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The thrombin receptors PAR1 and PAR4 and their relative role in platelet activation

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"Life is a mystery"
-Julien Offray de La Mettrie

To all my dear friends standing steady on earth or flying in the sky

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ABSTRACT

Many blood cell mechanisms in the human body are working all the time to maintain haemostasis in the blood vessels. Once a wound arises platelets are alerted via different substances to cover the wound and prevent loss of blood. Most of the times these mechanisms do stop the blood, and further heal the wound. During other circumstances the platelet-covering continues to form a thrombus, preventing the blood to flow and instead causes myocardial infarction or stroke. There are several risk factors triggering development of circulatory diseases such as obesity, lack of exercise, smoking, infection and stress.

This thesis describes the interaction between the two platelet thrombin receptors PAR1 and PAR4, together with the interaction of the oral pathogen *Porphyromonas gingivalis* (with thrombin-like gingipains), and the cross talk with the stress hormone epinephrine and its α_{2A} adrenergic receptor. Until now PAR1 is thought to be the most important thrombin receptor due to its high affinity for thrombin. From a phylogenetical and patophysiological point of view there must be a reason why platelets express two different thrombin receptors. Today PAR4 is considered less important, but this thesis implies that PAR4 plays an important role in platelet signaling and haemostasis.

The results show that bacteria pre-stimulated platelets, followed by epinephrine gives a strong and full aggregation and calcium mobilization, in both aspirinated and non-aspirinated human platelets. The amount of bacteria does not itself, or epinephrine alone give aggregation or calcium mobilization. This mechanism is dependent on both Rgp type gingipain released from *P. gingivalis*, and PARs in an interaction with the α_{2A} adrenergic receptor.

Further, results reveal that PAR4 interacts and cross talks with the platelet α_{2A} -adrenergic receptor in *aspirinated* platelets. Neither of the two platelet purinergic P2Y-receptors (P2Y₁₂ and P2Y₁) contribute to this action, but the purinergic P2X₁ does. In aggregation studies a low dose of PAR4 activating peptide (AP), but not PAR1-AP, followed by epinephrine results in a strong aggregation and in a calcium mobilization. ATP secretion measurements did reveal that ATP was released during epinephrine stimulation, which indicate that ATP and P2X₁ have a key role in this event. By blocking P2X₁ both aggregation and calcium mobilization were abolished, but not by blocking P2Y₁₂ and P2Y₁. Inhibition of PI3-kinase, both epinephrine-induced calcium mobilization and aggregation were significant reduced. In *non-aspirinated* platelets PAR1 synergizes with the α_{2A} adrenergic receptor and P2X₁.

In conclusion, this thesis suggests that PAR4 plays an intriguing and important role in platelets with inactivated cyclooxygenase 1. The results described in this thesis contribute to an increased knowledge of the platelet thrombin receptors.

SAMMANFATTNING

I människokroppen flyter blodet genom kärlen för att bibehålla hemostasen och andra jämvikter i balans. Ibland händer det att en skada uppstår inne i kärlväggen, och då samlas blodplättarna (trombocyterna) vid skadan och fäster samt sprider ut sig och bildar en plugg för att stoppa blödningen. När skadan är täppt så kommer läkningsprocessen att börja återställa alla kärlväggsceller och blodflödet igen. Ibland händer det att läkningsprocessen inte startar, utan att trombocytpluggen blir större och tillslut bildar en trombos, en blodpropp. Vad detta beror på kan vara att det finns pågående cirkulatoriska sjukdomar och förhöjd aktivitet i kärlen. Det finns olika riskfaktorer som kan utlösa hjärt-kärlsjukdomar såsom rökning, övervikt, stillasittande, dålig kosthållning, infektion och stress.

Det som beskrivs i denna uppsats är interaktionen mellan den orala patogenen *Porphyromonas gingivalis* (som har trombinliknande egenskaper) och de två trombinreceptorerna (PAR1 och PAR4) som befinner sig på trombocytens yta, samt korslänknings till stresshormonet adrenalin. Idag anses PAR1 vara den receptor som den viktigaste för det kroppsegna enzymet trombin på grund av dess starka förmåga att binda till trombin. Om man kollar fylogenetiskt och patofysiologiskt så borde det finnas en orsak varför trombocyt har två trombinreceptorer. I denna uppsats visar vi att den andra trombinreceptorn, PAR4, kan ha stor betydelse gällande trombocytens signalering, trombocytaktivering och i hemostasen.

Våra resultat visar att trombocyter föraktiverade med *P. gingivalis* följt av en adrenalinstimulering ger upphov till stark trombocyttaggregation och intracellulära kalciumhöjningar i både aspirin- och icke-aspirinbehandlade trombocyter. Mekanismerna som ligger bakom detta är bakteriefrisatta ämnet Rgp-gingipainin, och trombinreceptorerna i en interaktion med α_{2A} adrenerga receptorn.

Vi visar även att PAR4 interagerar och korslänkar med trombocytens α_{2A} adrenerga receptor i aspirinbehandlade trombocyter resulterade i trombocytaktivering. Ingen av de två purinerga trombocytreceptorerna P2Y₁₂ eller P2Y₁ bidrar med denna händelse, utan det visade sig att det var den tredje purinerga receptorn P2X₁ som har en avgörande roll. Aggregationsstudier visade att den PAR4-aktiverande peptiden följt av adrenalinstimulering gav stark aggregation och intracellulära kalciumhöjningar, vilket den PAR1-aktiverande peptiden följt av adrenalin inte gav. I samma studie undersökte vi även frisättningen av trombocytgranula, och en preaktivering av PAR4 följt av en adrenalinstimulering gav ATP-frisättning, vilket betyder att P2X₁ har stor betydelse för denna aktivering. Denna aktivering hämmades totalt vid inkubering av en P2X₁-hämmare. Ytterligare undersökning av den intracellulära signaleringen visade att inhibering av PI3-kinas reducerade den PAR4/adrenalinstimulerade aktiveringen hos aspirinbehandlade trombocyter.

Konklusionen av denna studie är att PAR4 följt av stresshormonet adrenalin spelar en stor roll hos trombocyter med inaktivt cyklooxygenas 1. Trombocytens PAR4 kan vara av stor betydelse för trombocytaktivering.

LIST OF PAPERS

This thesis is based on the following papers

- I. **The Periodontal Pathogen *Porphyromonas gingivalis* Sensitises Human Blood Platelets to Epinephrine**, Martina Nylander, Tomas L. Lindahl, Torbjörn Bengtsson, Magnus Grenegård, *Platelets* 2008; 19(5): 352-358
- II. **The ATP-gated P2X₁ Receptor Plays a Pivotal Role in Activation of Aspirin-treated Platelets by Thrombin and Epinephrine**, Magnus Grenegård, Karin Vretenbrant-Öberg, Martina Nylander, Stéphanie Désilets, Eva G. Lindström, Anders Larsson, Ida Ramström, Sofia Ramström, Tomas L. Lindahl, *JBC* 2008; 283 (27): 18493-18504

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Paper I: Informa Healthcare, London, UK

Paper II: ASBMB Publications, Bethesda, USA

ABBREVIATIONS

AC	adenylyl cyclase
ADP	adenosine 5'-diphosphate
ASA	acetyl salicylic acid
ATP	adenosine 5'-triphosphate
bFGF	basic fibroblast growth factor
cAMP	cyclic adenosine monophosphate
COX	cyclooxygenase
DVT	deep vein thrombosis
GP	glycoprotein
HEPES	N-[2-hydroxyethyl]-piperazine-N'-[2-ethanene-sulfonic acid]
Kgp	lysine-specific protease
NO	nitric oxide
OMV	outer membrane vesicles
PAI-1	plasminogen activator inhibitor 1
PAR	protease-activated receptor
PAR-AP	protease-activated receptor-activating peptide
PDGF	platelet-derived growth factor
PGI ₂	prostacyclin = prostaglandin I ₂
PRP	platelet rich plasma
Rgp-A,B	arginine-specific proteases
TRAP	thrombin receptor activating peptide
TXA ₂	thromboxane A ₂
VEGF	vascular endothelial growth factor
vWF	von Willebrand factor

BACKGROUND

Haemostasis

To maintain haemostasis in the body and in the circulatory system a series of complex actions must take place all the time. The meaning of the word haemostasis is to maintain equilibrium in the blood system and in the blood vessels. Blood is a very complex liquid solution consisting of different numbers of blood cells, plasma, and plasma proteins flowing through the vessels maintaining oxygen levels in tissues and to transport carbon dioxide away. Other functions of the blood are to maintain the glucose concentration and other important hormones and factors. Blood flowing in the vessels are always searching for wounds and other complications such as infection, inflammation, high or low concentrations of different substances. From a pathophysiological point of view insufficient platelet activation and coagulation leads to bleeding, whilst misdirected and powerful platelet activation and coagulation results in thrombosis which can stop blood flow and may elicit myocardial infarction or stroke.

Beyond normal haemostasis different blood disorders and sickness can be developed. One of the major health risks today in the western world is cardiovascular disease and thrombosis. There are different risk factors highlighted believed to trigger abnormal haemostasis such as smoking [1], excessive intake of alcohol [2], bad diet [3, 4], obesity [5], lack of exercise [6], infection [7] and stress. Why development of cardiovascular disease and thrombosis occur is yet not fully known. Not a single risk factor can be pointed out to be the cause, but adding several risk factors together could explain the correlation to these circulatory events. Powerful platelet activation is mostly related to artery thrombosis and is also connected to atherosclerosis in the vessel wall. Atherosclerosis dependent thrombosis (e.g. when a vulnerable atherosclerotic plaque ruptures) is one of the major medical problems today. There are still many question marks remaining concerning development of circulatory diseases which are causing high mortality. It is therefore of an importance that research in haemostasis and atherothrombosis is progressing and that involving mechanisms are clarified enabling improvements of therapy.

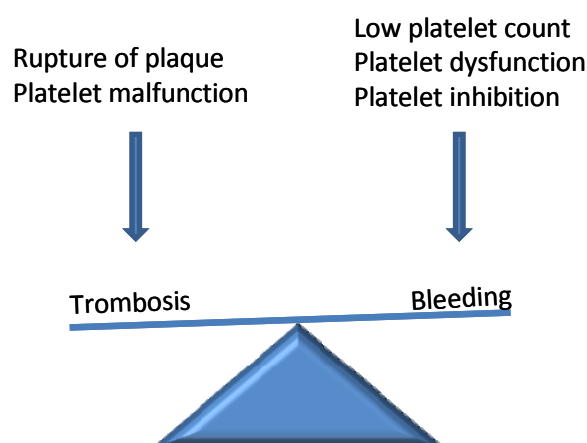


Figure 1. An illustrative image of the fragile balance between bleeding and thrombosis. To prevent thrombosis, there is always an increasing risk of bleeding, on the other hand, to prevent bleeding there is always an increasing risk of thrombosis.

Haemostasis and infection

It is known that infection is one of the risk factors contributing to and developing cardiovascular disease. There are many reports on pathogens infecting the circulatory system and the presence of pathogens to be correlated to cardiovascular disease. Both viruses and bacteria species have been detected in different groups of cardiovascular high risk patients. Pathogens found within these patient groups are often cytomegalovirus (CMV) [8, 9], herpes simplex virus (HSV) [10], *Chlamydia pneumoniae* [11], *Helicobacter pylori* [12], and the periodontal pathogen *Porphyromonas gingivalis* [13].

Haemostasis and stress

Stress is a possible risk factor of causing cardiovascular disease. During stress e.g. mental stress and exercise, levels of catecholamines will be elevated in the plasma [14-16]. von Känel and colleague [17] state that there is an increased incidence of thrombosis in patients with atherosclerosis due to increased sympathetic system. Exercise and epinephrine infusion can enhance thrombin-induced fibrinogen binding and aggregability of blood platelets according to Wallin *et al.* [14]. Platelet releasing factors due to platelet activation was found in eleven out of fifteen epinephrine-infusion studies (reviewed by [17]).

Platelets play an important role in haemostasis and thrombosis. Platelets are small cell fragments circulating in the outer area of the vessels and these cells are the first to detect and attach to a wounded vessel wall, recruiting even more platelets and other blood components to form a plug and to prevent blood loss. Eventually the formed plug must be dissolved and to allow the healing process. All these procedures are taking place in our circulatory system every now and then automatically. Almost every time all these mechanism work out perfectly well but there are a few times these mechanisms goes wrong and give complications.

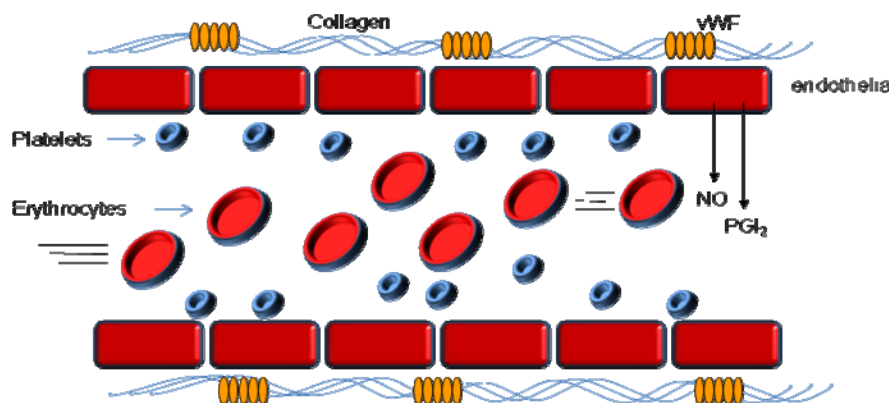


Figure 2. Normal haemostasis in blood vessel. Platelet inhibitors nitric oxide (NO), and prostaglandin I₂ (PGI₂) are secreted from endothelial cells to suppress platelet activation.

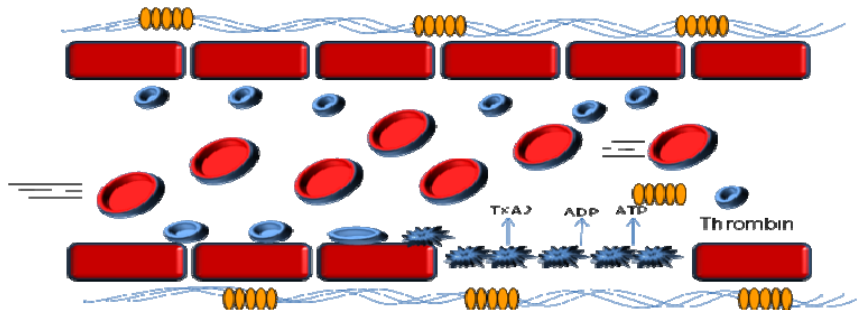


Figure 3. Collagen and von Willenbrand factor (vWF) are exposed during injury. Platelets adhere, activates, releases granule contents, and spread out to cover the wound.

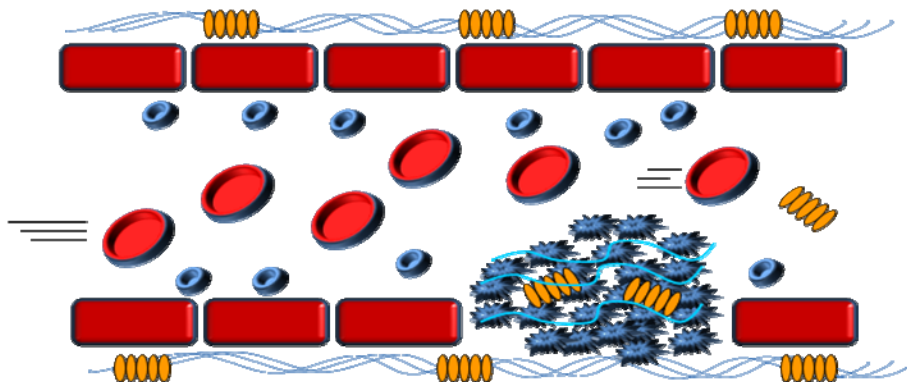


Figure 4. Platelets recruit more platelets to form a plug. Finally the platelet plug is stabilized due to formation of a fibrin network, and further blood loss is prevented.

Platelets

Platelets are small anuclear cell fragments derived from is progenitor megakaryocytes and platelets have a life span between seven and eleven days in the blood system. The concentration of platelets in the blood is between $150\text{-}350 \times 10^9$ cells/L, and the size is $3.6 \times 0.7 \mu\text{m}$ [18] and due to their small size and due to the shear force [19] platelets are pressed out to the outer area of the vessel and are the first cells to attach to the vessel wall when injury. Different compounds, ions, proteins and substances are stored in platelet granules. Today the storages are divided into two granules; dense bodies and α -granules. During platelet activation granules secrete their content to enhance platelet activation and to further signaling the response. Dense bodies contain for example adenosine 5'-diphosphate (ADP) [20-22], adenosine 5'-triphosphate (ATP), calcium, serotonin. α -granules contain larger proteins such as TSP1, vWF, fibronectin, and different coagulation factors (f V, VII, XI, XIII) [18]. Recently Italiano et al reported that there are at least two different sub alpha granules in platelets [23]. They suggest that one type of sub alpha granule contains and releases pro-angiogenetic (e.g. PDGF, bFGF) substances, and the other type releases anti-angiogenesis substances (e.g. endostatin, PF-4, PAI-1) depending on activation.

Platelets undergo different stages and different shapes due to activation. When platelets are in an inactivated stage the shape is discoid circulating in the blood in the peripheral area in the vessel. During the first stage of activation platelets undergo shape change and attach to (for example) the wounded surface and start to spread out with its pseudopodia, and finally platelets spread on the surface secreting their granule containing and recruits more platelets to form a layer of platelets covering the wounded site [24]. All these stages are depending on different receptors on the platelet surface or/and on the receptors on the vessel endothelial cells. Endothelial cells during normal physiological conditions releases platelet inhibitors such

as nitric oxide (NO) and prostacyclin (PGI₂) [25, 26] to maintain haemostasis. Platelets and other components linking platelets together play an important role during formation of an arterial thrombosis.

- Protease-activated receptors

The enzyme thrombin which also is a serine protease is an active form of coagulation factor II and has a central role in normal haemostasis and thrombus development by cleavage of soluble plasma protein fibrinogen to an insoluble fibrin gel and also to activate platelets. The activating effect on platelets is detectable at a thrombin concentration of piko-/nanomolar. This concentration is much lower than what it takes for thrombin to generate fibrin [27, 28]. Further, thrombin is known to be the most potent platelet agonist, acting via glycoprotein (GP) I β α and protease-activated receptors (PARs) 1 and 4 [24]. It has been reported that GPI β α acts as a cofactor for PAR1, but not for PAR4 [29, 30], the importance for this GPI β α /PAR interaction is still unclear.

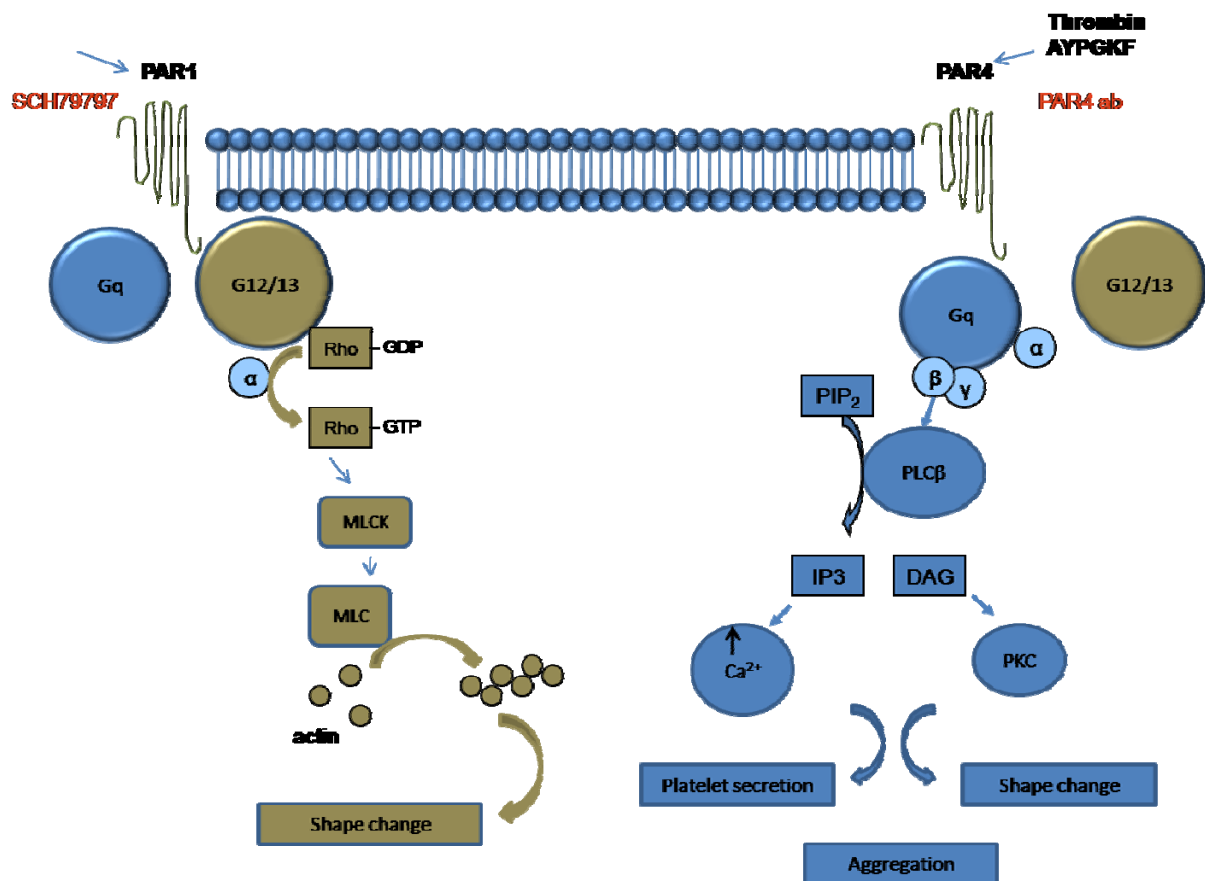


Figure 5. PAR1 and PAR4 signaling pathways in thrombin activation. PAR1 and PAR4 both signal downstream via Gq and G_{12/13}.

Thrombin activates PARs in an intriguing and fascinating way. The cleavage mechanism of thrombin is that the N-terminal exodomains of PARs are cleaved off by thrombin [31], and the new unmasked N-terminal serves as a tethered ligand [32] which in turn activates the receptor [33, 34]. The new exposed N-terminal of the receptor is formed and acts as its own agonist. Synthesised peptides for the two thrombin receptors mimicking the thrombin response and the new amino acid sequence called PAR-activating peptides are now frequently used for research [35, 36].

There are four known PARs (1-4) today, whereof two are found in platelets. Platelet thrombin receptors PAR1 and PAR4 belong to the G-protein coupled receptor family (GPCRs) and couple to $G\alpha_q$ and $G\alpha_{12/13}$ [30, 37, 38], which in turn leads to a rise in intracellular calcium concentrations via activation of phospholipase C, and activation via Rho-kinase and MLC-kinase leading to myosin rearrangement in the cytoskeleton and release of granule enhancing platelet activation [39]. It is known that thrombin has a higher affinity for PAR1 than PAR4 and that more PAR1 are expressed on platelet surface compared with PAR4 [24]. Due to the high PAR1 affinity lower concentrations of thrombin is acquired for activating PAR1 [39], contrarily our research group [40] showed that thrombin activates PAR4 in low doses. These scientific findings have contributed to that the role of PAR4 at platelet activation is uncertain and challenged. From a biological point of view it is unlikely that a cell express specific receptor protein without physiological function.

- Purinergic receptors

Platelets express three different kinds of purinergic receptors, and they are divided in to two groups; P2Y and P2X. The first purinergic receptors to be found and described are the P2Y receptors, the ADP receptors, designated P2Y₁ and P2Y₁₂. The P2Y receptors are G-protein coupled receptors. P2Y₁ activation leads to platelet activation via $G\alpha_q$ which in turn activates phospholipase C leading to intracellular calcium mobilization, shape change, and finally a transient platelet aggregation [41]. Activation of P2Y₁₂ leads to platelet activation via $G\alpha_{12}$ and inhibition of adenylyl cyclase and further suppresses cyclic AMP (cAMP) resulting in platelet aggregation [41-43]. To achieve full platelet activation and aggregation both P2Y₁ and P2Y₁₂ must be activated [44]. Inhibitors of P2Y receptors are believed to be therapeutic drugs for preventing thrombosis without major bleeding complications. The two most frequently inhibitors used in research today are clopidogrel and cangrelor. Clopidogrel is an irreversible inhibitor for P2Y₁₂, in clinical use since several years. A study of Storey *et al.* [45] showed that blood samples *ex vivo* from clopidogrel receiving patients, platelet aggregation was almost fully inhibited. Another drug, reversibly inhibiting P2Y₁₂, is cangrelor, which is a rapidly acting ATP analog thought to be one of the new breakthroughs of antiplatelet drugs. Cangrelor is at present in clinical trials.

The other type of purinergic receptors is the calcium ion channel P2X receptors. P2X₁ is expressed on smooth muscle cells and in human blood platelets [46-49]. Until year 2000 ADP was thought to be the agonist for P2X₁ and Mahaut-Smith *et al* were the first to show that ATP was the agonist, and not ADP [50]. Characteristic of P2X₁ is the rapid desensitization rate [51, 52]. The ion channel is formed by two transmembrane domains forming a trimer [53]. The role of ATP compared to ADP in platelet activation is less well established. The mechanism during ATP stimulation via P2X₁ is hard to study due to the fast desensitization of the receptor, and historically this is why P2X₁ with ATP as an agonist was found late. The breakthrough came when

the usage of apyrase (ATP-diphosphohydrolase; EC 3.6.1.5) was discovered and prevented desensitization of P2X₁. Further platelets were stimulated with the ATP-analogue α,β methyleneATP, a transient shape change could be visualised (Rolf et al 2001), whereas stimulation and activation of P2X₁ could not induce platelet aggregation alone [54]. However, the function of P2X₁ is suggested to potentiate other platelet activation pathways [50]. Hechler and colleagues have shown in vitro and in vivo studies that P2X₁ is necessary for thrombus formation in high shear rate blood flowing on collagen-coated surfaces. *In vivo* results showed that in P2X₁^{-/-} mice no spontaneous bleeding was observed, and bleeding time compared to WT mice were in almost all cases normal. In their two different thrombosis mice models infusion of a collagen-epinephrine mixture led to death in 14 of 16 WT mice whereas 8 mice in the P2X₁^{-/-} model survived up to 90 minutes, 6 mice died after 6 minutes, and one mice died after 13 minutes [55].

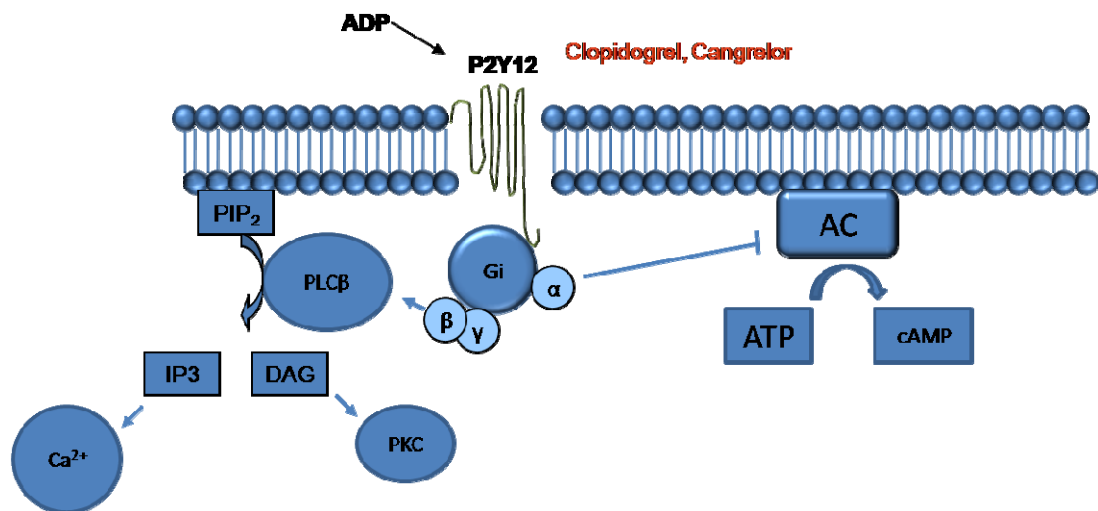


Figure 6. P2Y₁₂ and G_i signaling pathway in platelets.

- Adrenergic receptor

Epinephrine and norepinephrine are catecholamines released during a sympathetic activation such as myocardial infarction, and physical exercise. Epinephrine acts by binding to the G₂-coupled α_{2A} adrenergic receptor and by inhibiting adenylyl cyclase and the production of cAMP [56]. Epinephrine binds to the α_{2A} adrenergic receptor with a higher affinity than norepinephrine. Norepinephrine binds to the β adrenergic receptor. α_{2A} adrenergic receptors are found in many cells and tissues, and blood platelets [57].

Platelet-bacteria interactions

Many pathogens are known to interfere with blood cells and more specific to platelets. Characterization of bacteria interaction with platelets are i) binding directly to platelets via receptor binding and bacteria protein, or by ii) bacteria secreted virulence factors binding and activating platelets and both resulting in thrombus formation [58-60].

- *Porphyromonas gingivalis*

P. gingivalis is a well known bacterium causing the chronic periodontal disease, periodontitis. *P. gingivalis* is a Gram-negative, black pigmented, nonmotile anaerobic bacterium [61, 62]. This bacterium can be found in the oral cavity, in the gum, in the tooth pockets and the chin [63]. The growth of *P. gingivalis* requires iron, which often is supplied by hemin. Hemin is localized on the cell surface of the bacteria and gives the colonies its black pigmented colour [64]. When *P. gingivalis* colonizes the oral cavity, invasion of the epithelial cells is possible. This mechanism is an important strategy to evade the immune system of the host and causes damage to the tissue [62]. Reports of *P. gingivalis* have shown that the bacterium can, after attachment to available surfaces, degrade through their proteases extra cellular matrix components, and then eventually transmigrate to the blood system. Periodontal disease is known as a “gum-disease”, and there are two forms of this disease; gingivitis and periodontitis. The most concerned disease is periodontitis, which is initiated by bacterial infection. If this disease is left untreated it will lead to tooth loss in adults. Smoking, diabetes and genetic background are some of the risk factors leading to periodontitis [65]. Of the total population approximately 15% have the most severe form of periodontitis, and infection with *P. gingivalis* seems to also give complications other than tooth loss. In the past few years, *P. gingivalis* has been found in plaques within the cardiovascular systems [66], and is thereby related to cardiovascular disease. How *P. gingivalis* adheres, colonizes and grows in the oral cavity and evades the host immune response is not yet clear. The adherence to oral tissues like tooth-surface, epithelial cells and other bacteria in the sulcus occurs via e.g. fimbriae, hemagglutinins, and lipopolysaccharides. The colonization and growth is due to the hemagglutinins which bind to human erythrocytes with high affinity, and the proteases degrade the cell membrane to achieve the heme within the erythrocyte. Heme, amino acids and small peptides is needed for *P. gingivalis* to grow and colonize. To avoid the human immune response, *P. gingivalis* produces polysaccharide capsules which resist phagocytosis and killing from polymorphonuclear leucocytes. *P. gingivalis* may migrate through the epithelial cell barrier in order to hide from the immune response of the host [61, 67].

P. gingivalis contains cysteine proteases that cleave proteins to smaller peptides used for growth [68], to facilitate the invasion in host tissue, regulate the host immune defence [63] and to attach to human erythrocytes to achieve heme [69, 70]. These extracellular proteases are found in high concentrations in the cell surface membrane and in the OMV. R-gingipains (Rgp-A, Rgp-B, arginine-specific cysteine proteases) and K-gingipains (Kgp, lysine-specific cysteine proteases) are the most common gingipains [71]. It has been shown that gingipains exhibit “thrombin-like” activity, mimicking thrombin [66]. Gingipains seem to have different roles during colonization. Gingipains can down regulate the kallikrein system, the complement pathway and also the coagulation cascade. *P. gingivalis* and gingipains also have been shown to initiate the coagulation cascade by interacting with prothrombin, factor X and the protein C [72, 73].

Platelet-Stress interactions

An increased level of catecholamine might have correlations with cardiovascular disease [74]. The catecholamine epinephrine is elevated when exercising, during mental stress, and especially during myocardial infarction [75]. Epinephrine is known to be a weak platelet agonist, but together with a secondary platelet agonist epinephrine via α_{2A} -adrenergic receptors enhances platelet activation [74, 76]. Further it is known that epinephrine potentiates the effect of thrombin [77]. Platelets express α_{2A} -adrenergic receptors which are G_{ai} -coupled receptors. It is known that platelets in plasma are activated by epinephrine, whereas isolated platelets in buffer does not activate or aggregate by epinephrine stimulation [74].

Thrombosis

Thrombus formation is a series of events consisting of peripheral circulating platelets adhering very quickly to a wounded site exposing extracellular matrix into the circulation. One of the first adhesion steps is when the subendothelial substance collagen binds to platelet receptor glycoprotein (GP) Ib-IX-V via von Willenbrand factor (vWF) released from both endothelial cells and platelets. Further, another platelet receptor GPVI binds directly to exposed collagen enhancing the first adhesion step. The GPIa-IIa ($\alpha_2\beta_1$) receptor on platelet surface also binds collagen directly. The two latter collagen binding receptors are essential for platelet adhesion and aggregation [78]. This primary adhesion step induces activation in platelets followed by platelets releasing all their granule containing consisting of ADP [79], ATP, serotonin, and TXA_2 , and when this granule secretion has taken place even more platelets recruits to the wounded site. GPIIb-IIIa on the platelet surface binds to both fibrinogen and vWF acting as bridges between platelets, and finally an aggregate is created. To stabilize the formed thrombus thrombin is generated when prothrombin, also known as factor II is cleaved by factor X (Xa) and further degenerate fibrinogen to unsolvable fibrin. Recently Furie *et al.* developed a new method to study thrombus formation live in mouse model. $FeCl_3$ -induced injury, a laser creates a wound in the vessel wall and thrombus development could be visualized by fluorescent microscopy. In this type of mouse model it is now possible to find out how platelets attach to each other and how thrombus formation is developed [80].

There are two main different types of thrombosis; deep vein thrombosis (DVT) and arterial thrombosis. Formation of DVT may occur in veins when blood flow rate is decreased and also due to damage in the vessel wall. The second type of thrombosis, arterial thrombosis takes place in the arteries, and in most of the cases the thrombosis is due to a rupture of atheroma, therefore arterial thrombosis also is called atherothrombosis. Two different diseases belong to this type of thrombosis; stroke and myocardial infarction.

In this thesis we have focused on platelet thrombin receptors and different risk factors contributing to cardiovascular events. In these two papers we studied platelets and interactions with the periodontal pathogen *P. gingivalis* and the catecholamine epinephrine and how these two factors interplay with the thrombin receptors PAR1 and PAR4.

AIM

Paper I: The aim of paper I was to elucidate if *Porphyromonas gingivalis* through its thrombin-like gingipains and epinephrine interact synergistically to stimulate platelet aggregation.

Paper II: The aim of paper II was to elucidate the roles of PAR1 and PAR4 in the thrombin-epinephrine cross-talk.

MATERIALS AND METHODS

Materials

Cathepsin B inhibitor II (Calbiochem, San Diego, California, USA); HEPES solution (145mM NaCl, 5mM KCl, 1mM MgSO₄, 10mM glucose, 10mM HEPES, pH:7.4); Krebs-Ringer glucose buffer (KRG); 120mM NaCl, 4.9mM KCl, 1.2mM MgSO₄, 1.7mM KH₂PO₄, 8.3mM Na₂HPO₄, 1.1mM CaCl₂ and 10mM glucose, pH 7.3. The peptides TRAP-6 (SFLLRN) and AYPGKF, which are agonists of the thrombin receptor subtypes PAR1 and PAR4, respectively, were synthesized by the Biotechnology Centre of Oslo, Oslo University, Norway. The PAR4-blocking polyclonal chicken antibody was directed against a peptide that spans the thrombin cleavage site, which has the sequence GGDDSTPSILPAPRGYPGQVC. The peptide was synthesized by the Biotechnology Center of Oslo and used to immunize chickens. Chicken antibodies, in contrast to mammalian antibodies, do not cause platelet activation per se and are less likely to produce artifacts (16). Briefly, three laying white leghorn hens were immunized with the PAR4 peptide (2.0 mg) conjugated to hemocyanin (6.6 mg; obtained from *Concholepas concholepas*). The hens received one immunization with Freund's complete adjuvant and three booster injections with incomplete adjuvant. After the immunization period the eggs from the three hens were collected and the antibodies were purified from the egg yolk by the polyethylene glycol (PEG) method (17). Innovagen (Lund, Sweden) synthesized the cell-penetrating pepducin P4pal-i1 (palmitate-NH-ATGAPRLST), which resembles the first intracellular loop of PAR4 and can therefore selectively inhibit PAR4 activation by interfering with binding of the G-protein (18). The PAR1 antagonist SCH79797 dihydrochloride (N3-Cyclopropyl-7-[4-(1m-ethylethyl) phenyl] methyl-7H-pyrrolo [3,2-f]quinazoline-1,3-diamine dihydrochloride), a potent non-peptide PAR1 antagonist, was obtained from Tocris Cookson Ltd. (Bristol, UK). The polyclonal rabbit antibody (#9271) directed against serine437-phosphorylated Akt was purchased from Cell Signaling Technology (Danvers, MA, USA), and the phycoerythrinconjugated SPAN12 antibody was from Immunotech (Marseilles, France). The lysing solution came from Diapensia HB (Linköping, Sweden). Cangrelor (formerly AR-C69931MX; N6-(2-methylthioethyl)-2-(3,3,3-trifluoro propylthio)-β, γ-dichloromethylene ATP tetrasodium salt) was kindly provided by AstraZeneca (Dr. Michael Wayne, Wilmington, DE, USA) and the Medicines Company, (MA, USA). We also used the following drugs (all from Sigma Chemicals Co., St. Louis, MO, USA): ADP, aspirin, apyrase, α,β-Me-ATP, epinephrine, fura-2, ionomycin, LY 294002, luciferin/luciferase bioluminescent kit, MRS2159, MRS2179, NF449, prazosine, Ro318220, thrombin, UK 14.304, and yohimbine.

Isolation of Human Platelets

Venous heparinised blood was collected from blood donor volunteers at the local blood centre. The blood donors were informed about the purpose of the study and gave informed consent. The blood collection protocol was approved by the Ethics Committee at Linköping University Hospital. The blood was mixed (1/5; v/v) with an acid citrate dextrose solution (85mM tris-sodium citrate, 71mM citric acid, and 111mM glucose) and then centrifuged at 220 x g for 20 min. The resulting platelet-rich plasma (PRP) was collected and then incubated at room temperature with aspirin (100 mM) and apyrase (0.5 U/ml). The PRP was subsequently centrifuged again at 480 x g for 20 min, and the pellet containing platelets was resuspended in Hepes buffer supplemented with apyrase (1 U/ml). The platelet suspensions obtained in that manner were kept in plastic tubes and were used within 3 h. Extracellular Ca²⁺ concentration was set to 1mM immediately before each measurement. In some experiments, platelets were carefully isolated in the absence of aspirin.

Measurement of Platelet Aggregation

Aliquots (0.5 ml) of platelet suspensions (2.5×10^8 platelets/ml) were pre-incubated at 37°C for 2 min. Thereafter, platelet aggregation was induced by adding thrombin, PAR-activating peptides, and epinephrine. Changes in light transmission were recorded using a Chronolog Dual Channel lumi-aggregometer (Model 560, Chrono-Log, Haverston, PA, USA).

Measurement of Cytosolic calcium

Platelets were loaded with fura-2 by incubating PRP with 4 μ M fura-2-acetoxymethylester (from a 4 mM stock solution dissolved in DMSO) for 45 min at 20°C, and they were subsequently pelleted and resuspended as described under “*Isolation of human platelets*” before each measurement, 2 ml of platelet suspension ($1-2 \times 10^8$ /ml) was incubated at 37°C for 5 min and then exposed to different drugs. Fluorescence signals from platelet suspensions were recorded using a Hitachi F-2000 fluorescence spectrofluorometer specially designed to measure $[Ca^{2+}]_i$. Fluorescence emission was determined at 510 nm, with simultaneous excitation at 340 nm and 380 nm. $[Ca^{2+}]_i$ was calculated according to the general equation reported by Grynkiewicz *et al* [81]: $[Ca^{2+}]_i = Kd(R-R_{min})/(R_{max}-R) (F_o/F_s)$. Maximum and minimum ratios were determined by adding 0.1% Triton X-100 and 25 mM EGTA, respectively.

Measurement of Dense Granule Secretion

The amount of liberated ATP in platelet suspensions (0.5-ml aliquots; 2.5×10^8 platelets/ml) was registered using a luciferin/luciferase bioluminescent kit. Secretion of ATP was induced by adding thrombin or PAR-activating peptides alone or combined with epinephrine. The ATP-dependent increase in bioluminescence was recorded in the Chronolog lumi-aggregometer.

Analysis of Serine-phosphorylated Akt

Isolated platelets ($1-2 \times 10^9$ /ml; 100- μ l aliquots) were pre-warmed at 37°C for 3 min and then incubated with SFLLRN, AYPGKF, or thrombin for 5 min. The reaction was stopped by mixing platelet suspensions (1:2 v/v) with Laemmli buffer (Bio-Rad, Hercules, CA, USA: 62.5 mM Tris-HCl, 25% glycerol, 2% SDS, 0.01% bromophenol blue, and 5% mercaptoethanol; pH 6.8) and subsequently heating at 95°C for 5 min. Thereafter, the samples were stored at -70°C until used. To analyze serine473-phosphorylated Akt, the samples were again heated at 95°C for 5 min and then separated by 7.5% SDS-PAGE using a Mini-PROTEAN II Electrophoresis Cell (Bio-Rad). The proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore Corporation, Billerica, MA, USA) using a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad). To minimize unspecific binding, the membranes were blocked overnight at 4°C with 5% (w/v) dry milk and 0.1% (v/v) Tween 20 in PBS (pH 7.4) composed of 10 mM phosphate-buffer and 150 mM NaCl. To detect phosphorylated Akt, a polyclonal rabbit antibody against the serine437 position of Akt and a secondary horseradish peroxidase-conjugated antibody were used at dilutions of 1:1000 (Cell Signaling Technology, USA). The membranes were rinsed in PBS supplemented with 0.1% (v/v) Tween 20 between incubations, and then analyzed using ECL Western blotting detection reagents (Amersham Biosciences, UK) in a LAS-1000 Imaging Analyzer (Fuji Photo Film, Tokyo, Japan).

Culture and Preparation of Porphyromonas gingivalis

The facultative anaerobic bacteria *P. gingivalis* (ATCC 33277) was cultured in fastidious anaerobe broth pH 7.2 (29.7 g/L, Lab M, Lancashire, UK) and on fastidious anaerobe agar pH 7.2 (46.0 g/L with added L-tryptophan 0.1 g/L, Lab M, Lancashire, UK) in an atmosphere containing CO₂, N₂, and H₂ (80:10:10; Concept 400 Anaerobic Work Station, Ruskinn

Technology Limited, Leeds, UK). Suspension of *P. gingivalis* and fastidious anaerobe broth cultured for 48–72 hours was centrifuged and washed twice at 6000 x g for 30 min (4°C), the supernatant was poured away and further the pellet was resuspended in KRG. The washed bacteria was diluted in KRG to achieve an optical density (OD) of 1.7 at 600 nm, which corresponded to 1.5–2 x 10⁹ Colony Forming Units per ml (CFU/ml) as determined with viable count.

Experimental Designs

Paper I: Isolated platelet suspensions were incubated with *P. gingivalis* and changes in light transmission or fura-2 fluorescence were registered as described above. In some experiments, *P. gingivalis* was pre-treated with protease inhibitors for 45 min to establish the role played by gingipains [5]. Leupeptin (1 mM) and cathepsin inhibitor II (1 mM) was used as arg-specific gingipain inhibitor and lys-specific gingipain inhibitor, respectively. The importance of α 2- adrenergic receptors was elucidated by pretreating the platelets for 1 minute with the antagonist yohimbin (1 mM). The significance of the thrombin receptor subtypes protease-activating receptor (PAR) 1 and PAR-4 was evaluated by pretreating the platelets for 3 minutes with the PAR-1 antagonist SCH79797 (5 mM) and a PAR-4-blocking polyclonal chicken antibody (3 mg/ml), respectively.

Paper II: Platelets were exposed to various concentrations of thrombin (0.21-21 nM) and after 3 min followed by an addition of epinephrine (0.1-10 μ M). In one experimental design, the time to addition of epinephrine was varied from 15 s to 5 min. Platelets were also exposed to the PAR1- and PAR4-activating peptides SFLLRN (0.3- 12.5 μ M) and AYPGKF (30-300 μ M) followed by 10 μ M epinephrine. Another experiment used to evaluate the significance of PAR4 and PAR1 activation was to initially incubate platelets with a PAR4-blocking antibody (10 μ g/ml), or an unspecific IgY antibody (10 μ g/ml), or the PAR4-specific inhibitor pepducin P4pal-i1 (10 μ M), or the PAR1 antagonist SCH79797 (5 μ M) for 5 min and then expose them to thrombin followed by epinephrine. The role of secondary release of ADP was evaluated by pre-incubating platelets for 3 min with the P2Y1 antagonist MRS2179 (10-20 μ M) and/or the P2Y12 antagonist cangrelor (10-100 nM). Correspondingly, the role of released ATP was analyzed by pretreating platelets for 3 min with the P2X1 antagonists MRS2159 (0.01-10 μ M) and NF449 (0.1-10 μ M).

RESULTS

Paper I

In this paper we describe the interaction between the oral pathogen *P. gingivalis* and human isolated platelets and the stress hormone epinephrine. *P. gingivalis* secrete virulence factors called gingipains, known to have thrombin-like properties. We have used the classical method light-transmission aggregometry to study the platelet function and we have also utilized an intracellular calcium measurement method. *P. gingivalis* was introduced to platelets in a sub threshold dose which never gave platelet aggregation by itself. Epinephrine is known to be a weak platelet agonist, and never triggers aggregation in an isolated platelet suspension. Platelets were incubated with a *P. gingivalis* at a platelet/bacteria ratio which not gave an aggregation followed by addition of epinephrine (10 μ M). Full aggregation was achieved, in both aspirin-treated and non aspirin-treated platelets. Both thrombin receptors PAR1 and PAR4 were individually studied with PAR1 inhibitor SCH79797 (5 μ M) and a PAR4-blocking polyclonal chicken antibody.

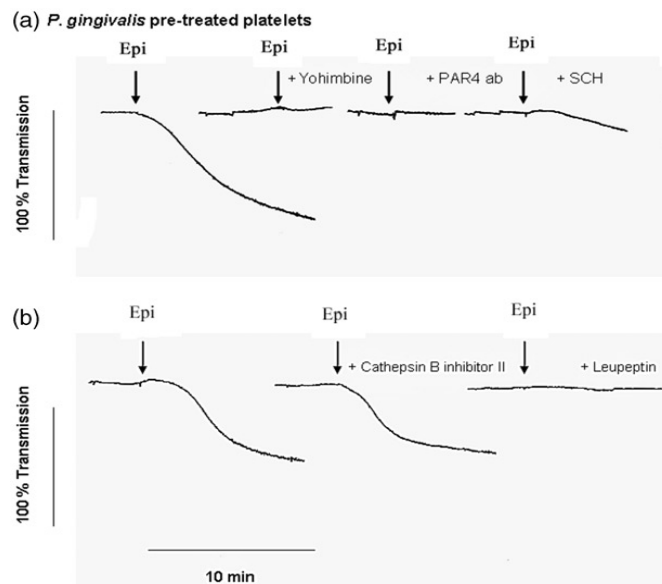


Figure 7. (a) Characterization of platelet aggregation induced by *P. gingivalis* combined with epinephrine. Platelets were exposed to sub-threshold number of *P. gingivalis* (one bacteria/60 platelets) and thereafter stimulated with epinephrine (Epi; 10 μ M), as indicated by arrows. From left to right, representative traces showing the effect of different receptor antagonism. Platelets were exposed to the α 2-adrenergic receptor antagonist yohimbin (1 mM), a PAR-4 blocking antibody (PAR4 ab; 3 μ g/ml), or the PAR-1 antagonist SCH79797 (SCH; 5 μ M) followed by *P. gingivalis*/epinephrine. Aggregation was measured as increase in light transmission through a platelet suspension. The traces shown are representative for at least three experiments. (b) Characterization of platelet aggregation induced by *P. gingivalis* combined with epinephrine. Platelets were exposed to sub-threshold number of *P. gingivalis* (one bacteria/60 platelets) and thereafter stimulated with epinephrine (Epi; 10 μ M), as indicated by arrows. From left to right, the traces illustrate the effect of inhibition of *P. gingivalis*-derived gingipains. *P. gingivalis* was pre-exposed for 45 min with leupeptin (inhibits arg-specific gingipains) or cathepsin B inhibitor II (inhibits lys-specific gingipains). Thereafter, *P. gingivalis* was co-incubated with platelets followed by the addition of epinephrine. Aggregation was measured as increase in light transmission through a platelet suspension. The traces shown are representative for at least three experiments.

Aggregation studies revealed that inhibition of PAR4 abolished the *P. gingivalis*/epinephrine stimulation, and that the PAR1 antagonist significantly reduced the *P. gingivalis*/epinephrine induced aggregation in samples from about half of the blood donors. To further study the epinephrine receptor α_2 adrenergic receptor on platelets a specific inhibitor for the α_{2A} adrenergic receptor, yohimbine, was used and results show that no platelet aggregation was detected when stimulation by the *P. gingivalis*/epinephrine combination. Both types of gingipains Rgp and Kgp (lys-specific and arg-specific) were also studied with two inhibitors, leupeptin (Rgp-inhibitor) and cathepsin II B (Kgp-inhibitor) and results show that Rgp lys-specific gingipain is responsible for platelet aggregation in the *P. gingivalis*/epinephrine stimulation. Further investigation was made by intracellular calcium studies. Addition of *P. gingivalis* resulted in no calcium rise in platelets, in contrast if followed by epinephrine there was a rise in intracellular calcium. An inhibition of PAR4 suppressed this action, as well as the inhibition of Rgp-gingipains.

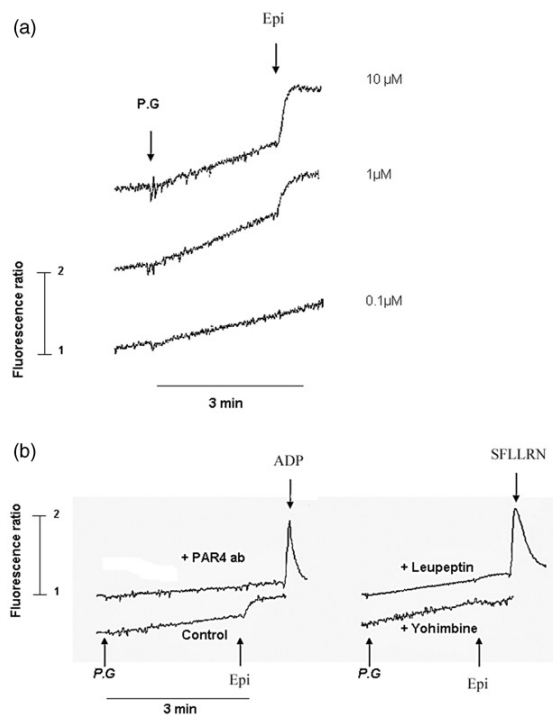


Figure 8. (a) Cytosolic Ca^{2+} responses obtained from fura-2-loaded platelets. Suspensions of platelets were stimulated with *P. gingivalis* followed by epinephrine (Epi; 0.1–10 μ M) as indicated by arrows. The traces shown are representative for at least three experiments. (b) Cytosolic Ca^{2+} responses obtained from fura-2-loaded platelets. Suspensions of platelets were stimulated with *P. gingivalis* followed by epinephrine (Epi; 10 μ M) as indicated by arrows. Suspensions of platelets were pre-treated with a PAR-4 blocking antibody (PAR4 ab; 3 μ g/ml) or the 2-adrenergic receptor antagonist yohimbine (1 μ M) followed by *P. gingivalis* and epinephrine, as indicated above each trace. Conversely, *P. gingivalis* was pre-treated with the arg-specific gingipain inhibitor leupeptin for 45 min before co-incubation with platelets. The responsiveness of drug-treated platelets was controlled by adding ADP (10 μ M) and SFLLRN, PAR-1 activating peptide. The traces shown are representative for at least three experiments.

Conclusions

- There is a direct interaction between the periodontal pathogen *P. gingivalis* and the stress hormone epinephrine.
- *P. gingivalis* derived Rgp-specific gingipains activates PARs on the surface of the platelets.
- The synergistic action of *P. gingivalis* and epinephrine occurred both in aspirin-treated and untreated platelets.

Paper II

In this paper we describe the potential synergetic interaction between the thrombin receptor PAR4 and the stress hormone epinephrine in aspirin-treated platelets. Methods used were light-transmission aggregometry, intracellular calcium measurements, western blot technique, and ATP-release measurement. Results shows that a sub-threshold concentration of thrombin followed by epinephrine gives strong and full platelet aggregation and calcium mobilization, In contrast a sub-threshold concentration of thrombin does not result in any aggregation or rise in intracellular calcium at all. Epinephrine alone does not stimulate aggregation in an isolated platelet suspension. Results concerning inhibition of PAR1 and PAR4, PAR4 inhibition with the PAR4-blocking polyclonal chicken antibody completely abolish platelet aggregation, whereas PAR1 blocking with SCH 79797 does not. Further inhibition of $\alpha 2$ adrenergic receptor with yohimbin also abolishes platelet aggregation.

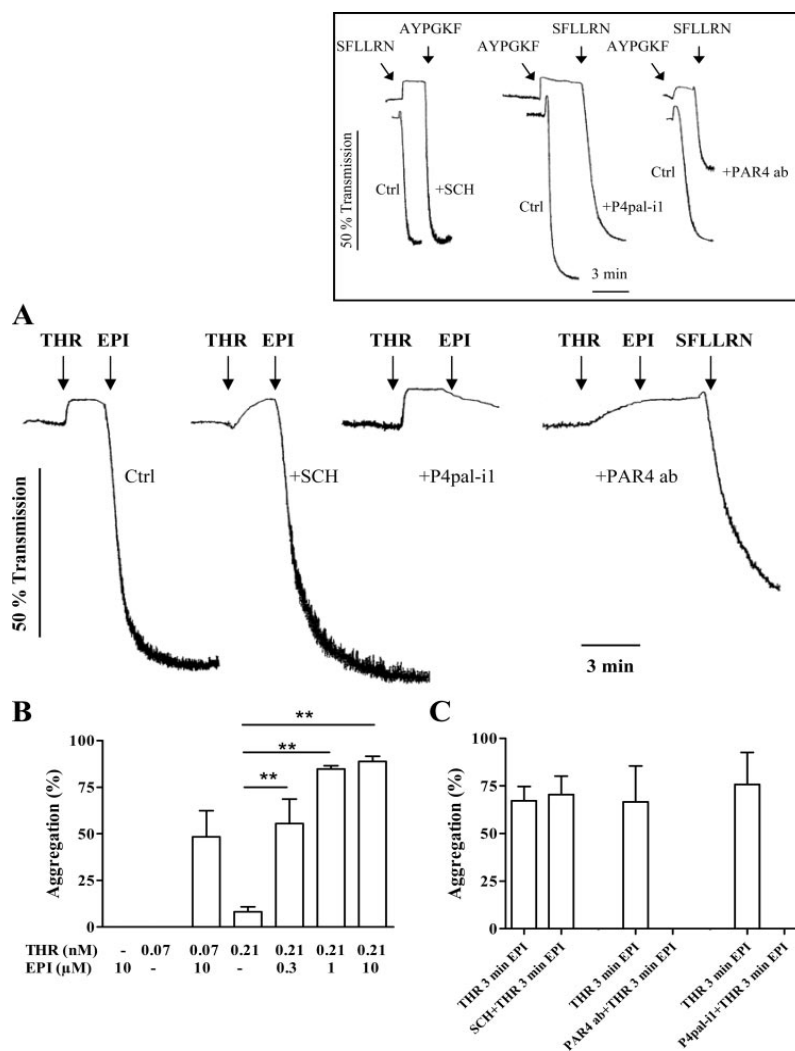


Figure 9. Platelet aggregation induced by thrombin and epinephrine.

A, original traces of platelet suspensions exposed to sub-threshold concentrations of THR (0.21 nM) and EPI (10 μ M), as indicated by the arrows. In some experiments the platelets were pretreated with the PAR-1 antagonist SCH79797 (5 μ M; SCH), the PAR4-specific pepducin P4pal-il (10 μ M) or the PAR4-blocking antibodies (PAR4 ab; 10 μ g/ml) for 5 min before introducing thrombin and epinephrine. The trace to the right shows that the PAR1-activating peptide SFLLRN (12.5 μ M) induced a strong aggregation response in the presence of the PAR4-blocking antibodies. Inset, SCH79797 (5 μ M) abolished platelet aggregation induced by SFLLRN (12.5 μ M), whereas the PAR4-blocking antibodies and the PAR4-specific pepducin P4pal-il eliminated aggregation caused by AYPGKF (100 μ M). Ctrl, control. B, summarized effects of thrombin and epinephrine on platelet aggregation. Platelets were pretreated for 3 min with sub-threshold concentrations of thrombin, and epinephrine was introduced 3 min later. C, summarized effects of SCH79797, the PAR4-blocking antibodies, and P4pal-il on thrombin/epinephrine-induced platelet aggregation. The platelets were treated with the inhibitors for 5 min after which a sub-threshold concentration of thrombin was added, and epinephrine (10 μ M) was introduced 3 min later. Bar graphs B and C show the maximum aggregation induced by thrombin and epinephrine (means \pm S.E., n = 3–8). Statistical significance was tested by analysis of variance (**, $p < 0.01$).

To further study the mechanism cross linking these receptors giving full aggregation response we tried to inhibit the two P2Y receptors; P2Y12 and P2Y1 with cangrelor and MRS2179, respectively. Our results show that inhibiting either of the P2Y receptors or both together the PAR4/epinephrine event cannot be abolished. We know that secondary granule release upon the first activation is important in platelets. We therefore studied the ATP granule release, and results shows that ATP is secreted during activation by epinephrine in PAR4 pre-stimulated platelets. ATP secretion is coupled to another purinergic receptor, P2X1, which is a rapid calcium cation channel with ATP as an agonist. Furthermore, we blocked the P2X1 receptor by using MRS2159 and NF449, and the results are very interesting; both aggregation and calcium mobilisation induced by PAR4/epinephrine stimulation was completely abolished by this blockade. Thus ATP activation of P2X1 is an important key event for the synergetic interaction between PAR4 and epinephrine.

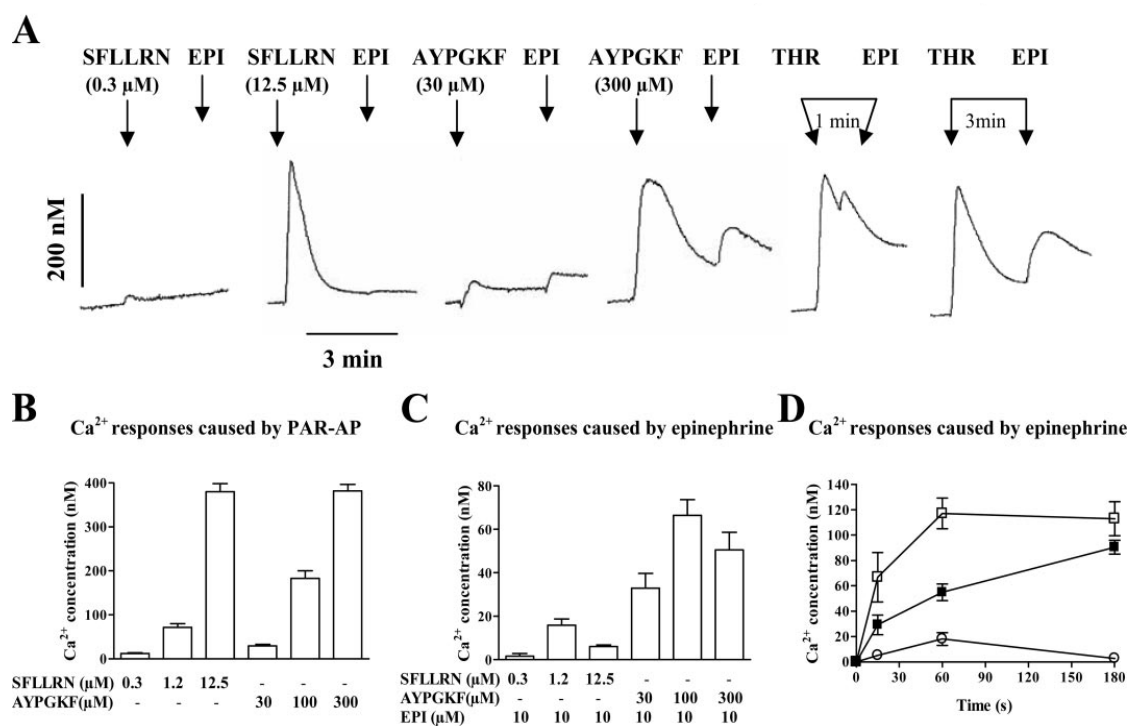


Figure 10. The Ca²⁺-mobilizing effect of PAR-activating peptides and epinephrine.

A, original traces of fura-2-loaded platelets exposed to the PAR1-activating peptide SFLLRN (0.3–12.5 μ M) and the PAR4-activating peptide AYPGKF (30–300 μ M). EPI (10 μ M) was introduced 3 min after the PAR-activating peptides. The two traces to the right show the Ca²⁺-mobilizing capacity of epinephrine introduced 1 or 3 min after THR (0.7 nM). B, summary of the peak rise in [Ca²⁺]_i induced by SFLLRN and AYPGKF. C, the peak rises in [Ca²⁺]_i induced by epinephrine in platelets pre-stimulated with SFLLRN or AYPGKF. D, time study of the peak rises in [Ca²⁺]_i induced by epinephrine (10 μ M) in platelets pre-stimulated with 0.7 nM thrombin (■), 100 μ M AYPGKF (□), or 12.5 μ M SFLLRN (○). Epinephrine was added from 15 s to 5 min after thrombin or the PAR-activating peptides. The data in bar graphs B–D represent the means \pm S.E. (n = 4–6).

To study the signal downstream we studied the phosphorylation of Akt on serine 473. A low dose of PAR4-AP, but not a low dose of PAR1-AP induced phosphorylation. One of the mechanisms underlying this synergetic action may be src-kinase and PI3/Akt signaling. We used the PI3-kinase inhibitor LY294002 which reduced the signal from the PAR4/epinephrine activation. In aspirin-treated platelets PAR4 and α_2 adrenergic receptor interacts via the purinergic receptor P2X1. In contrast, both PAR1 and PAR4 interact with α_2 adrenergic receptor and P2X1 in non-aspirinated platelets.

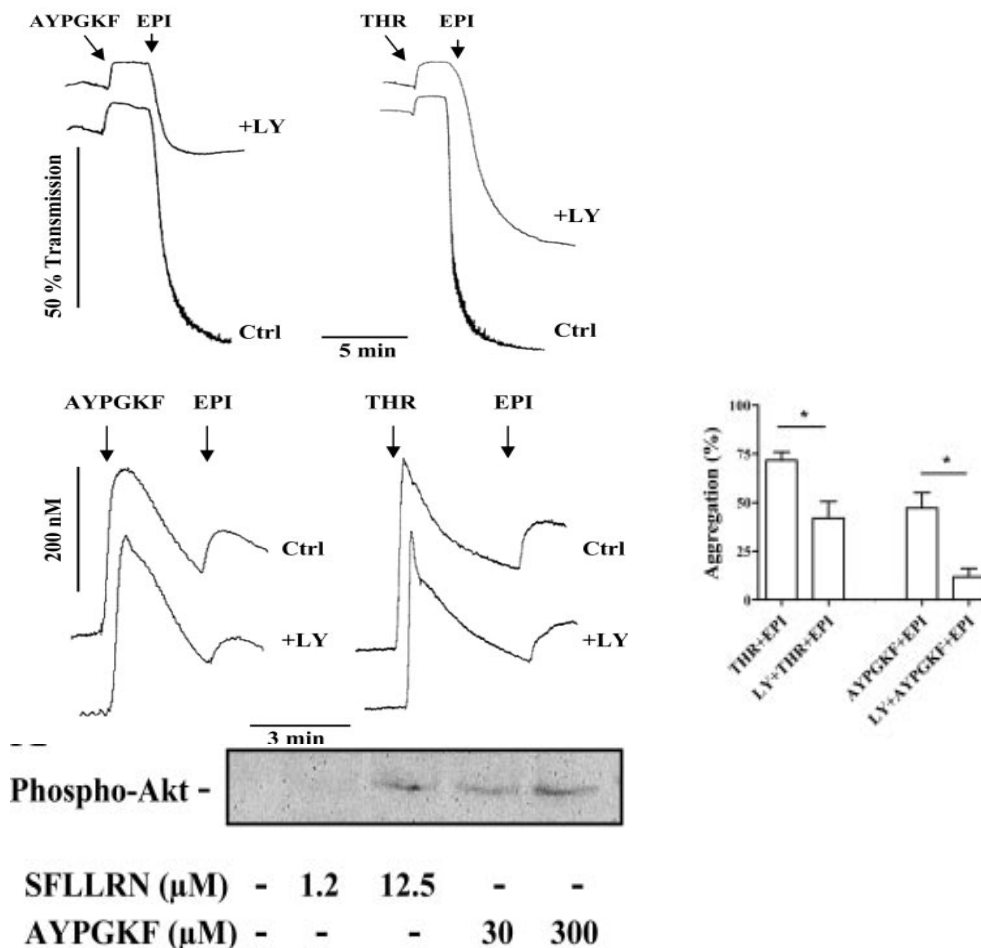


Figure 11. **The role of PI3-kinase in thrombin-epinephrine cross-talk.**

A, Western blot demonstrating phosphorylation of serine 473 on Akt. Platelet suspensions were treated with the PAR4-activating peptide AYPGKF (30 or 300 μM) or the PAR1-activating peptide SFLLRN (1.25 or 12.5 μM) for 5 min, and the reaction was subsequently stopped by adding Laemmli buffer. Untreated suspensions were analyzed in parallel. The illustrated blot is representative of four separate analyses. B, original traces of aggregation (top) and Ca^{2+} (below) measurements. Platelet aggregation was induced by adding THR (0.21 nM) or the PAR4-activating peptide AYPGKF (30 μM) followed by EPI (10 μM). Ca^{2+} responses were provoked by adding 0.7 nM thrombin or 300M AYPGKF followed by 10 μM epinephrine. Platelets were pretreated for 5 min with the PI3-kinase inhibitor LY294002 (10 μM ; LY). Untreated platelets were analyzed in parallel (Ctrl). Inset, summarized effect of LY294002 (10 μM) on platelet aggregation induced by a low concentration of thrombin (0.21 nM) or AYPGKF (30 μM) combined with epinephrine (10 μM). The results represent the maximum aggregation (means \pm S.E., n=6). Statistical significance was determined using Student's t test (*, $p < 0.05$).

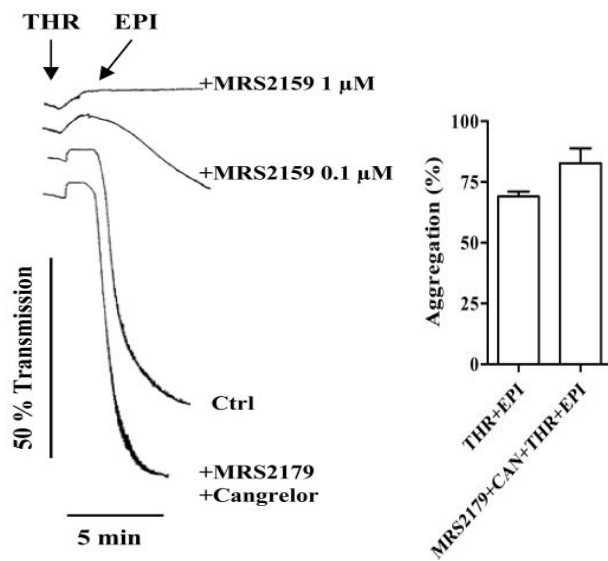


Figure 12. **The role of ADP and ATP in thrombin-epinephrine cross-talk.**

In some experiments the platelets were pretreated with the P2X1 receptor antagonist MRS2159 or the P2Y1/P2Y12 receptor antagonists MRS2179 (20 μM) and cangrelor (100 nM; CAN). Impact of the combination of MRS2179 (20 μM) and cangrelor (100 nM) on thrombin/epinephrine-induced platelet aggregation (means ± S.E., n=6).

Conclusions (paper II)

- PAR4 synergies with the α_{2A} -adrenergic receptor and induces strong activation in aspirin-treated platelets, in both aggregation and calcium mobilisation measurements.
- Secreted ATP via the P2X1 receptor is a powerful executor of aggregation and calcium mobilisation.
- The cross-talk between PAR4/ α_{2A} -adrenergic receptor/P2X1 circumvents the action of aspirin and ADP receptor antagonists.
- PI3-kinase is involved in the cross talk between PAR4 activation and epinephrine.

DISCUSSION

During my time as a PhD student I have tried to understand the platelet thrombin receptors. There must be a reason and physiological relevance why platelets express two different PARs. PAR1 was the first receptor to be discovered in platelets and is therefore more known compared to PAR4. Thrombin binds to and cleaves PAR1 with much higher affinity than PAR4 and is thought to be more important in the circulatory system. Very little is understood regarding the differences in how these receptors signal downstream.

We have learned how to exercise, we think we know how to eat, and we do know that we should neither smoke nor drink, but still we do. Can all or some of cardiovascular risk factors contribute to bad health such as cardiovascular disease, atherosclerosis and finally atherothrombosis? We know that the formation of atherosclerotic plaque starts in early age, but what is it that triggers plaque rupture? Is today's society too stressful which leads us to always walk around with elevated sympathetic activation? Do we spread too much pathogens or are we less resistant to pathogens than our forefathers? To understand this problem basic science is needed, and first to start with is to investigate blood platelets together with different outer threats.

Is it possible to develop cardiovascular disease from infection or and from stress? It is reported that viruses and bacteria are found in the circulatory system and in atherosclerotic plaques [82, 83]. The questions are; are these agents the triggers and reason for development of atherosclerosis, or is it the inflammatory assembly in an atherosclerotic environment enjoyable for pathogens?

Our first results in vitro showed that a low dose of *P. gingivalis* followed by epinephrine resulted in full and strong aggregation, whereas neither *P. gingivalis* nor epinephrine alone induced aggregation, which could lead to a new interesting mechanism involving infection and the stress hormone epinephrine. Naito et al [84] showed that strains of *P. gingivalis* lacking Kgp-type activity induced platelet aggregation in PRP, in as well as in strains lacking Rgp-activity. We show otherwise, but in our study we used isolated platelets, not PRP, and we used inhibitors towards Kgp- and Rgp gingipains. Rgp-gingipains are divided into two subtype groups; rgpA and rgpB, and Naito and colleagues also showed confirming results with ours; the strain which has rgpB-derived activity induced aggregation in isolated platelet suspension, which could be suppressed with leupeptin, an Rgp-specific inhibitor. Finally in our project we used a concentration of *P. gingivalis* that never induced platelet aggregation by itself.

Our conclusion concerning the crosstalk between PAR4 and α_{2A} -adrenergic receptor during thrombin stimulation is that ATP via P2X₁ is a key mediator for aspirin-treated platelets. Thrombin receptors and purinergic receptors have been investigated for many years, and are suggested as targets for new antithrombotic drugs. In one study of Moliterno *et al.* [85] an inhibitor of PAR1, SCH 530348 was given in a phase 2 trial. The PAR1 antagonist or placebo plus aspirin, clopidogrel, and antithrombin therapy were given. Results showed no differences in minor or major bleeding, but there was a trend for an increased risk of major adverse cardiac events with higher doses of the PAR1 antagonist. Hechler *et al.* [86] showed in a murine model that injections of an experimental antagonist of P2X₁, NF449, a decreased collagen and epinephrine-induced platelet aggregation intravascularly, and there was no increase in tail bleeding time. Contrarily they could not prevent TRAP-induced aggregation in human PRP incubated with NF449.

This thesis contributes with new information on different aspects of less known risk factors (e.g. infection and stress) contributing to cardiovascular disease together with the mechanisms of action of the two thrombin receptors in platelets.

Main conclusions and findings

Platelet thrombin receptors PAR1 and PAR4 have during the last decades been investigated in many physiological and biological aspects. Until now PAR1 has been considered a good target for prevention of myocardial infarction and stroke, since its properties are to have high affinity for thrombin, fast calcium mobilization upon activation, and a fast downstream signaling. PAR4 on the other hand is binding thrombin with lower affinity compared to PAR1, and therefore requires a higher dose of thrombin. PAR4 cleaves more slowly by thrombin, but has a more pronounced response and contributes to the most part of the thrombin signal [87], and is thought to be less important in circulatory events.

In our two papers we have shown that epinephrine via α_{2A} -adrenergic receptors causes Ca^{2+} mobilization in *i*) *P. gingivalis* pre-treated platelets and in *ii*) thrombin pre-treated platelets. This effect required *i*) *P. gingivalis*-derived arg-specific gingipains and preactivation of PARs and *ii*) preactivation of PAR4 in aspirinated platelets, and that P2X₁ receptor and release of ATP has a key role in the cross talk between the two receptors.

Contrary to other platelet activators (e.g. ADP, thromboxane A₂, thrombin, serotonin), epinephrine does not induce rises in $[\text{Ca}^{2+}]_i$ in isolated platelet suspensions. In fact, it has been known for decades that the α_{2A} -adrenergic receptor is coupled to a G_i-protein and that the subsequent intracellular signal transduction downstream involves inhibition of adenylyl cyclase activity [88].

Our data thus show that arg-specific gingipains facilitates for an alternative signal transduction pathway when platelets are exposed to epinephrine. In accordance, Keularts *et al.* [89] have previously reported that epinephrine causes Ca^{2+} mobilization in platelets pre-stimulated with thrombin. Consequently, we suggest that one mechanism underlying the marked aggregation response induced by *P. gingivalis*/epinephrine involves a capacity of epinephrine to provoke Ca^{2+} mobilization in both aspirin, and non-aspirinated platelets.

In conclusion, the first study points to a direct interaction between the periodontal pathogen *P. gingivalis* and the stress hormone epinephrine. We propose that *P. gingivalis*-derived arg-specific gingipains activates PARs on the surface of the platelets. This leads to an aggregation response and an unexpected Ca^{2+} mobilization when epinephrine binds to the α_{2A} -adrenergic receptor. Our results indicate for a novel mechanism that may contribute to pathological platelet activation. The relevance in a clinical context remains, however, to be determined.

Aspirin is used extensively and worldwide to manage cardiovascular diseases. In addition, particularly in clinical research, much attention has been focused on “aspirin resistance,” failure to inhibit cyclooxygenase 1, or treatment failure. We furthermore found that in aspirin-treated platelets the synergistic interplay of thrombin and epinephrine depends primarily on occupancy of PAR4 and the α_{2A} -adrenergic receptor, and we also noted that the P2X₁ receptor and ATP as an agonist is a key mediator during the cross-talk between those two receptors. This means that ATP can be a surprisingly effective inducer of platelet aggregation. Moreover, we suggest that PAR4-induced activation of PI3-kinase participates in the signaling cross-talk that leads to a more powerful platelet response.

Taking the two papers together, these findings imply that PAR4 plays an important and unique role in platelet signaling and activation.

TACK

Under mina två år på Farmakologen och Klinisk Kemi har jag jobbat ihop med otroligt trevliga och kunniga människor. Tack vare dem så blev denna avhandling klar i tid.

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