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Platelet activation and platelet- monocyte aggregate formation induced by the atherosclerotic plaque lipid lysophosphatidic acid

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Abbreviations

µM	micomolar

- A -

A3P5P	$P2Y_1$-receptor antagonist
ADP	adenosine- diphosphate
AR-C69931	$P2Y_{12}$- receptor antagonist
ASS	acetyl- salicic acid
ATP	adenosine- triphosphate

- B -

BSA	bovine serum albumin

- C -

CD	cluster or differentiation, a cell surface marker, i.e. CD41
COX-1	cyclooxygenase 1

- D -

DGPP	diacylglycerolpyrophosphate
DMSO	dimethyl-sulfoxide

- E -

EC	effective concentration
EDG	endothelial differentiation gene (former description for LPA- /S1P- receptors)
EDTA	ethylene- diamine- tetra- acetic acid

- G -

GP	membrane glycoprotein, i.e. GPIIb-IIIa = integrin $\alpha_{IIb}\beta_3$

- I -

IL-6	interleukin 6

- L -

LPA	lysophosphatic acid
LPAAT	LPA-acetyltransferase
LPC	lysophosphatidylcholine
LPP	lipidphosphate- phosphohydrolase
Lyso-PLD	lysophospholipase D (formerly autotaxin)

- M -

M	molar
MAG	monoacylglycerol
mM	millimolar
MMP	matrix-metalloproteinase
MRS2179	$P2Y_1$- receptor antagonist

- N -

NATyrPA	*N*-acyl-Tyrosine- phosphoic acid
NASerPA	*N*-acyl-Serine- phosphoric acid
NFκB	a transcriptionsfactor

- O -

Ox-LDL	oxidized- low-density- lipoprotein

- P -

$P2Y_{1/12}$	ADP-receptors
PA	phosphatidic acid
PAF	platelet activating factor
PBS	phosphate buffered saline
PC	phosphatidylcholine
PCR	polymerase-chain -reaction
PDGF	platelet derived growth factor
PLA	phospholipase A
PLD	phospholipase D
PPP	platelet poor plasma
PPAR	peroxisome proliferator-activated receptor (a transcription factor)
PRP	platelet rich plasma
PS	phosphatidylserine

- R -

RGDS	Arg-Gly-Asp-Ser (a fibrinogen receptor blocking peptide)

- S -

S1P	sphingosine-1-phosphate
SD	standard deviation

- T -

TF	tissue factor
TNF	tumor necrosis factor

- U -

U46619	a thromboxane receptor agonist

- V -

VCAM	vascular cell adhesion molecule
vWF	von Willebrand factor

1. Introduction

Platelets are anucleated blood cells that originate from the bone marrow by fragmentation of megakaryocytes (Hartwig and Italiano, 2003). Under conditions of regular blood flow platelets circulate freely at a concentration of 150 – 300 x 10^{9}/ l and they do not adhere to the vascular wall. In the event of vascular injury platelets adhere to the site of the lesion where they contribute to primary haemostasis and thus arrest haemorrhage after vascular tissue trauma. Acute vascular injury marks the physiological trigger for platelet function.

In atherosclerosis, plaque rupture leads to a similar sequence of platelet activation. Whether through acute injury or chronic lesions in atherosclerosis, platelets act in the same way changing their shape, aggregating and thus leading to the formation of a platelet- and fibrin-rich intravascular thrombus. Thus "erroneous" activation of intravascular haemostasis is of paramount importance in the pathogenesis of acute thrombotic intravascular occlusion in atherosclerosis. It is this basic event which precipitates acute ischemic syndromes such as unstable angina, myocardial infarction, peripheral arterial occlusion and stroke. The mechanism by which platelet activation is triggered after plaque rupture is of vital importance. Within atherosclerotic arteries one possible culprit for intravascular platelet activation are oxidized lipids being suddenly exposed to circulating platelets upon plaque rupture.

The following work will focus on one of those specific platelet- activating lipids, found after lipid oxidation, lysophosphatidic acid (LPA). Recently it has been recognized that LPA has thrombotic and atherogenic activities (Siess and Tigyi, 2004). Here we will focus on its platelet activating ability. LPA has been identified as a biologically active lipid in mildly oxidized LDL, human atherosclerotic lesions, and in the supernatant of activated platelets. It is known that LPA can cause platelet shape change and platelet aggregation. However, a systematic study concerning the mechanism of LPA- induced aggregation is missing. Moreover, it is unknown whether LPA induces platelet aggregation in whole blood.

2. State of Research

2.1. General mechanism of platelet activation and arterial thrombus formation

Under physiological conditions the main trigger for the formation of a haemostatic thrombus is the loss of the endothelial cell integrity, which causes platelets to come into contact with subendothelial matrix components mediating the initial adherence of platelets. Subendothelial components exposed locally (like collagen) bind circulating von Willebrand factor (vWF), which causes platelet adhesion, sustains platelet activation and may cause platelet aggregation (Savage et al., 1996). Circulating plasma factors and molecules released from platelets can cause positive feedback loops of platelet activation. Some of these plasma factors are: epinephrine, increased under conditions of stress, thrombin, formed by the coagulation system, thromboxane A_2 (TXA_2), ADP, and serotonin, released by the activation of platelets (Siess, 1989).

Upon activation platelets undergo several closely intertwined phases: shape, adhesion, secretion, and aggregation. Non- activated circulating platelets are mostly discoid in shape. However, depending on the stage of activation, other forms of platelets, for example spheroid forms, are circulating in plasma. Platelets can undergo shape change which involves platelets to become spheroid and form protuberances, which can be motile, extend and retract. These extensions are called pseudopods and can facilitate the adherence of platelets to injured endothelial surfaces and to each other.

Once adherent to a surface some of the platelets may flatten laterally extending their cell body between the pseudopods (=spreading). This process allows a platelet monolayer of activated platelets to firmly attach and seal an endothelial lesion.

Independent of whether activated platelets adhere and spread or whether they aggregate, they may secrete the contents of their dense granules (ADP and serotonin) and α-granules, or release thromboxane A_2 (TXA_2). These factors can amplify platelet activation leading to further platelet secretion and aggregation.

The exposure of an active fibrinogen receptor (glycoprotein GPIIb-IIIa complex, integrin $\alpha_{IIb}\beta_3$) is required for aggregation (Plow and Ginsberg, 1989). About 80 000 copies of the fibrinogen receptor exist on the surface of unstimulated platelets and bind soluble fibrinogen in its activated form leading to the formation of fibrinogen bridges between platelets. (Phillips et al., 1988; Plow and Ginsberg, 1989). This process requires divalent cations (Ca^{2+}, Mg^{2+}) as a cofactor. Distinct amino acid sequences, like RGD or KQAGDV, that are present in the fibrinogen molecule sequence, mediate the ligand receptor interaction and bind to specific extracellular regions of the integrin $\alpha_{IIb}\beta_3$. By inhibition of these amino acid sequences aggregation can be completely abolished (Phillips et al., 1988; Plow and Ginsberg, 1989). The integrin $\alpha_{IIb}\beta_3$ can also bind other

plasma constituents like von Willebrand factor (vWF), and fibronectin which leads to further recruitment of platelets (Ruggeri, 2002).

There are two types of aggregation: a transient, reversible form and an irreversible form. With the reversible form agonists stimulate, within seconds, the initial binding of adhesive ligands, like fibrinogen to the integrin $\alpha_{IIb}\beta_3$. This initial binding is reversible. With further platelet activation through TXA_2 generation and secretion of ADP and diverse proteins from α-granules (such as thrombospondin) reversible aggregation becomes irreversible (Gawaz, 2001). With irreversible aggregation the activation of the $\alpha_{IIb}\beta_3$ integrin leads to the immobilization of soluble fibrinogen, vWF, and fibronectin onto the surface of adherent platelets. Combined with the activation of the coagulation system on the surface of activated platelets a stable and permanent thrombotic occlusion occurs.

2.2. Arterial thrombus formation induced by atherosclerotic plaques

Under pathophysiological conditions haemostatic factors play a crucial role in generating a thrombotic plug at the site of vascular damage. Studies have revealed that there are several stages in atherosclerotic plaque formation. A lesion is initiated by lipid accumulation in the intima and inflammatory leukocyte recruitment by activated endothelial cells. Macrophages accumulate oxidized low-density lipoprotein (oxLDL) and become lipid rich foam cells. Migration of smooth muscle cells from the media to the intima and their proliferation leads to the synthesis of extracellular matrix. Lesion progression is characterized by degradation of the matrix and by weakening of the fibrous cap.

Plaque damage can occur by apoptosis of endothelial cells leading to erosion or rupture. In all cases thrombogenic plaque material is exposed to circulating blood. Some of these thrombogenic factors are tissue factor, which stimulates the coagulation system, and numerous molecules which activate platelets, such as subendothelial collagen, vWF, cholesterol sulfate, oxidized phosphatidyl choline and LPA (Katsuda and Kaji, 2003) ((Abi-Younes et al., 2000; Barnes and Farndale, 1999; De Meyer et al., 1999; Libby et al., 2002; Merten et al., 2001; Rajavashisth et al., 1999; Siess et al., 1999; Weidtmann et al., 1995).

Tissue factor (TF) is a transmembrane glycoprotein that serves as one of the primary initiators of blood coagulation. TF is present on fibroblasts, smooth muscle cells (especially those in atherosclerotic plaques), and under pathophysiological conditions expressed on endothelial cells and circulating monocytes (Camerer et al., 1996). TF binds to FVIIa of the coagulation cascade and enhances the FVIIa catalytic activity generating FIXa and FXa which leads to thrombin generation, subsequent platelet activation, and fibrin synthesis.

In response to various chemical (TNF, IL-1, MCP-1, or IL6) and mechanical stimuli TF activity is up-regulated by monocytes and endothelial cells (Lorenzet et al., 1998). It is known that the

interaction of monocytes with platelets facilitates TF expression on monocytes (Celi et al., 1994). In turn, TF expression on monocytes facilitates the interaction of monocytes with activated platelets via binding of P-selectin (Shebuski et al., 2002). The end result of the increased expression of TF on monocytes is the acceleration of the rate and extent of fibrin formation and deposition in a thrombus or plaque.

Upon lesion of the vessel wall, plaque rupture or erosion, platelet adhesion to collagen under arterial flow conditions (high shear rate) is critically dependent on the interaction of activated vWF with platelet glycoprotein GPIb (vWF-Receptor). Platelet glycoprotein Ib receptor binding of circulating vWF leads to a conformational change of vWF, which then binds to exposed collagen, namely type I, III, or VI (Dent et al., 1990; Ruggeri, 2002; Zimmerman et al., 1986).

Subendothelial collagen interacts with two membrane glycoproteins in platelets, integrin$_{\alpha 2\beta 1}$ (GPIa-IIa) and GPVI. Both, integrin$_{\alpha 2\beta 1}$ and GPVI, mediate platelet adhesion and generation of intracellular signals for platelet activation. Both collagen receptors can stimulate the cytosolic tyrosine kinase Syk, which phosphorylates and thereby activates phospholipase Cγ2 leading to an increase in cytosolic Ca^{2+} via production of inositol-1,4,5-trisphosphate (Keely and Parise, 1996; Tokumura et al., 1987). The activation of platelets by collagen is carefully balanced out by the platelet-endothelial cell adhesion molecule 1 (PECAM-1, CD 31), which serves as an inhibitory receptor (Patil et al., 2001).

Other substances described in atherosclerotic plaques which can directly activate platelets are oxidized phosphatidylcholine, cholesterol sulfate, PAF and LPA. PAF is a powerful mediator of inflammation apart from directly activating platelets and may contribute to plaque formation (Brocheriou et al., 2000; Lupia et al., 2003; Mueller et al., 1995).

Here we will focus on LPA, which has been shown to mediate platelet activation by the plaque-lipid rich core (Rother et al., 2003; Siess et al., 1999). Important in the context of this work is the accumulation of LPA in the intima of atherosclerotic lesions (Rother et al., 2003; Siess et al., 1999).

2.3. Lysophosphatidic acid

LPA activates platelets directly. It has been shown to be formed during mild oxidation of low-density lipoprotein (LDL) and to accumulate in the lipid rich core of human atherosclerotic plaques. LPA receptor antagonists can abrogate the platelet activating effects of not only LPA, but also mox-LDL and plaque core-lipids (Rother et al., 2003) (Siess et al., 1999). LPA binds to three or more specific plasma membrane receptors and elicits a wide range of biological effects including platelet activation, the stimulation of cell proliferation, migration, smooth muscle cell contraction, apoptosis, as well as the promotion of cell survival (Mills and Moolenaar, 2003; Tigyi and Parrill, 2003). These biological effects combined provide evidence that LPA might also

initiate and perpetuate pathophysiological processes such as inflammation and atherogenesis (Siess, 2002).

2.3.1. LPA in plasma and serum

LPA present in plasma ranges in a concentration from 100-500 nM (Baker et al., 2002; Bjerve et al., 1974; Sano et al., 2002), whereas the serum concentration of LPA is in the 1-10 μM range (Baker et al., 2001; Sano et al., 2002; Saulnier-Blache et al., 2000). This difference in concentration implies that LPA is formed during blood coagulation and platelet activation probably through the interaction of enzymes released from activated platelets and plasma phospholipids.

LPA structure

The basic LPA structure consists of a glycerol backbone, a phosphate group and a fatty acid chain. The variability of the LPA species lies in the length and saturation of the fatty acid chain, and in the link of the fatty acid chain (ester or ether link) at position C1. Plasma LPA is composed of varying fatty acid chains, C18:0, C16:0, C18:1 and C20:4 (Baker et al., 2001; Pages et al., 2001).

In this dissertation acyl-LPA (16:0), acyl-LPA (18:1) and alkyl-LPA (16:0) were used. For illustration see the Figure below.

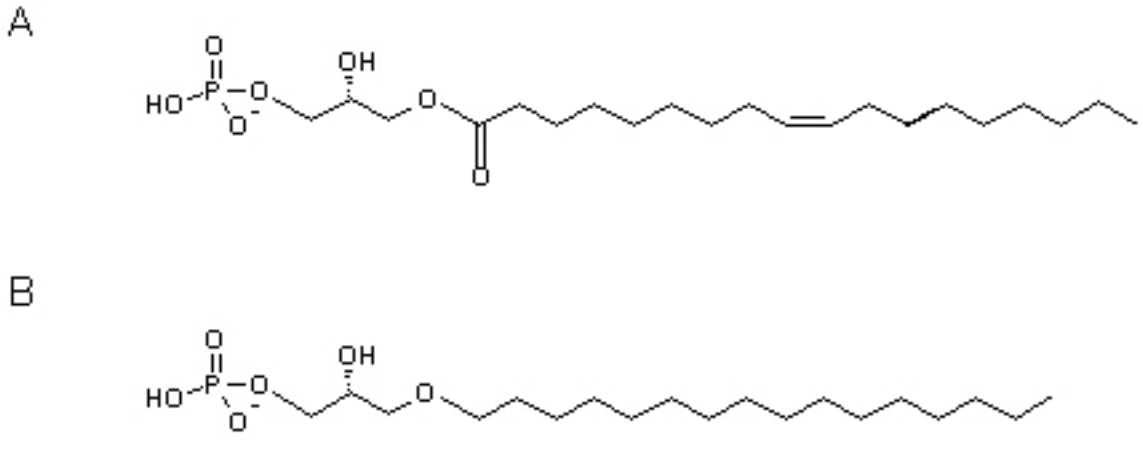


Fig 2.1

LPA structures for 1-acyl LPA (18:1) (A) and 1-alkyl LPA (16:0) (B)

LPA is the simplest phospholipid and consists of a glycerol backbone with a hydroxyl group at position sn-2 (or sn-1) attached to a phosphate group at position sn-3 and a fatty acid chain at position sn-1 (or sn-2).

Structure (A) shows oleoyl-Lysophosphatidic acid also called acyl-LPA (18:1), structure (B) shows mono-(16:0)-alkyl-glycerol-phosphate or alkyl-LPA (16:0), 1-acyl LPA has a double bond oxygen at C1 in contrast to 1-alkyl LPA.

2.3.2. LPA- Formation

A critical concentration of LPA has to be produced and transported to the extracellular medium, since LPA interacts with membrane receptors. LPA can be synthesized by a number of different cell types, including platelets (Moolenaar, 1995).

Here we will focus on LPA being formed by a number of different mechanisms, as an intermediate product in the intracellular lipid synthetic pathways or produced in a stimulus-coupled manner.

Intracellular LPA as a precursor in glycerolipid synthesis

In the chain of reactions in glycerolipid synthesis LPA formation can be catalyzed by the enzyme, glycerophosphate acyltransferase (GPAT), located in both endoplasmatic reticulum and mitochondria. GPAT acylates glycerol-3 phosphate into LPA as an intermediate product before LPA is then acylated by monoacylglycerolphosphate acyltransferase (MGAT) into phosphatidic acid (PA), the precursor of all glycerolipids (Haldar and Vancura, 1992).

An alternative pathway is the reduction of acyl dihydroxyacetone phosphate (acyl DHAP) in peroxysomes contributing to LPA formation in pancreatic islets exposed to high glucose concentrations (Dunlop and Larkins, 1985).

Further LPA can be synthesized by the action of monoacylgycerol kinase (MAG-kinase) on monoacylglycerol, as an important precursor of phosphatidylinositol synthesis(Simpson et al., 1991). MAG kinase has also been proposed to be involved in the formation of arachidonoyl-LPA in platelets (Gerrard and Robinson, 1989). However, whether LPA, produced during glycerolipid synthesis, can accumulate and contribute to the extracellular release of phospholipids is not yet resolved.

Extracellular LPA Generation by phospholipid hydrolysis

Hydrolysis of fatty acids at the *sn*-1 position by phospholipase A_1 (PLA_1) or at the *sn*-2 position by phospholipase A_2 (PLA_2) generates LPA from PA. Platelets and to a smaller degree red blood cells, have been identified as sources of LPA in blood (Eichholtz et al., 1993). PA was found to be rapidly generated in thombin-stimulated platelets and could subsequently be converted to LPA via PLA enzymes (Lapetina et al., 1981a, b). LPA generated through this mechanism appears within 15 min following thrombin stimulation and constitutes only a minor portions, estimated 10%, of LPA detected in serum (Aoki et al., 2002; Gaits et al., 1997; Sano et al., 2002).

A larger proportion of LPA has been shown to be produced in plasma by platelets releasing PLA_1 and PLA_2 that generate a de novo pool of lysophospholipids primarily lysophosphatidylcholine (LPC) from Phosphaditylcholine (PC) in plasma and membrane phospholipids (Sano et al.,

2002). LPC can then be further metabolized by the enzyme lysophospholipase D (lyso-PLD) identical to the tumor cell motility stimulating protein known as autotaxin (Tokumura et al., 2002) (See Fig 2.2 below). Autotaxin belongs to the family nucleotide pyrophosphatase/ phosphodiesterases and promotes tumor cell motility, progression, metastasis, and angiogenesis via a pertussis toxin-sensitive mechanism (Lee et al., 2002b; Nam et al., 2001; Stracke et al., 1997). These activities have also been described for LPA (Imamura et al., 1993; Imamura et al., 1996). LPA production due to lyso-PLD activity in human whole blood ex vivo has been described as fairly rapidly and amounts to about 1.2µM within 1 h (starting from approximately 130 nM) (Baker et al., 2001). Sano et al have used activated plasma and showed a 20-fold increase within the first hour (Sano et al., 2002).

However, this increase is not due to an increase in Lyso-PLD activity (Aoki et al., 2002; Sano et al., 2002), but has been proposed to be caused by PLA_1, PLA_2, capable of not only generating lysophosphatidylcholine (LPC), but also lysophosphatidylserine (LPS), and lysophosphatidylethanolamine (LPE) from plasma PC, PS, and PE. An important source of LPC, being the most abundant lysophospholipid (125-150µM) in plasma, is the enzyme lecithin-cholesterol acyltransferase (LCAT) (Croset et al., 2000; Tokumura et al., 1999).

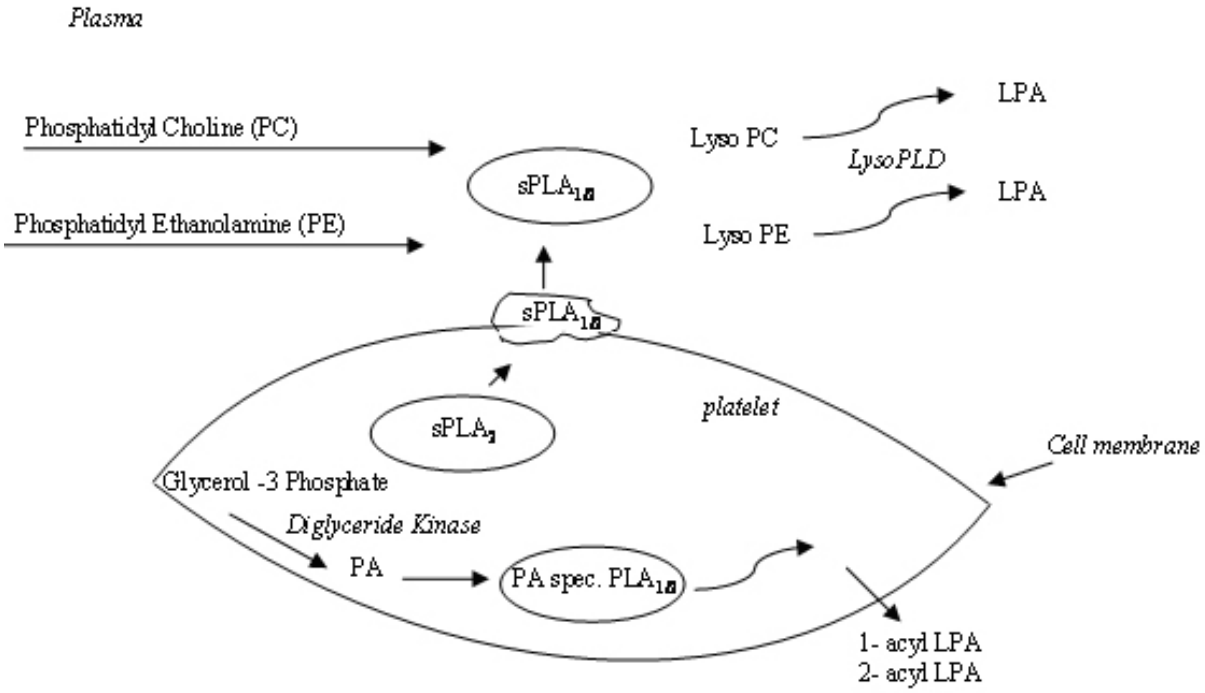


Fig 2.2
Schematic Model of LPA formation
PA = phosphatidic acid, $sPLA_{1/2}$ = secretory phospholipase $A_{1/2}$ LysoPLD= lysophospholipase D
Phosphatidylcholine and Phosphatidyl ehanolamine are located in plasma and on the outside cell membrane.

In addition, a secretory or type II PLA_2 ($sPLA_2$) with slightly different activity than PLA_2, has been implicated in LPA production from microvesicles shed by inflammatory cells (Gerrard and Robinson, 1989). $sPLA_2$ hydrolyzes phospholipids of lipoproteins and microvesicle membranes

that have altered membrane asymmetry that leads to PA accumulation in the outer leaflet of the plasma membrane (Eckey et al., 2004; Fourcade et al., 1995; Hurt-Camejo et al., 2001; Snitko et al., 1997). Especially aminophospholipids like phosphatidylethanolamine and phosphatidylserine are hydrolyzed by $sPLA_2$ generating the corresponding lysophospholipid substrate for lysophospholipase D cleavage (Fig 2.2) (Gaits et al., 1997).

LPA contained in atherosclerotic plaques can be formed by mild oxidation of LDL

Mild oxidation of LDL leads to a large increase of its LPA content as compared to native LDL. LPA exposed on mildly oxidized LDL has been shown to activate platelets and endothelial cells (Siess et al., 1999). Mild oxidation of LDL, demarcating the early phase of atherosclerotic lesion development, takes place mainly in the vascular intima, and is caused by superoxide anions formed by activated macrophages and endothelial cells (Holvoet and Collen, 1998; Ross, 1999; Sevanian et al., 1997). LPA also accumulates in the intima of human atherosclerotic lesions and is predominantly present in the lipid rich core (Siess et al., 1999).

LPA in atherosclerotic lesions may also be produced by the action of $sPLA_2$ which is secreted upon cell stimulation with pro-inflammatory cytokines in human plaques (Hurt-Camejo et al., 2001; Menschikowski et al., 2000).

Additionally contributing to the LPA content in atherosclerotic plaques is the release of LPA by activated platelets, imparted in advanced lesions.

2.3.3. LPA degradation

Enzymatic removal of LPA results in functional inactivation of LPA and transformation into other bioactive lipid mediators. There are three major pathways of LPA degradation. The first step is phosphate removal to form monoacylglycerol by phosphatase or phosphohydrolizing enzymes. The second step is conversion to phosphatidic acid (PA) by acyltransferases. The third step is the removal of the sn-1 acyl chain to form glycerol phosphate by LPA-specific lysophospholipases. Important for our consideration is the first pathway involving a class of integral membrane enzymes, called lipid phosphate phosphatases (LPPs), since they most efficiently catalyze the dephosphorylation of LPA.

LPP's function as ecto- enzymes, degrading LPA into monoacylglycerol, an LPA-receptor inactive molecule. LPP's are hydrophobic and localize to the plasma membrane and to intracellular membrane compartments. Studies published disagree widely on the mechanism and selectivity of the effect, but commonly conclude that overexpression of LPPs can attenuate cellular responses to LPA (Alderton et al., 2001; Hooks et al., 2001; Jasinska et al., 1999; Xu et al., 2000). Four isoforms of LPP's have been cloned and subdivided according to their function. LPP1 and LPP3 are both present in platelets, whereby LPP1 shows a 16-fold higher activity and

is regulated by platelet agonists (Smyth et al., 2003). LPP1 accounts for the major LPP activity in human platelets and most efficiently catalyzes the dephosphorylation of LPA, followed by PA, sphingosine 1- phosphate (S1P) and ceramide 1- phosphate (C1P) (Roberts et al., 1998; Waggoner et al., 1996). Interestingly, when platelets were stimulated with thrombin-receptor activating protein (TRAP), ADP, or LPA platelet activation was accompanied by a relocalization of LPP1 and LPP3 to the platelet surface (Smyth et al., 2003). Moreover, antagonizing LPP1 by a receptor inactive phosphoinositide analog of LPA could enhance LPA- and ADP- induced platelet aggregation, shape change, Rho-activation, and even thrombin stimulated LPA production (Smyth et al., 2003). Thus LPP1 appears to be an important and rapid inhibitor of agonist-induced platelet activation.

The second LPA degradation pathway involves the action of 1-acylglycerol 3–phosphate acyltransferase (AGPAT) enzymes, also called lysophosphatidic acid acyltransferases (LPAAT). These enzymes catalyze the formation of phosphatidic acid by transferring an acyl group from acyl-CoA to LPA. Proteins with LPAAT activity include a transmembrane family of enzymes and membrane associated proteins (Aguado and Campbell, 1998; Hannah et al., 1999; Leung, 2001). They include five different members and are proposed to mediate the majority of activity within most cells (Leung, 2001).

The third degradation pathway involves the hydrolysis of the fatty acyl group by the action of lysophopholipase A enzymes. The majority of these enzymes act on lysophosphatidyl choline (LPC) (Wang et al., 1999). However, an LPA specific lysophospholipase activity has been purified from rat brain (Thompson and Clark, 1994).

2.3.4. Transport- and binding- proteins of LPA: albumin, intracellular fatty acid binding proteins, and gelsolin

LPA intra- and extra- cellular transport depends on the presence of binding proteins which facilitate solubility and transport of an amphiphilic molecule like LPA through a hydrophilic medium. Due to the hydrophobic character of the fatty acid chain, LPA has a high affinity to other proteins, most importantly, albumin.

Albumin serves as an extracellular transport protein that binds LPA with an affinity constant, K_D, of 360 nM and a stoichiometry of 3 moles LPA to 1 mole albumin (Thumser et al., 1994). In fact LPA released from activated platelets into the extracellular medium has only been able to be detected in the presence of albumin demonstrating the high affinity of albumin to LPA (Eichholtz et al., 1993; Tigyi et al., 1991).

Albumin bound LPA is responsible for most heat stable and lysophospholipase-sensitive biological activities of serum (Ridley and Hall, 1992; Tigyi and Miledi, 1992). In addition LPA has been identified as the active molecule responsible for biological activity of serum albumin on

stimulation of Ca^{2+} efflux on *Xenopus oocytes*, actin stress fiber formation and cell proliferation (Ridley and Hall, 1992; Tigyi et al., 1991; Tigyi and Miledi, 1992).

In a recent study Hama et al found that LPA receptor activation induced by added LPA can be inhibited by albumin present in serum. High concentrations of BSA (>5% w/v, physiological plasma concentration: 4% w/v) completely inhibited LPA-induced activation of the three LPA receptors, LPA_3 being the most sensitive receptor (Hama et al., 2002).

Intracellular fatty acid binding proteins (FABP's) transport LPA from one organelle to the other with an affinity in the micromolar range. FABP's aid in LPA metabolism when transporting LPA from mitochondria to microsomes where LPA is further acylated to phosphatidic acid (Vancura and Haldar, 1992). Additionally, FABP's stimulate the enzyme glycerophosphate-acyltransferase which produces LPA from glycerolphosphate and fatty acid coenzyme A precursors (Jolly et al., 1997).

Another molecule, gelsolin, an approximately 85 kD/ Protein has been shown to be of potential intra- and extra- cellular importance. Extracellular gelsolin can bind to LPA and then either sequester it from or deliver it to its receptors. Intracellular gelsolin – a member of a family of actin binding proteins - binds and caps actin filaments and is thus involved in regulating the cytoskeleton architecture. Depending on the actin concentration gelsolin can either sever pre-existing filaments, promote filament formation and cap fast growing actin filaments. Therefore, gelsolin can either promote or stop actin filament growth.

The two forms of gelsolin, plasma and intracellular gelsolin have different structures due to alternative splicing (Kwiatkowski et al., 1986). Both forms contain a 730 amino acid core structure that is made up of six repeated domains. The six domains make up three distinct actin binding sites that have been localized within domains 1,2, and 4 (Kwiatkowski et al., 1986; Pope et al., 1995). A difference in structure and in activity between plasma and intracellular gelsolin has been shown (Huckriede et al., 1990; Wen et al., 1996).

Structurally, plasma gelsolin has an intradomain disulfide bond within domain 2 which links Cys^{188} and Cys^{201}, while in cytoplasmatic gelsolin all five cysteine residues are in their free thiol form (Wen et al., 1996) (See Fig. 2.3).

Functionally, when comparing plasma, recombinant, and cytoplasmatic gelsolin by using microinjection, only plasma gelsolin was active on stress fiber networks, suggesting that functional differences exist on the activity within cells (Huckriede et al., 1990).

Phosphoinositides, like PIP_2 and Ca^{2+}, regulate actin binding and severing activities of plasma gelsolin as measured by the intracellular effects in rat cardiomyocytes (Bucki et al., 2001). LPA binds to the same sites on gelsolin as PIP_2 and was therefore proposed to regulate gelsolin activity by the same mechanism as PIP_2 (Kwiatkowski, 1999; Meerschaert et al., 1998). Interestingly, the LPA/ PIP_2 binding site lies within domain 2, coinciding with the site of the disulfide bond which distinguishes the cytoplasmatic gelsolin from plasma gelsolin.

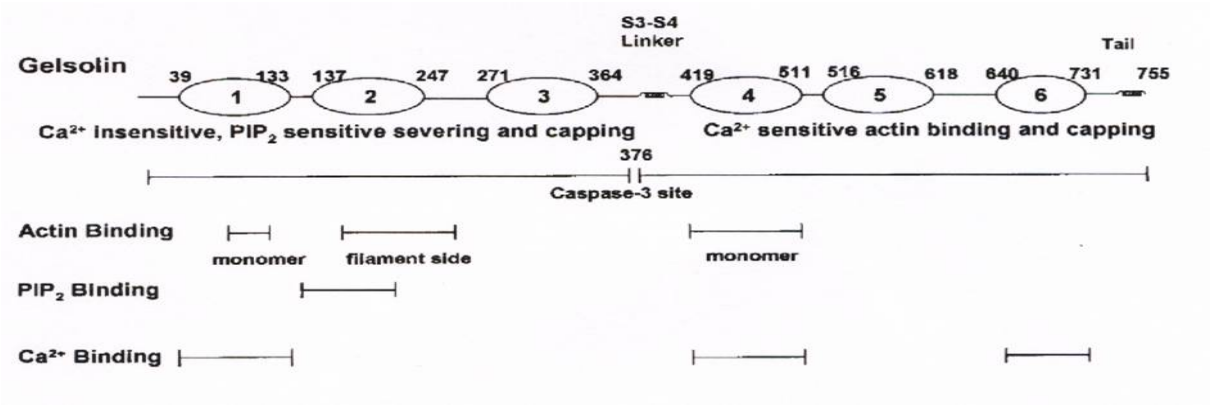


Fig 2.3
Schematic representation of domains of plasma gelsolin and their functions.
Amino acid positions are numbered as in human plasma gelsolin and segmental boundaries are based on the structural definitions defined by the gelsolin crystal. Actin, PIP_2, and Ca^{2+}-binding segments are shown. Gelsolin domains are circled (Sun et al., 1999).

Extracellular plasma gelsolin is present at concentrations of 100-250µg/ml (1.2- 2.9 µM) and binds LPA with high affinity close to that of LPA receptors (K_d 6 nM) and exceeding that of albumin (K_d 360 nM). Extracellular gelsolin may function as a high affinity plasma carrier (K_d of 32 nM), which protects a portion of circulating LPA from biodegradation and prevents LPA from binding to numerous possible low affinity proteins (Goetzl et al., 2000). Gelsolin concentrations of less than 10% of the plasma concentration were shown to increase LPA activity, whereas 20% or higher concentrations of those in plasma diminished biological effects of LPA in rat cardiac myocytes (Goetzl et al., 2000).

In the context of this work, only a potential effect of gelsolin on the outside cell surface may play a role.

2.3.5. Platelet shape change and aggregation induced by LPA

The first report on platelet aggregation induced by LPA was done in 1979 by Schumacher et al., who discovered that in plasma, incubated at 36ºC for 18-24 hours, a factor developed, which on intravenous injection in cats evoked platelet aggregation followed by an increase in pulmonary vascular resistance (Schumacher et al., 1979). They also provided evidence that PA and LPA, are the active components causing aggregation of human and feline platelets. Of interest is that LPA was unable to induce aggregation in dogs, rabbits, pigs, and rats (Mauco et al., 1978; Schumacher et al., 1979).

LPA (Oleoyl-LPA or 18:1) is a potent activator of platelets and induces shape change of human isolated platelets at very low concentrations (EC_{50} = 18 nM) via remodelling of the actin cytoskeleton, which is dependent on $G_{12/13}$- mediated activation of the small GTP- binding protein, Rho and subsequently Rho kinase (Bauer et al., 1999; Retzer and Essler, 2000). LPA also stimulates the tyrosine kinase Src during *shape change* causing subsequent activation of the

tyrosine kinase Syk (Maschberger et al., 2000). Src and Syk, which are most likely G_i activated in platelets, can elicit an increased exposure of fibrinogen binding sites on the integrin $\alpha_{IIb}\beta_3$ which is a prerequisite for subsequent aggregation (Bauer et al., 2001).

Higher concentrations of LPA were required to induce aggregation in isolated platelets (EC_{50} = 10µM) (Benton et al., 1982) (Maschberger et al., 2000). Aggregation of washed platelets was considerably influenced by the concentration of albumin, and by fibrinogen (Benton et al., 1982). Benton et al showed that 1mg/ml bovine serum albumin (=15µM) was able to inhibit the platelet aggregation caused by 3µM of LPA. Due to albumin present in plasma much higher concentrations of LPA are needed to induce aggregation in platelet rich plasma (20-100µM) (Tokumura et al., 1987). Studies on the effects of a number of LPA analogues have shown a higher potency for highly unsaturated and long chain acyl – LPAs (such as C20:4) than for the LPA analogues with a shorter C_{18} fatty acyl group, such as an oleoyl group (18:1). Ether-linked-LPAs had the strongest aggregating activity (Tokumura et al., 2002).

When platelets are stimulated by thrombin they mostly produce acyl- LPA (18:2) and acyl- LPA (20:4) (Sano et al., 2002). Acyl- LPA (20:4) is a more potent platelet activator in inducing shape change than other acyl- LPA species (Rother et al., 2003; Tokumura et al., 2002). Compared to acyl-LPA (16:0) the (20:4) acyl- LPA- species was 6.5-fold more effective in inducing platelet shape change (Rother et al., 2003). However, alkyl- LPA (16:0) has been shown to have the greatest potency in inducing shape change, 18.5-fold more effective than acyl- LPA (16:0) (Rother et al., 2003).

The study of Gueguen et al has determined varying extents of platelet aggregation for nine LPA analogues all showing cross-desensitization for the same LPA receptor (Gueguen et al., 1999).

Also in the study of Guegen platelets have shown a decreased response to LPA in a medium containing 1.3 mM calcium, contrasting previous observations of Sugiura et al who showed the necessity of calcium for LPA-induced aggregation (Alberghina et al., ; Gueguen et al., 1999; Sugiura et al., 1994). Thus in our studies experiments acyl-LPA (16:0) and alkyl-LPA (16:0) will be analysed for inducing aggregation with regard to calcium dependency.

2.3.6. LPA-receptors

It is still unknown which LPA receptors mediate platelet activation and aggregation. LPA-induced activation of platelets is possibly mediated by G- protein coupled LPA-receptors as they have been shown in a number of other cells. LPA activates possibly the three different types of G-protein coupled receptors, LPA_1, LPA_2, LPA_3 receptors, formerly termed endothelial cell differentiation gene (Edg-2, -4, -7). All three LPA receptors have been found to be expressed in platelets as detected by RT- PCR (Motohashi et al., 2000).

However, by studying the effect of LPA-receptor activation with various subtype selective agonists and antagonists, LPA responses of platelets have shown to be inconsistent with the pharmacological properties of LPA_{1-3} of other cells (Hooks et al., 2001).

The short chain phosphatidate, dioctanoyl glycerol pyrophosphate (DGPP 8:0), a selective antagonist of LPA_1 and LPA_3 with no effect on LPA_2, antagonizes platelet activation (Fischer et al., 2001; Rother et al., 2003). DGPP 8:0 inhibited both acyl- LPA and alkyl-LPA responses (Rother et al., 2003), indicating LPA might therefore induce platelet activation through LPA_1 and LPA_3 receptors. In another study where platelets from some donors were non-responsive to the highly potent alkyl-LPA (16:0), the expression of LPA_2 mRNA dominated over LPA_3 and LPA_1 in those non-responders. This also suggests that LPA_2 plays a less important role in LPA- induced platelet activation than LPA_1 and LPA_3 (Tokumura et al., 2002). This discrepancy could suggest that LPA responses in platelets are mediated by yet unidentified receptors or novel LPA receptors like LPA_4 (Noguchi et al., 2003).

Adding to the complexity is the discovery of the nuclear receptor PPARγ (peroxisome proliferator-activated receptor) as LPA-receptor (McIntyre et al., 2003). LPA-induced PPARγ activation was held responsible for LPA-induced formation of neointima in vivo in rat carotid arteries (Zhang et al., 2004).

2.4. Platelet-monocyte interaction

2.4.1. LPA-induced platelet-monocyte activation

It is becoming increasingly apparent that circulating monocytes may represent a principle player in the inflammatory and coagulative pathways. Monocytes can adhere to and migrate through the dysfunctional endothelium into the intima and can thus mediate inflammatory responses in atherosclerotic lesions (Ross, 1999).

High concentrations of LPA (0.1-0.3 mM) stimulate in human monocytes an increase in cytosolic Ca^{2+}, chemotaxis, and their haptotactic migration. These actions are possibly related to the activation of LPA membrane receptors (Zhou et al., 1995). More recently LPA has been shown to induce a rise in intracellular Ca^{2+} in Mono Mac 6 cells at much lower concentrations (EC_{50} = 52nM) (Fueller et al., 2003). These cells, a differentiated human monocytic cell line, expressed LPA_1 and LPA_2 receptors, similar as human blood monocytes and macrophages (Fueller et al., 2003). Minimally oxidized LDL and serum also induced a rise in intracellular Ca^{2+} in Mono Mac cells and this action was mediated by the activation of LPA receptors (Fueller et al., 2003). However, another study showed that human blood monocytes express only LPA_1, but not LPA_2 and LPA_3 receptors (Lee et al., 2002a). Thus circulating or locally produced LPA might possibly stimulate macrophages and monocytes by LPA receptor activation.

2.4.2. Mechanisms of platelet-monocyte interaction

Activated platelets may stimulate 1) inflammatory responses and 2) can have a procoagulant influence at the site of platelet activation. Upon activation, platelets can adhere to monocytes and thereby initiate monocyte activation (Larsen et al., 1989; Neumann et al., 1996; Ott et al., 1996; Rinder et al., 1991). It has first been demonstrated by Jungi et al that thrombin- stimulated platelets adhere to monocytes and neutrophils (Jungi et al., 1986). Monocytes have a competitive advantage over neutrophils when less potent agonists, like epinephrine and ADP, are used (Rinder et al., 1991). In this dissertation experiments on platelet-monocyte interaction in response to LPA were performed.

The process of platelet-monocyte attachment is mediated by the platelet's surface expression of P- selectin, an adhesion molecule contained in α-granules that appears on the membrane surface after degranulation. P- selectin is a member of a family of adhesive proteins including E- and L-selectin that are known to regulate transient interactions between endothelial cells and leukocytes (Lasky, 1992). P-selectin interacts with its natural ligand, P-selectin glycoprotein ligand-1 (PSGL-1), present on monocytes and neutrophils, providing an anchoring source for inflammatory cells on activated platelets (McEver and Cummings, 1997; Sako et al., 1993). Firm attachment is subsequently mediated by monocyte CD11/CD18- dependent platelet interactions (Cerletti et al., 1999). However, P- selectin may not be the only mode of attachment to leukocytes; additional molecules may be involved (Sarma et al., 2002).

Activated monocytes may perpetuate inflammation by releasing metabolites of arachidonic acid, leukotrienes and interleukins (Feuerstein and Hallenbeck, 1987). Moreover, platelet-activated monocytes can initiate the extrinsic pathway of coagulation via surface expression of tissue factor, may influence rapid fibrin formation and facilitate adhesion to the endothelium via expression of the β2- integrin Mac-1(Altieri et al., 1988; Drake et al., 1989; Zillmann et al., 2001). Experimental studies have demonstrated that platelet/leukocyte-interaction is involved in atherogenesis by neointima formation, luminal tissue factor expression and fibrin deposition (Kawasaki et al., 2001; Singh et al., 2001).

The possible clinical relevance of platelet- monocyte interaction was shown by Furma et al. who demonstrated elevated levels of platelets attached to monocytes in patients with acute coronary artery disease (Furman et al., 2001). In patients with acute myocardial infarction the increase in platelet-monocyte aggregates, having a half life of about 30 minutes in vivo, did not correlate with the number of P-selectin-positive platelets which were previously believed to be the "gold standard" marker of platelet activation (Michelson et al., 1996; Michelson et al., 2001; Michelson and Furman, 1999). In fact Michelson et al has demonstrated that platelet-monocyte aggregates are a more sensitive marker than P-selectin of platelet activation in vivo.

3. Key Questions addressed

The following thesis will present a functional study trying to disclose some of the mechanisms involved in LPA-induced platelet activation, in particular aggregation and platelet-monocyte interaction.

It is known that LPA can cause platelet shape change and platelet aggregation. However, a systematic study concerning the mechanism of LPA- induced aggregation in washed platelet suspensions and platelet rich plasma is missing; and it is unknown whether LPA induces platelet aggregation in whole blood.

The following issues will be addressed:

- Determining the potency of LPA as a platelet agonist in washed platelets, platelet-rich plasma and blood
- Establishing a possible donor- dependent LPA-induced platelet response
- Discovering which mediators are involved in LPA-induced platelet aggregation
- Discerning inhibitors of LPA-induced platelet activation
- Finding out whether LPA can induce platelet adhesion to monocytes and by which mechanism the adhesion could be mediated

4. Materials and methods

4.1. Materials

Adenosine-3´, 5´Diphosphate, Sodium Salt	Sigma
Adenosine-5´-Diphosphate (ADP), Disodium Salt	Sigma
Albumin, bovine; Fraktion V; Prod.-Nr. A2153	Sigma
Albumin, bovine; essentially fatty acid free; Prod. -No. A7511	Sigma
Apyrase; Prod.-No. A6535	Sigma
ARC-69931 MX; Code- No. 1-407-526, Batch-No. 4256J	Astra Zeneca
Calciumchloride ($CaCl_2 \times 2\ H_20$)	Merck
CAMP Correlate-EIA Kit; Prod.-No. 90066	Biotrend
CD 14- PE; clone: MϕP9, Prod.-No. 347497	BD, Biosciences
CD 41a- FITC; clone: HIP8 anti GPIIb; Prod. No. 555466	BD, Biosciences
Creatine phosphate / Creatine phosphate kinase	Sigma
Collagen (Kollagenreagens ”Horm“) and SKF Horm® Buffer	Nycomed Pharma Gmbh
Diacylglycerolpyrophosphate	Avanti Polar Lipids
Dimethylsulfoxide (DMSO)	Sigma
Ethane- N,N,N’,N’ Tetra- Acetic- Acid (BAPTA-AM); Prod.– No. A1076	Sigma
Ethanol, absolute	Merck
Ethylendiaminetetraacetate (EDTA); disodiumsalt, dihy-Drate	Sigma
Ethyleneglykol—bis(β-Aminoethylether)-N,N,N´,N´-Tetra-Acetate (EGTA)	Sigma
Fluorescein Isothiocyanate (FITC)- conjugated mouse IgG_1, κ monoclonal Immunoglobulin Isotype Standard, clone: MOPC-31C, Prod.-No.550616	BD, Biosciences
Formaldehyde-Solution at least 37%	Sigma
Gelsolin, from bovine plasma; Prod.-No. G8032	Sigma
Gelsolin, from human plasma; Prod.-No. G1583	Sigma
D(+)-Glucose-Monohydrate	Merck
Hepes, N-[2-Hydroxyethyl]piperazine-N´-[2-ethane- sulfonic-acid	Sigma
Iloprost ; Prod. No. SH-L-401A	Schering

Potassiumdihydrogenphosphate (KH_2PO_4)	Merck
Potassiumchloride (KCl)	Merck
Prostaglandin E_1, Prod. No. P5515	Sigma
Oleoyl-L-α-Lysophosphatidic Acid (C18:1, [cis]-9), Sodium Salt	Sigma
1-Palmitoyl-sn-glycero-3-phosphate (P-LPA; 1-acyl-LPA 16:0)	Alexis Corp. (San Diego, CA, USA).
Platelet activating factor (β-Acetyl-γ-O-Hexadecyl-L-α-Phosphatidylcholin)	Sigma
P-Selectin- Antibody, CD62p-FITC, subclass IgG_1 Prod.- No. MCA796F	Serotec
P-Selectin Monoclonal Blocking Antibody, Clone G-1, subclass IgG_1 Prod.-No. BMS 126	Bendermedsystems
PGE_1 [11α, 13E, 15S]-11,15- Dihydroxy-9-oxoprost-13-enoic acid; Prod.-No. P5515	Sigma
Red Blood Cell Lysing solution, Erythrolysis	Serotec Ltd
Refludan	Pharmion Ltd. c/o LOGOSYS Logistic GmbH
RGDS (Arg-Gly-Asp-Ser)	Sigma
SKF-96365, Hydrochlorid (1-[β-[3-(4-Methoxyphe-nyl)propoxy]-4-methoxyphenylethyl]-1H-imidazol)	Calbiochem
Tissue Factor- Antibody, TF-FITC, clone I, subclass IgG_1 , Prod.- Number: 9010-5079	Biotrend
Thrombin Receptor Activator Protein (TRAP), Prod. No S-7152	Sigma

4.2. Buffers

Acid- Citrate- Dextrose	85 mM	Trisodium Citrate
	111 mM	Glucose
	65 mM	Citric Acid
BSA- Buffer:	150 mM	NaCl
	10 mM	Hepes
	0,25 mM	Bovine Serumalbumine
Fixing Solution (pH 7.4)	150mM	NaCl
	4.6 mM	Na_2EDTA
	4.5 mM	Na_2HPO_2
	1.6 mM	KH_2PO_4
	0.16% w/v	Formaldehyde
Buffer B (pH 6.2) :	20 mM	Hepes
	138 mM	NaCl
	2.9 mM	KCl
	1 mM	$MgCl_2$
	0.36 mM	NaH_2PO_4
Buffer C (pH 7.2):	See Buffer B	
Solution I	16.0g	NaCl
	0.4g	KCl
	2.0g	NaHCO3
	0.116g	NaHPO4. H2O

4.3. Methods

4.3.1. Preparation of LPA

Both phospholipids palmitoyl-LPA and oleoyl-LPA were dissolved in 80% ethanol/ 20% H_2O solution to create a 1mM stock solution stored at -20°C. The ethanol containing LPA solution was used up to a final concentration of 3µM. Any concentration higher than 3µM of LPA was taken from BSA buffered solution LPA stock (see section above for BSA). BSA buffered LPA stock was obtained by vaporizing a certain volume of LPA dissolved in ethanol using Nitrogen gas. The LPA sediment was dissolved in an equal volume of BSA buffer to create a 1mM LPA solution.

4.3.2. Preparation and Incubation of other agonists and antagonists

All but the following agonists and antagonists were dissolved in 0.9% NaCl.

Collagen was dissolved in SKF Horm® Buffer at a concentration of 100µg/ml.

For experiments in whole blood, aspirin was dissolved at pH 7 (maximum solubility of 3.3g/l) in 0.9% NaCl and drawn together with the anticoagulant into a separate syringe to be mixed with whole blood to obtain a 1mM final concentration.

In experiments with washed platelets and PRP asprin was dissolved in absolute ethanol.

BAPTA- AM was dissolved in DMSO to create a 20mM stock.

The following platelet inhibiting antagonists were used: apyrase (10 U/ml), $P2Y_1$ receptor antagonists MRS2179 (100 µM) and A3P5P (200 µM), $P2Y_{12}$ receptor antagonist ARC69931 (1 µM), P- selectin antibody, Ca^{2+} binding molecule EGTA (1mM) and BAPTA-AM (10- 200 µM) and SKF 96365 (100µM), fibrinogen binding molecule RGDS (5 mM). All the ADP inhibitors were added 30 sec prior to adding the agonist. When working with a P- selectin antibody, the antibody was also added 30 sec prior to the agonist. The Ca2+ binding molecule, EGTA (1 mM), was added 10 sec before agonist addition. BAPTA-AM and SKF 96365 were pre-incuabted at 37 °C for 30 minutes and 10 minutes, respectively

In platelet- rich-plasma experiments and washed platelets aspirin was preincuabted at 37 °C for 10 min. In experiments with whole blood aspirin was directly dissolved in the anticoagulant when drawing blood. Therefore, incubation time in whole blood depended on the experiment, but was at least 20 minutes.

4.3.3. Blood Preparation

Blood from healthy volunteers not taking any medication was drawn by cubital venipuncture into propylene syringes containing either tri-sodium-citrate dihydrate (3.8% w/v) 1:10 or recombinant hirudin (Refludan®; 13µg/ml; 200U/ml). A winged infusion set (0.8 X 20 X 300 mm) was used and blood was immediately transferred into polystyrene tubes and kept at 20°C during platelet isolation. Experiments were completed within 2 hours after drawing blood.

4.3.4. Preparation of Platelet-Rich-Plasma (PRP)

For PRP whole blood was centrifuged at 180g for 20 min until platelet rich plasma (PRP) had separated from erythrocytes and leukocytes. The supernatant of PRP was transferred carefully without disturbing the buffy coat and red blood cell layer into a capped polystyrene tube and kept at room temperature. The remainining PRP was centrifuged at 1500g for 10 min to produce platelet poor plasma in order to gage the aggregometer to 100% light transmittance.

4.3.5. Preparation of washed platelets

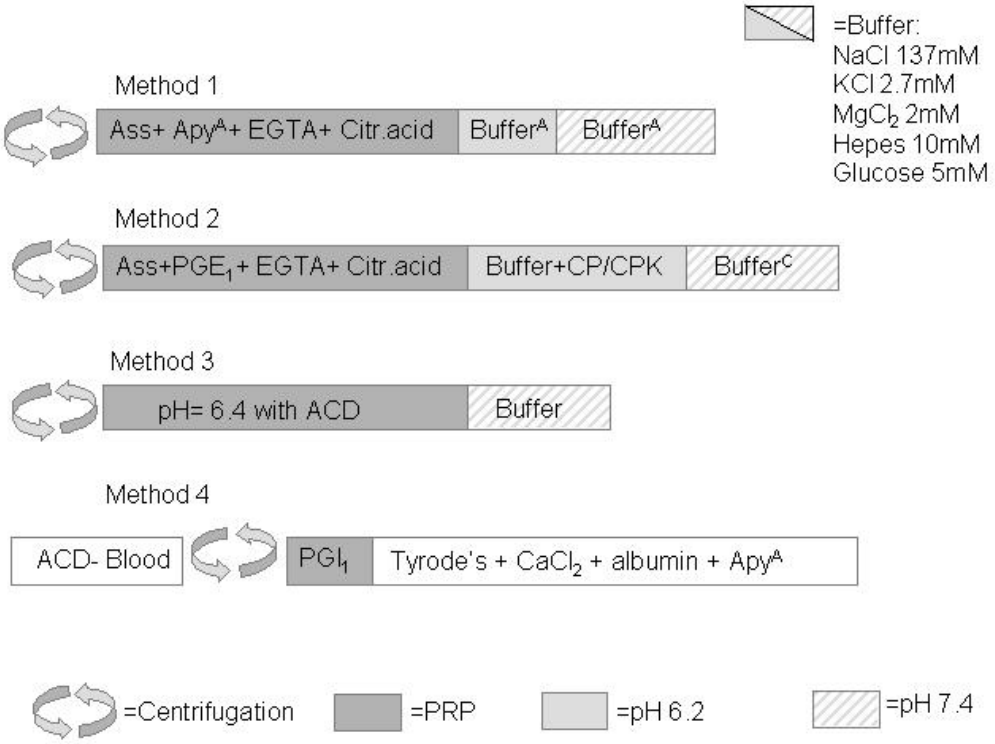


Fig 4.1

Summary of different platelet preparations

Apy^{A} or ^{A} alone stands for apyrase in a final concentration 0.3 U/ml. Buffer ^{C} stands for CP/CPK containing buffer. ACD stands for Acid-citrate- dextrose and functions similar to EGTA as a Ca^{2+} chelator.

4.3.5.1. Method 1

Whole blood anticoagulated with either tri-sodium-citrate dihydrate (3.8% w/v) 1:10 or recombinant hirudin (Refludan®; 13µg/ml; 200U/ml) was centrifuged at 180g for 20 min. The top layer of PRP was carefully separated with a large pipette into a separate 50 ml capped polystyrene tube. Then the COX-1 inhibitor, aspirin 1 mM, and ADP scavenger enzyme, apyrase 0.3 U/ml final concentration (f.c.) was added to PRP and incubated at 37°C for 15 minutes. Subsequently citric acid 9mM and EDTA 5mM (f.c.) were added in order to bind excess Ca^{2+} preventing the formation of ionized Ca^{2+} needed for clotting.

Following centrifugation at 800g for 20 min the supernatant was discarded and the platelet pellet resuspended in buffer B stored at 37 °C prior to suspension. The volume of buffer B was determined by 15% the original blood volume. 0.3 U/ml apyrase (f.c.) was dissolved in buffer B before resuspending the pellet. A final centrifugation at 800g for 20 min was needed to resuspend the platelets in buffer A also stored at 37°C. 0.3 U/ml Apyrase and 5 mM glucose were added to buffer B prior to suspension. The volume of buffer C was calculated to obtain a final platelet count of 400 000/µl.

4.3.5.2. Method 2

In addition, a modified version of platelet preparation of method 1 was implemented. The modified version followed the above platelet preparation closely up to the point of incubation of PRP. In addition to aspirin and apyrase PRP was incubated with PGE_1 (2μM) in order raise cAMP levels via inhibition of G_i. In any of the subsequent steps apyrase was replaced by Creatinphosphate (CP 1mM)/ Creatinphosphate Kinase (CPK 10U/ml), catalyzing the following reaction: Creatinphosphate + ADP ←→ Glucose-6-phosphate + ATP.

The platelet pellet was subsequently resuspended in buffer C, which equals buffer B excepts with a higher pH.

4.3.5.3. Method 3

In this method PRP was acidified to a pH of 6.4 with acid-citrate-dextrose and centrifuged at 800g for 20 min. The platelet pellet was resuspended in buffer C.

4.3.5.4. Method 4

Preparation of washed platelets for measuring aggregation and serotonin secretion. Platelets were prepared as described by Gachet et al (Cazenave JP, 1993). Blood was collected into a 50ml propylene syringe containing 1 volume of anticoagulant ACD (see buffers) for 6 volumes of blood (final pH 6.5 and citrate concentration 22 mM). Blood is centrifuged for 15 minutes at 1000 rpm (175 x g) to obtain PRP. After removing this portion and incubating for 15-30 minutes at 37°C, PRP is centrifuged at 3000 rpm (1570 x g) for a period of time depending on the plasma volume:

Plasma volume (ml)	15	20	25	30	35	40
Centrifugation time (min)	10	12	13	14	15	16

The supernatant, platelet-poor-plasma, is carefully and completely removed to avoid the generation of thrombin during subsequent washing steps. Platelets are then resuspended in the first wash solution, prewarmed to 37°C, with the addition of 1 μl/ml prostacyclin, PGI_2 1 mM. A wash volume of 10 ml is normally required for the platelet pellet derived from 50-100 ml of blood. The platelet suspension is transferred to a closed test tube and incubated at 37°C for 10 minutes. Then 1 μl/ml of PGI_2 is added followed by centrifugation for 8 minutes at 2600 rpm. The platelet pellet is then resuspended in the final suspension medium (20 ml), Tyrode buffer containing 2 mM $CaCl_2$, and 0.35 % fatty acid free human albumin at a density of $3x10^5$ platelets/μl. 0.02 U/ml of the ADP scavenger apyrase were added. Platelets were kept at 37°C throughout all experiments.

When required, cytoplasmatic labeling of platelets may be performed by incubation in the first wash slolution with [^{3}H]serotonin (0.2 μCi/ml) according to (Gachet et al., 1995; Ohlmann et al., 2000).

4.3.6. Measuring shape change and aggregation in washed platelets and platelet- rich- plasma (PRP)

All of the experiments measuring shape change and aggregation in washed platelets and PRP were performed in a LABOR® aggregometer (Fa. Fresenius, Bad Homburg, Germany) at 37°C under stirring (1000 rpm) by the turbidimetric method of Born. 400µl aliquots of PRP or platelet suspension were placed into transluscent plastic cuvettes containing magnetic stirring-bars and incubated for 1 min before being placed on the magnetic stirrer base of the aggregometer. After 30 to 90 seconds of stirring the different agonists were added directly to the samples of platelet suspension and platelet shape change or aggregation were recorded in % decrease or increase in light transmission with a fine-needle writer.

Light transmission of unstimulated PRP represents 0 % aggregation the light transmission of PPP 100 % aggregation. Platelet shape change was recorded as a reduction in light transmission caused by negative deflection of light.

4.3.7. Aggregation in blood

Whole blood platelet aggregation was measured using the single-platelet counting technique described by Heptinstall et al with the following modifications(Fox SC, 1982). Aliquots (400µl) of anticoagulated blood samples were placed into aggregometer cuvettes and incubated 2 min at 37 ° C. The samples were stirred in the LABOR® aggregometer (section 4.2.6) at 300 rpm for 30 sec. The inhibitor/antagonist was added and incubated for 30 sec before adding LPA (1µM-20µM, from a 1 mM stock solution dissolved in albumin buffer (section 4.1.2). Other antagonists except aspirin were added in the same manner than for PRP experiments.

Aliquots (15µl) were removed at time 0 for baseline count and at set time intervalls therafter. The 15µl were placed in 30µl of fixing solution. After dilution to 1 ml the samples were centrifuged at a speed set by the Sysmex® Platelet Centrifuge PC-800 (Toa Medical Electronics, Japan) and counted using a platelet counter (Sysmex® Platelet Counter PL-100, Toa Medical Electronics, Japan). The percentage aggregation calculated as percentage loss of single platelets compared to baseline count. All platelet counts were done in duplicates.

The samples were removed at predetermined time intervals in order to obtain a time dependent course of aggregation. The samples needed to remain at least 30 min in fixing solution before platelet count was performed.

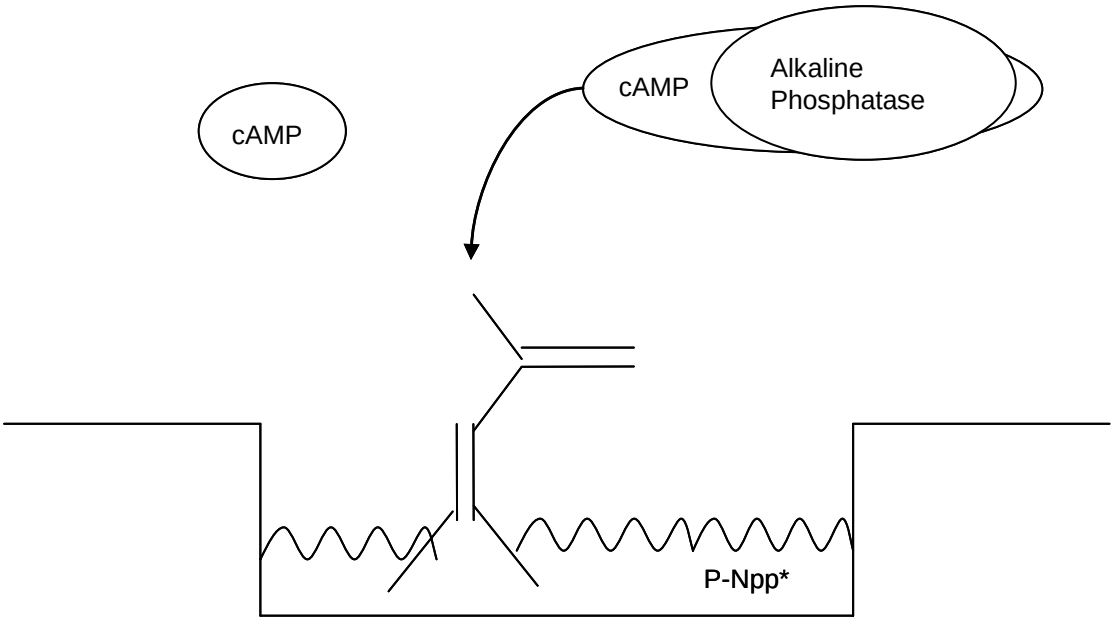
4.3.8. Measuring cAMP levels in platelets

In washed platelets according to method 1 cAMP levels were determined with an enzyme-immuno- assay (EIA) Kit (Assay Designs, Inc).

The platelet suspension, adjusted to 1 million cells/µl, was incubated with the stable prostacyclin analog, Iloprost (50 nM), for 5 minutes 37 °C in order to raise cAMP levels. Platelets were then stirred at 1000 rpm for 30 seconds until the platelet stimulant, LPA (0.1 – 40 µM), ADP (5 and 50 µM) or epinephrine (10 µM) was added. After 2 minutes of stirring the platelets were lysed in a 0.1M HCl (f.c.) solution. All samples were measured in duplicates. After 5 minutes all platelets had been been subject to lysis and the samples were centrifuged at 1000g for 5 minutes. The supernatant was placed into a 64-well microplate pre-coated with an antibody.

The principle of measuring cAMP can be seen in the figure below. cAMP in the supernatant from lysed platelets competes with cAMP conjugated to alkaline phosphatase. Alkaline phosphatase has the ability of splitting paranitrophenylphosphate producing a yellow color of the end product. The bound yellow color is inversely proportional to the concentration of cAMP.

The measured optical density is used to calculate the concentration of cAMP in pmol/ 10^6 platelets.



Alkaline Phosphatase acts as kinase to p-Npp (paranitrophenylphosphate)

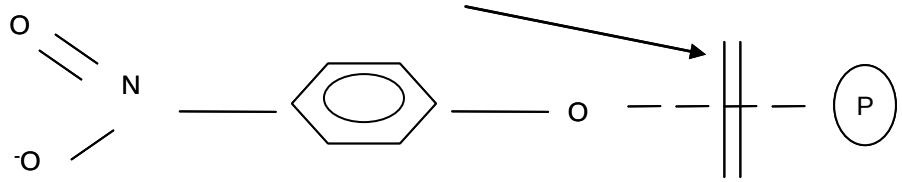


Fig 4.2

Principle of the enzyme immono assay (EIA) when measuring camp

cAMP in the supernatant from lysed platelets competes with cAMP conjugated to alkaline phosphatase. Alkaline phosphatase splits paranitrophenylphosphate producing a yellow color. The bound yellow color is inversely proportional to the concentration of cAMP.

4.3.9. Quantifying platelet aggregates and platelet-monocyte- aggregates (PMA) and P-selectin expression

4.3.9.1. Preparation of whole blood samples and measuring aggregation FACS analysis

400µl aliquots of hirudinized blood as described for blood. The stirrer was set to a speed of 1000 rpm and timed to 5 min. The agonist was added prior to stirring and the reaction stopped after 5 minutes by placing 100µl of blood sample in red blood cell (RBC) lysing solution. Centrifuging the lysed samples at 3000 rpm for 5 minutes allowed the removal of RBC debris. Washing the pellet with 1ml PBS, centrifuging at 3000rpm for 5 minutes, removed most of the lysing solution. This step was followed by incubation with antibodies. CD 14-PE binds to a monocyte specific glycoprotein marking all the monoctes. As a second antibody monoclonal CD 41a- FITC binding to GPIIb part of the fibrinogen receptor on platelets was used to stain platelets. The unspecific binding was determined with a FITC-conjugated IgG_1 isotype control antibody. All samples were incubated for 15 min at room temperature in the dark followed by resuspension in 600µl PBS. .

4.3.9.2. Measuring P-selectin expression in platelets isolated by method 4

For P-selectin expression, platelets (1.5×10^8) were incubated for 15 min with 0.5 mg anti-CD62-PE antibody, and 0.5 ml Tyrode's buffer containing 2 mM $CaCl_2$ and 0.35 % fatty acid free albumin was added. Mean fluorescence intensity was measured by FACS after collecting 10000 platelet events. The mean fluorescence intensity of isotype-matched IgG_1-PE was substracted

4.3.9.3. Principle of FACS analysis

The principle of Flow Cytometry is analyzing the light signals generated by particles as they flow through a liquid stream past a light beam. An argon laser beam emitting light at 488 nm constitutes the light source. The argon laser is focused to an elipse of 20µm by 60µm in size. The smaller height of 20 µm avoids the simultaneous analysis of two cells being in the beam at the same time. Two cells closer than 20µm together would be counted as one event, thus there is an ideal cell density of under 1 million cells/µm.

While cells are in the laser beam illumination signals and fluorochrome signals are collected simultaneously. The illumination signals are collected by the first lens positioned in the forward direction of the laser beam and the second lens positioned at a right angle to the laser beam. The light collected head on is not the laser light directly, but it is light that has been slightly refracted by the cell and strikes the forward positioned lens indicating the size of the cell. The laser light striking the forward positioned lens directly is being blocked by a centrally located obscuration bar. Light hitting the forward scatter lens is focused onto a photodiode where it is converted to an electrical current. In a similar manner the sideward positioned lens collects light that has been scattered to wide angles from the original direction of the beam. Irregularities in the cell cause this wide angle scatter indicating the granularity of the cell.

The type of cell was identified using fluorescently tagged antibodies, CD 41a and CD14, targeting surface molecules specific for platelets and monocytes. The fluorochromes conjugated onto the antibodies are transferred to a higher energy state and emit their defined fluorescent signals when exciting by the laser beam.

These signals are collected by the same right-angle lens that collects side scatter light. The light is subsequently partitioned according to its color, in this case, FITC - green and PE - red. Afterwards these light signals are converted into a data file via electrical current produced with the help of Photodiode detectors and Photomultipliers. The conversion from electrical current to the computer was done on a Mac Intosh Computer using Cellquest as a software program.

4.3.9.4. Statistical analysis

Statistical Analysis was completed with Excel 2000 (Microsoft). The results represent statistical mean and mean standard of deviation from which significance was calculated via student's t-test for paired values. $P<0.05$ was considered to be significant.

5. Results

5.1. Response of washed platelets to LPA: Comparison of different platelet isolation procedures

The purpose of testing washed platelets, separated from their physiological medium, was to demonstrate the direct effect of LPA on platelets excluding any cofactors from plasma or other cells. Moreover, washed platelets allowed us to define other plasma factors interfering with the action of LPA on platelets.

Since there are different biochemical structures of LPA, varying in biological activity, the term LPA refers to 1- acyl LPA (16:0) throughout the text unless mentioned otherwise.

5.1.1. LPA only induced shape change of platelets isolated according to method 1 and method 2

LPA induced the initial platelet activation, shape change, in washed platelets prepared by method 1 and method 2. Platelets, however, did not aggregate in response to low (5μM) or high (120 μM) LPA concentrations. The LPA concentrations tested ranged from 5μM to 120μM. Even the addition of fibrinogen (0.1-1 mg/ml), plasma (2-20 %), Ca^{2+} (1mM), alone and in combination of both or all three, did not show aggregation, only shape change as the sign for initial platelet activation (Fig5.1).

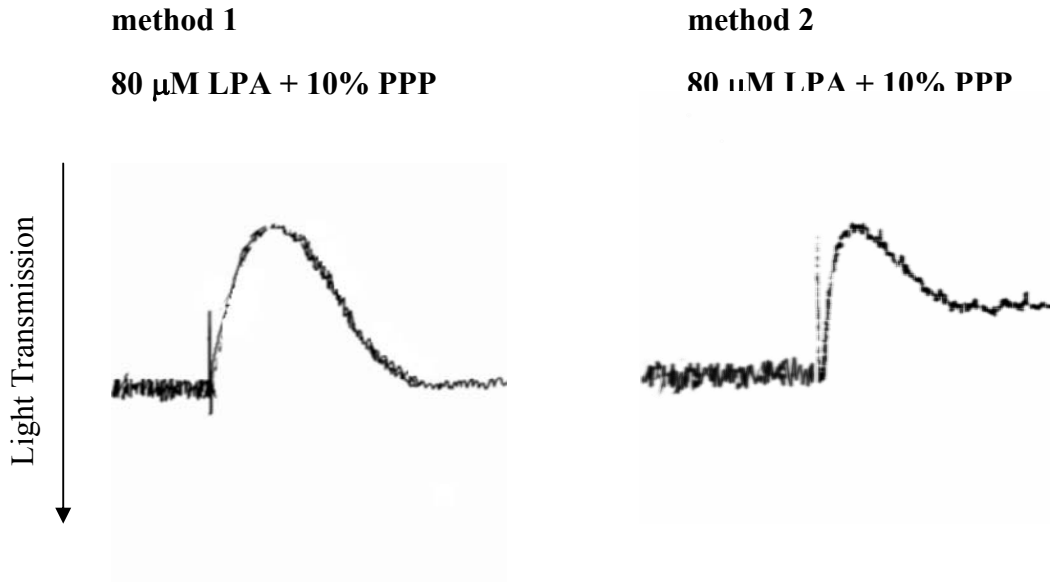


Fig 5.1

LPA induced shape change in platelets washed by method 1 and 2

Platelets isolated by method 1 and method 2 were either incubated for 2 minutes at 37°C and stirred for 1 minute at 1000 rpm at 37°C before 80 μM of LPA + 10 % PPP was added. LPA induced shape change but no aggregation in these platelets. Tracings recorded by aggregometry represent experiments of washed platelets from different donors (n=20).

In order to explore whether LPA might amplify the aggregation response induced by ADP (5 μM), ADP was added together with LPA in the presence of 5 % platelet poor plasma (PPP). As a result the ADP-induced aggregation was amplified (Fig 5.2). ADP in the absence of PPP did not induce platelet aggregation (data not shown).

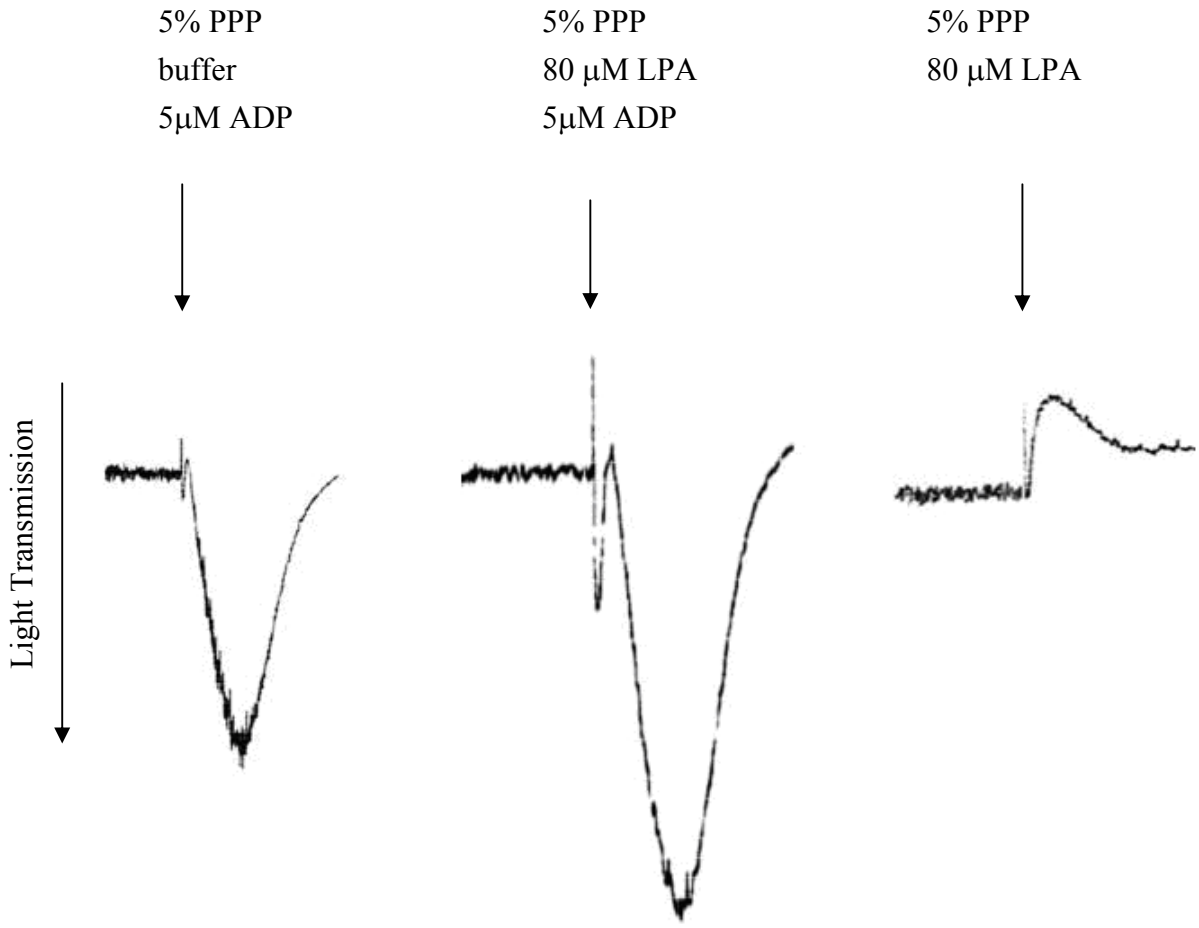


Fig 5.2

ADP induced aggregation in platelets isolated by method 1 is amplified by LPA

Washed platelets (method 1) were incubated for 2 minutes and stirred for 1 minute at 1000 rpm at 37°C in the presence of platelet poor plasma (PPP; 5%). ADP (5μM) was added alone or together with LPA (80 μM), simultaneously. Aggregation tracings are representative of 6 experiments.

5.1.2. LPA induced aggregation of platelets isolated according to method 3 and method 4

Platelets prepared according to method 3 showed irreversible platelet aggregation, which was observed after low concentrations of LPA (1μM) (Fig 5.3).

However, when looking at these aggregation tracings closely, no rapid oscillations at baseline were apparent and no shape change in response to LPA was observed. In addition the aggregation response to LPA declined 60 minutes after venipuncture and disappeared in most of the experiments with increasing time.

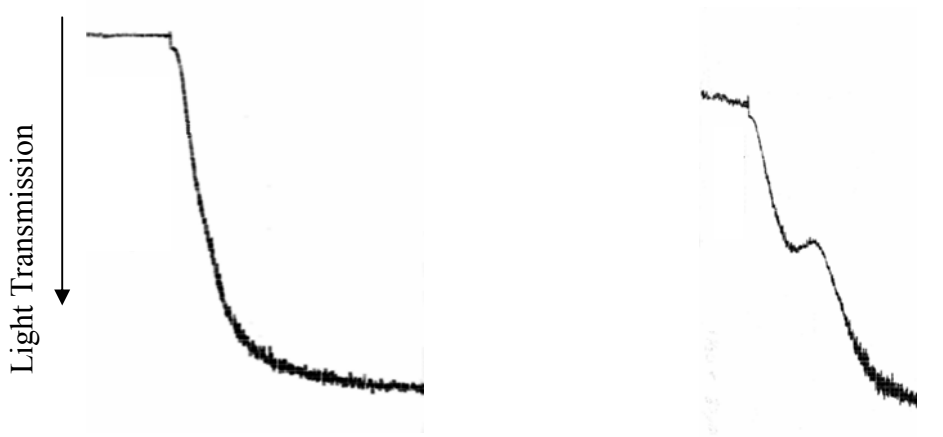


Fig 5.3

LPA induced aggregation in platelets isolated by method 3

Platelets were stirred at 1000 rpm for 1 minute at 37°C before the addition of LPA (1 μM). No plasma was added. Platelet aggregation was recorded by aggregometry within 30-60 minutes after venipuncture. Tracings are representative of 40 experiments.

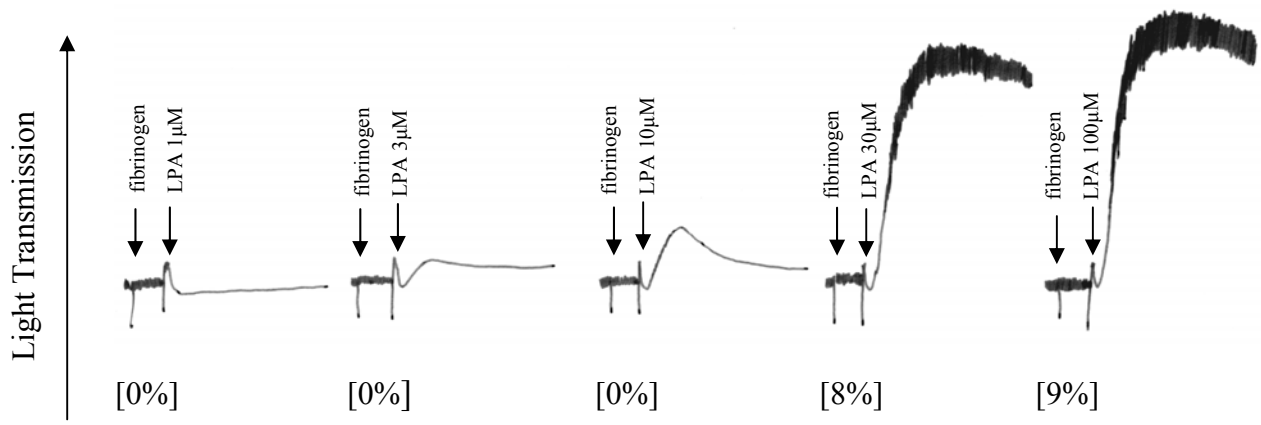


Fig 5.4

LPA induced shape change followed by aggregation in platelets isolated by method 4

Washed platelets (suspended in Tyrode with fatty acid free albumin 0.35% (w/v) $3x10^8$ platelets /ml) were stirred at 1000 rpm in the presence of fibrinogen and buffer at 37°C before 1-100μM LPA were added. Secretion of tritiated serotonin is indicated in brackets.

Platelets prepared according to method 4 showed shape change followed by aggregation in response to LPA. The platelet response did not diminish when the platelet suspension was kept at 37°C for a time period of 3 hours. In contrast to the platelet isolation procedure with method 3, platelets were washed in the presence of prostacyclin, heparin and resuspended in buffer containing apyrase to avoid platelet pre-activation and desensitization of the ADP receptor $P2Y_1$ (Baurand et al., 2000; Cazenave et al.).

Using this platelet preparation, a concentration of 1µM LPA induced shape change. 3µM LPA induced shape change and reversible aggregation. Higher concentrations (10, 30, 100µM) of LPA elicited maximal irreversible aggregation. Serotonin secretion increased proportional to the LPA concentration added (Fig 5.4).

Figure 5 shows the dose-response curve of aggregation induced by LPA in platelets prepared by method 4. Aggregation starts with only 3µM LPA and maximal aggregation is reached with 30µM (Fig 5.5).

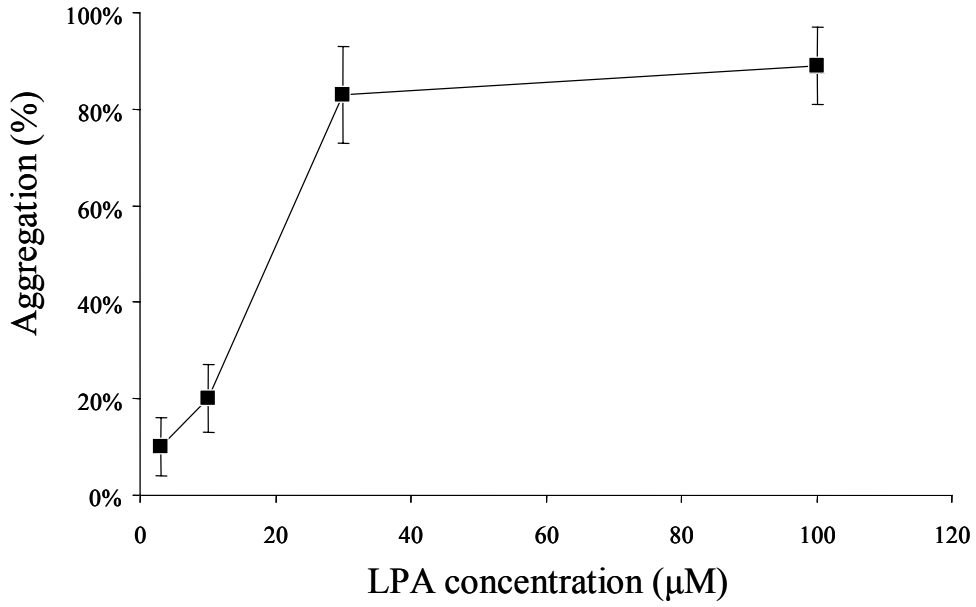


Fig 5.5

Dose –response of 1-acyl-LPA (16:0) induced aggregation in platelets isolated by Method 4

Washed platelets (suspended in Tyrode with fatty acid free albumin 0.35% (w/v) $3x10^8$ platelets /ml) were stirred at 1000 rpm in the presence of fibrinogen and buffer at 37°C before 1-100 µM LPA were added (mean ± SD, n=3).

5.2. LPA-induced shape change in washed platelets, PRP, and whole blood

5.2.1. Shape change induced by LPA in washed platelets and in the presence of albumin or plasma

It was previously observed that a 500-fold lower concentration of LPA was needed to induce platelet shape change in washed platelets isolated by method 1 (EC_{50} of 11nM) than in PRP (EC_{50} of 4µM) (Rother et al., 2003). Thus, we suspected inhibiting factors present in PRP to be responsible for this difference in activation. In searching for these factors we thought of two possible substances known to interact with LPA: albumin and gelsolin (Goetzl et al., 2000; Thumser et al., 1994); see section 1.3.4).

Here we compared shape change in washed platelets adding LPA (50 nM), together with albumin (1-65µM) or platelet poor plasma (PPP 1-10%). Shape change was measured under stirring conditions in an aggregometer. PPP was used for comparative analysis to determine whether albumin was the only inhibiting factor of plasma. The plasma volume added was calculated to contain the same amount of albumin as human fatty acid- free albumin that was added separately. The amount of albumin in PPP was calculated based on the average amount of albumin contained in plasma (45g/l; 0.66mM).

The results show that the LPA- induced shape change was inhibited by increasing concentrations of PPP or albumin. However, PPP inhibited the LPA-induced shape change to a greater degree than albumin alone. 60µM of albumin were required, when added alone (IC_{50} 13 µM), whereas 45µM albumin in plasma were required to inhibit shape change completely (IC_{50} 5 µM) (Fig 5.6).

Therefore, albumin seems to be an important, but not the only factor present in plasma inhibiting the LPA-induced platelet shape change.

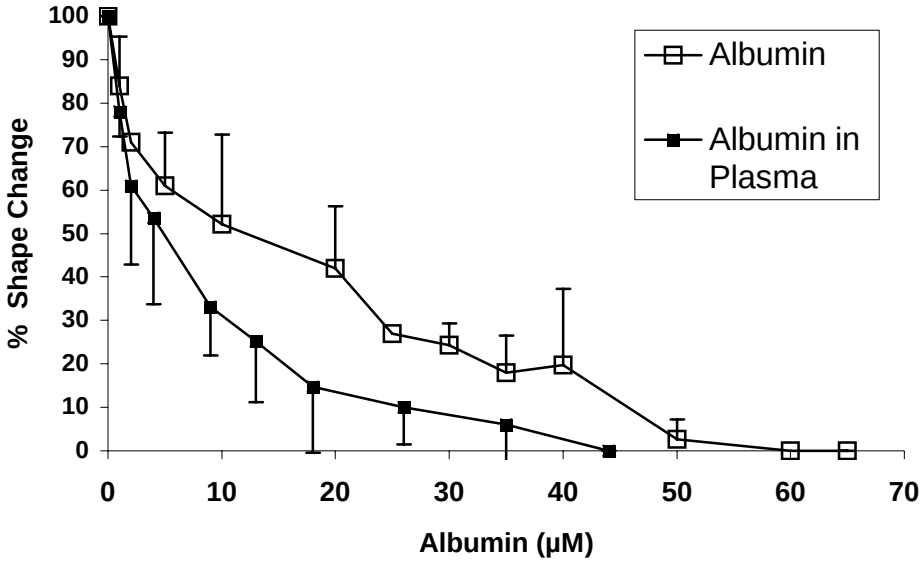


Fig 5.6

Dose- response curves comparing the inhibiting effect of albumin and albumin contained in plasma on LPA- induced platelet shape change in washed platelets

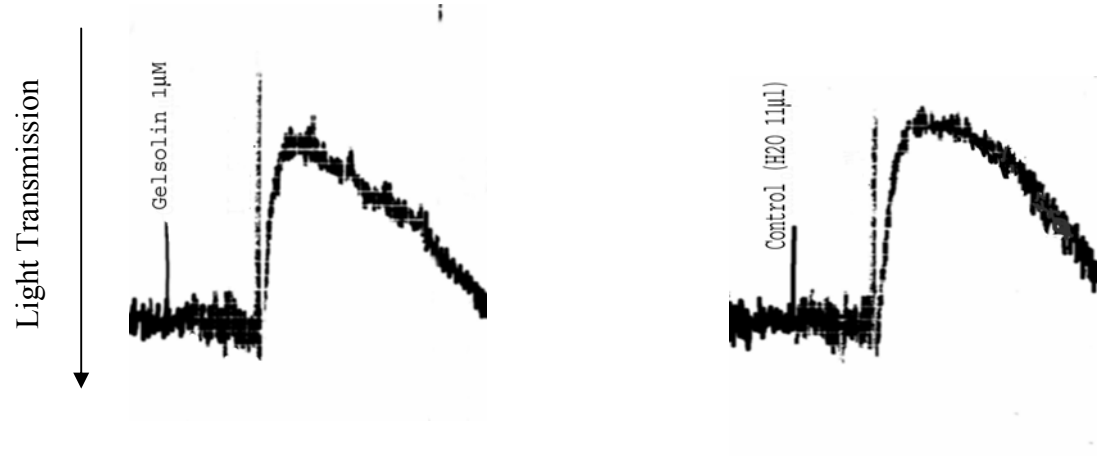
Washed platelets isolated according to method 1 were stirred for 1 minute at 1000 rpm at 37 °C before simultaneous addition of albumin + 50nM LPA or PPP (platelet poor plasma) + 50nM LPA (mean ± SD, n=22). The amount of albumin in plasma was calculated assuming the average plasma albumin concentration of 45g/l or 0.66mM.

5.2.2. **Shape change induced by LPA in washed platelets in the presence of gelsolin**

Since albumin does not seem to be the only factor responsible for the inhibition of shape change seen by plasma, we tried to find other plasma contents contributing to the effect. Gelsolin has been shown to strongly interact with LPA and has also been found in plasma (Goetzl et al., 2000); (see section 1.3.4).

Based on a previous study plasma gelsolin added in low concentrations, < 10% plasma level (< 0.12 μM), is expected to enhance LPA activity and added in high concentrations, > 20% of the plasma level > 0.59 μM), is expected to suppress LPA activity (Goetzl et al., 2000). When adding these concentrations prior to and simultaneously with LPA to washed platelets (method 1), neither concentration showed any effect on LPA- induced shape change.

Another study showed evidence that intracellular gelsolin activity is Ca^{2+} dependent. This raised the possibility that gelsolin may be capable of binding LPA and delivering it to its cellular receptors only in the presence of extracellular Ca^{2+}. Thus we carried out experiments in the presence of Ca^{2+}, to exclude a failure in gelsolin activity due to lack of Ca^{2+}. However, platelet shape change remained unchanged in response to 50nM LPA+ Ca^{2+} (1 mM). Even very high concentrations of gelsolin up to 1μM did not affect shape change (Fig 5.7). These results indicate that plasma gelsolin had no effect on the interaction of LPA with platelets.



Gelsolin (1 μM) + LPA (50 nM)

H_2O (11 μl) + LPA (50 nM)

Fig 5.7

Shape change induced by LPA in washed platelets remains uninfluenced in the presence of gelsolin

Washed platelets prepared by method 1 were incubated for 2 minutes and stirred for 1 minute at 1000 rpm at 37°C in the presence of gelsolin (1μM) before 50nM LPA was added. Shape change tracings measured by aggregometry representative of 6 experiments.

5.2.3. Shape change induced by LPA of washed platelets, PRP and blood is mediated by Rho-kinase activation – but independent of ADP receptors $P2Y_1$ and $P2Y_{12}$

Many platelet stimuli induce shape change independent of additional platelet activators such as ADP and thromboxane A_2. To explore whether LPA- induced shape change is independent of released ADP, the ADP receptor antagonists MRS2179 (a $P2Y_1$ receptor inhibitor) and ARC 69931MX ($P2Y_{12}$ receptor inhibitor) were used. They showed no inhibitory effect on platelet shape change in washed platelets and PRP (results not shown).

Shape change induced by LPA showed a similar dose response curve, whether this response was induced in PRP or in blood (Fig 5.8a). This indicates the independence from red blood cells and suggesting the independence from ADP. Indeed, the $P2Y_1$ receptor antagonist, MRS2179, and the $P2Y_{12}$ receptor antagonist, AR-C69931MX or the combination of both antagonists did not inhibit LPA-induced shape change in whole blood (Fig 5.8b). The control experiments show that – as expected- MRS2179 but not AR-C69931MX blocked the ADP-induced shape change in PRP and whole blood (Fig 5.8b). Together these results indicate that LPA induces shape change independent of synergistic interaction with ADP.

LPA-induced shape change of washed platelets is mediated by Rho-kinase activation (Retzer and Essler, 2000). Consistent with this result it was observed, that LPA-induced platelet shape change in blood could be inhibited by the Rho-kinase inhibitor Y27632.

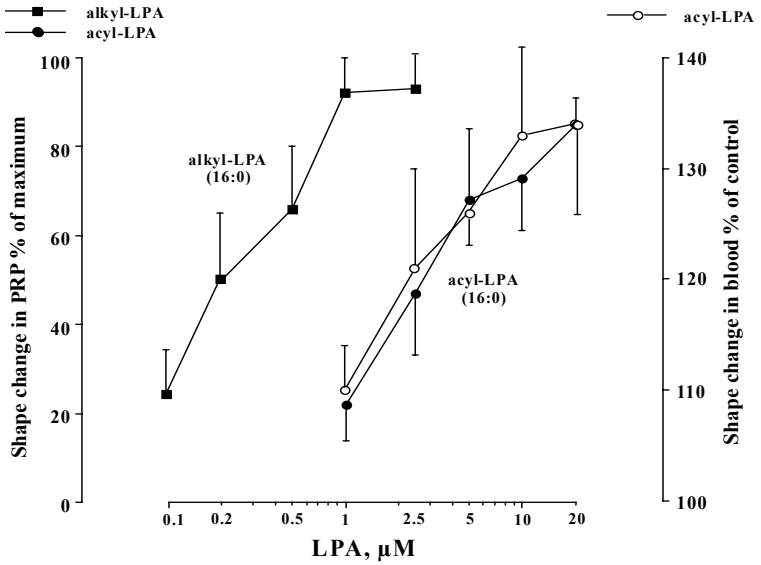
Also pre-treatment of platelets with aspirin had no effect on LPA-induced shape change, neither in washed platelets, PRP (results not shown), nor in blood (Fig 5.8b). Therefore LPA-induced shape change seems to be independent of thromboxane.

In contrast to the donor-dependent variation of LPA-induced aggregation (see section 5.3.1), LPA-induced shape change of PRP and blood was observed in all blood donors tested.

5.2.4. Comparison of 1-acyl-LPA (16:0) and 1-alkyl-LPA (16:0)- induced shape change in PRP and in whole blood

It has been shown recently that 80% acyl-LPA and 20% alkyl-LPA is present in atherosclerotic plaques (Rother et al., 2003). We determined the potency of the two different LPA species, 1-alkyl-LPA (16:0) and 1-acyl-LPA in inducing shape change and aggregation. The results identified 1-alkyl-LPA (16:0) to be almost 20- fold more potent than 1-acyl-LPA in inducing shape change of PRP (Fig 5.8a). 1-Alkyl-LPA (16:0) was also more potent than 1-acyl-LPA (16:0) in inducing shape change of washed platelets and in whole blood (data not shown and section 5.3.3).

5.8a



5.8b

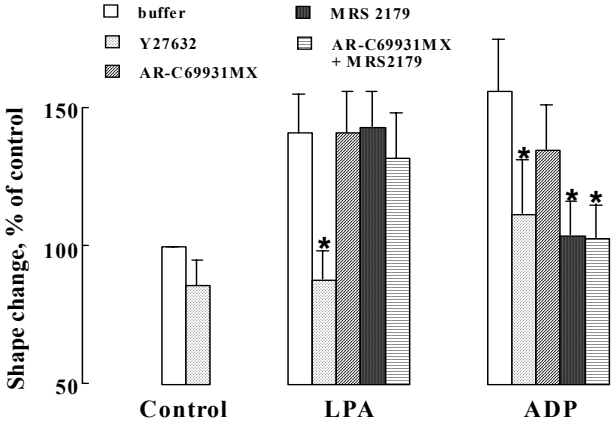


Fig 5.8

LPA induces platelet shape change directly in blood and in PRP. 1-alkyl-LPA is a more potent activator than 1-acyl-LPA in blood and in PRP.

a) Dose-response curves comparing the effect of 1-alkyl-LPA (16:0) and 1-acyl-LPA (16:0) in inducing shape change of PRP. Shape change in ACD-PRP was measured by aggregometry (left y-axis). Shape change induced by 1-acyl-LPA (16:0) in blood was measured by flow cytometry (right y-axis). Values are mean ± SD of 4-6 experiments. $P < 0.01$ for the shape change induced by all concentrations of LPA. b) Citrated blood was pre-incubated for 30 minutes with Rho kinase inhibitor Y27632 (20µM) or for 30 seconds with the $P2Y_{12}$ receptor antagonist AR-C69931 (1µM), $P2Y_1$ receptor antagonist MRS2179 (100µM), before addition of buffer (control), 20µM 1- acyl LPA (16:0), or 5 µM ADP for 5 minutes. Shape change was measured by flow cytometry. Values are mean ± SD of 4 to 5 experiments. * $P < 0.01$ as compared to buffer plus stimulus.

5.3. LPA -induced aggregation in PRP and whole blood

5.3.1. LPA induced platelet aggregation and ATP secretion in PRP: donor dependent variations

It was observed that the LPA concentrations needed to induce aggregation in PRP varied from donor to donor. Concentrations of 10 µM LPA were sufficient to induce aggregation in some donors but concentrations of 80 µM LPA were necessary to induce aggregation in others. The same donor platelets consistently showed the same strong or weak aggregation response to LPA.

Platelet concentrations were not adjusted. However individual platelet count differences did not correlate with sensitivity to LPA (data not shown).

Three distinct groups of LPA-induced aggregation response could be distinguished (Fig 5.9). Representative aggregation tracings above each dose response curve show a reversible aggregation in group 1 and 2 and an irreversible aggregation in group 3. The irreversible aggregation is known to be associated with granule release and subsequent amplification of aggregation.

In order to find out whether reversible aggregation correlates with granule release, we measured LPA- induced aggregation and simultaneous secretion of ATP. As shown in Fig 5.10, LPA induced reversible aggregation, which was paralleled by rapid ATP secretion.

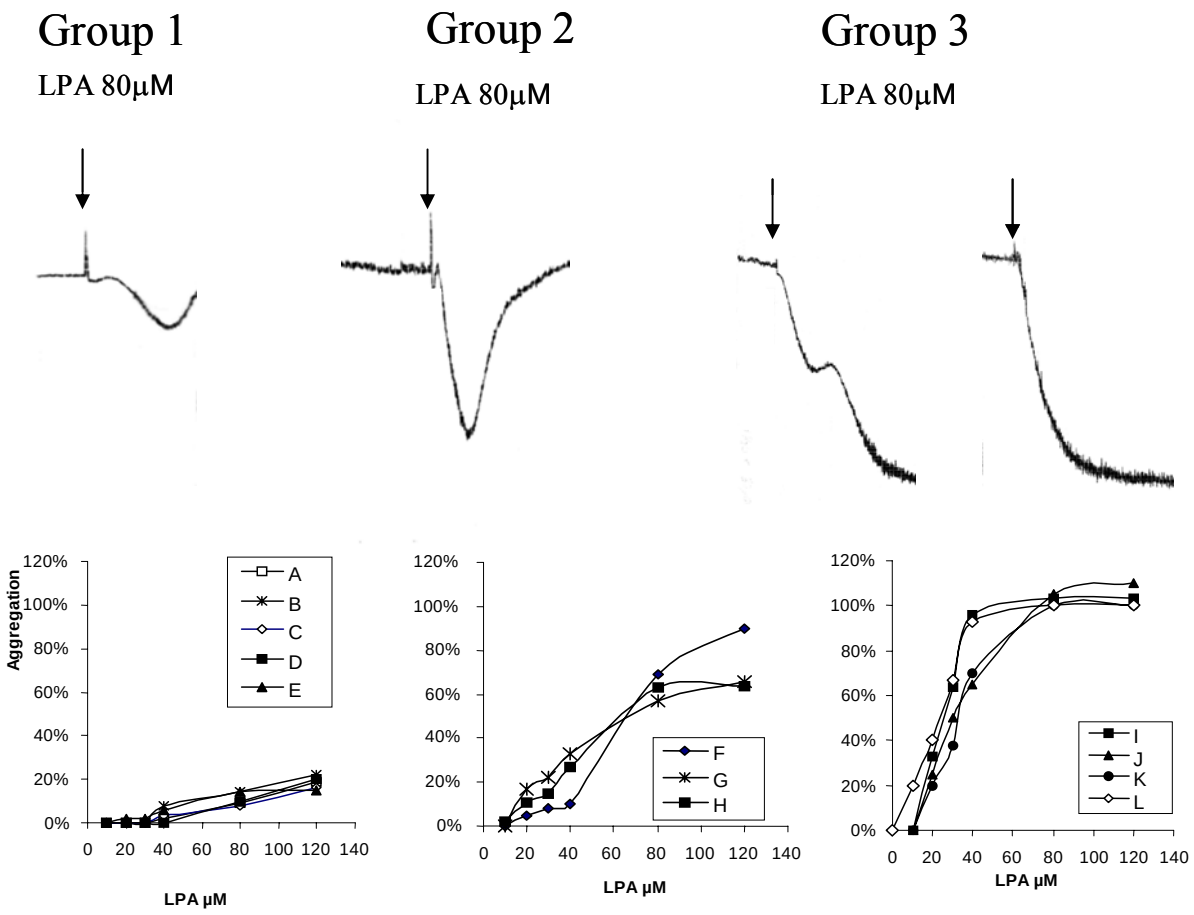


Fig 5.9

Above: Representative tracings from LPA-induced aggregation in PRP from different blood donors

PRP was stirred for 1 minute at 1000 rpm at 37°C before 80 µM LPA was added. Aggregation was recorded by aggregometry.

Below: Dose response curves of LPA-induced aggregation of PRP from different blood donors

LPA- induced maximal aggregation was recorded by aggregometry as % of maximum aggregation induced by 5µM ADP (100%). Each line represents the aggregation of a different donor (A-L).

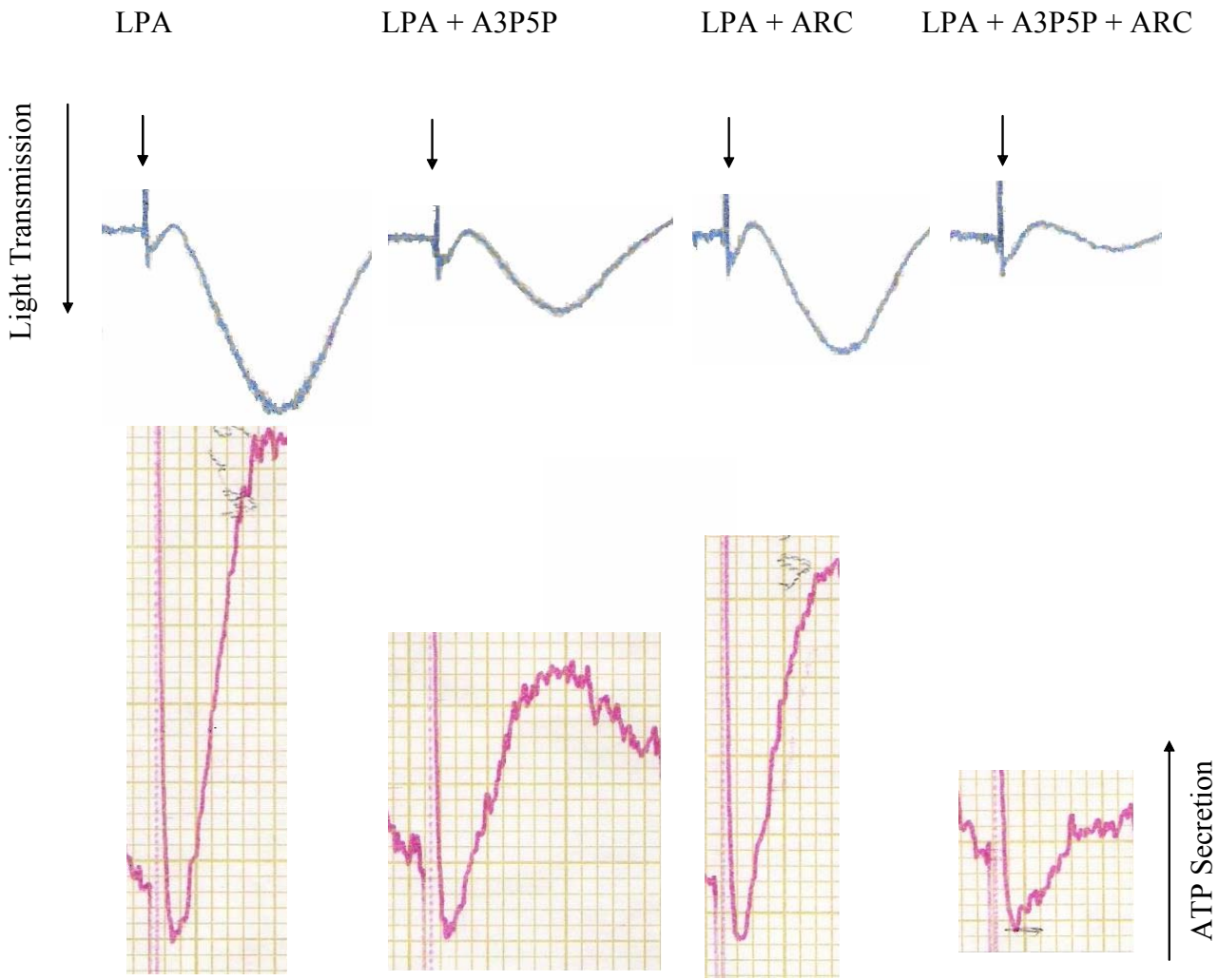


Fig 5.10

Representative LPA-induced aggregation tracings and secretion of ATP in PRP. Effect of ADP receptor-antagonists

PRP was stirred for 1 minute at 1000 rpm at 37°C before LPA (80µM) was added. The $P2Y_1$ and $P2Y_{12}$ ADP-receptor antagonists, A3P5P (200µM) and AR-C69931MX (1µM) (ARC) were added 15 seconds prior to LPA addition. Aggregation (% light transmission) and simultaneous ATP secretion were recorded in a Lumi-aggregometer.

5.3.2. Low concentrations of LPA induced aggregation in whole blood

When stirring whole blood at different speed (1000 rpm, 500 rpm, and 300 rpm) for 1 minute we observed different spontaneous aggregation responses, varying from 46± 6%, 29± 3%, to 17± 5 % (mean ± SD, n=3), respectively, after 1 minute of stirring (Fig 5.11). Therefore, we minimized spontaneous aggregation due to stirring by choosing the lowest stirring speed (300rpm) in all experiments in whole blood.

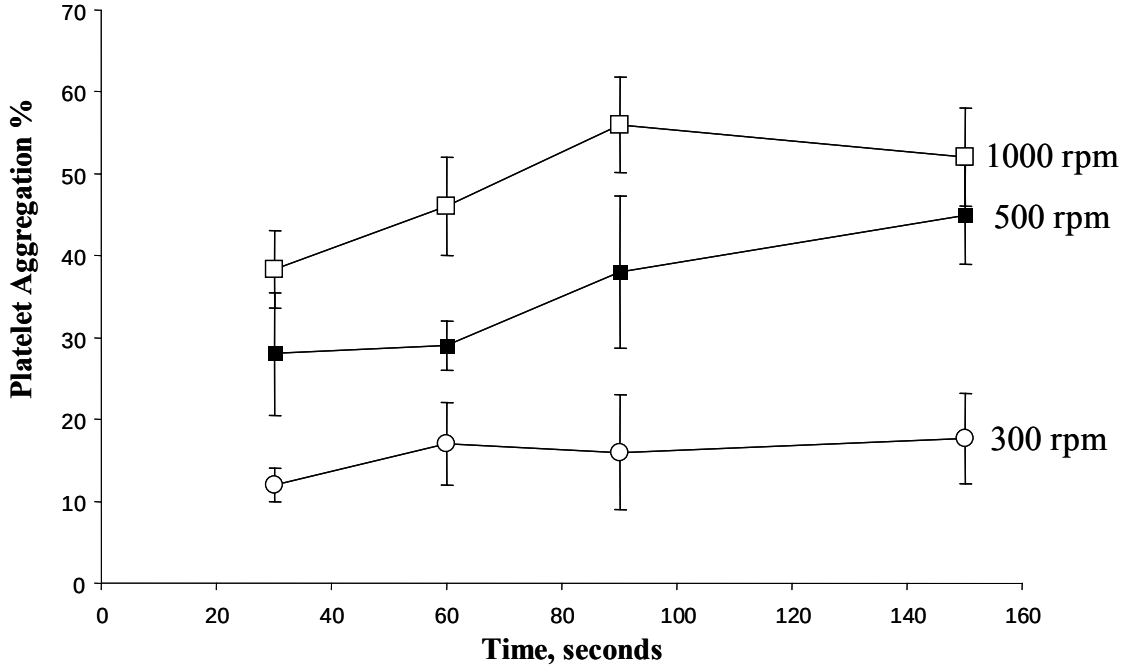


Fig 5.11

Time course of spontaneous aggregation at different stirring speed (1000 rpm, 500 rpm, and 300 rpm)

Whole blood anticoagulated with hirudin was stirred in the presence of buffer at different speeds. Platelet aggregation was measured at 30, 60, 90, and 150 seconds of stirring. Percent aggregation is defined by the loss of single platelets (0 single platelets =100% aggregation). Values are mean ± SD, n=3.

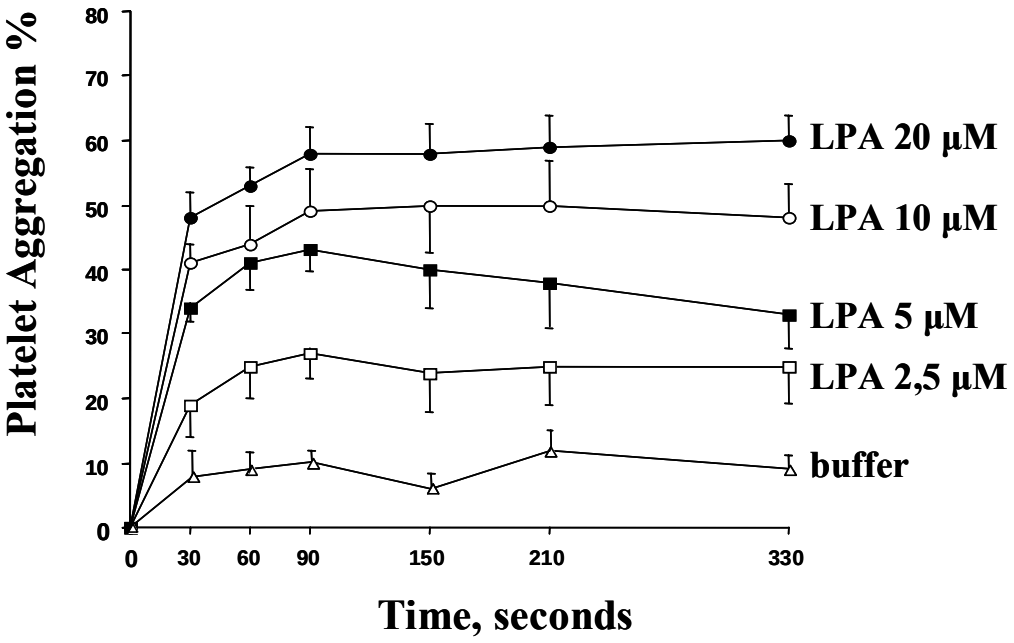


Fig 5.12

Time course of aggregation induced by different concentrations of 1-acyl-LPA (16:0) in LPA-sensitive donors

Whole blood anticoagulated with hirudin was stirred for 1 minute at 300 rpm at 37°C before different LPA concentrations were added. Values are mean ± SD, n=7.

Aggregation in whole blood could be induced by LPA concentrations about ten times lower (EC_{50} range 2-5 μM LPA) than in PRP (EC_{50} range, 40-65 μM LPA). LPA elicited platelet aggregation in whole blood in a time- and dose- dependent manner (Fig 5.12). Platelet aggregation started within 30 seconds and was irreversible. A concentration of 2.5 μM LPA induced already a significant aggregation response in whole blood. Maximum aggregation was reached with 20 μM LPA.

5.3.3. 1-acyl-LPA (16:0) versus 1-alkyl-LPA (16:0) induced aggregation in whole blood

When comparing the effect of 1- acyl-LPA (16:0) and 1-alkyl-LPA (16:0) on platelet aggregation in whole anticoagulated blood, the alkyl-LPA species was almost 20-fold more potent than the acyl-LPA species (Fig 5.13).

The effective concentration causing 50% of maximum aggregation (EC_{50}) for alkyl-LPA (16:0) was 0.3 μM versus 5μM for acyl-LPA (16:0). The lowest concentration that induced a significant platelet aggregation was 0.2 μM for alkyl-LPA (16:0) versus 2.5 μM for acyl-LPA (16:0) ($P< 0.01$ and 0.001).

1-acyl-LPA (16:0) and 1-acyl-LPA (18:1) demonstrated the same potency of platelet aggregation (results not shown).

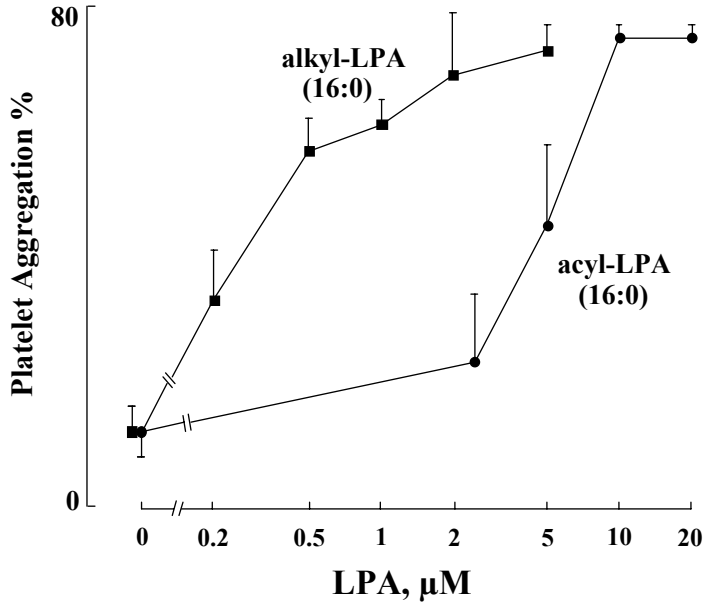


Fig 5.13

Dose response curves comparing the effect of 1-alkyl-LPA (16:0) and 1-acyl-LPA (16:0) in inducing platelet aggregation in whole blood

Whole blood was stirred for 1 minute at 300 rpm at 37°C before either 1-acyl-LPA (16:0) or 1-alkyl-LPA (16:0) was added. Platelet aggregation was measured 5 minutes after addition of LPA. Values represent mean ±SD, n=4.

5.3.4. LPA- induced platelet aggregation in whole blood is independent of the type of anticoagulant

Previous studies have shown that the type of anticoagulant can influence stimulus-induced platelet aggregation in whole blood. Therefore, we compared two types of anticoagulants, hirudin and citrate, and measured LPA-induced platelet aggregation in whole blood. In our experiments the type of anticoagulant used had no influence on LPA-induced aggregation. The time-course and concentration response curves of LPA-induced aggregation were similar in hirudin-and citrate-anticoagulated blood, indicating that the extracellular Ca^{2+} concentration was not critical for the aggregation response induced by LPA (Fig 5.14).

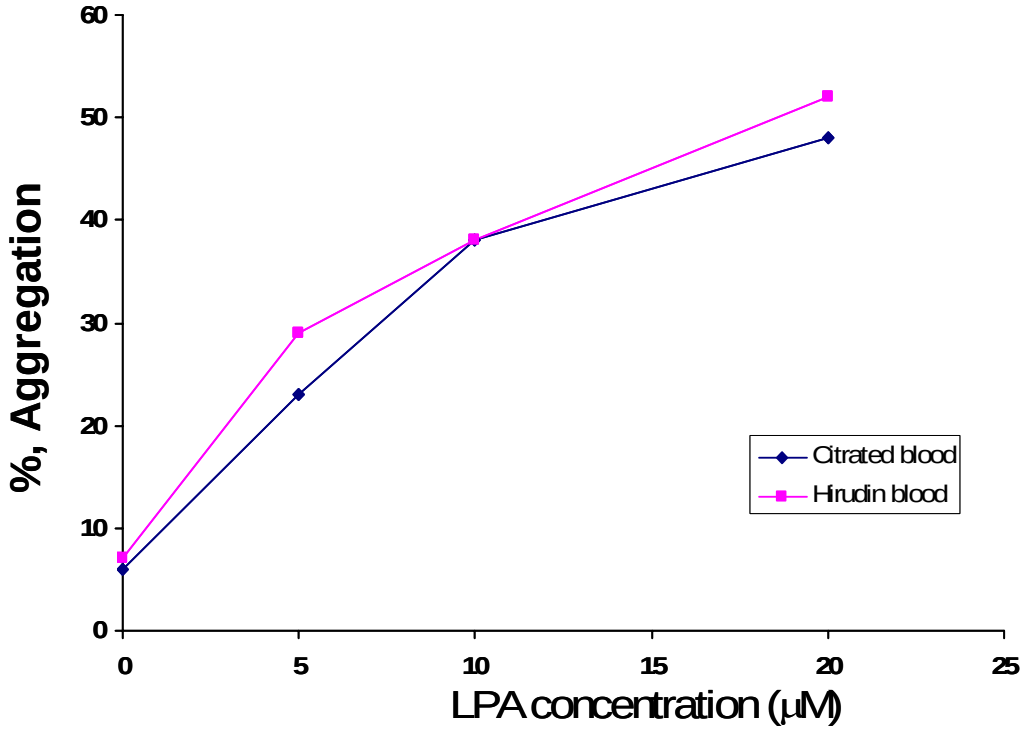


Fig 5.14

Concentration response curve of LPA induced aggregation in blood anticoagulated with either citrate or hirudin

Blood was anticoagulated with trisodium citrate (3.8% wt/v) or recombinant hirudin (200U/ml). LPA was added in different concentrations after 1 minute of stirring (300rpm) at 37°C. Aggregation was measured 90 seconds after adding LPA (mean ± SD, n=5).

Similar than in PRP donor- dependent differences in aggregation could be observed in whole blood. In some donors platelets aggregated after addition of 2,5 µM of LPA. Also the maximum aggregation after adding 20 µM LPA was double in group A compared to group B (Fig 5.15).

The distribution of the LPA- induced aggregation in blood is shown in Fig 5.16. Of 56 donors tested, 21 donors showed a platelet aggregation response to 20 µM LPA higher than 50 % with a mean ± SD of 70% ± 14 %. Others (25 donors) showed aggregation lower than 50 % with a mean ± SD of 26 ± 14 %. Ten donors showed no significant aggregation as compared to control.

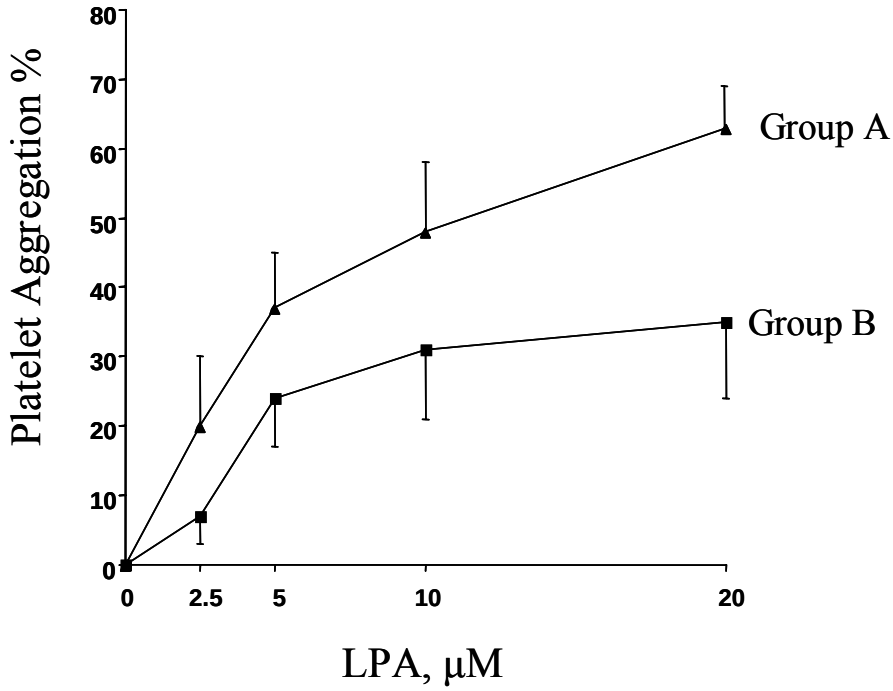


Fig 5.15

Donor dependent differences in aggregation induced by different concentrations of LPA in LPA-sensitive donors

Whole blood anticoagulated with hirudin was stirred for 1 minute at 300 rpm at 37°C before different LPA concentrations were added. Group A represents donors that showed a maximum aggregation > 50%, group B lower than 50% after adding 20μM LPA. Values are mean ± SD, n=4 for group A, n=5 for group B.

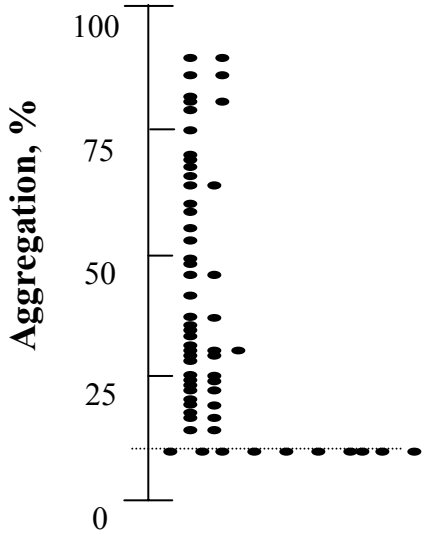


Fig 5.16

Distribution of LPA-sensitive donors in response to 20 μM of LPA

Whole blood anticoagulated with hirudin was stirred for 1 minute at 300 rpm at 37°C before LPA was added. Aggregation was measured 90 seconds after the addition of LPA. Each dot represents % aggregation of one donor (n=56). Dotted line indicates the average spontaneous aggregation after stirring 90 seconds.

In order to measure the intra- individual variation of platelet aggregation, four donors were tested repeatedly (3 to 5 times) on different days and it was found that the aggregation response was fairly consistent within the same blood donor (Table 5.1).

	Donor A				
Date	23.04.02	27.03.02	05.08.02	06.08.02	04.09.02
Control	33	4	5	47	17
LPA 20 µM	84	85	69	80	76
ADP 5 µM	84	78	78	82	85
	Donor B				
Date	19.03.02	27.05.02	29.08.02	12.08.03	
Control	2	14	28	12	
LPA 20 µM	23	60	48	42	
ADP 5 µM	89	84	80	78	
	Donor C				
Date	25.02.02	04.03.02	05.03.02	07.03.02	
Control	14	2	9	5	
LPA 20 µM	86	89	79	82	
ADP 5 µM	86	90	83	6	
	Donor D				
Date	18.09.03	02.04.02	25.04.02		
Control	3	16	16		
LPA 20 µM	46	27	35		
ADP 5 µM	75	59	84		

Table 5.1

Aggregation induced by LPA in LPA-sensitive donors

Whole blood anticoagulated with hirudin was stirred for 1 minute at 300 rpm at 37°C before LPA (20µM), ADP (5µM), or buffer was added. Different Donors (A- D) were tested on different dates. Values are shown in % aggregation.

Spontaneous aggregation did not correlate with LPA-induced aggregation response (Fig 5.17), nor did the spontaneous aggregation correlate with ADP-induced aggregation (data not shown).

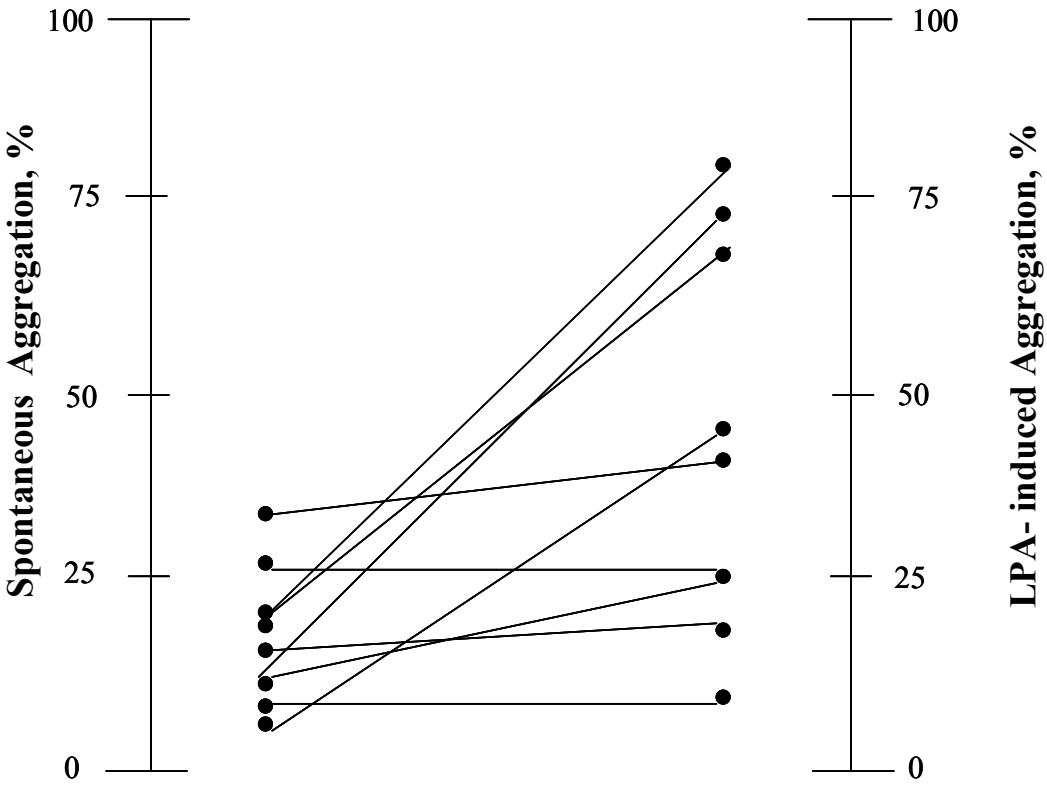


Fig 5.17

Spontaneous aggregation vs. LPA- induced aggregation

Whole blood anticoagulated with hirudin was stirred for 60 seconds at 300 rpm at 37°C in the presence of buffer (spontaneous aggregation) or LPA (10μM). Each dot represents % aggregation of individual donors (n=9). Shown here is the relationship between spontaneous and LPA-induced aggregation.

Together these results show that the aggregation response to LPA in blood was dependent mainly on the blood donor.

5.4. Mechanisms of LPA-induced platelet aggregation

5.4.1. LPA-induced platelet aggregation in washed platelets and in PRP is partly mediated by secreted ADP

5.4.1.1. Involvement of platelet ADP receptors

In order to determine which mediators play a role in LPA-induced aggregation in washed platelets (prepared by method 3), the following antagonists were tested for their effectiveness in inhibiting aggregation.

When the $P2Y_{12}$ receptor antagonist, ARC-69931-MX, was added 1 minute prior to adding LPA (1 μM), the aggregation response to LPA was completely abolished. A similar inhibitory effect was seen after addition of the ADP-scavenging enzyme, apyrase. The $P2Y_1$ receptor antagonist,

A3P5P, barely inhibited LPA-induced aggregation demonstrating a minor role. Results are shown in representative aggregation tracings (Fig5.18).

In a control- experiment for ADP receptor specificity, platelet aggregation induced by ADP (5μM) was also inhibited by A3P5P (10 μM), AR-C69931MX (50 nM), and

apyrase (5 U/ml).

Platelets prepared according to method 3 appeared to be pre-activated (see section 5.1.2); therefore, method 4 of platelet preparation was used. With this method AR-C69931MX ($P2Y_{12}$ antagonist) as well as MRS2179 ($P2Y_1$ antagonist) drastically reduced the LPA-induced irreversible platelet aggregation and converted it into reversible aggregation (Fig 5.19). A combination of both ADP receptor antagonists was only slightly more effective in inhibiting LPA-induced aggregation. This finding shows that ADP- mediated activation of the $P2Y_1$ and $P2Y_{12}$ receptor is required for LPA-induced secondary irreversible aggregation.

Table 3a summarizes the effects of MRS2179, AR-C69931MX, and aspirin on LPA-induced aggregation and serotonin secretion in platelets prepared by method 4. The reduction of LPA-induced aggregation of washed platelets was paralleled by the inhibition of serotonin secretion to less than 2% by AR-C69931MX, MRS2179, or a combination of both ADP receptor antagonists (Table 5.2a). Aspirin caused a reduction in serotonin secretion, but had no effect on platelet aggregation.

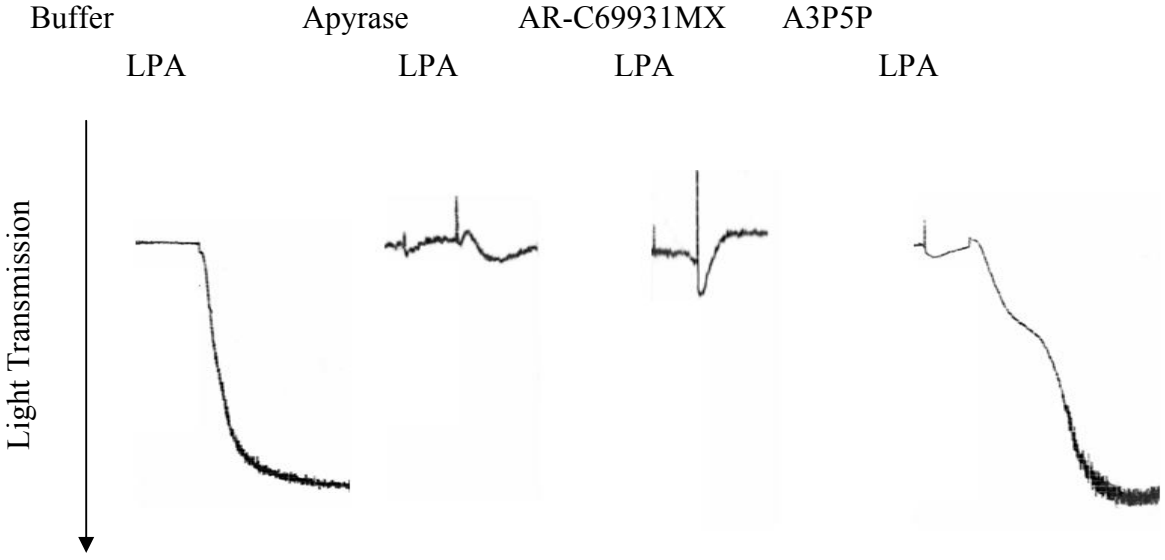


Fig 5.18

LPA-induced aggregation is dependent on ADP-mediated activation of the $P2Y_{12}$ receptor (in platelets washed by method 3)

Platelets prepared according to method 3 were stirred for 1 minute at 1000 rpm and 37°C in the presence of $P2Y_1$ receptor inhibitor A3P5P (200 μM), $P2Y_{12}$ receptor inhibitor AR-C69931MX (1μM), or apyrase (10 U/ml) before LPA was added. Tracings are representative of 6 experiments.

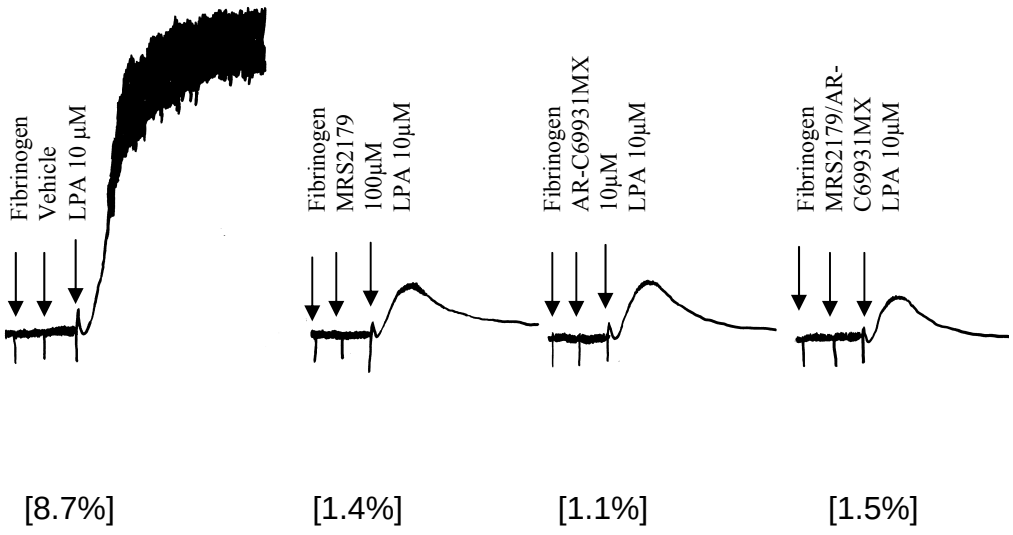


Fig 5.19

LPA- induced aggregation is dependent on ADP-mediated activation of $P2Y_1$ and $P2Y_{12}$ receptors (in platelets washed by method 4)

Platelets washed by method 4 were incubated with buffer, MRS2179 (100µM), AR-C69931MX (1 µM), or a combination of both before addition of 10 µM LPA. Serotonin secretion (percent of total) is indicated in brackets.

	Aggregation (A) and serotonin secretion (S)			
	Control		Aspirin	
	A (%)	S (%)	A (%)	S (%)
Buffer	0	0.3±0.2 (3)	0	0.5±0.5 (3)
LPA**, 10 µM	77 (2)	13 (2)	71 (2)	4.2 (2)
AR-C69931MX + LPA, 10 µM	28 (2)	1.1 (2)	36 (2)	1.4 (2)
MRS_{2179} + LPA, 10 µM	26 (2)	1.4 (2)	27 (2)	1.1 (2)
AR-C69931MX + MRS2179 + LPA	19 (2)	1.5 (2)	20 (2)	1.8 (2)
LPA, 30 µM	80±5 (3)	19±11 (3)	73±16 (3)	7.7±4.4 (3)
AR-C69931MX + LPA, 30 µM	36±16 (3)	0.8±0.6 (3)	33±14 (3)	1.4±1.3 (3)
MRS2179 + LPA, 30 µM	41±24 (3)	1.9±1.6 (3)	32±19 (3)	2.1±0.8 (3)
AR-C69931MX + MRS2179 + LPA	22±11 (3)	0.8±0.6 (3)	20±12 (3)	1.0±1.4 (3)

Table 5.2a

Effect of ADP- receptor antagonists on LPA-induced platelet aggregation (A) and dense granule secretion (S) of washed platelets (method 4)

Platelets washed by method 4 were pre- incubated with buffer or ASS (1mM) before MRS2179 (100µM), AR-C69931MX (1 µM), or a combination of both were added. Platelets were stimulated with 10 µM and 30 µM 1-acyl-LPA (16:0). Values are mean ± SD (n), A% = aggregation, S% = secretion.

In addition to serotonin secretion from dense granules, LPA can also induce P- selectin expression, released from α–granules. LPA (30 μM) induced P-selectin expression was inhibited by either one of the ADP-receptor antagonists. Both ADP- receptor antagonists together show no additional effect (Table 5.2b). Aspirin had no effect on aggregation, however, reduced secretion significantly in platelets washed according to method 4.

	P-Selectin Expression (P)	
	Control	Aspirin
	P- Selectin (MFI)	
LPA, 30 μM	73	85
AR-C69931MX + LPA, 30 μM	47	60
MRS2179 + LPA, 30 μM	50	62
AR-C69931MX + MRS2179 + LPA	47	56

Table 5.2b

Effect of ADP- receptor antagonists on LPA-induced P-Selectin expression of washed platelets (method 4)

Platelets washed by method 4 were pre- incubated with buffer or ASS (1mM) before MRS2179 (100μM), AR-C69931MX (1 μM), or a combination of both were added. Platelets were stimulated with 30μM LPA. P% = P-Selectin expression was measured fluorescence of CD62p-FITC conjugated antibody, (n=1), MFI = mean fluorescence intensity.

In order to test the effect of platelet inhibition in a more physiological medium we tested the same antagonists on LPA-induced aggregation in PRP.

In PRP high and low responders were distinguished by the amplitude and reversibility of aggregation (Table 5.3). Irreversible aggregation, seen in the high responders to LPA, could be reduced and converted into reversible aggregation by either the $P2Y_1$ or the $P2Y_{12}$ receptor antagonist. Reversible aggregation could be inhibited significantly only by both ADP-receptor antagonists together. Remarkably Aspirin showed no inhibition of aggregation (Table 5.3).

	Aggregation in %	
	high responders	low responders
LPA 80 µM	73±12 (12)	21±11 (8)
ASS PRP + LPA	71±12 (7)	22±12 (8)
AR-C69931MX + LPA	38±10 (8)	
A3P5P + LPA	39±9 (12)	
Apyrase	40±13 (10)	
A3P5P + AR-C69931MX + LPA		14±12 (8)

Table 5.3

LPA- induced aggregation in PRP - effect of ADP receptor-antagonists and ASS

PRP was pre – treated with Aspirin right after drawing blood. PRP was stirred for 1 minute at 1000 rpm at 37°C before LPA (80 µM) was added. High responders were donors from group 1 and low responders from group 2. ADP-receptor antagonists were added 15 seconds before LPA. Aggregation induced by ADP was set to 100%. Values are mean ± SD, n= 5-7.

The aggregation and ATP secretion seen in Fig 5.10 (section 5.3.1) represents the aggregation response of low responders. Aggregation could be inhibited by the $P2Y_1$ and $P2Y_{12}$ - ADP receptor antagonists, A3P5P and AR-C69931MX. Aggregation was reduced slightly by either one of the ADP-receptor antagonists. Both receptor antagonists together inhibited aggregation and ATP secretion almost completely. ATP secretion was proportional to the amplitude of aggregation (Fig 5.10).

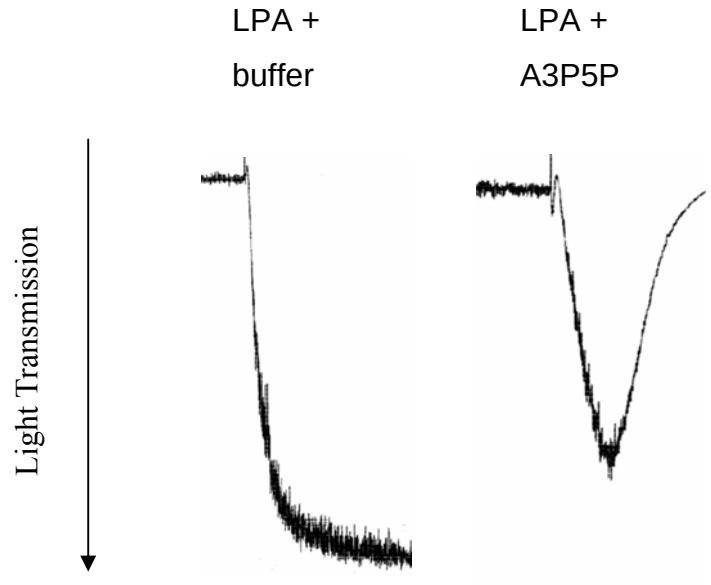


Fig 5.20

LPA-induced secondary irreversible aggregation mediated by ADP

PRP was stirred at 1000 rpm for 1 minute at 37°C in the presence of A3P5P (100µM), before addition of 80µM LPA to a high responding blood donor. Representative tracings are of one individual donor. 5 experiments with different donors were performed with the same results.

Irreversible platelet aggregation induced by 80 µM of LPA in high responders (Fig 5.20) was converted into reversible aggregation by apyrase, the $P2Y_{12}$ receptor antagonist AR-C69931MX, and the $P2Y_1$ -receptor antagonist A3P5P. The $P2Y_1$ -receptor antagonist MRS2179 had the same effect.

These results indicate that LPA- induced irreversible aggregation was dependent on ADP, whereas LPA- induced reversible aggregation and secretion of low responders was mainly mediated by ADP activation of both, $P2Y_1$ + $P2Y_{12}$, receptors.

5.4.1.2. No inhibition of LPA- induced aggregation by aspirin in PRP and washed platelets

Aspirin (1 mM) incubated for 10 minutes, did not inhibit LPA-induced irreversible or reversible aggregation, but reduced ATP secretion in PRP, (Table 5.3, Fig 5.21). Also no inhibition of aggregation, but a reduction of ATP secretion by aspirin was observed in washed platelets (method 4) (Table 5.2). In platelets washed according to method 3, aspirin had no effect on LPA-induced aggregation as well (Table 5.4). The results indicate that cyclooxygenase dependent pathways, like thromboxane A_2 (TX A_2) production, do not play a role in LPA- induced aggregation (see Fig 5.12)

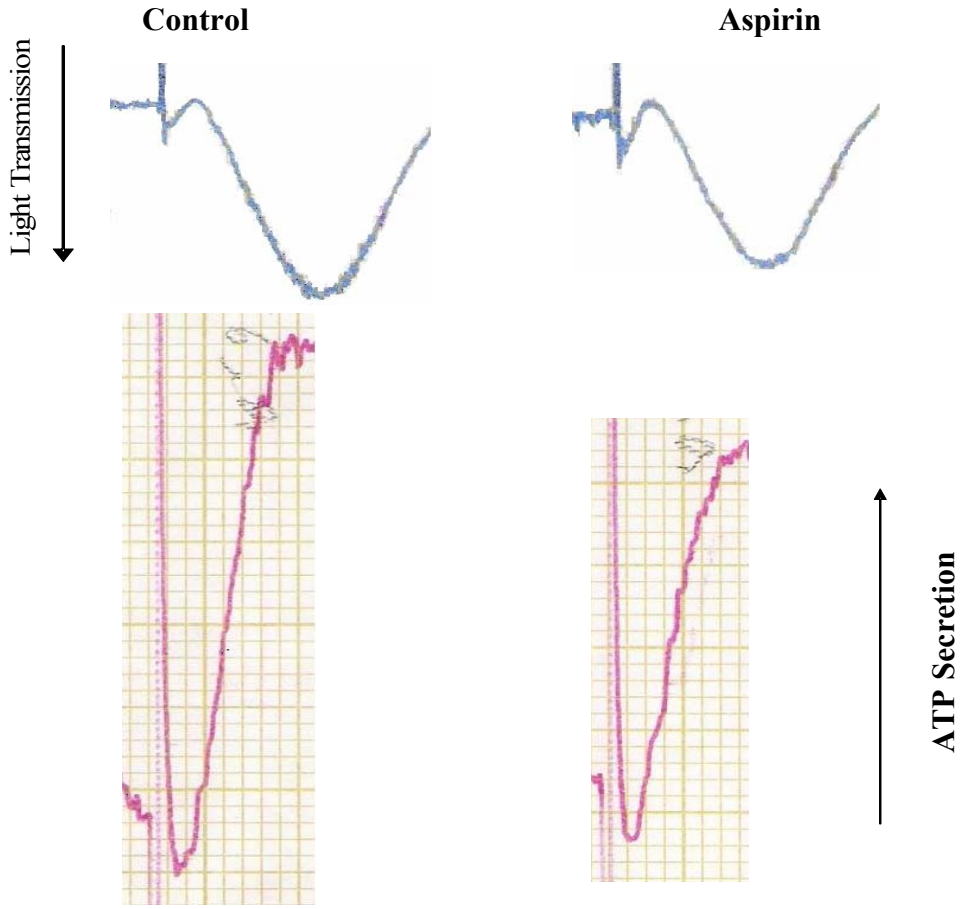


Fig 5.21

Effect of aspirin on LPA-induced aggregation and ATP secretion of PRP

PRP was incubated for 10 minutes with aspirin (1 mM) and stirred for 1 minute at 1000 rpm at 37°C before LPA (80µM) was added. Aggregation (% light transmission) and simultaneous ATP secretion were recorded in a Lumi-aggregometer.

5.4.1.3. Role of intracellular Ca^{2+}

The cytosolic Ca^{2+} chelator, BAPTA-AM, inhibited the LPA- induced aggregation completely in platelets washed by method 3. Similarly, the Ca^{2+} channel blocker, SKF 96365, could also inhibit the LPA-induced aggregation completely, indicating that LPA-induced aggregation in this type of washed platelet preparation is dependent on the influx of external Ca^{2+}. The solvent control (DMSO) did not influence aggregation (Fig 5.22).

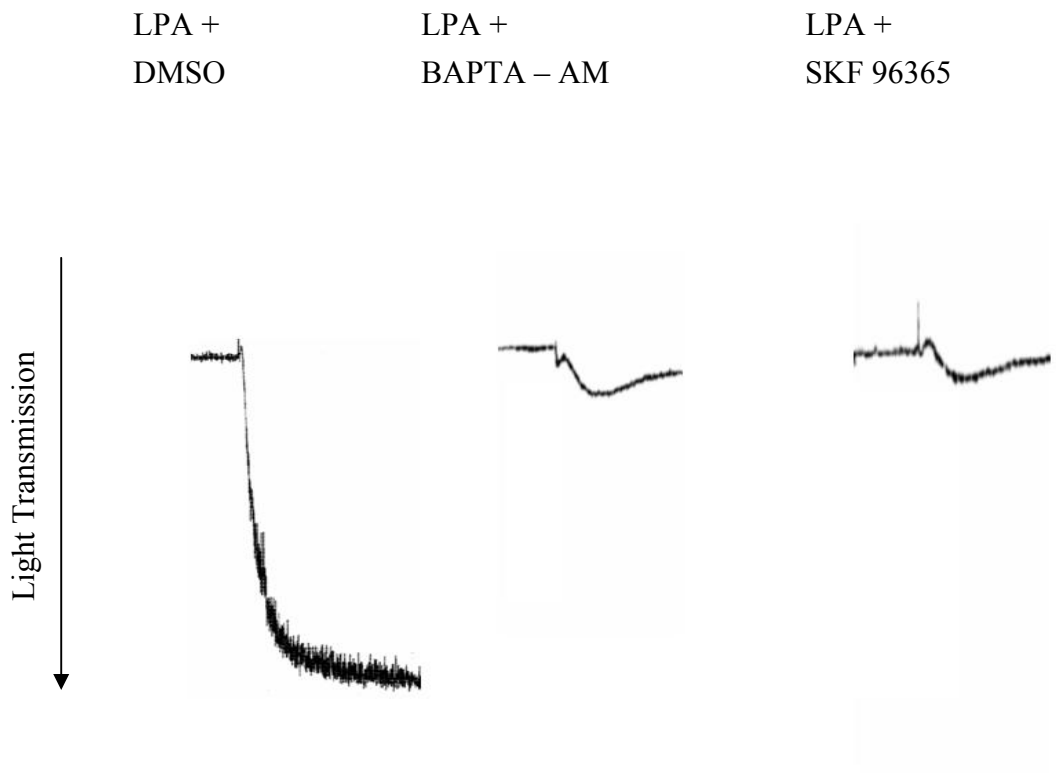


Fig 5.22

LPA- induced aggregation of platelets (washed by method 3) is Ca^{2+} -dependent

Platelets prepared according to method 3 were pre-incubated with DMSO (0.3%), BAPTA-AM (20 μM, dissolved in DMSO) for 20 min, or SKF96565 (100μM) for 10 min at 37°C before stirring for 1 minute at 1000 rpm and exposure to LPA (1 μM). Representative tracings of 5 experiments.

Table 5.4 shows a summary of the effect of all platelet inhibitors on LPA- induced platelet aggregation using three different methods of platelet preparation. Activation of the $P2Y_{12}$ and $P2Y_1$ receptors seem to be involved in irreversible aggregation, whereas Ca^{2+} is essential for reversible aggregation.

	Platelets washed by method 3	Platelets washed by method 4	PRP
	LPA 1 μM	LPA 30μM	LPA 80 μM
Buffer	++	++	++
Aspirin, 1mM	++	++	++
Apyrase, 10U/ml	+	+	+
MRS 2179, 100μM	+	+	+
A3P5P, 100μM	+		+
AR-C69931, 1μM	+	+	+
EGTA, 5 mM	-		-
BAPTA- AM, 200μM	-		
SKF 96365	-		-

Table 5.4

Summary showing the effect of platelet inhibitors on LPA-induced platelet aggregation of washed platelets (method 3 and method 4) and PRP

(++) indicates secondary aggregation (irreversible), (+) indicates primary aggregation (reversible), and (-) shows no aggregation.

5.4.2. Mediators of LPA -induced aggregation in whole blood

5.4.2.1. Dependency on platelet ADP receptors, but not thromboxane formation

Platelet aggregation in whole blood stimulated by LPA was entirely dependent on ADP- induced activation of the $P2Y_1$ and the $P2Y_{12}$ receptor as determined by the use of the ADP-scavenging enzyme apyrase and specific ADP receptor antagonists (Table 5.5, Fig 5.23).

The ADP receptor antagonists, AR-C69931MX and MRS2179 inhibited LPA-induced aggregation below baseline (buffer-stirred control) (Fig 5.23). As a control for ADP receptor specificity, platelet aggregation induced by ADP was also inhibited by MRS2179 and AR-C69931MX (Table 5.5). Notably, also in whole blood aspirin did not inhibit LPA-induced aggregation.

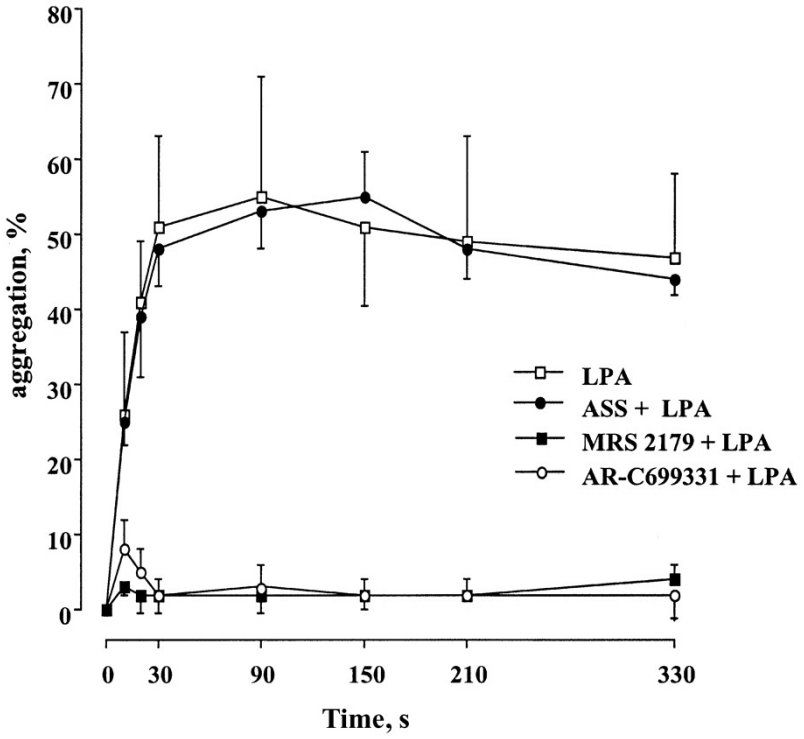


Fig 5.23

LPA- induced aggregation is completely inhibited by blocking either $P2Y_1$ or $P2Y_{12}$ receptor and is insensitive to inhibition by aspirin

Whole blood anticoagulated with hirudin was pre-incubated for 10 minutes at 37°C with aspirin (1mM) and/or stirred for 1 minute at 300 rpm in the presence of AR-C69931MX (1μM) and MRS2179 (100μM) before addition of 10μM LPA. Aggregation was measured at various times after adding LPA (mean ± SD, n=3).

Platelet aggregation, %

	control	LPA[2] 20 μM	ADP 5 μM
Buffer	18±12 (6)	72±26 (6)	89±18 (6)
Aspirin[1]	----------	72±13 (5)	----------
Apyrase[1]	----------	17±11 (6)*	----------
AR-C69931MX[1]	----------	7±3 (3)**	15±12(6)*
MRS2179[1]	----------	9±5 (3)**	28±16(6)*
AR-C69931MX + MRS2179[1]		10±4 (6)**	13±6 (6)**
RGDS[1]	----------	19±9 (6)*	----------
Anti-P-selectin[1]	----------	61±14 (4)	----------

Table 5.5

Effect of platelet inhibitors on LPA-and ADP-induced platelet aggregation in whole blood

Concentrations of platelet inhibitors[1] were aspirin 1 mM, AR-C69931MX 1 μM, MRS2179 100 μM, RGDS 5 mM, apyrase 10 U/ml, and anti-P-selectin 10 μg/ml. -------- indicates that these values have not been determined. Values are mean ± SD, (number)=n.

* $P< 0.05$ ** $P<0.01$.

5.4.2.2. No role of the Ca^{2+} dependent pathway in whole blood

Platelet aggregation was not significantly inhibited by the Ca^{2+} channel blocker, SKF96365, nor by the external Ca^{2+} chelator EGTA (Table 5.6).

Control	36 +/- 19 (4)
LPA	52 +/-9 (4)
EGTA[1] + LPA	61 +/-18 (4)
SKF 96365[1] + LPA	49 +/- 19 (4)
EGTA+ SKF[1] + LPA	66 +/- 20 (4)

Table 5.6

Effect of Ca^{2+} inhibitors on LPA- platelet aggregation in whole blood

Concentrations of platelet inhibitors[1] were SKF96365 100 μM (incubated for 5 minutes), and EGTA 5 mM (added 10 seconds before LPA, 20μM, addition). Values are mean ± SD (n).

5.4.2.3. LPA-receptor desensitization inhibits LPA- induced aggregation

To determine whether the LPA- induced aggregation was mediated by LPA- receptor activation, desensitization experiments were performed in whole blood. LPA (10 μM) was incubated for 10 minutes at 37°C followed by stimulation with LPA (20 μM) or other agonists, ADP (5 μM) and collagen (0.5 μg/ml). Platelet aggregation was diminished with subsequent LPA stimulation, but not with addition of ADP (Table 5.7).

Agonist	Pre-Ink 10' LPA + buffer	Pre-Ink 10 ' LPA + LPA	Pre- Ink 10' LPA + ADP	Pre-Ink 10 ' LPA + Collagen
Agg %	12 +/- 4	23 +/-2	70 +/-5	79 +/-2
Agonist	Pre-Ink 10' buffer + buffer	Pre-Ink 10' buffer + LPA	Pre-Ink 10' buffer + ADP	Pre-Ink 10' buffer + Collagen
Agg %	16 +/- 9	40 +/-7	71 +/- 10	77 +/-2

Table 5.7

LPA-receptor desensitization inhibits LPA-induced platelet aggregation in whole blood

Samples anticoagulated with hirudin were incubated at 37°C with 10 μM LPA (upper row) or with buffer (lower row). After 10 minutes either buffer, LPA 20 μM, ADP 5μM, or collagen 2.5 μg/ml, were added. Aggregation was measured after 90 seconds, (mean ± SD, n=3).

Interestingly, incubation of blood for 10 min at 37ºC reduced the LPA-induced aggregation, but not the ADP- induced aggregation.

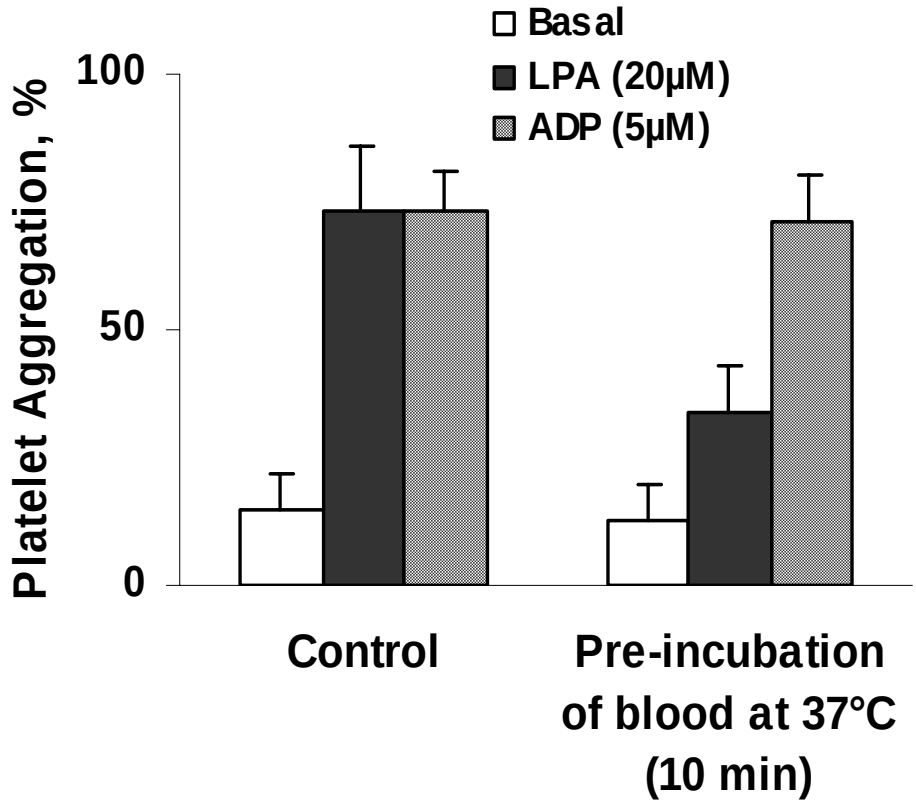


Fig 5.24

Incubation of blood at 37°C inhibits LPA- induced platelet aggregation in whole blood

Whole blood samples anticoagulated with hirudin were incubated at 37°C for 1 min (control) or 10 min. Then either buffer, LPA 20 µM, or ADP 5µM were added and aggregation was measured 1 minute after addition of the agonist (mean ± SD, n=6).

5.5. LPA synergizes with different platelet stimuli in inducing platelet aggregation in washed platelets and whole blood

Based on the fact that dose-response curves of LPA for platelet shape change and aggregation in blood were similar (compare Fig 5.8a and Fig 5.12), we hypothesized that LPA stimulates signal transduction pathways during platelet shape change that synergize with signalling pathways stimulated by other agonists leading to platelet aggregation in whole blood. It has been shown in the past and rediscovered recently that upon coactivation of platelet receptors that couple to different G proteins, their specific signal transduction pathways converge in inducing platelet aggregation (Dorsam et al., 2002; Nieswandt et al., 2002; Quinton et al., 2002).

LPA is known to activate in various cell types proteins of the $G_{12/13}$ family that couple to the Rho/Rho-kinase pathway. In platelets it is likely that only G_{13} will be activated by LPA, since G_{12}, albeit expressed in mouse platelets, is not functional (Moers et al., 2003). In the present

study we investigated whether LPA might also induce the activation of G_i that leads to a decrease in cAMP levels in platelets.

	Experiment 1	2	3	4	5	6	7	mean±SD
Base value without Iloprost	18			9	12	40	19	
Iloprost 50 nM	100	100	100	100	100	100	100	100
LPA 0.1µM	118	114	44	87	78	100	88	90 ± 25
LPA 0.5µM	94			44	95	100	91	85 ± 23
LPA 1 µM	112	165	100	87	117	73	72	102 ± 32
LPA 5µM	71			86	12	73	84	65 ± 30
LPA 10 µM	94	78	24	100	48	40	63	64 ± 28
LPA 20 µM				116	77	70	72	84 ± 22
LPA 40 µM	94	104	48	107	116	40		83 ± 32
LPA 80 µM				93	91	40		75 ± 30
ADP 5 µM	47					23		
ADP 50 µM		65	24	59	91	40	24	50 ± 26
Epi 10 µM	12	10	12	20	12	49	24	21 ± 14

Table 5.8
cAMP in % of the initially raised cAMP levels with prostacyclin analogue Iloprost
Samples from platelets isolated by method 1 incubated in the presence of Iloprost 37°C for 5 minutes in order to raise cAMP levels. Platelets were then stirred at 1000 rpm for 30 seconds until the platelet stimulant, LPA (0.1 – 80 µM), ADP (5 and 50 µM) or epinephrine (10 µM) was added. Values (in % of initially raised cAMP) are measured in duplicates, mean ±SD (n).

	Aggregation, %
Buffer	4±4 (4)
LPA, 20 µM	14±11 (4)
ADP, 0.5 µM	26±12 (4)
LPA + ADP	74±10 (4)

Table 5.9

LPA synergizes with ADP in inducing platelet aggregation in whole blood

Samples anticoagulated with hirudin, incubated at 37°C and stirred for 1 min, were stimulated by LPA (20µM) or ADP (0.5µM) alone or in combination. Values are mean ±SD (n).

Levels of cAMP in unstimulated platelets were raised from 8 ± 1 pmol /10^8 platelets (n=5) to 54 ± 24 pmol / 10^8 platelets (n=7) by the prostacyclin analogue iloprost. In platelets treated with a large concentration range of LPA (0.1-80 μM) there was no significant change in the concentration of cAMP (n=7), whereas epinephrine (10 μM) showed a decrease in cAMP to 10 ± 5 pmol/ 10^8 platelets (n=7), respectively. Therefore, platelet LPA receptors are unlikely to couple to G_i. We also have previously obtained evidence that LPA receptors activated during shape change do not couple to G_q (Maschberger et al., 2000). Therefore, the only G protein activated by LPA during shape change seems to be G_{13}.

Next, we investigated whether LPA receptor-coupled G_{13} activation might synergize with other platelet receptors that couple to G_i or G_q in inducing platelet aggregation. Stimulation of washed platelets by LPA together with epinephrine, known to selectively activate G_i in platelets, or together with ADP, activating G_i through $P2Y_{12}$ receptor, synergistically induced platelet aggregation (Fig 5.25a,b)

Moreover, also LPA and serotonin, which selectively stimulates G_q and subsequent Ca^{2+} mobilization in platelets, synergized in inducing platelet aggregation (Fig 5.25c). Interestingly, the synergistic platelet aggregation of LPA plus serotonin, but not of LPA plus epinephrine, was inhibited by the ADP receptor antagonists, indicating different dependency of the aggregation response on released ADP (Fig 5.25b-c). We also observed that LPA synergized with ADP, epinephrine, and serotonin in inducing platelet aggregation in blood (Table 5.8 and data not shown).

To explore the signal transduction pathway stimulated by LPA receptor G_{13} activation that underlies the synergism, we studied the involvement of the Rho /Rho kinase pathway, which is known to mediate LPA-induced shape change in washed platelets.

Although LPA-induced shape change in whole blood was blocked by pre-treatment with the Rho kinase inhibitor Y-27632 (Fig 5.7b), aggregation induced by LPA plus low concentrations of ADP (0.5 and 1 μM) in blood was not inhibited by Y-27632, excluding a role of the Rho/Rho kinase pathway in the synergistic aggregation response (Fig 5.26).

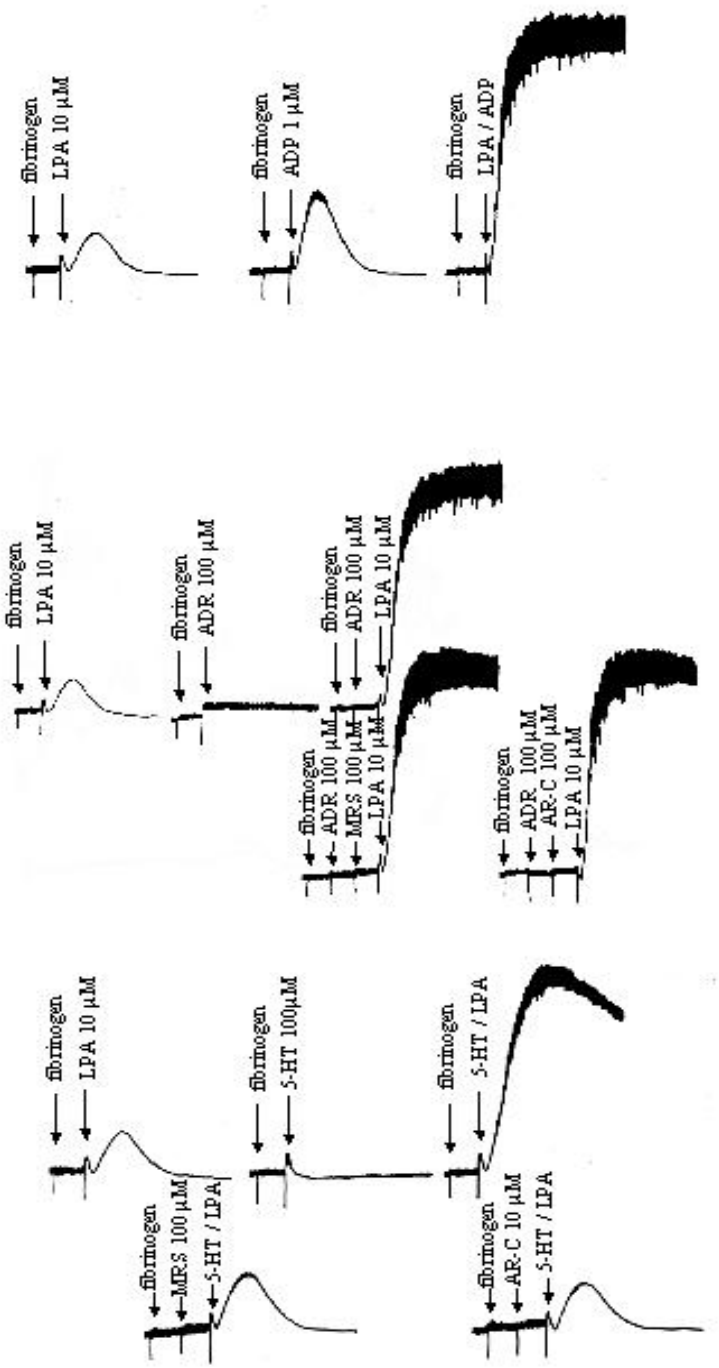


Fig 5.25a,b,c

LPA synergizes with ADP, epinephrine, and serotonin in inducing aggregation of washed platelets (method 4)

Washed platelets were stimulated by LPA, epinephrine (ADR), or serotonin (5-HT) alone or in combination. The synergistic aggregation induced by LPA plus serotonin, but not LPA plus epinephrine was inhibited by MRS2179 or AR-C69931.

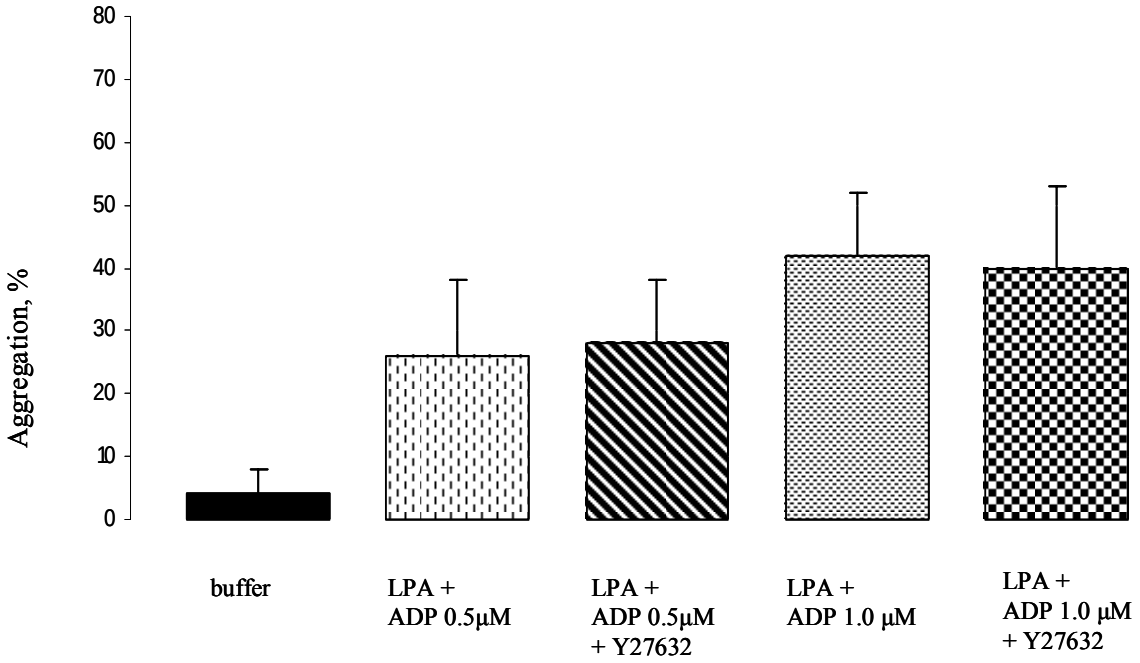


Fig 5.26

LPA– induced aggregation in synergy with ADP- was not inhibited by the Rho kinase inhibitor Y27632 in whole blood

Whole blood anticoagulated with hirudin, incubated at 37°C and stirred for 1 min, was stimulated by LPA (20µM) in combination with ADP (0.5µM) or ADP (1.0µM). Samples were incubated for 30 min at 37°C with the Rho kinase inhibitor, Y27632 (20µM). Values are mean ±SD, n = 3.

5.6. Role of LPA produced by platelets in thrombin- or collagen-stimulated platelet aggregation

LPA produced by thrombin or collagen- aggregated platelets might play a role as a positive feedback mediator of platelet activation. Washed platelets produce about 5 ng LPA/ 10^9 cells after aggregation with thrombin (2 U/ml) for 2 minutes that will lead to a maximal concentration of 10 nM LPA assuming a complete release of LPA from platelets to the extracellular medium (Gerrard and Robinson, 1989).

With the aim of analyzing the possible role of endogenous LPA as a positive feedback loop for aggregation, washed platelets (method 4) were pre-treated with the LPA- receptor antagonist NPSerPa and then stimulated with different concentrations of collagen and thrombin. NPSerPa (10 µM), which completely blocked shape change induced by 0.1 to 0.5 µM LPA (data not shown), had no effect on thrombin- or collagen – induced platelet aggregation (Table 5.10).

These results indicate that LPA formed by activated platelets does not mediate or support stimulus- induced platelet aggregation.

	Aggregation, %	
	Control	NPSerPA
Collagen, 1 µg/ml	85±9 (4)	60±30 (4)
Collagen, 2.5 µg/ml	80±6 (4)	86±16 (4)
Thrombin 0.1 U/ml	67±23 (4)	68±27 (4)
Thrombin 0.2 U/ml	88±16 (4)	89±12 (4)

Table 5.10

Effect of the LPA-receptor antagonist NPSerPA on collagen- and thrombin- induced aggregation of washed platelets

Washed platelets (0.4 ml) prepared according to method 4 were pre-incubated with albumin (0.25 mM)-buffer (12µl, control) or NPSerPA (10 µM) for 10 min at 37 °C before stimulation with collagen or thrombin. Aggregation was measured 2 min after addition of the stimuli.

5.7. LPA- induced platelet-monocyte interaction

Platelet-monocyte interaction was measured by flow cytometry in counting the number of monocytes that were positive for FITC-labelled platelet marker CD 41a, thus recording the number of monocytes with adherent platelets as a percentage of all monocytes.

The graph below shows the right shift of the curve in response to 20 µM of LPA. The degree of right shift is about equal to the degree of shift seen after the addition of a strong agonist, such as collagen.

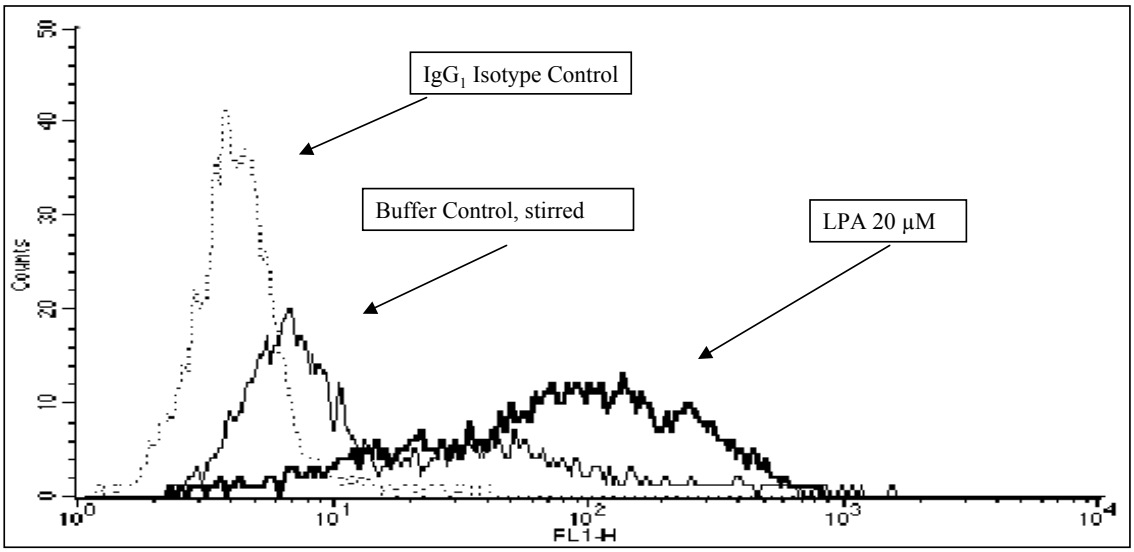


Fig 5.27

Gated monocytes with increasing CD41a-PE indicate platelet adhesion to monocytes

Increasing fluorescence-1 (right shift) indicates the increasing occurrence of CD41a-PE on monocytes. Blood samples were stirred for 5 minutes 300 rpm 37°C after addition of buffer or LPA (20µM).

5.7.1. Mechanism of LPA-induced platelet-monocyte aggregate formation

LPA (20 μM) stimulated the formation of platelet-monocyte aggregates in anticoagulated blood from 32% ± 13.5% (control, stirred samples) to 89% ± 19% (mean ± SD, n= 13, Fig 27). A similar increase was seen after addition of 5 μM ADP, 72% ± 31% (values are mean ± SD, n=11).

The stimulation of platelet-monocyte aggregate formation was dose- dependent (Fig 5.27a). Platelet-monocyte aggregate formation was significantly increased after 2.5 μM LPA and was maximal after 20 μM LPA, 87% ± 12% (Fig 5.27a).

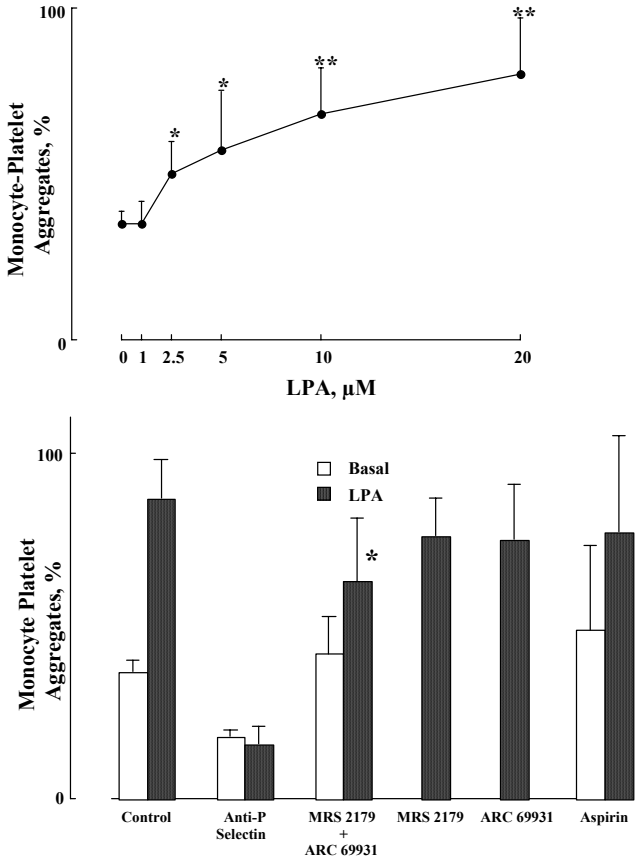


Fig 5.27

LPA- induced platelet- monocyte aggregate formation is dose-dependent and P-selectin mediated

a) dose response curve of LPA-induced platelet-monocyte aggregate formation

Platelet-monocyte aggregates in stirred blood samples at 300 rpm 37°C were measured 5 minutes after incubation with LPA by FACS analysis. Values represent the mean ± SD, n=7-19, *P<0.05 **P<0.01.

b) Platelet-monocyte aggregate formation is mediated via P-selectin

Effects of ADP receptor antagonists and aspirin. Blood samples were stirred for 5 minutes 300 rpm 37°C after addition of buffer or LPA (20μM). A blocking anti-P-selectin antibody (10 μg/ml) or the ADP- receptor antagonists MRS2179 (100 μM) and AR-C69931 (1 μM) alone or in combination was added 30 seconds before the addition of buffer or LPA. Blood was incubated with aspirin (1 mM) for 30 to 45 minutes prior to treatment with LPA. Values represent mean ± SD, n=8-10. *P<0.05 as compared to control.

Pre-incubation with a blocking P-selectin antibody completely inhibited LPA-induced platelet-monocyte aggregate formation. These results indicate that platelet-monocyte aggregate formation in LPA-stimulated platelets is mediated by P-selectin, an adhesion molecule that is expressed on the platelet surface upon platelet activation. LPA-induced platelet aggregation was not inhibited (Table 5.5, section 5.4.2), indicating, firstly, that P-selectin antibody did not unspecifically inhibit LPA-induced platelet activation and, secondly, that platelet aggregation was not required for platelet-monocyte conjugate formation.

The $P2Y_1$ receptor antagonist MRS2179 (100 μM) and the $P2Y_{12}$ receptor antagonist AR-C69931MX (1 μM), did not significantly inhibit the LPA-induced platelet-monocyte aggregate formation in whole blood (Fig 5.27b). Only both ADP- receptor antagonists together significantly reduced the LPA-induced platelet-monocyte aggregate formation ($p<0.05$), indicating that the concomitant stimulation of both ADP- receptors plays a role in the LPA- induced platelet-monocyte adhesion.

Partially supporting these results is the fact that in washed platelets (method 4), we observed an inhibition of P-selectin expression by either one of the ADP-receptor antagonists. But no additional inhibition could be seen by the combination of both ADP-receptor antagonists in washed platelets stimulated by LPA (30μM) (section 5.4.1, Table 2b).

The fibrinogen receptor antagonist RGDS (5 mM) completely inhibited LPA- induced platelet aggregation (Fig 5.28 and Table 5.4, section 5.4.1), but not platelet- monocyte aggregate formation (66% ± 26% versus 68% ± 35%, mean ± SD, n=9). The basal levels of platelet-monocyte aggregates were even enhanced after RGDS (59% ± 30% versus 32% ± 13%, n=8, $P < 0.05$) (Fig 5.28).

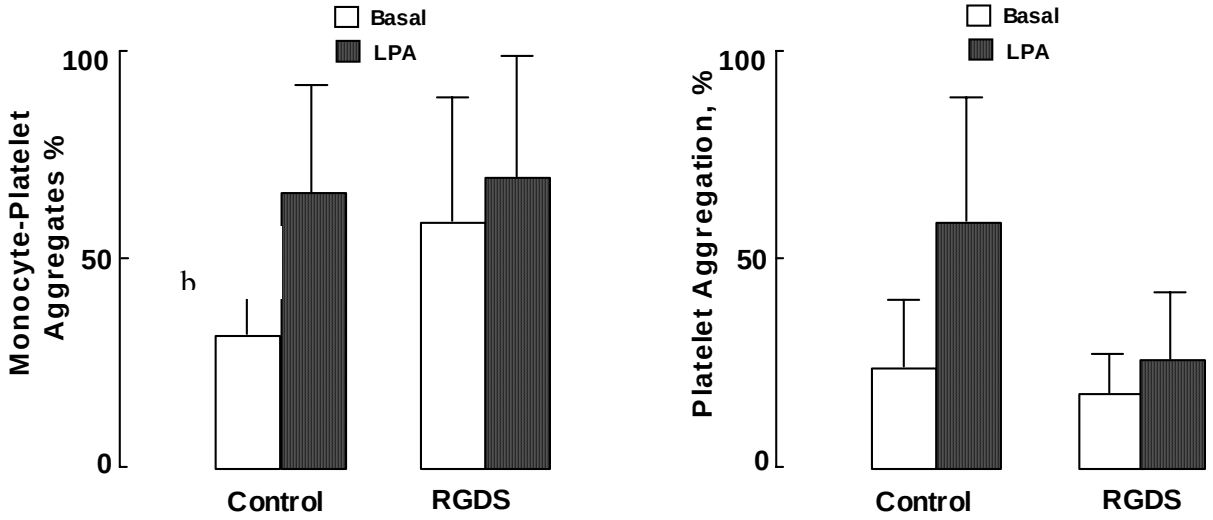


Fig 5.28

LPA- induced platelet- monocyte aggregate formation is not inhibited by RGDS

Platelet-monocyte aggregates in stirred blood samples at 300 rpm 37°C were measured 5 minutes after incubation with LPA. The fibrinogen receptor antagonist, RGDS (5mM) was added 30 seconds prior to LPA. Values represent the mean ± SD, n=9.

Platelet- monocyte formation could also be inhibited by EGTA 5 mM. EGTA was added just prior to LPA (20 μM) to avoid any alteration of the GP IIb/ IIIa fibrinogen receptor. Platelet aggregation, which is dependent on a functional fibrinogen receptor, was not inhibited by EGTA in the same samples (Table 5.11).

EGTA (5 mM) inhibited the LPA-induced platelet-monocyte formation significantly, resulting in a reduction from 52 ± 9 % to 17 ± 13 %. SKF 96365 (100 μM) also showed a reduction of LPA-stimulated platelet-monocyte-conjugate formation; however, this effect was non-significant (Table 5.11).

Stirred control sample	LPA	EGTA + LPA	SKF + LPA	EGTA + SKF + LPA
28 +/- 5	52 +/- 9	17 +/- 13 **	34 +/- 15	27 +/- 20*
		**p> 0.005		* p< 0.05

Table 5.11

Inhibition of platelet- monocyte aggregation by the external Ca^{2+} chelator EGTA and or the Ca^{2+} entry inhibitor SKF 96365

Platelet-monocyte aggregates in stirred blood samples at 300 rpm 37°C (anticoagulated with hirudin) were measured 5 minutes after incubation with 1-acyl-LPA (16:0). The external Ca^{2+} chelator, EGTA 5 mM, was added 10 seconds before the addition of buffer or LPA, the Ca^{2+} channel blocker SKF 96365 was incubated for 5 minutes at 37°C prior to LPA addition. Values represent mean ± SD (n=4).

	% Platelet- monocyte aggregation		
	Donors		
	A	B	C
Experiment 1	84	53	92
Experiment 2	52	71	96
Experiment 3	95	58	92
Experiment 4	69		70
Mean	75	61	88
± SD	19	9	12
Total mean ± SD	75± 16 (n=11)		

Table 5.12

Inter – and intra- individual variations of platelet- monocyte aggregation induced by LPA

Platelet-monocyte aggregates in stirred blood samples, 300 rpm at 37°C, were measured 5 minutes after incubation with LPA by FACS analysis. Blood of individual donors (A-C) [1] was tested in different experiments. Values are mean ±SD, n= 3-4.

Pre-incubation of whole blood with aspirin (1mM) did not inhibit the LPA-induced platelet-monocyte aggregate formation (Fig 5.27b). Under the same conditions aspirin reduced the collagen (0.1µg/ml)-induced platelet aggregate formation significantly (n=8, $p<0.05$), indicating that the treatment with aspirin was effective. Aspirin was also found to be ineffective in inhibiting P-selectin exposure of washed platelets stimulated by LPA (section 4.4.1, Table 5.2b).

Whereas LPA-induced platelet aggregation in blood was donor-dependent, we observed that platelet- monocyte aggregate formation was not. Variations observed were non- donor- specific, shown in Table 5.12.

5.7.2. Specific desensitization of the LPA-receptor mediated platelet- monocyte aggregate formation in whole blood

Similarly to the results shown for LPA-induced platelet aggregation, LPA-stimulated platelet-monocyte aggregate formation could be specifically desensitized by incubating the samples with 10 µM LPA for 10 minutes. The LPA desensitized sample showed a platelet- monocyte aggregation which is close to the baseline (Table 5.13, results shown in bold), whereas the sample incubated with buffer still showed a significant platelet- monocyte aggregation after stimulation with LPA.

Stimulation with collagen or ADP of the LPA-densitized sample, showed no inhibition of platelet-monocyte formation, demonstrating the specific desensitization of the LPA-receptor (Table 5.13).

Agonist	Ink 10'LPA + buffer	Ink 10 ' LPA + LPA	Ink 10' LPA + ADP	Ink 10 ' LPA + Collagen
platelet-monocytes, %	34 +/- 5	**35 +/- 6**	90 +/-5	93 +/-3
Agonist	Ink 10' buffer buffer	+Ink 10' buffer + LPA	Ink 10' buffer + ADP	Ink 10' buffer + Collagen
Platelet-monocytes, %	**27 +/- 6**	**50 +/-16**	73 +/- 16	96 +/-1

Table 5.13

Specific desensitization of LPA- induced platelet monocyte aggregate formation

Samples were incubated at 37°C with 10 µM LPA (row 1) or with buffer (row 2). After 10 minutes either buffer or the agonist, LPA 20µM, ADP 5µM, or collagen 2.5µg/ml, were added. Platelet-monocyte aggregates in stirred blood samples were measured 5 minutes after the addition of LPA by FACS analysis (mean ± SD, n=3).

6. Discussion

6.1. LPA induced shape change in washed platelets, PRP, and whole blood

We observed that about a 500-fold lower LPA concentration was required for platelet shape change in isolated platelets compared to that in PRP. From the several LPA- binding proteins albumin is the major plasma protein responsible for the inhibition of LPA-induced shape change (Fig 5.6). The action of LPA was reported to be inhibited by albumin in sf9 insect cells expressing specifically the LPA_3 receptor (Hama et al., 2002). Thus albumin blocking LPA-induced shape change in isolated platelets implies that LPA-induced shape change could be LPA_3 mediated. Such a conclusion would be consistent with results applying the LPA-receptor antagonist, 8:0 -diacyglycerolphosphate (DGPP). DGPP (8:0) has a higher affinity for the LPA_3 receptor than the LPA_1 receptor and inhibits platelet shape change induced by LPA (Rother et al., 2003).

Since plasma was more potent than albumin in inhibiting LPA-induced shape change (Fig 5.6), other LPA-binding proteins in plasma, such as gelsolin, were suspected to have an additional inhibiting effect. However, no inhibition was seen with gelsolin. In a previous study by Goetzl et al., gelsolin concentrations of less than 10% of the plasma concentration were shown to increase LPA activity, whereas 20% or higher concentrations of those in plasma diminished biological effects of LPA in rat cardiac myocytes (Goetzl et al., 2000). In this study LPA-induced gene activation was measured which required much longer incubation times (24-30 hours) (Goetzl et al., 2000) than in the present study measuring LPA- induced platelet activation (15-30 sec). The longer incubation time may have been necessary to allow for effective LPA-gelsolin binding. It is also possible that LPA in our experiments could not readily bind to gelsolin, since both substances were added almost simultaneously to platelets. Another possible explanation is the fact that Goetzl et al. has shown by western blot analysis that only LPA_1 and LPA_2 receptors are expressed in rat cardiac myocytes. If shape change would be LPA_3-mediated, as proposed earlier, this receptor may not be influenced by gelsolin-delivered LPA. Therefore, it still remains open which plasma factors in addition to albumin inhibit LPA-induced shape change.

The dose-response curves for LPA- induced platelet shape change in PRP and blood were almost identical, indicating that platelet stimuli possibly released from blood cells, such as ADP from red cells, did not synergize with LPA in inducing platelet shape change in blood. Moreover, the LPA-induced shape change in blood was not inhibited by ADP receptor antagonists showing independence of a synergy with ADP and implying a direct LPA-receptor mediated activation. In

PRP and blood LPA-induced shape change could be inhibited by the Rho-kinase inhibitor Y27632, indicating that the G_{13} pathway leading to Rho activation was also required for platelet shape change in blood as previously shown for platelets in suspensions (Bauer et al., 1999; Retzer et al., 2000). The fact that dose-response curves for shape change and aggregation in whole blood were similar suggests that analogous signaling pathways were activated by LPA in PRP and blood.

6.2. Platelet aggregation in washed platelets, PRP, and whole blood

6.2.1.Difference in aggregation using different isolation procedures of washed platelets

The principle advantage of isolating platelets is that platelets can be studied in the absence of red blood cells and plasma proteins, which could influence platelet activation. In addition platelets can be prepared in large quantities and the platelet concentrations and divalent cations (Ca^{2+}, Mg^{2+}) can be adjusted to physiological levels.

LPA induced the initial platelet response, shape change, in washed platelets prepared by method 1 and method 2. Platelets, however, did not aggregate in response to low (5μM) or very high (120 μM) LPA concentrations. Even the addition of fibrinogen (0.1-1 mg/ml), plasma (2-20 %), Ca^{2+} (1mM), alone and in combination of both or all three, did not enable platelet aggregation in response to LPA. Methods 1 and 2 use two washing steps and apply ADP scavengers, apyrase, or CP/ CPK.

Platelets prepared according to method 3 showed irreversible platelet aggregation, which was observed after low concentrations of LPA (1μM). In method 3 no washing step and no ADP scavengers were used. It is likely that by using this method, LPA synergized with ADP in inducing aggregation as observed previously (Benton et al., 1982; Gueguen et al., 1999). ADP can be released during platelet centrifugation and by resuspending the platelet pellet. Indeed, when the $P2Y_{12}$ receptor antagonist, ARC-69931-MX, or apyrase was added, the aggregation response to LPA was completely abolished indicating that the ADP-induced $P2Y_{12}$ activation mediates the synergistic effect with LPA in this platelet preparation. The $P2Y_1$ receptor antagonist, A3P5P, did not inhibit LPA-induced aggregation. It has been shown that the $P2Y_1$ receptor, but not the $P2Y_{12}$ receptor is desensitized after exposure of platelets to ADP (Gachet, 2000). Thus a possible explanation is that the $P2Y_1$ receptor is already desensitized during platelet preparation by method 3 due to the release of ADP during platelet centrifugation and resuspension.

By the fact that no shape change was seen and platelets lost their reactivity after 30- 60 minutes, we can further conclude that these platelets were pre-activated possibly due to the ADP release. In a method similar to method 3 Benton et al used only one centrifugation step to obtain the

platelet pellet and excluded the use of apyrase (Benton et al., 1982; Gueguen et al., 1999). They were the first to show LPA-induced platelet shape change and aggregation in washed platelets. However, they used acid citrate dextrose (ACD) anticoagulated blood, followed by dilution with ACD to PRP in a 1:1 ratio, thus chelating much more of the extracellular Ca^{2+} and Mg^{2+}, than in our method 3, and thereby possibly preventing platelet pre-activation during the subsequent centrifugation step.

Platelets prepared according to method 4 were not pre-activated and showed shape change followed by aggregation in response to LPA. The reaction of platelets to LPA using this method was similar to platelet activation in PRP. The platelets were washed in the presence of prostacyclin (PGI_2) and heparin and resuspended in buffer containing apyrase to avoid desensitization of the ADP receptor $P2Y_1$ (Baurand et al., 2000; Cazenave et al.). PGI_2 prevents dense granule release during centrifugation. No dense granule secretion as measured by serotonin release occurred until platelets were stimulated by LPA. LPA in this platelet preparation caused irreversible aggregation via ADP release from dense granules. This can be concluded by the inhibition of LPA-induced platelet aggregation by either ADP-receptor antagonist (Fig5.19).

6.2.2. Aggregation in PRP and whole blood

LPA induced platelet aggregation in whole blood at very low concentrations approaching those found in vivo (0.5- 1μM) (Fig 5.12) (Baker et al., 2000). In comparison much higher LPA concentrations were required in platelet rich plasma (Fig 5.9). Therefore, we suspected platelet stimulating factors to be present in whole blood. Using different platelet inhibitors for studying the possible mechanism we found that blockade of either the $P2Y_1$ or the $P2Y_{12}$ receptor completely inhibited LPA-induced platelet aggregation in whole blood, demonstrating a complete dependence on ADP possibly released from red blood cells or from platelets. LPA on its own induced very little dense granule secretion (1-2%) in washed platelets, but LPA together with ADP, once secreted, effectively induced dense granule release. Remarkably, blockade of either the $P2Y_1$ or the $P2Y_{12}$ receptor reduced LPA-induced dense-granule secretion. Our results indicate that coactivation of the LPA-receptors with either one of the two purinergic ADP receptors is sufficient to induce full platelet response. Both ADP receptors show equal importance in mediating LPA-induced aggregation in whole blood (Fig 5.23) and aggregation and dense granule secretion of washed platelets (method 4) (Fig 5.19). Most likely both ADP receptors are activated simultaneously by released ADP, and the synergism of signaling pathways induced by the $P2Y_1$ receptor (G_q, Ca^{2+}) and the $P2Y_{12}$ receptor (G_i) is required for platelet aggregation. Indeed, exogenous ADP also induces platelet aggregation in whole blood which is blocked by either $P2Y_1$ or $P2Y_{12}$ inhibition (Table 5.5). The situation is different for ADP-induced platelet activation in suspensions or PRP, where the $P2Y_1$ receptor has been responsible for the initial activation and the $P2Y_{12}$ receptor for amplification (Storey et al., 2001).

In the presence of ADP receptor antagonists washed platelets (method 4) still showed a small aggregation in response to LPA, whereas platelets in blood show no aggregation. This may either be due to the inhibition of LPA by albumin present in blood or due to a faster degradation of LPA, possibly via the ectoenzymes LPP1 and LPP2 expressed on blood cells.

Platelets in whole blood and PRP showed different LPA- aggregation responses according to the blood donor (Fig 5.9, Fig 5.15, Fig 516, Table 5.1), whereas shape change, induced by direct LPA-receptor activation was similar in all donors. This indicates that the different aggregation responses of individual donors are not due to different binding of LPA to its receptors, or activation of platelet LPA receptors. Thus, donor-dependent variations in the concentration of albumin which binds LPA, are unlikely to explain the different LPA aggregation responses. The difference is also unlikely to be explained by environmental and endogenous factors, such as age, cigarette smoking, alcohol consumption, and dietary fat, that have been shown to influence inter-individual variability in aggregation responses to other platelet stimuli (Mori et al., 1997; Renaud et al., 1992; Terres et al., 1989; Terres et al., 1991). No correlation between these factors and platelet aggregation could be seen in our donors.

Since LPA-induced aggregation in blood was completely and in PRP partly dependent on the synergism with released ADP, it is possible that platelets from LPA-responsive donors could have a lower threshold for ADP secretion in response to LPA. Another possibility is that released ADP might activate ADP receptors in LPA-responsive donors better than in non-responsive donors. Indeed donor-dependent, inter-individual differences have been described in response to ADP (Feng et al., 2001; Michelson et al., 2000; O'Donnell et al., 2001). Although ADP–induced (5μM) aggregation was maximal in LPA-non-responders, we can not exclude this possibility since only a maximal concentration of ADP was tested and no ADP dose-response curves for aggregation in blood were carried out.

Additionally, the presence of highly active LPP1 in plasma, platelets and blood cells could be held responsible for this difference. LPP1 expressed highly in platelets has been shown to effectively antagonize LPA-induced platelet activation. LPP1 inhibition has shown to increase LPA-induced aggregation 2-3 fold in 6 of 9 donors in gel-filtrated platelets (Smyth et al., 2003). 3 donors showed no enhanced response in the presence of the LPP1 antagonist (Smyth et al., 2003). Thus, if some donors show a higher LPP1 activity or higher LPP1 expression than others, it would explain why LPA induces less aggregation in those donors.

6.2.3. Activation by different LPA species

Different LPA molecular species have been detected in mox-LDL and atherosclerotic plaques (Rother et al., 2003; Zhang et al., 2004). Acyl- LPA (20:4) makes up 15%, alkyl-LPA makes up 36 % of the total LPA contained in the lipid rich core of atherosclerotic plaques (Rother, 2005). Acyl- LPA (20:4) has shown a 6.5- times higher activity than acyl– LPA (16:0) when inducing

platelet shape change (Rother, 2005). Our results show that alkyl- LPA (16:0) induced a much stronger aggregation in blood than acyl- LPA (16:0) (Fig 5.13). Supporting these findings alkyl-LPA (16:0) showed an 18-fold higher potency than acyl-LPA (16:0) when inducing platelet shape change in PRP (Fig 5.8). Simon et al. demonstrated even a 30-fold higher potency of the alkyl species when inducing platelet aggregation of washed platelets (Simon et al., 1984). However, observations by Bandoh et al. in sf9- insect cells overexpressing either one of the LPA receptors (LPA_1, LPA_2 or LPA_3) show that acyl-LPA had a higher potency than alkyl- LPA (Bandoh et al., 2000). Moreover, Tokumura et al. published that platelets from 2 out of 30 blood donors didn't react at all to alkyl-LPA, but only in response to acyl-LPA (18:1 as well as 20:4) (Tokumura et al., 2002). We did not observe complete non-responders when comparing the two LPA species. The higher potency of the alkyl-LPA species for activating platelets and the reported absence of alkyl-LPA response in two platelet donors suggest the existence of a yet unknown LPA-receptor (Sugiura et al., 1994).

6.2.4. LPA-induced aggregation in blood and PRP- independence of the anticoagulant

LPA is capable of inducing platelet aggregation in PRP and blood independently of the anticoagulant used when comparing hirudin and citrate (Fig 5.14 for whole blood). Hirudin is a direct thrombin inhibitor leaving physiological Ca^{2+} ion levels of 1.1- 1.2 mM. Citrate lowers ionized Ca^{2+} levels to 40-50μM (Phillips et al., 1997). Citrate anticoagulation and low Ca^{2+} conditions are known to facilitate thromboxane A_2 synthesis and granule secretion (Packham et al., 1987). In fact some platelet agonists such as ADP and platelet –activating factor are known to stimulate thromboxane A_2 formation, thromboxane A_2–dependent dense granule secretion and the second wave of aggregation under low concentrations of extracellular Ca^{2+} (Packham et al., 1989; Siess and Lapetina, 1989). LPA- induced platelet aggregation of washed platelets, PRP and blood was independent of platelet cycloogygenase activity, as shown by the lack of inhibition by aspirin (Table 5.2a, 5.2b, Table 5.3, Table 5.4, Table 5.5, Fig 5.21, Fig 5.23). This could a reason, why LPA-induced platelet aggregation was similar in citrate- and hirudin- anticoagulated PRP and blood.

Concerning the influence of extracellular Ca^{2+} concentration on LPA-induced platelet aggregation using washed platelets there are contradictory results in previous studies. Guegen et al showed that Ca^{2+}, 1.3 mM, had an inhibitory effect on LPA-induced aggregation of washed platelets. Other studies by Suguira et al and Benton et al showed that the presence of Ca^{2+} in the platelet resuspending buffer increased LPA-induced aggregation of washed platelets ((Eichholtz et al., 1993; Gueguen et al., 1999; Sugiura et al., 1994). On the other hand Jalink et al clearly illustrated the ability of LPA to form insoluble complexes with Ca^{2+} with a subsequent decrease in the availability of LPA and thus diminishing LPA-induced platelet response (Eichholtz et al., 1993; Gueguen et al., 1999; Jalink et al., 1995; Sugiura et al., 1994).

6.2.5. Synergistic interaction of LPA with serotonin, epinephrine and ADP

Activation by serotonin of the $5HT_{2A}$ receptor and activation by ADP of the $P2Y_1$ receptor are known to couple to mobilization of calcium from intracellular stores through activation of phospholipase C and formation of inositol triphosphate by coupling to the heterotrimeric G protein G_q (Leon et al., 1997; Schachter et al., 1996). The $P2Y_{12}$ receptor and the epinephrine receptor couples to adenlylate cyclase via the G protein G_i (Cusack and Hourani, 1982; Hourani and Hall, 1994). It has previously been demonstrated that epinephrine and serotonin together are able to induce aggregation in isolated platelets (isolated similar as in method 3) (Jin and Kunapuli, 1998). Concomitant activation of the same pathway produces no synergistic platelet aggregation: Activation of the $P2Y_1$ receptor plus serotonin induced only shape change, also activation of the $P2Y_{12}$ receptor plus stimulation by epinephrine had no synergistic effect (Fig 5.25) (Jin and Kunapuli, 1998). We observed that LPA synergistically induced platelet aggregation of washed platelets with serotonin as well as epinephrine. Interestingly, the synergistic platelet aggregation of LPA plus serotonin, but not of LPA plus epinephrine, was inhibited by the ADP receptor antagonists indicating a different dependency of the aggregation response on released ADP (Fig 5.25). We also observed that LPA synergized with ADP, epinephrine, and serotonin in inducing platelet aggregation in blood (Table 5.9 and data not shown). We found that LPA does not activate G_i. The synergism inducing platelet aggregation may be explained by the activation of different G-protein signalling in platelets: LPA which activates G_{13} synergized with epinephrine and $P2Y_{12}$ which stimulates G_i, or with serotonin and $P2Y_1$ activation stimulating Gq (Fig 6.1).

To consider the role of Rho-kinase, which is downstream of LPA-induced $G_{12/13}$ activation, we used the Rho-kinase inhibitor Y-27632. Although LPA-induced shape change in whole blood was blocked by pre-treatment of platelets with Y-27632, aggregation induced by LPA or by the combination of LPA plus ADP was not affected by this drug. These results indicate that Rho-kinase is not involved in the signalling pathway of LPA that mediates platelet aggregation. Thus LPA which does not activate G_i (present study) may only couple to G_{13}. And activation of G_{13} triggered by LPA combined with simultaneous activation of G_q or G_i can cause platelet aggregation (see Fig 6.1).

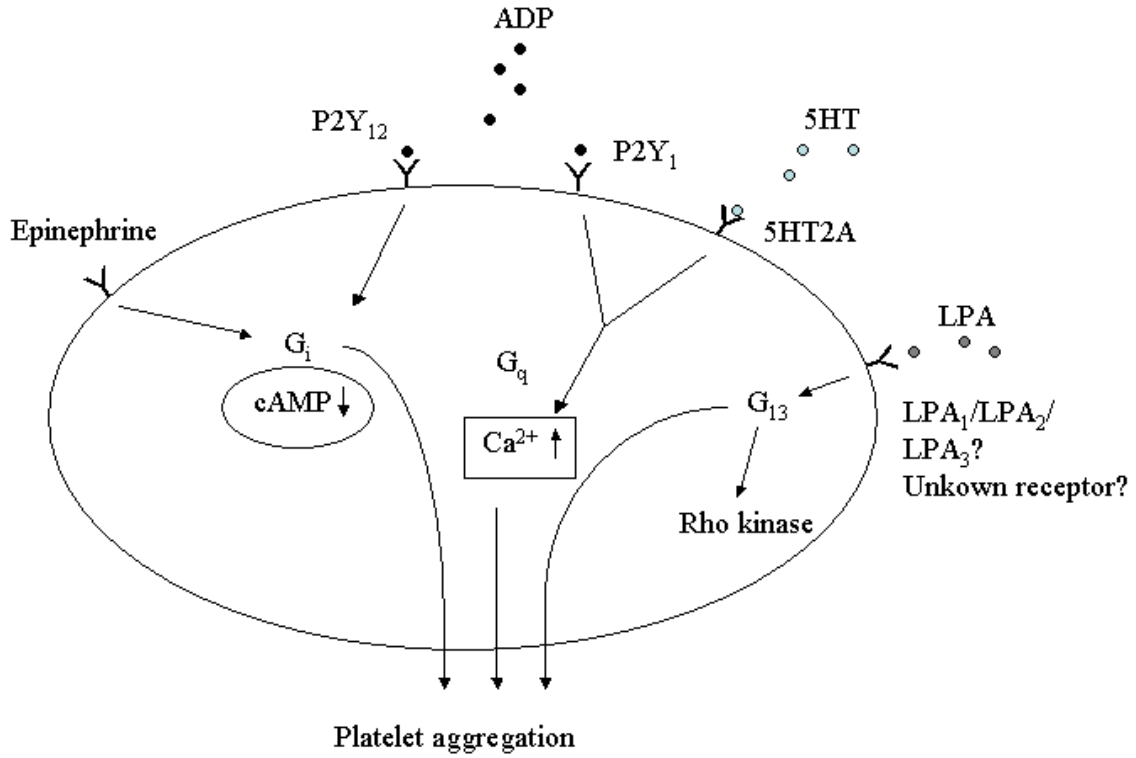


Fig 6.1
Mechanism for platelet aggregation
At least two signal-transduction pathways can synergize with LPA to induce irreversible platelet aggregation. Synergism has been demonstrated for the G_q/ Ca^{2+} pathway and G_i pathway.

6.2.6. LPA is not a positive feed-back mediator of platelet activation

LPA formed during platelet aggregation does not seem to be a positive feedback mediator of stimulus-induced platelet aggregation. Washed platelets produce about 5 ng LPA/ 10^9 cells after aggregation with thrombin (2 U/ml) that will lead to a calculated maximal concentration of 10 nM LPA, assuming a complete release of LPA from platelets to the extracellular medium (Gerrard and Robinson, 1989). Our results show no significant inhibition of collagen- or thrombin–induced aggregation by LPA-receptor antagonists (Table 5.10). LPA could be too weak of an agonist or the released amount of LPA could be too low to enhance aggregation induced by a strong stimulus such as thrombin or collagen. Therefore, LPA which is present in oxidized LDL and atherosclerotic plaques, seems to be unique in being only pathophysiological, but not physiological platelet agonist.

6.2.7. Perspective: Preventing LPA-induced platelet aggregation

The complete independence of platelet aggregation of cylooxygenase-derived prostaglandin endoperoxides and thromboxane A_2 is not known for other platelet stimuli, except for ADP. Although either ADP-receptor antagonist by itself could inhibit LPA-induced aggregation, the significance of inhibiting specific LPA-receptors lies in the attempt to influence only pathophysiological events without influencing physiological thrombus formation. In the event of plaque rupture or platelet activation the local LPA concentration could reach a level slightly higher than the circulating plasma level, leading to platelet aggregation. Thus LPA-specific receptor antagonists could be a new strategy in the prevention of ischemic events under pathophysiological conditions, without the side effects of causing increased bleeding. We suggest that ADP-receptor antagonists, which in contrast to aspirin effectively inhibited LPA-induced platelet aggregation, might also reduce LPA-triggered thrombus formation *in vivo*. Based on our study we would expect that particularly patients with platelets that are highly sensitive to LPA should benefit from this type of antiplatelet drugs.

6.3. LPA- induced platelet-monocyte adhesion in whole blood

LPA also stimulated the formation of platelet-monocyte aggregates in whole blood. The dose-response curve of acyl- LPA was similar to the one for whole blood platelet aggregation. However, within the same experiment the extent of platelet aggregation and platelet-monocyte aggregate formation often did not correlate suggesting that these two LPA induced-responses are regulated differently.

Concurring with previous studies it was found that LPA-induced platelet-monocyte adhesion was P-selectin mediated. P-selectin (GMP 140, PADGEM, CD 62P) is expressed on the surface of activated platelets after fusion of α-granules with the plasma membrane of the surface connected canalicular system (Stenberg et al., 1985 J of Cell Biol 1985). The $P2Y_1$-antagonist and the $P2Y_{12}$–antagonist separately did not significantly inhibit LPA-induced platelet-monocyte aggregate formation in whole blood and P-selectin exposure of washed platelets. Incubation of blood with both ADP- receptor antagonists only modestly reduced LPA-stimulated platelet-monocyte aggregate formation. These results indicate that LPA can stimulate platelet P-selectin exposure and subsequent platelet-monocyte aggregate formation through a mechanism independent of a synergistic interaction with ADP. Therefore, the mechanism differs from LPA-induced aggregation in that there is only a minor role of ADP in platelet-monocyte adhesion (Fig 5.27). These results are congruent with the data from Zhao et al who found that PAF- and ADP-induced platelet-monocyte formation could only be inhibited by the combination of $P2Y_{12}$ receptor antagonist and dypiridamole (Zhao et al., 2001).

Our results with RGDS, a fibrinogen receptor blocking peptide (Arg-Gly-Asp-Ser), that blocked aggregation, but not platelet-monocyte aggregate formation, show that these two LPA-induced responses are regulated independently of each other (Fig 5.28). These results are in agreement with prior studies showing that the fibrinogen receptor does not play a role in platelet-monocyte adhesion (Sarma et al., 2002). Platelets respond to LPA either with secretion of ADP from dense granules leading to a synergistic aggregation response, or with P-selectin expression and platelet-monocyte adhesion (Fig 6.2).

Moreover, platelet-monocyte attachment was Ca^{2+} dependent and blood donor-independent (Table 5.11, Table 5.12), further demonstrating the lack of correlation between the mechanism of aggregation and platelet-monocyte formation. Fig 6.2 summarizes the possible mechanism of platelet aggregation versus platelet-monocyte attachment.

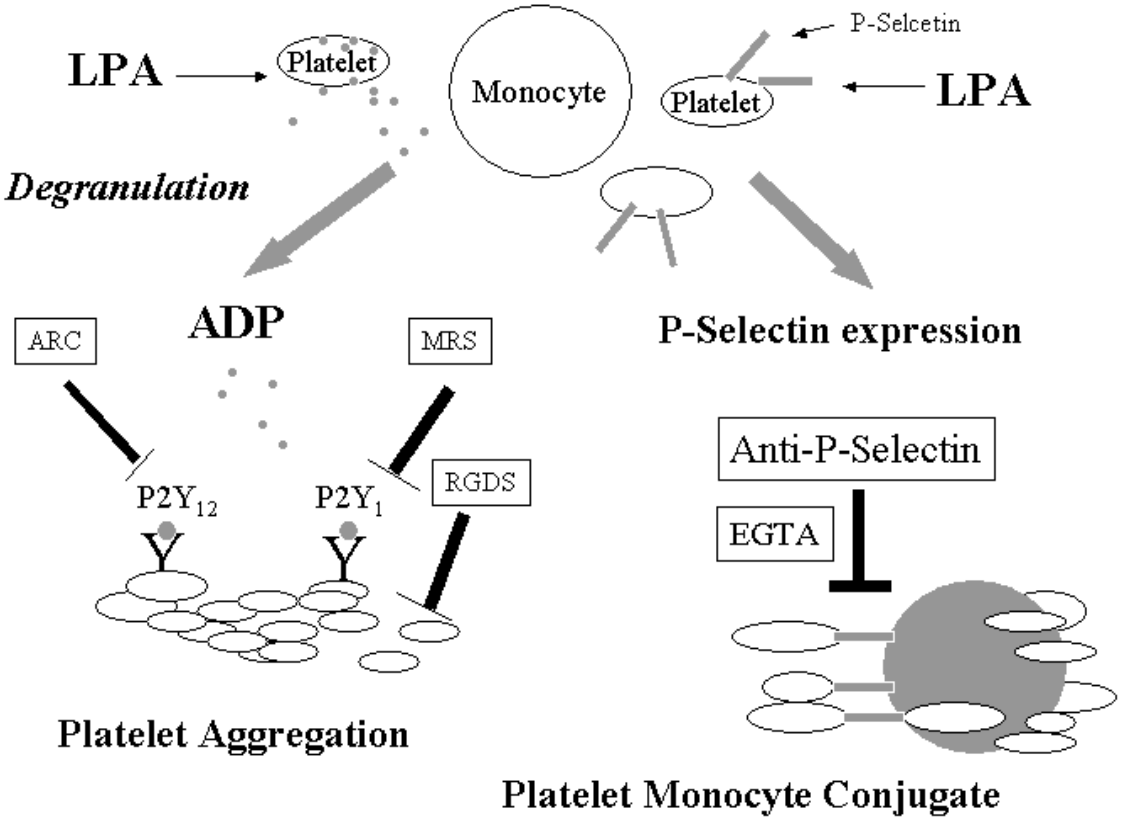


Fig 6.2
Schematic Representation of the possible mechanism of platelet-monocyte formation and platelet aggregation
LPA causes platelet degranulation of dense granules. The ADP contained in dense granules can then amplify LPA-induced platelet aggregation. Meanwhile LPA also induced P-selectin expression on platelets, which leads to the Ca^{2+} -dependent attachment of platelets to monocytes.

Interestingly though, in patients with acute myocardial infarction platelet-monocyte adhesion was divalent cation-independent, raising the possibility of a new cation-independent mechanism of platelet-moncyte adhesion that could be triggered after myocardial injury (Sarma et al., 2002). One possibility is that the glycoprotein Ibα (GP Ibα) is involved. GP Ibα was shown recently to interact with P-selectin. Platelets expressing the GPIb in a complex with GPIX-V constitute a

physiological Ca^{2+} independent counterreceptor for P-selectin (Romo et al., 1999). However, GP Ib-IX-V has not yet been identified on monocytes; therefore, it remains to be seen whether binding of P-selectin to GPIbα is an alternative link between platelets and monocytes.

6.3.1. Perspective: Prevention of LPA-induced platelet-monocyte aggregate formation

The finding that low LPA concentrations stimulate the formation of platelet-monocyte aggregates in whole blood is of possible pathophysiological relevance. Oxidatively modified LDL, that contains LPA and is elevated in plasma of patients with coronary artery disease, might facilitate the formation of platelet-monocyte aggregates(Holvoet and Collen, 1998; Sevanian et al., 1997). These are a more sensitive indicator of *in vivo* platelet activation than platelet P-selectin and have been recently recognized as an early marker of acute myocardial infarction (Furman et al., 2001; Michelson et al., 2001). Moreover, the adherence of platelets to monocytes stimulates the expression of tissue factor on monocytes, promotes fibrin formation and might accelerate intravascular thrombosis (Celi et al., 1994). Platelet-monocyte interaction also stimulates the synthesis of cytokines such as interleukin-6 that increases the hepatic production of C-reactive protein, a risk factor of acute myocardial infarction (Berk et al., 1990; Weyrich et al., 1996).

The contribution of platelet-monocyte interaction to intra-arterial thrombosis and ischemia-reperfusion injury causing myocardial infarction is unknown. In cat models of myocardial ischemic reperfusion injury it has been shown that disruption of platelet-neutrophil interactions is beneficial (Hayward et al., 1999). In baboons, platelets rapidly formed platelet-monocyte aggregates after being stimulated in vitro by thrombin and being re-infused (Michelson et al., 2001).

Based on the results of this dissertation, conventional anti-platelet therapy, including aspirin, GP $\alpha_{IIb}\beta_3$ antagonists, and also clopidogrel will not influence LPA- induced platelet–monocyte conjugate formation. LPA-stimulated patelet-monocyte adhesion and P-selectin exposure of platelets, just as platelet aggregation, was insensitive to inhibition by aspirin. Thus in patients with acute coronary syndromes, most of them taking aspirin, platelet-monocyte aggregate formation, as early marker of myocardial infarction, will be unaffected.

The LPA-induced platelet-monocyte adhesion could be of pathological significance in that LPA locally released from activated platelets, either present in circulating oxidized LDL, or exposed upon plaque disruption by stimulating platelets can attract monocytes to sites of plaque lesions. This process might lead to pro-inflammatory feedback mechanisms and progression of atherogenesis. LPA-specific receptor antagonists could therefore also be a strategy to attenuate inflammation and atherogenesis.

7. Summary

Oxidized LDL and platelets play a central role in the pathogenesis of atherosclerosis and ischemic cardiovascular diseases. Lysophosphatidic acid (LPA) is a thrombogenic substance that accumulates in mildly-oxidized LDL and in human atherosclerotic lesions, and is responsible for the initial platelet activation, shape change, induced by mildly-oxidized LDL and extracts of lipid-rich atherosclerotic plaques (Siess et al., 1999 Proc Natl Acad Sci USA 1999). LPA directly induced platelet shape change in blood and platelet-rich plasma (PRP) obtained from all blood donors. Albumin was one of the main inhibiting factors of platelet shape change in plasma. Interestingly LPA, at concentrations slightly above plasma levels, induced platelet shape change and aggregation in blood. 1-alkyl-LPA (16:0) was almost 20-fold more potent than 1-acyl-LPA (16:0). LPA-stimulated platelet aggregation in blood and PRP was donor-dependent.

LPA-induced aggregation in blood could be completely blocked by the ADP- scavenging enzyme, apyrase, and antagonists of the platelet ADP-receptors $P2Y_1$ and $P2Y_{12}$. These substances also inhibited LPA-induced aggregation of platelet-rich plasma, and aggregation and serotonin secretion of washed platelets. These results indicate a central role for ADP-mediated $P2Y_1$ and $P2Y_{12}$ receptor activation in supporting LPA-induced platelet aggregation and show that LPA synergistically with ADP induces platelet aggregation in blood. Thus antagonists of platelet $P2Y_1$ and $P2Y_{12}$ receptors, especially in donors highly sensitive to LPA, might be useful in preventing LPA-elicited thrombus formation in patients with cardiovascular diseases.

The mechanism of LPA plus ADP-induced aggregation was independent of the Rho/Rho kinase pathway which mediated LPA-induced platelet shape change in blood. LPA, activating G_{13}, but not G_i or G_q synergized also with epinephrine, activating G_i, and serotonin, activating G_q, in amplifying LPA-induced platelet aggregation in washed platelets. LPA/serotonin-induced aggregation was blocked by either ADP-receptor antagonist whereas synergistic aggregation induced by LPA/epinephrine was independent from ADP-receptor antagonists. The latter results demonstrate an additional mechanism for aggregation independent of $P2Y_1$ and $P2Y_{12}$. Most surprising, LPA-induced platelet aggregation was insensitive to inhibition by aspirin.

LPA at low concentrations, starting slightly above plasma level, was also capable of eliciting platelet-monocyte conjugate formation. LPA-induced platelet-monocyte formation was independent of the blood donor. ADP mediated $P2Y_1$ and $P2Y_{12}$ receptor activation played only a

minor role. Platelet-monocyte aggregate formation stimulated by LPA was P-selectin-mediated and insensitive to inhibition by aspirin.

Pathophysiolocical events such as sudden plaque rupture or progressive enrichment of circulating oxidized LDL at critical sites of turbulent flow might lead to higher local blood concentration of LPA. This can induce platelet shape change, platelet aggregation and platelet-monocyte formation at atherosclerotic sites. LPA receptor antagonists could be of possible benefit not only preventing arterial thrombosis, but also retarding vascular inflammation in patients with cardiovascular disease.

8. Zusammenfassung

Oxidiertes LDL und Thrombozyten spielen eine zentrale Rolle in der Entstehung von Atherosklerose und von ischämischen kardiovaskulären Ereignissen. In milde oxidiertem LDL und in atherosklerotischen Läsionen akkumuliert Lysophosphatidsäure (LPA), welche als thrombogene Substanz, die initiale Thrombozytenaktivierung, den Gestaltwandel, verursachen kann. Es wurde gezeigt, dass milde oxidiertes LDL und Extraktionen aus lipid-reichen atherosklerotischen Plaques der Gestaltwandel (shape change) der Thromozyten induzieren kann (Siess et al., 1999 Proc Natl Acad Sci USA 1999). Gleichermaβen kann LPA kann den Gestaltwandel von Thrombozyten im Blut und in Plättchen-reichem Plasma (PRP) induzieren, wobei sich Albumin als einer der Hauptinhibitoren in PRP zeigte. Bemerkenswerterweise konnte LPA schon ab Konzentrationen gering über der LPA Plasma Konzentration den Gestaltwandel und die Aggregation von Thrombozyten induzieren. Als besonders potent gilt 1- Alkyl-LPA (16:0), welches in 20-fach schwächerer Konzentration als 1-Acyl- LPA (16:0) den Gestaltwandel in PRP und die Aggregation im Blut verursachte. Die LPA-stimulierte Thrombozyten-Aggregation im Blut und im PRP waren interessanterweise abhängig von dem jeweiligen Blutspender.

Die LPA-induzierte Aggregation im Blut konnte durch das ADP- hydrolisierende Enzym, Apyrase, die Inhibition von den ADP- Rezeptoren $P2Y_1$ und $P2Y_{12}$, komplett gehemmt werden. Ebenfalls konnte die LPA-induzierte Aggregation in Plättchen-reichem Plasma, wie auch die Aggregation und die Serotonin Sekretion in isolierten Thrombozyten durch Apyrase und die ADP-Rezeptoren komplett gehemmt werden. Diese Ergebnisse unterstreichen die zentrale Rolle der ADP-Rezeptor ($P2Y_1$ und $P2Y_{12}$) vermittelten Aktivierung, in der durch LPA induzierten Thromboztenaktivierung. Gleichzeitig zeigt dieser Synergismus, dass LPA zusammen mit ADP die Thrombozyten Aktivierung im Blut unterhält. Aus diesem Grund sind Antagonisten des $P2Y_1$ and des $P2Y_{12}$ Rezeptors, besonders bei Blutspendern, die hochsensibel auf LPA reagieren, sinnvoll in der Prävention von LPA-induzierter Thrombus-Formation in Patienten mit kardiovaskulären Erkrankungen.

Im Gegensatz zu dem LPA-vermittelten Gestaltwandel im Blut, war die LPA- plus ADP-vermittelte Aggregation im Blut Rho/Rho kinase unabhängig. LPA welches G_{13}, aber nicht G_i oder G_q aktiviert, konnte zusammen mit Epinephrine, welches G_i aktiviert, und mit Serotonin, welches G_q aktiviert, in Synergismus treten und die LPA-induzierte Aggregation in gewaschenen Thrombozyten potenzieren. Die LPA/Serotonin-induzierte Aggregation konnte durch den

jeweiligen ADP- Rezeptor Antagonisten gehemmt werden, hingegen war die synergistisch induzierte Aggregation durch LPA/ Epinephrine unabhängig von beiden ADP- Rezeptor Antagonisten. Die letzteren Ergebnisse suggerieren, das ein zusätzlicher Mechanismus für die LPA-induzierte Aggregation, unabhängig von $P2Y_1$ und $P2Y_{12}$, existiert. Überraschenderweise konnte die LPA induzierte Thrombozyten Aggregation nicht durch Aspirin gehemmt werden.

Niedrige LPA- Konzentrationen, gering über der Plasma Konzentration, lösten auch die Formation von Thrombozyten- Monozyten- Konjugaten aus. Die LPA- induzierte Thrombozyten-Monozyten- Formation war unabhängig von dem jeweiligen Blutspender. Im Vergleich zur LPA-induzierten Thrombozyten Aggregation, spielte die ADP- vermittelte Aktivierung des $P2Y_1$ und des $P2Y_{12}$ Rezeptors nur eine untergeordnete Rolle. Die Thrombozyten- Monozyten- Formation war P- Selektin- vermittelt und war auch durch Aspirin nicht hemmbar.

Pathophysiologische Ereignisse, wie die Ruptur eines atherosklerotischen Plaques oder die Anreicherung von zirkulierendem oxidiertem LDL an kritischen Stellen, z.B. Gefäßbifurkationen, mit turbulenten Strömungsverhältnissen kann zu lokal erhöhten Blutkonzentrationen von LPA führen. Dies kann zur Thrombozytenaktivierung in Form von Gestaltwandel, Aggregation, und Thrombozyten- Monozyten Formation an Atherosklerotischen Plaques führen. LPA- Rezeptor Antagonisten können dabei eine maßgebliche Rolle in der Prävention, nicht nur von arteriell thrombotischen Ereignissen, sondern auch von vaskulär entzündlichen Prozessen in Patienten mit kardiovaskulären Ereignissen, darstellen.

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List of publications

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Presentations

Haserück, N., Rother, E., Corrinth, C., and Siess, W.. Comparison of platelet shape change and aggregation induced by lysophosphatidic acid and mildly oxidized LDL in platelet-rich plasma and washed platelets. 8th Erfurth Conference on Platelets, European Platelet Group, Erfurt, Germanz, June 25th-28th, 2000. *Platelets* (2000) 11, 344.

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Haserück, N., Gachet, C., Siess, W. "Low concentrations of lysophosphatidic acid stimulates platelet aggregation in whole blood through $P2Y_1$ and the $P2Y_{12}$ receptor." XVIII. Congress of International Society on Thrombosis and Haemostasis, Paris, France. July 6th-July 12th, 2001. *Thromb Haemostas*. (2001) Suppl. 86/ 1

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