THESIS ON NATURAL AND EXACT SCIENCES B72

Inhibition of platelet aggregation with combination of antiplatelet agents

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Declaration:
Hereby I declare that this doctoral thesis, my original investigation and achievement, submitted for the doctoral degree at Tallinn University of Technology has not been submitted for any academic degree.

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Trombotsüütide agregatsiooni inhibeerimine antiagregatiivsete ainete kombineerimisega

VILJA MARDLA

CONTENTS

LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to in the text by their roman numerals. Some unpublished data are also presented.

I. Mardla V., Kobzar G., Rätsep I., Lõhmus M., Järving I., Samel N. (2001) Sensitivity of platelets to prostaglandins in patients with coronary artery disease. Proc. Estonian Acad. Sci. Biol. Ecol., 50, 1, 33-36. Short communications.

II. Kobzar G., Mardla V. (2002) Inhibition of platelet aggregation by pyridoxine. Nutr. Res., 22, 997-1001.

III. Mardla V., Kobzar G., Samel N. (2004) Potentiation of antiaggregating effect of prostaglandins by α-tocopherol and quercetin. Platelets, 15, 5, 319-324.

IV. Kobzar G., Mardla V., Samel N. (2005) Effects of α-tocopherol, L-arginine, and quercetin on aggregation of human platelets. Nutr. Res., 25, 269-575.

V. Mardla V., Kobzar G., Rätsep I., Samel N. Effects of combining four antiplatelet vitamins on platelet aggregation. Manuscript.

Abstracts of reports on the subject of the thesis:

I. Kobzar G., Mardla V., Rätsep I., Raukas R., Samel N. (2002) Inhibition of platelet aggregation by alpha-tocopherol in healthy subjects and patients with coronary artery disease. Abstracts of Nottingham Platelet Conference incorporating the 5th UK meeting on platelets and 9th Erfurt Conference on platelets. Nottingham, UK. Platelets, 13, 343.

II. Kobzar G., Mardla V., Samel N. (2004) Combined effect of prostaglandins with alpha-tocopherol and quercetin. Abstracts of 10th Erfurt Conference on platelets. Erfurt, Germany. Platelets, 15, 496.

ABBREVIATIONS

AA	arachidonic acid
ABP	actin binding protein
AC	adenylate cyclase
ADP	adenosine diphosphate
AMP	adenosine monophosphate
ATP	adenosine triphosphate
bFGF	basic fibroplast growth factor
C	vitamin C, ascorbic acid
CAD	coronary artery disease
cAMP	adenosine cyclic monophosphate
cAMP-PK	adenosine cyclic monophosphate protein kinase
cGMP	guanosine cyclic monophosphate
cGMP-PK	guanosine cyclic monophosphate protein kinase
COX	cyclooxygenase
DAG	diacylglycerol
DOG	1,2-dioctanoyl-sn-glycerol
ECGF	endothelial cell growth factor
EDNO	endothelium-derived nitric oxide
EDTA	ethylendiaminetetraacetate
GC	guanylate cyclase
GDP	guanosine diphosphate
GP	glycoprotein
H_2O_2	hydrogen peroxide
IC_{50}	the concentration causing half-maximal inhibition
IGF-I	insulin-like growth factor-I
IP_3	inositoltriphosphate
LDL	low-density lipoproteins
LPO	lipid peroxidation
NMMA	N^G-monomethyl-L-arginine
NO	nitric oxide
NOS	nitric oxide synthase
O_2-	superoxide radical
Ox-LDL	oxidised low-density lipoproteins
PAF	platelet activating factor
PAR	G-protein-coupled protease receptors
PDE	phosphodiesterase
PDGF	platelet-derived growth factor
PG	prostaglandin
PGE_1	prostaglandin E_1
PGE_2	prostaglandin E_2
8-iso-PGE_2	8-iso-prostaglandin E_2
PGE_3	prostaglandin E_3

8-iso-$PGF_{2\alpha}$	8-iso-prostaglandin $F_{2\alpha}$
PGG_2	prostaglandin G_2
PGH_2	prostaglandin H_2
PGI_2	prostaglandin I_2, prostacyclin
PIP_2	phosphatidylinositolbiphosphate
PKC	protein kinase C
PL	pyridoxal
PLA_2	phospholipase A_2
PLC	phospholipase C
PLP	pyridoxal phosphate
PM	pyridoxamine
PN	pyridoxine
PPP	platelet-poor plasma
PRP	platelet-rich plasma
Q	quercetin
SOD	superoxide dismutase
TGF-β	transforming growth factor-β
TxA_2	thromboxane A_2
TxB_2	thromboxane B_2
VASP	vasodilator-stimulated phosphoprotein
VEGF	vascular endothelial growth factor
vWF	von Willebrand factor
WP	suspension of washed platelets

INTRODUCTION

All blood cells originate and mature in the bone marrow. They begin in stem cells and then differentiate into red cells, white cells and platelets. Platelets are the smallest anucleated cells of human blood (diameter 2-4μm). Platelets play a crucial role in the blood clotting process by forming a platelet plug. This is a two-step process. First, single platelets bind to the site of the wound (adhesion) and become activated. Next, the platelets bind to each other forming platelet aggregates. When platelets become activated, they undergo a shape change from the discoid form to the globular form and release agents, which activate the surrounding platelets.

Under normal physiological conditions when a blood vessel is damaged, the task of platelets within the circulation is to arrest the loss of blood. This process ultimately culminates in the formation of a platelet plug temporarily sealing off the damaged vessel wall. In contrast, in pathological conditions, such as atherosclerosis, arterial thrombus formation may limit the blood supply to nearby tissues, thus causing local ischemia and/or the progression of the atherosclerotic lesion.

Platelets can be activated by a number of compounds found in blood and blood vessels such as adenosine diphosphate (ADP), platelet-activating factor (PAF), thrombin, collagen, etc., on the other hand, the inhibitors of platelet activation such as prostacyclin (PGI_2), nitric oxide (NO) and some others prevent platelets to aggregate. Besides the above-mentioned two groups of compounds, which cause platelet aggregation or inhibition, there are factors that modulate aggregation. They themselves induce only a small aggregation or do not at all, but may essentially amplify or weaken the aggregating effect of other compounds.

The inhibitors of platelet activation are of more interest for medicine since they can be used for the inhibition of thrombus formation. There are a number of antiplatelet drugs used in medicine at present. However, all they have unwanted side effects and are not effective in all patients. Some food components such as vitamin E, vitamin B_6, L-arginine and flavonoids inhibit platelet aggregation. They have mild if any side effects when administered, even at doses, which essentially exceed recommended daily intake. However, each of these agents alone is a weak inhibitor of platelet aggregation.

On the other hand, there can be several antiplatelet compounds such as vitamins, prostacyclin, NO in blood simultaneously and they can interact with each other. The effects from the combination of two or more agents may be significantly greater than the sum of the effects of each agent administered alone (synergism) or a concurrent action of an agent at a concentration that is not effective may increase the effect of another agent (potentiation). There is a little information on the effects of the combination of several antiplatelet agents together. Different antiaggregants may act through different signalling pathways. As a rule, the antiplatelet drugs used in medicine at present block only one of several biochemical pathways of platelet activation, leaving others untouched. The use of the combination of different weak antiplatelet agents, which inhibit platelet aggregation through different signalling

pathways, might give a stronger effect. Using the photometrical method, we studied how the interaction of vitamins with each other and vitamins with endogenous antiplatelet compounds such as prostaglandins affect platelet function.

Interaction with intact vascular endothelium, which produces a number of antiplatelet compounds, contributes to the protection of platelets from activation. However, interaction with dysfunctional endothelium is found to cause platelet activation. α-Tocopherol was shown to protect endothelium from the factors causing its damage. The effect of α-tocopherol on vascular endothelium is largely based on the studies of vascular endothelial cells in culture. In this work instead of endothelium cells in culture, we used preparations of umbilical vein and studied the effect of α-tocopherol on its endothelium and the effect of endothelium on platelet function.

1. REVIEW OF THE LITERATURE

1.1. Platelet activation and their role in the formation of thrombi

1.1.1. Platelets and their role in thrombosis

Platelets are anuclear secretory blood cells that contain a complex network of membrane structures, including the plasma membrane that extends through the multiple channels of the surface-connected canalicular system. The dense tubular system is the equivalent of the smooth endoplasmatic reticulum in the other cells. There are numerous organelles dispersed in the cytoplasm including mitochondria, lysosomes and peroxisomes. α-Granules and dense granules are platelet specific storage granules. There are also glycogen particles (Willoughby et al., 2002).

Platelets play a key role in the first phases of the haemostatic process and arterial thrombosis. Arterial thrombi mainly consist of platelets. For example, especially dangerous are coronary artery thrombi because they may cause heart infarction. Whenever a blood vessel is damaged, platelets will adhere to the vessel wall (adhesion) and to each other (aggregation) (Willoughby et al., 2002). Thrombus is rapidly formed, which often includes some leukocytes; there are platelet-platelet aggregates and platelet-monocyte aggregates (Fig. 1). The size of thrombus depends on a complex equilibrium between factors, which aggregate platelets such as ADP, thrombin, collagen, etc. Moreover factors which disaggregate platelets such as NO, PGI_2, etc. Normally, the thrombus partially dissociates and only the necessary platelet mass is left on nonthrombogenic surface. Parts of a lost thrombus may break off and be swept by the circulation as an embolus. Normally, they rapidly dissociate into single platelets, but under pathological condition for example in the case of atherosclerosis, they can block smaller vessels (Clemetson and Clemetson, 1998). Elevated circulating levels of platelet-leukocyte aggregates have been reported in cardiac patients and individuals with chronic physiological stress (Brydon et al., 2006).

The platelets activated in the thrombus formation, release their granule contents (Table 1), including chemokines (platelet factor 4, β-thromboglobulin) and growth factors (PDGF, TGF-β, ECGF, VEGF, IGF-1, bFGF), which influence platelets, endothelial cells, smooth muscle cells and other cells from surrounding tissue (Gawaz, 2004).

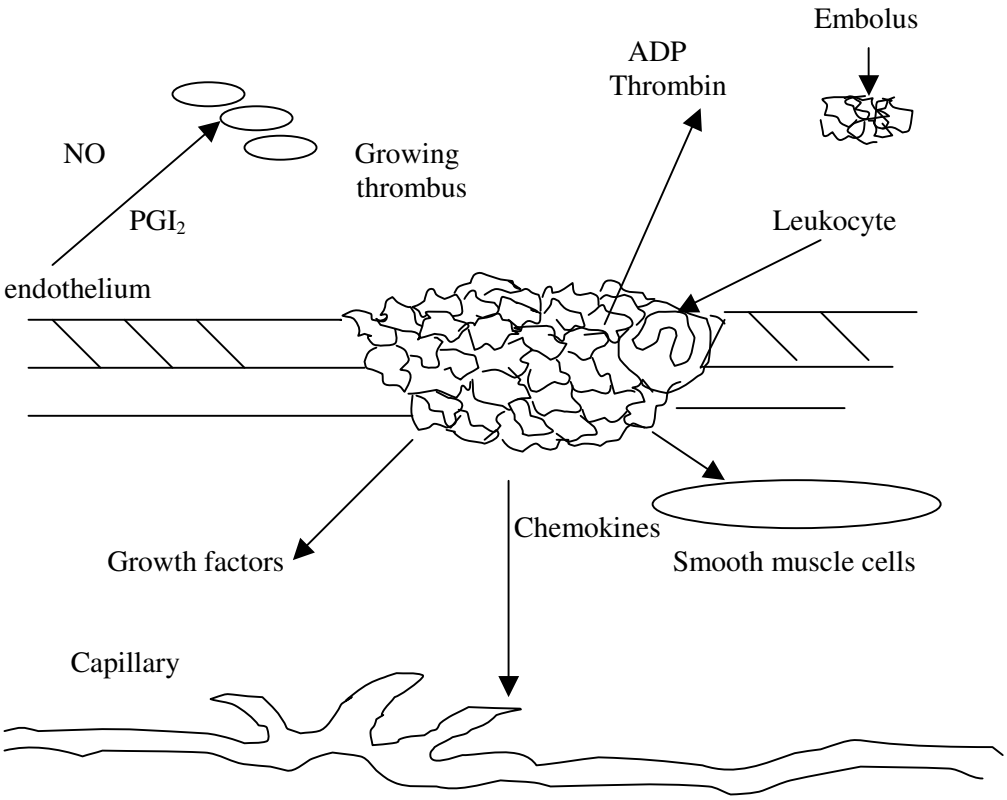


Fig 1. The first stages in the repair of damaged vessel wall. Platelets are brought into contact with the exposed subendothelium, and a thrombus is rapidly formed which often includes leukocytes. The size reached by the thrombus depends on a complex equilibrium between feedback factors such as ADP and thrombin, which have a platelet aggregating effect, and factors from the surrounding endothelial cells such as NO and PGI_2, which have a platelet antiaggregating and vascular relaxing effect. The platelets activated in the thrombus release their granule contents, including chemokines and growth factors, which influence the cells from the surrounding tissue. The growth factors increase the extension of new blood vessels (Modified from Clemetson and Clemetson, 1998).

α-Granules	Dense granules	Lysosomes
vWF	ADP	Glycohydrolases
Platelet factor 4	ATP	Acid proteasis
β-Thromboglobulin	GDP	
PDGF	GTP	
TGF-β	Serotonin	
ECGF	Calcium	
VEGF	Histamine	
IGF-1	Antiplasmin	
bFGF		
Fibrinogen		
Factor VIII		
Factor V		
Thrombospodin		
Albumin		
Fibronectin		
P-selectin		

Table 1. Platelet granules content.

Platelets contain three types of granules (α-granules, dense granules and lysosomes), which may release their contents upon platelet activation. α-Granules contain mainly proteins such as fibrinogen, von Willebrand factor (vWF), platelet factor 4, β-thromboglobulin, platelet derived growth factor (PDGF), etc. Dense granules contain adenine and guanine nucleotides, serotonin, calcium, antiplasmin, histamine. Lysosomes contain enzymes such as acid proteases and glycohydrolases that may participate in inflammation and extravasation of leukocytes through their cytotoxic and proteolytic activity at sites of platelet accumulation at inflamed tissue (Gawaz et al., 2004).

Dense granule contents are easily secreted, α-granule release requires higher agonist concentrations and lysosomal granule secretion only occurs with potent activating agents (Willoughby et al., 2002).

In addition to the contents of the granules, platelets will also produce and secrete pharmacologically active substances such as thromboxane A_2 (TxA_2) and platelet activating factor (PAF) in the case of strong agonists such as thrombin and high dose collagen during their activation and aggregation, establishing a positive feedback system (Gawaz et al., 2004).

Coagulation

The platelet plug provides a nidus around which the blood clots, forming a more definitive haemostatic plug. The fundamental reaction in the formation of the blood clot is the conversion of the soluble plasma protein fibrinogen into insoluble fibrin under the action of thrombin. Thrombin splits off two polypeptide chains from the fibrinogen molecule to form fibrin monomer, which polymerizes to form a meshwork of strands. Initially, the strands are held together weakly by hydrogen bonds, but the activation of factor XIII by thrombin leads to the formation of covalent cross-bridges between fibrin chains, thus strengthening those (Minors, 2007).

The conversion of prothrombin, the inactive precursor of thrombin, to thrombin involves a series of plasma serine proteases (collectively referred to as clotting factors) that normally exist an inactive, proenzyme form, becoming activated in a cascade sequence (Fig. 2). The activation of the clotting factors proceeds down two pathways that converge at factor X to a common pathway. The intrinsic pathway can be activated *in vivo* by the exposure of blood to collagen in the subendothelium. This activates the first factor in this pathway factor XII. The extrinsic pathway is activated by the release of lipoproteins, referred to as tissue thromboplastin or tissue factor, from damaged tissue. Factor VII formed in the extrinsic pathway can directly activate factor IX, thus bypassing the early stages in the intrinsic pathway. In addition, thrombin has positive feedback effects, because thrombin activates factors V and VIII (which act as co-factors in the formation of thrombin and factor Xa, respectively) and factor XI. Blood platelets also play an essential role in clotting since they provide phospholipids PF_3, and the activation of factor X by factor IX and the conversion of prothrombin to thrombin by factor X take place, in part, on the surface of platelets in association with PF_3. In this association, the relevant co-factor (V or VIII) in the reaction is bound to PF_3 along with calcium ions, and the activity of the co-factor is greatly increased (Minors, 2007).

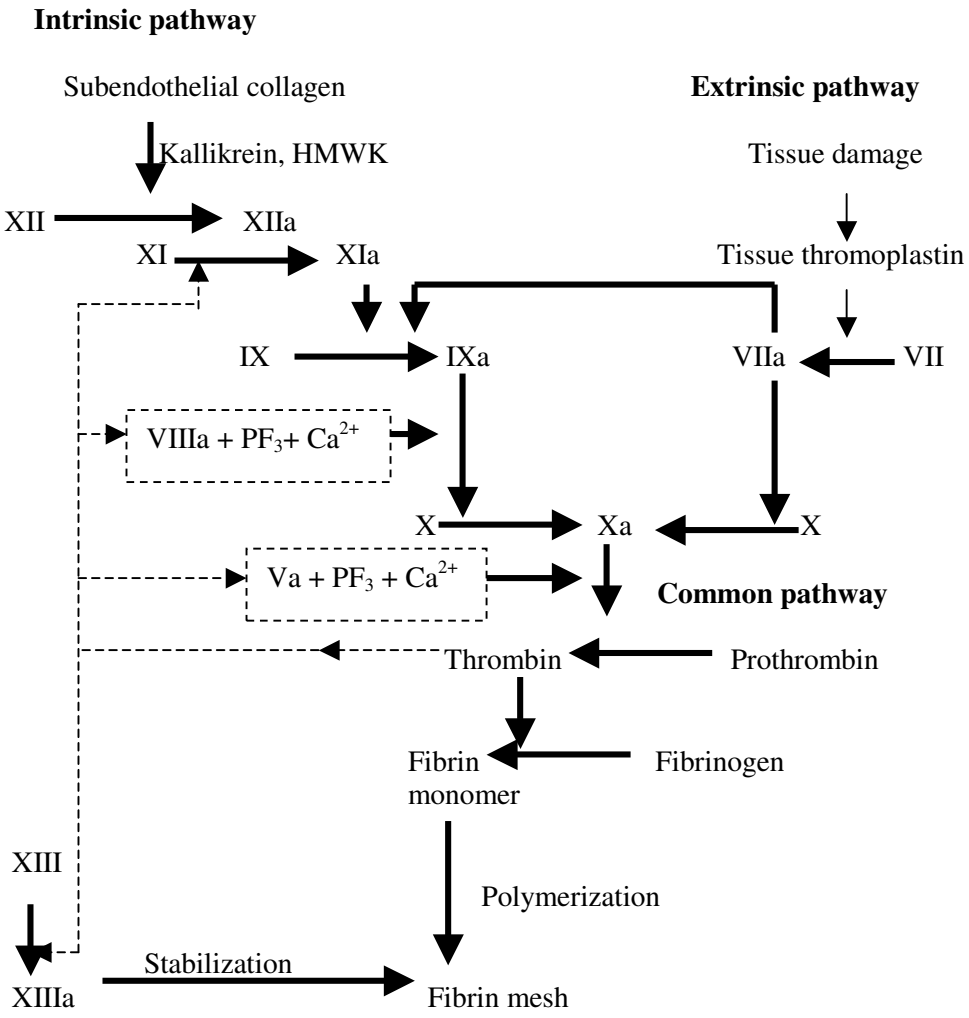


Fig. 2. The clotting cascade. The active form of the various clotting factors is suffixed 'a'. Interrupted arrows show some of the positive feedback actions of thrombin. Reactions shown in boxes take place on the surface of platelets in association with the platelets, platelet factor 3 (PF_3), and calcium ions. HMWK - high molecular weight kininogen (Minors, 2007).

1.1.2. Biochemical pathways of platelet activation

Activation of platelet function as evidenced by aggregation, adhesion and secretion has usually been associated with classical primary agonist such as ADP, thrombin and collagen. On the other hand, „ secondary" agonists like adrenaline do not induce aggregation on their own, but have long been known to promote effective platelet aggregation in the presence of low levels of primary agonists which themselves induce only minimal platelet activation.
All platelet agonists are believed to exert their effects through the interaction with specific receptors on the platelet plasma membrane. There are many target receptors in platelets, but the fibrinogen receptor is essential for linking platelets in aggregation. The size of growing thrombus is dependent on the degree to which fibrinogen receptor is activated in the outer layers of the thrombus (Clemetson and Clemetson, 1998).

Many of the receptors for the stimulatory as well as inhibitory mediators are coupled via G-proteins to adenylate cyclase (AC), followed by the modulation of cytoplasmatic cAMP-levels and subsequent intracellular calcium levels, since cAMP regulates the uptake of free intracellular calcium to the dense tubular system, the platelet storage pool for calcium and inhibits phosholipase A_2 (PLA_2) and cyclooxygenase (COX). Other ways of the calcium mobilisation involve the activation of phospholipase C (PLC) for example by thrombin. PLC catalyses the breakdown of phosphatidylinositol (4,5) biphosphate (PIP_2) into diacylglycerol (DAG) and inositol (1,4,5) triphosphate (IP_3). DAG activates protein kinase C (PKC) and IP_3 induces a release of Ca^{2+} from dense tubular system (Willoughby, 2002).

Last but not least, there exists a substance NO that stimulates guanylate cyclase (GC). GC regulates cytoplasmatic cAMP via the modification of phosphodiesterase (PDE). This enzyme is responsible for the restitution of cAMP to ADP, thus influencing cytoplasmatic calcium levels. If cytoplasmatic calcium increases, calcium-dependent PLA_2 liberates arachidonic acid (AA) from membrane phospholipids. AA is a substance of COX, ending up in the production of TxA_2. In addition, increased cytoplasmatic calcium causes platelet shape change and release of granule contents. Upon activation, platelets release ADP and TxA_2 (which activate additional platelets), serotonin, phospholipids, lipoproteins and other proteins important for the coagulation cascade. The primary effects of agonists are often enhanced by secondary effects attributable to the synthesis of TxA_2 from released AA and to the secretion of ADP (Blockmans et al., 1995).

1.1.3. Key platelet agonists

ADP

Platelet ADP receptors are potential targets for antithrombotic drugs, since ADP plays a crucial role in platelet activation. ADP is an important agonist in its own right and amplifies responses to the agonists. *In vitro,* it causes platelet shape change, aggregation and release of granule contents and plays a role in the secondary aggregation induced by other aggregating agents (Blockmans et al., 1995).

Jin et al. (1998) demonstrated the existence of two distinct G-protein-coupled ADP receptors on platelets, one coupled to PLC, $P2Y_1$, and the other to AC, $P2Y_{12}$ (also known as $P2T_{AC}$ receptor). ADP-induced aggregation shows that there must be simultaneous activation of both receptors (Savi et al., 1998). Both are purinergic receptors, which recognise ADP and ATP. ATP is an antagonist to ADP on platelets. $P2Y_{12}$ receptor couples to inhibitory G-protein (G_i) and upon activation by ADP causes the inhibition of AC. This receptor is essential for ADP-induced platelet aggregation, but does not play a significant role in platelet shape change. $P2Y_1$ receptor couples to Gq and causes intracellular calcium mobilisation from intracellular stores through the activation of PLC and IP_3 formation (Kunapuli et al., 2003).

Evidence suggests that no single receptor can mediate the full response of platelets to ADP. Additionally, ADP acts synergistically to other, even weak, agonist such as serotonin, adrenaline or chemokines (Rozalski et al., 2005). The third ADP receptor P2X1 is an ion channel that upon activation causes an influx of calcium. Even though the activation of this receptor by itself does not lead to aggregation, it causes platelet shape change and aids the activation process of other agonists (Murugappa and Kunapuli, 2006). It is important for thrombus formation under shear conditions in small arteries. ADP receptors on blood platelets are potential new targets for antiplatelet therapy (Rozalski et al., 2005).

PAF

PAF is a phospholipid (Fig. 3), which is a potent platelet agonist. Platelets, monocytes and endothelial cells produce PAF. PAF is released from platelets following stimulation by thrombin or collagen, but not by ADP, AA or PAF itself. Its biological activities are multiple, including an effect on smooth muscular cell proliferation, agonist activity on neutrophils and monocytes and direct toxicity on endothelial cells (Ngugĕn et al., 1995). PAF has been ascribed a potential role in various pathologies such as inflammation, allergy and cardiovascular diseases (Montrucchio et al., 2000).

PAF acts by binding to a G-protein-coupled seven transmembrane receptor, and activates multiple intracellular signalling pathways. PAF induces platelet shape change, aggregation and release of granule contents by the stimulation of the PLC,

intracellular Ca^{2+} mobilisation and the release of AA (Agarwal et al., 1994). PAF stimulates not only PLC but also extracellular signal regulated kinases (Chen et al., 2003). Platelet aggregation induced by PAF is only weakly inhibited by aspirin, whereas collagen-or AA-induced platelet aggregation is strongly inhibited by aspirin.

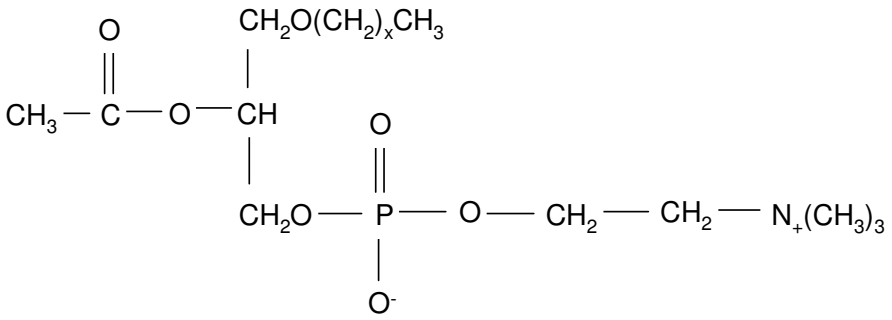


Fig. 3. Structural formula of platelet activating factor (1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine). Where x = 13 to 17.

Thrombin

Thrombin is a serine protease and very powerful platelet agonist, causing platelet shape change, aggregation and secretion from the dense granules, α-granules and lysosomes. Thrombin, ADP and PAF not only cause primary aggregation of platelets but also induce secondary aggregation by ADP released from the storage granules. Thrombin-induced platelet activation is accompanied by G-protein mediated activation of PLC leading to the activation of PKC and increase in the intracellular calcium. Thrombin also activates GP IIb-IIIa, which results in binding fibrinogen and vWF (Ramasamy, 2003). Thrombin signalling is mostly mediated by a family of G protein-coupled protease receptors (PAR). PAR1 and PAR4 have been detected in human platelets. PAR1 is activated when thrombin binds its own receptor and cleaves at the arginine41-serine42 peptide bond giving a tethered peptide ligand capable of activating the receptor. PAR4 is relatively insensitive to thrombin, with EC_{50} for the protease approximately 50-fold higher than the corresponding figure for PAR1. PAR1 is the major mediator of thrombin signalling at low concentrations of thrombin. At high concentrations of thrombin, both PAR1 and PAR4 contribute to platelet activation and counter-regulate the release of VEGF and endostatin from platelets (Ma et al., 2005).

TxA_2

TxA_2 (Fig. 4) is a potent platelet agonist and act through TP receptors. TxA_2 and its mimetics induce a wide array of platelet responses including aggregation, shape change and increase in the expression of GP IIb/IIIa receptors (Halushka, 2000).

The stimulation of TP receptors in platelets leads to an increase in intracellular free calcium, activation of PLC, generation of IP_3 and DAG and activation of PKC. Pharmacological studies suggest that there is heterogeneity of TP receptors in the blood platelets (Tsuboi et al., 2002). TxA_2 stimulates two subtypes of TP receptors Tpalpha and Tpbeta, but its effect in platelets is mediated predominantly through the alpha isoform (Murugappan et al., 2004). Platelet TxA_2 synthesis is markedly increased in a variety of thrombotic cardiovascular diseases.

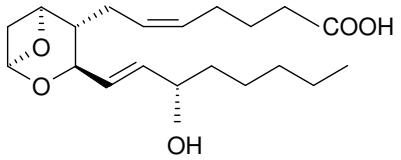


Fig. 4. Chemical structure of TxA_2.

Collagen

Platelets have multiple binding sites for collagen. Platelet receptors for collagen are GP Ia/IIa, GP VI and GP IV (Nurden, 1996). Receptors occupancy is associated with phospholipase C activation and increase in intracellular calcium. Collagen induces platelet shape change, dense granule release and aggregation. Collagen-induced platelet aggregation is a complex process and involves synergistic action of integrins and G-protein-coupled receptors and their ligands (Nieswandt et al., 2001).

Over the past years, GP VI has been established as the central platelet collagen receptor that is essential for platelet adhesion and aggregation. It was demonstrated that direct GP VI-collagen interaction is crucial for initial platelet tethering and subsequent stable platelet adhesion and aggregation at sites of arterial injury (Massberg et al., 2003). Collagen stimulates platelet nitric oxide synthase activation through a specific calcium-dependent GP VI receptor signalling cascade (Riba et al., 2005). Under high shear condition, GP VI and GP Ib-IX-V complex bind collagen and vWF, respectively, and are critical for the initial interaction of circulating platelets with the injured vessel wall (Andrews and Berndt, 2004).

1.2. Antiplatelet agents and their biochemical mechanism of action

1.2.1. Signal pathways of platelet inhibition

Platelet aggregation is at the origin of thrombotic complications of atherosclerosis, especially cerebral and coronary artery diseases. It is thus of great importance to prevent early platelet activation and aggregation, and antiplatelet agents (antiaggregants) are used to prevent the apparition or recurrence of a thrombotic process. The ideal antiaggregant should not inhibit primary haemostasis, which

would result in bleeding complications. It is therefore essential to understand the exact mechanism of the action of antiaggregants. The development of antiaggregants has followed different approaches. The inhibition of platelet function can be reached by increasing intracellular cyclic nucleotides (cAMP, cGMP), either inhibiting phosphodiesterases (PDEs) or by stimulating AC or GC.

The biochemical mechanisms of platelet inhibition are summarized by Schwartz et al. (2001). The elevation of platelet cyclic nucleotides interferes with basically all known platelet activatory signalling pathways and effectively blocks complex intracellular signalling networks, cytoskeletal rearrangements, fibrinogen receptor activation and platelet degranulation. The major target molecules of cyclic nucleotides in platelet are cyclic nucleotide-dependent protein kinases that mediate their effects through phosphorylation of specific substrates. They directly affect receptor/G-protein activation and interfere with a variety of signal transduction pathways, including the PLC, PKC and mitogen-activated PKC pathways. The regulation of these pathways blocks several steps of cytosolic Ca^{2+} elevation and controls a multitude of cytoskeleton-associated proteins.

Cyclic nucleotide levels in human platelets

The elevation of intracellular cAMP and cGMP is the most potent endogenous mechanism of platelet aggregation inhibition. Cyclic nucleotide levels are up-regulated by synthesis through ACs and GCs and down-regulated by degradation through PDEs (Fig. 5). Major target enzymes of cyclic nucleotides involve cAMP-protein kinase (cAMP-PK) and cGMP-protein kinase (cGMP-PK), which mediate their effect through phosphorylation of specific substrate proteins.

ACs are integral membrane glycoproteins that catalyse the synthesis of cAMP from ATP, leading to an increased level of intracellular cAMP. Platelet AC is activated by the α subunit of the stimulatory G-protein ($G\alpha_S$) and inhibited by the α subunit inhibitory G-protein ($G\alpha_i$). Binding prostaglandins (PGI_2, PGE_1, PGE_2) to their receptor, which is coupled to G_S, leads to the stimulation of cAMP formation. Receptors for adenosine and β-adrenergic agents are also coupled to G_S and activate AC. Some platelet activators such us thrombin, adrenaline or ADP induce the release of $G\alpha_i$, thereby down-regulating cAMP levels.

GCs exist in membrane-bound and soluble forms. To date, in platelets is found only the soluble form of GC, which located in the cytoplasm. There is no evidence that platelets also contain membrane-bound GC. Soluble GC contains heme as a prosthetic group and activated by NO-generating agents.

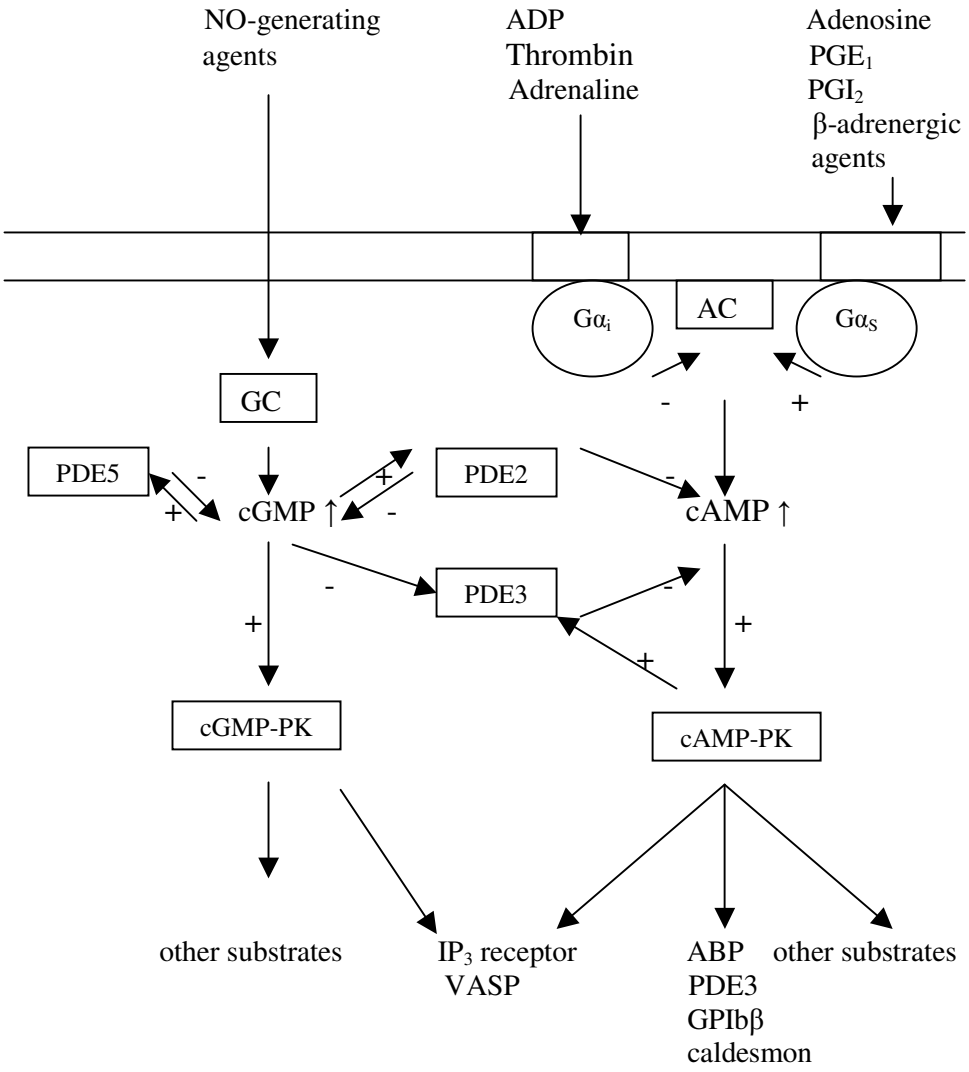


Fig. 5. Regulation and known effectors sites of cyclic nucleotides in platelets (Modified from Schwartz et al., 2001).

PDEs

PDEs are a large group of enzymes consisting of several isoenzyme families that hydrolyse the 3′-phosphoester bond on cAMP and/or cGMP, converting them into biologically inactive 5′-nucleotide metabolites. Platelets contain at least three different types of PDEs (Fig. 5): the cGMP-stimulated PDE2, the cGMP-inhibited PDE3 and cGMP-binding cGMP-specific PDE5. PDE2 hydrolyses both cAMP and

cGMP. PDE3 preferentially hydrolyses cAMP. PDE3 activity is increased by direct cAMP-PK-catalysed phosphorylation. This appears to be a negative feedback loop, which rapidly returns elevated cAMP levels back to basal. PDE5 is highly specific for cGMP hydrolysis.

Inhibitors of PDEs regulate platelet cAMP and cGMP levels via decreased degradation of cyclic nucleotides. The clinical use of specific inhibitors of platelet PDEs, especially in combination with low doses of cyclic nucleotide-elevating vasodilators, could be very useful in situations where specific inhibition of platelet function is desired without a side effect on vascular smooth muscle cells. PDE3 inhibitors have been described as useful antiplatelet agents that also inhibit vascular smooth muscle cell proliferation (Kondo et al., 1999; Hirose et al., 2000; Manns et al., 2002). PDE3 lowers cAMP even in resting platelets and thereby controls thrombus formation (Feijge et al., 2004).

Inhibition of intracellular Ca^{2+} elevation

The increase of intracellular free Ca^{2+} plays a key role during platelet activation, since it regulates multiple Ca^{2+}-dependent enzymes, like Ca^{2+}-dependent PKC isoforms, Ca^{2+}/calmodulin-dependent myosin light chain kinase, cytosolic phospholipase A_2 and the small GTPase Rap1b. Increased levels of cAMP and cGMP antagonise the activator-evoked Ca^{2+} release from intracellular stores and secondary store-mediated Ca^{2+} influx. IP_3 receptors, which mediate the release of Ca^{2+} from the dense tubular system and probably also the secondary store-related influx are directly phosphorylated by cAMP-PK and cGMP-PK in human platelets (El-Daher et al., 2000).

1.2.2. Antiplatelet agents

Inhibition of platelet function by many clinically used antiaggregants blocks only a certain function of platelet activation, leaving others untouched. Inhibition of platelet aggregation includes a variety of signal transduction cascades and this is a very complex process. New platelet inhibitors could be considered in view of *in vitro* results obtained on enzymes involved in platelet activation (Marder et al., 2004).

Clinically available antiplatelet drugs include aspirin, epoprostenol (synthetic prostacyclin), ticlopidine and clopidogrel (which inhibit expression of GP IIb/IIIa receptors in response to ADP) and monoclonal antibody against the GP IIa/IIIb receptor (abciximab). The sites of action of antiplatelet drugs are shown in Fig. 6 (Rang et al., 2001).

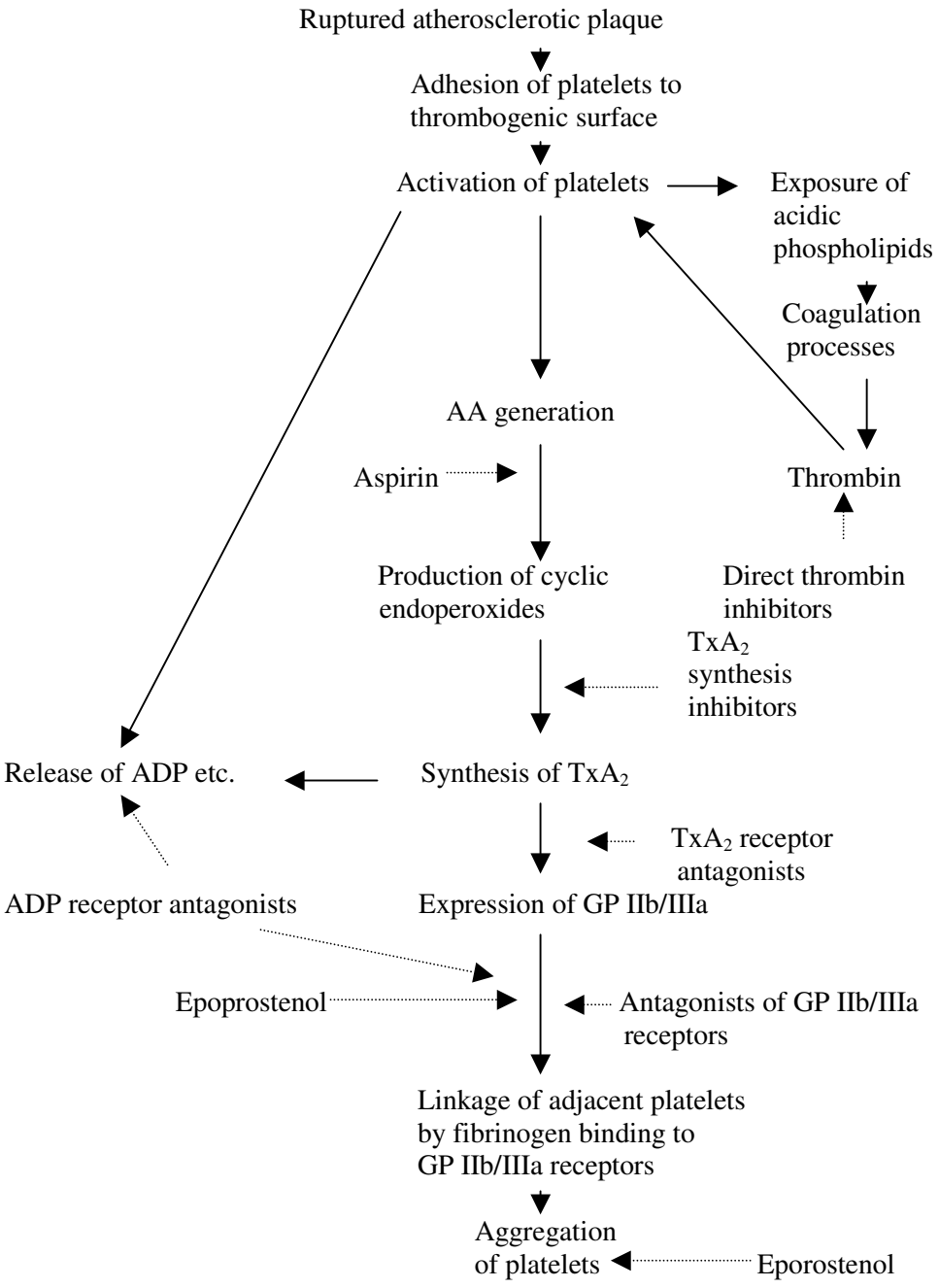


Fig. 6. Sites of action of antiplatelet drugs (Rang et al., 2001).

It is clear that the most important problem in the clinical use of antiplatelet drugs including aspirin is their side effects and in many cases, they are not sufficiently effective. Oral GP IIb/IIIa inhibitors were developed but did not become mainstream therapy due to their cost and the side effect of bleeding (Skinner M. P., 2007). Aspirin is the most widely used antiplatelet drug at present.

Clinical pharmacology of aspirin

Aspirin inhibits platelets by acetylating COX-1, thereby blocking the production of TxA_2. TxA_2 activates platelets, constricts blood vessels, and promotes atherogenesis by inducing the proliferation of vascular smooth muscle cells (Roth and Calverley, 1994). Inhibition of COX-1 by aspirin is irreversible and lasts for the life of the platelet because platelets are anucleate and cannot synthesize new protein. COX-1 is constitutively expressed in platelets (Marcus et al., 2002).

The clinical pharmacology of aspirin has been characterised by measuring arachidonic acid-induced platelet aggregation, which reflects thromboxane-dependent platelet function; and by measuring serum and urinary thromboxane, which reflects the inhibitory effect of aspirin on thromboxane production. Urinary concentration of 11-dehydro thromboxane B_2 reflects total body thromboxane A_2 production, which is believed to be derived primarily from platelets (COX-1) but can also be derived from extra-platelet sources (Fig. 7).

Major adverse effects of aspirin are the ones from gastrointestinal tracts and those resulting from prolonged bleeding time (Shrör, 1995). Another problem with aspirin is that not all patients who are treated with aspirin derive benefit from the drug; some patients still suffer thrombosis despite aspirin therapy (Sane et al., 2002; Patrignani, 2003). Patrignani (2003) suggested that an incomplete suppression of platelet TxA_2 biosynthesis has been assumed to participate in the phenomenon of aspirin resistance.

Mechanism of aspirin resistance

Aspirin resistance refers to less than expected suppression of thromboxane A_2 production by aspirin and has been reported to be independently associated with an increased risk of adverse cardiovascular events. Aspirin treatment completely blocks platelet COX-1 activity, but TxA_2 biosynthesis enhances, presumably through an increased generation of COX-2-dependent PGH_2 in plaque monocytes or activated vascular cells. COX-2 was shown to be expressed in platelets from patients undergoing a coronary artery bypass grafting (Weber et al., 2002). Bhatt (2004) showed some of the possible mechanisms of aspirin resistance: cellular factors (insufficient suppression of COX-1, over-generation of 8-iso-$PGF_{2\alpha}$), clinical factors and genetic polymorphisms. Also, potential causes of aspirin resistance include inadequate dose, drug interaction and other pathways of platelet activation: non-platelet thromboxane production, increased platelet turnover, shear stress, stimulation by collagen, von Willebrand factor, ADP, thrombin, adrenaline

receptors (Hankey and Eikelboom, 2006; Tran et al., 2007). Using *in vitro* tests of platelet function to define and diagnose aspirin resistance may be problematic because these tests measure the effect of aspirin on platelet function in a non-physiologic setting. *In vitro* tests do not take into account the possibility that the production of thromboxane from extra-platelet sources can activate platelets (Fig. 7). Aspirin selectively inhibits COX-1, but there are some reports suggesting that platelet-inhibiting effects of aspirin are not only related to prostaglandin blockade. It has been reported that aspirin inhibits GP IIb/IIIa, P-selectin, vitronectin, CD63, CD107a receptor expression on human platelets (Malinin et al., 2003). Antiplatelet properties of aspirin are probably multi-factorial.

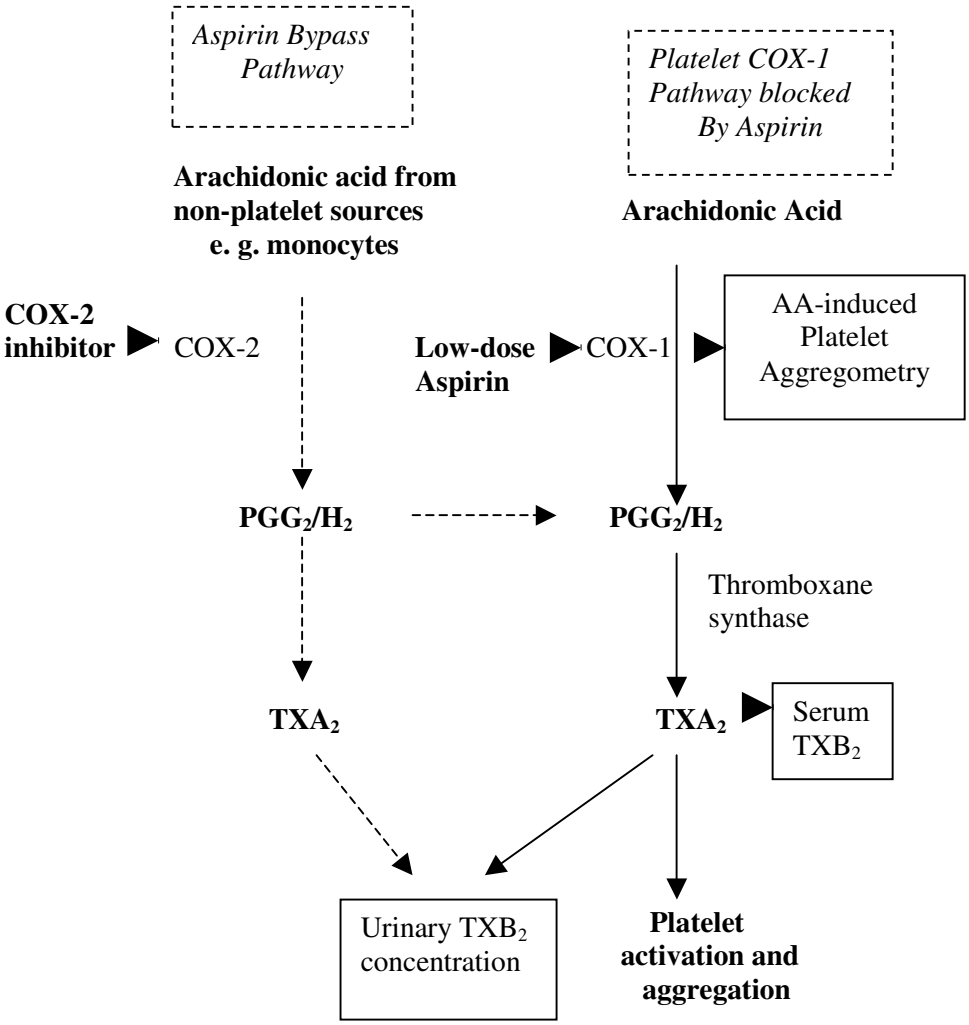


Fig. 7. Sources of thromboxane production and laboratory measures of antiplatelet effect of aspirin. (Hankey and Eikelboom, 2006).

1.2.3. Antiplatelet prostaglandins (PGs)

Antiplatelet prostaglandins are formed by arachidonic acid (AA) following its mobilization from the sn-2 position of membrane phospholipids by different stimuli (Fig 8). In platelets, AA, released in response to primary agonists, such as thrombin and ADP, is offered to COX-1, for its transformation to prostaglandin endoperoxide PGH_2 (Capone et al., 2007). In fact, COX-1 is the major COX isoform expressed in platelets; mature platelets express only COX-1 (Patrignani et al., 2005).

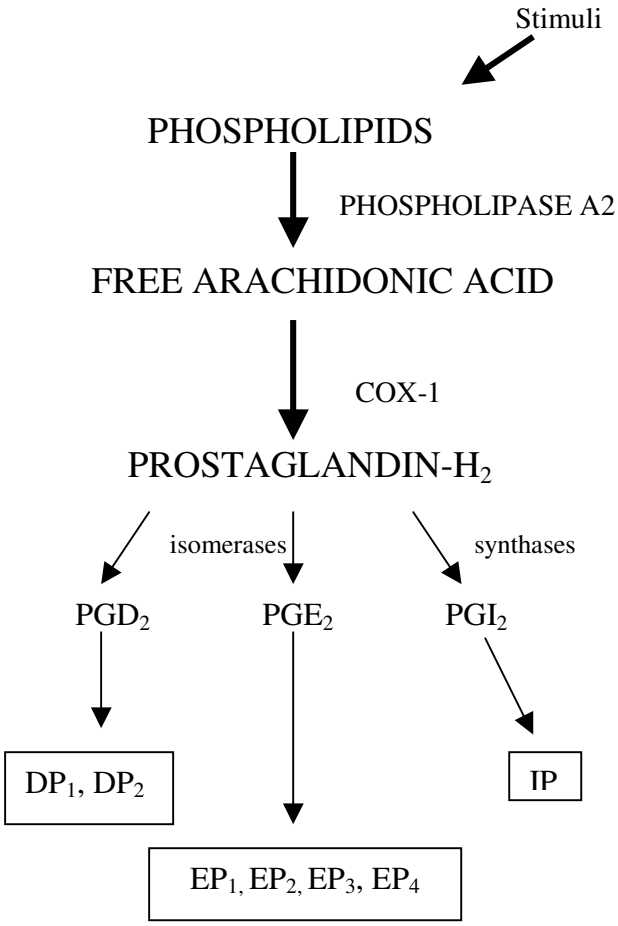


Fig. 8. Pathway of antiplatelet prostaglandins biosynthesis and their specific receptors

The most studied antiplatelet prostaglandin is prostacyclin (PGI_2) (Fig. 9). Several studies have shown that PGI_2 has its own IP receptor, while prostaglandin E1 (PGE_1) (Fig. 9) acts via the IP receptor (Fig. 8). The IP receptor shows high binding activity to prostacyclin analogues such as iloprost, PGI_3 and PGE_1 (Tsuboi et al., 2002), which inhibit platelet aggregation by increasing cAMP levels. All these analogues of prostacyclin are very effective inhibitors of platelets (Kobzar et al., 1993; Kobzar et al., 2001).

The IP receptor agonists act as physiological antagonists, inhibit all aspects of aggregation and can even reverse aggregation, irrespective of the nature of the activating agent. The major drawback of these compounds is profound vasodilation that they induce. At present, PGI_2 (epoprostenol) is used in hospitals as an injectable agent to limit platelet aggregation. However, epoprostenol in continuous infusion has adverse side effects (Pickles and O'Grady, 1982; Sueta et al., 1995; Takahashi et al., 2002). Recent studies have demonstrated that PGI_2 protects against atherothrombosis. Further clinical investigations of orally stable prostacyclin analogues for treatment of cardiovascular diseases may now be warranted (Arehart et al., 2007).

PGD_2 is also synthesised by platelets (Fig. 9) and is an inhibitor of platelet aggregation. PGD_2 acts via DP receptor (DP_1, DP_2) (Fig. 8). PGD_2 inhibits platelet aggregation by increasing cyclic AMP levels, as it is the case for PGI_2. The possible antithrombotic activity of PGD_2 or the DP mimetics has been limited by the side effects, for example facial flushing and headache. The existence of subtypes and isoforms of the DP and IP receptors has not been reported (Armstrong, 1996; Narumiya et al., 1999).

PGE_2 (Fig. 9) has a dual action on human platelets. At high concentration, PGE_2 inhibits platelet aggregation by cAMP-dependent mechanism resulting from the interaction with platelet IP receptor. At low concentration, the direct effect of PGE_2 is the potentiation of platelet aggregation induced by other agents. PGE_2 does not directly aggregate platelets, but rather enhances the extent of aggregation. PGE_2 interacts with two platelet receptors: IP and EP receptors (Armstrong, 1996).

The pharmacology of PGE_2 is complicated by the identification of at least four receptor subtypes (EP_1, EP_2, EP_3, EP_4) (Fig. 8), which often mediate opposing effect in cell. EP_3 is the receptor that mediates decreases in cAMP, EP_2 and EP_4 mediates increases in cAMP. EP_1 mediates increases in Ca^{2+} (Narumiya et al., 1999).

Isoprostanes are family of PG isomers, which are formed from arachidonic acid by free radical or COX-dependent manner. They exert their effect via TP receptors or TP-receptor-like isoprostane receptors. However, there is no evidence to date that specific isoprostane receptors exist (Cracowski and Ormezzano et al., 2004). Wilson et al. (2006) have confirmed that dimerization of the human TP isoforms, TPα and TPβ enhances isoprostane-mediated signal transduction. Some isoprostanes act on platelets as agonists or antagonists, depending on the concentrations (Pratico et al., 1995; Leitinger et al., 1997; Cranshaw et al., 2001). We found that low concentration of 8-iso-PGE_2 could amplify or weaken platelet

aggregation induced by various aggregatory agents in human and rabbit platelets (Kobzar et al., 1997). Vasalle and co-workers (2003) reported that the plasma level of 8-isoprostanes enhances in patients with coronary artery disease. Isoprostanes are currently the more valuable biomarkers of lipid peroxidation and potential mediators in cardiovascular diseases.

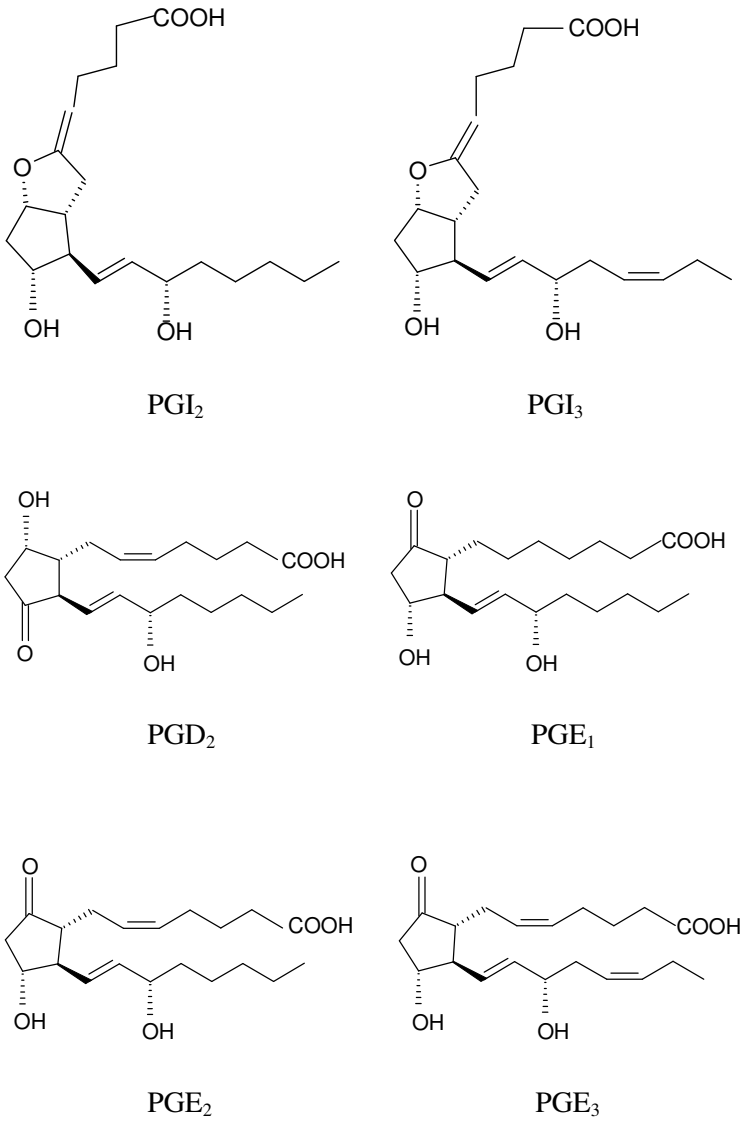


Fig. 9. The chemical structure of the antiplatelet prostaglandins.

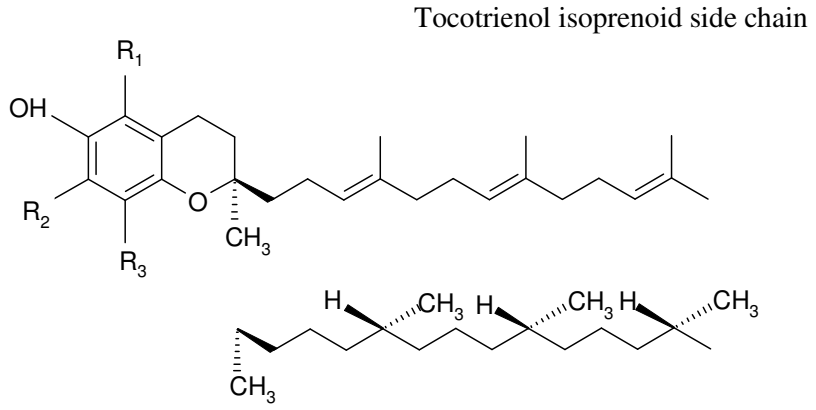
1.2.4. Antiplatelet vitamins

Some vitamins are natural antithrombotic and antioxidant food components. Under normal circumstances, there are large numbers of antioxidants within the body that form a natural defence against free-radical induced damage. Antioxidants react with the free radicals to neutralise their effect by donating electrons, thereby forming much less reactive radicals. The imbalance between protective antioxidants and damaging free radicals is termed oxidative stress. Free radicals are formed in large amounts as an unavoidable by-product of many biochemical processes and in some instances deliberately such as in activated neutrophils. Free radicals contain unpaired electrons. Common examples of free radicals include the hydroxyl radical, superoxide anion and nitric oxide radical (Iuliano et al., 1997).

It is known that oxidative stress is an important factor in the development of vascular disease and thrombosis. Free radicals attack normally functioning cells, including platelets and vascular endothelial cells. Free radicals induce platelet aggregation followed by thrombi formation. Oxidative stress in blood platelets expresses by generation of superoxide radicals (O_2^-) and other reactive oxygen species: H_2O_2, singlet oxygen and organic radicals (Olas et al., 2002). It was shown that collagen, but not ADP or thrombin increased O_2^- formation in washed human platelets. Collagen activation induces nicotinamide adenindinucleotide oxidase-dependent O_2^- release in platelets, which in turn enhances the availability of released ADP from platelet granules (Krötz et al., 2002).

It was known that different dietary components affect platelet function and prevent atherosclerosis. Vitamin E is a powerful lipid-soluble antioxidant present in cell membranes. It prevents vascular endothelial dysfunction (Keaney et al., 1993) and inhibits the aggregation of human platelets (Freedman et al., 1996; Pignatelli et al., 1999; Bakaltcheva et al., 2001). Natural vitamin E includes two groups of closely related fat-soluble compounds, the tocopherols and tocotrienols, each with the four α-, β-, γ-, δ-analogues (Fig. 10).

The main attention of researches was focused on biological activity of α-tocopherol. One reason why non-α-tocopherols such as γ-and δ-tocopherol have been considered to be less important than α-tocopherol is their low plasma concentration compared with α-tocopherol. The concentration of γ-tocopherol in blood is only about 10% of that of α-tocopherol (Packer et al., 2001). However, not only plasma concentration, but also tissue concentrations are important. Burton et al. (1998) found that γ-tocopherol represents 31% of vitamin E stored in human adipose tissue, 38% in muscle tissue, and 53% in the skin. The high tissue concentration of γ-tocopherol is likely due to the lipoprotein lipase-mediated catabolism of chylomicron particles, in which part of the chylomicron-bound vitamin E is transported to peripheral tissues (Saldeen and Saldeen, 2005).



tocotrienol isoforms

	R_1	R_2	R_3
α:	CH_3	CH_3	CH_3
β:	CH_3	H	CH_3
γ:	H	CH_3	CH_3
δ:	H	H	CH_3

Fig. 10. Molecular structure of vitamin E stereoisomers. Tocotrienols consist of a chromanol nucleus and a lipophilic isoprenoid chain. Tocopherols differ only in the side chain (phytyl). The naturally occurring isoforms, α, β, γ and δ, have methylation patterns as indicated (Packer et al., 2001).

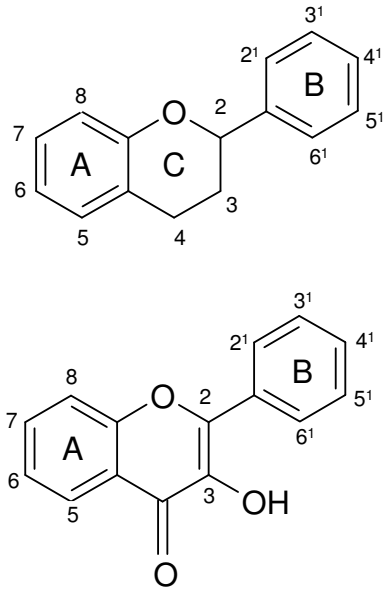
Vitamin E molecular mechanism is associated with its radical-scavenging function. Ricciarelli and co-workers (2001) showed that vitamin E also acts by mechanisms that are independent of its antioxidant properties. The inhibition of PKC activity by α-tocopherol represents the first observation of a non-antioxidant action of α-tocopherol. It inhibits the aggregation of human platelets by a PKC-dependent mechanism both *in vitro* and *in vivo* (Freedman et al., 2000; Mabile et al., 1999).

α-Tocopherol does not inhibit PKC by binding to it, but it dephosphorylates PKC via the activation of protein phosphatase 2A (Ricciarelli et al., 2001). γ-Tocopherol decreases platelet aggregation more potently than α-tocopherol (Saldeen et al., 1999). The ability of vitamin E to modulate signal transduction and gene expression has been observed in numerous studies; however, the detailed molecular mechanisms involved are often not clear. Vitamin E modulates the activity of several enzymes involved in signal transduction, such as protein kinase C, protein kinase B, protein tyrosine kinases, 5-, 12-and 15-lipoxygenases, cyclooxygenase-2, phospholipase A_2, protein tyrosine phosphatase, and diacylglycerol kinase (Zingg, 2007).

Several vitamins interact with each other. The intake of one vitamin can influence the plasma concentration of others. The major antioxidant of the aqueous phase is vitamin C, which acts on the first line of defence during oxidative stress. Vitamin C is also important for maintaining levels of antioxidant vitamin E by reducing its radical (the oxidised form of vitamin E). In addition, it has been shown that vitamin C decreases platelet aggregation *in vivo* and *in vitro* (Wilkinson et al., 1999; Cordova et al., 1982). Savini and co-workers (2007) found that besides its role during platelet aggregation, vitamin C might also have important effects during post-aggregatory events. It can regulate the thrombus viscoelastic properties.

Flavonoids are polyphenolic compounds that occur in plants (Fig. 11). The biological properties, antioxidant activity, specific interaction with cell receptors and enzymes are related to the chemical structure of polyphenols.

The intakes of dietary flavonoids quercetin, kaempferol, myricetin and morin are associated with a reduced risk for cardiovascular disease. In addition, flavonoids reduce platelet-dependent thrombosis. Certain flavonoids possess inhibitory effects on several enzyme systems intimately connected to cell activation processes such as PKC, phospholipase A_2, cyclooxygenase and others (Middelton, 1998; Chi et al., 2001). It was suggested that flavonoids inhibit platelet aggregation by the inhibition of cyclooxygenase activity (Jenssen et al., 1998; Chi et al., 2001) and by antagonising the intracellular production of hydrogen peroxide (Pignatelli et al., 2000). However, flavonoids isolated from purple grape juice inhibit platelet superoxide release and enhance platelet-derived NO release (Freedman and Keaney, 2001). Like α-tocopherol, flavonoids (kaempferol, quercetin and myricetin) inhibit PKC in different cells, too (Gamet-Payrastre et al., 1999). The antiplatelet activity of rutin (glycoside of quercetin) involves the following pathways: rutin inhibits the activation of phospholipase C, inhibits PKC, TxA_2 formation and intracellular Ca^{2+} mobilisation, finally resulting in the inhibition of platelet aggregation (Sheu et al., 2004).



	5	7	2^1	3^1	4^1	5^1
Quercetin	OH	OH	-	OH	OH	-
Kaempferol	OH	OH	-	-	OH	-
Myricetin	OH	OH	-	OH	OH	OH
Morin	OH	OH	OH	-	OH	-

Fig. 11. The chemical structure of favonoids. The lower part of the Figure shows the chemical structure of flavonols.

In addition, it was found that polyphenol resveratrol (Fig. 12) inhibits platelet aggregation (Zhirong et al., 2002) *in vivo* and *in vitro* and reduces oxidative stress in blood platelets (Olas and Wachowicz, 2002). It is interesting to note that resveratrol effectively inhibits collagen-and epinephrine-induced aggregation of platelets from aspirin resistant patients, which may contribute to cardioprotective effect in high-risk cardiac patients (Stef et al., 2006).

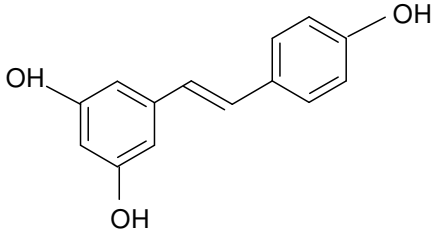


Fig. 12. Chemical structure of resveratrol.

Dobrydneva et al. (1999) showed that resveratrol inhibits calcium influx in thrombin-stimulated platelets. It can be concluded from these findings that the antiplatelet flavonoids are inhibitory at multiple points in biochemical pathways.

Vitamin B_6 comprises of a group of six related compounds: pyridoxal, pyridoxine, pyridoxamine and their respective 5′-phosphates. The chemical structures of vitamin B_6 vitamers are shown in Fig. 13. Vitamin B_6 is non-toxic at normal intake level. It was found that vitamin B_6 in the form of pyridoxal phosphate (PLP) inhibits platelet aggregation (Krishnamurthi et al., 1982; Shoene et al., 1986).

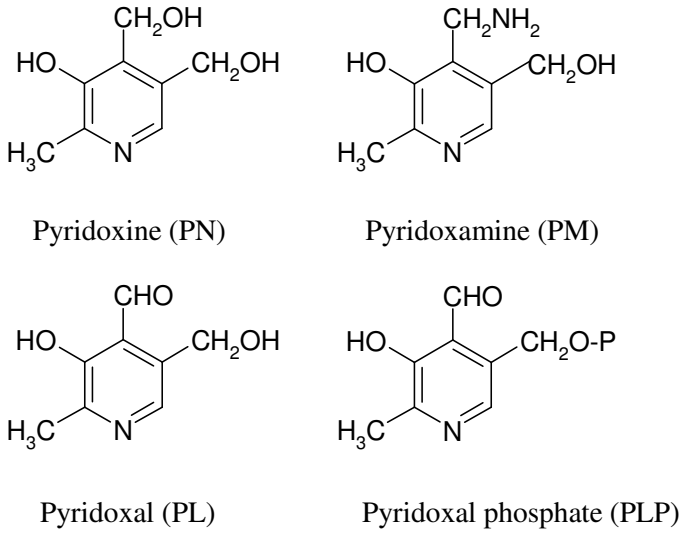


Fig. 13. The chemical structure of vitamin B_6 vitamers.

The inhibitory effects of PN and PLP are believed to be caused by the occupation of GPIIb/IIIa and the inhibition of fibrinogen binding to GPIIb/IIIa (Chang et al., 2002). PLP was also shown to increase the PGE_1-induced inhibition of platelet aggregation (Zahavi et al., 1984).

On the other hand, the vitamers of vitamin B_6 have the structural similarity to dihydropyridine, a calcium channel blocker and it was found that vitamin B_6 inhibits Ca^{2+} influx into the intracellular compartment of vascular smooth muscle (Dakishinamurti et al., 1998). Therefore, it can be suggested that the mechanism of action of vitamin B_6 on platelets also may be similar: the blockage of channels with the inhibition of Ca^{2+} influx into platelets.

PLP is unable to cross the cell membrane and must first be converted enzymatically to pyridoxal (PL) by membrane-associated alkaline phosphatases. In contrast, PL and pyridoxine readily diffuse through the cell membrane and are converted in cells to respective 5′-phosphates by intracellular PL kinase enzyme

(Brin et al., 1978). The borderline vitamin B_6 deficiency (plasma PLP concentration < 30 nmol/l) is strongly associated with the risk of coronary artery disease (Lin et al., 2006).

The design of novel antiplatelet agents using pyridoxine as a template has led to the discovery of a class of novel antiplatelet agents. Zhang and co-workers described the synthesis of several pyridoxine derivatives, which reduced platelet aggregation induced by agonist collagen, ADP and thrombin receptor-activating peptide (Zhang et al., 2004). In addition, pyridoxine was shown to have effect on vascular endothelial cells. Pyridoxine prevents dysfunction of endothelial cell nitric oxide production in response to low-density lipoprotein. This has important potential therapeutic implication for cardiovascular disease prevention (Ji et al., 2006).

1.3. Vascular endothelial injury and thrombosis

The initial step in atherosclerosis is the targeting of leukocytes and platelets to the sites of endothelial injury. Endothelial injury has been implicated in the pathogenesis of many cardiovascular diseases (Maxwell, 2000; Landmesser and Drexler, 2005).

Oxidant stress is a key determinant of atherothrombosis. Free radical or oxidative stress-mediated endothelial injury induces the expression of adhesion molecules, which promote monocyte, neutrophil and platelet adhesion. Free radicals and oxidant stress activate platelets and increase platelet aggregation on injured endothelium (Loscalzo, 2003; Pratico, 2005).

1.3.1. Nitric oxide, vascular endothelial injury and platelets

The role of nitric oxide (NO) is the vascular system is complex and diverse. NO has been shown to serve many vasoprotective roles, including the inhibition of platelet aggregation and adherence to the site of vascular injury, inhibition of leukocyte adherence and stimulation of endothelial cell growth (Naseem, 2005). Additionally, NO can protect the endothelial cells from apoptosis (Pratico, 2005). NO has also been shown to affect endothelial cell pathophysiology during the injury response (Kibbe et al., 1999). The loss of endothelial ability to constitutively synthesise and release NO has been suggested to be the inciting event in atherogenesis. In contrast, NO can also interact with superoxide to produce peroxynitrite and other potentially toxic oxidants that may be involved in vascular injury. NO and PGI_2, both released from the endothelium, act synergistically to inhibit platelet aggregation and adhesion, suggesting that the activity of both mediators is required for the exertion of full antiplatelet activity (Radomski et al., 1987; Broeders et al., 2001).

NO is a diffusible substance with a short half-life of a few seconds. The formation of NO occurs via nitric oxide synthase (NOS). Three different isoforms of NOS have been described: neorohumoral (nNOS), inducible (iNOS) and

endothelial (eNOS) (Moncada et al., 1997). Most of eNOS appear to be bound to endothelial cell membranes, whereas only a small fraction is of cytosolic origin. Receptor-dependent agonists (acetylcholine and platelet-derived products such as thrombin and ADP) increase intracellular free Ca^{2+}, which in turn activates eNOS and elicits endothelium-dependent relaxation. Endothelium-derived nitric oxide (EDNO) inhibits platelet adhesion and aggregation, which is mediated by the stimulation of soluble guanylyl cyclase in platelets. Therefore, EDNO causes both vasodilation and platelet deactivation, and thereby represents an important antithrombotic feature of the endothelium (Tomasian et al., 2000).

A number of studies have shown the presence of constitutive NO synthase (cNOS) protein and activity in human platelets (Chen et al., 1997; Freedman et al., 1996). L-arginine is the substrate for NO production. Vascular endothelium dysfunction is attributed to L-arginine deficiency or the presence of L-arginine endogenous inhibitors. L-arginine has antiatherogenic actions reducing oxidative stress and the formation of vascular injury. Beneficial effects of L-arginine are thought to result from increased NO production (Goumas et al., 2001). Oral L-arginine supplement improved endothelial function and reduced LDL oxidation in CAD (Gornik and Creager, 2004; Yin et al., 2005).

1.3.2. Antioxidant vitamins, vascular endothelial injury and platelets

Free radicals cause vascular endothelial injury, but the body has evolved major antioxidant defence mechanisms to protect it from free radical attack. These defences can be confidently considered as cellular, membrane, and extracellular mechanisms. Antioxidants such as vitamin E, β-carotene, and coenzyme Q are present within cell membranes. The lipophilic vitamin E is a highly effective antioxidant when incorporated in the lipid core of cell membranes. It has the ability to scavenge intermediate peroxyl radicals and, therefore, interrupt the chain reaction of lipid peroxidation. For this reason, vitamin E is described as chain-breaking antioxidant (Betteridge, 2000). Antioxidant vitamins, such as vitamin C and E, may have an important role in preventing vascular injury (Molyneux et al., 2002). Most investigators have focused on three main dietary antioxidant vitamins (vitamin E, vitamin C and β-carotene) (Gotto, 2003). It was shown that combined supplementation of vitamin E and C in reasonable doses slowed common carotid atherosclerosis (Salonen et al., 2000).

In addition, observational studies suggest that B vitamins may reduce the extent of carotid atherosclerosis. Title et al. (2000) showed that folic acid supplementation, alone or with antioxidant vitamins, will reduce the risk of cardiovascular disease.

The endothelial dysfunction and increased platelet aggregation are present before and throughout the development of atherosclerosis, and particularly during plaque rupture. It is widely appreciated that the oxidatively modified form of LDL is a critical factor in atherogenesis and platelet aggregation (Jialal and Devaraj, 1996). There is evidence that plaque stability and a tendency to thrombosis is the

subject to modification by specific antioxidants. It has been shown that antioxidants increase the collagen content and stabilize atherosclerotic plaque and restore endothelial function (Orbe et al., 2003). Cellular antioxidants inhibit monocyte adhesion, protect against the cytotoxic effect of oxidised LDL (ox-LDL) and inhibit platelet activation. Vitamin E and other antioxidants have been shown to reduce thrombus formation (Mehta et al., 1999) and the extent of atherosclerosis (Chen et al., 1997).

In contrast, other studies have demonstrated that antioxidants provided by dietary intake or the addition of antioxidants in the form of vitamin supplements do not reduce the risk of atherosclerosis. A number of studies are currently going on and will evaluate combinations of antioxidants and longer-term use in different populations, including those more susceptible to have early vascular damage. Antioxidants that studied may not be potent enough or doses used may be too low (Lonn, 2001; Gotto 2003; Violi and Cangemi, 2005).

1.3.3. Vitamin E, vascular endothelial injury and platelets

Vitamin E prevents the development of vascular endothelial dysfunction. It breaks the chain of free radical LDL oxidation and prevents cell wall damage (Frei et al., 1994). The levels of superoxide, ox-LDL and lipid peroxidation products can be decreased by vitamin E (Fig. 14). In addition, vitamin E has a role in preserving the biological activity of endothelium-derived nitric oxide (Carr and Frei, 2000).

Endothelium-derived nitric oxide (EDNO) has antiatherogenic and antithrombotic effect. EDNO is synthesised from L-arginine through the action of the enzyme endothelial nitric oxide synthase (eNOS). ENOS is regulated by intracellular calcium and its activity is enhanced by shear stress and a number of receptor-mediated agonists, such as acetylcholine, serotonin, and thrombin (Fig. 14). EDNO is a membrane-diffusible molecule that readily interacts with the heme group of the cytosolic enzyme guanylate cyclase in platelets and smooth muscle cells. Subsequent formation of cGMP results in the inhibition of platelet aggregation and adhesion (Fig. 14) and in the stimulation of vasodilation (Carr and Frei, 2000).

In patients with CAD decreased EDNO bioactivity has been observed. Vitamin E is likely to improve endothelial vasodilation function by its inhibitory effect on lipid peroxidation or PKC inhibition. Vitamin E acts in the vascular wall by inhibiting PKC activation by ox-LDL, hence decreasing superoxide production (Keaney et al., 1996).

Some studies indicate that vitamin E may provide cardiovascular protection (Emmert and Kirchner, 1999; Spencer et al., 1999; Nutall et al., 1999). It was shown that supplementation with vitamin E restores normal endothelial function reduces superoxide anion formation and increases the expression of soluble guanylyl cyclase in rats with heart failure (Bauersachs et al., 2001). The toxicity of vitamin E is low and in large human studies with oral supplementation, few side effects have been reported.

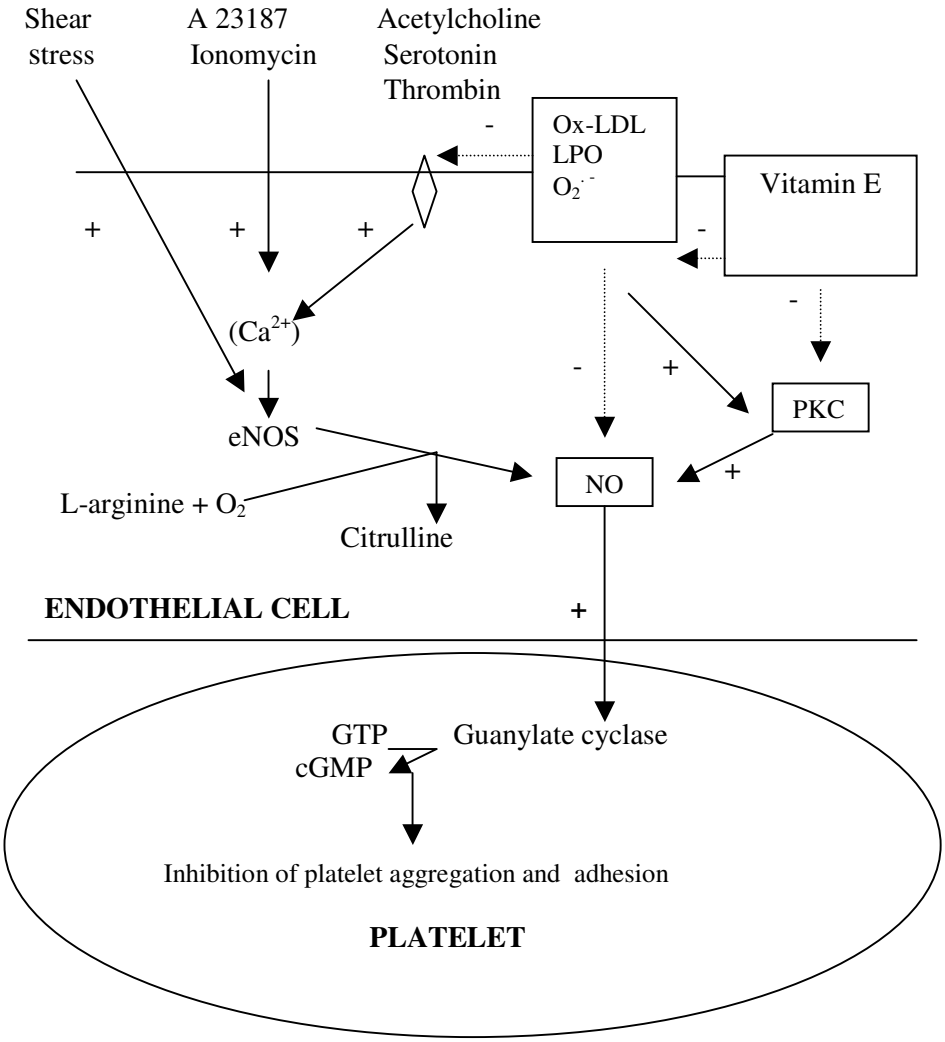


Fig. 14. The interaction of oxidants and antioxidant vitamin E with endothelial-derived nitric oxide. Stimulation of endothelial nitric oxide synthase (eNOS) by shear stress, calcium ionophores (A23187 and ionomycin) or receptor-mediated agonist (acetylcholine, serotonin, thrombin) causes the conversion of L-arginine to citrulline and NO. NO diffuses out of the endothelial cells and stimulates guanylate cyclase in platelets, causing the inhibition of platelet adhesion and aggregation. The biological activity of NO is attenuated by reaction with superoxide radicals ($O_2^{\cdot -}$) and oxidised low-density lipoproteins (Ox-LDL). Ox-LDL and plasma membrane lipid peroxidation (LPO) can also interrupt agonist-induced NO synthesis. The levels of superoxide, Ox-LDL and LPO products can be decreased by vitamin E. Vitamin E also inhibit endothelial PKC (Modified from Carr and Frei, 2000).

α-Tocopherol, the principle form of vitamin E in human plasma prevents the development of endothelial dysfunction (Keaney et al., 1993; Stewart-Lee et al., 1994). It was shown that α-tocopherol increases eNOS activity in concentration-dependent manner in human cultured endothelial cells (Heller et al., 2004). In addition, eNOS activation was higher after supplementation with the mixed tocopherol preparation, but not with α-tocopherol alone. The preparation of mixed tocopherols, containing γ-, δ-, and α-tocopherol showed to have better antioxidant and anti-inflammatory action that α-tocopherol alone (Saldeen and Saldeen, 2005; Reiter et al., 2007). In addition, Wu and co-workers (2005) found that vitamin E increases production of vasodilator prostanoids PGI_2 and PGE_2 in human aortic endothelial cells through opposing effects on COX-2 and phospholipase A_2. This was associated with a dose-dependent upregulation of cytosolic phospholipase A_2 expression and arachidonic acid release. In contrast, vitamin E dose-dependently inhibited COX activity but did not affect the expression of either COX-1 or COX-2, indicating that the effect of vitamin E on COX activity was post-translational. Thus, vitamin E had opposing effect on the 2 key enzymes in prostanoid synthesis.They suggested that vitamin E-induced increase in PGI_2 and PGE_2 production might contribute to beneficial effect in preserving endothelial function**.**

2. AIMS OF THE PRESENT STUDY

The main purpose of this work was to study the effects of vitamins in combination with each other and with other antiplatelet agents on platelet aggregation, to investigate the effect of α-tocopherol-incubated vascular endothelium on platelet activation. The specific aims of the study were:

- To study the antiplatelet effect of α-tocopherol, quercetin, pyridoxine and vitamin C on platelet aggregation
- To compare the effects of α-tocopherol and α-tocopherol acetate on platelet aggregation
- To study the effect of α-tocopherol on 1,2-dioctanoyl-sn-glycerol (DOG)-induced platelet aggregation
- To study the antiplatelet effect of α-tocopherol in combination with L-arginine
- To study the antiplatelet effect of α-tocopherol in combination with vitamin C, pyridoxine, pyridoxal phosphate and quercetin
- To study the antiplatelet effect of α-tocopherol in combination with prostaglandins (PGI_2, PGE_1, iloprost)
- To study the antiplatelet effect of quercetin in combination with prostaglandins (PGI_2, PGE_1, iloprost)
- To study the effect of the interaction of vascular endothelium with platelets on their activation

3. RESULTS AND DISCUSSION

3.1. Effect of antiplatelet vitamins on platelets

3.1.1. Comparison of antiaggregatory effect of α-tocopherol and α-tocopherol acetate

Previously the effect of α-tocopherol on platelets was studied mainly with such inductors of platelet aggregation as arachidonic acid and collagen (Pignatelli et al., 1999; Freedman et al., 1996). ADP is also an important physiological activator of platelets and exerts its effect by acting on two subtypes of cell surface P2 receptors. The blockade of the ADP receptor is a mode of newer classes of antithrombotic drugs in coming era (Murugappan et al., 2004). Therefore, in this work we used ADP to induce platelet aggregation.

It was found that α-tocopherol affects the platelet through a PKC-dependent mechanism and also through the neutralisation of free radicals. The latter are released by activated platelets and potentiate platelet aggregation (Leo et al., 1997). It was found that oxygen free radicals could activate PKC in different cell types, including platelets (Pratico et al., 1999) and so potentiate platelet aggregation (Pratico et al., 1993). α-Tocopherol inhibits platelets through PKC and, being antioxidant, also through the neutralisation of oxygen free radicals, which are released by activated platelets. We controlled a role of these two mechanisms of the inhibition for ADP-induced platelet aggregation. For this, the effects of both α-tocopherol and α-tocopherol acetate were studied. The latter isoform in contrast to the former has only minimal antioxidant activity (Freedman and Keaney, 2001).

For these experiments, the platelets of both healthy volunteers and patients suffering from CAD were used. α-Tocopherol at concentration of 1 mM inhibited platelet aggregation by 63.6 ± 8.8 % and 33.46 ± 6.5 % in CAD patients and healthy subjects, respectively. The inhibitory effect of 2 mM of α-tocopherol was 57.8 ± 13.3 % and 43.3 ± 8.5 %, respectively (Fig. 15). α-Tocopherol acetate inhibited platelet aggregation at a higher concentration than α-tocopherol (Fig. 16). At a concentration of 2 mM, it inhibited platelet aggregation by 28.9 ± 4.9 and 19.5 ± 3.4 % in CAD patients and healthy subjects, respectively. At a concentration of 4 mM, the inhibitory effect of α-tocopherol acetate was 42.3 ± 4.1 % and 33.5 ± 4.8 %, respectively. The effect of both α-tocopherol and α-tocopherol acetate was higher in the PRP of CAD patients than in healthy subjects. α-Tocopherol acetate inhibited platelet aggregation at higher concentration than α-tocopherol. Thus, the higher potency of α-tocopherol indicates that a part of the inhibition of ADP-induced aggregation is caused by the neutralisation of free radicals.

Since PKC is located inside platelets, α-tocopherol must be transported through plasma membrane. Freedman and co-workers (1996) suggested that the effect of α-tocopherol depended on its levels in platelets rather than in blood plasma. The transfer of α-tocopherol from plasma to cells occurs by the known mechanisms of

lipid transport between lipoprotein and cells: 1) uptake facilitated by lipid transfer protein and lipases, 2) receptor-mediated lipoprotein endocytosis, and 3) selective lipid uptake (Mardones and Rigotti, 2004). The inhibitory effect of α-tocopherol was more effective in patients with CAD. It is known that CAD patients have a higher plasma lipid level. Due to this, the loading of platelets with α-tocopherol may be more effective.

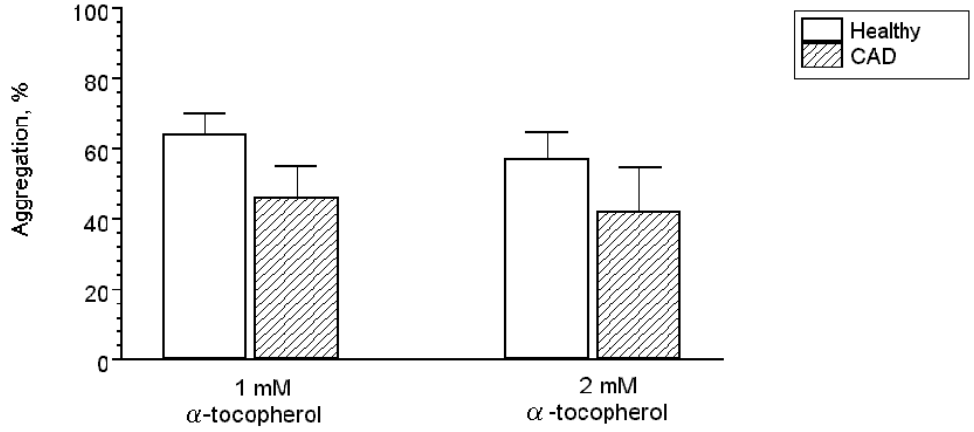


Fig. 15. Inhibition of ADP-induced platelet aggregation in the presence of α-tocopherol (1 mM and 2 mM) in PRP. α-Tocopherol was incubated in PRP for 5 minutes. The aggregation induced by ADP in the absence of α-tocopherol was taken as 100%. The data are expressed as an arithmetic mean ± standard error (SEM) of separate experiments with the PRP of 14 healthy subjects and 8 CAD patients. The statistical significance of difference between patients and healthy subjects was determined using t-test for unpaired experiment ($P < 0.05$ for both 1 and 2 mM).

It is necessary to note that the effect of α-tocopherol on platelet function is largely based upon *in vitro* studies and there is evidence that *in vivo* α-tocopherol may act by a number of different mechanisms. α-Tocopherol does not act on platelet and vascular endothelium only by the mechanism that involves PKC but also increases platelet-derived NO (Saldeen et al., 1999) and endothelium-derived NO (Carr and Frei, 2000) release. It was shown that endogenous NO and antiaggregatory PGI_2 synergistically inhibit blood platelets *in vitro* (Radomski et al., 1987). In the same way, this synergism is also effective in the inhibition of thromboembolism in arterioles (Broeders et al., 2001).

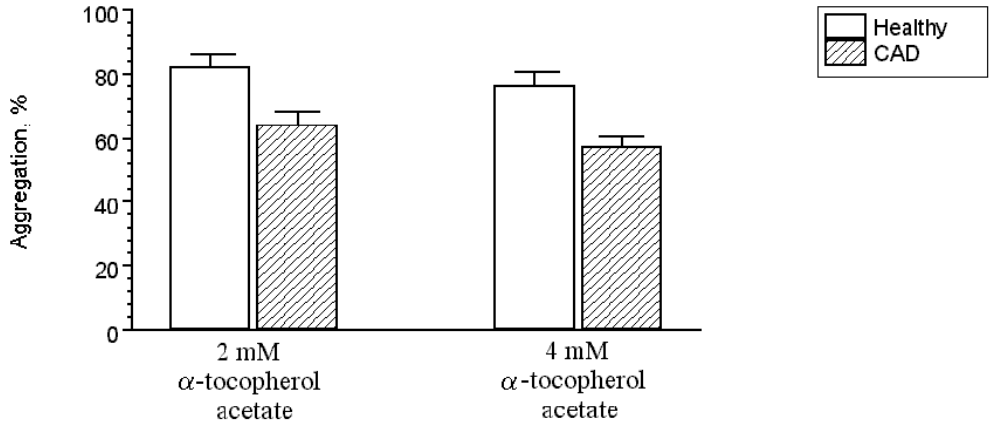


Fig. 16. Inhibition of ADP-induced platelet aggregation in the presence of α-tocopherol acetate (2 mM and 4 mM) in PRP. α-Tocopherol acetate was incubated in PRP for 5 minutes. The data are expressed as an arithmetic mean ± SEM of separate experiments with the PRP of 13 healthy subjects and 10 CAD patients. The statistical significance of difference between patients and healthy subjects was determined using t-test for unpaired data ($P < 0.05$ for both 2 and 4 mM).

3.1.2. DOG-induced platelet aggregation and inhibitory effect of α-tocopherol on DOG-induced platelet aggregation

Since inhibition of platelet aggregation by α-tocopherol is mediated through PKC inhibition, the effect of PKC agonist 1,2-dioctanoyl-sn-glycerol (DOG) was studied on platelet aggregation both in healthy subjects and patients suffering from CAD, though in the latter it was two times less effective (Fig. 17). α-Tocopherol inhibited platelet aggregation induced by DOG. However, it was essentially less effective on DOG-induced platelet aggregation in CAD patients compared to that of healthy subjects (Fig. 18).

These results are in accordance with the suggestion that α-tocopherol induces its effect through the inhibition of PKC. At the same time, we show that PKC of patients suffering from CAD is less sensitive to activating effect of DOG and inhibiting effect of α-tocopherol. It is known that patients suffering from CAD are at a state of heightened oxidative stress (Guzik et al., 2005). On the other hand, it was shown that oxidative inactivation of PKC occurred in intact cells exposed to oxidative stress (Gopalakrishna and Anderson, 1987).

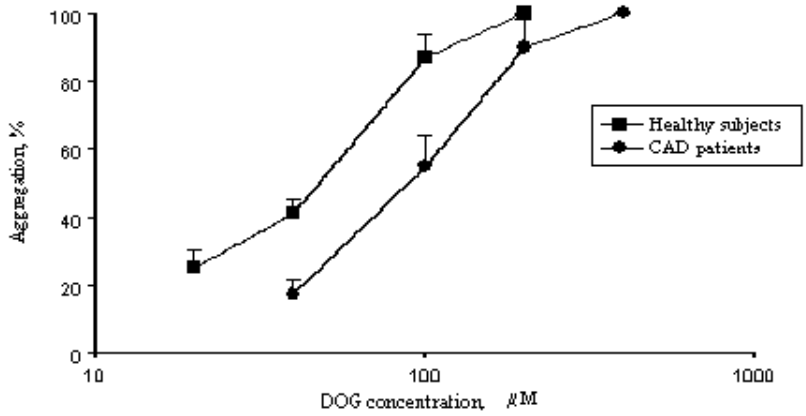


Fig. 17. 1,2-Dioctanoyl-sn-glycerol (DOG)-induced platelet aggregation in healthy subjects and patients with CAD. P = 0.0335 < 0.05. Data were expressed as an arithmetic mean ± standard error (SEM) of separate experiments with PRP of n subjects (n = 6 healthy subjects; n = 6 CAD patients). Vertical lines were shown SEM.

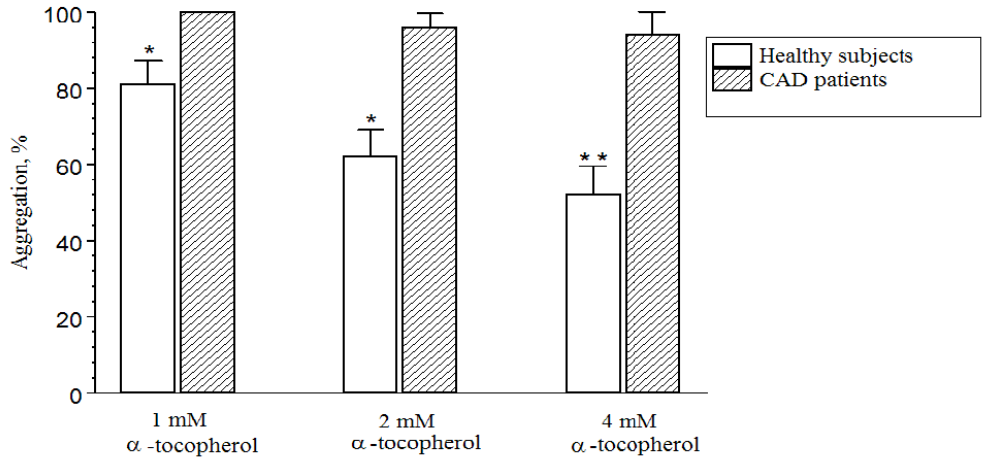


Fig. 18. Inhibition of DOG-induced platelet aggregation in the presence of α-tocopherol (1 mM; 2 mM; 4 mM). PRP was incubated in the presence of α-tocopherol for 5 minutes and aggregation was induced by DOG. Aggregation induced by DOG in the absence of α-tocopherol was taken as 100%. Data were expressed as an arithmetic mean of separate experiments with PRP of n subjects. Platelet aggregation (%) in the presence of α-tocopherol (1 mM) was 81 ± 6.4 (n =7) and 100 (n = 7) in healthy control and patients with CAD, respectively. Platelet aggregation (%) in the presence of α-tocopherol (2 mM) was 62 ± 7.4 (n = 7) and 96 ± 3.7 (n = 7) in healthy control and in patients with CAD, respectively. Platelet aggregation (%) in the presence of α-tocopherol (4 mM) was 52 ± 7.4 (n = 7) and 94 ± 6 (n = 7) in healthy control and patients with CAD, respectively. The differences between CAD patients and healthy subjects were compared with t-test. * - P < 0.05, ** - P < 0.01.

This can explain the lower sensitivity of platelets to DOG and α-tocopherol in the CAD patients. PKC regulates activation and transcription of a large number of proteins involved in the inflammatory process. α-Tocopherol and γ-tocopherol have significant anti-inflammatory activity. While both tocopherols exhibit anti-inflammatory activity *in vitro* and *in vivo*, supplementation with mixed tocopherols seems to be more potent than supplementation with α-tocopherol alone (Reiter et al., 2007). The relationship between oxidative stress parameters and inflammatory species suggest their strong mutual involvement in atherosclerosis development that leads to CAD progression (Kotur-Stevuljevic et al., 2007).

3.1.3. Inhibitory effect of quercetin on platelets

Quercetin caused a dose-dependent inhibition of platelet aggregation. Quercetin at concentrations of 20 and 40 μM inhibited ADP-induced platelet aggregation in healthy subjects by 23.9 ± 4.9 % and 37.0 ± 5.2 %, respectively (n = 12) (Fig. 19).

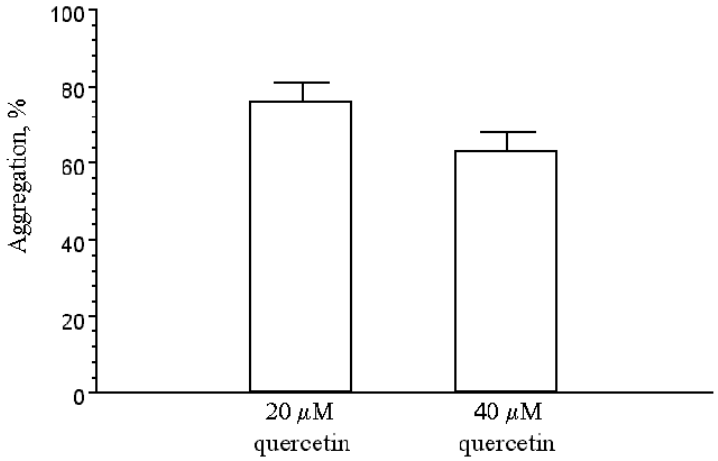


Fig. 19. Inhibition of ADP-induced platelet aggregation by quercetin. Quercetin at concentrations of 20 and 40 μM inhibited ADP-induced platelet aggregation in healthy subjects by 23.9 ± 4.9 % and 37.0 ± 5.2 %, respectively. Quercetin was added 1 min before ADP. The data are expressed as mean ± SEM of separate experiments with the PRP of 12 healthy subjects. The difference was compared by t-test for paired data, ($P < 0.05$ for 20 μM vs. 40 μM).

Effective antiplatelet concentrations of quercetin were early found to vary from 2500 μM (Janssen et al., 1998) to 10 μM (Pignatelli et al., 2000). This variation

might be dependent on differences in experimental procedure. According to Pignatelli and co-workers (2000), the high potency of quercetin in their work was caused by the incubation of quercetin with platelets for 30 min before the addition of the agonist collagen. This incubation time was much longer than that previously (about 10 min) in studies on the effects of flavonoids on platelet function. We found that 20 μM of quercetin, incubated with platelets only for 1 min, inhibited platelet aggregation by 24.6 ± 5.9 %. However, with longer incubation time (10 min), the effect was also significantly greater, namely 43.3 ± 6.4 % (t-test for paired data, $P < 0.05$, n= 7) (Fig. 20). As distinct from Pignatelli and co-workers (2000), we used ADP instead of collagen to induce platelet aggregation and it was added at a concentration that caused the submaximum aggregation of platelets.

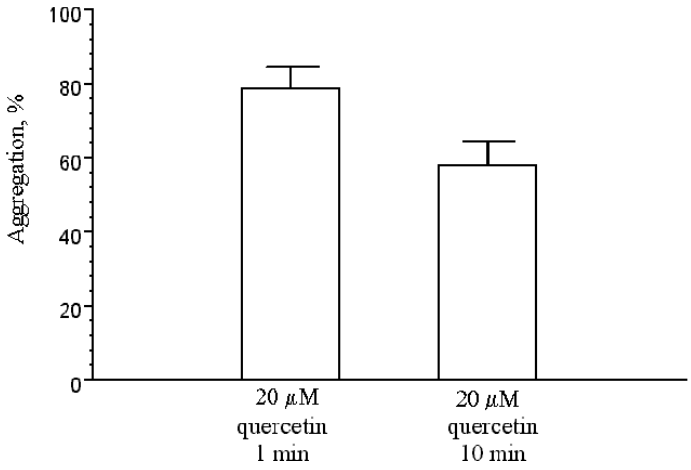


Fig. 20. Dependence of inhibition of ADP-induced platelet aggregation by quercetin on incubation time. Platelet aggregation induced by ADP in the presence of quercetin (20 μM) for 1 min and 10 min. The data are expressed as an arithmetic mean ± SEM of separate experiments of 7 subjects. The difference was compared by t-test for paired data, $P < 0.05$ for 1 min vs. 10 min.

3.1.4. Inhibitory effect of pyridoxine (vitamin B_6) on platelets

Pyridoxine induced a concentration-dependent inhibition of platelet aggregation in both PRP and WP (see article II; Fig. 2). The samples of PRP might be subdivided into two groups depending on sensitivity to pyridoxine: the high sensitive group and the low sensitive one with the mean values of IC_{50} equal to 1.7 ± 0.5 μM and 4000 ± 800 μM, respectively (see article II; Fig. 1). The sensitivity did not depend on the gender, age or AB0 blood-group status of the volunteers. In the preparation of WP, pyridoxine induced an inhibition of platelet aggregation only at high

concentrations. The sensitivity of WP to pyridoxine was low irrespective of whether WP was prepared from high sensitive or low sensitive PRP.

In four experiments, the values of IC_{50} for pyridoxine in the samples of PRP with low sensitivity and of WP prepared from the same PRP were compared. They were found to be 3500 ± 1200 μM and 3000 ± 500 μM, respectively and did not differ significantly from each other (t-test for paired data, $P < 0.05$).

The experimental data show that pyridoxine can inhibit platelet aggregation at micromolar concentrations (see article II; Fig. 2; Fig. 1, left curve). Schoene and co-workers (1986) studied the effect of the other form of vitamin B_6, pyridoxal 5′-phosphate (PLP), on platelet aggregation. The effective concentration of PLP was three orders of magnitude higher than for pyridoxine.

The reason for the low effectiveness of PLP (Shoene et al., 1986) on platelets may be its binding by plasma proteins. Indeed, it was found that PLP was essentially more potent in WP than in PRP (Schoene et al., 1986). Under physiological conditions, PLP is bound to blood proteins, predominantly to albumin (Lumeng et al., 1974). Antiplatelet effect can be explained *in vitro* by the fact that millimolar concentrations of PLP exceed the binding capacity of albumin and it can interact with platelets. In our experiments, the effect of pyridoxine did not depend on the presence of plasma; the values of IC_{50} were similar in PRP and WP. It shows that plasma proteins do not bind pyridoxine. Pyridoxine not only inhibits platelet aggregation, but it was shown to prevent dysfunction of endothelial cell nitric oxide production in response to low-density lipoprotein (Ji et al., 2006). Pyridoxine may be a useful therapeutic agent to prevent platelet aggregation and endothelial dysfunction in cardiovascular diseases.

3.1.5. Inhibitory effect of vitamin C on platelets

Vitamin C, the main water-soluble antioxidant in plasma, caused a weak inhibition of platelet aggregation. Vitamin C at concentrations of 2 mM inhibited ADP-induced platelet aggregation by 35 ± 8.84 % (Fig 21).

The inhibitory effect of vitamin C may be caused by the reduction of reactive oxygen species (Olas and Wachowicz, 2002), the regulation of platelet-derived NO production (Takajo et al., 2001), the inhibition of thromboxane B_2 formation (Ho et al., 1976), the stimulation of prostaglandin E_1 production (Srivastava, 1985), or the inhibition of platelet CD40L expression (Pignatelli et al., 2005).

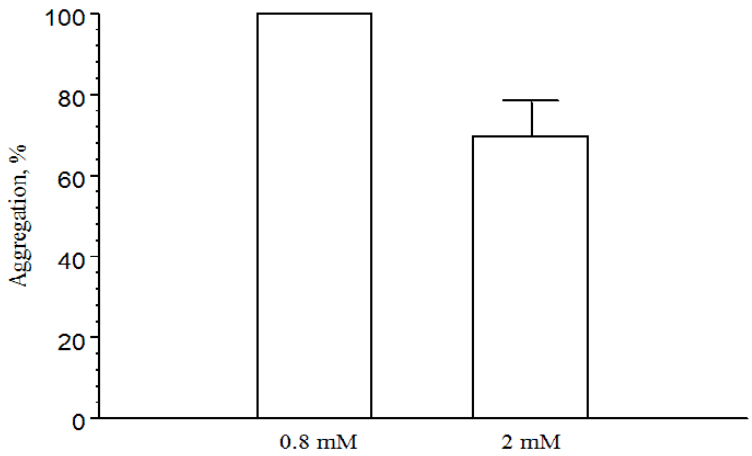


Fig. 21. Inhibition of ADP-induced platelet aggregation in the presence of vitamin C (0.8 mM and 2 mM) in PRP. Vitamin C was incubated in PRP for 1 minute. The aggregation induced by ADP in the absence of vitamin C was taken as 100%. The data are expressed as an arithmetic mean ± standard error (SEM) of separate experiments with PRP of 4 healthy subjects. The difference was compared by t-test, sample. $P < 0.05$.

3.2. Effect of combination of antiplatelet agents

Several antiplatelet agents when acting together on platelets may amplify the inhibitory effect of each other. For example, a reciprocal potentiation of antiaggregatory activity was observed between low concentration of PGI_2 and NO (Radomski et al., 1987). In the same way, this synergism is also effective in the inhibition of thromboembolism in arterioles (Broeders et al., 2001). In addition, the antiaggregatory action of L-arginine is potentiated by prostacyclin (Radomski et al., 1990).

There is a little data on the effects of the combination of antiplatelet vitamins with each other and with other antiplatelet agents. It was found that the mostly used antiplatelet drug aspirin and vitamin E synergistically inhibit platelet aggregation. Vitamin E can potentiate the antiplatelet activity of aspirin by inhibiting the early events of platelet activation pathways induced by collagen (Celestini et al., 2002) and by increasing the neutrophil nitric oxide production (Gonzalez-Correa et al., 2005). This finding provides a rationale for combining aspirin and vitamin E to prevent thrombotic complications in atherosclerotic patients. Moreover, a combination of aspirin and vitamin E might be useful for prevention of endothelial injury in cardiovascular disease. At subthresold concentrations, vitamin E had a potentiating effect on endothelial protection by aspirin (Podhaisky et al., 1997).

In addition, it was found that mixed tocopherols are more potent in preventing platelet aggregation than is α-tocopherol alone. The effect of mixed tocopherols is associated with increased NO release, NOS activation and SOD protein content in platelets (Li et al., 2001; Liu et al., 2003). In addition, flavonoids quercetin and catechin synergistically inhibit platelet function. The combination of 5 μM quercetin and 25 μM catechin significantly inhibited collagen-induced platelet aggregation (Pignatelli et al., 2000) and platelet CD40L expression (Pignatelli et al., 2005). However, neither of these flavonoids at such concentrations had any effect on platelet function when used alone. Of other flavonoids, the mixture of four anthocyanins (delphinidin-3-rutinoside, cyanidin-3-rutinoside, cyanidin-3-glucoside, and malvidin-3-glucoside) was effective when used at ten times lower concentrations than whose which caused inhibition of platelet function when each of these compounds was added to platelet alone (Rechner and Kroner, 2005).

In the next part of this work, we studied the effect of antiplatelet vitamins in combination with each other and with L-arginine and antiplatelet prostaglandins.

3.2.1. Potentiation of α-tocopherol by L-arginine

We found that L-arginine at concentrations up to 1 mM, added to a cuvette with platelets, did not inhibit platelet aggregation. Anfossi and co-workers (1999) obtained a similar result. These authors recorded a small inhibition of ADP-induced platelet aggregation only with a higher concentration of L-arginine or when it was incubated with platelets alone at a concentration of 1 mM for a longer time (20 min). However, when we combined only 100 μM L-arginine with a subthresold concentration of α-tocopherol (200 μM), platelet aggregation decreased by 29.3 ± 4.5% (n = 6) (see article IV; Fig. 1A). This inhibition could be abolished by N^G-monomethyl-L-arginine (NMMA), an inhibitor of nitric oxide synthase (NOS) (see article IV; Fig. 2B) suggesting that L-arginine acted through NOS. It was found that vitamin E enhances nitric oxide release and PKC-dependent NOS phosphorylation in platelets (Freedman and Keaney, 2001). However, the precise mechanism through which PKC inhibition modulates NOS activity in platelets is unknown.

3.2.2. Effects of combination of several vitamins together on platelet aggregation

Futher we studied the effects of the combination of several vitamins together. α-Tocopherol (α-T, 200 μM), ascorbic acid (C, 20 μM), pyridoxine (PN, 0.2 μM) (or pyridoxal 5'-phosphate (PLP, 0.02 μM)) and quercetin (Q, 2 μM) in pairwise combinations and three and four together were added into PRP of CAD patients (article V). The compounds singly at these concentrations were not effective. However, pairwise combinations of α-T, Q, C and PN at the same concentrations inhibited platelet aggregation (Fig. 22).

The inhibitory effect of the combinations of Q with PN and C with PN were very weak, only 4.3 ± 1.9% and 6.4 ± 2.6%, respectively. α-T together with C inhibited platelet aggregation by 13.9 ± 4.3%. The combinations of α-T with Q and α-T with PN were most effective inhibiting platelet aggregation by 16.1 ± 4.6% and 16.2 ± 4.4%, respectively. The inhibitions caused by the combination of the three combounds together were stronger than those caused by pairwise combinations (Fig. 23), although, the differences between the most effective pairwise combination (α-T+Q and α-T+PN) and the least effective combination of three vitamins, α-T+C+Q, which inhibited the platelet aggregation by 21.1 ± 7.2% were not stastistically significant ($P > 0.05$). The combination of α-T, C and PN was the strongest one of three vitamins inhibiting platelet aggregation by 38.6 ± 5.9%. The combination of α-T + Q + PN inhibited the aggregation by 33.0 ± 5.0%. The strongest effect was caused by the combination of four vitamins, which inhibited platelet aggregation by 51.1 ± 4.2%.

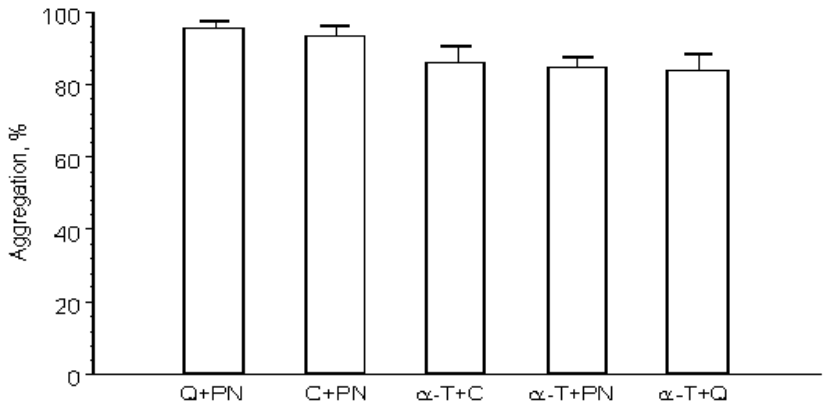


Fig. 22. Inhibition of ADP-induced platelet aggregation by pairwise combinations of vitamins. Data are mean ± SEM (n = 8) for ADP-induced platelet aggregation in the presence of vitamin combinations. None of the vitamins was effective alone. Effects were evaluated with analysis of variance (ANOVA) with *post hoc* Newman-Keuls comparison. The group means are significantly different ($P < 0.05$). ADP-induced aggregation is taken as 100%. α-T - 200 μM α-tocopherol, Q - 2 μM quercetin, C - 20 μM ascorbic acid, PN - 0.2 μM pyridoxine.

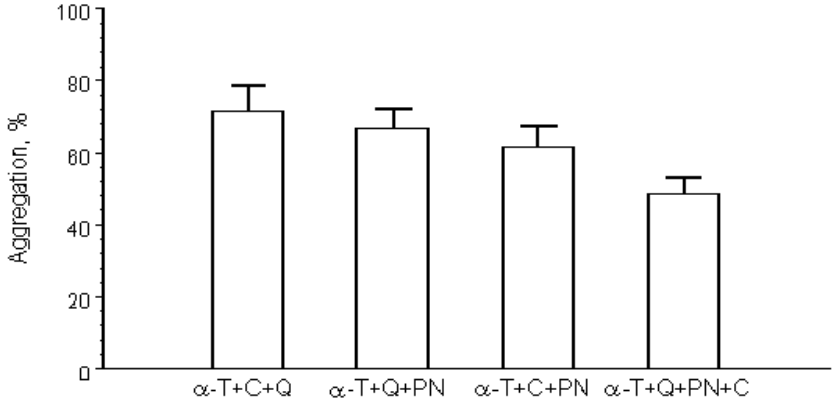


Fig. 23. Inhibition of ADP-induced platelet aggregation by α-tocopherol, quercetin, ascorbic acid, and pyridoxine in the combination of three or four together. Data are mean ± SEM (n = 8) for ADP-induced platelet aggregation in the presence of vitamin combinations. None of the vitamins was effective alone. Effects were evaluated with analysis of variance (ANOVA) with *post hoc* Newman-Keuls comparison. The group means are significantly different ($P < 0.05$). ADP-induced aggregation is taken as 100%. α-T- 200 μM α-tocopherol, Q - 2 μM quercetin, C - 20 μM ascorbic acid, PN - 0.2 μM pyridoxine.

In the separate series of experiments, the principal form of vitamin B_6 in human blood PLP was used (Driskell et al., 1991) instead of PN. PLP was taken at 0.02 μM, e.g. ten-times lower concentration than PN. The results are shown in Fig. 24. The combinations of α-T+Q+PLP, α-T+C+PLP, and α-T+Q+PLP+C inhibited platelet aggregation by 18.2 ± 2.8%, 25.0 ± 4.9% and 42.9 ± 2.5%, respectively. As in the previous series of experiments, the combination of four compounds was the strongest. It should be noted that concentration of PLP was especially low. In combination with other vitamins, it was effective at a concentration of 0.02 μM. It is equal to the basal concentration of PLP detected in blood before the administration of vitamin B_6 (Bor et al., 2003).

Besides vitamins, some antiplatelet drugs have also synergistic effect. The combination of them is successfully used in medicine. In patients with vascular disease, the combination of aspirin with dipyridamol (Diener et al., 1996) and aspirin with clopidogrel (Muller et al., 2000) was found to give better results than either of the drugs alone. It is necessary to note that combinations of drugs may inhibit the formation of not only aggregates of platelets but also of the aggregates of platelets with other cells. This effect was found for the combination of AR-C69931, an analogue of clopidogrel directly acting on P2T receptors, with

dipyridamole and aspirin, which significantly inhibited platelet-leukocyte conjugate formation besides the inhibition of platelet aggregation (Zhao et al., 2001).

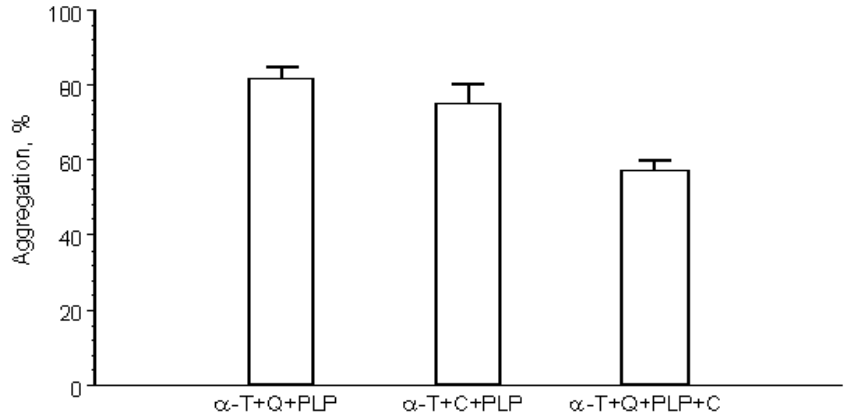


Fig. 24. Inhibition of ADP-induced platelet aggregation by α-tocopherol, quercetin, ascorbic acid, and pyridoxal 5'-phosphate in the combination of three or four together. Data are mean ± SEM (n = 8) for ADP-induced platelet aggregation in the presence of vitamin combinations. None of the vitamins was effective alone. Effects were evaluated with analysis of variance (ANOVA) with *post hoc* Newman-Keuls comparison. The group means are significantly different ($P < 0.05$). ADP-induced aggregation is taken as 100%. α-T- 200 μM α-tocopherol, Q - 2 μM quercetin, C - 20 μM ascorbic acid, PLP - 0.02 μM pyridoxal 5'-phosphate.

3.2.3. Effects of antiplatelet prostaglandins in healthy subjects and patients suffering from CAD

PGI_2, PGE_1 and their analogues acting through the same receptor type and effectively inhibit platelet aggregation. However, a clinical use of these compounds for the treatment of cardiovascular disease is restricted to unwanted side effects that are caused by the interaction of these prostaglandins with the receptors of blood vessels and other tissues. Moreover, the sensitivity of platelets to these prostaglandins is significantly lower in patients suffering from CAD (see article I). We compared the effects of PGE_1, 13, 14-dihydro-PGE_1 and 5,6-dihydro- PGE_3 on platelets of patients suffering from CAD with that of healthy subjects and found that they were about two times less effective on platelets of patients (Fig. 25). Mehta et al. (1980) found the same effect for PGI_2. The effect may be explained by the fact that the synthesis of PGI_2 is increased in blood vessels of the patients (Fish

et al., 1997) and long-term exposure to PGI_2 may result in the desensitization of PGI_2 receptors.

To lower the side effects of PGs one may use them at a lower concentration. However, in this case it is necessary to increase their effectiveness on platelets. This can be achieved by combining PGs with other antiplatelet agents. Therefore, we studied the effect of PGs in combination with α-tocopherol and with quercetin on platelet aggregation.

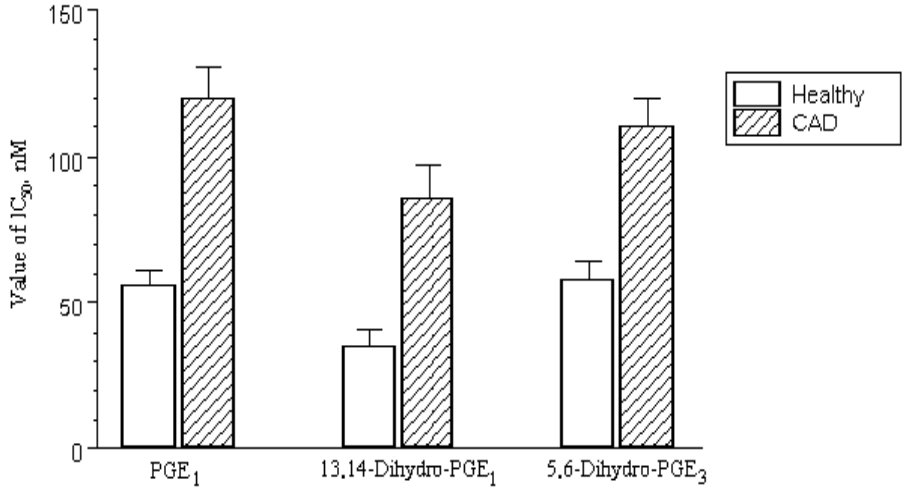


Fig. 25. Inhibition of platelet aggregation with prostaglandins in healthy subjects (n = 11) and patients suffering from CAD (n = 11). In patients suffering from CAD, prostaglandins are about two times less effective than in healthy subjects. The statistical significance of difference for healthy donors vs. patients was determined with Student's t-test for unpaired data. $P < 0.01$ for all three PGs.

3.2.4. Potentiation of antiaggregating effect of prostaglandins by α-tocopherol

In this series of experiments, a subthreshold concentration (200 μM) of α-tocopherol was used together with PGs (see article III). α-Tocopherol significantly increased the inhibitory effects of PGs (Table 2). The strongest effect was obtained with iloprost, the value of IC_{50} decreased 4.3 times in the presence of α-tocopherol.

The characteristic actions of PGs studied are mediated through specific cell-surface receptors, the activation of which leads to the elevation of intracellular cyclic adenosine monophosphate (cAMP) through Gs protein coupling to adenylate cyclase (Wise and Jones, 1996). cAMP, in turn, activates cAMP-dependent protein kinase and this causes the inhibition of platelet aggregation (Schwartz et al., 2001).

α-Tocopherol acts inside platelets through the inhibition of PKC (Freedman et al., 1996), which might decrease the threshold for platelet inhibition by PGs.

Table 2. Potentiation of the antiaggregatory effects of PGs by α-tocopherol

PG	IC_{50} (nM) ± SEM		
	In the absence of α-tocopherol (I)	In the presence of α-tocopherol 0.2 mM (II)	(I)/(II)
PGE_1	50 ± 8.1 (7)	29 ± 5.4** (7)	1.7
PGI_2	1.4 ± 0.2 (5)	0.58 ± 0.07** (5)	2.4
Iloprost	1.3 ± 0.1 (12)	0.30 ± 0.07*** (12)	4.3

The data are presented as means ± standard error of mean (SEM) of the concentration of prostaglandin (PG) that causes the inhibition of platelet aggregation by 50% (IC_{50}). Platelet aggregation was induced by adenosine diphosphate. The number of experiments is shown in parenthesis. ** - $P < 0.01$, *** - $P < 0.001$.

3.2.5. Potentiation of antiaggregating effect of prostaglandins by quercetin

Quercetin potentiated the effect of PGs at a 10 times lower concentration than it was effective alone (see article III). At a concentration of 2 μM, it increased the effects of PGs studied (Table 3). This concentration is comparable with that found in plasma after supplementation of humans with food rich in quercetin (McAnlis et al., 1999). This indicates that comparative potentiating effect might take place *in vivo* after quercetin supplementation.

Quercetin interacts with a number of cell enzymes (Middelton et al., 2000) and the mechanism of its inhibitory effect on platelet aggregation may be more complicated. Antiplatelet effect of flavonoids might be the inhibition of TxA_2 receptors together with the inhibition of TxA_2 formation in platelets (Tzeng et al., 1991; Guerrero et al., 2005). The latter may be caused by the inhibition of cyclooxygenase (Chi et al., 2001). It was also suggested that the antiplatelet effect of quercetin might be caused by the inhibition of nucleotide phosphodiesterase leading to the increase of cAMP (Lanza et al., 1987). In addition, quercetin inhibits the activity of PKC and phosphatidylinositol 3- kinase (Agullo et al., 1997; Gamet-Payrastre et al., 1999). The latter is important for the control of the reversible

aggregation of platelets induced by a low concentration of an agonist (Lauener et al., 1999).

Table 3. Potentiation of the aggregatory effect of PGs by quercetin

PG	IC_{50} (nM) ± SEM		
	In the absence of quercetin (I)	In the presence of quercetin 2 μM (II)	(I)/(II)
PGE_1	32 ± 0.9 (4)	19 ± 1.3*** (4)	1.7
PGI_2	1.4 ± 0.2 (7)	0.8 ± 0.2* (7)	1.8
Iloprost	1.7 ± 0.2 (12)	0.68 ± 0.09*** (12)	2.1

The data are presented as mean ± standard error of mean (SEM) of the concentration of prostaglandin (PG) that causes the inhibition of platelet aggregation by 50 % (IC_{50}). Platelet aggregation was induced by adenosine diphosphate. The number of experiments is shown in parenthesis. * - $P < 0.05$, *** - $P < 0.001$.

A correlation was found between the consumption of vitamin E (Ricciarelli et al., 2001; Azzi et al., 2003) or flavonoids (Dogne et al., 2002) and lower incidence of cardiovascular disease. Some of this beneficial effect was linked to the ability of α-tocopherol and flavonoids to inhibit platelet aggregation. At the same time, discrepancy between a low concentration of this compound in plasma and a much higher one necessary to inhibit platelet aggregation *in vitro* experiments casts doubt on the physiologic relevance of *in vitro* effects. However, several *ex vivo* studies showed that the administration of E vitamin to humans did cause the inhibition of platelet function (Freedman et al., 1996; Mabile et al., 1999). To explain the discrepancy, Freedman and co-workers (Freedman et al., 1996) suggested that the effect of α-tocopherol depends on its levels in platelets rather than in plasma and supraphysiological concentrations are required *in vitro* experiment to effectively load the platelets with α-tocopherol. Our results show that the effect of weak inhibitors of platelet aggregation may be enhanced by subthreshold concentrations of other weak antiaggregants. In addition, subthreshold concentrations of weak exogenous antiaggregants may potentiate the effect of endogenous inhibitors of platelets such as PGs (Kobzar et al., 2004). One might speculate that the potentiation of endogenous PGI_2 might take place *in vivo* in blood vessels and this might, in part, explain the discrepancy between *in vitro* and *ex vivo* experiments with weak antiplatelet compounds.

In our work, we found that L-arginine, which acts through NO/cGMP signalling pathway, potentiated the antiplatelet effect of α-tocopherol. α-Tcopherol and quercetin enhanced the effects of prostaglandins (PGI_2, PGE_1, iloprost), which act through cAMP pathway**.** In addition, α-tocopherol, vitamin C, PN (PLP) and quercetin potentiated each others. Three compounds together caused stronger effect than the combination of two, and the combination of four was even more effective. Simultaneous effect through different inhibitory intracellular pathways allows to suppress the activity of platelets more completely. This might explain the beneficial effect of vitamin E and flavonoid consumption on cardiovascular disease.

Overall, the effect of each separate vitamin *in vitro* is relatively weak. However, they can potentiate the inhibitory effect of each other. They enhance also the effect of antiplatelet prostaglandins. Thus, they might inhibit platelet aggregation and thrombi formation more effectively.

3.3. Effect of α-tocopherol-incubated vascular endothelium on platelet activation

In this series of experiments, we studied the effect of the interaction of vascular endothelium with platelets on their activation *in vitro* experimental condition. One segment of a human umbilical vein was treated with 2 mM α-tocopherol in Krebs solution for two hours and another stayed in Krebs solution without α-tocopherol. Then PRP was dropped through the vein segments and ADP-induced platelet aggregation was measured (Kobzar et al., 2002). Platelets dropped through the vein segment treated with α-tocopherol were less sensitive to ADP than those dropped through the control segment of vein (Fig. 26) indicating that the dysfunction of endothelium took place in the endothelium of the control vein segment. These changes might be caused by oxidation processes in tissues due to a long period of staying of the vein in artificial conditions out of the organism. α-Tocopherol inhibited these changes and so protected platelets from activation by dysfunctional endothelium.

Earlier Podhaisky et al. (1997) showed that preincubation with α-tocopherol protects cultured bovine endothelial cells from H_2O_2-induced toxicity. This protection is caused by the increase in intracellular glutathione level and as a result in H_2O_2 degradation activity (Shimpuku et al., 2000).

ATP from vessel cells is converted to ADP by endothelial ADPase. It was shown that in the case of endothelial injury ADPase activity of the vessel is reduced (Marcus et al., 1995) and the local level of ADP in such a vessel is higher. This increase of ADP might cause the desensitisation of ADP receptors of platelets passing through vessels and lead to the decrease of platelet sensitivity to ADP.

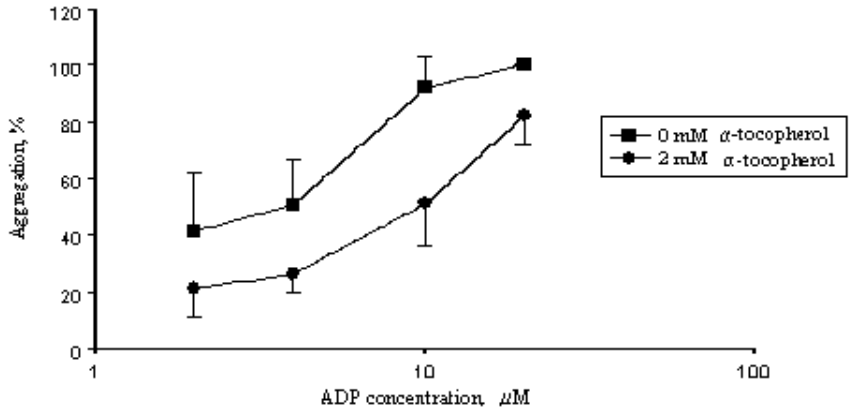


Fig. 26. ADP-induced aggregation of platelets dropped through umbilical vein treated with α-tocopherol or without α-tocopherol. Statistical significance was determined using t-test for a paired experiment ($P < 0.05$; $n = 10$). Vertical bars were shown SEM.

It is necessary to note that the effect of α-tocopherol on vascular endothelium is largely based on the studies of vascular endothelial cells in culture. For this purpose, we used an intact vein and found that in such an experimental model α-tocopherol protects platelets from activation by dysfunctional vascular endothelial cells.

4. CONCLUSION

In this work, the protection of platelet from activation with some vitamins and prostaglandins alone and in combination with each other has been demonstrated. The following conclusions are derived from the work.

- α-Tocopherol, quercetin, pyridoxine and vitamin C each alone at comparatively high concentrations inhibited ADP-induced platelet aggregation.

- α-Tocopherol acetate having only minimal antioxidant activity inhibited ADP-induced platelet aggregation significantly less than α-tocopherol, which has strong antioxidant properties. This suggests that only a part of the inhibitory effect of α-tocopherol on platelets can be explained by antioxidative properties.

- α-Tocopherol inhibited platelet aggregation induced by PKC activator 1,2-dioctanoyl-sn-glycerol (DOG). These results are in accordance with the suggestion that α-tocopherol induces its effect through the inhibition of PKC. At the same time, we show that PKC of patients suffering from CAD is less sensitive to the activating effect of DOG and inhibiting effect of α-tocopherol.

- L-arginine potentiates the effect of α-tocopherol.

- α-Tocopherol, vitamin C, PN (PLP) and quercetin potentiated the effects of each other. Three compounds together caused a stronger effect than the combination of two, and the combination of four was even more effective.

- α-Tocopherol and quercetin potentiated the antiaggregatory effects of prostaglandins I_2, E_1 and iloprost. Especially effective was the combination of α-tocopherol with iloprost.

- The combination of antiplatelet vitamins with each other and with antiplatelet prostaglandins allows to use these compounds at essentially lower concentrations to inhibit platelet aggregation and might decrease the adverse side effects of the latter.

- α-Tocopherol protected platelets from activation by vascular endothelium. Platelets dropped through the vein treated with α-tocopherol were less activated than platelets dropped through the untreated vein. Thus, α-tocopherol can protect platelets from activation not only directly but also indirectly acting on vascular endothelium.

INHIBITION OF PLATELET AGGREGATION WITH COMBINATION OF ANTIPLATELET AGENTS

ABSTRACT

Platelets play a key role in the first phase of the haemostatic process. These highly reactive cell fragments are involved in several pathological conditions, in particular arterial thrombosis, which can lead to the occlusion of coronary vessels and myocardial infarction.

Platelet activation is regulated by a number of compounds produced by blood cells and vessels. A number of compounds found in blood and blood vessels, such as ADP, PAF, thrombin, thromboxane A_2 and many others promote the aggregation of platelets. Other compounds inhibit this process. The most effective among them are PGI_2 and NO. The upset of balance between proaggregatory and antiaggregatory influences leads to different disorders. The prevailing of proaggregatory factors is a cause of many diseases such as cardiovascular diseases, stroke and others.

There are a number of drugs used in medicine to prevent platelet aggregation. However, they have unwanted side effects and are not effective in all patients. There is a need for more safe and effective antithrombotic drugs.

Some food components such as vitamin E, vitamin B_6, L-arginine and flavonoids inhibit platelet aggregation. They have mild if any side effects when administered at doses, which exceed recommended daily intake essentially. However, each of these agents alone is a weak inhibitor of platelet aggregation *in vitro*.

On the other hand, a correlation was found between the consumption of vitamins or flavonoids and a lower incidence of cardiovascular disease. Some of this beneficial effect was linked to the ability of vitamins and flavonoids to inhibit platelet aggregation. At the same time, there is a discrepancy between a low concentration of these compounds in plasma and a much higher one necessary to inhibit platelet aggregation *in vitro* experiments. This casts doubt on the physiologic relevance of *in vitro* effects.

However, the possibility of the interaction of different antiplatelet agents such as vitamins, prostacyclin and others, which can be in circulating blood simultaneously, has not been considered and there is little information on the effects of antiplatelet compounds in combination with each other. The present research was focused on the antiplatelet properties of vitamins: α-tocopherol, quercetin, pyridoxine and their effect in combination with each other, with L-arginine and prostaglandins.

It was shown that α-tocopherol, quercetin and pyridoxine each alone at comparatively high concentrations inhibited ADP-induced platelet aggregation. α-Tocopherol acetate having only minimal antioxidant activity inhibited ADP-induced platelet aggregation significantly less than α-tocopherol, which has strong

antioxidant properties. This suggests that only a part of the inhibitory effect of α-tocopherol on platelets can be explained by antioxidant properties.

α-Tocopherol inhibited platelet aggregation induced by PKC activator 1, 2-dioctanoyl-sn-glycerol (DOG). These results are in accordance with the suggestion that α-tocopherol induces its effect through the inhibition of PKC. At the same time, we show that PKC of patients suffering from CAD is less sensitive to the activating effect of DOG and inhibiting effect of α-tocopherol. α-Tocopherol, quercetin and pyridoxine potentiated the antiplatelet effect of each other at subthreshold concentrations. The inhibition of platelet aggregation by α-tocopherol was also potentiated by L-arginine. Besides, α-tocopherol and quercetin potentiated the antiaggregatory effect of endogenous antiplatelet agents prostaglandin E_1 and prostacyclin and also a synthetic analogue of the latter, iloprost. Thus, the combination of vitamins can be effective at essentially lower concentrations compared with each alone and can potentiate also endogenous antiplatelet agents. This allows to use these compounds at essentially lower concentrations to inhibit platelet aggregation and might decrease the adverse side effect of the latter.

Platelets dropped through the preparated umbilical vein segment treated with α-tocopherol were less sensitive to ADP than those dropped through the control segment of the vein indicating that the dysfunction of endothelium took place in the endothelium of the control vein segment. These changes might be caused by oxidation processes in tissues due to a long period of staying of the vein in artificial condition out of the dysfunctional endothelium. Thus, α-tocopherol can protect platelets from activation not only directly but also indirectly acting on vascular endothelium.

TROMBOTSÜÜTIDE AGREGATSIOONI INHIBEERIMINE ANTIAGREGATIIVSETE AINETE KOMBINEERIMISEGA

KOKKUVÕTE

Trombotsüütidel on tähtis roll hemostaasi protsesside esimeses faasis. Need suure reaktiivsusega rakufragmendid osalevad mitmetes patoloogilistes protsessides, eriti arteriaalses tromboosis, mis võib lõpptulemusena viia südameveresoonte sulgumise ja müokardiinfarktini.

Trombotsüütide aktivatsioon on reguleeritav erinevate ainete poolt, mida produtseerivad vererakud ja veresooned. Veres ja versoontes on leitud mitmeid aineid, nagu ADP, PAF, trombiin, tromboksaan A_2 ja paljud teised, mis kutsuvad esile trombotsüütide agregatsiooni. Samas aga teised ained inhibeerivad seda protsessi. Kõige efektiivsemad viimaste seas on PGI_2 ja NO. Tasakaalu häirumine agregatiivsete ja antiagregatiivsete mõjude vahel viib erinevatele kõrvalekalletele. Juhul kui ülekaalus on agregatiivsed faktorid põhjustab see mitmeid haigusi nagu südameveresoonkonna haigused, insult jt.

Meditsiinis kasutatakse mitmeid ravimeid, mis hoiavad ära trombotsüütide agregatsiooni. Kahjuks esineb nendel ravimitel mitmeid soovimatuid kõrvaltoimeid ja nad pole efektiivsed kõigil patsientidel. Seetõttu eksisteerib vajadus ohutumate ning efektiivsemate antitrombootiliste ravimite järgi.

Mõned toidus esinevad ained nagu vitamiin E, vitamiin B_6, L-arginiin ja flavonoidid inhibeerivad trombotsüütide agregatsiooni. Nende kõrvaltoimed on väikesed või puuduvad hoopis, isegi juhul kui neid aineid manustatakse päevaseks tarbimiseks ettenähtud kogustest tunduvalt suuremates doosides. Sellest hoolimata on nad eraldivõetuna nõrgad trombotsüütide agregatsiooni inhibiitorid *in vitro*.

Teisest küljest on leitud seos vitamiin E või flavonoidide tarbimise ja madala südameveresoonkonnahaiguste esinemise vahel. Osa sellest kasulikust efektist on seotud vitamiinide ja flavonoidide võimega inhibeerida trombotsüütide agregatsiooni. Samal ajal eksisteerib vastuolu nende ühendite madalate kontsentratsioonide esinemisel vereplasmas ja tunduvalt suuremate kontsentratsioonide vahel, mis on vajalikud inhibeerimaks trombotsüütide agregatsiooni *in vitro* eksperimentides. See paneb kahtluse alla *in vitro* efektide füsioloogilise tähtsuse.

Senini pole piisavalt tähelepanu pööratud erinevatele antiagregatiivsete ainete vahelistele interaktsioonidele nagu vitamiinid, prostatsükliin jt. Vastavad ained tsirkuleerivad veres üheaegselt ja vähe on informatsiooni nende koostoimeefektide kohta. Antud töös on uuritud vitamiinide (α-tokoferool, kvertsetiin ja püridoksiin) antiagregatiivseid omadusi, nende omavahelisi koostoimeefekte ning samuti ka nende koostoimeefekte L-arginiini ja prostaglandiinidega.

Selles töös on näidatud, et α-tokoferool, kvertsetiin ja püridoksiin eraldivõetuna inhibeerivad ADP-indutseeritud trombotsüütide agregatsiooni suhteliselt suurtes

konsentratsioonides. α-Tokoferool atsetaat, millel on minimaalne antioksüdatiivne aktiivsus inhibeerib ADP-indutseeritud trombotsüütide agregatsiooni tunduvalt vähem kui tugevate antioksüdatiivsete omadustega α-tokoferool. See näitab, et α-tokoferooli trombotsüüte inhibeeriv efekt on ainult osaliselt seletatav tema antioksüdatiivsete omadustega.

α-Tokoferool inhibeerib trombotsüütide agregatsiooni, mis on indutseeritud PKC aktivaatori 1,2-dioktanoüül-sn-glütserooli (DOG) poolt. Need tulemused on vastavuses oletusega, et α-tokoferool toimib PKC inhibitsiooni kaudu. Samal ajal on koronaartõvega patsientidel PKC vähem tundlik DOG aktiveeriva efekti suhtes ja α-tokoferooli inhibeeriva efekti suhtes. α-Tokoferool, kvertsetiin ja püridoksiin potentseerivad lävikontsentratsioonides üksteise antiagregatiivseid efekte trombotsüütidele. Vereliistakute agregatsiooni inhibeerimist α-tokoferooli poolt võib potentseerida ka L-arginiiniga. Lisaks sellele potentseerivad α-tokoferool ja kvertsetiin endogeensete trombotsüütide agregatsiooni inhibiitorite toimet. Seega võib öelda, et vitamiinide kombineerimine madalates konsentratsioonides võib olla efektiivsem kui vitamiinide eraldi kasutamine ja samas võivad nad potentseerida endogeenseid trombotsüütide agregatsiooni inhibiitoreid. See võimaldab viimati mainitud antiagregatiivseid aineid kasutada madalates kontsentratsioonides inhibeerimaks vereliistakute agregatsiooni ja lisaks sellele vähendada viimaste ebasoodsaid kõrvaltoimeid.

Trombotsüüdid, mis on lastud läbi α-tokoferooliga töödeldud nabaväädi veeni segmendist on vähem tundlikud ADP suhtes, kui need trombotsüüdid, mis on läbinud kontrollveeni segmendi. See viitab kontrollveeni segmendi endoteeli düsfunktsioonile. Neid muutusi võivad põhjustada koes toimuvad oksüdatsiooni protsessid, kuna veen on pika perioodi kestel väljaspool organismi kunstlikes tingimustes. α-Tokoferool inhibeerib neid muutusi ja kaitseb trombotsüüte düsfunktsionaalse endoteeli poolt põhjustatud aktivatsiooni eest. Seega, α-tokoferool kaitseb trombotsüüte aktivatsiooni eest mitte ainult otseselt, vaid ka kaudselt toimides veresoone endoteelile.

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APPENDIX I

Materials and methods

Blood collection

Venous blood from healthy volunteers and patients with CAD was used for experiments. Blood samples were taken from antecubital vein. Blood was anticoagulated with one-tenth volume of 3.8% trisodium citrate solution.

Preparation of platelet-rich plasma (PRP) and washed platelets (WP)

PRP was obtained after centrifugation of citrated blood at 160 *x* g for 10 min at room temperature. The remaining blood was recentrifuged at 4000 *x* g for 10 min to prepare platelet-poor plasma. Platelet cell count in PRP was measured by impedance method in Swelab counter.

The suspension of WP was prepared as follows. Indomethacin (14 μM) and EDTA (12 mM) were added to PRP and centrifuged at 110 *x* g for 10 min to sediment red and white cells remaining in PRP. The resulting PRP was separated. After adding 750 μl Tyrode solution, EDTA (12 mM) and indomethacin (14 μM) was recentrifuged at 600 *x* g for 10 min. The supernatant was decanted and the pellet was resuspended in 8 ml of Tyrode solution by gently sucking and blowing with a plastic pipette. Then EDTA and indomethacin were added again and the suspension was centrifuged at 600 *x* g for 10 min. The final suspension was prepared in calcium-free Tyrode solution by adding a volume to obtain a platelet count in the range of 230-270 x 10^8 platelet per ml. Before aggregation studies $CaCl_2$ (0.9 mM) was added.

Tyrode's solution used to prepare the final suspension of washed platelets contained (mM): NaCl 136.9; KCl 2.68; $CaCl_2$ 0.9; $MgCl_2$ 1.05; $NaHCO_3$ 11.0; NaH_2PO_4 0.42; glucose 5.55; gassed with a mixture of O_2 (95%) and CO_2 (5%), pH 7.4.

Platelet aggregation

Platelet aggregation was measured photometrically in Chrono-log aggregometer. An aggregometer cuvette containing 0.25 ml of an aliquot of PRP or WP was maintained at 37^0C and stirred with a teflon-coated bar. Aggregation was induced by ADP that was added to platelets at a concentration just sufficient to induce submaximum effect. It varied from 2 to 10 μM and from 10 to 20 μM in different samples of PRP and WP, respectively. Inhibition of platelet aggregation was determined by incubation vitamins and prostaglandins in cuvette with PRP, α-tocopherol and α-tocopherol acetate 5 min, quercetin 1 or 10 min, pyridoxine 1 min, PG-s 1 min prior to the addition of ADP. PGs were dissolved in ethanol, kept at -20^0C and diluted with saline before the experiment. PGE_1 and PGI_2 were

obtained from Kemasol Ltd. (Tallinn, Estonia). ADP was dissolved in saline, α-tocopherol in ethanol and quercetin in 1 M NaOH solution. The Na salt of PGI_2 was dissolved in glycine buffer (pH 10.4) before each experiment and kept on ice. All the compounds were diluted in saline.

Umbilical vein

Umbilical cords were collected after vaginal and caesarean deliveries of pregnant women and were immediately placed in cold modified Krebs solution and transported to the laboratory. Then two small vein segments with intact endothelium (2 cm length) were prepared. The vein segments were set up in Krebs solution on two canyls and bubbled with a mixture of O_2 (95%) and CO_2 (5%) at 37^0C. One vein segment was incubated without α-tocopherol as control for two hours and second vein segment was incubated with α-tocopherol (2 mM) for two hours and then washed by Krebs solution. PRP was prepared as described above. PRP was dropped slowly through the vein segment, collected in a plastic tube and then platelet aggregation was measured.

Krebs solution of the following composition (g/l) was used: NaCl 7.1; KCl 0.44; $CaCl_2$ 0.29; $MgCl_2$ x 6 H_2O 0.2; NaH_2PO_4 x H_2O 0.16; glucose 2.18; $NaHCO_3$ 1.4; pH 7.4; was gassed with mixture of O_2 (95%) and CO_2 (5%).

Statistics

The values of IC_{50} were calculated from concentration-effect curves obtained from the blood sample of each subject separately as the concentration necessary to reduce the ADP-induced aggregation by 50%. Data were expressed as arithmetic mean ± standard error of separate experiments with PRP or WP of n subjects. The statistical significance of differences was calculated by paired or unpaired Student's t-test, as appropriate. The results were considered to be statistically significant at a probability level of $P < 0.05$.

Concentration-effect curves for the combined effect of vitamins and prostaglandins were evaluated with the analysis of variance (ANOVA) with a *post hoc* Newman-Keuls comparison. The effect of PG in the presence and absence of α-tocopherol and quercetin was measured with the sample of PRP.

Student's t-test for paired data was used to determine statistical differences between the points for the same concentration of PG in the absence and presence of α-tocopherol or quercetin. The results were considered to be statistically significant at a probability level of $P < 0.05$.

The study was approved by the Tallinn Medical Research Ethics Committee: decisions number 484 and 1087.

APPENDIX II

CURRICULUM VITAE

1. Personal data

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Date and place of birth: may 11, 1963, Tallinn

2. Contact information:

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Phone: 6525046
E-mail: Vilja@chemnet.ee

3. Education

Educational institution	Graduation year	Education (field of study/degree)
Tallinn Technical University	1999	master's degree
Tartu University	1987	biology

4. Languages

Language	Level
Estonian	fluent
Russian	average
English	average

5. Special Courses

Period	Educational or other organisation
14.03.02-6.06.02	Language Centre, Tallinn University of Technology
01.12.05-02.12.05	Infotechnology Centre, Tallinn University of Technology
14.10.06-18.10.06	EMI Language Centre

6. Professional Employment

Period	Organisation	Position
2002-2008	Tallinn University of Technology, Faculty of Natural Sciences	researcher
1997-2001	Tallinn Technical University, Institute of Chemistry	researcher
1988-1996	Institute of Chemistry	engineer

7. Scientific work

Publications:

Kobzar G., Mardla V., Rätsep I., Samel N. Platelet activity before and after coronary artery bypass grafting. Platelets 2006, 17, 5, 289-291.
Kobzar G., Mardla V., Samel N. Effect of α-tocopherol, L-arginine, and quercetin on aggregation of human platelets. Nutr. Res. 2005, 25, 569-575.
Mardla V., Kobzar G., Samel N. Potentiation of antiaggregating effect of prostaglandins on platelets by alpha-tocopherol and quercetin. Platelets 2004, 15, 5, 319-324.
Kobzar G., Mardla V., Rätsep I., Raukas R., Samel N. Inhibition of platelet aggregation by alpha-tocopherol in healthy subjects and patients with coronary artery disease. Platelets 2002, 13, 5/6.
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Kobzar G., Mardla V., Järving I., Samel N., Lõhmus M. Effect of 8-iso-PGE_2 on platelets and smooth muscles. Pharmacol. Toxicol., 1995, 76, Supplement III, 34.
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8. Defended theses

Masters' degree work in biotechnology

9. Main areas of scientific work

The study of the effects of biologically active compounds on the function and biochemical processes in cells, mechanism of interaction of drugs with plasma membrane receptors.

10. Other research projects

V. Mardla participated in the project "Mechanisms Underlying Coronary Arterial Thrombosis and the Means for Dispersion and Inhibition of such Thrombi" granted by European Commission.

APPENDIX III

ELULOOKIRJELDUS

1. Isikuandmed
   Ees-ja perekonnanimi: Vilja Mardla
   Sünniaeg ja-koht: 11.05.1963, Tallinn
   Kodakondsus: eesti

2. Kontaktandmed
   Aadress: Sõpruse pst. 235-51, Tallinn, 13420
   Telefon: 6525046
   E-posti aadress: Vilja@chemnet.ee

3. Hariduskäik

Õppeasutus	Lõpetamise aeg	Haridus (eriala/kraad)
TTÜ	1999	loodusteaduste magister
TRÜ	1987	bioloogia

4. Keelteoskus

Keel	Tase
Eesti	kõrg
Vene	kesk
Inglise	kesk

5. Täiendusõpe

Õppimise aeg	Täiendusõppe läbiviija nimetus
14.03.02-6.06.02	TTÜ keeltekeskus
01.12.05-02.12.05	TTÜ IT täiendõppekeskus
14.10.06-18.10.06	EMI keelekeskus

6. Teenistuskäik

Töötamise aeg	Tööandja nimetus	Ametikoht
2002-2008	TTÜ matemaatika-loodusteaduskond	teadur
1997-2001	TTÜ Keemia Instituut	teadur
1988-1996	Eesti TA Keemia Instituut	tehnoloogiainsener

7. Teadustegevus

Publikatsioonid:

Kobzar G., Mardla V., Rätsep I., Samel N. Platelet activity before and after coronary artery bypass grafting. Platelets 2006, 17, 5, 289-291.
Kobzar G., Mardla V., Samel N. Effect of α-tocopherol, L-arginine, and quercetin on aggregation of human platelets. Nutr. Res. 2005, 25, 569-575.
Mardla V., Kobzar G., Samel N. Potentiation of antiaggregating effect of prostaglandins on platelets by alpha-tocopherol and quercetin. Platelets 2004, 15, 5, 319-324.
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Kobzar G., Mardla V., Järving I., Samel N. Comparison of antiaggregatory effect of PGI_3, PGI_2 and Iloprost on human and rabbit platelets. Cell. Physiol. Biochem. 2001, 11, 279-284.
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Kobzar G., Mardla V., Järving I., Samel N., Lõhmus M. Effect of 8-iso-PGE_2 on platelets and smooth muscles. Pharmacol. Toxicol., 1995, 76, Supplement III, 34.

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Kobzar G., Mardla V., Järving I., Lõhmus M., Vahemets A., Samel N., Lille Ü. Comparison of the inhibitory effect of E-prostaglandins in human and rabbit platelet-rich plasma and washed platelets. Comp. Biochem. Physiol., 1993, 106C, 489-494.

8. Kaitstud lõputööd

Magistritöö biotehnoloogias

9. Teadustöö põhisuunad

Bioaktiivsete ainete toime uurimine raku funktsioonidele ja biokeemilistele protsessidele. Ravimite interaktsioonimehhanismid plasmamembraani retseptoritega.

10. Teised uurimisprojektid

V. Mardla osales projektis “Mechanisms Underlying Coronary Arterial Thrombosis and the Means for Dispersion and Inhibition of such Thrombi” rahastatud Euroopa Komisjoni poolt.

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ARTICLE I

Mardla V., Kobzar G., Rätsep I., Lõhmus M., Järving I., Samel N. (2001)
Sensitivity of platelets to prostaglandins in patients with coronary artery disease.
Proc. Estonian Acad. Sci. Biol. Ecol., 50, 1, 33-36. Short communications.

Proc. Estonian Acad. Sci. Biol. Ecol., 2001, **50**, 1, 33-36

SENSITIVITY OF PLATELETS TO PROSTAGLANDINS IN PATIENTS WITH CORONARY ARTERY DISEASE

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Abstract. The platelet sensitivity to the antiaggregatory prostaglandins (PGE_1, 13,14-dihydro-PGE_1, and 5,6-dihydro-PGE_3) was studied in patients with coronary artery disease. Platelets of healthy subjects were used as control. The sensitivity was tested in vitro by inhibiting the adenosine diphosphate-induced platelet aggregation with these prostaglandins of various concentrations. In the patients the antiaggregatory potency of the prostagiandins was significantly iower than in healthy subjects.

Key words: platelets, prostaglandins, coronary artery disease.

Abbreviations: PG = prostaglandin; PGE_l = prostaglandin E_1; 13,14-dihydro-PGE_1 = prostaglandin 13,14-dihydro-E_1; 5,6-dihydro-PGE_3 = prostaglandin 5,6-dihydro-E_3; PGI_2 = prostaglandin I_2, prostacyclin; ADP = adenosine diphosphate; CAD = coronary artery disease; PRP = platelet-rich plasma.

INTRODUCTION

Prostaglandin E_l (PGE_1) and its natural analogues prostaglandin 13,14-dihydro-E_1 (13,14-dihydro-PGE_1 and prostaglandin 5,6-dihydro-E_3 (5,6-dihydro-PGE_3) interact with the same platelet receptor as prostacyclin (PGI_2). These prostaglandins (PGs) are synthesized in the organism in very low quantities, but are very potent inhibitors of platelet aggregation (Kobzar et al., 1993). Sinzinger et al. (1998) showed that PGE_1 and 13,14-dihydro-PGE_1 inhibit platelet adhesion to the vessel wall as well as platelet thrombus formation and

induce thromboresistance in experimental animals and in man. There are no data in the literature on the effect of 13,14-dihydro-PGE_1, and 5,6-dihydro-PGE_3 on platelets of patients suffering from coronary artery disease (CAD). Therefore in this work we compared the antiaggregatory potency of these PGs in patients suffering from advanced CAD with that of age and sex matched healthy subjects.

MATERIALS AND METHODS

The studied patients suffered from CAD and underwent coronary artery bypass grafting. Preliminary coronary angiography showed $\geq$ 75% lumen narrowing in at least one coronary artery. All patients gave informed consent and the local ethics committee approved the study. Donors of the local blood bank served as control. Blood samples were taken from the radial artery through an indwelling catheter before cardiopulmonary bypass in the patients and from the antecubital vein in the healthy subjects.

Blood was collected into 10-mL plastic centrifuge tubes containing 1 mL of 3.8% trisodium citrate. Platelet-rich plasma (PRP) and platetet-poor plasma were prepared by centrifugation of the citrated blood at 160 x *g* or 4000 x *g* for 10 min at room temperature, respectively.

Aggregation was measured photometrically in a Chrono-Log aggregometer according to the method of Born (1962) by detecting the transmission of the light through PRP. An aggregometer cuvette containing 0.5 mL of an aliquot of PRP was maintained at 37 °*C* and stirred with a Teflon coated stirring bar at 1200 rpm. Inhibition of platelet aggregation was determined by incubation of PGs with PRP.

The IC_{50} was calculated from dose-effect curves as a concentration necessary to reduce the ADP-induced aggregation by 50% of its control amplitude. Data were expressed as arithmetic mean ± standard error (SEM) of separate experiments with PRP of *n* subjects. Statistical significance of healthy donors versus CAD patients was determined using the Student's *t*-test for an unpaired experiment.

PGs were dissolved in ethanol, kept at -20 °C, and diluted with saline before the experiment. PGE_1 was obtained from Kemasol Ltd. (Tallinn, Estonia). 5,6-Dihydro-PGE_3 was separated by repeated HPLC-purification from the mixture of natural E-PGs extracted from ram seminal vesicles (Lõhmus et al., 1990). 13,14-Dihydro-PGE_1 was synthesized from PGE_1 by the reduction of the 13,14 double bound with H_2 on the Pd(C) catalyst. The purity of the PGs used for the platelet antiaggregatory study was more than 99.9% by HPLC.

RESULTS

The chemical structure of the PGs studied is shown in Fig. 1. All three compounds were about two times less effective in CAD patients than in control (Table 1). These data are in accordance with the earlier studies, which showed

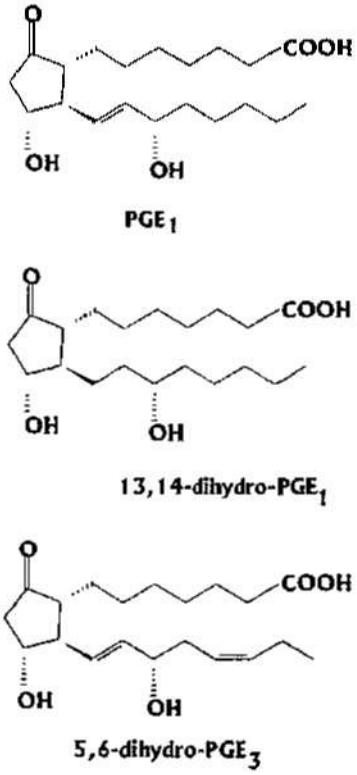

Fig. 1. Chemical structure of the prostaglandins under study.

Table 1. Inhibition of ADP-induced platelet aggregation in healthy subjects and patients with CAD

Compound	Healthy subjects		CAD patiens		*P*-value
	IC_{50}, nM	*n*	IC_{50}, nM	*n*	
PGE_1	56±5	11	120±20	11	<0.01
13,14-Dihydro-PGE_1	35±6	11	86±12	11	<0.01
5,6-Dihydro-PGE_3	58±6	10	110±9	10	<0.01

Data are expressed as arithmetic mean ± SEM of separate experiments with PRP of *n* subjects. IC_{50} is the concentration causing 50% inhibition of ADP-induced platelet aggregation. The statistical significance of healthy donors versus CAD patients was determined with unpaired Student's *t*-test.

a lower sensitivity of platelets of CAD patients to PGI_2 (Mehta et al., 1980) and to PGE_1 (Sinzinger et al., 1981). This effect may be explained by the fact that PGI_2 synthesis is increased in blood vessels of CAD patients (Fisch et al., 1997) and long-term exposure of platelets to PGI_2 may result in desensitization of PGI_2

receptors. The lower sensitivity of platelets to 13,14-dihydro-PGE_1 and 5,6-dihydro-PGE_3, as well as to PGE_1 and PGI_2, may be used as an indicator of CAD.

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KORONAARTÕVEGA PATSIENTIDE TROMBOTSÜÜTIDE TUNDLIKKUS PROSTAGLANDIINIDE SUHTES

Vilja MARDLA, Gennadi KOBZAR, Indrek RÄTSEP, Madis LÕHMUS, Ivar JÄRVING ja Nigulas SAMEL

Eksperimentaalselt on uuritud koronaartõvega patsientide trombotsüütide tundlikkust antiagregatiivsete prostaglandiinide (PGE_1, 13,14-dihüdro-PGE_1 ja 5,6-dihüdro-PGE_3) suhtes. Tervete doonorite trombotsüüte on käsutatud kontrolliks. Trombotsüütide tundlikkust on testitud *in vitro* inhibeerides prostaglandiinidega adenosiindifosfaadi toimel indutseeritud trombotsüütide agregatsiooni. Koronaarhaigusega patsientidel oli nende prostaglandiinide antiagregatiivne aktiivsus tunduvalt madalam võrreldes tervete kontrollgrupiga.

ARTICLE II

Kobzar G., Mardla V. (2002) Inhibition of platelet aggregation by pyridoxine. Nutr. Res., 22, 997-1001.

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Inhibition of platelet aggregation by pyridoxine

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Abstract

Vitamin B_6 in the form of pyridoxal-5′-phosphate (PLP) has been previously shown to inhibit *in vitro* platelet aggregation induced by several different agonists. However, this inhibitory effect took place only at millimolar concentrations of PLP that is too high to have physiological relevance. The aim of this work was to study the anti-aggregatory effect of vitamin B_6 in the form of pyridoxine (PN) hydrochloride. Experiments were performed on platelets separated from the human blood. Aggregation was measured photometrically. The values of PN HCl concentration causing 50% inhibition of ADP-induced platelet aggregation (IC_{50}) were obtained from concentration-effect curves. PN HCl inhibited platelet aggregation at low concentrations, IC_{50} being 1.7 ± 0.5 μM. This indicates that the antiplatelet effect of PN HCl may have physiological relevance.

Keywords: Platelet aggregation; Pyridoxine; Vitamin B_6

1. Introduction

Oral supplementation of vitamin B_6 to volunteers significantly inhibits ADP- or epinephrine-induced platelet aggregation [1,2], which plays a central role in thrombi formation. A biochemical mechanism for the antiplatelet effect of vitamin B_6 was suggested by Chang and co-workers [3]. They found that vitamers of vitamin B_6 pyridoxal (PL), pyridoxine (PN) and pyridoxal-5′-phosphate (PLP) down-regulate transient expression of glycoprotein IIb (GPIIb) in human erythroleukaemia cells with transfected GPIIb promoter region. GPIIb is

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the α-subunit of the platelet membrane receptor GPIIb/IIIa, which plays a major role in platelet aggregation. The authors proposed that down-regulation effect of vitamin B_6 on GPIIb promoter activity might lead to a reduction of GPIIb protein expression and thus be detrimental to platelet aggregation. Since platelets do not have nuclear DNA, vitamin B_6 must affect megakaryocytes, the cells from which platelets are derived.

On the other hand, it was found that vitamin B_6 could affect platelet aggregation directly. PLP added in a cuvette with platelets at millimolar concentrations has been shown to inhibit the platelet aggregation induced by a number of different agonists [4]. However, effective concentrations of PLP *in vitro* experiments exceeded its blood concentration by several orders of magnitude. Therefore, PLP may not be able to function in organism as direct antithrombotic agent [5].

In this work, we studied the antiplatelet effect of vitamin B_6 in the form of PN hydrochloride and found that it induces the inhibition of platelet aggregation *in vitro* at concentrations of three orders of magnitude lower than that previously found for PLP.

2. Materials and methods

Venous blood from 11 male and 4 female healthy volunteers of Estonian nationality aged 20–55 (mean 31) year was used for experiments. Blood was anticoagulated with one-tenth volume of 3.8% trisodium citrate solution. Platelet-rich plasma (PRP) was obtained after the centrifugation of the citrated blood at $160 \times g$ for 10 min at room temperature. The suspension of washed platelets (WP) was prepared as described previously [6] using EDTA and indomethacin to protect platelets from activation during washing procedure.

Platelet aggregation was measured photometrically in a Chrono-log aggregometer. An aggregometer cuvette containing 0.25 ml of an aliquot of PRP or WP was maintained at 37°C and stirred with a teflon-coated bar. Aggregation was induced by adenosine diphosphate (ADP). Inhibition of platelet aggregation was determined by incubation of PN HCl in an aggregometer cuvette 1 min prior to the addition of ADP. The values of IC_{50} were calculated from concentration-effect curves obtained from the blood sample of each subject separately as the concentrations necessary to reduce the ADP-induced aggregation by 50%. Data were expressed as mean $\pm$ S.E.M. of the values of IC_{50} of different subjects.

ADP, indomethacin, and PN hydrochloride were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A). Tyrode's solution used to prepare the final suspension of washed platelets contained (mM): NaCl 136.9; KCl 2.68; $CaCl_2$ 0.9; $MgCl_2$ 1.05; $NaHCO_3$ 11.0; NaH_2PO_4 0.42; glucose 5.55; gassed with a mixture of O_2 (95%) and CO_2 (5%), pH 7.4.

3. Results

PN HCl induced a concentration-dependent inhibition of platelet aggregation in both PRP and WP. The samples of PRP might be subdivided into two groups depending on sensitivity to PN HCl: high sensitive group and low sensitive one with the mean values of IC_{50} equal to 1.7 ± 0.5 μM and 4000 ± 800 μM, respectively (Fig. 1). The sensitivity did not depend

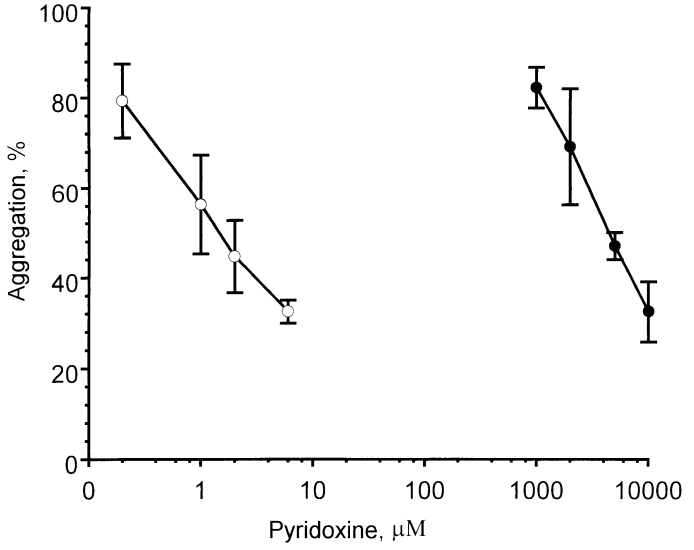

Fig. 1. Inhibition of adenosine diphosphate (ADP)-induced platelet aggregation by pyridoxine (PN) hydrochloride in high (○) and low (●) sensitive platelet-rich plasma. The curves in figure are means of separate curves obtained in independent samples. Samples from 5 healthy subjects were used to obtain left and 6 other subjects to obtain right curve. Aggregation induced by ADP in the absence of PN hydrochloride is taken as 100%. Vertical bars show standard error of mean.

on the gender, age or ABO blood-group status of volunteers. In the preparation of WP, PN HCl induced an inhibition of platelet aggregation only at high concentrations. The sensitivity of WP to pyridoxine was low independently of whether WP were prepared from high sensitive or low sensitive PRP.

In four experiments, the values of IC_{50} for PN HCl in the samples of PRP with low sensitivity and of WP prepared from the same PRP were compared. They were found to be 3500 ± 1200 and 3000 ± 500 μM, respectively and did not differ significantly from each other (paired t-test, $P > 0.05$).

4. Discussion

Our experimental data show that PN HCl can inhibit platelet aggregation at micromolar concentrations (Fig. 1, left curve; Fig. 2). It is three orders of magnitude less than that earlier found for PLP, the major form of vitamin B_6 in blood. The effective concentrations of PN HCl in our work were about two orders of magnitude higher than its concentration in blood plasma [7]. However, it is necessary to keep in the mind that we used the turbidometric method, which is not sufficiently sensitive to detect small aggregate formation in the early phase of aggregation. It was found that the aggregates of small size are formed at the lower concentrations of agonists [8]. Also, *in vivo*, the antiaggregatory effect of PN HCl may be enhanced by the synergistic effects of other dietary components having similar biological activity [9].

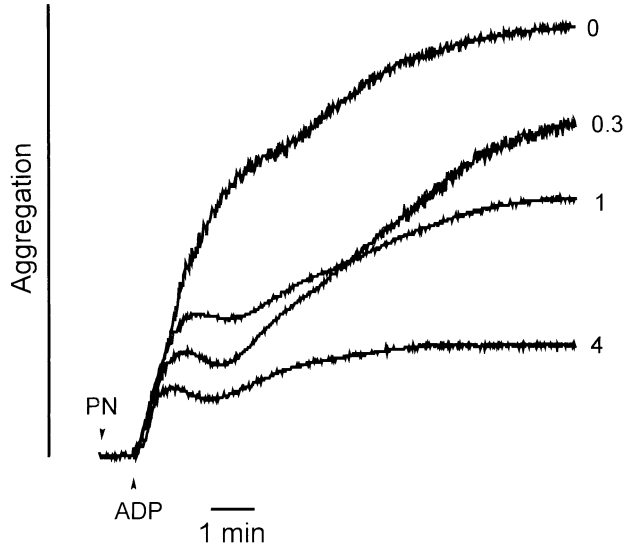

Fig. 2. Tracings showing inhibition of platelet aggregation by pyridoxine (PN) hydrochloride. The tracings were obtained in the sample of platelet-rich plasma (PRP) of one subject. Aggregation was induced by 4 μM adenosine diphosphate (ADP). PRP was incubated with PN for 1 minute before ADP addition. Arrows show PN and ADP addition to PRP. Numbers near tracings show PN concentration in μM. Data are representative of 5 independent experiments each with PRP of different subjects.

The reason for low effectiveness of PLP on platelets may be its binding by plasma proteins. Indeed, it was found that PLP was essentially more potent in WP than in PRP [5]. Under physiological conditions, PLP is bound to blood proteins, predominantly to albumin [10]. Antiplatelet effect *in vitro* can be explained by the fact that millimolar concentrations of PLP exceed the binding capacity of albumin and it can interact with platelets. In our experiments, the effect of PN HCl did not depend on the presence of plasma; the values of IC_{50} were similar in PRP and WP. This shows that plasma proteins do not bind PN HCl.

A part of PRP samples was sensitive to PN HCl at micromolar and other part at millimolar concentrations. The further investigation is needed to clarify whether the difference in sensitivity is a characteristic of the individual, from which PRP sample was prepared.

The carbonyl group of PLP binds to proteins as a Schiff's base with the ϵ-amine lysine. The formation of Schiff's base with the proteins or glycoproteins of platelet membrane was suggested to be responsible for the antiplatelet effect of PLP [4]. PLP is unable to cross the cell membrane and must first be converted enzymatically to PL by membrane-associated alkaline phosphatases. In contrast, PL and PN readily diffuse through the cell membrane and are converted in cells to respective 5′-phosphates by intracellular PL kinase enzyme (EC 2.7.1.35) [11].

It was found recently that vitamin B_6 inhibits Ca^{2+} influx into the intracellular compartment of the vascular smooth muscle of rat [12]. The vitamers of vitamin B_6 have the structural similarity to dihydropyridine, a calcium channel blocker. This suggests that vitamin B_6 can block calcium channels. Thus, the mechanism of action of vitamin B_6 on

platelets may be also the blockage of channels with the inhibition of Ca^{2+} influx into platelets and as a result the inhibition of platelet aggregation.

In conclusion, PN hydrochloride at low concentrations inhibits platelet aggregation in human PRP, IC_{50} being 1.7 ± 0.5 μM. It is three orders of magnitude higher potency than that previously found for PLP, another form of vitamin B_6. The results show that the antiplatelet effect of PN hydrochloride may have physiological importance.

Acknowledgments

G. Kobzar has designed this work, prepared the washed platelets, made aggregation measurements, and written the manuscript. V. Mardla has prepared platelet-rich plasma. Blood was obtained from the volunteers in the North-Estonian Blood Centre. The work was supported by Estonian Science Foundation grant 3736.

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ARTICLE III

Mardla V., Kobzar G., Samel N. (2004) Potentiation of antiaggregating effect of prostaglandins by α-tocopherol and quercetin. Platelets, 15, 5, 319-324.

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Potentiation of antiaggregating effect of prostaglandins by α-tocopherol and quercetin

Vilja Mardla, Gennadi Kobzar, Nigulas Samel

Prostaglandin (PG) I_2 (prostacyclin), PGE_1 and their analogues are effective inhibitors of platelet aggregation. However, a clinical use of these compounds for the treatment of cardiovascular diseases is restricted due to unwanted side effects. α-Tocopherol and quercetin are weak antiplatelet agents. At the same time, they have mild if any side effects when consumed medicinally. The aim of this work was to study the possibility to decrease the effective antiplatelet concentrations of PGs combining them with α-tocopherol or quercetin. Platelet-rich plasma (PRP) was prepared from human blood. The inhibition of adenosine diphosphate-induced platelet aggregation was caused by PGs in the presence and absence of α-tocopherol or quercetin and corresponding concentration–effect curves were obtained. At a subthreshold concentration 200 and 2 μM, respectively, both α-tocopherol and quercetin essentially increased the antiplatelet effects of PGI_2, PGE_1 and iloprost. Especially effective was the combination of α-tocopherol with low concentrations of iloprost. Thus, combination of PGs with α-tocopherol or quercetin allows the use of prostaglandins at lower concentrations to inhibit platelet aggregation.

Introduction

Prostaglandin (PG) I_2 (prostacyclin) and PGE_1 are found in different body tissues and body fluids. They are effective inhibitors of platelet aggregation. PGI_2 derived from vascular endothelium plays an important role in the local control of vascular tone and thrombus formation.[1] These features have made these PGs attractive substances for the treatment of cardiovascular diseases. However, the clinical use of PGI_2 and its pharmacological analogues is restricted due to their adverse side effects.

α-Tocopherol, the main form of vitamin E,[2,3] and quercetin, together with some other flavonoids,[4,5] were also found to inhibit platelet aggregation *in vitro*. Furthermore, the consumption of α-tocopherol was associated with cardiovascular protection[6] and it was found that the oral intake of vitamin E can inhibit platelet aggregation.[3,7] The intake of dietary flavonoids was also associated with a reduced risk of ischemic heart disease and the incidence of myocardial infarction.[8] In addition, flavonoids were found to reduce platelet-dependent thrombosis.[9] α-Tocopherol and quercetin are essentially weaker inhibitors of platelet aggregation than PGs. At the same time, the combination of PGs with these compounds might increase their antiplatelet effect and so might decrease the therapeutic concentrations of PGs. To test this possibility, we studied the combined effect of PGI_2, PGE_1 and iloprost with α-tocopherol or quercetin and found that at subthreshold concentration the two latter compounds essentially potentiate the effect of the PGs *in vitro*.

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Materials and methods

Materials

ADP, α-tocopherol and quercetin were obtained from Sigma Chemical Co. (St. Louis, MO, USA), Na salt of PGI_2 from Kemasol Ltd. (Tallinn, Estonia). (*E*)-(3a*S*, 4*R*, 5*R*, 6a*S*)-hexahydro-5-hydroxy-4-[(*E*)-(3*S*, 4*RS*)-2-hydroxy-4-methyl-1-octan-6-ynyl]-$\Delta^{2(1H),\delta}$-pentalenevaleric acid (iloprost) was a generous gift of Schering AG (Berlin, Germany).

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ADP was dissolved in saline, α-tocopherol in ethanol and quercetin in 1 M NaOH solution. The Na salt of PGI_2 was dissolved in glycine buffer (pH 10.4) before each experiment and kept on ice. All the compounds were diluted in saline.

Subjects and preparation of platelet-rich plasma

Blood of 27 healthy volunteers of both genders, aged from 19 to 53 years, was obtained through the North-Estonian Blood Center. All subjects gave their informed consent.

Blood was anticoagulated with one-tenth volume of 3.8% trisodium citrate solution. Platelet-rich plasma (PRP) was obtained after the centrifugation of the citrated blood at $160 \times g$ for 10 min at room temperature.

Platelet aggregation

Platelet aggregation was measured photometrically in a Chrono-log aggregometer. An aggregometer cuvette containing 0.25 ml of an aliquot of PRP was maintained at 37°C and stirred with a teflon-coated bar. Aggregation was induced by adenosine diphosphate (ADP), which was added to platelets at a concentration just sufficient to induce submaximum aggregation (95–85% of maximum). This concentration varied from 2 to 10 μM in different samples of PRP. The inhibition of platelet aggregation was determined by the incubation of α-tocopherol for 5 min, quercetin, PGI_2, PGE_1 and iloprost for 1 min prior to the addition of ADP into a cuvette with PRP. The values of IC_{50} (the concentration necessary to reduce the ADP-induced aggregation by 50%) were obtained from concentration–effect curves for PGs in the presence and absence of α-tocopherol or quercetin.

Statistical analysis

Data were expressed as a mean ± SEM. Concentration–effect curves were evaluated with analysis of variance (ANOVA) with a post hoc Newman–Keuls comparison. The effects of PG in the presence and absence of α-tocopherol or quercetin were measured with the same sample of PRP. Each point was obtained as a mean of measurements at least in four samples.

Student's *t*-test for paired data was used to determine statistical differences between the points for the same concentration of PG in the absence and presence of α-tocopherol or quercetin. Results were considered to be statistically significant at a probability level of $P < 0.05$.

Results

α-Tocopherol alone at a concentration of 200 μM added to PRP did not inhibit ADP-induced platelet aggregation but caused a parallel shift of effect–concentration curves for PGI_2 and PGE_1 to the left and decreased the corresponding values of IC_{50} 2.4 and 1.7 times (Figure 1A,C). At the same concentration, α-tocopherol potentiated the inhibitory effect of iloprost. However, the shift of the concentration–effect curve was not parallel (Figure 1B). The inhibition of platelet aggregation by 20% in the presence and absence of α-tocopherol was achieved with 0.02 and 0.6 nM of iloprost, respectively; difference in the concentrations of iloprost was 30 times. At the same time, it was necessary to add 1 nM of iloprost together with α-tocopherol and 3 nM of iloprost without α-tocopherol to inhibit platelet aggregation by 60%. In this case, the difference of the concentrations of iloprost in the presence and absence of α-tocopherol was only 3 times. A subthreshold concentration (2 μM) of quercetin caused about the same shift of concentration–effect curves of all the three PGs to the left (Figure 2). In the presence of quercetin, the values of IC_{50} decreased 1.8, 1.7 and 2.5 times for PGI_2, PGE_1, and iloprost, respectively.

Discussion

In several works, it was found that α-tocopherol and quercetin are able to inhibit platelet aggregation *in vitro*. The effective concentrations were found to vary from 50 μM[10] to 1 mM[2] for α-tocopherol and from 10 μM[5] to 2500 μM[11] for quercetin. The difference in concentrations depends on an agonist used to induce aggregation, the incubation time of α-tocopherol or quercetin and the method of platelet preparation. In our experimental conditions, α-tocopherol or quercetin, when used alone, inhibited platelet aggregation at concentration 1 mM and 20 μM, respectively (not shown). In combination with PGs, we used 5 and 10 times lower concentrations of α-tocopherol and quercetin, respectively. At these concentrations, neither of the compounds inhibited of platelet aggregation when used alone, but essentially potentiated the inhibition induced by each of three PGs. α-Tocopherol caused a parallel shift of effect–concentration curve for PGI_2 and PGE_1 to the left. However, the shift of effect–concentration curve for iloprost was much greater at lower concentrations of iloprost than at higher. Quercetin potentiated the effects of each of the three PGs at roughly equal measure.

A correlation was found between the consumption of vitamin E[6,12] or flavonoids[8] and a lower incidence of cardiovascular disease. Some of this beneficial effect was linked to the ability of α-tocopherol and flavonoids to inhibit platelet aggregation. At the same time, discrepancy between a low concentration of this compound in plasma and much higher one necessary to inhibit platelet aggregation in *in vitro* experiments casts doubt on the physiological relevance of *in vitro* effects. However, several *ex vivo*

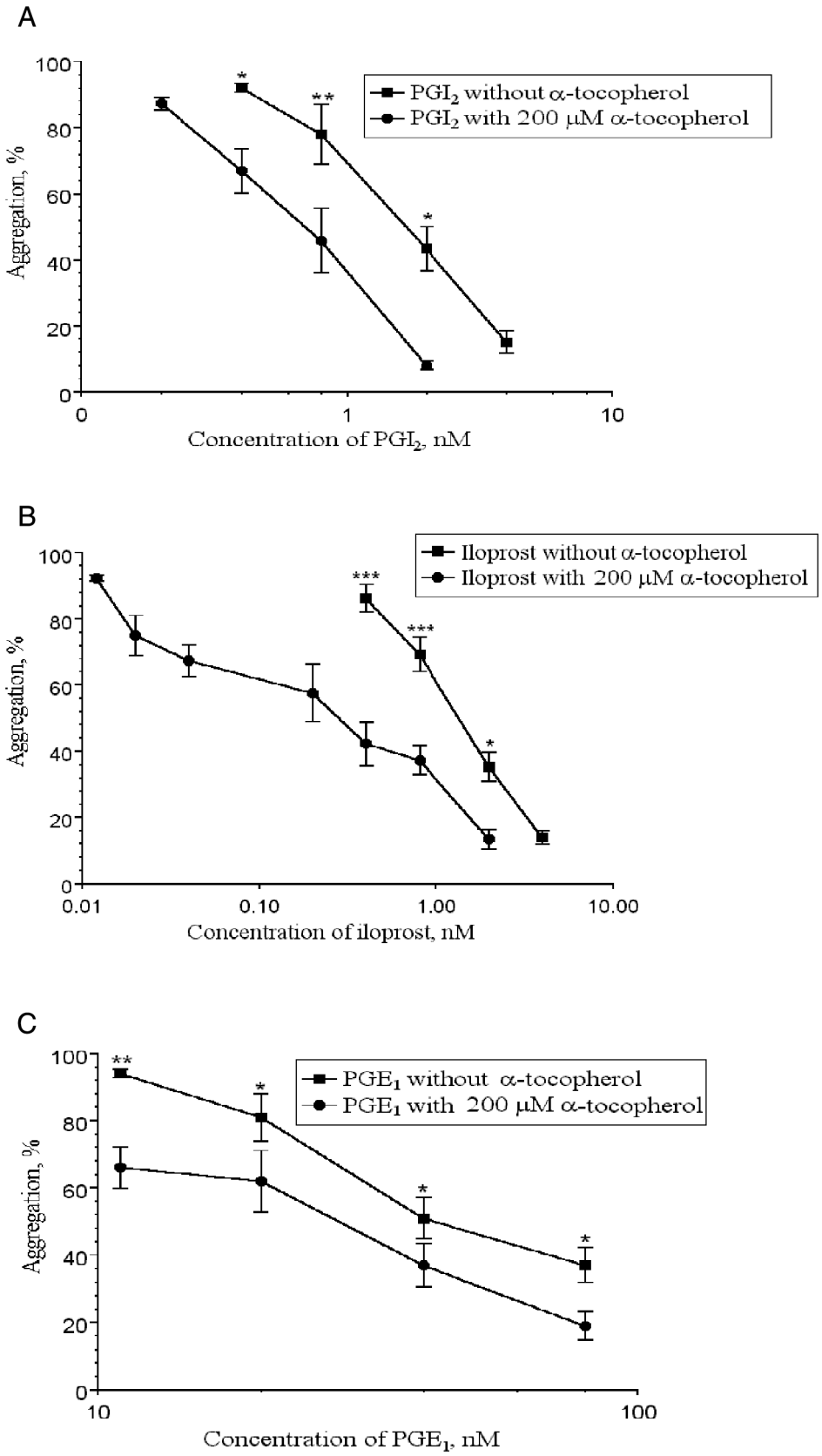

Figure 1. Inhibition of ADP-induced platelet aggregation by prostacyclin (PGI_2) (A), iloprost (B) and prostaglandin (PG) E_1 (C) in the absence and presence of α-tocopherol. Concentration–effect curves were evaluated with analysis of variance (ANOVA) with post hoc Newman–Keuls comparison, $P < 0.001$ for all curves. The comparison between the aggregation caused by the same concentrations of each PG in the absence and presence of α-tocopherol was performed by Student's *t*-test for paired data. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. *n* in A, B and C is 4, 10 and 5, respectively. ADP-induced aggregation is taken as 100%.

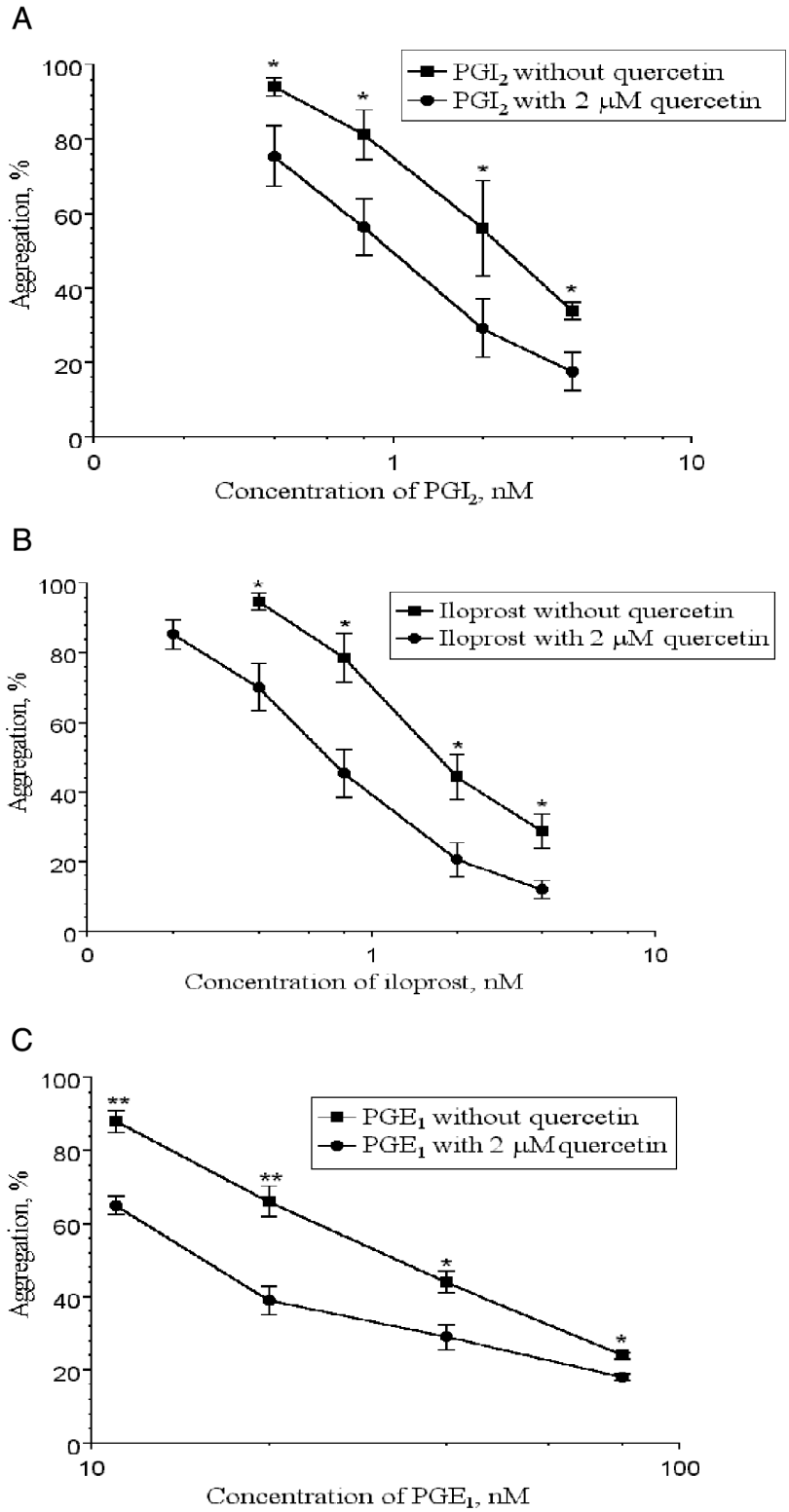

Figure 2. Inhibition of ADP-induced platelet aggregation by prostacyclin (PGI_2) (A), iloprost (B) and prostaglandin (PG) E_1 (C) in the absence and presence of quercetin. Concentration–effect curves were evaluated with analysis of variance (ANOVA) with post hoc Newman–Keuls comparison, $P < 0.001$ for all curves. The comparison between aggregation caused by the same concentrations of each PG in the absence and presence of quercetin was performed by Student's *t*-test for paired data. * $P < 0.05$; ** $P < 0.01$. *n* in A, B and C is 5, 8 and 4, respectively. ADP-induced aggregation is taken as 100%.

studies showed that the administration of vitamin E to humans did cause the inhibition of platelet function.[3,7] To explain the discrepancy, Freedman *et al.*[3] suggested that the effect of α-tocopherol depends on its levels in platelets rather than in plasma and supraphysiological concentrations are required in *in vitro* experiment to effectively load the platelets with α-tocopherol. Our results showed that α-tocopherol potentiated the effect of PGs at essentially lower concentration than that necessary to

inhibit platelet aggregation when used alone. One might speculate that the potentiation of endogenous PGI_2 might take place *in vivo* in blood vessels and this might, in part, explain the discrepancy between *in vitro* and *ex vivo* experiments with α-tocopherol.

After supplementation to humans of food rich in quercetin,[13] its level in plasma reached a value comparable with that used in our work to potentiate PG effects. However, Goldberg *et al.*[14] recently found that, after absorption in humans, a major part of quercetin forms glucuronide and sulfate conjugates and the free form of quercetin in serum ranged from 17.2 to 26.9% of total values. On the other hand, the antiplatelet effect of quercetin depends on incubation time with platelets.[5] This indicates that the antiplatelet effect of quercetin, as that of α-tocopherol, might depend on its level in platelets rather than in serum and after quercetin supplementation this level may be sufficient to potentiate effect of PGs.

The characteristic actions of PGs studied are mediated by specific cell-surface receptors, the activation of which leads to the elevation of intracellular cyclic adenosine monophosphate (cAMP) through Gs protein coupling to adenylate cyclase.[15] cAMP, in turn, activates cAMP-dependent protein kinase and this causes the inhibition of platelet aggregation.[16] α-Tocopherol is found to act inside the platelets through the inhibition of protein kinase C (PKC).[3] The latter is a key enzyme in signal transduction, the activation of which leads to platelet aggregation. PKC inhibition might decrease the threshold for platelet inhibition by PGs.

Quercetin interacts with many cell enzymes[17] and the mechanism of its inhibitory effect on platelet aggregation may be more complicated. Tzeng *et al.*[18] concluded from the result of their work that the major antiplatelet effect of flavonoids might be the inhibition of TxA_2 receptors together with the inhibition of TxA_2 formation in platelets. The latter may be caused by the inhibition of cyclooxygenase.[19] It was also suggested that the antiplatelet effect of quercetin may be caused by the inhibition of nucleotide phosphodiesterase leading to the increase of cAMP.[20] In addition, quercetin inhibits the activity of PKC and phosphatidylinositol 3-kinase.[21,22] The latter is important for the control of the reversible aggregation of platelets induced by a low concentration of an agonist.[23]

α-Tocopherol and quercetin are weak antiplatelet agents. However, they have mild if any side effects when administered at doses, which essentially exceed recommended daily intake. A long-term supplementation of vitamin E at a dose of 727 mg was found to be safe in healthy older adults.[24] Also, in most cases, extensive feeding studies of animals did not show any adverse effects of quercetin.[25] Quercetin is consumed as a supplement within a typical dose range recommended by manufacturers of between 500 and 1000 mg/day, which is 10 to 20 times higher than can be consumed in a vegetarian diet. Thus, these compounds can be used medicinally at high doses.

This study confirms and extends the previous evidence about the antiplatelet effect of α-tocopherol and quercetin and demonstrates that the subthreshold concentrations of α-tocopherol and quercetin potentiate the antiplatelet effects of PGI_2, PGE_1 and iloprost. Especially effective is the combination of α-tocopherol with the lower concentrations of iloprost. The combination of α-tocopherol or quercetin allows the use of PGs at lower concentrations to inhibit platelet aggregation and might decrease adverse side effects of the latter.

Acknowledgements

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ARTICLE IV

Kobzar G., Mardla V., Samel N. (2005) Effects of α-tocopherol, L-arginine, and quercetin on aggregation of human platelets. Nutr. Res., 25, 269-575.

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Effects of α-tocopherol, L-arginine, and quercetin on aggregation of human platelets

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Abstract

Some nutrients such as vitamin E, L-arginine, and flavonoids inhibit platelet aggregation; however, the effects of these compounds separately are weak. In food, these compounds may be presented all together. The aim of this work was to study the antiplatelet effect of vitamin E in combination with L-arginine or the flavonoid quercetin and also with prostacyclin, an endogenously formed antiplatelet agent of the human being. The inhibition of adenosine diphosphate-induced platelet aggregation was measured photometrically in human platelet-rich plasma. α-Tocopherol alone inhibited platelet aggregation at a concentration of 1 mmol/L and higher. The combination of 100 μmol/L of L-arginine with 200 μmol/L of α-tocopherol caused a significant decrease of adenosine diphosphate–induced platelet aggregation. At these concentrations, neither of the compounds were effective alone. In addition, quercetin, at a concentration of 2 μmol/L, was ineffective alone. However, in combination with 200 μmol/L of α-tocopherol, quercetin reduced platelet aggregation. Furthermore, α-tocopherol at a subthreshold concentration potentiated the antiplatelet effect of prostacyclin. Thus, α-tocopherol at a subthreshold concentration enhanced the inhibitory activity of other natural agents of platelet aggregation.

Keywords: α-Tocopherol; L-Arginine; Quercetin; Platelet aggregation; Prostacyclin

1. Introduction

The excessive activation of platelets, which leads to their aggregation, is, in part, responsible for thromboembolic diseases such as myocardial infarction, stroke, unstable

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angina, and peripheral artery disease [1]. The inhibition of platelet activation is an approach for treating these diseases. Platelet aggregation is prevented by several antiplatelet drugs, the best known of which is aspirin. However, all drugs, including aspirin, have adverse side effects.

Some nutrients also have antiplatelet actions. Several studies have shown that the supplementation of vitamin E is associated with a significant reduction in cardiovascular events [2]. It has also been found that vitamin E inhibits collagen-, phorbol myristate acetate-, arachidonic acid-, or adenosine diphosphate (ADP)-induced platelet aggregation [3-5]. However, in in vitro studies, the effective concentration of α-tocopherol was much higher than the general concentrations in blood plasma. This fact and the early studies with vitamin E supplementation, which failed to inhibit platelet function, led to the conclusion that the effect of the vitamin was not physiologically relevant [6]. More recent studies, however, showed that dietary intakes of vitamin E does inhibit platelet function [5,7].

In in vivo experiments, the inhibition of platelet aggregation was achieved with vitamin E supplementation during 2 to 8 weeks at comparatively high doses that exceeded the recommended 12- to 15-mg daily intake by at least 3-fold [5,7]. In addition to vitamin E, other compounds such as L-arginine and flavonoids in food may act as inhibitors of platelet aggregation. Dietary components may act in conjunction with endogenous inhibitors of platelet aggregation in blood vessels. The aim of this research was to study the antiplatelet effect of vitamin E in combination with L-arginine or the flavonoid quercetin and also with the natural antiplatelet agent prostacyclin (PGI_2), which is formed in blood vessels.

2. Methods and materials

Blood was taken from healthy volunteers (18 men and 9 women) aged 21 to 56 years (mean, 41 ± 2.7 years) and combined with anticoagulant (one-tenth volume of 3.8% trisodium citrate solution). Platelet-rich plasma (PRP) was obtained after the centrifugation of the citrated blood at 160*g* for 10 minutes at room temperature. Platelet aggregation was measured photometrically in a aggregometer (Chrono-log Co, Havertown, PA). An aggregometer cuvette containing 0.25 mL of an aliquot of PRP was maintained at 37°C and stirred with a Teflon-coated bar. Aggregation was induced by the addition of ADP, which was added to platelets at a concentration just sufficient to induce submaximum aggregation (95%-85% of maximum). This concentration varied from 2 to 10 μmol/L in different samples of PRP. Further, the amplitude of this submaximum aggregation was taken as 100% to calculate the inhibitory effect of the compounds studied. The inhibition of platelet aggregation was determined by the incubation of α-tocopherol for 5 min, L-arginine for 3 min, PGI_2 for 1 minute, and quercetin for 1 or 10 minutes before the addition of ADP in a cuvette with PRP.

Adenosine diphosphate, α-tocopherol, L-arginine, N^G-methyl-L-arginine (NMMA), and quercetin were obtained from Sigma Chemical Co (St Louis, Mo). Na salt of PGI_2 was obtained from Kemasol Ltd (Tallinn, Estonia). Adenosine diphosphate, L-arginine, and NMMA were dissolved in saline. The Na salt of PGI_2 was dissolved in glycine buffer (pH 10.4) before each experiment and kept on ice. In this condition, the PGI_2 was stable throughout an experiment. α-Tocopherol was dissolved in ethanol and quercetin in 1 mol/L NaOH solution, and both solutions were diluted in saline. Solvent controls were performed in

each experiment. The controls did not affect platelet aggregation at the final concentration of PRP tested.

All data are presented as mean ± SEM. Student *t* test for paired data was used when 2 measures had to be compared. Statistical significance was accepted at $P < .05$.

3. Results

α-Tocopherol at concentrations of 1 mmol/L and 2 mmol/L inhibited ADP-induced platelet aggregation by 23.8% ± 5.3% (n = 15) and 39.7% ± 5.8% (n = 14), respectively. L-arginine at concentrations up to 1 mmol/L added to a cuvette with platelets did not inhibit platelet aggregation. However, the combination of 100 μmol/L L-arginine with a subthreshold concentration of α-tocopherol (200 μmol/L) resulted in a decrease of ADP-induced platelet aggregation by 29.3% ± 4.5% (n = 6) (Fig. 1A). This inhibition could be abolished by NMMA, an inhibitor of nitric oxide synthase (NOS) (Fig. 1B).

Quercetin caused a dose-dependent inhibition of platelet aggregation. At a concentration of 20 μmol/L, quercetin decreased aggregation by 36.3% ± 7.4% compared with α-tocopherol. α-Tocopherol potentiated the effect of quercetin. The combined effect of α-tocopherol at a

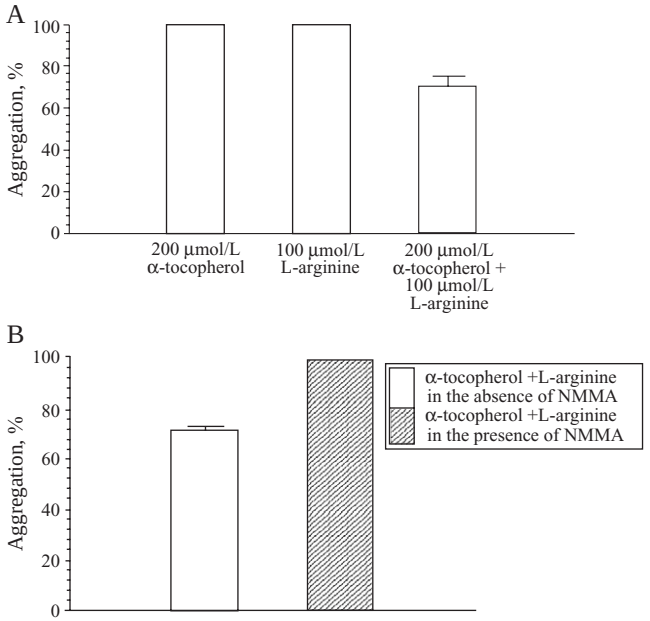

Fig. 1. A, Effect of α-tocopherol and L-arginine on ADP-induced platelet aggregation. α-Tocopherol (200 μmol/L) or L-arginine (100 μmol/L) each alone did not have effect on ADP-induced platelet aggregation. However, they inhibited aggregation significantly (*t* test for paired data; $P = .0013$; n = 6) when added together into PRP. B, Abolishing of the combined effect of α-tocopherol and L-arginine on platelet aggregation by NMMA. The inhibition of platelet aggregation was caused by the combination of 200 μmol/L α-tocopherol and 100 μmol/L L-arginine. NMMA, at a concentration of 0.4 μmol/L, abolishes the inhibitory effect (*t* test for paired data; $P = .0009$; n = 3). Adenosine diphosphate-induced aggregation was taken as 100%. Each sample was prepared of the blood of a different healthy volunteer.

subthreshold concentration (200 μmol/L) and 20 μmol/L of quercetin was significantly lower, namely, 51.7% $\pm$ 3.1% (t test for paired data; P = .0283; n = 4) (Fig. 2A). Even when both the compounds at subthreshold concentrations were added into PRP, they caused the inhibition of platelet aggregation. The combination of 200 μmol/L α-tocopherol with 2 μmol/L of quercetin also resulted in the inhibition of platelet aggregation by 33.8% $\pm$ 5.9% (n = 5) (Fig. 2B), compared with the treatment of α-tocopherol or quercetin above.

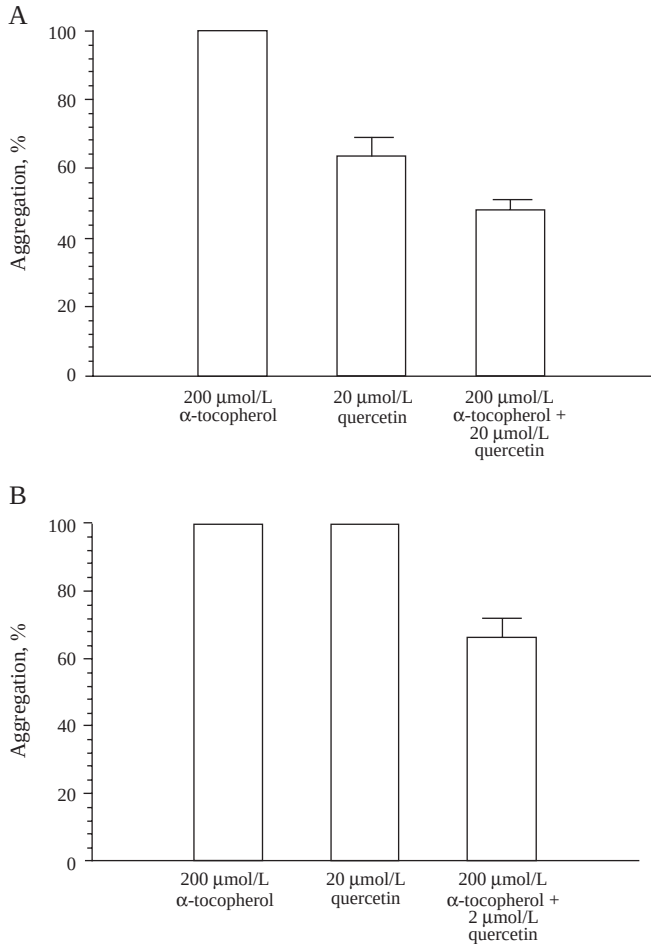

Fig. 2. A, Potentiation of the inhibitory effect of quercetin by α-tocopherol on ADP-induced platelet aggregation. α-Tocopherol alone, at a concentration of 200 μmol/L, did not inhibit platelet aggregation. Quercetin alone, at a concentration 20 μmol/L, significantly decreased ADP-induced aggregation (t test for paired data, P = .0089, n = 4). α-Tocopherol (200 μmol/L), when added together with quercetin (20 μmol/L), significantly increased the effect of the latter (t test for paired data; P = .0283; n = 4). B, Combined effect of α-tocopherol and quercetin on ADP-induced platelet aggregation. α-Tocopherol (200 μmol/L) and quercetin (2 μmol/L) each alone did not have effect on platelet aggregation. However, they decreased ADP-induced aggregation significantly when added together into platelet-rich plasma (t test for paired data; P = .0013; n = 5). Adenosine diphosphate-induced aggregation is taken as 100%. Each sample was prepared of the blood of a different healthy volunteer.

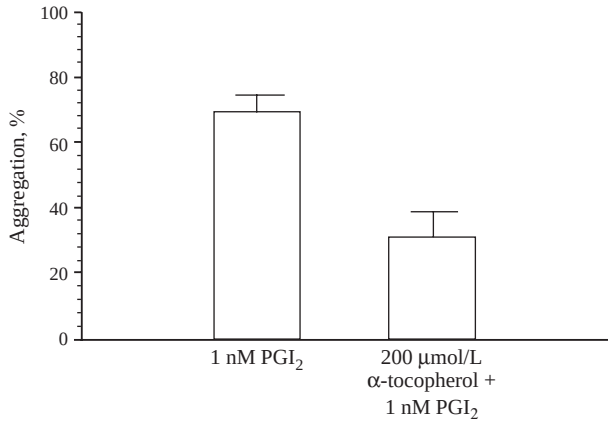

Fig. 3. Potentiation of antiaggregating effect of PGI_2 by α-tocopherol. α-Tocopherol, at a concentration 200 μmol/L, significantly increased the inhibitory effect of PGI_2 (t test for paired data; $P = .0025$; n = 4). Aggregation induced by ADP is taken as 100%. Each sample was prepared of the blood of a different healthy volunteer.

α-Tocopherol also potentiated the inhibition of platelet aggregation induced by PGI_2. At the subthreshold concentration (200 μmol/L), α-tocopherol caused a 2.5-fold increase of PGI_2 on preventing platelet aggregation (from 22.0% ± 9.2% to 54.2% ± 9.8%; Fig. 3) or a 50% decrease in platelet aggregation.

4. Discussion

This study showed that besides its direct antiplatelet activity, α-tocopherol, at a subthreshold concentration, potentiated the inhibitory effect of other antiplatelet compounds. Acting alone, α-tocopherol inhibited ADP-induced platelet aggregation at a concentration of 1 mmol/L, which is compatible with the results of other investigators [5]. However, in combination with 100 μmol/L of L-arginine or 2 μmol/L of quercetin, α-tocopherol was effective at an essentially lower concentration, namely, 200 μmol/L to reduce platelet aggregation. At this concentration, α-tocopherol potentiated the effect of PGI_2 as well. The latter compound, which is distinct from L-arginine and quercetin, is a natural endogenous inhibitor of platelet aggregation in blood vessels. L-arginine or quercetin, when added to platelets each alone at the aforementioned concentrations, was ineffective, but they inhibited platelet aggregation at a higher concentration. When 100 μmol/L of L-arginine was tested in combination with 2 μmol/L of quercetin, platelet aggregation was not inhibited (not shown). Thus, α-tocopherol and L-arginine or α-tocopherol and quercetin potentiated each other, whereas L-arginine and quercetin did not. Other investigators reported that another flavonoid catechin potentiated the antiplatelet effect of quercetin [8].

In our work, L-arginine alone, at a concentration of 1 mmol/L, did not inhibit platelet aggregation when incubated for 3 minutes. Anfossi et al [9] reported a similar result. These authors observed a modest inhibition of ADP-induced platelet aggregation only with the higher concentration of L-arginine or when it was incubated with platelets alone at a concentration of 1 mmol/L for a longer time of 20 minutes. In this study, NMMA abolished

the effect on platelets, suggesting that L-arginine acted through NOS. It was also found that vitamin E enhanced nitric oxide release and protein kinase C-dependent NOS phosphorylation in platelets [10]; however, the precise mechanism through which PKC inhibition modulates NOS activity in platelets is not known.

As distinct from L-arginine, quercetin alone inhibited platelet aggregation. Effective antiplatelet concentrations of quercetin were reported to vary from 2500 μmol/L [11] to 10 μmol/L [8]. This variation might be dependent on differences in experimental procedure. According to Pignatelli et al [8], the high potency of quercetin in their work was caused by the incubation of quercetin with platelets for 30 minutes before the addition of the agonist collagen. This incubation time was much longer than that used previously (about 10 minutes) on platelet function in studies with flavonoids. In a separate series of experiments, we found that 20 μmol/L of quercetin incubated with platelets for only 1 minute inhibited platelet aggregation by 24.6% $\pm$ 5.9%; however, with a longer incubation time (10 minutes), the effect was also significantly greater (43.3% $\pm$ 6.4%; t test for paired data; $P = .0382$; n = 7; data not shown). In contrast to Pignatelli et al [8], we used ADP instead of collagen to induce platelet aggregation and at a concentration that caused the submaximal aggregation of platelets.

In our work, the effective concentration of L-arginine (100 μmol/L) in combination with α-tocopherol was not higher than that found in plasma by Walker et al [12]. They reported that with supplementation of humans with 5 g of L-arginine 3 times a day for 2 weeks, the concentration in plasma increased from 80 to 117 μmol/L.

The concentration of α-tocopherol in human blood after oral supplementation during 2 weeks reached 75 [4] or 46.9 μmol/L [7]. These concentrations are also essentially lower than what was effective in previous in vitro experiments [4,5] and in our work both when α-tocopherol was used alone and in combination with L-arginine or quercetin. Nevertheless, it has been shown that, after oral supplementation of α-tocopherol in humans, platelet aggregation was significantly lower than in the control group not supplemented with α-tocopherol [5,7]. To explain the discrepancy between the in vitro and ex vivo experiments, it has been suggested that the effect of α-tocopherol depends on its level in platelets rather than in plasma; supraphysiological concentrations are required for in vitro experiments to effectively load the platelets with α-tocopherol [5]. Indeed, α-tocopherol levels in platelets achieved with in vitro loading were comparable with those measured after α-tocopherol supplementation [5]. This suggests that although the effective concentration of a platelet inhibitor for in vitro experiments may be essentially higher than its concentration in plasma, it does not exclude a possible physiological relevance in vivo.

Goldberg et al [13] found that the concentration of free quercetin in serum reached 25 μg/L (0.07 μmol/L) after its oral supplementation. This concentration is 28.5 times lower than what was effective in our experiments. However, a recent investigation has shown that after oral ingestion, most of quercetin in human serum and urine is in the form of glucuronide and sulfate conjugates [14]. Total quercetin level can increase to 248 μg/L in blood plasma of man following a meal containing onion, which is a rich source of quercetin [15]. At present, the effects of conjugated forms of quercetin on platelet aggregation are not known.

In conclusion, α-tocopherol, in combination with quercetin or L-arginine, causes an antiaggregating effect at much lower concentrations than each of these compounds does

separately. α-Tocopherol also potentiates the inhibition of platelets by PGI_2, an antiplatelet compound formed in blood vessels.

Acknowledgments

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ARTICLE V

Mardla V., Kobzar G., Rätsep I., Samel N. Effects of combining four antiplatelet vitamins on platelet aggregation. Manuscript.

Effects of combining four antiplatelet vitamins on platelet aggregation

Vilja Mardla, Gennadi Kobzar, Indrek Rätsep, Nigulas Samel

Some vitamins have been shown to inhibit platelet aggeregation. However, their effects are weak when they are used singly. The combination of these compounds together may give synergistic effects and permit to use them at lower concentrations. The effect of 200 μM α-tocopherol (α-T), 20 μM ascorbic acid (C), 0.2 μM pyridoxine (PN)(or 0.02 μM pyridoxal 5'-phosphate (PLP)) and 2 μM quercetin (Q) in pairwise combinations and three and four together were studied *in vitro* on ADP-induced platelet aggregation by the photometrical method. The compounds singly at these concentrations were not effective. However, all pairwise combinations of α-T, Q, C and PN at the same concentrations significantly inhibited platelet aggregation ($P<0.05$). The inhibition caused by the combination of the three compounds together was stronger than the pairwise combinations. The strongest effect was caused by the combination of all four compounds. Vitamin B_6 in the form of PLP was effective in combination with other two or three compounds at the concentration as low as 0.02 μM that corresponds to the basal level of that compound in blood. Thus, the combinations of α-T, C, PN (PLP) and Q inhibit platelet aggregation *in vitro* at much lower concentrations than each of these compounds does singly. Four compounds together caused higher effect than the combination of two or three of them.

Key words: α-tocopherol, ascorbic acid, platelets, pyridoxine, quercetin.

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Introduction

The multiple factors contribute to the development of coronary vascular disease (CVD). The final manifestation of the process, i.e. the acute coronary syndromes, is known to be thrombotic in nature. Platelets play a key role in the arterial thrombosis. They adhere at the site of vascular lesions and activated endothelium, release agents, which interact with receptors on the surrounding platelets, and cause the activation of signalling pathways in platelets leading to the functional expression of GPIIb-GPIIIa and the aggregation of platelets. Platelet activation is regulated by a number of endogenous and exogenous compounds promoting platelet activation or inhibition.[1] Antiplatelet therapy is widely used in the treatment of thrombosis. However, the drugs used in medicine have unwanted side effects and are not effective in all of patients.[2]

The consumption of fruits, vegetables, and other plant-based food rich in some vitamins[3,4,5,6] has a beneficial effect on cardiovascular disease (CVD). This is at least partly attributed to the effects of the compounds on the platelet function. α-Tocopherol (α-T),[7,8,9] pyridoxal 5'-phosphate (PLP), pyridoxine (PN),[10,11,12] quercetin (Q),[13, 14, 15] and ascorbic acid (C)[16] were shown to inhibit platelet aggregation. These food-derived compounds have no side effects. However, the antiplatelet effects of these compounds are weak when they are used singly. Effects from the combination of two agents may be synergistic, i.e. significantly greater than the sum of the effects of each agent administrated alone or potentiating, i.e. the increase of the effect of a compound by the concurrent action of another agent at a concentration that is not effective.

Thus, Pignatelli with co-workers[17] found that the combination of 5 μM Q and 25 μM catechin significantly inhibited collagen-induced platelet aggregation. However, neither of these flavonoids at such concentrations had any effect on the platelet function when used singly. α-T and Q at subthreshold concentrations were also shown to potentiate the antiplatelet effects of each other.[18]

Here we studied the *in vitro* effects of α-T, C, PN, and Q on platelet aggregation to find the most effective combinations. The compounds were used, in pairwise combinations and three or four together.

Materials and Methods

ADP, α-T, Q, PN, PLP and C were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). ADP, PN, and PLP were dissolved in saline. α-T was dissolved in ethanol and Q in 1 M NaOH solution. All the compounds were diluted in saline. Solvent controls were performed in each experiment. They did not affect platelet aggregation at final concentrations.

Patients suffering from CVD were included in the study. The study was approved by the local ethical committee. Venous blood was collected in tubs with 3.8% trisodium citrate solution. Platelet-rich plasma (PRP) was obtained after the centrifugation of the citrated blood at 160 x g for 10 min at room temperature. Platelets were counted in a Swelab cell counter and adjusted to a final concentration of 2.5 x 10^{11} cells/L. Platelet aggregation was measured by photometrical method in a Chrono-log aggregometer. An aggregometer cuvette containing 0.25 ml of an aliquot of PRP was maintained at 37°C and stirred with a teflon-coated bar. Aggregation was induced by adenosine diphosphate (ADP), which was added to platelets at a concentration just sufficient to induce submaximum aggregation (95- 85 % of the maximum). This concentration varied from 2 to 10 μM in different samples of PRP. The amplitude of this submaximum aggregation was taken as 100 % to calculate the inhibitory effect of the compounds studied. Vitamins were added into cuvette with PRP 1 min before ADP.

Results were expressed as the arithmetic mean ± SEM. Effects were evaluated with the analysis of variance (ANOVA) with post hoc Newman-Keuls comparison. The statistical significance of differences was determined using the *t*-test. $P < 0.05$ was considered statistically significant.

Results

The inhibition of ADP-induced platelet aggregation by the combinations of 200 μM α-T, 2 μM Q, 20 μM C, and 0.2 μM PN or 0.02 μM PLP was studied. The compounds singly at these concentrations were not effective. However, pairwise combinations of α-T, Q, C and PN at the same concentrations inhibited platelet aggregation (Fig. 1). The inhibitory effects of the combinations of Q with PN and C with PN were very weak, only 4.3 ± 1.9 % and 6.4 ± 2.6 %, respectively. α-T together with C inhibited platelet aggregation by 13.9 ± 4.3 %. The combinations of α-T with Q and α-T with PN were most effective inhibiting platelet aggregation by 16.1 ± 4.6 % and 16.2 ± 4.4 %, respectively.

The inhibitions caused by the combination of three compounds together were stronger than those caused by pairwise combinations (Fig. 2), although, the differences between the most effective pairwise combinations (α-T + Q and α-T + PN) and the least effective combination of three vitamins, α-T + C + Q , which inhibited the platelet aggregation by 21.1 ± 7.2 %, were not statistically significant ($P > 0.05$). The combination of α-T, C and PN was the strongest one of three vitamins inhibiting platelet aggregation by 38.6 ± 5.9 %. The combination of α-T + Q + PN inhibited the aggregation by 33.0 ± 5.0 %. The strongest effect was caused by the combination of four vitamins, which inhibited platelet aggregation by 51.1 ± 4.2 %.

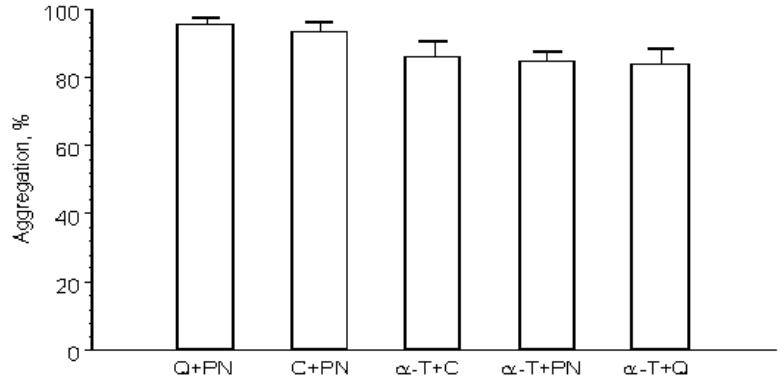

Fig. 1. Inhibition of ADP-induced platelet aggregation by pairwise combinations of vitamins. Data are mean ± SEM (n = 8) for ADP-induced platelet aggregation in the presence of vitamin combinations. None of the vitamins was effective alone. Effects were evaluated with analysis of variance (ANOVA) with *post hoc* Newman-Keuls comparison. The group means are significantly different ($P < 0.05$). ADP-induced aggregation is taken as 100 %. α-T - 200 μM α-tocopherol, Q - 2 μM quercetin, C- 20 μM ascorbic acid, PN - 0.2 μM pyridoxine.

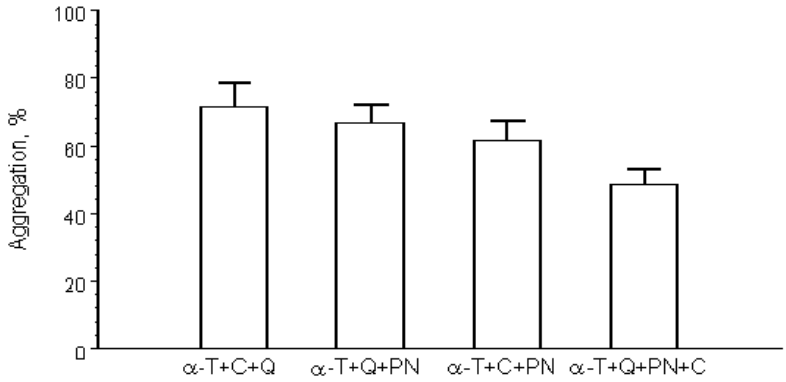

Fig. 2. Inhibition of ADP-induced platelet aggregation by α-tocopherol, quercetin, ascorbic acid, and pyridoxine in the combination of three or four together. Data are mean ± SEM (n = 8) for ADP-induced platelet aggregation in the presence of vitamin combinations. None of the vitamins was effective alone. Effects were evaluated with analysis of variance (ANOVA) with *post hoc* Newman-Keuls comparison. The group means are significantly different ($P < 0.05$). ADP-induced aggregation is taken as 100 %. α-T - 200 μM α-tocopherol, Q - 2 μM quercetin, C- 20 μM ascorbic acid, PN - 0.2 μM pyridoxine.

In the separate series of experiments, the principal form of vitamin B_6 in human blood, PLP[19] was used instead of PN. PLP was taken at 0.02 μM, i.e. ten times lower concentration than PN. The results are shown in Fig. 3. The combinations of α-T + Q + PLP, α-T + C + PLP, and α-T + Q + PLP + C inhibited platelet aggregation by 18.2 ± 2.8 %, 25.0 ± 4.9 % and 42.9 ± 2.5 %, respectively. As in the previous series of experiments, the combination of four compounds was the strongest.

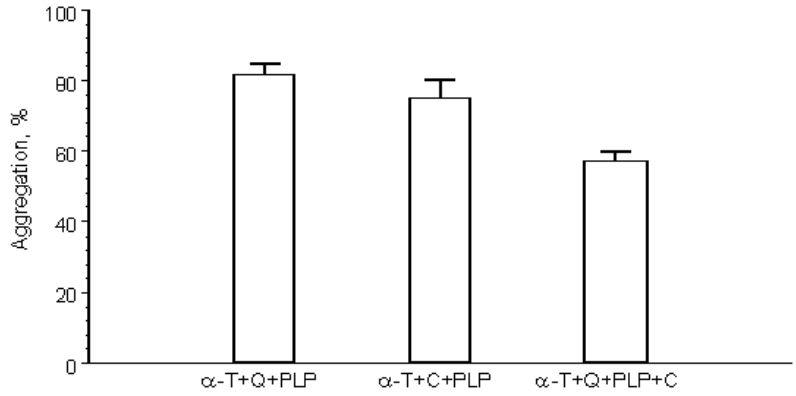

Fig. 3. Inhibition of ADP-induced platelet aggregation by α-tocopherol, quercetin, ascorbic acid, and pyridoxal 5'-phosphate in the combination of three or four together. Data are mean ± SEM (n =8) for ADP-induced platelet aggregation in the presence of vitamin combinations. None of the vitamins was effective alone. Effects were evaluated with analysis of variance (ANOVA) with *post hoc* Newman-Keuls comparison. The group means are significantly different ($P < 0.001$). ADP-induced aggregation is taken as 100 %. α-T - 200 μM α-tocopherol, Q - 2 μM quercetin, C - 20 μM ascorbic acid, PLP- 0.02 μM pyridoxal 5'-phosphate.

Discussion

In this work, α-T, Q, C, PN or PLP at used concentrations were not effective when used singly. However, in combinations, they inhibited ADP-induced aggregation. Thus, each compound potentiated others. Three compounds together caused stronger effect than the combination of two, and the combination of four was even more effective. The compounds synergistically increased the inhibition of platelet aggregation.

It should be noted that the concentration of PLP was especially low in this work. In combination with other vitamins, it was effective at a concentration of 0.02 μM. This is four orders of magnitude as low as that inhibiting platelet aggregation when PLP was

used singly[20] and is equal to the basal concentration of PLP detected in blood before the administration of vitamin B_6.[21]

Besides *in vitro* effects, the administration of α-T to humans causes the inhibition of platelet function.[7,8,22,23,24] However, in some cases a dietary supplementation with α-T was not effective.[25,26] Liu and co-workers[24] suggested that the absence of tocopherols other than α-T in the preparation used in the clinical studies might account for the conflicting results, They found that the supplementation of humans with mixed tocopherols might be more effective.

The concentration of α-T in human blood after oral supplementation during two weeks reached 75 μM[8] or 46.9 μM[23]. These concentrations are lower than in our work and *in vitro* experiments of other authors.[7,8] Nevertheless, it has been shown that after oral supplementation of humans with α-T, platelet aggregation was significantly lower than in the control group.[7,23] It has been suggested that the effect of α-T depends on its levels in platelets rather than on its plasma concentration.[7] Indeed, α-T levels in platelets achieved with *in vitro* loading were comparative with those measured after α-T supplementation.[7] Thus, although effective concentration of a platelet inhibitor *in vitro* may be essentially higher than its concentration in plasma after supplementation, this does not exclude its physiological relevance.

In blood, Q is mainly in conjugated form. Thus, Goldberg and co-workers[27] found that after absorption in humans, a major part of Q forms glucuronide and sulfate conjugates and the free form of Q in serum ranged from 17.2 to 26.9% of total values. However, the supplementation of conjugated forms of Q to humans is also effective. The ingestion of onion soup high in Q[28] and 150-mg or 300-mg doses of Q-4'-O-β-D-glucoside[29] has been found to inhibit platelet aggregation in humans.

It was found that the oral supplementation of PN to volunteers significantly inhibited ADP- or epinephrine-induced platelet aggregation.[30,31] The oral administration of vitamin C has also been shown to reduce platelet aggregation in humans.[32,33] The concentrations of PN and C in our work were lower than their physiological values in plasma.[21,34]

In different cells, the eight natural vitamin E analogues were shown to modulate the activity of several enzymes involved in signal transduction, such as protein kinase C, protein kinase B, protein tyrosine kinases, 5-, 12-, and 15-lipoxygenases, cyclooxygenase-2, phospholipase A2, protein phosphatase 2A, protein tyrosine phosphatase, and diacylglycerol kinase.[35]

Only some of these effects were found on platelets. The inhibition of PKC in platelets is one of them[7] (Freedman et al. 1996). Besides, vitamin E may inhibit collagen-induced platelet activation by blunting hydrogen peroxide formation[8] and increasing platelet-derived NO.[36] *In vivo*, a major mechanism underlying the effect of tocopherols on platelet aggregation may be the increase of endothelium-derived NO.[24,37]

The major antiplatelet effect of Q might be the inhibition of thromboxane A_2 (TxA_2) receptors together with the inhibition of TxA_2 formation in platelets.[13,15] The latter may be caused by the inhibition of cyclooxygenase (COX).[38,39] Although Q does not cause significant changes in the basal level of platelet cyclic adenosine monophosphate (cAMP), it modifies prostacyclin (PGI_2) stimulated increase in platelet cAMP.[38]

Q inhibits the intracellular mobilization of Ca^{2+} as well as the collagen-stimulated platelet protein tyrosine phosphorylation of the Fc receptor γ-chain, the tyrosine kinas Syk, the adaptor protein LAT, and phospholipase Cγ2.[40] In addition, it also suppresses the activity of the kinases Fyn, Lyn and phosphoinositide 3-kinase.[40]

The inhibitory effect of C may be caused by the reduction of reactive oxygen species,[41] the inhibition of thromboxane B_2 formation,[42] or the stimulation of prostaglandin E_1 production.[43] An *in vitro* study demonstrated that C dose-dependently inhibited platelet CD40L expression caused by collagen.[44] At least partly, the antiplatelet effect of C seems to be indirect in whole blood. The addition of ascorbate to the polymorphonuclear leukocyte (PMN) suspension augmented NO release, which then activated the guanylyl cyclase and elevated the platelet guanosine monophosphate (cGMP) in the platelets preventing their activation.[45]

The occupancy of GPIIb/IIIa has been suggested to play a major role in antiplatelet effects of B_6 vitamers.[12] It has also been found that PLP potentiates the cAMP accumulation induced by prostaglandin E_1 (PGE_1) although without PGE_1 it did not increase cAMP concentration in platelets.[10]

Some other antiplatelet compounds of food have also been found to act synergistically on platelet aggregation. The mixture of anthocyanins delphinidin-3-rutinoside, cyanidin-3-rutinoside, cyanidin-3-glucoside, and malvidin-3-glucoside and four phenolic acids were effective at ten times lower concentrations than each of them when used singly.[46] A combination of α-, δ-, γ-tocopherols in ratio 1:2:5 was shown to inhibit synergistically platelet aggregation *in vitro*[9]. Moreover, after supplementation to humans, the same mixed tocopherol preparation inhibited ADP–induced platelet aggregation.[24] α-T and Q were also shown to potentiate each other[18] and prostaglandins PGI_2 and PGE_1,[47] antiaggregating compounds that can be formed endogenously in blood vessels. In addition, Q may increase the effect of α-T in organism by elevating the amount of α-T in the plasma and the liver due to the inhibition of the metabolism of vitamin E.[48] PLP was also shown to increase the PGE_1-induced inhibition of platelet aggregation[10].

Besides vitamins, some antiplatelet drugs have also synergistic effect. The combination of them is successfully used in medicine. Thus, the combination of aspirin with dipyridamol[49] and aspirin with clopidogrel[50] was found to give a better result than either of the drugs alone. It is necessary to note that the combination of drugs may inhibit the formation of not only the aggregates of platelets but also of the aggregates of platelets with other cells. This effect was found for the combination of AR-C69931,

an analogue of clopidogrel directly acting on P2T receptors, with dipyridamole and aspirin, which significantly inhibited platelet-leukocyte conjugate formation besides the inhibition of platelet aggregation.[51]

In conclusion, the combination of α-T, C, PN (or PLP) and Q inhibits platelet aggregation *in vitro* at much lower concentrations than each of these compounds does singly. Four compounds together caused a higher effect than the combination of two or three of them**.** It may be suggested that a diet with an appropriate combination of vitamins or their supplementation could have an antithrombotic effect.

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