$\alpha_1$-acid glycoprotein modulates the function of human neutrophils and platelets

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"The world, chico, and everything in it."
-Tony Montana, in the movie *Scarface*, 1983
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List of papers
This thesis is based on the following papers.

I. The acute-phase protein α1-acid glycoprotein (AGP) induces rises in cytosolic Ca$^{2+}$ in neutrophil granulocytes via sialic acid binding immunoglobulin-like lectins (Siglecs)

II. Sialic acid residues play a pivotal role in α1-acid glycoprotein (AGP) induced generation of reactive oxygen species in chemotactic peptide pre-activated neutrophil granulocytes

III. Characterisation of GEA 3175 on human platelets; comparison with S-nitroso-N-acetyl-D,L-penicillamine

IV. α1-acid glycoprotein (AGP)-induced platelet shape change involves the Rho/Rho kinase signalling pathway
Gunnarsson, P., Levander, L., Påhlsson, P., and Grenegård, M., *Thrombosis and Haemostasis*, accepted for publication
Abstract

The acute-phase protein α1-acid glycoprotein (AGP; orosomucoid) was initially identified and characterised in the 1950s. The normal plasma concentration is around 0.5-1 mg/ml but during inflammation the concentration increase several fold and the carbohydrate composition of the protein changes. AGP is a highly glycosylated protein with 45% of the molecular weight consisting of glycans. These glycans are believed to be of importance for the function of the protein. However, the precise physiological role of AGP is still unclear.

The present thesis reveals that AGP at physiological concentration induce calcium elevation in human neutrophils and platelets. In neutrophils this response was enhanced several fold if surface L-selectin was pre-engaged. Our results showed that this L-selectin-mediated amplification was abolished if the neutrophils were pre-treated with Src or phosphoinositide 3-kinase (PI3K) inhibitors. AGP alone did not induce production of reactive oxygen species (ROS) in neutrophils. However, if the neutrophils were activated by the chemotactic peptide N-formyl-methionyl-leucyl-phenylalanine (fMLP) a subsequent addition of AGP caused a prominent ROS response. Moreover, both the calcium rise and the ROS response were depending on sialic acid residues on AGP. In the case of calcium elevation we defined the receptor as sialic-acid-binding immunoglobulin-like lectin (Siglec)-5 on the neutrophil.

In platelets, AGP induced a Rho-kinase dependent phosphorylation of myosin phosphatase target subunit-1 (MYPT1) and a minor calcium response. This resulted in a prominent platelet shape change (i.e. spherical shape and granule centralization) recorded as change in light transmission and by differential interference contrast (DIC) microscopy. The shape change caused by AGP was strongly suppressed by inhibition of Rho-kinase and abolished by Rho-kinase inhibition combined with chelation of intracellular calcium. No other manifestations of platelet activation like aggregation or secretion were registered. Opposite to neutrophils the effect of AGP on platelets was not mediated by an interaction between sialic acid and siglec molecules. However, the results indicated that AGP may bind to a collagen/thrombospondin-1 surface receptor. Endogenous inhibitors like nitric oxide (NO) and adenosine abolished the AGP-induced platelet shape change. The antagonizing action of NO on shape change caused by AGP was long acting. In comparison, other aspects of agonist-induced platelet activation (e.g. intracellular calcium elevations) are only transiently suppressed by NO. This indicates that endothelium-derived NO may play a crucial role to counter balance the effect of AGP in vivo.

Take together the results in this thesis reveal that AGP can initiate intracellular signalling and modulate functional responses in neutrophils and platelets.
Populärvetenskaplig sammanfattning


Våra resultat visade att isolerade humana neutrofiler som stimulerats med 0,5 mg/ml AGP (vilket är den normala koncentrationen i blodet) reagerade med en lite ökning av kalciumjoner inuti cellen. Vidare upptäckte vi att inbindning av en monoklonal antikropp till celladhesionsmolekylen L-selectin på neutrofilens yta innan neutrofilerna stimuleras med AGP förstärker kalciumsignalen i cellen. Sockret sialinsyra på AGP visade sig binda till Siglec-5 vilken tillhör en relativt ny typ av molekyler på neutrofilens yta. I den aktiverande signalen som AGP ger upphov till är två olika intracellulära enzym, Src kinasa och PI3 kinasa, helt nödvändiga. Om neutrofilen blivit föraktiverad av bakteriepeptiden fMLP ger, förutom ökning i intracellulärt kalcium, AGP upphov till produktion av bakteriedödande reaktivt syremetaboliter. Sockermolekylerna på AGP visade sig även vara involverade i denna förmåga att aktivera produktionen av syremetaboliter.

Effekten av AGP på trombocyter visade sig var att trombocyterna ändrar form från diskformade till en aktiverad sfärisk form. Denna effekt på trombocyter är, till skillnad från effekten på neutrofiler, inte beroende av sockret sialinsyra på AGP. Den intracellulära signalmolekul som är ansvarig för AGPs formförändrande effekt är enzymet Rho-kinas som inaktiverar myosinofosfatas vilket i sin tur gör att den lättå kedjan av myosin ökar sin fosforylering med formförändring av trombocyten som följd. Denna AGP inducerade formförändring hämmas av kväveoxid (NO) och adenosin vilka båda normalt produceras av kärlväggen. NO var särskilt effektivt att inhibera formförändringen vilket är något överraskande då andra NO medierade effekter, som hämning av intracellulär kalcium ökning, är relativt kortlivade. Våra resultat visade vidare att det inte är nödvändigt att en substans frisätter mätbara mängder NO molekyler för att ha NO medierade effekter. Detta visas med skillnaden mellan de två kväveoxid innehållande substanserna S-nitroso-N-acetyl-D,L-penicillamine (SNAP) och GEA 3175. Båda substanserna har effekter som kan liknas med NO dock är det så att bara SNAP frisätter mätbara mängder NO i en fysiologisk saltlösning.
**Abbreviations**

AGP  /\alpha_1/-acid glycoprotein (orosomucoid)
cGMP  cyclic guanosine 3’5’-monophosphate
DIC  differential interference contrast
DPI  diphenyleneiodonium chloride
EC50  effective concentration 50%
FMLP  N-formyl-methionyl-leucyl-phenylalanine
ITAM  immunoreceptor tyrosine-based activation motif
ITIM  immunoreceptor tyrosine-based inhibition motif
KRG  Krebs-Ringer glucose
MLC  myosin light-chain
MLCK  myosin light-chain kinase
MYPT1  myosin phosphatase target subunit-1
NADPH  nicotinamide adenine dinucleotide phosphate
NeuAc  N-acetylneuraminic acid (sialic acid)
NO  nitric oxide
PAR  protease-activated receptor
PI3K  phosphoinositide 3-kinase
PKB  protein kinase B (Akt)
PKC  protein kinase C
PKG  protein kinase G
PLC  phospholipase C
PMA  phorbol 12-myristate 13-acetate
PMN  polymorphonuclear granulocytes
PRP  platelet-rich plasma
RIA  radioimmunoassay
ROS  reactive oxygen species
sGC  soluble guanylyl cyclase
Siglecs  sialic acid-binding immunoglobulin-like lectins
SL  sialyllactose
sLex  sialyl Lewis X
SNAP  S-nitroso-N-acetyl-D,L-penicillamine
SYK  spleen tyrosine kinase
VASP  vasodilator stimulated phosphoprotein
WB  Western blot
Introduction and aims

α-acid glycoprotein (AGP; orosomucoid) is a normal constituent of human plasma and an acute-phase reactant. After 50 years of research its function in a physiological context is still not established. Several reports have pointed to an immunomodulatory role for AGP. However, few specific mechanisms explaining this have been presented. The aim of this thesis was to investigate specific effects and mechanisms of action of AGP on human blood platelets and neutrophil granulocytes.

Specific aims were to:

- Investigate if AGP could induce intracellular signalling in neutrophils and platelets.
- Establish the corresponding surface receptors, mechanisms and the functional consequences following the exposure of platelets and neutrophils to AGP.
- Characterize the pharmacology of the NO-containing drugs SNAP and GEA 3175 on platelets and investigate their effect on AGP-stimulated platelets.
Review of the literature

General aspects of inflammation and hemostasis

The human body has a well developed defense systems to injury and infection. The role of this is to maintain the integrity of the organism. In case of injury it is crucial to stop the bleeding in order to prevent blood loss. This response is called hemostasis which is often separated into a primary and a secondary phase. The first part comprises of vessel constriction, platelet adhesion and aggregation whereas the second phase consists of blood coagulation. The platelets makes a new, temporary lining of the vessel wall. This hemostatic plug is however not sufficient to permanent stop the bleeding and coagulation is needed. On the surface of the adhered platelets negatively charged phosphatidylserine is exposed on which the coagulation takes place. Blood coagulation is the enzymatic cascade reaction in which tissue factor from subendothelial cells starts the cascade that ends in the formation of a fibrin network. When all this take place in a blood vessel that has not sustained acute injury it is a pathological process called thrombosis.

Inflammation is characterized by the five cardinal signs. The first four, rubor (redness), tumor (swelling), calor (heat), dolor (pain) were described by Celsus (1st century). These all together leads to the fifth cardinal sign functio laesa (loss of function) which was presumably founded by Galen (2nd century) but the origin of that expression is still unclear [1]. The inflammatory process is the protection to an infection or other potentially harmful mechanical injury. If a foreign particle has breached the body’s outer defense, the skin or intestinal mucosa, the second line of defense will be activated resulting in an inflammatory response. First, cells at the place of intrusion (macrophages, dendritic cells, natural killer cells) and recruited neutrophils try to destroy the invading pathogen together with exudated plasma proteins (i.e. complement system). This is the unspecific part of the immune system and called the innate immunity. The specific part (acquired immunity) mainly consist of different sub-types of T-cells and B-cell derived antibodies. This part of the immune system will respond after a couple of days but on the other hand it has a memory function in case of recurrent infection by the same pathogen.

\(\alpha\)-acid glycoprotein

During the inflammatory response the liver increases its production of several plasma proteins, often denoted acute-phase proteins or acute-phase reactants. Most of the research has been carried out in the regard of using these proteins as diagnostic markers and the most well known among them is C reactive protein (CRP). The \(\alpha\)-acid glycoprotein (AGP), also known as orosomucoid is another acute-phase protein. This protein was identified and initially characterized in the 1950s [2,3]. As other acute-phase glycoproteins, AGP is mainly synthesized by the liver under influence of interleukin (IL)-1, IL-6 and corticosteroids (the biology of the acute-phase proteins is reviewed in [4]). However production from other cells as neutrophils, epithelial cells, macrophages,
monocytes and endothelial cells has been reported [5-10]. The protein is made up of a single polypeptide chain, consisting of 183 amino acids [11] with a molecular weight of 41-43 kDa [12]. AGP is highly glycosylated with five asparagine-linked carbohydrate chains (N-glycans) at positions: Asn-15, -28, -54, -75 and -85 [12,13] (Figure 1). The glycans can be either bi-, tri- or tetra-antennary all with the capability of expressing terminal sialic acids. In fact, glycans constitute 45 % of the entire molecular weight [14]. The normal plasma concentration in healthy humans is around 0.5-1.0 mg/ml [15] (0.36-1.46 mean 0.77 mg/ml [16] and 0.52-1.17 mg/ml [17]), and during inflammation the concentration can rise up to five-fold [14]. Elevated levels have been seen in cancer, during acute inflammation, pregnancy, ulcerative colitis and rheumatoid arthritis [18-20]. Changes in AGP glycosylation, such as increased fucose content and altered branching of the N-glycans, are also commonly associated with inflammation [21-23]. For example in rheumatoid arthritis and asthma the glycans on AGP are more branched [24] and in rheumatoid arthritis the glycans also have a higher amount of fucose compared to normal [18,25]. On the contrary, during acute inflammatory conditions the branching is reduced [22,24,26]. The relevance of this change is still largely unknown. In summary, the molecular aspects of AGP have been thoroughly described but its role in cell biological and in a physiological context is poorly defined.

**Polymorphonuclear granulocytes of neutrophilic type (Neutrophils)**

Neutrophils participate in the first line of defence to invading pathogens. Neutrophils and other polymorphonuclear (PMN) granulocytes (i.e. basophils and eosinophils) were discovered by Schultze in 1865 [27] and further characterized by Metchnikov in 1882 who used a staining technique developed by Ehrlich [28]. Neutrophils evolve from the myeloid stem cell in the red bone marrow under influence of several mediators (e.g. IL-3, granulocyte-colony stimulating factor (G-CSF) and granulocyte–macrophage colony-stimulating factor (GM-CSF)) [29-31]. The maturation takes about 11 days before the neutrophils migrate out to the blood stream [32]. Under normal conditions, the turnover rate is $1.63 \times 10^9$ neutrophils per kg body weight per day [32]. When entering the blood the neutrophils are terminally differentiated unlike the monocyte that has to be differentiated into a macrophage when entering the tissue to get phagocyte capabilities. The neutrophil is about 10 μm

![Figure 1. Illustration of the acute-phase protein AGP showing the normal glycosylation. (Asn=asparagine)](image-url)
in diameter and has a characteristic multi lobulated nucleus (Figure 2). As the full name indicates the neutrophil contain various types of granules [33]. Early in the maturation in the bone marrow the azurophil granules starts to develop and since this is the first to appear it is also named primary granules. These granules contain in the fully developed state defensins, myeloperoxidase (MPO), cathepsin G and elastase. Later in the maturation of neutrophils specific granules (or secondary granules) start to develop. In these granules are lactoferrin, elastase and MPO stored. The last type of granule to appear is the tertiary granules containing cathepsin and gelatinase [34-38]. Besides these granules the neutrophil has secretory vesicle containing plasma proteins, alkaline phosphatase and receptors. Once in the circulation, the neutrophils patrol in average for 7 hours [32,35]. After entering the tissue the neutrophil exist for 1-2 days before undergoing apoptosis [31,39]. Among the leukocytes, the neutrophils are the most numerous with 1500 to 7700 cells per microliter of blood [32]. However, the main role for the neutrophil is outside the circulation. In order to reach the place of pathogen invasion the neutrophils need to pass through the endothelium and the entire blood vessel. The endothelial cells covers the inside of all the blood vessels. In order to attract neutrophils upon inflammation the endothelial cells display several receptors. This interaction is in great part dependent of carbohydrate recognition.

**Lectins**

Proteins recognising and binding carbohydrates are denoted lectins. They appear both as soluble proteins and membrane bound on the surface of various cell types. In humans, lectins mainly participate in cell adhesion and in pathogen recognition [40-42]. However, much research has to be done before their complete roll in innate immunity is elucidated. Below follows a description of lectins that were found to be of main importance in the studies presented in this thesis.
**Selectins**

The attraction of neutrophils to the site of tissue damage is mainly regulated by the endothelium. This is done by expression of different cell surface molecules on which the neutrophil can attach. This interaction involves in the first step selectins on which the neutrophils tether, slow down and start rolling. Selectins are calcium dependent carbohydrate binding proteins (C-type lectin). The selectin family consists of P-, E-, and L-selectin where P- is found on platelets, P- and E- are expressed on endothelium and the L-type is expressed on leukocytes [43]. P-selectin is constitutively expressed in the Weibel-Palade bodies of the endothelial cells and in α-granules in platelets. Activation results in a rapid increase in P-selectin surface expression on both endothelial cells and platelets [44]. Increased E-selectin expression on endothelial cells requires protein synthesis which can be induced by IL-1β, tumor necrosis factor-α and bacterial lipopolysaccharide [45,46]. In vitro the peak expression of E-selectin is reached 4 hours after stimulation with IL-1β [47]. L-selectin which is the third type of selectin is constantly present on the surface of circulating neutrophils [48,49]. When neutrophils are activated, L-selectin is shed of by cellular proteases [50]. All types of selectins are glycoproteins and consists of a short cytoplasmic tail, two to nine complement regulatory repeats, epidermal growth factor (EGF)-like motif and N-terminally a calcium dependent lectin domain [46]. P-and L-selectins are responsible for tethering the neutrophil to the endothelium whereas E-selectin is important for slowing down the rolling velocity [51,52].

Selectins may also cause outside-in signalling subsequent to carbohydrate binding. For example signalling consequences by L-selectin binding to its ligand include calcium elevation [53,54], tyrosine phosphorylations and mitogen-activated protein kinases (MAPK) (e.g. p38, JNK, ERK1/2) activation [55,56]. In lymphocytes L-selectin engagement triggers activation of Ras through the Src kinase (56Lck) leading to reactive oxygen species (ROS) production [57,58].

**Glycans are ligands for selectins**

The ligands for selectins are still not completely understood and no single ligand for respective selectin has been described. Protein carbohydrate binding are general weak interactions demanding multi-valency for binding [59]. There are several theoretical variants of multi-valency in selectin binding. One theory involves the polypeptide backbone in the binding resulting in protein-carbohydrate-protein interaction. Another theory involves multiple binding of ligands to one selectin. However, the carbohydrates of the sialylated fucosylated lactose-amine family, where the epitope sialyl Lewis X (sLex) is the simplest example, are important binding epitopes for selectins. The sLex determinant (NeuAcα2,3Galβ1,4(Fucβ1,3GlcNacβ1-R) (Figure 1) has been found as a general ligand to all three selectins and is expressed on various cell surface structures [60]. Furthermore, there is also some evidence that selectin-selectin binding is possible. For example L-selectin on neutrophils has been show to bind E-selectins on activated endothelium [61]. This is not surprising since E-selectin has 11 glycosylation sites and N-liked carbohydrate accounts for about 30 % of its molecular weight [62]. Many ligands to selectins have been described, but their
physiologic relevance is still uncertain. However, during inflammation P-selectin glycoprotein-1 (PSGL-1) expression on endothelium and on leukocytes together with CD44 on leukocytes are considered as the most important [63]. In the case of lymphocyte homing mucosal addressin cell adhesion molecule 1 (MAdCAM-1) and to a lesser extent glycosylation-dependent cell adhesion molecule 1 (GlyCAM-1) and CD34 expression on high endothelial venules (HEV) (in Peyer’s patch) are considered important [63]. Other selectin ligands are expressed on various malignant cells or in tissues were their biological significance remains to be established.

**Other lectins**

Besides the well-studied selectins, the neutrophils express other carbohydrate binding structures on their cell surface. Lectins on the surface of neutrophils except selectins comprise S-type (Galectins), P-type (Mannose-6-P receptors), I-type (Immunoglobulin-type), L-type and R-type [64,65]. A novel group of lectins are the I-type sialic-acid-binding immunoglobulin-like lectins (Siglecs). The rapid evolving group of Siglecs comprise of 14 different sub-types (Siglec-1 to 11 and -14 to -16) [66,67]. The Siglecs are type 1 membrane proteins displaying an amino-terminal V-set immunoglobulin domain that binds sialic acids and variable numbers of C2-set immunoglobulin domains. Human neutrophils express Siglec-5 and -9 which are denoted as CD33-related Siglecs [68,69]. Siglec-5 signalling is reported to be inhibitory with immunoreceptor tyrosine-based inhibition motif (ITIM) domains recruiting phosphatases. Very recently it has been shown that Siglec-5 have 99 % similarity in the first two immunoglobulin domains with Siglec-14 and that Siglec-14 is expressed on granulocytes [70,71]. Conversely, it has been shown that Siglec-14 has activating capacity through interaction with DAP-12 and phosphoinositide 3-kinase (PI3K). No definite cellular function has been proposed for Siglec-9 but it been associated with neutrophil viability [72].

**Diapedesis, chemotaxis, phagocytosis and respiratory burst**

After the initial carbohydrate dependent adherence to the endothelium the neutrophil extravasate in its chase for pathogens. Neutrophils travel in the tissue towards a pathogen by moving in the direction of an increasing concentration of a substance. This process is called chemotaxis. Neutrophils express on their surface several chemotactic receptors that direct the movement of the cell in the gradient of a specific substance with a maximum speed of 20 μm/min [73]. End target chemoattractant as C5a [74-76] (complement cascade) and N-formyl-methionyl-leucyl-phenylalanine (fMLP) [77,78] (bacteria) and other formylated peptides [79,80] (mitochondria) together with host-derived, also denoted intermediate, chemoattractant IL-8 [81] (macrophages, endothelial cells, fibroblasts) and leukotriene B4 (LTB4) (macrophages, PNM, mast cells) [82] produced at the site of injury are responsible for guiding the neutrophils in the right direction. The general molecular events leading to directed cell movement mainly comprise PI3K or Rho activation [83]. Once the neutrophils are at the right location they start working by engulfing the pathogens. This process can either be mediated by Fc receptors (e.g. CD16, CD32 and after
interferon priming also CD64) in the case of antibody opsonisation or by C3bi via the activated integrin CD11b/CD18 (MAC-1) [84]. However, neutrophils are also able to phagocytose non-opsonized prey. Structures on the pathogen for recognition comprise mannan in the yeast cell wall, formylated peptides from bacteria and damaged cells and lipopolysaccharides and lipoteichoic acids on the surface of bacteria [83,85-87]. The engulfed pathogen will end up in what is called a phagosome where it will be killed and degraded [39]. One of the main killing mechanisms is the generation of reactive oxygen species (ROS). Neutrophils produce vast amount of ROS (e.g. superoxide anion and hydrogen peroxide) to kill encountered microorganisms. The production of superoxide anion is mediated by the nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase enzyme system and the oxygen radical is further converted spontaneously or via superoxide dismutase to hydrogen peroxide. The functional NADPH-oxidase consists of the membrane bound and heme containing flavocytochrome b composed of p91phox (β-chain, 91 kDa, glycosylated, 4-6 transmembrane regions) and p22phox (α-chain, 22 kDa, nonglycosylated, 2-3 transmembrane regions) and cytosolic proteins p47phox, p67phox, p40phox and small G proteins Rac2 and RapA1 [88-91]. Upon neutrophil activation protein kinase C (PKC), p38MAPK, p21 activated kinase (PAK), protein kinase B (PKB; Akt), casein kinase 2, ERK1/2 or a phosphatidic acid-activated kinase may phosphorylate p47phox which is then translocated together with p67phox and p40phox to the flavocytochrome b part of the enzyme. The functional NADPH-oxidase is attached to a cellular membrane which either could be the phagocytic granule or the outer cell-membrane. The first case will give rise to what is called intracellular production of ROS whereas in the second case the production will be extracellular. Furthermore, several reports have proposed intracellular calcium concentration as also an important regulator of NADPH-oxidase [92-97]. Calcium elevation has been shown to be connected with intracellular ROS production as especially the granule localized NADPH-oxidase seems to be calcium sensitive [98,99]. However, the calcium rise by itself is not responsible for the NADPH-oxidase assembly [100]. The role of calcium in neutrophil ROS production is excellently reviewed by Brechard et al. [101].

**Formyl receptor signalling**

As previously described one major chemotactic factor is the bacteria derived peptide fMLP which was found to activate human neutrophils (Figure 2) in the second part of the 1970s [77,102,103] and the receptor was cloned by Boulay et al. in 1990 [104,105]. Human neutrophils express two types of receptors to fMLP identified as; formyl peptide receptor (FPR) and FPR like-1 (FPRL1) [106]. The third homologue FPRL2 is not expressed on neutrophils and does not bind fMLP [107]. FPR is considered to be a high-affinity receptor with a Kd of 1 nM and FPRL1 is characterised as a low-affinity receptor with a Kd of 1.5 μM [108-111]. Noteworthy, the FPRL1 receptor has been characterized as a high-affinity receptor for lipoxin A₄ [112,113]. The FPRL1 receptor has also been shown to bind serum amyloid A [114]. The receptors are 7-transmembrane classical G-protein coupled (pertussis toxin sensitive) inducing intracellular calcium elevation through βγ-subunit activation of phospholipase C (PLC)β hydrolysis of phosphatidylinositol 4,5 bisphosphate (PIP₂) to
inositol 1,4,5-triphosphate (IP$_3$) and diacylglycerol (DAG) \cite{115-118}. The initial calcium rise is elicited by IP$_3$ binding to receptors on intracellular stores resulting in a rapid increase in calcium concentration from the basal level of 100 nM \cite{119}. Endoplasmatic reticulum (ER) is considered to be the main intracellular store of calcium in neutrophils. At least two distinct cellular locations for calcium stores in neutrophils appear to utilize different molecular/biochemical mechanisms of calcium mobilization \cite{120}. One site is located peripherally under the plasma membrane and appears to be involved in the activation of β$_2$-integrins, while the other is localized more centrally in the cell and releases its calcium in response to chemoattractants such as fMLP \cite{120}. These more centralised stores were earlier denoted calciosomes \cite{121}. During activation, a slower and more sustained calcium elevation takes place due to store-operated calcium entry (SOCE) over the plasma membrane \cite{122-125}. Interestingly, a minor increase in IP$_3$ (15% of maximum) is sufficient to cause maximum calcium elevation \cite{119,126}. The restoration of intracellular calcium takes several minutes and is regulated by the extrusion of calcium ions by calcium ATPases. Activation of PKC by PLC/β generated DAG is regarded to be the other main intracellular signal in fMLP activation. Human neutrophils express the conventional isoforms of PKC (α, βI and β), the novel isoform PKCδ and the atypical form PKCζ \cite{117}. This isoform is not dependent on the classical activation pathway involving DAG but might be activated by Rho signalling pathway \cite{127} or via 3′-phosphoinositide-dependent kinase-1 (PDK-1) \cite{128}. PDK-1 also phosphorylates PKCδ but this action is less pronounced \cite{128}. Beside the calcium signal and activation of PKC isoforms, PI3K/γ (PI3K IB) plays an important role in neutrophil functional responses \cite{129,130}. PI3K/γ is activated by the βγ subunit of heterotrimeric G proteins \cite{131}. The PI3K isoforms α, β and δ (class IA) are also expressed in neutrophils \cite{132}. These isoforms are however regulated by tyrosine kinases and can be activated through integrin activation \cite{133}. PI3Ks are responsible for production of the signalling lipid PIP$_3$ and the following binding and activation of signal enzymes like PKB \cite{131,134}. Furthermore, PI3K is also linked to MAPK cascade activation \cite{135}.

**Biology of the platelet**

Human platelets were probably first described in 1780 by Hewson \cite{136} but no convincing evidence were presented until 1865 when Schultz reported an accurate description of the platelet, or more definitely in 1882 when Bizzozero described the platelet and its function in hemostasis \cite{137,138}. Platelets are the smallest blood cells in man measuring 2-4 μm in diameter (Figure 3). The normal concentration range in the circulation spans from 150000 to 400000 per μl. The wide range of normal platelet concentration mocked the scientists when they first tried to determine the normal platelet concentration \cite{139}. The platelet originates from the megakayocytes by cytoplasmic fragmentation and after leaving the red bone marrow the platelet persists in average for 10 days in the circulation. The platelet is an anucleated cell fragment that has mainly two types of cytoplasmic granules. The α granule, average 80 granules per platelet and with a size of 200-500 nm contain P-selectin, von Willebrand factor, thrombospondin, fibrinogen, integrins α$_{2β}$ and α$_{β3}$, fibronectin and several other proteins \cite{140}. The dense granules are 10-times fewer than α granules and they
enclose for example Ca$^{2+}$, adenosine diphosphate (ADP), adenosine triphosphate (ATP) and serotonin (5’hydroxytryptamine; 5’HT). Platelets also contain lysosomes with a content of acid hydrolases, cathepsins and lysosomal membrane proteins (LAMP-1 (CD107a), LAMP-2 (CD107b) and LAMP-3 (CD63) [141]. Platelet granule constituents are formed by the megakaryocyte or endocytosed from blood plasma [142,143].

The main function of the platelet is to participate in primary hemostasis. In case of vascular damage the subendothelial matrix (most important collagen type III) triggers platelet activation and adherence. The initial event consists of the platelet receptor GP Ib-IX-V binding to von Willebrand factor (vWF) bound to collagen [144]. This slows the platelet down and further activation by the collagen receptor GP VI and firm adhesion is possible through collagen binding integrin αβ₃ [145]. Other integrins involved in adhesion are αβ₁ (binding to vitronectin), αβ₃ (binding to laminin) and αβ₁ (binding to fibronectin) [146,147]. Upon activation the platelets release their granule constituents where α-granules are the first ones to be released. The platelets also start to synthesize thromboxane A₂ (TXA₂) from arachidonic acid by the enzyme cyclooxygenase (COX)-1 (target of acetylsalicylic acid (ASA; aspirin)) and thromboxane synthase [148-150]. Produced TXA₂ together with ADP are the most important autocrine platelet activators. Once the platelets is activated the fibrinogen binding integrin αIIbβ₃ change its affinity to a RGD binding state which allows platelets to aggregate with fibrinogen as linking molecule [151-154]. Besides ADP (receptors; P2Y₁ and P2Y₁₂) and TXA₂ (TP receptor) thrombin (receptors; GP Ibα, PAR-1 and -4 (G₁₂/G₁₃-coupled)) is regarded the most powerful soluble platelet activator [147]. The serine protease thrombin is locally generated as one of the final steps in the coagulation cascade. Alongside these activators there are several other weaker activators or modulators of platelet responses. They comprise among others

![Image](image_url)

**Figure 3.** DIC microscopy pictures of human platelets in three different stages of activation. The picture to the left is showing unstimulated platelets, the picture in the middle is showing platelets undergoing shape change and the picture to the right is showing platelet aggregation. The scale bar indicate 5 μm.
serotonin, ATP and adrenaline [155,156]. The 5’HT$_{2A}$ receptor [157,158] (G$_q$-coupled) is the main receptor for serotonin, the P2X$_1$ receptor (ligand gated cation channel) is activated by ATP [159] and most interestingly the platelet has both $\alpha_{2A}$ [160] (G$_{\alpha}$-coupled) and $\beta_2$ [161] (G$_{\beta\gamma}$-coupled) adrenergic receptors (platelet receptors and activation is reviewed in [147,162]. Hence, adrenalin may both have adenylyl cyclase (AC) activating and inhibiting effects on platelets. However, in experimental studies the $\alpha_{2A}$-effect is the most prominent in human platelets. Besides these examples of platelet activators, there are numerous more endogenous molecules that will partially activate or at least modulate platelet responses. It is likely that several are yet to be discovered and characterized.

**Platelet inhibitors**

Activation of platelets is almost as much about lack of platelet inhibition as presence of activators. The endothelium is the main factor in remaining the platelets in a quiescent state. This is obtained by the mere presence and thereby prevents contact between platelets and the sub-endothelial matrix. However, the endothelial cells also more actively produce platelet inhibitors. One mechanism is membrane bound ADPases (CD39) that degrades endogenously released adenine nucleotides (ADP and ATP) from the platelets and other blood cells. Furthermore, production of cyclic adenosine monophosphate (cAMP) elevating agents such as adenosine and prostacyclin (PGI$_2$) together with cyclic guanosine monophosphate (cGMP) elevating nitric oxide (NO) are other important platelet inhibitors derived from the endothelium. Adenosine has been known to inhibit platelet aggregation since 1963 [163]. Platelets possess G$_i$ coupled, adenosine binding, A$_{2A}$ receptors on the external membrane leading to AC activation and cAMP elevation which inhibit calcium influx and calcium mobilization from internal stores together with inhibition of aggregation [164-168]. PGI$_2$ binds to the G$_i$ coupled IP-receptor and thereby also generates cAMP [169,170]. AC activation leading to platelet inhibition is associated with phosphorylation of several proteins among others, actin binding protein (ABP) [171], vasodilator-phosphoprotein (VASP) [172], caldesmon [173], GP I$\beta$/I$\gamma$ [174], IP$_3$ receptor [175], G$_{13}$ [176] and Rab11b [177]. Endogenous regulation of platelets is further reviewed by Jin et al [178].

**Nitric oxide (NO)**

The first description of using NO in treatment appeared in 1867 when Brunton reported about patients relieve from angina pectoris when inhaling amyl nitrite [179]. However the vasorelaxing effect of NO had been observed earlier in 1859 as flushing upon inhalation of amyl nitrite [180]. The true nature of NO as a vasorelaxant was not described until Murad, Furschott and Ignarro presented their works in the 1980s. Their work resulted in the Nobel Prize in 1998.

NO can appear in three redox forms (NO$^-$, NO$^+$ and NO$^+$). It has been reported that the only form capable to activate soluble guanylyl cyclase (sGC) is the uncharged NO radical; NO$^-$ (denoted NO
In this thesis) [181]. In cells, this radical is produced by NO-synthase (NOS) of which there are three different types (nNOS, eNOS, iNOS). The names of the different NOSs are correlated to their tissue of origin or characteristics. nNOS is found in neural tissue, iNOS is inducible and eNOS is found in endothelial cells. NOS catalyse the reaction in which L-arginine act as a substrate resulting in L-citrulline and NO as products. In this reaction the active NOS tetramer is formed by two NOS protein and two calcium-calmodulin molecules with NADPH and O₂ as co-substrates and (6R)-tetrahydrobiopterin (BH₄), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN) and iron protoporphyrin IX (haem) as co-factors [182]. Vascular endothelial expressed eNOS (NOS III) (1203aa, 133 kDa UniProtKB/Swiss-Prot P29474) [183,184] produce NO upon acetylcholine and bradykinin stimulation [185,186] to regulate vascular tone and platelet activity. nNOS (NOS I) (1434 aa, 161 kDa UniProtKB/Swiss-Prot P29475) produces NO that acts as a signalling molecule in neural transmission [182]. iNOS (NOS II) (1153 aa, 131 kDa UniProtKB/Swiss-Prot P35228) differs from the other two forms since NO produced from iNOS is not mainly for signalling purpose but to act as a cytotoxic agent [182]. The action of iNOS is believed to be of significance in macrophage cytotoxicity.

NO exerts most of its effects as a signalling molecule through the heme containing protein sGC [187]. Human sGC consist of an α part and a β part each of the two subunits can exist in two different forms, the α forms are denoted, α2 (82 kDa) and α3 (77 kDa) (α3 also denoted α1 [188]) and the β subunits are called β1 and β2 (both 70 kDa) [189]. NO diffuses into the cell and activate sGC through binding to the prosthetic heme group and thereby increasing catalytic activity regarding cGMP production from guanosine triphosphate (GTP) by 400 times [190,191]. Produced cGMP induces protein phosphorylation by activating protein kinase G (PKG) [191]. There are several molecular targets for NO some of which will be described below.

**Effect of nitric oxide on neutrophils**

The effects of NO on neutrophils are far from being completely understood. However, NO has an inhibitory action on neutrophils regarding LTB₄ production [192], chemotaxis [192], ROS production [89,192-194] and adherence to endothelium [195,196]. However, phagocytosis is a neutrophil response reported to be unaffected by NO-donors [197]. It is only the NO-mediated inhibition of neutrophil adherence to endothelium that shows dependence of cGMP elevation and the other cellular effects may be explained by oxidation, nitration, nitrosylation and radical scavenging. Nevertheless, the most prominent cellular consequence is the pro-apoptotic effect of NO that also is due to a mechanism unrelated to sGC [198-200].

**Effect of nitric oxide on platelets**

NO was established in the 1980s to be a platelet inhibitor [201,202]. The main mechanism of platelet inhibition is through the sGC/cGMP/PKG pathway. However other mechanisms,
presumably nitration and nitrosylation (nitrosation), may contribute to the inhibition especially at NO concentrations above 40 nM [203,204]. The molecular targets for PKG in platelets comprise the IP3 receptor [175], vasodilator stimulated phosphoprotein (VASP) [205,206], the adrenergic α2A receptor [207], the purinergic P2Y12 [207], heat-shock protein (hsp)-27 [208], TXA2 receptor [209], LIM and SH3 protein (LASP) [210] and Rap1b [211].

**Nitric oxide donors**

Substances able to release NO have been used since decades. Today NO-donors are still the first treatment in *angina pectoris*. There are several groups of NO donors of which the classical are considered sodium 3-morpholinosydnonimine (SIN-1), nitroprusside (SNP), organic nitrates (e.g. glyceryl trinitrate (GTN), isosorbide mononitrate (ISMN), pentaerythritol tetranitrate (PETN)), diazeniumdilates (NONOates, e.g. DEA/NO) and S-nitrosothiols (e.g. S-nitroso-glutathione (GSNO) and S-nitroso-N-acetyl-D,L-penicillamine (SNAP)) and a more recent group of NO hybride drugs (e.g. NO-NSAIDs) [212-214]. The NO donors can either spontaneously release NO (e.g. SNAP, SIN-1, DETA/NO) or demand tissue present (e.g. GTN, SNP). Despite the fact that NO exerts inhibiting effects on many aspects of platelet activation, no NO-donor has been developed and used clinically to prevent platelet activation.
Material and methods

Desialylation of AGP (neuraminidase and periodate) (Paper I, II and IV)

Desialylation was performed by incubating AGP with neuraminidase from *Clostridium perfringens* (100 mU/mg protein) at 37°C over night. AGP with modified sialic acid residues without changing the charge of the molecule, was produced by treatment with mild periodate. Briefly, AGP was incubated with 2 mM NaIO$_4$ dissolved in PBS, and then 10% (v/v) ethylene glycol and 20 mM NaBH$_4$ were added. The samples were desalted using PD-10 columns (GE Healthcare Bio-Sciences, Little Chalfont, UK), then lyophilized and stored at -70°C. The protein was reconstituted in ultra pure water before use.

Sialic acid analysis (Paper I and IV)

Sialic acid content of desialylated AGP was analysed by releasing the sialic acids by mild hydrolysis in 2 M acetic acid at 80°C for 3 h as described by Varki and Diaz [215]. The amount of released sialic acids was analysed by high pH anion exchange chromatography (Dionex Carbopack PA-100 column at 15°C) with pulsed amperometric detection (HPAEC-PAD) (Dionex, Sunnyvale, CA, USA). N-Glycolylneuraminic acid was used as an internal standard at a fixed concentration in the range of 42.4-62.2 μg/ml.

Isolation of neutrophils (Paper I, II and IV)

Isolation of neutrophils was performed essentially as described by the manufacturer of Polymorphprep™ (Axis Shield PoC AS, Oslo Norway). Heparinized peripheral blood from healthy blood donors was centrifuged through a density gradient of Polymorphprep™ according to manufacturer’s instructions. Separated PMNs were washed once in PBS before remaining erythrocytes were lysed through brief hypotonic lysis with ultra pure water at 4°C. When measuring intracellular calcium concentration neutrophils were incubated with 4 μM Fura-2-acetometylster (Fura-2-AM) for 30 minutes at 37°C. After the isolation procedure the neutrophils were resuspended in Krebs-Ringer glucose buffer and kept cold until used.

Culture of HL-60 cells (Paper I)

Human acute leukaemia myeloid (HL-60) cells were obtained from ATCC (Manassas, VA, USA) and cultured to undergo neutrophil-like differentiation as described by Sjögren et al. [216]. Briefly, the cells were grown in RPMI-1640 medium supplemented with penicillin-streptomycin (50 U/ml; 50 μg/ml) and heat inactivated foetal calf serum (10%). The differentiation of HL-60 cells towards a neutrophil-like phenotype was induced by 1.3% dimethyl sulfoxide (DMSO) for 5-6 days. HL-60 cells were loaded with Fura-2-AM (4 μM) at 37°C for 30 minutes.
Measurement of cytosolic calcium concentration (Paper I, III and IV)
Fluorescence signals from 2 ml aliquots of suspensions of Fura-2 loaded neutrophils, HL-60 cells or platelets were detected at 37°C under constant stirring using a Hitachi spectrofluorometer (model F-2000 or F-7000) (Hitachi Ltd., Tokyo, Japan) designed to measure intracellular calcium concentration. Fluorescence emission was registered at 510 nm during simultaneous excitation at 340 nm and 380 nm. The intracellular calcium concentration was calculated using the general equation described by Grynkiewicz [217]: 
\[
[Ca^{2+}]_i = \frac{K_d (R - R_{max})}{(R_{max} - R)(F_o/F_s)}
\]

Affinity chromatography (Paper I and IV)
Platelets and neutrophils were lysed using lysis buffer containing protease inhibitors (pepsstatin, leupeptin aprotinin and phenylmethylsulfonyl fluoride (PMSF)). Neutrophil lysate were pre-cleared through a 1 ml High-Trap NSH-activated column with immobilized BSA. Neutrophil and platelet lysate diluted in starting buffer (50 mM Tris-HCl supplemented with 50 mM CaCl₂; pH 7.5) were subsequent run through a column with immobilized AGP using an AKTAprime™ system (GE Healthcare Bio-Sciences). Non bound proteins were eluted with starting buffer and bound proteins were eluted with a 50 mM Tris-HCl buffer (pH 7.5) supplemented with 2.5 mM EDTA followed by a 100 mM glycin-HCL buffer (pH 2.2). PD-10 columns were used to desalt eluted proteins before lyophilization.

SDS-PAGE and Western blot (Paper I, III and IV)
Polyacrylamine gels (Bio-Rad, Hercules, CA, USA) (4-20% Paper I, 7.5% Paper III and 10% Paper IV) were used to separate proteins. Platelet reactions were stopped with an equal volume of Laemml sample buffer and by heating the samples to 95-97°C for at least 5 minutes. Separated proteins were transferred to immobilion-P (polyvinylidene fluoride (PVDF)) membranes (Millipore, Bedford, MA, USA) and blocked using 5% dry milk. Proteins of interest were detected using specific antibodies combined with biotinylated secondary antibodies and the membranes were developed with Western blot Luminol reagent (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Flow cytometry analysis of Siglec-5 expression (Paper I and II)
Neutrophil and HL-60 expression of Siglec-5 were detected by using a monoclonal Siglec-5 antibody (R&D Systems, Abingdon, UK) conjugated with Zenon labelling kit (Molecular Probes, Carlsbad, CA USA). The samples were kept on ice until analyzed on FACSCalibur (BD Biosciences, San Jose, CA, USA).

Chemiluminescence (ROS) (Paper II)
Neutrophil production of ROS was detected by luminol and horseradish peroxidase (HRP) enhanced chemiluminescence. ROS generation from 1x10⁶ cells/ml was detected using a Multi
biolumat LB 9505 C (Berthold, Wildbad, Germany) at 37°C or from 1x10^7 cells/ml in the FL-600 microplate reader with KC4 software (BioTek Instruments, Winooski, VT, USA). In the latter experimental set up the cells were activated with IgG opsonized zymosan.

**Isolation of human platelets (Paper III and IV)**

Human platelets were isolated from heparinized blood obtained from the blood bank at Linköping University hospital. Five parts of blood were mixed with one part acid-citrate dextrose (ACD)-solution (85 mM Na-citrate, 71 mM citric acid and 111 mM glucose) and centrifuged at 220g for 20 minutes to obtain platelet-rich plasma (PRP). To prevent endogenous activation of the platelets during the isolation procedure 100 μM ASA and 0.5 U/ml apyrase were added to the PRP. The platelets were isolated by centrifugation and subsequent resuspended in Hepes or Krebs-Ringer glucose (KRG)-buffer supplemented with 1 U/ml apyrase. Platelets were loaded with Fura-2-AM for 45 minutes at room temperature.

**Measurement of nitric oxide (Paper III)**

Amperometric measurement of NO from S-nitroso-N-acetyl-D,L-penicillamine (SNAP) and 1,2,3,4-Oxatriazolium, 3-(3-chloro-2-metylphenyl)-5-[(4-methylphenyl)sulfonyl] amino]-, hydroxide inner salt (GEA 3175) (GEA Pharmaceuticals, Copenhagen, Denmark) was performed with the ISO-NO mark II NO-meter (World Precision Instruments, Sarasota, FL, USA). The instrument was calibrated according to manufacturer’s instructions. The experiments were conducted in Hepes-buffer at both 37°C and room temperature.

**Determination of platelet cGMP content (Paper III)**

Platelet suspensions (2.5x10^8/ml) were exposed to different concentrations of SNAP and GEA 3175 and the reaction was stopped at different time points by adding 8.3% ice-cold trichloroacetic acid. The samples were centrifuged for 15 minutes at 4000g and supernatants were extracted with water-saturated diethyl ether. The aqueous phase was lyophilized and the reconstituted in Na-acetate (50 mM; pH 6.2). The levels of cGMP were determined by radioimmunoassay (RIA) previously described by Axelsson et al. [218].

**Whole blood aggregometry (Paper III)**

Platelet aggregation in whole blood was recorded as increase in impedance using a Chrono-Log lumiaggregometer (Chrono-Log, Haverston, PA, USA). Heparinized blood was mixed with NaCl (0.9%) in 1:1 ratio and heated under constant stirring (800 rpm) to 37°C. The effect of SNAP and GEA 3175 was recorded as reduction of ADP (40 μM) or PAR-1 activating peptide (SFLLRN) (10 μg/ml) induced aggregation.
**Measurement of platelet shape change, aggregation, P-selectin expression and ATP-secretion (Paper IV)**

Platelet shape change and aggregation was recorded as decrease or an increase in light transmission essentially described by Born [219]. The measurements were done in a suspension of platelets using a Chrono-Log lumiaggregometer at 37°C under constant stirring. ATP-secretion was monitored as light emission from luceferin under the influence of luciferase. In some experiments the PAR-4 activating peptide AYPGKF was used to induce aggregation. The P-selectin expression was analysed through flow cytometry using a FITC-conjugated anti-CD62P (BD Pharmingen, Franklin Lakes, NJ, USA) according to manufacturer’s instruction.

**Light microscopy (Paper IV)**

Platelet morphology was visualized by differential interference contrast (DIC) microscopy. The platelet samples were fixed by adding paraformaldehyde (4%) and subsequently mounted on glass slides and evaluated using a Zeiss Axiovert 200 microscope (Carl Zeiss, Jena, Germany).

**Apoptosis analysis (Paper IV)**

Isolated human neutrophils were incubated with AGP (0.5 mg/ml) for 4 hours in 37°C (O2 95% and CO2 5%). Apoptosis was measured using an Annexin V-FITC kit (R&D Systems) and the cells were kept on ice until analysed by flow cytometry using a FACSCalibur (BD Biosciences).

**Purification of AGP from human plasma (Paper IV)**

AGP was isolated from human plasma using dye-ligand chromatography followed by affinity chromatography [220,221]. The dye-ligand chromatography column consisted of Affi-Gel Blue Gel 100-200 mesh (Bio-Rad) packed in a FPLC column (XK26/20) (GE Health Bio-Sciences) which was connected in series to an AGP affinity column. Plasma samples were dialyzed against starting buffer (20 mM Na2HPO4/NaH2PO4; pH 5.8) over night. Precipitates were removed by centrifugation and supernatants were applied to the columns. AGP was eluted with 100 mM Glycine-HCl, pH 2.2. The collected fractions were neutralized with 200 mM Na2HPO4/NaH2PO4; pH 8.3 before dialyzed against sterile water and lyophilization.

**Statistical methods (Paper I, II, III and IV)**

Results are presented as mean with standard error of the mean (SEM) or standard deviation (SD) as indicated. Statistical significances between groups were calculated using analysis of variance (ANOVA) or student’s t-test. Repeated measurements were used when applicable. Data were analysed using GraphPad Prism 4 software (GraphPad Software Inc., San Diego, CA USA).
Results and discussion

Paper I and II

Several studies have shown a modulating effect of the acute-phase protein AGP on neutrophil functional responses [222-225]. The mechanism behind these effects is however largely unknown. We investigated the effect of AGP on human neutrophils regarding the capacity to induce intracellular signalling and ROS production (main findings are summarized in Figure 4). First we evaluated if AGP had any effect on isolated human neutrophils by measuring intracellular calcium mobilization. The results showed that AGP at the physiological concentration of 0.5 mg/ml induced an intracellular calcium rise in Fura-2 loaded human neutrophils, but the magnitude of the calcium response was however very small. It is well-known that carbohydrate containing molecules such as sulfatides are able to generate L-selectin mediated calcium signalling [53]. This mechanism is supposed to be mediated by glycans binding to and cross-linking L-selectin [226]. Since AGP is a highly glycosylated protein with a potential L-selectin binding capacity (i.e. expression of sialylated glycans like sLex) (Figure 1) we hypothesised that the calcium rise observed was mediated by AGP/L-selectin binding. This was tested by pre-incubating the neutrophils with the L-selectin antibody DREG-56. However, DREG-56 pre-treatment resulted in an unexpected enhancement of the AGP-induced calcium response. When pre-treated with DREG-56 the neutrophils responded to a concentration of AGP as low as 0.001 mg/ml with an EC50 value of 0.009 mg/ml. This enhancement of the AGP induced calcium response was not seen when neutrophils were pre-incubated with sulfatides, CD18 antibody (alone or cross-linked) or with the L-selectin antibody FMC 46. However, cross-linking of the L-selectin antibody FMC 46 sensitized the neutrophils to AGP. On the other hand both sulfatides and treatment with FMC 46 without cross-linking antagonized the DREG-56 enhancement of the AGP-induced calcium response. This could be regarded as a verification of a role of L-selectin in amplifying the following response of neutrophils to AGP. The results indicate that L-selectin is not the AGP-receptor and that a L-selectin mediated signal transform the neutrophils to a more AGP sensitive state.

The leukaemia cell-line HL-60 is often used as a model system in phagocytic research [227-229]. Since undifferentiated cells do not express L-selectin in contrast to DMSO differentiated HL-60 cells [216], we used HL-60 cells as a model for the role of L-selectin interaction. We showed that undifferentiated HL-60 cells do not respond with calcium elevation when stimulated with AGP (in the presence and absence of DREG-56 pre-treatment). However, HL-60 cells differentiated towards a neutrophil-like phenotype responded almost identical as normal neutrophils to AGP treatment. These HL-60 based results conclude that the effect attributable to AGP is amplified by L-selectin and that the AGP effect was not a mere phenomenon observed after cell isolation from whole-blood. The results conclude that AGP triggers intracellular calcium elevations in neutrophils and that pre-engagement of L-selectin is of main importance to enhance this response. L-selectin is, however, not the putative AGP receptor.
Figure 4. Summary of the main findings regarding AGP-induced signalling leading to increase in intracellular calcium concentration and ROS production in human neutrophils. Compounds used are shown in dotted boxes. Unclear signalling pathways are indicated with dotted lines. A line ending with a crossbar is indicating inhibition whereas an arrow means activation.
It has been shown that L-selectin engagement triggers calcium signalling and tyrosine phosphorylation of several proteins (e.g. Src family kinases and p38MAPK) [55-58,230]. Our results revealed that a calcium elevation per se, did not make the neutrophils more sensitive to AGP. The DREG-56 mediated enhancement of calcium mobilization caused by AGP was highly dependent on functional Src kinase tested by using the inhibitors PP2 and SU 6656 whereas the p38MAPK inhibitor SB 203580 did not have any effect. Further characterization revealed that PI3K inhibition with either LY 294002 or wortmannin significantly inhibited the AGP induced calcium rise whereas inhibition of SYK by piceatannol did not alter the AGP response. These results indicate that PI3K and Src family kinases are central parts of the L-selectin dependent enhancement of the AGP response. Moreover, our results showed that a high concentration of fMLP enhanced the calcium signalling capacity of AGP. Chemotactic peptide-mediated signal transduction has in fact been associated with both PI3K and Src activation [73,231-236]. Taken together the results indicate that L-selectin ligation by using DREG-56 induces Src and PI3K activation which is crucial for the amplification of the AGP response.

The most common pathway for receptor mediated calcium rise is through G-protein coupled activation of PLC, resulting in generation of IP3 which subsequently mediates calcium liberation from intracellular stores [237-242]. We tested the involvement of this signal pathway by inhibiting PLC prior to AGP activation. This was done by using the PLC inhibitor U73122 or by pre-activation of PKC. Specifically it has been shown that activated PKC desensitizes PLC signalling by inhibiting the coupling to the activated G protein [243]. To achieve this, the phorbol ester phorbol 12-myristate 13-acetate (PMA) was utilized. The results revealed that the AGP-induced calcium elevation was abolished by U73122 and greatly reduced by PMA. Taken together these results give evidence for a receptor-dependent PLC activation in AGP stimulated neutrophils.

The glycans on AGP are rich in terminal sialic acids (Figure 1) and L-selectin did not appear to be the AGP receptor we focused on the sialic acid binding lectins denoted Siglecs as potential receptors for AGP. It has previously been described that human neutrophils express Siglec-5 and Siglec-9 [68,69]. Most recently neutrophils have also been shown to express a third type of siglecs namely Siglec-14 [244]. Affinity chromatography of neutrophil lysate by using an AGP column revealed that Siglec-5, but not Siglec-9, binds to AGP. The AGP/Siglec-5 interaction was verified by the fact that Siglec-5 antibodies and sialyllactoses (3’sialyllactose (3’SL) (NeuAcα2,3Galβ1,4Glc) and 6’sialyllactose (6’SL) (NeuAcα2,6Galβ1,4Glc)) with Siglec-5 binding properties antagonized the AGP-induced calcium rise both in neutrophils and neutrophil-like HL-60 cells. The importance of sialic acids on AGP was verified by the minor calcium mobilizing capacity of two desialylated forms of AGP. We also found that HL-60 cells were positive for Siglec-5 and that they increase their expression of Siglec-5 during DMSO-induced differentiation (unpublished results) (Figure 5). Moreover, sialyllactoses did not only antagonize AGP induced calcium elevation
but also stimulated calcium rise on their own. Hence sialyllactoses may be classified as partial agonists for Siglec-5. Furthermore, flow cytometry analysis of Siglec-5 expression on neutrophils revealed a minor Siglec-5 up-regulation after fMLP activation, but no change was caused by the L-selectin mAb DREG-56. Consequently, a rapid up-regulation of Siglec-5 can not explain the DREG-56-mediated enhancement of AGP-induced calcium mobilization. Together the results suggest that sialic acid residues on AGP bind to Siglec-5 on the surface of neutrophils and this is followed by calcium signalling.

Src tyrosine kinases plays a pivotal role in neutrophil intracellular signalling pathways and phosphorylates among others immunoreceptor tyrosinebased activation motifs (ITAMs) and immunoreceptor tyrosinebased inhibitory motifs (ITIMs) [245,246]. Siglec-5 comprises ITIM-domains but the relation to cellular function is however unclear [68]. For example, an ITIM unrelated inhibition of calcium elevation in rat basophilic leukaemia cells has been shown upon Siglec activation [247]. Furthermore, another study show that Siglec-5 is able to enhance chemotactic peptide-induced respiratory burst in human neutrophils [248]. Other published reports are showing an inhibitory effect on intracellular calcium signalling obtained by cross-linking Siglec antibodies [247]. The experimental set-up in those studies differs significantly from ours and the results are therefore not fully comparably. Our experimental design regarding intracellular calcium concentration provides strong evidence of a L-selectin mediated enhancement of Siglec-5 signalling capacity. Due to the rapid onset of the AGP-induced calcium response, phosphatase recruiting ITIM activation is probably not involved in the calcium rise seen in our experiments. The intracellular domain of Siglec-5 also contain a signalling lymphocyte activation molecule (SLAM)-associated protein (SAP) binding motif which could explain the differences in calcium regulation observed [249,250]. Another explanation for the somewhat contradictory results is the fact that a Siglec-14 with almost identical extra-cellular characteristics and glycan binding properties as Siglec-5 has

Figure 5. Histogram from flow cytometry analysis of Siglec-5 expression on HL-60 cells at different stages of differentiation. The figure is showing one representative histogram out of five. (Unpublished results)
recently been described to be expressed on neutrophils [71,244]. This novel Siglec-14 is associated with the activating adaptor protein DAP-12 and hence could work in an activating fashion.

One important issue was to correlate the AGP-induced calcium response with functional responses. Intracellular calcium elevation has been coupled to ROS generation in neutrophils [92,96-99,251,252]. However, the small calcium rise generated by AGP (0.5 mg/ml) did not elicit ROS generation neither did DREG-56 pre-treatment followed by AGP cause any production of ROS. Apparently, the AGP induced calcium rise after DREG-56 stimulation was not sufficient to activate the NADPH-oxidase. Furthermore, pre-treatment with AGP or DREG-56 followed by AGP did not prime the neutrophils to a more powerful fMLP-induced ROS response nor was the zymosan induced ROS response amplified by AGP pre-incubation. However, in experiments in which the neutrophils were activated with fMLP (100 nM) a subsequent addition of AGP induced a prominent ROS generation. This indicates that if the NADPH-oxidase had been assembled and activated by a strong activator as fMLP the subsequent calcium rise elicited by AGP was sufficient to reactivate the oxidase. Our data using the NADPH-oxidase inhibitor diphenyleneiodonium chloride (DPI) verified that the observed ROS response was actually caused by the NADPH-oxidase. Furthermore, it was found that AGP introduced 3-10 minutes after fMLP elicited the maximum ROS response and when the time gap between fMLP and AGP was prolonged to 30 minutes the ROS generation was almost abolished, probably due to disassembly of the NADPH-oxidase subunits. Interestingly, an AGP concentration of 0.05 mg/ml, which is ten times lower than the normal plasma concentration, was sufficient to evoke maximal ROS generation in neutrophils. This indicates that the mechanism behind this ROS production might be very sensitive. The importance of sialic acid residues was also observed when measuring ROS production in fMLP pre-stimulated neutrophils. Those results showed that desialylated AGP had a significant lower ROS generating capacity. Furthermore, AGP-induced ROS response was reduced by pre-treatment with sialyllactoses. The AGP-induced calcium rise might consequently be responsible for the reactivation of a functional, but quiescent NADPH-oxidase in fMLP stimulated neutrophils. In summary, AGP cannot by itself evoke ROS production in unstimulated neutrophils nor could AGP amplify fMLP-induced ROS production. However, the addition of AGP after fMLP showed a sialic acid dependent ROS generation and this is most likely linked to the previously described calcium rise. Together, these results revealed that AGP directly modulates the outcome of a functional consequence of neutrophil activation. AGP may thus be considered as a biological active component in inflammation responses.

**Paper III and IV**

When isolated platelets were exposed to AGP they respond with a rapid and prominent shape change with no other manifestation of platelet functional responses. To our knowledge this is the first evidence for a direct effect of AGP on platelet functional responses. Earlier studies have reported that AGP inhibits agonist-induced aggregation [253-255]. Indeed we also found that the
platelet aggregation response to a PAR-4 activating peptide was reduced in AGP pre-treated platelets. The antagonizing effect of AGP on PAR-4-induced aggregation was however small. Furthermore, earlier studies have reported that AGP has anti-apoptotic effects under some specific conditions [256-258]. However, AGP treatment did not alter the viability of platelets and neutrophils tested by Annexin V binding on human neutrophils or by monitoring intracellular calcium concentration in platelets after long-time AGP exposure. The Annexin V binding was identical in AGP (0.5 mg/ml; 4 h) treated neutrophils compare to untreated cells. Furthermore, platelets exposed to AGP (0.5 mg/ml; 30 minutes) showed stable basal calcium concentration and responded just as well as control cells to PAR-1 activating peptide (10 μg/ml) stimulation. In conclusion, AGP stimulated platelet shape change, but no alteration in cell-viability was observed observed upon exposure of isolated platelets or neutrophils to AGP.

More specifically we found that AGP, at the physiological concentration of 0.5 mg/ml was able to induce shape change detected by light transmission measurement (the main findings are summarized in Figure 6). DIC microscopy clearly showed sphering of the platelets and centralization of granules, called internal contraction which is a cellular response that is a part of platelet activation (Figure 3) [259]. However, AGP did not cause dense- or α-granule secretion or aggregation. Affinity change of the αIIbβ3 integrin is a prerequisite for platelet aggregation [260,261]. Our result showed that AGP did not induce aggregation in platelets even in the presence of external fibrinogen. This suggests that the acute-phase protein lacks the capacity to change the integrin αIIbβ3 to its high affinity state that allows fibrinogen binding and to cause platelet degranulation. Furthermore, these results also indicate that the integrin αIIbβ3 is not the receptor for AGP.

Platelet shape change is the first morphological sign of activation and is mediated by increased phosphorylation of myosin light chain (MLC) [262]. This phosphorylation can occur either through calcium/calmodulin-dependent activation of myosin light chain kinase (MLCK) or inactivation of myosin light chain phosphatase induced by Rho-kinase phosphorylation [263-268]. AGP provoked a minor calcium rise in Fura-2 loaded isolated platelets. However, this minor calcium rise was not the main mechanism behind the shape change since BAPTA/AM pre-treatment abolish the calcium rise but the shape change could still be detected. On the contrary Rho-kinase inhibition by Y-27632 reduced significantly the AGP induced shape change. When combining BAPTA/AM and Y-27632 the shape change was abolished, demonstrating that calcium at least contributed to the response. Notably, inhibitors to SYK, Src or PI3K did not affect the AGP induced shape change indicating for restricted intracellular signalling mainly through Rho/Rho-kinase and to lesser extent through intracellular calcium mobilization. Taken together AGP can transmit two signals leading to platelet shape change, one weaker; a minor calcium elevation and a more powerful; Rho/Rho-kinase. The involvement of Rho-kinase was confirmed by studying the Rho-kinase specific phosphorylation of myosin phosphatase target subunit-1 (MYPT1) [269,270]. This phosphorylation of Thr696 in
Figure 6. Summary of the main findings in platelets regarding AGP and nitric oxide. Compounds used are shown in dotted boxes and methods used are indicated in solid boxes. Unclear pathways are indicated with dotted lines. A line ending with a crossbar is indicating inhibition whereas an arrow means activation.
MYPT1 inhibits the enzyme and thereby activates myosin light chain by increasing its phosphorylation leading to platelet shape change [271,272]. Western blot verified that a Rho-kinase dependent MYPT1 phosphorylation was caused by AGP. Actually, AGP was equally effective in inducing MYPT1 phosphorylation as a high dose of thrombin (0.1 U/ml). Furthermore, the phosphorylation was completely suppressed by the Rho-kinase inhibitor Y-27632. Taken together, AGP activates the Rho-kinase signalling pathway and induce small calcium responses and this is followed by spherical platelet shape.

No receptor for AGP has previously been defined on platelets. Based on our findings on neutrophils (Paper I) and observations of another research group [254], we hypothesised that terminal sialic acid residues might be important for the shape change induced by AGP. However, desialylated AGP (neuraminidase-or periodate-treated AGP) did not reduce the degree of platelet shape change, moreover we could not detect Siglec-5 in platelet lysate. Therefore we conclude that sialic acid and Siglec-5 are not involved in the AGP induced platelet shape change. Hence, the effects of AGP on neutrophils and platelets represent two entirely different mechanisms. The results in the present thesis also exclude aαβ3 and GPIbα (the vWF receptor) as receptors for AGP on platelets. The platelet express several collagen-binding proteins, such as GPVI, aαβ1 and CD36 [145]. In this regard, sub-aggregatory concentrations of collagen and thrombospondin-1 reduced the shape changing capacity of AGP. This indicates that AGP might interact with the same receptor as collagen and thrombospondin-1 or alternatively that collagen/thrombospondin-1 interferes with the intracellular signalling pathways used by AGP (i.e. pre-activation or inhibition of the Rho/Rho-kinase signalling pathway). In conclusion, the precise receptor(s) contributing to the AGP-induced shape change remains to be determined.

To put the effect of AGP in a more physiological context we treated the platelets with endogenous inhibitors. Indeed we found that the NO-donor SNAP or adenosine abolished the AGP-induced shape change. Additionally, pre-incubation of platelets for prolonged time with the drugs revealed that the inhibitory effect of adenosine was diminished whereas the effect of the NO-donor still was prominent. This finding was rather surprising since the inhibition of NO on some aspects of platelet activation can be short-lasting (Paper III). However, in our experiment VASP phosphorylation represent a long-lasting effect of NO and VSAP is known to be a key molecule in platelet shape change [273]. Based on this, it is tempting to speculate that the persistent VSAP phosphorylation seen in NO stimulated platelets might explain its strong antagonizing effect on AGP-induced shape change. This remains however to be elucidated. Furthermore, the NO/sGC/cGMP/PKG signal pathway may also cause Rho phosphoryletion on Ser188 residue [274]. In smooth muscle cells and glial cells this phosphorylation has been shown to inhibit the Rho/Rho-kinase pathway leading to suppression of transcription [275,276]. This mechanism has so far not been established in platelets. Furthermore, in vascular smooth muscular cells (VSMC) NO/sGC/cGMP/PKG mediates phosphorylations at Ser692, Ser695 and Ser852 residues on MYPT1 [277].
phosphorylation prevents inactivation of the phosphatase by the Rho/Rho-kinase dependent Thr696 phosphorylation. However, none of these PKG specific phosphorylations of MYPT1 have been described in platelets. In conclusion, all these molecular targets for the NO/sGC/cGMP/PKG pathway (i.e. VASP, Rho and MYPT1) may explain the long-lasting inhibition by the NO-donor SNAP on AGP-induced shape change. Moreover, our findings imply that endothelial cells, by releasing NO and adenosine may constitutively and totally counter balance the modulating effect of AGP on platelets.

NO releasing drugs (NO-donors) are widely used both in vivo and in vitro experimental systems. However, the detailed pharmacological profiles of these are not fully understood. We investigated the characteristics of two chemically different NO-donors, SNAP and GEA 3175 (Table I). The results showed that the well-known NO-donor SNAP released NO in an aqueous solution detected by the NO-sensor ISO-NO mark II. The kinetics of the NO release from S-nitrosothiols are depending on light and metal ions in the solution [278-280]. All physiological buffers contain transition metals and thereby stimulate NO generation from S-nitrosothiols [279]. When experiments were done under the same conditions NO could not be detected in the solution from the oxatriazole derivative GEA 3175 up to a concentration of 100 μM. The presence of human plasma (10 %) did not alter the absence of NO release from GEA 3175. This is in accordance with earlier studies stating that tissue has to be present to be able to detect NO from GEA 3175 [281]. However, when turning to radioimmunoassay (RIA) measurements the results showed that the two drugs were equally effective to induce cGMP generation in human platelets. The cGMP peak after SNAP (10 μM) or GEA 3175 (10 μM) addition appeared already after 20 seconds. Both SNAP and GEA 3175 had the capacity to reduce thrombin-induced elevation of intracellular calcium already after 5 seconds of pre-incubation in isolated human platelets. This concludes that a measurable amount of NO is not a prerequisite for cGMP elevations and inhibition of agonist-induced calcium responses.

**Table I. Summarized effects of SNAP and GEA 3175 in platelets.**

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<thead>
<tr>
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<th>SNAP</th>
<th>GEA 3175</th>
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<tr>
<td>NO release</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>cGMP</td>
<td>++</td>
<td>+</td>
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<tr>
<td>[Ca^{2+}]i inhibition (short time)</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>[Ca^{2+}]i inhibition (long time)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>GC dependent</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>p-VASP</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Whole blood aggregometry</td>
<td>+</td>
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</tr>
<tr>
<td>Inhibition of AGP response</td>
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Earlier studies have actually indicated for a higher potency of GEA 3175 than SNAP [282-284]. Indeed, when using a pre-incubation time of 2 minutes GEA 3175 was significantly more effective in reducing thrombin-induced calcium elevation compared to SNAP. This might be explained by the higher amounts of cGMP initially produced by SNAP-stimulation, leading to a more powerful phosphodiesterase (PDE)-5 activation and thereby a more rapid negative feedback mechanism [285]. It is well-known that NO inhibits agonist-induced calcium increase in platelets and most of this effect is mediated via cGMP elevation and subsequent PKG activation [286]. The sGC inhibitor ODQ abolished the calcium lowering effect induced by GEA 3175 indicating for an exclusive role of sGC and cGMP. This means that the oxatriazole derivative solely acts through sGC without marked NO release. The reduction in thrombin-induced calcium influx from the extracellular environment was greater in platelets treated with GEA 3175 than SNAP at both incubation times used (2 and 30 minutes). It was also found that GEA 3175 was more effective in reducing liberation of calcium from intracellular stores than SNAP at shorter incubation times (2 minutes). However, it is evident that the calcium lowering effect of both SNAP and GEA 3175 decline over time and that they are in that aspect rather short acting. Furthermore, we conclude that NO-donors are more effective in inhibiting influx of calcium compared to calcium released from intracellular stores.

The NO/sGC/cGMP/PKG pathway induces phosphorylation of VASP and this is assumed to be a key feature for platelet inhibition. Western blot analysis showed that both SNAP and GEA 3175 were equally effective in inducing Ser239 phosphorylation of VASP. Interestingly the VASP phosphorylation was equally pronounced 2 minutes after NO donor addition as after a incubation time of 30 minutes. This indicates for a persistent molecular modification induced by the NO-donors. This stands in contrast to the more short duration of the inhibition of thrombin-induced calcium elevation. It is known that VASP phosphorylation correlates with platelet inhibition, however, no connection to intracellular calcium regulation has been shown in platelets [172]. The effectiveness of NO-donors in experiments using whole blood is very variable. We performed impedance measurements in whole blood as a method to evaluate the effect of the drugs on platelet aggregation in a more complex and in vivo-like environment. SNAP had a greater capacity than GEA 3175 to reduce platelet aggregation when using ADP as activator. If the PAR-1 activating peptide was used as activator neither SNAP nor GEA 3175 were able to reduce the aggregation. Blood contain several components that may interfere with NO mediated signalling. The most obvious is the presence erythrocytes; as haemoglobin is a well-characterised scavenger of NO [287]. Our hypothesis that an unstable NO-donor like SNAP might be more susceptible to scavenging action in whole blood and thereby a less effective platelet inhibitor turned out wrong. Why the more stable NO-donor GEA 3175 was ineffective in whole blood aggregation remains to be investigated. In summary our results show that the duration of effects attributable to NO may vary significantly. Moreover, the present findings also propose that release of NO is not a prerequisite for a marked activation of sGC in platelets.
Conclusions
The principal finding in this thesis is that the acute-phase protein AGP exerts modulator roles on both neutrophils and platelets. These effects were previously unknown. The exact roles of the findings in this thesis are a subject of further investigation.

The research in the present thesis specifically shows that;

- AGP causes intracellular calcium rise in human neutrophils. This effect is dependent on terminal sialic acid residues on AGP and Siglec-5 on the surface of neutrophils. This mechanism is significantly enhanced when L-selectin is pre-engaged.
- The calcium rise is mediated by activation of PLC in the neutrophil. The interplay between L-selectin/Siglec-5 involves Src-kinase and PI3K.
- AGP induces ROS production in fMLP pre-treated neutrophils. This effect is also dependent on the sialic acid residues on AGP.
- AGP mediates platelet shape change through a Rho-kinase dependent MYPT1 phosphorylation.
- The platelet shape change by AGP is abolished by pre-treatment with adenosine or NO. The inhibition triggered by the latter compound is surprisingly long-lasting.
- GEA 3175, in contrary to SNAP, does not spontaneously release detectable amounts of NO in a buffer solution, but exerts cGMP-dependent inhibitory actions in platelets.
- SNAP is more effective than GEA 3175 in stimulating cGMP production in human platelets during a short time basis (seconds) of incubation.
- SNAP and GEA 3175 are equally effective in antagonizing thrombin provoked intracellular calcium rise but the effect of the latter drug was more long lasting. Both the NO containing compounds are more effective in inhibiting calcium influx than release from intracellular stores.
- Both SNAP and GEA 3175 provoke a long-lasting Ser239 phosphorylation of VASP.
Acknowledgement

I would like to show my appreciation to:

Dr. Magnus Grenegård, my supervisor, who with a never ending enthusiasm supported me through this work.

My co-workers, Prof. Peter Påhlsson and Louise Levander for fruitful collaboration in the field of glycobiology.

Dr. Anna Asplund-Persson for all the work within the nitric oxide area. It was pure enjoyment working with you.

Prof. Rolf Andersson for putting the facilities of the department at my disposal and for learning me all about the use of egg-cups.

My friends, Dr. Johan Paulsson for biomedical discussions at the gym and Dr. Martin Tinnerfelt-Winberg for helping me with the flow cytometry.

My fellow PhD-students, Caroline Skoglund and Dr. Andreas Eriksson for general discussions about platelets but also for the not so scientific movie discussions.

All the students passing through the lab over the years, it has been very educational. A special thanks to Louise Fornander for contributing with excellent data in paper II.

My families, new and old, for support and encouragement.

My wife, Lina, without whom this book may not have been written.
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