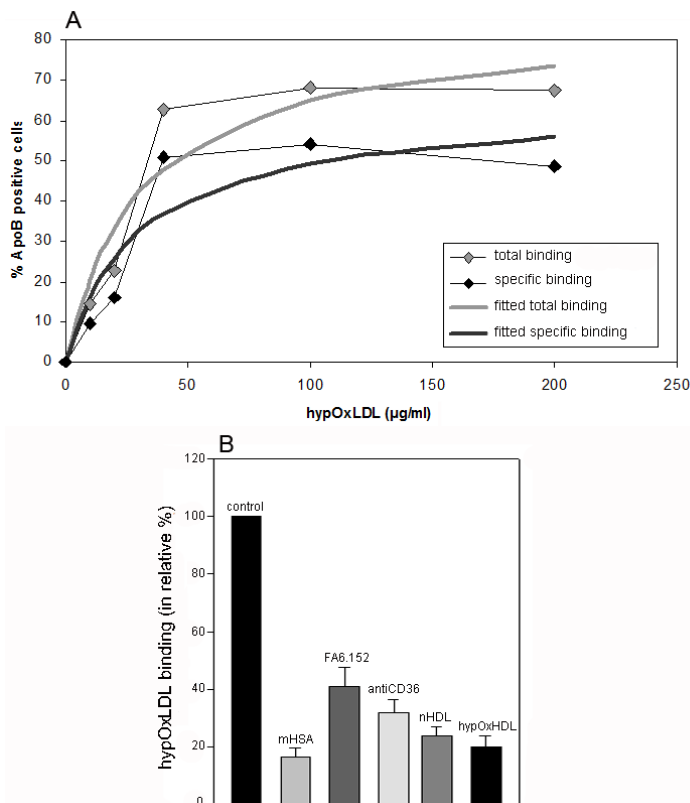

Figure 51: hypOxLDL: total binding to platelets determined by Apo B detection and displacement
    A: total and specific binding of hypOxLDL (10μg/ml-200μg/ml) to platelets: hypOxLDL from 6 different donors; means of results from 14 experiments, specific binding calculated by displacement with polyclonal CD36 antibody (3μg/ml)
    B: displacement of hypOxLDL (100μg/ml) binding to platelets by various competitors: mHSA (500μg/ml), nHDL (400μg/ml), FA6.152 (4μg/ml); anti CD36 (=polyclonal CD36 antibody (3μg/ml)); hypOxLDL from 4 different donors; means and standard deviations of results of 8 experiments

4   Discussion

Blood platelets are of central importance to the process of (primary) hemostasis and coagulation. Therefore, abnormalities in platelet function (resulting in thrombosis or bleeding) result in severe and potentially lethal consequences.

Inadvertent platelet activation can be observed in diverse diseases that coincide with inflammation and systemic oxidative stress – for example atherosclerotic disease that is the major source of morbidity and mortality in the Western world.

As platelet activation causes degranulation of platelets, resulting in the release of several growth factors as well as pro-inflammatory and pro-thrombotic mediators, platelet activation is associated with accelerated atherosclerosis and correlates with severity of this disease in humans.

Lipoproteins, especially low density lipoproteins (LDL) and high density lipoproteins (HDL), play a crucial and ambiguous role in the development of atherosclerotic disease.

A wealth of evidence indicates that high plasma concentrations of LDL favour the onset and propagation of atherosclerosis and thrombosis, while plasma concentrations of HDL are inversely correlated with the occurrence of these events.

Interestingly, both LDL and HDL have been also shown to be able to directly influence platelet function[123] and increasing evidence indicates that lipoproteins are able to induce different intracellular signalling pathways in platelets, although the entire mechanisms are still poorly understood.

A large body of evidence supports the view that oxidative stress is closely related to atherogenesis and results of several studies were able to show that circulating markers of inflammation are predictive of both atherosclerosis and the clinical events associated with this disease[124-128]. According to the *oxidative response to inflammation hypothesis of atherosclerosis*[129], inflammation is a primary process in atherosclerosis. Accordingly, inflammation represents the source of oxidative stress which in turn sets up a vicious circle by exacerbating inflammation.

Lipoproteins have been identified as preferred targets of such oxidative stress and MPO and oxidants generated by this enzyme seem to play a central role in the pathogenesis of atherosclerosis.

Hypochlorite/hypochlorous acid (HOCl), the major strong oxidant generated by MPO, has been implicated in the in vivo oxidation of LDL[22] and lipoproteins have been shown to acquire several pro-atherogenic and pro-thrombotic properties upon oxidative modification by hypochlorite.

Interestingly, the influence of oxidatively modified (low density) lipoproteins on platelet function has been shown to depend on the underlying oxidation procedure: hypochlorite-oxidised LDL are able to trigger platelet aggregation and this can not be observed with LDL oxidised by trace metal, even when both OxLDL species are oxidised to a comparable extent[97]. Furthermore (and this might also serve as an explanation for these findings), hypochlorite shows a strong predilection for the protein moiety of lipoproteins and in contrast to the situation observed with one-electron oxidants, lipoprotein oxidation by hypochlorite does not result in the formation of lipid hydroperoxides[130] or thiobarbituric acid reactive substances (TBARS)[97].

In light of the central importance of platelets to the atherosclerotic process, it was the aim of this work to investigate and characterise the impact of LDL and HDL – both in their native state as well as after oxidative modification – on several aspects of platelet function and to establish the molecular mechanisms that form the basis for the observed lipoprotein-mediated effects.

Platelets and high density lipoproteins

Plasma HDL cholesterol levels inversely correlate with platelet hyperreactivity[131]. Also in vitro studies were able to show an inhibitory effect of HDL on platelet function, although the underlying mechanism is not fully understood (reviewed by SURYA et al[123]).

In this work shows that native HDL mediate an inhibitory effect on platelet aggregation and on platelet activation induced by submaximal concentrations of classical agonists (ADP, thrombin and collagen). Nevertheless, HDL fail to show an impact on platelet activation when activation induced by these agonists

reaches a maximum extent. This platelet inhibiting impact of native HDL confirms and extends previously published findings[103, 131, 132]. Since many biochemical events that are critical for platelet activation are known to be affected by HDL, it is difficult to identify the mechanisms by which HDL mediate a protective impact on submaximal but not on fully activated platelets.

It has been shown in platelets that HDL_3 induce activation of protein kinase C (PKC) and consequently phosphoinositide-specific phospholipase C (PI-PLC), an important signal transduction mediator of thrombin, collagen and (to a minor extent) ADP, is inhibited[133]. Moreover HDL interaction with platelets accounts for the induction of nitric oxide synthesis[134]. Nitric oxide (NO), amongst many other actions, induces phosphorylation of platelet vasodilator stimulated phosphoprotein (VASP) by NO dependent activation of guanylyl cyclase and subsequent stimulation of cGMP dependent protein kinases. In its phosphorylated state, VASP is important to the polymerization of actin.

Somewhat surprisingly, HDL fail to show an impact on VASP phosphorylation in resting platelets. Nevertheless, upon coincubation with submaximal concentrations of prostaglandin E_1 (PGE_1), HDL enhance intracellular VASP phosphorylation in a dose dependent way. Since HDL have no influence on PGE receptor binding nor on prostaglandin stability[65], direct effects of HDL on PGE_1 seem unlikely.

Instead, the evoked effects might reflect the fact that NO production by platelets is not sufficient to induce VASP phosphorylation, but is able to synergistically increase VASP phosphorylation in the presence of other agonists.

Since Apo E was shown to markedly elevate platelet NO synthase activity and intra-platelet levels of cGMP, NO release induced by HDL is suggested to be mediated by Apo E[88,83]. Nevertheless, it could be shown that also apoE-rich subclass HDL_2 is not able to induce VASP phosphorylation on its own. Surprisingly HDL_3, which is reported to contain less Apo E, seems to have a stronger influence on VASP phosphorylation upon addition of submaximal concentrations of PGE_1 than HDL_2. Taken together, it can be stated that the effects of HDL on VASP phosphorylation may not be attributable to the Apo E content of the lipoproteins. VASP phosphorylation, which is an established

marker of NO-bioavailability and closely correlates with the *activation of pathways* that are responsible for inhibition of platelet function, does not increase upon incubation with HDL. This is especially surprising as the same preparations of HDL were able to augment platelet aggregation and platelet degranulation. To further investigate if the observed increase in VASP phosphorylation induced by HDL and PGE_1 is NO dependent or if other mechanisms are involved, experiments in the presence of an inhibitor of nitric oxide synthesis are planned for the future.

There is still disagreement which receptor is responsible for platelet interaction with native HDL. LDL receptor-related protein 8 (LRP8) has been identified on human platelets and has also been proven to bind Apo E particles and thereby HDL[83]. Nevertheless, it is still unknown if LRP8 is the only platelet receptor for native HDL, since also GPIIb/IIIa and CD36 represent potential receptor candidates[73, 135].

Binding studies performed within this work were able to show specific and saturable binding of native HDL to human platelets and it was possible to calculate specific binding and dissociation constant (2.4×10^{-7} M), which is fairly similar to results obtained by radiolabelling techniques (reviewed by KOLLER et al.[84]). It could be shown that maleylated human serum albumin (mHSA) is able to compete with HDL for binding to the platelet surface. This is especially interesting in light of the fact that mHSA has originally been shown to block binding of oxidatively (and chemically) modified LDL to virtually all scavenger receptors. Therefore, the possibility exists that HDL might mediate their effects on platelet function in vivo also by competing with oxidised LDL for their binding to platelets.

As oxidative stress originating from inflammation is of central importance to atherogenesis and as platelets represent redox-sensitive cells, it is of special interest that HDL also possess anti-oxidative and anti-inflammatory properties that are only in part attributable to enzymatic activities residing within the lipoprotein particle[136]. Due to their antioxidative exertion, HDL become unavoidably oxidised themselves and since they represent an easier target for oxidation than LDL, they are even more likely to get oxidised[137].

Given its accepted protective functions, oxidative modification of HDL is mainly seen as an unavoidable side-effect of its function to protect other targets (e.g., LDL) from oxidative damage. Nevertheless, recent literature has provided compelling evidence that upon oxidative modification of HDL, these lipoproteins not only lose important protective functions, but also acquire severe pro-inflammatory and pro-thrombotic properties. In detail, it could be shown that oxidatively modified HDL interfere with reverse cholesterol transport, activate mitogen-activated protein kinase and upregulate the expression of cyclooxygenase-2, plasminogen activator inhibitor-1 and matrix-degrading proteases in endothelial cells (reviewed by ANSELL et al.[138])

In light of the redox-sensitive nature of platelet function and as there are only very limited data concerning the impact of oxidatively modified HDL on platelets, it was the main aim of this work to also ascertain the effects of hypochlorite oxidised HDL on platelet function.

This work shows that upon oxidative modification HDL not only lose their platelet-inhibitory properties but even gain platelet activating properties. Hypochlorite-modified HDL significantly amplify degranulation induced by classical agonists (ADP, thrombin, collagen), whereas nHDL from the same donor display augmenting effects on agonist-induced platelet activation.

Subsequently it could be shown that hypOxHDL per se are able to trigger platelet aggregation and platelet activation in a dose- and modification-dependent way.

HDL modification with a 75-fold molar excess of hypochlorite over HDL is sufficient to invert their protective impact on platelet activation.

Upon oxidation by hypochlorite, HDL are able to induce GPIIb/IIIa activation and immediate intraplatelet calcium influx as well as platelet aggregation and degranulation (proven by surface expression of P-selectin and granulophysin). Moreover, hypOxHDL are able to reduce the amount of PGE_1-induced Ser 239 VASP phosphorylation.

All these platelet activating effects induced by hypOxLDL can be impaired by native HDL or by elimination of chloramines in hypOxHDL by means of methionine treatment. Furthermore, maleylated human serum albumin that also

interferes with binding of hypOxHDL to the platelet surface strongly augments the ability of hypOxHDL to induce platelet activation.

In line with these findings that strongly argue for a role of specific (and presumably chloramine-mediated) binding in the observed platelet-stimulating effects it could also be shown that platelet aggregation as well as activation induced by hypOxHDL can be totally inhibited by blocking an epitope on CD36 responsible for binding of oxidised LDL, indicating that interaction of hypOxHDL with CD36 is necessary for platelet activation.

In line with results of binding studies performed in other cell types where CD36 has been shown to bind (copper) oxidised HDL[139], preliminary results of binding studies in platelets suggest that CD36 might be the only binding site for hypOxHDL. Moreover, oxidised HDL seem to bind to the same domain of CD36 as oxidised LDL.

Taken together it can be stated that native HDL have a protective impact on platelet aggregation and activation. These effects invert upon oxidative modification of HDL and oxidised HDL per se are able to trigger platelet aggregation and activation.

The finding that oxidation by the in vivo occurring oxidant hypochlorite converts HDL into a strong platelet agonist represents an important finding. Myeloperoxidase and myeloperoxidase-derived hypochlorite play a central role in atherosclerosis and (other forms of) systemic inflammation and these diseases coincide with enhanced platelet reactivity. Local concentrations of hypochlorite at sites of acute inflammation have been calculated to be 340µM and higher[140]. Therefore, the extent of oxidative modification of hyp-OxHDL used in this study would be estimated to be roughly comparable to the degree of (subendothelial) HDL modification that might occur in vivo.

The in vivo formation of oxidised HDL might contribute to platelet hyperreactivity observed in conditions of systemic oxidative stress and might also play an important role in thrombus formation upon atherosclerotic plaque rupture.

Platelets and low density lipoproteins

It has been known for many years that platelets from hyperlipidemic patients are hyperreactive[141] and that LDL from patients with homozygous familial hypercholesterolemia show enhanced susceptibility to oxidative modification[142]. The fact that oxidative modification renders LDL atherogenic is undoubted but the role of native LDL is still unacknowledged. In vitro studies with human platelets revealed controversial results of the actions of native LDL. Therefore, their actions remain still unclear.

The results demonstrated in this work show that LDL are able to impair platelet activation induced by submaximal concentrations of classical agonists in a dose dependent way. Moreover, in the presence of nLDL, platelets show an increase in PGE_1-induced VASP phosphorylation. These results suggest that unmodified LDL do not bear any platelet activating properties, in contrast even inhibiting actions on human platelets could be ascertained.

Upon oxidative modification LDL are able to trigger platelet activation per se. Amounts as little as 5µg/ml of oxidised LDL are able to activate platelets and a 100-fold molar excess of hypochlorite over LDL is sufficient to invert their protective function and stimulate platelets.

Our findings that hypOxLDL independently trigger platelet aggregation and induce P-selectin surface expression is in line with other reports[23, 77, 99]. Moreover, hypochlorite-modified LDL elicit GPIIb/IIIa activation and induce surface expression of CD40L in human platelets. Furthermore, oxidised LDL are also able to counteract PGE_1–induced VASP phosphorylation.

Upon treatment with methionine or coincubation with native HDL the platelet activating properties of hypOxLDL are strongly reduced, whereat HDL_2, which contains more Apo E, seems to bear stronger platelet inhibiting capacities than HDL_3. This is in line with results of experiments on ADP induced platelet aggregation by other groups[87], which characterised the Apo E content as the responsible factor for the inhibitory action of HDL on ADP-induced platelet aggregation.

Moreover the effects of hypOxLDL could be inhibited by blocking binding of hypOxLDL to the platelet surface by means of mHSA. Furthermore, blocking of

amino acids Gln155 to Lys183 on CD36 by monoclonal antibody FA6.152 inhibits the actions of hypOxLDL, which reveals the importance of this scavenger receptor for intracellular signal transduction triggered by hypOxLDL.

Also in pre-activated platelets which are reported to express LOX-1[79], another potential receptor candidate for oxidised LDL, an essential role of CD36 in the process of hypOxLDL induced platelet activation is without controversy.

Moreover binding characteristics of oxidised LDL to human platelets were investigated.

The dissociation constant of hypOxLDL binding to human platelets was calculated to be 10.3 x 10^{-8}M, what represents a virtually identical affinity as that reported for copper oxidised LDL (9.6 x 10^{-8}M)[122].

Binding of oxidised LDL to human platelets could be successfully displaced by native HDL, mHSA and polyclonal anti CD36 antibody as well as a monoclonal antibody directed against the domain of amino acids 155-183 of CD36. Our results clearly argue for a central role of CD36 for the interaction of platelets with OxLDL.

Taken together these observations suggest that LDL generate platelet activating properties only upon oxidative modification. In their native form LDL are able to inhibit platelet activation, while oxidised LDL act as strong platelet agonists by inducing activation, granule secretion and platelet aggregation. The fact that hypOxLDL upregulate platelet CD40L expression is of special interest since CD40L is a powerful stimulus for oxidative stress[143]. Our findings further underline the central importance of platelets and (hypochlorite) oxidised LDL to atherogenesis, as CD40L is able to elicit inflammatory and pro-thrombotic responses which favour and accelerate atherosclerotic progression[144].

5   Summary

Besides their important function in primary haemostasis and coagulation, platelets and their activation state play a pivotal role in the initiation and progression of atherosclerotic disease. The haem-enzyme myeloperoxidase and the myeloperoxidase-derived oxidant hypochlorite play a central role in atherosclerosis and in (other forms of) systemic inflammation that coincide with enhanced platelet reactivity. Once activated, platelets elicit inflammation as well as thrombus formation and subsequent occlusion of blood vessels.

A wealth of evidence indicates that lipoproteins, especially low density lipoproteins (LDL) and high density lipoproteins (HDL), directly influence the activity state of platelets. It is generally accepted that high plasma levels of HDL inversely correlate with platelet hyperreactivity, whereat plasma levels of LDL show the opposite effect. Since hypercholesterolemic patients also show enhanced susceptibility to oxidative lipoprotein modification, it remains unclear if the underlying mechanism of platelet activation in these patients is a consequence of lipoprotein oxidation. In vitro studies on the effects of native LDL on platelet function revealed inconsistent results, while oxidised LDL are accredited platelet activating functions.

Results of this study indicate that not only native HDL but also native LDL have inhibitory effects on platelet activation. Both classes of lipoproteins are able to impair agonist-induced platelet aggregation and degranulation. Moreover they render platelets more sensitive towards prostaglandins that impede platelet function, proven by enhanced PGE_1-induced VASP phosphorylation. Upon oxidative modification of lipoproteins, which was performed by the in vivo occurring oxidant hypochlorite, not only LDL but also HDL invert their function and acquire the ability to independently trigger platelet aggregation, degranulation, GPIIb/IIIa activation and decrease of VASP phosphorylation. Moreover, hypochlorite-oxidised LDL upregulate expression of platelet CD40L. The latter is of special interest since (soluble) CD40L elicits inflammatory and pro-thrombotic responses that favour and accelerate the progression of atherosclerosis.

The results shown within this work clearly argue for a central role of scavenger receptor CD36 in the interaction of platelets with oxidised lipoproteins, since blocking of this receptor leads to a strong attenuation of all platelet-activating effects. Amino acids 155-183 of CD36 seem to be the responsible domain for these effects. Moreover, specific binding of these oxidised lipoproteins could be demonstrated, which seems prerequisite for subsequent signal transduction.

These novel findings further support the model that oxidative stress is closely related to potentially atherogenic events and that the interaction of platelets and lipoproteins might therefore play a pivotal role in the progression of these events. Notably, local concentrations of hypochlorite at sites of acute inflammation have been calculated to be 340µM and higher. Therefore, the extent of oxidative modification of the lipoproteins used in this study would be estimated to be roughly comparable to the degree of (subendothelial) lipoprotein modification that might occur in vivo.

6 Zusammenfassung

Neben ihrer zentralen physiologischen Bedeutung für die primäre Hämostase und die plasmatische Blutgerinnung spielen Thrombozyten und ihr Aktivierungszustand eine entscheidende Rolle in der Entstehung und dem Fortschreiten atherosklerotischer Erkrankungen.

Das Enzym Myeloperoxidase und das von diesem Enzym gebildete starke Oxidans Hypochlorit spielen eine zentrale Rolle in der Genese der Atherosklerose und anderer Formen inflammatorischer Erkrankungen, welche auch mit einer erhöhten Thrombozytenreaktivität einhergehen. Dies ist vor allem insofern interessant, als aktivierte Thrombozyten selbst Entzündungsreaktionen hervorrufen können.

Eine Vielzahl an Befunden weist darauf hin, dass Lipoproteine - speziell LDL und HDL – imstande sind, die Thrombozytenaktivierung direkt zu beeinflussen. Hohe Plasmakonzentrationen von HDL gehen mit einer verminderten Thrombozytenreaktivität einher, während erhöhte Plasmakonzentrationen von LDL eng mit Thrombozytenhyperreaktivität korrelieren.

Da jedoch Lipoproteine von hypercholesterinämischen Patienten eine erhöhte Anfälligkeit für oxidative Modifizierung zeigen bleibt unklar, ob die in diesen Patienten zu beobachtende Thrombozytenaktivierung primär eine Folge der erhöhten LDL Konzentrationen oder eine Konsequenz der Lipoproteinoxidation ist.

In vitro Studien zeigten bislang widersprüchliche Effekte von nativen LDL auf die Thrombozytenfunktion, während für oxidierte LDL eindeutig thrombozyten-aktivierende Wirkungen nachgewiesen werden konnten.

In dieser Arbeit konnte gezeigt werden, dass nicht nur native HDL sondern auch native LDL einen inhibierenden Effekt auf Thrombozyten ausüben. Beide Lipoproteinklassen sind im Stande die durch verschiedene Agonisten verursachte Thrombozytenaggregation und Degranulierung abzuschwächen. Darüber hinaus verstärken native Lipoproteine die Thrombozyten-hemmende Wirkung von Prostaglandin, was durch Steigerung der PGE_1-induzierte VASP Phosphorylierung nachgewiesen werden konnte.

Nach Oxidation der Lipoproteine durch das in vivo vorkommende Oxidans Hypochlorit verlieren sowohl LDL als auch HDL ihren hemmenden Einfluss auf die Thrombozytenfunktion und entwickeln die Fähigkeit unabhängig von anderen Agonisten Thrombozytenaggregation, Degranulierung und GPIIb/IIIa Aktivierung hervorzurufen, wie auch die intrazelluläre VASP Phosphorylierung zu reduzieren. Darüber hinaus sind Hypochlorit-oxidierte LDL im Stande, die Expression von CD40L an der Thrombozytenoberfläche zu induzieren. Dieser Befund ist auch insofern von großem Interesse, als CD40L Entzündungen und prothrombotische Antworten hervorruft, welche für das Fortschreiten von atherosklerotischen Geschehnissen mitverantwortlich sind.

Die in dieser Arbeit präsentierten Daten weisen darauf hin, dass oxidierte Lipoproteine eine spezifische und sättigbare Bindung an die Thrombozyten-oberfläche zeigen und dies dürfte die Grundlage für die beobachtete Signaltransduktion darstellen. Der Scavenger Rezeptors CD36 spielt offensichtlich eine zentrale Rolle in der Interaktion von Thrombozyten mit oxidierten Lipoproteinen, da das Blocken dieses Rezeptors zu einer starken Abschwächung der biologischen Wirkung oxidierter Lipoproteine auf die Thrombozyten führt. Konkret konnte eine Domäne auf CD36 (Aminosäuren 155 bis 183) identifiziert werden, welche für den Bindungsvorgang und daran anschließende Signaltransduktion essentiell ist.

Die hier vorgestellten Befunde unterstützen das Modell, dass oxidativer Stress in engem Zusammenhang mit (potentiell) pro-thrombotischen und pro-inflammatorischen Folgereaktionen steht und dass hierbei Thrombozyten wie auch Lipoproteine eine entscheidende Rolle einnehmen.

Vor dem Hintergrund von Berechnungen der lokalen Konzentrationen von Hypochlorit an Orten akuter Entzündung (340µM und höher) ist festzuhalten, dass das Ausmaß der Modifizierung der in dieser Arbeit verwendeten oxidierten Lipoproteine im Bereich des sich aus diesen Berechnungen für in vivo Verhältnisse ergebenden (subendothelialen) Modifikationsgrades liegt.

7   Table of figures

122

8 References

(1) Ross R, Glomset JA. Atherosclerosis and Arterial Smooth-Muscle Cell. Science 1973;180(4093):1332-9.

(2) Williams KJ, Tabas I. The Response-To-Retention Hypothesis of Early Atherogenesis. Arteriosclerosis Thrombosis and Vascular Biology 1995 May;15(5):551-61.

(3) Steinberg D, Parthasarathy S, Carew TE, Khoo JC, Witztum JL. Low-Density Lipoprotein and Atherogenesis - Reply. New England Journal of Medicine 1989 October 26;321(17):1196-7.

(4) Falk E, Shah PK, Fuster V. Coronary Plaque Disruption. Circulation 1995 August 1;92(3):657-71.

(5) Brown MS, Goldstein JL. A proteolytic pathway that controls the cholesterol content of membranes, cells, and blood. Proceedings of the National Academy of Sciences of the United States of America 1999 September 28;96(20):11041-8.

(6) Murphy JE, Tedbury PR, Homer-Vanniasinkam S, Walker JH, Ponnambalam S. Biochemistry and cell biology of mammalian scavenger receptors. Atherosclerosis 2005 September;182(1):1-15.

(7) Brown MS, Goldstein JL. Lipoprotein Metabolism in the Macrophage - Implications for Cholesterol Deposition in Atherosclerosis. Annual Review of Biochemistry 1983;52:223-61.

(8) Young IS, McEneny J. Lipoprotein oxidation and atherosclerosis. Biochemical Society Transactions 2001 May;29:358-62.

(9) Gordon DJ, Rifkind BM. High-Density Lipoprotein - the Clinical Implications of Recent Studies. New England Journal of Medicine 1989 November 9;321(19):1311-6.

(10) Connelly MA, Williams DL. Scavenger receptor BI: A scavenger receptor with a mission to transport high density lipoprotein lipids. Current Opinion in Lipidology 2004 June;15(3):287-95.

(11) Mineo C, Deguchi H, Griffin JH, Shaul PW. Endothelial and antithrombotic actions of HDL. Circulation Research 2006 June 9;98(11):1352-64.

(12) Griffin JH, Kojima K, Banka CL, Curtiss LK, Fernandez JA. High-density lipoprotein enhancement of anticoagulant activities of plasma protein S and activated protein C. Journal of Clinical Investigation 1999 January;103(2):219-27.

(13) Kontush A, Chantepie S, Chapman MJ. Small, dense HDL particles exert potent protection of atherogenic LDL against oxidative stress. Arteriosclerosis Thrombosis and Vascular Biology 2003 October;23(10):1881-8.

(14) Francis GA. High density lipoprotein oxidation: in vitro susceptibility and potential in vivo consequences. Biochimica et Biophysica Acta-Molecular and Cell Biology of Lipids 2000 January 17;1483(2):217-35.

(15) Sies H. Oxidative stress: Oxidants and antioxidants. Experimental Physiology 1997 March;82(2):291-5.

(16) Demirbag R, Yimaz R, Kocyigit A. Relationship between DNA damage, total antioxidant capacity and coronary artery disease. Mutation Research-Fundamental and Molecular Mechanisms of Mutagenesis 2005 March 1;570(2):197-203.

(17) Carr AC, Mccall MR, Frei B. Oxidation of LDL by myeloperoxidase and reactive nitrogen species - Reaction pathways and antioxidant protection. Arteriosclerosis Thrombosis and Vascular Biology 2000 July;20(7):1716-23.

(18) Ohgushi M, Kugiyama K, Fukunaga K, Murohara T, Sugiyama S, Miyamoto E, Yasue H. Protein-Kinase-C Inhibitors Prevent Impairment of Endothelium-Dependent Relaxation by Oxidatively Modified Ldl. Arteriosclerosis and Thrombosis 1993 October;13(10):1525-32.

(19) Harats D, Mulkins MA, Sigal E. A Possible Role for 15-Lipoxygenase in Atherogenesis. Trends in Cardiovascular Medicine 1995 January;5(1):29-36.

(20) Harats D, Shaish A, George J, Mulkins M, Kurihara H, Levkovitz H, Sigal E. Overexpression of 15-lipoxygenase in vascular endothelium accelerates early atherosclerosis in LDL receptor-deficient mice. Arteriosclerosis Thrombosis and Vascular Biology 2000 September;20(9):2100-5.

(21) Chen JW, Breckwoldt MO, Aikawa E, Chiang G, Weissleder R. Myeloperoxidase-targeted imaging of active inflammatory lesions in murine experimental autoimmune encephalomyelitis. Brain 2008 April;131:1123-33.

(22) Heinecke JW. Pathways for oxidation of low density lipoprotein by myeloperoxidase: tyrosyl radical reactive aldehydes, hypochlorous acid and molecular chlorine. Biofactors 1997;6(2):145-55.

(23) Volf I, Bielek E, Moeslinger T, Koller F, Koller E. Modification of protein moiety of human low density lipoprotein by hypochlorite generates strong platelet agonist. Arteriosclerosis Thrombosis and Vascular Biology 2000 August;20(8):2011-8.

(24) Shimano H, Yamada N, Ishibashi S, Mokuno H, Mori N, Gotoda T, Harada K, Akanuma Y, Murase T, Yazaki Y, Takaku F. Oxidation-Labile Subfraction of Human Plasma Low-Density-Lipoprotein Isolated by Ion-Exchange Chromatography. Journal of Lipid Research 1991 May;32(5):763-73.

(25) Bowry VW, Stanley KK, Stocker R. High-Density-Lipoprotein Is the Major Carrier of Lipid Hydroperoxides in Human Blood-Plasma from Fasting Donors. Proceedings of the National Academy of Sciences of the United States of America 1992 November 1;89(21):10316-20.

(26) Zheng LM, Nukuna B, Brennan ML, Sun MJ, Goormastic M, Settle M, Schmitt D, Fu XM, Thomson L, Fox PL, Ischiropoulos H, Smith JD, Kinter M, Hazen SL. Apolipoprotein A-I is a selective target for myeloperoxidase-catalyzed oxidation and functional impairment in subjects with cardiovascular disease. Journal of Clinical Investigation 2004 August;114(4):529-41.

(27) Pankhurst G, Wang XL, Wilcken DE, Baernthaler G, Panzenbock U, Raftery M, Stocker R. Characterization of specifically oxidized apolipoproteins in mildly oxidized high density lipoprotein. Journal of Lipid Research 2003 February;44(2):349-55.

(28) Upston JM, Niu XW, Brown AJ, Mashima R, Wang HJ, Senthilmohan R, Kettle AJ, Dean RT, Stocker R. Disease stage-dependent accumulation of lipid and protein oxidation products in human atherosclerosis. American Journal of Pathology 2002 February;160(2):701-10.

(29) Ansell BJ, Fonarow GC, Fogelman AM. The paradox of dysfunctional high-density lipoprotein. Current Opinion in Lipidology 2007 August;18(4):427-34.

(30) Peschel A. How do bacteria resist human antimicrobial peptides? Trends in Microbiology 2002 April;10(4):179-86.

(31) Weber C. Platelets and chemokines in atherosclerosis - Partners in crime. Circulation Research 2005 April 1;96(6):612-6.

(32) Scheuerer B, Ernst M, Durrbaum-Landmann I, Fleischer J, Grage-Griebenow E, Brandt E, Flad HD, Petersen F. The CXC-chemokine platelet factor 4 promotes monocyte survival and induces monocyte differentiation into macrophages. Blood 2000 February 15;95(4):1158-66.

(33) Sachais BS, Nassar T, Akkawi S, Kowalska MA, Bdeir K, Cines DB, Poncz M, Higazi AAR. Platelet factor 4 enhances the binding of oxidized-low density lipoprotein to vascular wall cells. Blood 2002 November 16;100(11):679A.

(34) Sachais BS, Nassar T, Williams KJ, Kuo A, Lietersdorf E, Shah N, Kowalska MA, Jarett L, Poncz M, Cines DB, Higazi AAR. Platelet factor 4 (PF4) may promote atherosclerosis by two distinct mechanisms. Blood 2000 November 16;96(11):242A.

(35) von Hundelshausen P, Weber C. Platelets as immune cells - Bridging inflammation and cardiovascular disease. Circulation Research 2007 January 5;100(1):27-40.

(36)   Pitchford SC, Momi S, Giannini S, Casali L, Spina D, Page CP, Gresele P. Platelet P-selectin is required for pulmonary eosinophil and lymphocyte recruitment in a murine model of allergic inflammation. Blood 2005 March 1;105(5):2074-81.

(37)   Diacovo TG, Puri KD, Warnock RA, Springer TA, vonAndrian UH. Platelet-mediated lymphocyte delivery to high endothelial venules. Science 1996 July 12;273(5272):252-5.

(38)   Kehrel BE, Jurk K. Platelets at the interface between hemostasis and innate immunity. Transfusion Medicine and Hemotherapy 2004;31(6):379-86.

(39)   Gawaz M, Langer H, May AE. Platelets in inflammation and atherogenesis. Journal of Clinical Investigation 2005 December;115(12):3378-84.

(40)   Hermann A, Rauch BH, Braun M, Schror K, Weber AA. Platelet CD40 ligand (CD40L) - subcellular localization, regulation of expression, and inhibition by clopidogrel. Platelets 2001 March;12(2):74-82.

(41)   Ni HY, Denis CV, Subbarao S, Degen JL, Sato TN, Hynes RO, Wagner DD. Persistence of platelet thrombus formation in arterioles of mice lacking both von Willebrand factor and fibrinogen. Journal of Clinical Investigation 2000 August;106(3):385-92.

(42)   Henn V, Slupsky JR, Grafe M, Anagnostopoulos I, Forster R, Muller-Berghaus G, Kroczek RA. CD40 ligand on activated platelets triggers an inflammatory reaction of endothelial cells. Nature 1998 February 5;391(6667):591-4.

(43)   Langer HF, Gawaz M. Platelet-vessel wall interactions in atherosclerotic disease. Thrombosis and Haemostasis 2008 March;99(3):480-6.

(44)   Celik S, Langer H, Stellos K, May AE, Gawaz MP, Dengler TJ. Platelet-associated LIGHT (TNFSF14) mediates adhesion of activated platelets to human vascular endothelium. Circulation 2006 October 31;114(18):181.

(45)   Heeschen C, Dimmeler S, Hamm CW, van den Brand MJ, Boersma E, Zeiher AM, Simoons ML, Capture S, I. Soluble CD40 ligand in acute coronary syndromes. New England Journal of Medicine 2003 March 20;348(12):1104-11.

(46)   Liu GZ, Fang LB, Hjelmstrom P, Gao XG. Enhanced plasma levels of LIGHT in patients with acute atherothrombotic stroke. Acta Neurol Scand 2008 March 31.

(47)   Springer TA. Traffic Signals for Lymphocyte Recirculation and Leukocyte Emigration - the Multistep Paradigm. Cell 1994 January 28;76(2):301-14.

(48)   May AE, Langer H, Seizer P, Bigalke B, Lindemann S, Gawaz M. Platelet-leukocyte interactions in inflammation and atherothrombosis. Seminars in Thrombosis and Hemostasis 2007 March;33(2):123-7.

(49)   Siegel-Axel DI, Gawaz M. Platelets and endothelial cells. Seminars in Thrombosis and Hemostasis 2007 March;33(2):128-35.

(50)   Kinlay S, Libby P, Ganz P. Endothelial function and coronary artery disease. Current Opinion in Lipidology 2001 August;12(4):383-9.

(51)   Furchgott RF, Zawadzki JV. The Obligatory Role of Endothelial-Cells in the Relaxation of Arterial Smooth-Muscle by Acetylcholine. Nature 1980;288(5789):373-6.

(52)   Mehta JL, Chen LY, Kone BC, Mehta P, Turner P. Identification of Constitutive and Inducible Forms of Nitric-Oxide Synthase in Human Platelets. Journal of Laboratory and Clinical Medicine 1995 March;125(3):370-7.

(53)   Bredt DS, Snyder SH. Nitric-Oxide - A Physiological Messenger Molecule. Annual Review of Biochemistry 1994;63:175-95.

(54)   Alderton WK, Cooper CE, Knowles RG. Nitric oxide synthases: structure, function and inhibition. Biochemical Journal 2001 August 1;357:593-615.

(55)   Papapetropoulos A, Rudic RD, Sessa WC. Molecular control of nitric oxide synthases in the cardiovascular system. Cardiovascular Research 1999 August 15;43(3):509-20.

(56)   Azuma H, Ishikawa M, Sekizaki S. Endothelium-Dependent Inhibition of Platelet-Aggregation. British Journal of Pharmacology 1986 June;88(2):411-5.

(57)   Freedman JE, Loscalzo J, Barnard MR, Alpert C, Keaney JF, Michelson AD. Nitric oxide released from activated platelets inhibits platelet recruitment. Journal of Clinical Investigation 1997 July 15;100(2):350-6.

(58)   Hanafy KA, Krumenacker JS, Murad F. NO, nitrotyrosine, and cyclic GMP in signal transduction. Med Sci Monit 2001 July;7(4):801-19.

(59)   Jin RC, Voetsch B, Loscalzo J. Endogenous mechanisms of inhibition of platelet function. Microcirculation 2005 April;12(3):247-58.

(60)   Horstrup K, Jablonka B, Honigliedl P, Just M, Kochsiek K, Walter U. Phosphorylation of Focal Adhesion Vasodilator-Stimulated Phosphoprotein at Ser157 in Intact Human Platelets Correlates with Fibrinogen Receptor Inhibition. European Journal of Biochemistry 1994 October 1;225(1):21-7.

(61)   Moncada S, Gryglewski R, Bunting S, Vane JR. Enzyme Isolated from Arteries Transforms Prostaglandin Endoperoxides to An Unstable Substance That Inhibits Platelet-Aggregation. Nature 1976;263(5579):663-5.

(62)   Marcus AJ, Weksler BB, Jaffe EA, Broekman MJ. Synthesis of Prostacyclin from Platelet-Derived Endoperoxides by Cultured Human-Endothelial Cells. Journal of Clinical Investigation 1980;66(5):979-86.

(63) Caughey GE, Cleland LG, Penglis PS, Gamble JR, James MJ. Roles of cyclooxygenase (COX)-1 and COX-2 in prostanoid production by human endothelial cells: Selective up-regulation of prostacyclin synthesis by COX-2. Journal of Immunology 2001 September 1;167(5):2831-8.

(64) Escudero I, Martinez-Gonzalez J, Alonso R, Mata P, Badimon L. Experimental and interventional dietary study in humans on the role of HDL fatty acid composition in PGI(2) release and Cox-2 expression by VSMC. European Journal of Clinical Investigation 2003 September;33(9):779-86.

(65) Tsai AL, Hsu MJ, Patsch W, Wu KK. Regulation of Pgi2 Activity by Serum-Proteins - Serum-Albumin But Not High-Density-Lipoprotein Is the Pgi2 Binding and Stabilizing Protein in Human Blood. Biochimica et Biophysica Acta 1991 December 6;1115(2):131-40.

(66) Gorman RR, Bunting S, Miller OV. Modulation of Human Platelet Adenylate-Cyclase by Prostacyclin (Pgx). Prostaglandins 1977;13(3):377-88.

(67) Wise H, Wong YH, Jones RL. Prostanoid signal integration and cross talk. Neurosignals 2002 January;11(1):20-8.

(68) Shattil SJ, Bennett JS, Colman RW, Cooper RA. Abnormalities of Cholesterol-Phospholipid Composition in Platelets and Low-Density Lipoproteins of Human Hyper-Betalipoproteinemia. Journal of Laboratory and Clinical Medicine 1977;89(2):341-53.

(69) Korporaal SJ, Akkerman JW. Platelet signaling induced by lipoproteins. Cardiovasc Hematol Agents Med Chem 2006 April;4(2):93-109.

(70) Siegel-Axel D, Daub K, Seizer P, Lindemann S, Gawaz M. Platelet lipoprotein interplay: trigger of foam cell formation and driver of atherosclerosis. Cardiovascular Research 2008 April 1;78(1):8-17.

(71) Moore KJ, Freeman MW. Scavenger receptors in atherosclerosis: Beyond lipid uptake. Arteriosclerosis Thrombosis and Vascular Biology 2006 August;26(8):1702-11.

(72) Ohgami N, Nagai R, Ikemoto M, Arai H, Kuniyasu A, Horiuchi S, Nakayama H. CD36, a member of the class B scavenger receptor family, as a receptor for advanced glycation end products. Journal of Biological Chemistry 2001 February 2;276(5):3195-202.

(73) Calvo D, Gomez-Coronado D, Suarez Y, Lasuncion MA, Vega MA. Human CD36 is a high affinity receptor for the native lipoproteins HDL, LDL, and VLDL. Journal of Lipid Research 1998 April;39(4):777-88.

(74) Han JH, Hajjar DP, Febbraio M, Nicholson AC. Native and modified low density lipoproteins increase the functional expression of the macrophage class B scavenger receptor, CD36. Journal of Biological Chemistry 1997 August 22;272(34):21654-9.

(75) Tontonoz P, Nagy L, Alvarez JGA, Thomazy VA, Evans RM. PPAR gamma promotes monocyte/macrophage differentiation and uptake of oxidized LDL. Cell 1998 April 17;93(2):241-52.

(76) Podrez EA, Byzova TV, Febbraio M, Salomon RG, Ma Y, Valiyaveettil M, Poliakov E, Sun M, Finton PJ, Curtis BR, Chen J, Zhang R, Silverstein RL, Hazen SL. Platelet CD36 links hyperlipidemia, oxidant stress and a prothrombotic phenotype. Nature Medicine 2007 September;13(9):1086-95.

(77) Korporaal SJA, Van Eck M, Adelmeijer J, Ijsseldijk M, Out R, Lisman T, Lenting PJ, Van Berkel TJC, Akkerman JWN. Platelet activation by oxidized low density lipoprotein is mediated by Cd36 and scavenger receptor-A. Arteriosclerosis Thrombosis and Vascular Biology 2007 November;27(11):2476-83.

(78) Volf I, Moeslinger T, Cooper J, Schmid W, Koller E. Human platelets exclusively bind oxidized low density lipoprotein showing no specificity for acetylated low density lipoprotein. Febs Letters 1999 April 23;449(2-3):141-5.

(79) Chen MY, Kakutani M, Naruko T, Ueda M, Narumiya S, Masaki T, Sawamura T. Activation-dependent surface expression of LOX-1 in human platelets. Biochemical and Biophysical Research Communications 2001 March 23;282(1):153-8.

(80) Calvo D, GomezCoronado D, Lasuncion MA, Vega MA. CLA-1 is an 85-kD plasma membrane glycoprotein that acts as a high-affinity receptor for both native (HDL, LDL, and VLDL) and modified (OxLDL and AcLDL) lipoproteins. Arteriosclerosis Thrombosis and Vascular Biology 1997 November;17(11):2341-9.

(81) Imachi H, Murao K, Cao W, Tada S, Taminato T, Wong NCW, Takahara J, Ishida T. Expression of human scavenger receptor B1 on and in human platelets. Arteriosclerosis Thrombosis and Vascular Biology 2003 May;23(5):898-904.

(82) Urban S, Zieseniss S, Werder M, Hauser H, Budzinski R, Engelmann B. Scavenger receptor BI transfers major lipoprotein-associated phospholipids into the cells. Journal of Biological Chemistry 2000 October 27;275(43):33409-15.

(83) Riddell DR, Vinogradiv DV, Stannard AK, Chadwick N, Owen JS. Identification and characterization of LRP8 (apoER2) in human blood platelets. Journal of Lipid Research 1999 October;40(10):1925-30.

(84) Koller E, Volf I, Gurvitz A, Koller F. Modified low-density lipoproteins and high-density lipoproteins. Pathophysiology of Haemostasis and Thrombosis 2006;35(3-4):322-45.

(85) Nofer JR, Tepel M, Kehrel B, Wierwille S, Walter M, Seedorf U, Zidek W, Assmann G. Low-density lipoproteins inhibit the Na+/H+ antiport in human platelets - A novel mechanism enhancing platelet activity in hypercholesterolemia. Circulation 1997 March 18;95(6):1370-7.

(86) Lerch PG, Spycher MO, Doran JE. Reconstituted high density lipoprotein (rHDL) modulates platelet activity in vitro and ex vivo. Thrombosis and Haemostasis 1998 August;80(2):316-20.

(87) Oravec S, Demuth K, Myara I, Hornych A. The effect of high density lipoprotein subfractions on endothelial eicosanoid secretion. Thrombosis Research 1998 October 15;92(2):65-71.

(88) Riddell DR, Graham A, Owen JS. Apolipoprotein E inhibits platelet aggregation through the L-arginine:nitric oxide pathway - Implications for vascular disease. Journal of Biological Chemistry 1997 January 3;272(1):89-95.

(89) Ou JS, Wang JL, Xu H, Ou ZJ, Sorci-Thomas MG, Jones DW, Signorino P, Densmore JC, Kaul S, Oldham KT, Pritchard KA. Effects of D-4F on vasodilation and vessel wall thickness in hypercholesterolemic LDL receptor-null and LDL receptor/apolipoprotein A-I double-knockout mice on western diet. Circulation Research 2005 November 25;97(11):1190-7.

(90) Drew BG, Fidge NH, Gallon-Beaumier G, Kemp BE, Kingwell BA. High-density lipoprotein and apolipoprotein AI increase endothelial NO synthase activity by protein association and multisite phosphorylation. Proceedings of the National Academy of Sciences of the United States of America 2004 May 4;101(18):6999-7004.

(91) Yuhanna IS, Zhu Y, Cox BE, Hahner LD, Osborne-Lawrence S, Marcel YL, Anderson RGW, Mendelsohn ME, Hobbs HH, Shaul PW. High-density lipoprotein binding to scavenger receptor-BI activates endothelial nitric oxide synthase. Nature Medicine 2001 July;7(7):853-7.

(92) Mineo C, Yuhanna IS, Quon MJ, Shaul PW. High density lipoprotein-induced endothelial nitric-oxide synthase activation is mediated by Akt and MAP kinases. Journal of Biological Chemistry 2003 March 14;278(11):9142-9.

(93) Ramet ME, Malzone A, Ramet M, Karas RH. High density lipoprotein increases the abundance of eNOS protein in human vascular endothelial cells by increasing its half-life. Circulation 2002 November 5;106(19):174.

(94) Koller E, Koller F, Binder BR. Influence of Ldl on Fibrinogen Binding to Human-Platelets. Thrombosis and Haemostasis 1989 August 19;62(1):261.

(95) Relou IAM, Gorter G, Ferreira IA, van Rijn HJM, Akkerman JWN. Platelet endothelial cell adhesion molecule-1 (PECAM-1) inhibits low density lipoprotein-induced signaling in platelets. Journal of Biological Chemistry 2003 August 29;278(35):32638-44.

(96)   Opper C, Schussler G, Sattler W, Malle E. Effect of hypochlorite (HOCl)-modified low density lipoproteins and high density lipoproteins on platelet function. Platelets 1998;9(5):339-41.

(97)   Volf I, Roth A, Cooper J, Moeslinger T, Koller E. Hypochlorite modified LDL are a stronger agonist for platelets than copper oxidized LDL. Febs Letters 2000 October 20;483(2-3):155-9.

(98)   Volf I, Roth A, Moeslinger T. Stimulating effect of biologically modified low density lipoproteins on ADP-induced aggregation of washed platelets persists in absence of specific binding. Thrombosis Research 2000 March 15;97(6):441-9.

(99)   Coelman LG, Polanowska-Grabowska RK, Marcinkiewicz M, Gear ARL. LDL oxidized by hypochlorous acid causes irreversible platelet aggregation when combined with low levels of ADP, thrombin, epinephrine, or macrophage-derived chemokine (CCL22). Blood 2004 July 15;104(2):380-9.

(100) Zabe M, Feltzer RE, Malle E, Sattler W, Dean WL. Effect of hypochlorite-modified low-density and high-density lipoproteins on intracellular Ca2+ and plasma membrane Ca2+-ATPase activity of human platelets. Cell Calcium 1999 December;26(6):281-7.

(101) Ardlie NG, Selley ML, Simons LA. Platelet Activation by Oxidatively Modified Low-Density Lipoproteins. Atherosclerosis 1989 April;76(2-3):117-24.

(102) Takahashi Y, Chiba H, Matsuno K, Akita H, Hui SP, Nagasaka H, Nakamura H, Kobayashi K, Tandon NN, Jamieson GA. Native lipoproteins inhibit platelet activation induced by oxidized lipoproteins. Biochemical and Biophysical Research Communications 1996 May 15;222(2):453-9.

(103) Nofer JR, Walter M, Kehrel B, Wierwille S, Tepel M, Seedorf U, Assmann G. HDL3-mediated inhibition of thrombin-induced platelet aggregation and fibrinogen binding occurs via decreased production of phosphoinositide-derived second messengers 1,2-diacylglycerol and inositol 1,4,5-tris-phosphate. Arteriosclerosis Thrombosis and Vascular Biology 1998 June;18(6):861-9.

(104) Walkowiak B, Michalak E, Koziolkiewicz W, Cierniewski CS. Rapid Photometric-Method for Estimation of Platelet Count in Blood-Plasma Or Platelet Suspension. Thrombosis Research 1989 December 15;56(6):763-6.

(105) Walkowiak B, Kesy A, Michalec L. Microplate reader - A convenient tool in studies of blood coagulation. Thrombosis Research 1997 July 1;87(1):95-103.

(106) Arnhold J, Wiegel D, Richter O, Hammerschmidt S, Arnold K, Krumbiegel M. Modification of Low-Density Lipoproteins by Sodium-Hypochlorite. Biomedica Biochimica Acta 1991;50(8):967-73.

(107) Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein Measurement with the Folin Phenol Reagent. Journal of Biological Chemistry 1951;193(1):265-75.

(108) Noble RP. Electrophoretic Separation of Plasma Lipoproteins in Agarose Gel. Journal of Lipid Research 1968;9(6):693-&.

(109) Bohlen P, Stein S, Dairman W, UDENFRIE.S. Fluorometric Assay of Proteins in Nanogram Range. Archives of Biochemistry and Biophysics 1973;155(1):213-20.

(110) Riddles PW, Blakeley RL, Zerner B. Reassessment of Ellman Reagent. Methods in Enzymology 1983;91:49-60.

(111) Inwald DP, Peters MJ, Walshe D, Jones A, Davies EG, Klein NJ. Absence of platelet CD40L identifies patients with X-linked hyper IgM syndrome. Clinical and Experimental Immunology 2000 June;120(3):499-502.

(112) Battye FL. Web servlet-assisted, dial-in flow cytometry data analysis. Cytometry 2001 February 1;43(2):143-9.

(113) Walkowiak B, Michalak E, Koziolkiewicz W, Cierniewski CS. Rapid Photometric-Method for Estimation of Platelet Count in Blood-Plasma Or Platelet Suspension. Thrombosis Research 1989 December 15;56(6):763-6.

(114) Jialal I, Devaraj S. Low density lipoprotein oxidation, antioxidants, and atherosclerosis: A clinical biochemistry perspective. Clinical Chemistry 1996 April;42(4):498-506.

(115) Virgolini I, Li S, Yang Q, Banyai M, Koller E, Angelberger P, Sinzinger H. Binding of 111In-Labeled Hdl to Platelets from Normolipemic Volunteers and Patients with Heterozygous Familial Hypercholesterolemia. Arteriosclerosis and Thrombosis 1992 July;12(7):849-61.

(116) Yamakado T, Hidaka H. Mepacrine-Induced Inhibition of Human-Platelet Cyclic-Gmp Phosphodiesterase. Folia Pharmacologica Japonica 1984;83(5):25.

(117) Mccrea JM, Robinson P, Gerrard JM. Mepacrine (Quinacrine) Inhibition of Thrombin-Induced Platelet Responses Can be Overcome by Lysophosphatidic Acid. Biochimica et Biophysica Acta 1985;842(2-3):189-94.

(118) Chrzanowska-Wodnicka M, Smyth SS, Schoenwaelder SM, Fischer TH, White GC. Rap1b is required for normal platelet function and hemostasis in mice. Journal of Clinical Investigation 2005 March;115(3):680-7.

(119) Sohn HY, Krotz F. Cyclooxygenase inhibition and atherothrombosis. Current Drug Targets 2006 October;7(10):1275-84.

(120) Assinger A, Schmid W, Eder S, Schmid D, Koller E, Volf I. Oxidation by hypochlorite converts protective HDL into a potent platelet agonist. Febs Letters 2008 March 5;582(5):778-84.

(121) Demers A, McNicoll N, Febbraio M, Servant M, Marleau S, Silverstein R, Ong H. Identification of the growth hormone-releasing peptide binding site in CD36: a photoaffinity cross-linking study. Biochemical Journal 2004 September 1;382:417-24.

(122) Pedreno J, Decastellarnau C, Cullare C, Sanchez J, Gomezgerique J, Ordonezllanos J, Gonzalezsastre F. Ldl Binding-Sites on Platelets Differ from the Classical Receptor of Nucleated Cells. Arteriosclerosis and Thrombosis 1992 November;12(11):1353-62.

(123) Surya II, Akkerman JWN. The Influence of Lipoproteins on Blood-Platelets. American Heart Journal 1993 January;125(1):272-5.

(124) Hwang SJ, Ballantyne CM, Sharrett AR, Smith LC, Davis CE, Gotto AM, Jr., Boerwinkle E. Circulating adhesion molecules VCAM-1, ICAM-1, and E-selectin in carotid atherosclerosis and incident coronary heart disease cases: the Atherosclerosis Risk In Communities (ARIC) study. Circulation 1997 December 16;96(12):4219-25.

(125) Libby P. Inflammation in atherosclerosis. Nature 2002 December 19;420(6917):868-74.

(126) Malik I, Danesh J, Whincup P, Bhatia V, Papacosta O, Walker M, Lennon L, Thomson A, Haskard D. Soluble adhesion molecules and prediction of coronary heart disease: a prospective study and meta-analysis. Lancet 2001 September 22;358(9286):971-6.

(127) Pradhan AD, Rifai N, Ridker PM. Soluble intercellular adhesion molecule-1, soluble vascular adhesion molecule-1, and the development of symptomatic peripheral arterial disease in men. Circulation 2002 August 13;106(7):820-5.

(128) Ridker PM, Hennekens CH, Roitman-Johnson B, Stampfer MJ, Allen J. Plasma concentration of soluble intercellular adhesion molecule 1 and risks of future myocardial infarction in apparently healthy men. Lancet 1998 January 10;351(9096):88-92.

(129) Stocker R, Keaney JF. Role of oxidative modifications in atherosclerosis. Physiol Rev 2004 October;84(4):1381-478.

(130) Hazell LJ, Vandenberg JJM, Stocker R. Oxidation of Low-Density-Lipoprotein by Hypochlorite Causes Aggregation That Is Mediated by Modification of Lysine Residues Rather Than Lipid Oxidation. Biochemical Journal 1994 August 15;302:297-304.

(131) Aviram M, Brook JG. Platelet Interaction with High and Low-Density Lipoproteins. Atherosclerosis 1983;46(3):259-68.

(132) Higashihara M, Kinoshita M, Teramoto T, Kume S, Kurokawa K. The Role of Apoe in Inhibitory Effects of Apoe-Rich Hdl on Platelet-Function. Febs Letters 1991 April 22;282(1):82-6.

(133) Nofer JR, Walter M, Kehrel B, Wierwille S, Tepel M, Seedorf U, Assmann G. HDL3-mediated inhibition of thrombin-induced platelet aggregation and fibrinogen binding occurs via decreased production of phosphoinositide-derived second messengers 1,2-diacylglycerol and inositol 1,4,5-tris-phosphate. Arteriosclerosis Thrombosis and Vascular Biology 1998 June;18(6):861-9.

(134) Riddell DR, Vinogradov DV, Stannard AK, Chadwick N, Owen JS. Identification and characterization of LRP8 (apoER2) in human blood platelets. J Lipid Res 1999 October;40(10):1925-30.

(135) Koller E, Koller F, Binder BR. Purification and Identification of the Lipoprotein-Binding Proteins from Human-Blood Platelet Membrane. Journal of Biological Chemistry 1989 July 25;264(21):12412-8.

(136) Nofer JR, Kehrel B, Fobker M, Levkau B, Assmann G, von Eckardstein A. HDL and arteriosclerosis: beyond reverse cholesterol transport. Atherosclerosis 2002 March;161(1):1-16.

(137) Garner B, Witting PK, Waldeck AR, Christison JK, Raftery M, Stocker P. Oxidation of high density lipoproteins - I. Formation of methionine sulfoxide in apolipoproteins AI and AII is an early event that accompanies lipid peroxidation and can be enhanced by alpha-tocopherol. Journal of Biological Chemistry 1998 March 13;273(11):6080-7.

(138) Ansell BJ, Fonarow GC, Fogelman AM. The paradox of dysfunctional high-density lipoprotein. Current Opinion in Lipidology 2007;18(4):427-34.

(139) Thorne RF, Mhaidat NM, Ralston KJ, Burns GF. CD36 is a receptor for oxidized high density lipoprotein: Implications for the development of atherosclerosis. Febs Letters 2007 March 20;581(6):1227-32.

(140) Katrantzis M, Baker MS, Handley CJ, Lowther DA. The oxidant hypochlorite (OCl-), a product of the myeloperoxidase system, degrades articular cartilage proteoglycan aggregate. Free Radic Biol Med 1991;10(2):101-9.

(141) Carvalho AC, Colman RW, Lees RS. Platelet-Function in Hyperlipoproteinemia. New England Journal of Medicine 1974;290(8):434-8.

(142) Napoli C, Postiglione A, Triggiani M, Corso G, Palumbo G, Carbone V, Ruocco A, Ambrosio G, Montefusco S, Malorni A, Condorelli M, Chiariello M. Oxidative structural modifications of low density lipoprotein in homozygous familial hypercholesterolemia. Atherosclerosis 1995 December;118(2):259-73.

(143) Sanguigni V, Ferro D, Pignatelli P, Del Ben M, Nadia T, Saliola M, Sorge R, Violi F. CD40 ligand enhances monocyte tissue factor expression and thrombin generation via oxidative stress in patients with hypercholesterolemia. Journal of the American College of Cardiology 2005 January 4;45(1):35-42.

(144) Andre P, Nannizzi-Alaimo L, Prasad SK, Phillips DR. Platelet-derived CD40L - The switch-hitting player of cardiovascular disease. Circulation 2002 August 20;106(8):896-9.

Curriculum Vitae

Alice Assinger, born on the 18th of January 1982, in Klagenfurt, Austria

CONTACT

Mailing address	Institut für Physiologie
	Schwarzspanierstraße 17
	1090 Vienna; Austria
Phone	+43 676 3593009
Email	alice.assinger@meduniwien.ac.at

EDUCATION

1988-1992	Elementary school Spittal/Drau, Austria
1992-2000	Grammar school "Porcia", Spittal/Drau, Austria
Jan.-Jun.1999	Duringthon Sixthform College, Worthing, Great Britain
Jun.2000	final examinations: grammar school "Porcia", Spittal/Drau, Austria
2000-2006	Diploma in nutritional sciences, University of Vienna, Austria
2001-2007	Diploma in biology, University of Vienna, Austria
Since 2006	PhD programme nutritional sciences, University of Vienna, Austria
Since 2007	Master programme genetics, University of Vienna, Austria

WORK EXPERIENCE

October 2000	Internship: Acurvedic Clinic Cochin, India
September 2002	Internship: bacteriological laboratory, Kärntnermilch; Austria
Oct.2002-Jun.2003	Internship: Fessel GfK (marketing research), Austria
Aug.2003-Dec.2003	Diploma project: nutritional status of children in rural and urban Ecuador; Ecuador
September 2004	Internship: bacteriological laboratory, Kärntnermilch; Austria
Since April 2006	Scientific assistant at the Institute of Physiology; Medical University of Vienna, Austria

PUBLICATIONS

Assinger,A., Schmid,W., Eder,S., Schmid,D., Koller,E. & Volf,I. (2008) Oxidation by hypochlorite converts protective HDL into a potent platelet agonist. *Febs Letters* 582, 778-784.

Pollreisz,A., **Assinger,A.,** Hacker,S., Hoetzenecker,K., Schmid,W., Lang,G., Wolfsberger,M., Steinlechner,B., Bielek,E., Lalla,E., Klepetko,W., Volf,I. & Ankersmit,H.J. (2008a) Intravenous immunoglobulins induce CD32-mediated platelet aggregation in vitro. *British Journal of Dermatology* 159, 578-584.

Schmid,W., **Assinger,A**., Lee,A., Bielek,E., Koller,E. & Volf,I. (2008) Platelet-stimulating effects of oxidized LDL are not attributable to toxic properties of the lipoproteins. *Thrombosis Research* 122, 630-639.

CONGRESS CONTRIBUTIONS

Assinger, A., Schmid,W., Eder,S., Koller,E., Volf,I. (2007) Oxidized high density lipoproteins are able to trigger platelet aggregation. *9thUK-1th Netherlands Platelet meeting, September 2007, Kings College London*

Schmid,W., Lee, A., **Assinger, A**., Koller,E., Volf,I. (2007) Platelet stimulating effects of oxidized LDL are not mediated by toxic lipid peroxidation products. *9thUK-1th Netherlands Platelet meeting, September 2007, Kings College London*

Pollreisz,A., Hacker,S., Hoetzenecker,K., **Assinger,A.,** Kebschull,M., Volf,I. & Ankersmit,H.J. (2008) Intravenous immunoglobulins induce CD32-mediated platelet aggregation in vitro. *Arteriosclerosis Thrombosis and Vascular Biology Annual Conference 2008, Atlanta; cited in Arteriosclerosis Thrombosis and Vascular Biology 28, E71.*