Mechanisms of Action and Targets for Actual and Future Antiplatelet Drugs

Kathleen Freson*,[1], Chantal Thys[1], Christine Wittevrongel[1] and Chris Van Geet[1,2]

[1]*Center for Molecular and Vascular Biology and* [2]*Department of Pediatrics, University of Leuven, Leuven, Belgium*

Abstract: Platelets are key players in arterial thrombosis and have become important targets in the primary and secondary prevention of atherothrombosis. Antiplatelet drugs are primarily directed against platelets and inhibit platelet activation by a number of different mechanisms. They are used for the prevention and treatment of thrombotic processes, especially in the arterial vascular system. Antiplatelet drugs in clinical use and experimental drugs are discussed.

INTRODUCTION

Platelets play a central role in the hemostatic process and consequently are similarly involved in the pathological counterpart, thrombosis. They are discoid cells, derived from megakaryocytes in the bone marrow that under normal conditions neither adhere to each other nor other circulating and endothelial cells. When blood vessels are damaged at their luminal side, platelets adhere to the exposed subendothelium. This adhesion is mediated by collagen and von Willebrand factor (vWF) both of which are exposed at the subendothelial surface. Adherent platelets release various factors, which activate other nearby platelets resulting in the recruitment of more platelets at the site of vascular injury. Initially, activated platelets change their shape, an event immediately followed by the secretion of platelet granule contents (including ADP, fibrinogen and serotonin) as well as by platelet aggregation. Aggregation of platelets is mediated by fibrinogen or vWF [1]. They connect platelets by bridging complexes of glycoprotein GPIIb/IIIa (integrin $\alpha IIb\beta 3$) on adjacent platelets, forming a stable platelet aggregate. These platelet-rich thrombi are important for the acute occlusion of stenotic vessels and ischemic injury to heart and brain [2, 3]. The activation of platelets with exposure of negatively charged phospholipids (e.g., phosphatidylserine and phosphatidic acid) facilitates the assembly of coagulation factors on the activated platelet membrane, leading to generation of thrombin and subsequent fibrin deposition to stabilize the initial platelet plug. Anticoagulants inhibiting the coagulation cascade are widely used to prevent acute occlusion of arteries but are not discussed upon in this review.

Antiplatelet drugs are primarily directed against platelets and inhibit platelet activation by a number of different mechanisms. A range of anti-platelet drugs are currently used, both prophylactically and therapeutically, in regimens to manage thrombo-embolic disorders. Three main classes of antiplatelet agents are currently available for clinical use: cycloxygenase inhibitors, thienopyridines, and intravenous GPIIb/IIIa antagonists. The discovery of the ultimate antiplatelet therapy that selectively targets pathological thrombus formation without undermining hemostasis remains elusive, although recent progress in unraveling the molecular events regulating thrombosis has provided promising new avenues to solve this long-standing problem. Potentially interesting novel targets are put forward such as inhibitors of vWF-GPIb activation, inhibitors of thrombin receptors, inhibition of collagen receptors and more. We recently found that the pituitary adenylyl cyclase-activating polypeptide (PACAP) modulates platelet function by stimulating its Gs receptor VPAC1 and thereby generating the platelet inhibitory second messenger cAMP [4]. Many of the currently available antiplatelet drugs and some novel approaches in treatment of cardiovascular disease are briefly discussed upon.

THE CYCLOOXYGENASE INHIBITOR ASPIRIN AND THE ARACHIDONIC ACID CASCADE

Aspirin was originally discovered by Felix Hoffman in 1897 and marketed to the public by the Bayer AG company two years later. It was introduced in 1899 as a non-steroidal anti-inflammatory drug (NSAID) and in the early 1970s it was also found to be an antiplatelet agent as it inhibits the arachidonic acid metabolism [5, 6]. Over the past 50 years the mechanism of the antiplatelet effect of aspirin has been elucidated in detail and a database consisting of over 100 clinical trials of aspirin prophylaxis in a wide range of cardiovascular disorders now provides a solid basis for assessing the balance between benefits and risks of drugs in the whole spectrum of atherothrombosis [7, 8].

Aspirin or acetylsalicylic acid is a synthetic compound that becomes acetylated at the hydroxyl group of salicylic acid (o-hydroxybenzoic acid) (Fig. **1**). Aspirin inhibits the cyclooxygenase (COX) activity of the enzyme prostaglandin H-synthase (PGHS), which catalyses the conversion of arachidonic acid and oxygen to PGH2, the first committed step in prostanoid synthesis. PGH2 is the precursor of PGD2, PGE2, PGF2α, and TXA2 (thromboxane A2) [9]. The aspirin-induced COX blockade eventually leads to a permanent defect in the TXA2-dependent platelet function. Two isozymes are known, called PGHS-1 or COX1 and PGHS-2 or COX2, which are both heme-containing, glycosylated proteins with two catalytic domains [10]. COX1 is ubiquitously expressed, including in platelets, and COX2 expression is rapidly induced in monocytes and endothelial cells after induction with various inflammatory and mitogenic mediators. The molecular mechanism of

*Address correspondence to this author at the Center for Molecular and Vascular Biology, University of Leuven, Herestraat 49, B-3000 Leuven, Belgium; Tel: 32-16-345775; Fax; 32-16-345990;
E-mail: Kathleen.freson@med.kuleuven.ac.be

1389-5575/06 $50.00+.00

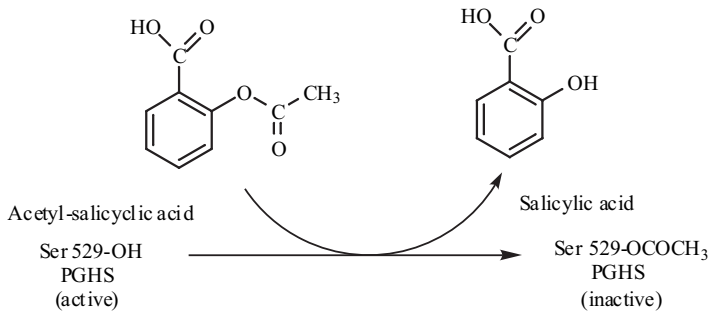

Fig. (1). Aspirin (acetylsalicylic acid) irreversible inhibits PGHS activity by a selective acetylation of the hydroxyl group of serine residue 529.

permanent inactivation of COX activity by aspirin is related to blockade of the COX channel as a consequence of acetylation of the hydroxyl group of a strategically located serine (serine-529 residue of human COX1 and serine-516 in human COX2), that prevents access of the substrate to the catalytic site of the enzyme [11] (Fig. **1**). X-ray crystallographic analysis of the aspirin-acetylated enzyme revealed that when serine-529 is acetylated by aspirin, the acetyl group protrudes into the cyclooxygenase channel at a critical site for arachidonic acid interaction with the tyrosine residue responsible for initiating catalysis [11]. The inhibition of COX2 by aspirin is similar but much higher aspirin concentrations are needed compared to the amount for COX1 inhibition. This may account at least in part for the substantial different daily dose requirements for the analgesic/anti-inflammatory (300 mg/2000 to 5000 mg) versus antiplatelet effects (30 to 75 mg) of this drug.

Aspirin is potent inhibitor of platelet aggregation induced by arachidonic acid but it is a relatively weak antiplatelet agent when platelets are stimulated by a number of other agonists, including ADP and thrombin. Aspirin does not prevent the α-granule release in response to different agonists, nor does it inhibit the epinephrine-induced platelet aggregation [12]. Several other stimuli, including shear forces and increased plasma catecholamines, can activate platelets and contribute to acute arterial thrombosis despite aspirin therapy. Though aspirin is not the perfect antiplatelet drug, it is obvious that aspirin can be used as primary and secondary prevention against cardiovascular disease [13, 14]. Because platelets are enucleated cells and only express COX1, irreversible inhibition of this enzyme prevents TXA2 formation for the rest of the platelet's life (~ 10 days). Only new platelets can therefore regain the ability to synthesize platelet TXA2. After a single dose of aspirin (325 mg), total whole blood COX activity recovers by 10 % per day as a function of platelet turnover [15].

After oral uptake, aspirin is rapidly adsorbed by the mucosa of the stomach and upper intestine, with a systemic bioavailability approaching 50% for single doses in the range of 20 to 1300 mg [16]. It is detectable in blood after 20 minutes of uptake and reaches a peak plasma level within one hour [17]. Enteric-coated aspirin preparations are more slowly absorbed in the small intestine, and have less bioavailability compared to the regular aspirin. Plasma half-life of aspirin is about 20 minutes but due to the fact that the COX1 inhibition is permanent, the effect of a single aspirin dose lasts for the lifetime of the platelet. Hemostasis may be normal with a normal bleeding time within 3 to 4 days after uptake of a single dose.

The relatively new but still vaguely defined term 'aspirin resistance' has been used to describe a number of different phenomena, including the inability of aspirin to: (1) protect individuals from thrombotic events, (2) cause a prolongation of the bleeding time, or (3) produce an anticipated effect on one or more *in vitro* platelet functional tests [18, 19]. Depending on the method used for assessing platelet function, about 40 % of either healthy persons or individuals with known cardiovascular disease may be resistant to the antiplatelet effects of aspirin [18, 20, 21]. Though it is generally accepted that conventional doses of aspirin may fail to inhibit the platelets of some individuals, the mechanisms underlying this problem are still unknown. In at least some individuals, aspirin resistance may result from increased baseline platelet reactivity, capable of overriding the inhibitory effects of aspirin, particularly given at a low dose. The platelet hyperreactivity may result from either a genetic factor (as functional polymorphisms in the genes coding for the different enzymes involved the arachidonic pathway or for the main platelet receptors) [22] or it is an acquired condition. There is a significant circadian variation in platelet reactivity and several daily-life activities, such as exercise, caffeine consumption, or cigarette smoking may increase the risk. Estrogen levels and hormone replacement therapy may also induce platelet hyperreactivity. Finally, aspirin resistance may also occur secondarily to pharmacological interventions as with certain non-steroidal anti-inflammatory drugs [23-25].

THIENOPYRIDINES AS PURINERGIC RECEPTOR ANTAGONISTS AND THE ADP SIGNALING PATHWAY

Ticlopidine and clopidogrel are structurally related thienopyridines with similar platelet-inhibitory properties and are the only platelet purinergic receptor antagonists currently available for clinical use [26, 27]. Both drugs bind irreversibly to the P2Y12 receptor on platelets and selectively inhibit the ADP-induced platelet aggregation for the rest of the platelet's life (Fig. **2A**) [28]. In addition, because P2Y12 participates in the amplification of platelet aggregation induced by other agonists as thrombin, chemokines, epinephrine, or TXA2, thienopyridines indirectly inhibit platelet aggregation induced by these other agonists besides ADP [29]. The first thienopyridines were

developed as antiflogistic agents in 1972. Three years later ticlopidine was introduced and in 1980 it was used to prevent platelet aggregation and thrombus formation in cardiac surgery with extracorporal circulation [30]. In 1986 clopidogrel was developed as successor of ticlopidine [31].

Ticlopidine

The chemical name for ticlopidine is 3-[(2-chlorophenyl)methyl]-7-thia-3-azabicyclo[4.3.0]nona-8,10-diene hydrochloride (or C14H15Cl2NS) with a molecular weight of 300.247 (Fig. **2B**). Side effects are relatively common with ticlopidine and about 20 % of patients are forced to discontinue it. Ticlopidine treatment can induce hypercholesterolemia, neutropenia, aplastic anemia, thrombocytopenia and the serious thrombotic thrombocytopenic purpura (TTP).

Clopidogrel

Clopidogrel is a chemically derivative of ticlopidine and contains a carboxymethyl group in the benzylic position that improves its pharmacological activity compared to its mother compound. Chemically it is methyl 2-(2-chlorophenyl)-2-(7-thia-3-azabicyclo[4.3.0]nona-8,10-dien-3-yl)acetate; sulfuric acid (Fig. **2b**). The empirical formula of clopidogrel bisulfate is C16H18ClNO6S2 and its molecular weight is 419.9. Clopidogrel is a chiral drug: the S-enantioner requires metabolic activation in the liver *in vivo* while the R-enantiomer is inactive. In contrast to ticlopidine, clopidogrel has demonstrated an excellent side-effect report and is well-tolerated even using high doses (600 mg). There are no reported cases of clopidogrel-induced neutropenia and the incidence of TTP is 100-fold less than with ticlopidine [32].

Both thienopyridines are pro-drugs that lack antiplatelets effects in vitro, requiring first passage through the liver to become biologically active. The active metabolite of ticlopidine that inhibits the ADP-induced platelet aggregation is PCR3787 [33]. Clopidogrel is metabolized by the hepatic cytochrome P-450-1A to a recently identified active metabolite [34,35]. Both drugs are orally administrated but clopidogrel is more active compared to ticlopidine on a weight basis and the antiplatelet activity is

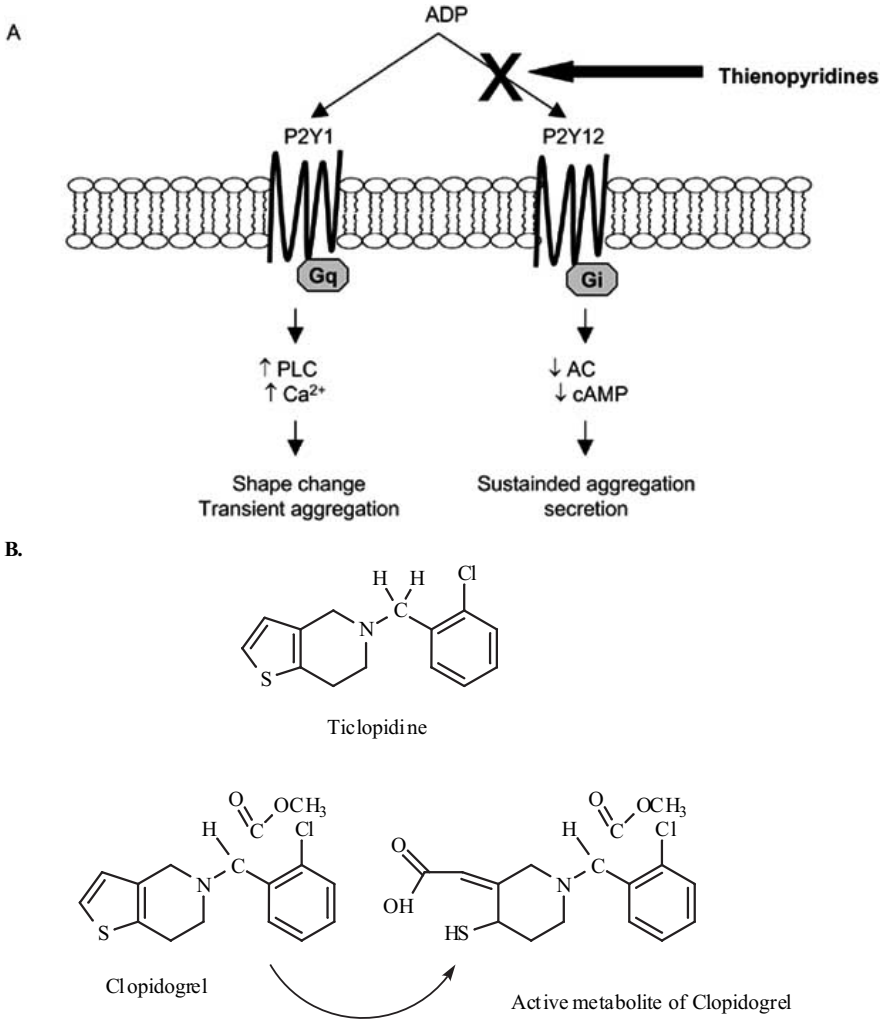

Fig. (2). A. The platelet membrane contains two ADP purinergic receptor subtypes classified as P2Y1 and P2Y12. ADP induces platelet aggregation *via* activation of both P2Y1 and P2Y12. ADP stimulation of the Gq-coupled P2Y1 receptor activates phospholipase C causing internal calcium mobilization and a platelet shape change. Activation of the Gi-coupled P2Y12 receptor leads to inhibition of adenylyl cyclase mediating a drop in the cAMP level. The P2Y12 receptor, which is the target of thienopyridines, sustains ADP-induced platelet aggregation. B. Chemical structures of ticlopidine, clopidogrel and the active metabolite of clopidogrel.

much faster after single administration and more stable after repeated administration. The rather slow onset of action is due to the fact that the in vivo activity of thienopyridines relate to their metabolism: platelet inhibition reaches a plateau after three to five days with ticlopidine and after four to seven days with clopidogrel. A rapid therapeutic effect can only be achieved after administration of a high initial loading dose of 500 mg for ticlopidine and 600 mg for clopidogrel. The recent CREDO trial suggests that at least 6 hours may be required for a 300 mg bolus dose of clopidogrel to exert any clinically meaningful effect [36].

Different clinical trials have been designed to establish the pathophysiologic conditions in which thienopyridines could be safer and useful. Ticlopidine treatment was shown to be effective in neurologic ischemic events, in secondary prevention of unstable angina, acute myocardial infaction, percutaneous transluminal coronary angioplasty and intermittent claudication [37-39]. However due to its numerous side effects and the enhanced risk of bleeding when it is used in combination with aspirin, ticlopidine was replaced by clopidogrel.

The CAPRIE trial involving 19185 patients divided in three groups: subjects with a history of recent ischemic stroke, subjects suffering from myocardial infarction and subjects with symptomatic peripheral vascular disease [40]. A marginal but significant clinical superiority of clopidogrel (75 mg daily) over aspirin (325 mg daily) was demonstrated for the secondary prevention of cardiovascular events [40]. After a mean follow up period of 1.91 years, patients treated with clopidogrel had a 8.7 % relative risk reduction in the composite end point of vascular death, myocardial infarction or ischemic stroke compared to the aspirin treatment group. Clopidogrel further emerges from the CAPRIE study to be equally tolerated as aspirin. Other studies showed that clopidogrel can also be used in association with different other antiplatelet drugs to explore the efficacy on the inhibition of different pathways of platelet activation. In the CURE trial, including 12500 patients, clopidogrel (300 mg loading dose and 75 mg daily) was added to background aspirin therapy (75 to 325 mg daily) for 3 to 12 months following an acute coronary syndrome [41]. After a mean follow up of 9 months, the combination of clopidogrel with aspirin reduced the composite endpoint (vascular death, myocardial infarction or ischemic stroke) with 20 % compared to aspirin therapy alone, although at the prize of bleeding. The PCI-CURE and CREDO trials prescribe the combination of clopidogrel with aspirin as the golden therapy for patients undergoing percutanerous transluminal coronary angioplasty with stent implantations [42,43]. Studies on the association of clopidogrel with GPIIbIIIa antagonists are ongoing.

GLYCOPROTEIN IIB/IIIA ANTAGONISTS

Platelet-platelet interactions are responsible for the formation of hemostatic plugs and other pathological thrombi. In unstimulated platelets, the major platelet integrin glycoprotein IIb/IIIa (GPIIb/IIIa) is maintained in an inactive conformation and functions as a low affinity receptor for fibrinogen [44]. After stimulation, the interaction between fibrinogen and GPIIb/IIIa forms intracellular bridges between platelets leading to platelet aggregation (Fig. **3A**). A conformational change in the extracellular domain of GPIIb/IIIa enables the high affinity binding of soluble plasma fibrinogen as a result of a complex network of inside-out signaling events [45]. This reversible phase of platelet aggregation is precipitated by a series of extremely complicated signaling pathways [46]. Patients with Glanzmann thrombastenia have a severe bleeding tendency due to mutations in the genes encoding GPIIb or GPIIIa. Consistent with this notion, molecules that block GPIIb/IIIa completely prevent platelet aggregation regardless of the platelet agonist. Different compounds with GPIIb/IIIa antagonistic activity have been developed but only three of them were approved for clinical use.

Abciximab

Abciximab (ReoPro®) is a chimeric mouse-human monoclonal antibody directed against the GPIIb/IIIa receptor (K_D 5 nmol/l); its mechanism of action is thought to be steric hindrance of the receptor itself as opposed to direct binding to the RGD binding site of the receptor [47]. It also inhibits leukocyte integrin $\alpha M\beta 2$ and the vitronectin ($\alpha v\beta 3$) receptor, which mediates platelet activation as well as endothelial and vascular smooth muscle cell proliferation [48]. The drug binds rapidly to platelets, blocking about 80 % of surface GPIIb/IIIa, and has a very short plasma half-life of 30 minutes after intravenous administration of the drug.

Tirofiban

Tirofiban (Aggrastat®) is a synthetic non-peptide small molecule developed as a mimic of an RGD-containing disintegrin, echistatin [49] (Fig. **3B**). This drug is selective for GPIIb/IIIa (K_D 15 nmol/l) and does not react with the vitronectin receptor. The clinical relevance of abciximab's cross-reactivity with other integrins remains entirely speculative. Following intravenous injection the number of unbound drug molecules relative to the number of platelet GPIIb/IIIa is very high and the mean plasma half-life of tirofiban is about 2 hours. Tirofiban's antiplatelet effects may therefore be effectively reversed by stopping the medication and waiting for about 4 hours for platelet aggregation to return to 50 % of its baseline.

Eptifibatide

Eptifibatide (Integrilin®) is an RGD containing cyclic synthetic heptapeptide modeled on the active site of barbourin, a disintegrin isolated from the snake venom that contains the KGD instead of RGD sequence in its active site [50] (Fig. **3B**). This compound is only reactive against GPIIb/IIIa (K_D 120 nmol/l). Again very high levels of free, unbound drug are attained following intravenous injection and eptifibatide had a plasma half-life of 2.5 hours. Stopping the medication results in a 50 % recovery of the platelet activity within 4 hours.

The differential expression of a ligand-induced binding site on platelets by the three antagonists, the ability to block other integrins by abciximab, and the faster recovery of platelet function when infusion is discontinued with tirofiban and eptifibatide compared to abciximab, have all

been advocated to explain for possible differences in clinical efficacy of these drugs but no proof of the superiority of one compound over the others has yet been provided [51]. Several receptor-binding pharmacodynamic studies have shown that a degree of receptor occupancy of 80 % is required to obtain a complete platelet inhibition [52]. All data thus far indicate that the main therapeutic advantage of GPIIb/IIIa antagonists is obtained in patients undergoing percutaneous coronary intervention [53]. Whether there are significant differences between abciximab, tirofiban or eptifibatide in their clinical efficacy in patients undergoing percutaneous coronary intervention, is still an ongoing debate. However, all data thus far indicate that all three of them are probably similar in the long-term reduction of death, myocardial infarction and the need for urgent target vessel revascularization [54]. In contrast, for the medical management of patients with non-ST segment elevation acute coronary syndromes, only eptifibatide and tirofiban proved clinically safe but only moderately effective [55] while abciximab may be associated with worse clinical outcomes [56]. Subgroup analysis have sought to determine if specific groups of patients derive either greater or lesser benefit from GPIIb/IIIa antagonist therapy compared with the general population but recognizing these subgroups and the particular effects that therapies may have on them is a extremely difficult task. In addition, excessive bleeding remains an important problem with these agents and efforts must be made to eliminate this side effect.

POTENTIAL TARGETS FOR ANTIPLATELET DRUGS

Mainly through studies in knockout mice or patients with platelet defects, many novel platelet signaling pathways and proteins were discovered with a potential use for

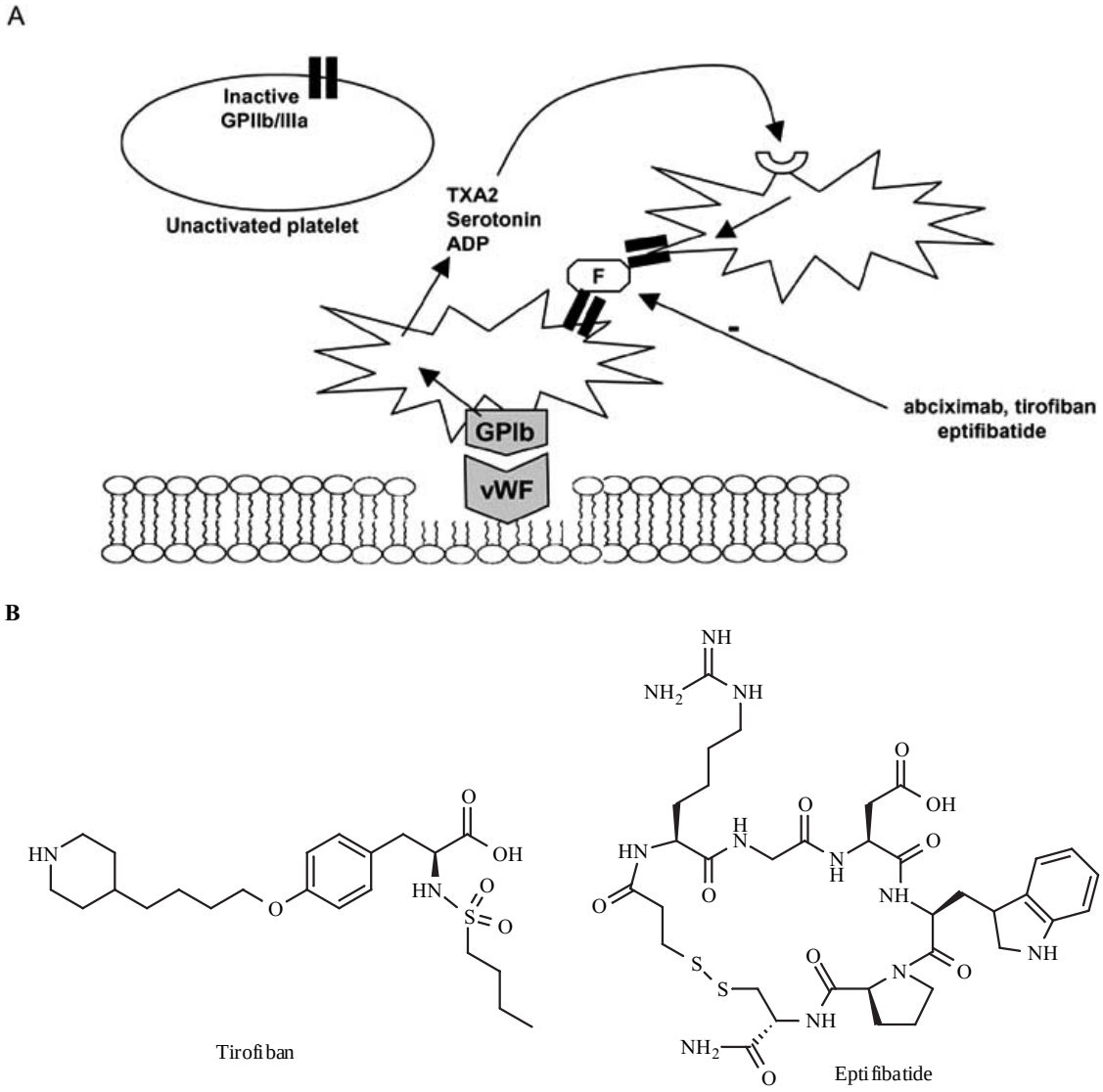

Fig. (3). . A. Vascular injury exposes subendothelial vWF to the circulating blood. Platelets adhere to the site of the injury after binding of the platelet GPIb receptor to vWF. This triggers a network of insite-out signaling events causing the release of TXA2, serotonin and ADP, which induces the local activation of platelets by interaction with different receptors. The subsequent step is the conformational change of the GPIIb/IIIa receptor enabling the high affinity binding of fibrinogen. GPIIb/IIIa antagonists inhibit the GPIIb/IIIa-inuced platelet activation and further platelet recruitment. B. Chemical structures of the GPIIb/IIIa antagonists tirofiban and eptifibatide.

antiplatelet therapy. A few novel antiplatelet approaches are briefly mentioned below.

Inhibitors of vWF-GPIb Activation

The selective inhibition of the vWF-GPIb interaction on platelets can be achieved by either blockade of the GpIb receptor or the identification of compounds that bind to specific regions of the vWF protein. The first approach using monoclonal antibodies against human GPIb was accomplished by a F(ab)2 fragment of the murine monoclonal antibody 6B4 that seems effective in a baboon model of arterial thrombosis [57] and by the monoclonal antibody AJvW-2 that proved to be antithrombotic and prevented neointima formation [58]. The second approach is aimed at developing recombinant fragments of the vWF protein that exclusively express the GPIb binding region. Two such fragments, the peptide VCL (from Leu504 to Lys728) and the recombinant fragment AR545C (from Ala444 to Asp730 with mutation Arg545Cys) have been tested in animals with optimistic results [59,60,61]. Both approaches leading to GPIb-vWF inhibition result is a slight prolongation of the bleeding time with antithrombotic doses of these substances [57,58,61], which suggest that other platelet membrane glycoproteins (as GPIa/IIa and GPIIb/IIIa) are involved in the formation of a hemostatic plug and that selective inhibition of GPIb could be a safe antithrombotic approach. Since GPIb blockers do not abolish thrombus formation [57,62], these pharmacological agents could be developed in combination with known antiplatelet drugs as aspirin.

Thrombin Receptors

Thrombin is a serine protease that catalysis the cleavage of fibrinogen and of the platelet thrombin receptors (protease-activated receptors or PAR receptors). Thrombin cleaves the N-terminal domain of these PAR receptors to unmask the new N-termini, which further acts as tethered ligands to activate the receptor [63]. As a result thrombin activates both the coagulation pathway and platelets. Approaches to inhibit the thrombin-induced mechanisms of thrombosis were designed at the level of thrombin itself or against its receptor. Antithrombins are used as anticoagulants and include the protease inhibitors hirudin and D-Phe-Pro-Arg-chloromethylketone that block the thrombin catalytic activity. The second approach consists of the development of inhibitors against the platelet G protein-coupled receptors PAR1 and PAR3. Thrombin receptor antagonists act differently from the specific thrombin protease inhibitors and their effect is limited to platelet inhibition without affecting the clotting system. Attempts being made are the use of receptor-blocking antibodies, the development of peptide-mimetic antagonists for the tethered receptor and the development of naturally occurring or synthetic thrombin antagonists. Anti-PAR1 monoclonal antibodies failed to fully prevent the thrombin-induced platelet aggregation [64]. In contrast, anti-PAR1 polyclonal antibodies, which probably cross-react with PAR3, have provided protection from thrombosis in non-human primates [65]. Peptide-mimetic antagonists for the tethered ligand are potent thrombin receptor antagonists, such as C186-65 and FR171113 [66,67]. The undecapeptide C186-65 is a specific inhibitor of the thrombin-mediated platelet activation [66] and FR171113 is a selective non-peptide thrombin receptor antagonist, that is 50 times more potent than C168-65 in inhibiting the thrombin-induced platelet aggregation [67]. The chemical name for FR171113 is 3-(4-chlorophenyl)-2-(2,4-dichlorobenzoylimino)-5-(methoxycarbonylmethylene)-1,3-thiazolidin-4-one (or C19H11N2O4SCl3) with a molecular weight of 469,7. The PAR antagonists are still in experimental phase and have not yet been tested in clinical trials.

Collagen Receptors

The activation of platelets by collagen necessarily requires the exposure of vessel wall collagens following trauma, surgical intervention or disease processes. Most collagens directly interact with the platelet surface through two receptors, glycoprotein VI and integrin $\alpha 2\beta 1$ and trigger adhesion, activation, secretion and the exposure of activated GPIIb/IIIa receptors [68]. Several approaches are followed to modulate platelet activation by interfering with either one of these collagen receptors. The identification of sequences in the native collagen that recognize the $\alpha 2\beta 1$ integrin [69] were the first step to design receptor- or ligand-blocking peptides, or peptide mimetics. Due to the rather widespread expression of $\alpha 2\beta 1$ in different cells, it is not yet possible to predict whether $\alpha 2\beta 1$ inhibition may be harmful. Theoretically appealing is the approach to block GPVI, a member of the immunoglobulin superfamily, thought to be the collagen receptor most relevant for starting intraplatelet signaling but also contributing to adhesion and to expression of the procoagulant activity by platelets [70]. Platelets in which the GPVI receptor is blocked by a monoclonal antibody failed to induce thrombus formation in a parallel perfusion flow chamber, indicating a role of GPVI as an antiplatelet target for prevention of acute arterial thrombosis [70]. In addition, the depletion of platelets GPVI by the monoclonal JAQ1 antibody produced a sustained antithrombotic activity in mice with only a moderate prolongation of the bleeding time [71]. Further *in vivo* studies are needed to define whether modulation of GPVI signaling could be a safe antithrombotic strategy.

Endothelial Ecto-Adenosine Phosphatase

When platelets are in proximity to endothelial cells, their function can be affected by nitric oxide and PGI2, as well as by the activity of endothelial cell CD39, an ATP diphosphohydrolase or ecto-ADTPase-1 that converts extracellular ATP and ADP to AMP [72]. By metabolizing ADP, CD39 can inhibit and/or reverse the ADP induced platelet aggregation and can prevent platelet recruitment following secretion of dense granule ADP. Moreover, since plasma contains measurable amounts of ADP and ATP, CD39 may modulate platelet and vascular reactivity [73]. Consistent with this possibility, platelets in CD39-deficient mice were unresponsive to ADP as a result of desensitization of their P2Y1 receptors, and there was increased fibrin deposition in the vasculature of multiple organs [73]. Based on these observations, a soluble, enzymatically active, recombinant form of CD39 has been synthesized and found to decrease platelet and fibrin deposition and infarct volume in a murine stroke model [74]. Accordingly, soluble CD39,

or a drug with ADTPase activity, could potentially be useful in the setting of acute arterial thrombosis.

Pituitary Adenylyl Cyclase-Activating Peptide

The pituitary adenylyl cyclase-activating peptide (PACAP) is a neuropeptide of the vasoactive intestinal peptide/secretin/glucagon superfamily and is located on human chromosome 18p [75]. The biological function of PACAP has been investigated in many organs and tissues such as endocrine glands, central nervous system, respiratory system, cardiovascular system, gastrointestinal tract [76] and recently also in platelet function [4]. Studies in two related patients with a partial trisomy 18p and monosomy 20p revealed three copies of the PACAP gene and elevated PACAP concentrations in plasma [4]. The patients suffer from severe mental retardation; have a bleeding tendency with mild thrombocytopenia, and their fibroblasts show increased PACAP mRNA levels. The PACAP type-I receptor (VPACI) in platelets and fibroblasts is coupled to adenylyl cyclase activation. Accordingly, we found increased basal cAMP levels in patients' platelets and fibroblasts, providing a basis for the reduced platelet aggregation with collagen in these patients. In contrast, PACAP antagonist PACAP(6-38) dose-dependently enhanced the collagen-induced aggregation of normal platelets by reducing adenylyl cyclase activation. Further studies are needed to gain more information concerning the mechanism involved in PACAP- and/or cAMP-mediated platelet function and eventually to detect any therapeutical potential to manage arterial thrombosis or bleeding by administration of PACAP mimetics or inhibitors, respectively.

CONCLUSION

Ischemic cardiovascular diseases represent the most common cause of mortality and morbidity in the western world, and atherothrombosis occupies a central role in their pathophysiology. Venous thrombi, which form under low shear conditions, are predominantly composed of fibrin and red cells, while arterial thrombi form under high shear conditions and are composed primarily of platelet aggregates held together by fibrin strands. Several successful strategies targeting specific steps in coagulation (anticoagulants) and platelet function (antiplatelets drugs) or their interaction have been developed to prevent or treat atherothrombotic disorders. Intense research is currently underway in an effort to develop more safe and effective compounds, such that novel antithrombotics are emerging to target specific steps in the coagulation cascade, as well as in pathways of platelet adhesion, activation and aggregation.

RESEARCH GRANTS

This work was supported by research grants G.0453.05 and G.0445.06 from the FWO-Vlaanderen (Belgium) and GOA/2003/09 from Research Council of the University of Leuven (Onderzoeksraad K.U. Leuven, Belgium).

REFERENCES

[1] Kulkarni, S.; Dopheide, S.M.; Yap, C.L.; Ravanat, C.; Freund, M.; Mangin, P.; Heel, K.A.; Street, A.; Harper, I.S.; Lanza, F.; Jackson, S.P. *J. Clin. Invest*., **2000**, *105*, 783.

[2] Fitzgerald, D.J.; Roy, L.; Catella, F.; FitzGerald, G.A. *N. Engl. J. Med.* **1986**, *315*, 983.

[3] Schafer, A.; Eigenthaler, M.; Bauersachs, *J. Clin. Lab*., **2004**, *50*, 559.

[4] Freson, K.; Hashimoto, H.; Thys, C.; Wittevrongel, C.; Danloy, S.; Morita, Y.; Shintani, N.; Tomiyama, Y.; Vermylen, J.; Hoylaerts, M.F.; Baba, A.; Van Geet, C. *J. Clin. Invest*., **2004**, *113*, 905.

[5] Roth, G.J.; Majerus, P.W. *J. Clin. Invest*. **1975**, *56*, 624.

[6] Burch, J.W.; Stanford, N.; Majerus, P.W. *J. Clin. Invest*., **1978**, *61*, 314.

[7] Antithrombotic Trialists' Collaboration. *BMJ*., **2002**, *324*, 71.

[8] Hennekens, C.H. *Am. J. Manag. Care*., **2002**, *8*, S691.

[9] Smith, W.L. *Am. J. Physiol*., **1992**, *263*, F181.

[10] Smith, W.L.; DeWitt, D.L.; Garavito, R.M. *Annu. Rev. Biochem*., **2000**, *69*, 145.

[11] Loll, P.J.; Picot, D.; Garavito, R.M. *Nat. Struct. Biol*., **1995**, *2*, 637.

[12] Rinder, C.S.; Student, L.A.; Bonan, J.L.; Rinder, H.M.; Smith, B.R. *Blood*, **1993**, *82*, 505.

[13] Lewis, H.D. Jr.; Davis, J.W.; Archibald, D.G.; Steinke, W.E.; Smitherman, T.C.; Doherty, J.E. 3rd.; Schnaper, H.W.; LeWinter, M.M.; Linares, E.; Pouget, J.M.; Sabharwal, S.C.; Chesler, E.; DeMots, H. *N. Engl. J. Med*., **1983**, *309*, 396.

[14] de Gaetano, G. *Lancet*, **2001**, *357*, 89.

[15] Burch, J.W.; Stanford, N.; Majerus, P.W. *J. Clin. Invest*., **1978**, *61*, 314.

[16] Pedersen, A.K.; FitzGerald, G.A. *N. Engl. J. Med*., **1984**, *311*, 1206.

[17] Patrono, C.; Coller, B.; Dalen, J.E.; Fuster, V.; Gent, M.; Harker, L.A.; Hirsh, J.; Roth, G. *Chest*, **1998**, *114*, 470S.

[18] Gum, P.A.; Kottke-Marchant, K.; Poggio, E.D.; Gurm, H.; Welsh, P.A.; Brooks, L.; Sapp, S.K.; Topol, E.J. *Am. J. Cardiol*., **2001**, *88*, 230.

[19] Eikelboom, J.W.; Hirsh, J.; Weitz, J.I.; Johnston, M.; Yi, Q.; Yusuf, S. *Circulation*, **2002**, *105*, 1650.

[20] Buchanan, M.R.; Brister, S.J. *Can. J. Cardiol*., **1995**, *11*, 221.

[21] Grotemeyer, K.H.; Scharafinski, H.W.; Husstedt, I.W. *Thromb. Res*., ***1993***, *71*, 397.

[22] Michelson, A.D.; Barnard, M.R.; Krueger, L.A.; Valeri, C.R.; Furman, M.I. *Circulation*, **2001**, *104*, 1533.

[23] Rao, G.H.; Johnson, G.G.; Reddy, K.R.; White, J.G. *Arteriosclerosis*, **1983**, *3*, 383.

[24] Catella-Lawson, F.; Reilly, M.P.; Kapoor, S.C.; Cucchiara, A.J.; DeMarco, S.; Tournier, B.; Vyas, S.N.; FitzGerald, G.A. *N. Engl. J. Med*., **2001**, *345*, 1809.

[25] Ray, W.A.; Stein, C.M.; Hall, K.; Daugherty, J.R.; Griffin, M.R. *Lancet*, **2002**, *359*, 118.

[26] Quinn, M.J.; Fitzgerald, D.J. *Circulation*, **1999**, *100*, 1667.

[27] Savi, P.; Herbert, J.M. S*emin. Thromb. Hemost*., **2005**, *31*, 174.

[28] Pereillo, J.M.; Maftouh, M.; Andrieu, A.; Uzabiaga, M.F.; Fedeli, O.; Savi, P.; Pascal, M.; Herbert, J.M.; Maffrand, J.P.; Picard, C. *Drug Metab. Dispos*., **2002**, *30*, 1288.

[29] Gachet, C. *Thromb. Haemost.*, **2001**, *86*, 222.

[30] Renner, C.; Guilmet, D.; Curtet, J.M. *Nouv. Presse Med*., **1980**, *9*, 3249.

[31] Herbert, J.M.; Tissinier, A.; Defreyn, G.; Maffrand, J.P. *Arterioscler. Thromb*., ***1993***, *13*, 1171.

[32] Bennett, C.L.; Connors, J.M.; Carwile, J.M.; Moake, J.L.; Bell, W.R.; Tarantolo, S.R.; McCarthy, L.J.; Sarode, R.; Hatfield, A.J.; Feldman, M.D.; Davidson, C.J.; Tsai, H.M. *N. Engl. J. Med*., **2000**, *342*, 1773.

[33] Vincent, J.E.; Zijlstra, F.J.; de Wit, C.M.; Bonta I.L. *Prostaglandins Leukot. Med*., **1984**, *16*, 279.

[34] Savi, P.; Combalbert, J.; Gaich, C.; Rouchon, M.C.; Maffrand, J.P.; Berger, Y.; Herbert, J.M. *Thromb. Haemost*., **1994**, *72*, 313.

[35] Savi, P.; Pereillo, J.M.; Uzabiaga, M.F.; Combalbert, J.; Picard, C.; Maffrand, J.P.; Pascal, M.; Herbert, J.M. *Thromb. Haemost*., **2000**, *84*, 891.

[36] Steinhubl, S.R.; Berger, P.B.; Mann, J.T. 3rd; Fry, E.T.; DeLago, A.; Wilmer, C.; Topol, E.J. *JAMA*, **2002**, *288*, 2411.

[37] Gent, M.; Blakely, J.A.; Easton, J.D.; Ellis, D.J.; Hachinski, V.C.; Harbison, J.W.; Panak, E.; Roberts, R.S.; Sicurella, J.; Turpie, A.G. *Lancet*, **1989**, *1*, 1215.

[38] Hass, W.K.; Easton, J.D.; Adams, H.P. Jr.; Pryse-Phillips, W.; Molony, B.A.; Anderson, S.; Kamm, B. *N. Engl. J. Med.*, **1989**, *321*, 501.

[39] Balsano, F, Rizzon, P.; Violi, F.; Scrutinio, D.; Cimminiello, C.; Aguglia, F.; Pasotti, C.; Rudelli, G. *Circulation*, **1990**, *82*, 17.
[40] CAPRIE Steering Committee. *Lancet*, **1996**, *348*, 1329.
[41] Yusuf, S.; Zhao, F.; Mehta, S.R.; Chrolavicius, S.; Tognoni, G.; Fox, K.K. *N. Engl. J. Med.*, **2001**, *345*, 494.
[42] Mehta, S.R.; Yusuf, S.; Peters, R.J.; Bertrand, M.E.; Lewis, B.S.; Natarajan, M.K.; Malmberg, K.; Rupprecht, H.; Zhao, F.; Chrolavicius, S.; Copland, I.; Fox, K.A.; Clopidogrel in Unstable angina to prevent Recurrent Events trial (CURE) Investigators. *Lancet*, **2001**, *358*, 527.
[43] Steinhubl, S.R.; Berger, P.B.; Mann, J.T. 3rd; Fry, E.T.; DeLago, A.; Wilmer, C.; Topol, E.J. *JAMA*, **2002**, *288*, 2411.
[44] Savage, B.; Ruggeri, Z.M. *J. Biol. Chem*., **1991**, *266*, 11227.
[45] Naik, U.P.; Patel, P.M.; Parise, L.V. *J. Biol. Chem*., **1997**, *272*, 4651.
[46] Clemetson, K.J. *Thromb. Haemost*., **1995**, *74*, 111.
[47] Coller, B.S. *J. Clin. Invest*., **1997**, *100*, S57.
[48] Plescia, J.; Conte, M.S.; VanMeter, G.; Ambrosini, G.; Altieri, D.C. *J. Biol. Chem*., **1998**, *273*, 20372.
[49] Lynch, J.J. Jr; Cook, J.J.; Sitko, G.R.; Holahan, M.A.; Ramjit, D.R.; Mellott, M.J.; Stranieri, M.T.; Stabilito, I.I.; Zhang, G.; Lynch, R.J. *J. Pharmacol. Exp. Ther*., **1995**, *272*, 20.
[50] Scarborough, R.M.; Naughton, M.A.; Teng, W.; Rose, J.W.; Phillips, D.R.; Nannizzi, L.; Arfsten, A.; Campbell, A.M.; Charo, I.F. *J. Biol. Chem*., **1993**, *268*, 1066.
[51] Quinn, M.J.; Plow, E.F.; Topol, E.J. *Circulation*, **2002**, *106*, 379.
[52] Gold, H.K.; Gimple, L.W.; Yasuda, T.; Leinbach, R.C.; Werner, W.; Holt, R.; Jordan, R.; Berger, H.; Collen, D.; Coller, B.S. *J. Clin. Invest*., **1990**, *86*, 651.
[53] Lincoff, A.M.; Califf, R.M.; Moliterno, D.J.; Ellis, S.G.; Ducas, J.; Kramer, J.H.; Kleiman, N.S.; Cohen, E.A.; Booth, J.E.; Sapp, S.K.; Cabot, C.F.; Topol, E.J. *N. Engl. J. Med*., **1999**, *341*, 319.
[54] Moliterno, D.J.; Yakubov, S.J.; DiBattiste, P.M.; Herrmann, H.C.; Stone, G.W.; Macaya, C.; Neumann, F.J.; Ardissino, D.; Bassand, J.P.; Borzi, L.; Yeung, A.C.; Harris, K.A.; Demopoulos, L.A.; Topol, E.J. *Lancet*, **2002**, *360*, 355.
[55] Boersma, E.; Harrington, R.A.; Moliterno, D.J.; White, H.; Theroux, P.; Van de Werf, F.; de Torbal, A.; Armstrong, P.W.; Wallentin, L.C.; Wilcox, R.G.; Simes, J.; Califf, R.M.; Topol, E.J.; Simoons, M.L. *Lancet*, **2002**, *359*, 189.
[56] Simoons, M.L. *Lancet*, **2001**, *357*, 1915.
[57] Cauwenberghs, N.; Meiring, M.; Vauterin, S.; van Wyk, V.; Lamprecht, S.; Roodt, J.P.; Novak, L.; Harsfalvi, J.; Deckmyn, H.; Kotze, H.F. *Arterioscler. Thromb. Vasc. Biol*., **2000**, *20*, 1347.
[58] Kageyama, S.; Yamamoto, H.; Yoshimoto, R. *Arterioscler. Thromb. Vasc. Biol*., **2000**, *20*, 2303.
[59] Gralnick, H.R.; Williams, S.; McKeown, L.; Kramer, W.; Krutzsch, H.; Gorecki, M.; Pinet, A.; Garfinkel, L.I. *Proc. Natl. Acad. Sci. USA*, **1992**, *89*, 7880.
[60] Gurevitz, O.; Goldfarb, A.; Hod, H.; Feldman, M.; Shenkman, B.; Varon, D.; Eldar, M.; Inbal, A. *Arterioscler. Thromb. Vasc. Biol*., **1998**, *18*, 200.
[61] McGhie, A.I.; McNatt, J.; Ezov, N.; Cui, K.; Mower, L.K.; Hagay, Y.; Buja, L.M.; Garfinkel, L.I.; Gorecki, M.; Willerson, J.T. *Circulation*, **1994**, *90*, 2976.
[62] Azzam, K.; Garfinkel, L.I.; Bal dit Sollier, C.; Cisse Thiam, M.; Drouet, L. *Thromb. Haemost*., **1995**, *73*, 318.
[63] Coughlin, S.R. *J. Thromb. Haemost*., **2005**, *3*, 1800.
[64] Klement, P.; Borm, A.; Hirsh, J.; Maraganore, J.; Wilson, G.; Weitz, J. *Thromb. Haemost.,* **1992**, *68*, 64.
[65] Cook, J.J.; Sitko, G.R.; Bednar, B.; Condra, C.; Mellott, M.J.; Feng, D.M.; Nutt, R.F.; Shafer, J.A.; Gould, R.J.; Connolly, T.M. *Circulation*. **1995**, *91*, 2961.
[66] Scarborough, R.M. *Circulation*, **1992**, *86*(Suppl. I), 1.
[67] Kato, Y.; Kita, Y.; Nishio, M.; Hirasawa, Y.; Ito, K.; Yamanaka, T.; Motoyama, Y.; Seki, J. *Eur. J. Pharmacol*., **1999**, *384*, 197.
[68] Watson, S.P. *Thromb. Haemost*., **1999**, *82*, 365.
[69] Emsley, J.; Knight, C.G.; Farndale, R.W.; Barnes, M.J.; Liddington, R.C. *Cell*, **2000**, *101*, 47.
[70] Goto, S.; Tamura, N.; Handa, S.; Arai, M.; Kodama, K.; Takayama, H. *Circulation*, **2002**, *106*, 266.
[71] Nieswandt, B.; Schulte, V.; Bergmeier, W.; Mokhtari-Nejad, R.; Rackebrandt, K.; Cazenave, J.P.; Ohlmann, P.; Gachet, C.; Zirngibl, H. *J. Exp. Med*., **2001**, *193*, 459.
[72] Marcus, A.J.; Broekman, M.J.; Drosopoulos, J.H.; Islam, N.; Alyonycheva, T.N.; Safier, L.B.; Hajjar, K.A.; Posnett, D.N.; Schoenborn, M.A.; Schooley, K.A.; Gayle, R.B.; Maliszewski, C.R. *J. Clin. Invest*., **1997**, *99*, 1351.
[73] Enjyoji, K.; Sevigny, J.; Lin, Y.; Frenette, P.S.; Christie, P.D.; Esch, J.S. 2nd.; Imai, M.; Edelberg, J.M.; Rayburn, H.; Lech, M.; Beeler, D.L.; Csizmadia, E.; Wagner, D.D.; Robson, S.C.; Rosenberg, R.D. *Nat. Med.*, **1999**, *5*, 1010.
[74] Marcus, A.J.; Broekman, M.J.; Drosopoulos, J.H.; Olson, K.E.; Islam, N.; Pinsky, D.J.; Levi, R. *Semin. Thromb. Hemost*., **2005**, *31*, 234.
[75] Hosoya, M.; Kimura, C.; Ogi, K.; Ohkubo, S.; Miyamoto, Y.; Kugoh, H.; Shimizu, M.; Onda, H.; Oshimura, M.; Arimura, A. *Biochim. Biophys. Acta*, **1992**, *1129*, 199.
[76] Vaudry, D.; Gonzalez, B.J.; Basille, M.; Yon, L.; Fournier, A.; Vaudry, H. *Pharmacol. Rev*., **2000**, *52*, 269.

Received: October 26, 2005 Revised: November 30, 2005 Accepted: December 05, 2005