UNIVERSITA' DEGLI STUDI DEL PIEMONTE ORIENTALE

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FACOLTA' DI MEDICINA E CHIRURGIA

Tesi di Dottorato di Ricerca in MEDICINA MOLECOLARE

XXIII ciclo – 2007/2010

ROLE OF ESTROGENS AND PHYTOESTROGENS ON MEGAKARYOCYTE DIFFERENTIATION AND PLATELET FUNCTION

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SUMMARY

During my PhD I focused my attention on the role of estrogens and estrogen-like molecules in megakaryocyte differentiation and platelet aggregation.

First of all I evaluated the expression of classical estrogen receptors and GPER during megakaryocytic differentiation.

Megakaryocytes are myeloid cells whose primary function is to produce and release platelets into the bloodstream, through long cytoplasmatic processes called proplatelets. Growing evidence suggest that estrogens and their classical receptors ERα and ERβ may play a crucial role during megakaryopoiesis. However, the potential role of GPER, a novel estrogen receptor, ERα and ERβ has not yet been investigated. In this context, I evaluated the expression of classical estrogen receptors and GPER in human CD34+ hematopoietic stem cells and how their expression was modulated during megakaryocyte differentiation. My findings demonstrated that all estrogens receptors were expressed in CD34+ cells and that their expression decreased dramatically during *in vitro* differantiation. Moreover, in mature CD61+ megakaryocytes, GPER and ERα expression was no more detectable by Real Time PCR. Taken together, these findings of a rapid decrease of ERα, ERβ1, ERβ4 and GPER expression during the first stages of megakaryocytic differentiation maylead to conclude that the estrogen receptors could be not crucial in megakaryocyte maturation and proplatelet formation. However, we cannot firmly exclude that these receptors can eventually play a role in the first stages of megakaryocytic differentiation. Moreover they could play some role in the maintenance of hematopoietic stem cells undifferentiation and in preventing megakaryocytic differentiation.

Subsequently I analyzed the effects of 8-prenylnaringenin, one of the most potent phytoestrogens, on platelet activation and aggregation.

Platelets are small anucleated cell fragments which play a pivotal role in haemostasis, thrombosis, and inflammation. The modulation of platelet activity using specific pharmacological agents has proven to be a successful strategy for the prevention of thrombosis. Recently, a number of dietary sources have been shown to inhibit platelet function, including phytoestrogens and flavonoids. 8-prenylnaringenin (8-PN) is one of the most potent phytoestrogens in hops that has been used for centuries as a preservative and flavouring agent in beer. Human exposure to 8-PN occurs not only through the consumption of beer, but also via an increasing number of dietary supplements containing hop extracts. In order to evaluate the possible role of 8-PN in modulating platelet function, herein I studied the effects of this potent phytoestrogen on platelet activation and aggregation.

My data demonstrated for the first time that 8-PN exerts antiaggregatory and antiadhesive effects on human platelets, independently of estrogen receptors. To investigate the mechanisms by which 8-PN inhibited platelet activation and aggregation, I analyzed the phosphorylation state of VASP, that partially correlates with platelet inhibition. Platelet treatment with 8-PN caused a sustained phosphorylation of VASP in both Ser239 and Ser157 and increased cAMP and cGMP levels. However the use of sGC and NOS inhibitors was not able to block the effects of 8-PN on platelet aggregation. These data suggest that the inhibition of platelet aggregation mediated by 8-PN does not rely on the activation of the platelet NO/cGMP/PKG pathway. Therefore I focused my attention on protein phosphorylation. My results showed that 8-PN treatment did not have any effect on the basal state of protein phosphorylation. Nevertheless 8-PN inhibited the collagen-induced phosphorylation in tyrosine and the activation of several kinases: Pyk2, Akt, and Erk 1/2.

In conclusion, taken together these data demonstrate that 8-PN exerts antiaggregatory and antiadhesive effects on human platelets, independently of estrogen receptors. However the molecular mechanisms involved are not completely characterized.

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ANALYSIS OF THE EXPRESSION OF CLASSICAL ESTROGEN RECEPTORS AND GPER DURING MEGAKARYOCYTE DIFFERENTIATION

INTRODUCTION

MEGAKARYOCYTES

Megakaryocytes (MKs) are highly specialized precursor cells that live in the bone marrow and differentiate to produce blood platelets (*Radley & Scurfield*, 1980). During differentiation, megakaryocytes enlarge and become polyploid throught repeated cycles of DNA replication without cell division, called endomitosis (*Ravid et al*, 2002). This DNA amplification is ultimately used to direct protein and lipid synthesis that increases the cell size and fills it with platelet-specific granules, cytoskeletal proteins, and an extensive internal membranous labyrinth, called the demarcation membrane system, that supplies membrane during proplatelet formation (*Zucker-Franklin*, 1970). The maturation culminates with the assembly of long cytoplasmic protrusions called proplatelets that release platelets into circulation. Mature MKs move from the osteoblastic niche to the bone marrow venous sinusoids where nascent platelets are released from proplatelet ends (*Avecilla et al*, 2004).

Megakaryocytopoiesis

According to the monophyletic theory of hematopoiesis, a pluripotent stem cell CD34+ multiplies to produce more pluripotent stem cells, thus ensuring the steady and lasting supply of stem cells. CD34+ hematopoietic stem cells are the 1-3% of bone marrow mononuclear cells, the 0.1-0.2% of peripheral blood cells and the 0.8-1.2% of cord blood cells. Some of the pluripotent stem cells differentiate into precursor cells that are at least partially committed to become one type of mature blood cells. Indeed the hematopoietic stem cell can differentiate into lymphoid progenitors, that can generate lymphocytes and natural killers, or myeloid progenitors, that can generate granulocytes, monocytes, erythrocytes and megakaryocytes (*Ogawa*, 1993).

During maturation, megakaryocyte precursors undergo endomitosis and become polyploid (**Fig.1**); in particular human megakaryocytes may reach a DNA content of at least 16N (*Therman et al,* 1983). This presumably facilitates the increase in cell mass required to assemble hundreds of individual platelets. The endomitotic process results in prematurely terminated mitosis in which the nuclear envelope breaks down with each cycle and cells complete anaphase A (*Vitrat et al*, 1998). However, spindle poles fail to move outward during anaphase B, telophase and cytokinesis are aborted, and the nuclear envelope reassembles around the entire set of sister chromatids, forming a single enlarged and multilobed nucleus. Cytoplasmic maturation begins during endomitosis and accelerates significantly after all DNA synthesis has ceased (*Paulus*, 1970). Cytoplasmatic maturation results in the accumulation of distinctive components: platelet-specific proteins, dense granules, α-granules, lysosomes and the demarcation membrane system (*Yamada*, 1957). The biogenesis of secretory granules is a cardinal feature of thrombopoiesis. They originate from the Golgi complex, are present in maturing MKs from early stages (*Youssefian & Cramer*, 2000), and acquire their contents through a combination of endogenous synthesis and uptake of plasma components by receptor mediated endocytosis and pinocytosis (*Handagama et al*, 1987). The demarcation membrane system is derived from the megakaryocyte plasma membrane, that builds and elaborates a network of membrane channels composed of flattened cisternae and tubules that serve as a membrane reservoir for platelet formation and retain contact with the cell exterior (*Shaklai & Tavassoli*, 1978).

Proplatelet formation and platelet release

Proplatelet are pseudopodial extensions that have the appearance of beads linked by thin cytoplasmic bridges to the megakaryocyte body and constitute the essential intermediate structures in platelet release. The process of forming and elongating proplatelets begins with the erosion of

one pole of the MK (**Fig. 1**) to generate large pseudopodial-like structures that elongate, thin, and branch to yield slender tubular tree-like structures (*Italiano et al*, 1999).

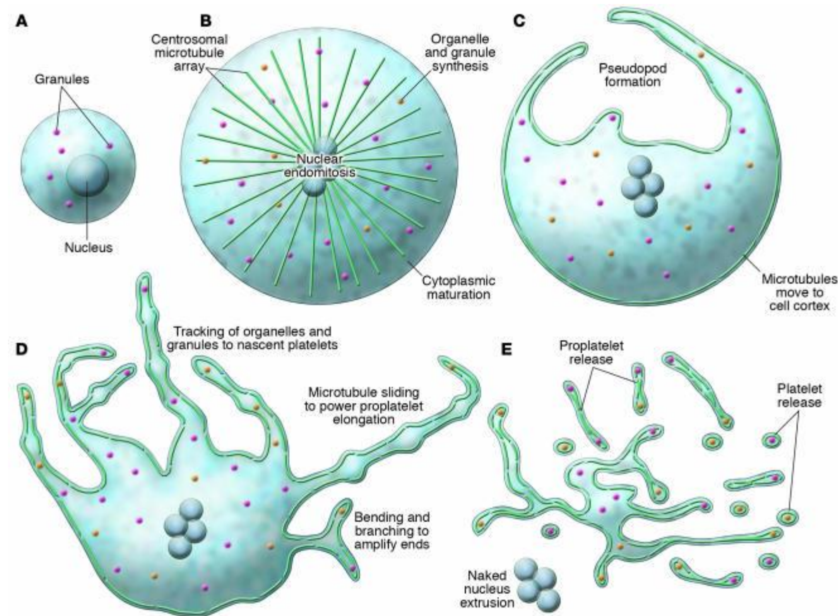

Fig. 1: *Mechanism of megakaryopoiesis and piastrinogenesis* (*Patel et al*, 2005)

This process is characterized by a repetitive dynamic bending and branching that bifurcates the shaft multiple times and thereby increases the number of free proplatelet ends. In the proplatelet tips, a single microtubule, derived from the microtubule bundles of the proplatelet shaft, rolls up into a circumferential coil that defines the territory of an individual platelet. Once the coil has been established, the nascent platelet fills with its content of granules and organelles. The filling of proplatelets with platelet-specific granules begins early during the proplatelet elaboration process. Once delivered into the proplatelets, granules track back and forth slowly over microtubules in the shafts (*Richardson et al*, 2005) and when they reach the ends of proplatelets are trapped into the nascent platelet buds. Once the platelet has been filled with its content of intracellular materials, a single microtubule is rolled into a coil, and the platelet is released.

The regulation of megakaryocytopoiesis

The megakaryocytopoiesis occurs into the bone marrow microenvironment where chemotactic factors, cytokines and adhesive interactions play an essential role (*Avecilla et al*, 2004).

Among cytokines, thrombopoietin (TPO), which is produced in the liver and marrow stroma, is the main regulator of megakaryocyte differentiation (*Kaushansky*, 1995). The word “thrombopoietin” was used for the first time in 1958 to describe a humoral substance able to induce an increase in megakaryocyte number, size and ploidy (*Kelemen*, 1970). The thrombopoietin is a glycoprotein of 332 aminoacids that sustains hematopoietic stem cell expansion and survival (*Kaushansky*, 2005). Moreover TPO promotes megakaryocytes proliferation and differentiation. When the thrombopoietin interacts with its receptor c-Mpl, causes the activation of JAK2 and the subsequent phosphorylation events involved in cell survival and proliferation.

Another important cytokine is the stromal cell-derived factor 1 (SDF-1), a CXC chemokine whose main receptor is CXCR4. It is synthesized essentially by stromal cells located in the marrow, endothelial, dendritic cells, and also megakaryocytes (*Massberg et al*, 2006). SDF-1 is the major chemokine involved in the homing of hematopoietic cells and, even more, in their egress from the marrow. CXCR4 expression increases with MK maturation (*Hamada et al,* 1998). It seems that the major role of CXCR4/SDF-1 lies in the MKs migration from the osteoblastic to the endothelial niche during maturation. This could provide one mechanism for retaining in the marrow immature MKs and later permitting them to migrate toward endothelial cells and liberate platelets in the circulation. In addition increasing evidences suggest that proplatelet formation is tightly regulated by the stroma and the extracellular matrix. In the marrow, collagen inhibits proplatelet formation through RhoA activation (*Sabri et al*, 2004). When a MK begins to migrate towards the endothelium, it interacts with other compounds of the extracellular matrix such as fibrinogen or

vonWillebrand factor, which may increase proplatelet formation (*Larson & Watson*, 2006). Regulation of these late stages of differentiation is mediated by integrins α2β1, αIIbβ3 and GPIb.

In addition to chemotactic factors, cytokines and adhesive interactions, many transcription factors are also involved in the regulation of megakaryocytopoiesis (*Larson & Watson*, 2006). Among these, GATA-1 plays a major role in MK development by functioning both as an activator and repressor of transcription. Indeed the main MK specific genes such as GPIIb, GPIb, GPIX or GPV are regulated by GATA-1 (*Martin et al*, 1993). In addition to regulating MK maturation by activation of platelet-specific gene transcription, GATA-1 regulates polyploidization through its downstream effector STAT1 (*Huang et al*, 2007). Three other transcription factors, RUNX1/AML-1, p45NF-E2, and MKL1, are implicated in terminal MK differentiation. RUNX-1/AML-1 is a DNA-binding subunit of the core binding factor (CBF) transcription complex. It may cooperate with GATA-1, but plays also a role independently of GATA-1 (*Waltzer et al*, 2003). One of the main target genes of RUNX1 is c-MPL, that codes for the thrombopoietin receptor (*Onodera et al*, 2000). p45NF-E2 is associated with proteins of the MAF family to form an active transcription factor complex (*Heller et al*, 2005). Three target genes of p45NF-E2, β1-tubulin, 3β-HSD and Rab27b, are involved in proplatelet formation (*Nagata et al*, 2003). MKL1 also plays a role in regulating MK migration and acts as a link between transcription and extracellular signals. MKL1 nuclear localization and function are related to actin polymerization by the small G proteins of the Rho family (*Cheng et al*, 2009).

ESTROGENS

Estrogen receptors

The main estrogenic hormone, 17β-estradiol (17β-E2), plays important regulatory roles in a wide variety of biological processes including reproduction, differentiation, cell proliferation, apoptosis, inflammation, cardiovascular functions, bone integrity, cellular homeostasis, behavior and brain functions (*Simpson et al*, 2005). Traditionally estrogens, like other steroid hormones, act as nuclear transcription factors, by modulating target genes through complex interactions with coactivator or corepressor proteins, histone-modifying enzymes, and proteins comprising basal transcriptional machinery. These actions generally involve the entry of a free steroid into a target cell by passive diffusion through the plasma membrane, subsequently the steroid can bind its receptor with high affinity (*Manavathi & Kumar*, 2006). Nuclear actions of 17β-E2 are mediated by two main nuclear estrogen receptor subtypes, ERα and ERβ, which are members of a superfamily of nuclear receptors that function as ligand- or hormone-dependent transcription factors. They consist of a C-terminal steroid ligand-binding domain, a centrally located DNA binding domain, and an N-terminal domain with less well characterized function. Additionally, ER contains two autonomous transcriptional activation domains: the AF-1 domain located at the N-terminus, and AF-2 located within the ligand-binding domain. Steroid receptors are latent transcriptional activators that require ligand binding for activation. The binding of estrogen to its receptor triggers specific conformational changes that result in receptor dissociation from a protein chaperone complex, dimerization of steroid receptors, and binding of the receptor dimer to specific hormone responsive elements located at the 5' regulatory region of primary steroid responsive target genes. Through interaction with AF-1 or AF-2, the activated DNA-bound receptor mediates assembly of a productive transcription complex at the promoter (*McKenna & O'Malley*, 2002). The two estrogen receptor subtypes have a well conserved DNA binding domain and ligand-binding domain, whereas there is considerable

divergence between receptor subtypes at the N-terminus. Moreover, they have distinct expression patterns in tissues: ERα is primarily involved in reproductive functions and it is abundant in mammary glands, uterus and vagina, while ERβ is almost ubiquitous in the central nervous system, in the cardiovascular system, in the immunity system, in the urogenital apparatus, in the gastroenteric apparatus, in the kidney and the lung (*Zhang & Trudeau*, 2006). Such regulatory interactions result in the synthesis of specific mRNAs and, in turn, the synthesis of proteins responsible for the hormone cellular effects. In general, these actions induce biological responses that develop in 30 to 60 minutes and are known as genomic or classical actions of steroid hormones (*Manavathi & Kumar*, 2006).

It has been reported that different ERβ isoforms exist. ERβ1 is the well characterized and the only full-function isoform. The other ERβ isoforms differ only for the last exon, which encodes an isoform specific C-terminus tail of reduced length. On the basis of these analysis, ERβ2, β4 and, β5 should have an AF-2 domain different from that of ERβ1 (*Moore et al*, 1998). Moreover it has been shown that ERβ1 prefers to heterodimerize with ERβ isoforms, particularly ERβ4 and β5 and that ERβ1 serves as the ''obligatory partner'' of a functional dimeric complex, whereas ERβ2, β4, or β5 act as the ''variable dimer partners'' and serve as enhancers (*Leung et al*, 2006).

Recently, it has been reported that, in addition to these classical actions, estrogens can also induce rapid, non-genomic effects in different cell types (**Fig. 2**).

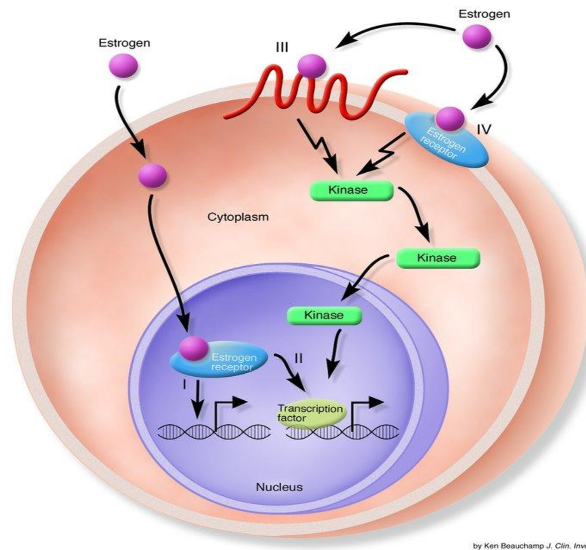

Fig. 2: *Genomic and non-genomic action of classical estrogen receptors* (*Lorenzo*, 2003)

These effects cannot be explained by their nuclear action alone and thus suggest the existence of alternative mechanisms involving short-term rapid cytoplasmic signaling. These responses, possibly explained by the existence of signals generated by cytoplasmic and cell surface steroid receptors, are generally known as non-classical or non-genomic signals. Non-genomic signaling is characterized by responses very rapid (taking only few seconds or minutes), insensitive to inhibitors of mRNA and protein synthesis, found in highly specialized cells that do not accomplish mRNA and protein synthesis and in cells lacking steroid nuclear receptors, and activated by steroids coupled to high-molecular-weight substances, such as estrogen-bovine serum albumin conjugates, that do not pass across the plasma membrane. Non-genomic signaling of estrogen involves a series of events that include mobilization of second messengers, interaction with membrane receptors, such as insulin like growth factor-1-receptor and epidermal growth factor receptor, and stimulation of effector molecules, such as the tyrosine kinase Src, PI3-K, Akt and MAPKs in various cell types (*Manavathi & Kumar*, 2006). The rapid actions of estrogen can be divided into two major categories: classical receptor mediated responses, which are mediated by ERs, and non-classical, non-ERs mediated responses, which are mediated by proteins other than ERs, such as GPER. The

results of several biochemical and microscopic analyses have suggested the existence of different pools of ER in the cellular environment, including the plasma membrane, the mitochondria, and the endoplasmic reticulum (*Govind & Thampan*, 2003). In general, about 80% of ERs localizes in the nucleus in absence of estrogen. Since many estrogen-stimulated nongenomic pathways are thought to be initiated at the plasma membrane, researchers have made several attempts to identify a membrane-associated ER. The membrane-binding activity of estrogen was first identified in 1977, but the precise nature of the receptors at those sites are still unknown, and direct evidence supporting their existence has not yet been found. Plasma membrane ERs appear to be identical to nuclear ERs, according to results of immunochemistry analysis with a panel of antibodies against multiple epitopes of nuclear ERs (*Ropero et al*, 2002; *Watson et al*, 2002). Biochemical and microscopic evidence of a membrane ERα has also proven to be controversial because classical ERs contain no hydrophobic transmembrane stretches, have no intrinsic kinase or phosphatase activity, and lack myristoylation sites to be recruited to the membrane. Nevertheless, emerging data have revealed several post-translational ERα modifications that in principle could contribute to membrane localization of the receptor *(Razandi et al*, 2003).

Estrogens, megakaryocytes, and platelets

An increasing number of evidence suggest that estrogens may play an important role in the regulation of megakaryopoiesis and platelet function and that megakaryocytes and platelets express classical estrogen receptors (*Tarantino et al*, 1994; *Khetawat et al*, 2000).

Indeed it has been reported that high estrogen concentrations promote an increase in megakaryocyte number in murine models (*Perry et al*, 2000). The same results have been observed in the bone marrow of postmenopausal women who had received previous estrogen treatment (*Bord et al*, 2000). Moreover *in vitro* studies have demonstrated that megakaryocytes differentiated from murine embrionic stem cells are able to synthesize estradiol, important in the stimulation of proplatelet

formation (*Nagata et al*, 2003). Bord and colleagues have also reported that classical estrogen receptors espression is strongly modulated during megakaryocytic differentiation in presence of estrogen stimulation (*Bord et al*, 2004). In addition it has been shown that estrogen treatment causes an early increase in ERα mRNA and protein during megakaryocyte differentiation. However, in mature megakaryocytes ERβ, but not ERα transcript, was detected and demonstrated to show a predominantly cytoplasmic location (*Khetawat et al*, 2000). These results were confirmed also in platelets, where ERβ is the most abundant ER and it is almost 3.7 kDa larger than ERβ protein from normal breast and prostate tissues. The bigger dimensions and the reduced electrophoretic mobility of platelet ERβ respect to the other cell lines, are ascribed to a platelet-specific glycosylation process: in fact, the platelet ERβ is N-glycosylated (*Nealen et al*, 2001). However, the role of sex steroid hormones in the regulation of platelet function has been investigated for years, even if conflicting results have been reported (*Elkeles et al*, 1968; *Ross & Glomset*, 1976; *Fuster et al*, 1992; *Bar et al*, 2000). Platelets represent one of the possible targets of the estrogenic action: effects of estrogen in the cardiovascular system could be the result of a direct modulation of platelet function by estrogen itself. Moreover, these anucleated cells represent an excellent and unique model to selectively investigate the signaling pathways mediating the non-genomic effects of estrogens. However, the signal transduction pathways and the physiological consequences of ERα and ERβ engagement in platelets has not yet been investigated.

Although sex hormones were first reported to affect platelets more than 30 years ago, contrasting data are present in literature concerning the effects of estrogen on platelet functions. It was observed an estrogen inhibitory effect on aggregation and ATP release induced by ADP in human platelets (*Bar et al*, 2000), calcium fluxes (*Miller et al*, 1995), and cGMP levels (*Anwaar et al,* 2000). Moreover, clinically evident coronary artery disease occurs on average 10-15 years later in women than men; however, prognosis is worse for women after myocardial infarction or coronary revascularization. Women also have an increased risk of arterial and venous thromboembolism

during pregnancy and with the use of oral contraceptives. In our laboratory we have previously demonstrated that estrogen may have pro-aggregatory effects on human platelets caused by a rapid and transient tyrosine phosphorylation of Src, and the formation of a membrane-associated Src-dependent signaling complex, which includes ERβ, Src, Pyk2 and PI3-K. Moreover, we have found that in platelets stimulated with low concentrations of thrombin 17β-E2 caused a strong potentiation of integrin αIIbβ3 activation and platelet aggregation, through a mechanism depending on ERβ engagement and Src kinase activation (*Moro et al*, 2005). This estrogen signaling pathway is coordinated by membrane lipid rafts. Indeed ERβ reversibly translocated to the lipid raft fractions in a hormone-dependent manner, and promoted the rapid and transient recruitment and activation of the tyrosine kinases Src and Pyk2 within the membrane raft domains (*Reineri et al*, 2007).

AIM

The molecular events involved in megakaryocytic maturation, in proplatelet formation and platelet release are not fully understood. It has been reported that gene expression modulation during megakaryocyte differentiation involves humoral factors, such as hormones and growth factors. An increasing number of evidences suggest that estrogens may play an important role during megakaryocytopoiesis and platelet formation. Accordingly, it has been shown that high levels of estrogens and conventional hormone replacement therapies increase the number of megakaryocytes in mice (*Perry et al*, 2000) and in postmenopausal women (*Bord et al*, 2000). Moreover Nagata and colleagues have shown that estradiol can be synthesized within murine megakaryocytes and that estradiol positively affects proplatelet formation (*Nagata et al*, 2003). In addition, Bord et al have reported that estrogens can promote megakaryocyte proliferation, differentiation and maturation, thereby modulating the expression of the classical estrogen receptors ERα and ERβ (*Bord et al*, 2004). Previous studies in our laboratory have provided evidence that estrogens can potentiate platelet aggregation through a rapid and reversible ERβ-mediated signaling (*Moro et al*, 2005) and that membrane lipid rafts coordinate this pathway (*Reineri et al*, 2007).

Megakaryocytes and platelets are known to express ERβ (*Khetawat et al*, 2000). Genomic effects of estrogens in megakaryocytes have been suggested to contribute gendering differences in platelet function. However, estrogens can also induce rapid, non-genomic effects through interaction with classical ERs, localized on the plasma membrane, and through a member of the 7-transmembrane G protein-coupled receptor family, GPR30 (*Cheskis et al*, 2007), now known as G protein-coupled estrogen receptor 1 (GPER).

Despite the potential importance of a non-genomic signaling, its exact role in megakaryocyte differentiation has not yet been determined.

In particular, the relationship between GPER and the classical estrogen receptors is not clarified. Moreover the different ERβ isoforms and GPER may play an important role in megakaryocyte differentiation and maturation. In this context we sought to evaluate whether the expression of GPER and ERβ isoforms could change during megakaryocytic differentiation.

METHODS

MEGAKARYOCYTE DIFFERENTIATION FROM HUMAN CORD BLOOD-DERIVED CD34+ CELLS

Human umbilical cord blood (CB) was collected after normal pregnancies at the “Ospedale Maggiore della Carità”, Novara, Italy, upon informed consent of parents. The samples were processed within 48 hours. Mononuclear cells were separated by layering CB onto Lympholyte (density<1077 g/ml, Cedarlane, Hornby, Canada) and centrifuging for 30 min at 425 x g at 20°C. The resulting nucleated cells were washed twice in phosphate-buffered saline (PBS) and suspended in RPMI medium. Mononuclear cells were plated 6 x 10^6/ml and cells were let adhere for 30 minutes at 37°C in a 5% CO_2 fully-humidified atmosphere. CD34+ cells were obtained from nonadherent cells by isolation with immunomagnetic beads technique (Miltenyi-Biotec, Bergisch Gladbach, Germany) according to the manufacturer’s instructions. Subsequently, CD34+ cells were plated 1 x 10^6/ml in StemSpan Serum-Free Expansion Medium (Stem-Cell Technologies, Vancouver, BC, Canada) supplemented with 10 ng/ml thrombopoietin, interleukin (IL)6 and IL11 (all from PeproTech EC Ltd, London, UK). Cultures were maintained for up to 13 days at 37°C in 5% CO_2 fully-humidified atmosphere to induce megakaryocyte differentiation. After 13 days, CD61+ mature megakaryocytes were isolated by immunomagnetic beads technique (Miltenyi-Biotec, Bergisch Gladbach, Germany) according to the manufacturer’s instructions.

IMMUNOFLUORESCENCE ANALYSIS

Mature megakaryocytes were cytospun onto glass coverslips after 13 days of culture. Cells were fixed in 3% paraformaldehyde for 20 min at RT. Upon washing with PBS, cells were

permeabilized with 0.5% Triton X-100 for 5 min and subsequently blocked with 3%BSA in PBS for 1 h at RT. Cells were then incubated with goat polyclonal anti-CD61 for 1 h at RT (Santa Cruz, Heidelberg, Germany). After washing with PBS, cells were incubated with 10 μg/ml of the appropriate secondary antibody conjugated with Alexa Fluor 488 (Invitrogen, Milan, Italy) in PBS for 1 h at RT. Nuclear counterstaining was performed with Hoechst 33258 100 ng/ml in PBS for 3 min at RT. Specimens were mounted in Mowiol 4-88. Samples were immediately analyzed by microscopy or stored at 4°C. Conventional fluorescence microscopy was performed through an Axioscope 2 Plus microscope (Carl Zeiss, Göttingen, Germany), and a 63X/1.25 or a 100X/1.30 Plan Neofluar oil-immersion objective.

RNA ISOLATION, QUANTIFICATION AND RETROTRANSCRIPTION

Total RNA from each sample was extracted by RNAqueous kit (Ambion Inc, Foster City, CA, USA) according to the manufacturer's instructions. The RNA concentration was determined by Qubit fluorimeter (Invitrogen Life Technologies, Paisley, UK).
750 ng/reaction of RNA were retrotranscribed using the QuantiTect Reverse Transcription Kit (Qiagen, Milan, Italy) in a final volume of 20 μl according to the manufacturer's instructions.

REAL TIME PCR

Real-time PCR was carried out using ABI Prism Sequence Detection System 7000 (Applied Biosystems, Foster City, CA 94404, USA). The reaction was performed in a volume of 20 μl using a TaqMan gene-expression master mix (Applied Biosystems, Foster City, CA 94404, USA), primers forward and reverse 900 nM each, 250 nM TaqMan probe (all from Applied Biosystems, Foster City, CA 94404, USA), 4 μl of cDNA, and sterile water. Specific TaqMan assays for GAPDH, ERα, and GPER were performed. We have also used specific primers and

probes for ERβ isoforms previously designed in our laboratory. In particular, we have used 100 nM primers forward for ERβ1 and ERβ4, and 450 nM primers reverse for ERβ1 and ERβ4.

STATISTICS

Unpaired, two-tailed Student's t test was performed to analyze data, with a significant difference set at $P < 0.05$. Data are presented as the means ± SEM.

RESULTS AND DISCUSSION

To investigate the possible effects of estrogen on megakaryocytic commitment and proplatelet formation, we have examined the expression of ERα, ERβ1, ERβ4, and GPER during in vitro megakaryocyte differentiation.

CD34+ cells were isolated from human cord blood and cultured in StemSpan Serum-Free Expansion Medium supplemented with 10 ng/ml thrombopoietin, IL6 and IL11 at 37°C in 5% CO_2 to induce megakaryocyte differentiation. Cells were harvested, cytospun on glass coverslips, and stained with goat polyclonal anti-CD61 and secondary antibody conjugated with Alexa Fluor-488 after 13 days of culture. Nuclear counterstaining was performed with Hoechst 33258. Specimens were mounted in Mowiol 4-88. Conventional fluorescence microscopy was performed through an Axioscope 2 Plus microscope, using a 63X/1.25 or a 100X/1.30 Plan Neofluar oil-immersion objective. At least 100 cells were evaluated for each specimen. The percentage of mature megakaryocytes positive for CD61 staining and with polyploid nuclei at the end of culture was 70 ± 15% (**Fig. 3**).

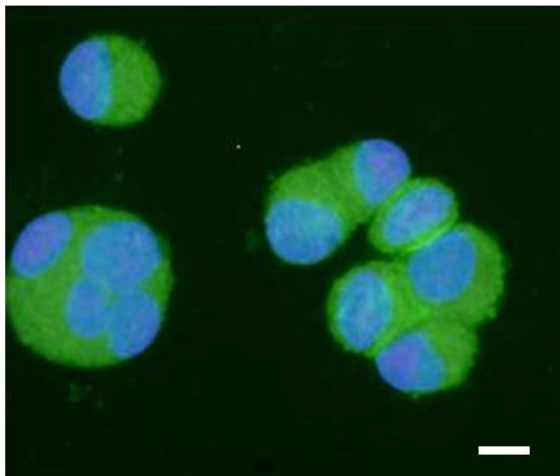

Fig. 3*:*

Representative immunofluorescence image of mature megakaryocytes derived from CD34+ cells*,*

as revealed by staining for CD61 (scale bar = 20 μm).

To evaluate estrogen receptors expression during megakaryocyte differentiation, three independent experiments were performed, all using a different pool of three cord blood samples (a total of nine cord blood samples was used), to avoid any biological variability.

ERα, ERβ1, ERβ4, and GPER expression was assessed in cells on days 0 (CD34+), and 7 of differentiation, as well as in mature megakaryocytes isolated at day 13 by CD61 immunomagnetic beads technique. Moreover GPER expression was also evaluated at day 3 of differentiation. Total RNA was extracted and reverse transcription reactions were performed. Real-time polymerase chain reaction was carried out on an ABI 7000 thermal cycler using the TaqMan chemistry. GAPDH was used as endogenous control and CD34+ cells at day 0 of culture were used as calibrator. Each sample was analyzed in triplicate and the results were consistent. ERα, ERβ1, ERβ4 and GPER expression in CD34+ cells was arbitrarily set to 100.

Our data showed that the expression of all estrogen receptors was decreased during megakaryocytic differentiation. In particular ERα expression after 7 days of differentiation was 13.4 ± 4.98 % (**$p<0.01$) with respect to calibrator and in mature megakaryocytes (CD61+) its relative expression was not detectable (***$p<0.001$) (**Fig. 4**).

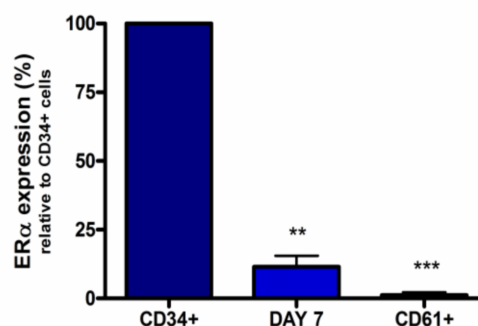

Fig. 4:

Differential expression of ERα during megakaryocyte differentiation. *Results are expressed as the percentage of ERα expression compared with the expression levels in CD34+ cells (100%). Each sample was analyzed in triplicate. We performed gene expression analysis using the comparative ΔΔCT method. Values are the means ± SEM of three independent experiments.*

A similar pattern of expression was observed for both ERβ isoforms analyzed. Indeed at day 7 of differentiation, ERβ1 expression was 5.15 ± 1.26% and ERβ4 expression was 2.05 ± 1.4% with respect to calibrator (***p<0.001). Moreover in mature megakaryocytes (CD61+ cells) the expression of ERβ1 was 1.75 ± 0.28%, while ERβ4 was no more detectable (**Fig. 5**).

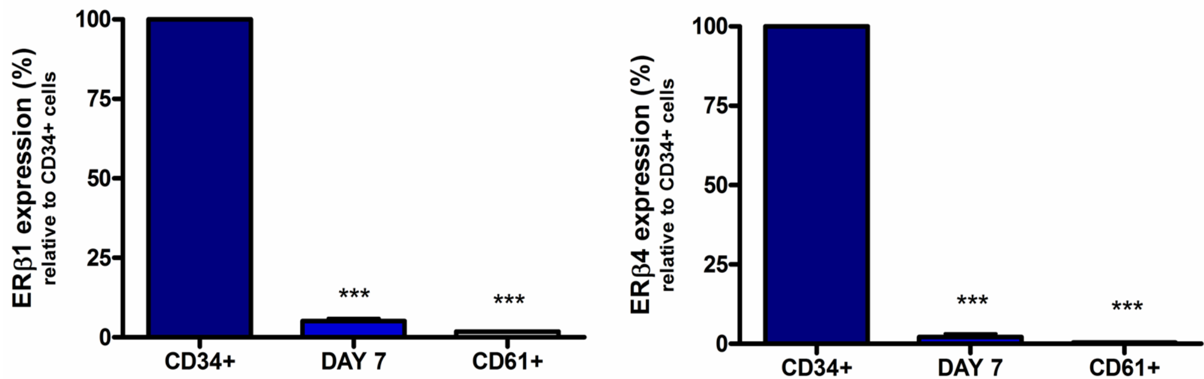

Fig. 5:

Differential expression of ERβ isoforms during megakaryocyte differentiation. *Results are expressed as the percentage of ERβ1 and ERβ4 expression compared with the expression levels in CD34+ cells (100%). Each sample was analyzed in triplicate. We performed gene expression analysis using the comparative ΔΔCT method. Values are the means ± SEM of three independent experiments.*

Concerning GPER, its expression decreased dramatically at day 3 (35.83 ± 13.25% with respect to calibrator, **p<0.01). Moreover, GPER was no longer detectable both at day 7 and in mature megakaryocytes (CD61+ cells) (***p<0.001) (**Fig. 6**).

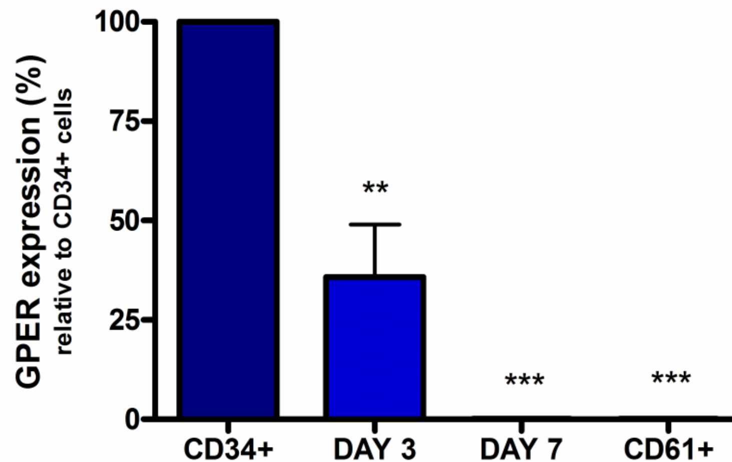

Fig. 6:

Differential expression of GPER during megakaryocyte differentiation. *Results are expressed as the percentage of GPER expression compared with the expression levels in CD34+ cells (100%). Each sample was analyzed in triplicate. We performed gene expression analysis using the comparative ΔΔCT method. Values are the means ± SEM of three independent experiments.*

Taken together, these findings of a rapid decrease of ERα, ERβ1, ERβ4 and GPER expression during the first stages of megakaryocytic differentiation lead to the conclusion that these estrogen receptors could be not important in megakaryocyte maturation and proplatelet formation. However, we cannot firmly exclude that these receptors can eventually play a role in the first stages of megakaryocytic differentiation. Moreover they could play some role to maintain hematopoietic stem cells undifferentiated and to prevent megakaryocytic differentiation.

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EFFECTS OF 8-PRENYLNARINGENIN ON HUMAN PLATELET ACTIVATION AND AGGREGATION

INTRODUCTION

PLATELETS

Platelets are small, discoid, anucleated cellular fragments with a diameter of about 3 µm and a thickness of 1 µm, that originated from large bone marrow megakaryocytes (*Radley & Scurfield*, 1980). In man, platelets circulate at a concentration of 250000-350000 cells/µl of blood and have a life span of 7 to 10 days. Although platelets are the smallest cellular elements in blood and lack the nucleus, they possess a metabolic and functional complexity similar to larger nucleated blood cells.

Their primary function is to prevent hemorrhage from a damaged blood vessel wall by adhering and forming an aggregate at the site of injury. They also participate in reactions of primary haemostasis, blood coagulation, inflammation and wound healing (*Bouchard & Tracy*, 2001).

Platelet structure

Platelets have a discoid shape with flat, featureless surfaces interrupted only by pitlike openings into the open canalicular system. The surface-connected open canalicular system is an extensive system of internal membrane conduits that serves as a passageway to the outside world. It also serves as a reservoir of membrane receptors, proteins and plasma membranes for cell spreading (**Fig. 1**).

Platelet plasma membrane presents an asymmetric distribution of phospholipids: sphingomyelin and phosphatidylcholine are abundant in the outer leaflet, phosphatidylethanolamine and phosphatidylserine are confined to the cytoplasmic side where they may serve as substrates for phospholipases (*Blockmans et al*, 1995). The membrane

proteins can be wholly or partly inserted into the lipid bilayer and some of them have covalently linked multibranched carbohydrate chains to form the so called “glycocalyx”. This dynamic structure serves as site of first contact, sensing changes in the vascular compartment requiring the hemostatic response of platelets at sites of vessel injury. Indeed the glycocalyx is covered by major and minor glycoprotein receptors necessary to facilitate platelet adhesion to damaged surfaces, to trigger full platelet activation and aggregation, interaction with other cellular elements and to accelerate the process of clot retraction (*Okumura & Jamieson*, 1976).

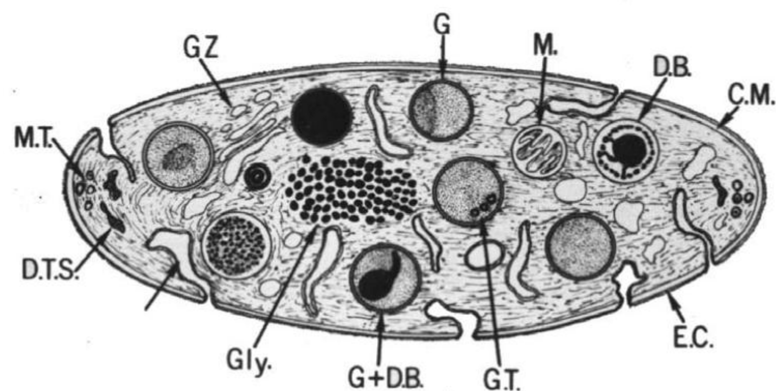

Fig 1: *Longitudinal section of a resting platelet. The indicated structures are the plasma membrane (C.M.), the open canalicular system (C.S.), the microtubules (M.T.), the glycogen granules (Gly) the mitochondria (M.), the α-granules (G.), the dense bodies (D.B.) and the dense tubular system (D.T.S.).*

(White et al, 1994*)*

Most of the platelet surface membrane glycoproteins span the lipid bilayer with their carbohydrate chains associated with the N-terminal extracellular domains. Transmembrane segments of glycoproteins, which are rich in hydrophobic residues, anchor the protein into the lipid bilayer. Most of them have a short cytoplasmic C-terminal region which in some cases makes either permanent or transient links with cytoskeletal proteins juxtaposed to the inner leaflet of the lipid bilayer. Membrane glycoprotein and cytoskeletal protein interactions may

restrain movements of other constituents in the bilayer and could be important in the maintenance of platelet shape (*Blockmans et al*, 1995).

A small thin zone of cytoplasm separates the plasma membrane of the resting platelets from a marginal microtubule coil and the general intracellular space, which contains all inclusion bodies and the internal cell cytoskeleton. Resting platelets present a well-defined cytoskeleton that allows the maintenance of the platelet discoidal shape. Moreover, when the platelet undergoes activation, the cytoskeleton permits the transmission of membrane mechanical forces, causing shape change (*Fox et al,* 1993), and plays an important role during release reaction of platelet granules and clot retraction.

The cytoskeleton contains two proteins of the contractile system, actin-binding protein and α-actinin, that are membrane-associated proteins and act as linkage molecules between membrane and cytoskeleton. Two other proteins, talin and vinculin, are involved in organizing actin near the platelet surface. They are associated with the adhesion plaques and may be responsible for localizing actin in these centers (*White*, 1994). The conversion of actin into masses of filaments shortly after activation of resting platelets constitutes one of the major early responses of the cell: the initial assemblies of actin are irregular networks of small protrusions extending from the discoid cell. These changes precede the development of early dendritic forms, which cause the loss of platelet discoid shape, and the extension of long, filiform processes.

Another form of parallel actin filament association in platelets are the stress fibers that radiate from the centre toward the periphery and end into pseudopodia. These structures help to stabilize the irregular form of activated dendritic and spread platelets. Actin filaments are also arranged in concentric layers around the central region where microtubule coils are abundant and play an important role in granules centralization (*White*, 1994). Upon platelet activation, myosin associates with the actin filaments, thus generating the tension required for the

centralization of granules. The platelet cytoskeleton is also composed of a microtubular coil, just beneath the platelet membrane. This microtubular coil, composed of tubulin, is involved in the maintenance of discoid shape of the resting platelets. The loss of the discoid shape is associated with the disappearance of the circumferential band of microtubules, and the recovery with the reformation of the bundle in its usual position under the cell surface (*White & Escolar,* 1991).

Platelets contain no rough endoplasmic reticulum or Golgi complex, indeed they do not synthesize proteins and polypeptides destined for granule storage and exocytotic release. The various procoagulant proteins and mitogenic factors present in the protein storage granules, as well as many of the enzymes contained in the lysosomes, peroxisomes and mitocondria, are produced by the megakaryocytes.

The mature platelet contains an extensive smooth endoplasmic reticulum, called dense tubular system. On enzymatic and functional profiles it resembles both the classical smooth endoplasmic reticulum and the sarcoplasmic reticulum of skeletal muscle cells (*White*, 1994). Indeed it contains both the NADH/cytocrome c reductase and the Ca^{2+}/Mg^{2+} ATPase. This latter enzyme operates as a calcium pump which functions optimally at a cytosolic Ca^{2+} concentration in the range of 0.1-1.0 μM. The smooth endoplasmic reticulum is believed to contain the major intracellularly releasable storage pool for Ca^{2+} in the platelet and, through operation of the uptake pump, the cytoplasmic level of this cation is maintained in the range of 50-100 nM in the resting platelet.

The intracellular membrane system also contains the fully enzymatic potential for the liberation of arachidonate from phospholipids, and for conversion of this fatty acid to prostaglandin endoperoxides and thromboxane A_2. Since thromboxane A_2 is a potent excitatory agonist, its rapid release from the platelet amplifies the haemostatic response by binding to surface membrane receptors on other platelets (*Praticò & Dogné*, 2009).

The dense tubular system is physically connected to the canaliculi of the open canalicular system: their association is usually restricted to one or two areas of the cytoplasm where the elements of the open canalicular systems are gathered in clusters or groups. Dispersed in the platelet cytoplasm there are numerous organelles, such as mitochondria, glycogen particles, lysosomes and peroxisomes. Platelet mitochondria are comparable to those found in other cells: they contain the enzymes of the tricarboxylic acid cycle and of fatty acid oxidation together with the systems required for coupled oxidative phosphorylation. Glycogen granules are a prominent feature in the platelet cytosol and provides the fuel reserve which enables the platelet to meet the energy requirements imposed by cellular activation. Platelet lysosomes contain a range of glycosidases, and a number of proteases, such as cathepsins D and E, and a neutral protease. In platelet peroxisomes are present catalases and other oxidases (*White*, 1994).

Moreover platelets present platelet-specific storage granules, that are α-granules and dense bodies. The α-granules contain coagulation factors, such as fibrinogen, factor V, high molecular weight kininogen, von Willebrand factor (vWF), antiheparins, platelet factor 4, β-thromboglobulin, platelet basic protein, growth-promoting factors and mitogens, such as PDGF and thrombospondin. α-granules also contain low concentrations of all plasma proteins. Many of the proteins found in platelet α-granules are packaged after synthesis in the precursor megakaryocyte; a number of α-granule proteins is synthesized *de novo* by megakaryocytes including coagulation factor V, platelet factor 4, and vWF. Other proteins including IgG, albumin and fibrinogen are taken up from plasma by an endocytotic mechanism. Dense bodies are storage sites of serotonin, ATP, ADP, divalent cations, as Ca^{2+} and Mg^{2+}; other constituents, like GTP and pyrophosphate, are present at much lower concentrations.

Platelet integrins and immunoreceptors

Integrins

Integrins are a family of heterodimeric proteins, composed of non-covalently associated α and β subunits. Each subunit consists of a large extracellular domain, a single-span transmembrane domain and a short cytoplasmic domain composed of roughly 20-60 amino acids (*Hynes*, 2002).

Platelets express five integrins: αIIbβ3, αVβ3, α2β1, α5β1, and α6β1. αIIb, αV, α5, and α6 are proteolytically cleaved during synthesis in the megakaryocyte to form a mature subunit consisting of an extracellular heavy chain linked to membrane spanning and intracellular light chains that is disulfide linked (*Hynes*, 2002). Both of the β3 and α5β1 integrins engage extracellular matrix ligands that contain the canonical Arg-Gly-Asp (RGD) motif.

Ligand binding to integrins is regulated by receptor conversion from a low-affinity to a high-affinity state in a cellular process referred to as "inside-out" signaling. In addition, the binding of multivalent extracellular matrix ligands can be promoted through valency regulation by clustering or oligomerization of integrin heterodimers (*Carman & Springer*, 2003). Binding of such ligands triggers "outside-in" signals that cooperate with signals resulting from agonist occupancy of immunoreceptors or G protein-coupled receptors to induce anchorage-dependent responses, including actin polymerization and reorganization.

αIIbβ3 is the most abundant surface-expressed integrin in platelets, with an additional pool that can be recruited from α-granules and from the open canalicular system upon platelet activation (*Shattil et al*, 1985). αIIbβ3 can bind several RGD ligands, including fibrinogen, fibrin, von Willebrand factor, vitronectin, fibronectin and thrombospondin. αIIbβ3 is required for platelet aggregation, spreading on extracellular matrix and clot retraction. Since fibrinogen is a symmetrical molecule, platelet aggregates are formed by cross-links of αIIbβ3 on adjacent

platelets by soluble fibrinogen at low shear rates or by vWF at high shear rates (*Savage et al*, 1998). Interestingly, under conditions of extremely high shear stress, vWF binding to its alternate receptor, GPIb-IX-V, may be sufficient to support activation-independent platelet aggregation (*Ruggeri et al*, 2006). In unstimulated platelets αIIbβ3, as the other integrins, is in a low-affinity state and is unable to bind soluble ligands. *In vitro*, αIIbβ3 can become activated by platelet stimulation with one or more excitatory agonists or by activating antibodies, by manganese chloride, or by the binding of RGD ligands. Soluble agonists such as adenosine diphosphate, thrombin and thromboxane A_2, initiate the “inside-out signaling” through heptahelical G protein-coupled receptors, while immobilized agonists such as vWF or collagen initiate the process by interacting with GPIb-IX-V or collagen receptors GPVI and α2β1, respectively. These “inside-out signals” are Ca^{2+}-dependent and involve conformational changes in both the ligand-binding extracellular region and the cytoplasmic tails of the receptor (**Fig. 2A**). They are thought to ultimately modulate αIIbβ3 affinity by regulating the interaction of specific proteins, such as talin, with integrin cytoplasmic tails (*Vinogradova et al*, 2002).

As previously mentioned, ligand binding to αIIbβ3 stimulates “outside-in signaling” (**Fig. 2B**) to promote platelet adhesion and spreading on extracellular matrix, fibrin clot retraction, development of platelet procoagulant activity and microparticle generation in response to stimuli, such as collagen and thrombin.

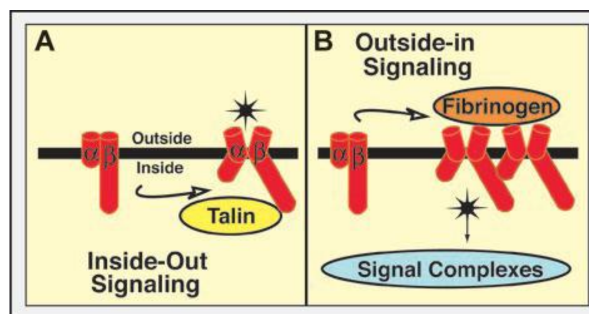

Fig. 2: ***Integrin activation is bidirectional and reciprocal.*** *The IIb3 equilibrates between resting and activated states. Conversion from resting to activated does not imply a single, abrupt change but rather a series of coordinated and linked conformational transitions. Figure **A** shows the inside-out signaling, figure B shows the outside-in signaling. (Shattil & Newman,* 2004)

One of the first steps involves receptor oligomerization and a complex regulation by protein tyrosine kinases and phosphatases to activate c-Src and other Src family kinases as well as Syk (*de Virgilio et al*, 2004). These initial reactions lead to the assembly of a larger integrin-based signaling complex made up of additional enzymes, adapter molecules and substrates, including Src homology-2 (SH2)-domain containing leukocyte-specific phosphoprotein of 76 kDa (SLP-76), Vav1, phospholipase C γ (PLCγ), adhesion- and degranulation-promoting adapter protein, Nck and cdc42, which promote actin polymerization and reorganization. Although αIIbβ3 is required for the growth of stable thrombi (*Bergmeier et al*, 2006), the interaction between immobilized fibrinogen and αIIbβ3 promotes only minimal aggregation and thrombus formation *ex vivo*. Thus, other thrombogenic matrices such as vWF and collagen likely fulfill this role (*Savage et al*, 1996).

In particularly the two platelet collagen receptors, GPVI and integrin α2β1, play an essential role in promoting platelet adhesion and aggregation. α2β1 is composed of a 150 kDa α2-chain and a 130 kDa β1-chain. α2 is the only platelet subunit to contain a domain involved in the

binding to the GFOGER motifs of triple helical peptide of collagen I, VI and XI in a Mg^{2+} and Mn^{2+} dependent manner (*Tuckwell et al*, 1995). Optimal α2β1 function requires activation. Three activation states have been proposed: unactivated, fully activated, and intermediate (*Van de Walle et al*, 2005). Signaling through α2β1 in collagen adherent platelets induces ADP release (*Jung & Moroi*, 2000; *Atkinson et al*, 2003) and includes phosphorylation of Src family kinases, SLP-76, Syk and PLCγ2, as well as FcγRIIA in human platelets (*Keely & Parise*, 1996).

Immunoreceptors

Originally described in the context of immune cell development and function, immunoreceptors are now known to be essential in all hematopoietic cells, including platelets. Immunoreceptor signaling is built on intracellular tyrosine phosphorylation. Active receptor acts through motifs called immunoreceptor tyrosine-based activation motifs (ITAMs) (*Reth*, 1989), which are phosphorylated by Src family kinases during receptor signaling (*Gibbins et al*, 1996). Once phosphorylated, the ITAM binds the tandem SH2 domains of the intracellular tyrosine kinase Syk or ZAP-70 (z-associated protein of 70 kDa), activating downstream signals. While the signals downstream are complex, effectors with defined roles in platelets include the adapters SLP-76 (*Clements et al*, 1999) and linker for activated T cells (LAT) (*Pasquet et al*, 1999), the Bruton's tyrosine kinase (*Quek et al*, 1998), the guanine nucleotide exchange factor Vav (*Pearce et al*, 2004), and the PLCγ2 (*Wang et al*, 2000). Activation pathways are often balanced by inhibitory receptors that harbor immunoreceptor tyrosine based inhibitory motifs (ITIMs) that bind the SH2 domains of downstream phosphatases such as SH2-domain-containing tyrosine phosphatase-1 (SHP-1), and SHP-2 (*Pasquet et al*, 2000). ITAMs and ITIMs are integral parts of the receptor, located either in the intracellular domain

of the ligand-binding receptor itself or in an associated, specialized, ITAM-containing subunit such as the FcγR subunit.

One of the most important immunoreceptors in platelets is GPVI. GPVI is expressed exclusively in platelets and mature megakaryocytes, where it is associated with the ITAM-containing transmembrane adapter protein FcγR. GPVI-FcγR is the only collagen receptor capable of activating platelets. Indeed, when circulating platelets come in contact with subendothelial collagen following vessel injury, GPVI-FcγR appears to be the receptor that generates the first collagen signal (*Chen & Kahn*, 2003). While it is known that stimulation through collagen can induce fibrinogen binding (*Lecut et al*, 2004), it has often been difficult to discriminate between signaling responses triggered through α2β1 and GPVI. Several GPVI agonists that do not bind α2β1 have been employed to sort this out, such as GPVI-activating antibodies, the collagen related peptide (CRP) that contains a repeated Gly-Pro-Hys sequence, and the snake venom convulxin. Binding of GPVI-FcγR by these molecules appears to activate the receptor in a manner analogous to related immune receptors (**Fig. 3**).

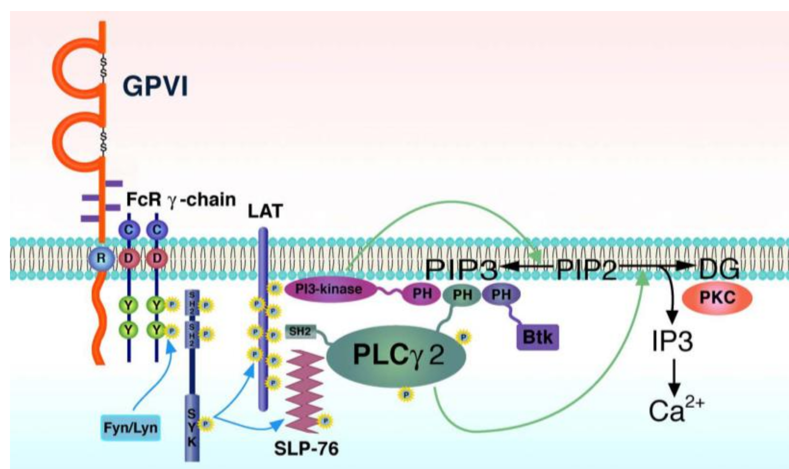

Fig. 3: *Model of the activation pathways induced by GPVI*. (*Moroi & Jung*, 2004)

The FcγR ITAM of clustered receptors is phosphorylated by Src family kinases and then binds and activates the Syk kinase (*Gross et al*, 1999). Syk drives downstream signaling by phosphorylating a number of substrates, including the adapters SLP-76 and LAT and ultimately activating PLCγ2. Collagen stimulation through GPVI-FcγR may further amplify platelet activation by inducing dense granules release and TxA2 generation (*Nakamura et al*, 1998). Activation of Rap1b also occurs in response to GPVI agonists, by a mechanism that in part relies on released ADP and PI3K. However, as PKC may regulate Rap1b activity and has also been implicated in αIIbβ3 affinity modulation through GPVI (*Quinton et al*, 2002), GPVI-induced αIIbβ3 affinity modulation may proceed through the common PKC-Rap1b-RIAM-talin pathway to integrin activation (*Han et al*, 2006).

Another important receptor involved in platelet aggregation is GPIb-IX-V complex of the leucine-rich repeat family that consists of four transmembrane proteins: GPIbα and GPIbβ are disulfide-linked and non-covalently associated with GPIX and GPV at a 2:2:2:1 ratio (*Lopez & Dong*, 1997). This glycoprotein complex represents, after αIIbβ3 integrin, the second receptor mostly expressed on platelet surface.

A well-established ligand for GPIb-IX-V is vessel wall vWF. This interaction is essential for slowing circulating platelets and allowing the formation of arterial thrombi (*Savage et al*, 1998). Different studies indicate that the molecular basis of vWF-GPIb-IX-V signal transduction may result in platelet activation through the FcγR ITAM in a manner similar to GPVI (*Kasirer-Friede et al*, 2004).

Platelet activation and aggregation

Platelets play an essential role in the first phases of the haemostatic process. When a blood vessel is damaged at its luminal side, subendothelial elements are exposed and come in contact with the blood elements. When platelets are exposed to non-endothelial surface they undergo a rapid shape change, from a disc to a spiny sphere with long and fine filopodia, adhere, flatten, and spread on the surface (**Fig. 4**).

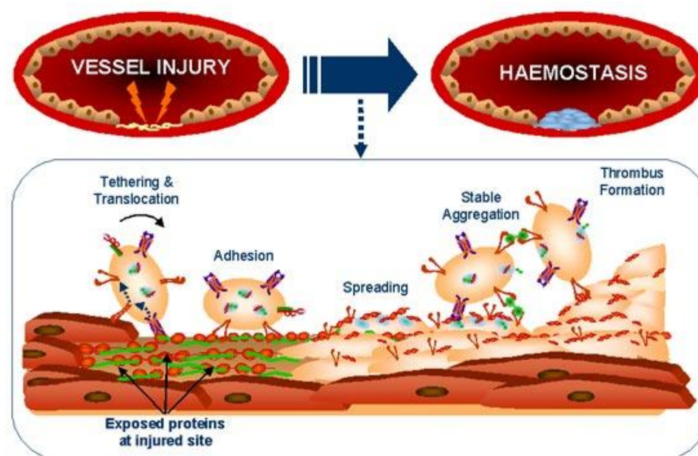

Fig. 4: *Platelet activation and aggregation*

The most important subendothelial structures to which platelets can adhere with their specific receptors are collagen, fibrinogen, fibronectin, and von Willebrand factor. At high shear stresses, platelet adhesion is largely dependent on immobilized vWF, which interacts with GPIb-IX-V. This interaction results in platelet activation and in generation of an intraplatelet signal necessary to activate integrin αIIbβ3, leading to irreversible platelet adhesion and aggregation.

After adhesion to a subendothelial surface, platelets undergo spreading. During spreading, platelet secretion also occurs: platelets secrete α-granule and dense body contents, and only partially lysosomal enzymes. Strong agonists result in the secretion of 70% to 90% of α-

granule and dense body contents. Secretion allows the release, in blood microenvironment, of molecules that, at first, contribute to the formation of the haemostatic plug and then to the dissolution of platelets-fibrin aggregates. Platelets granule secretion requires the fusion of granular membranes with the plasma membrane or with membranes of the surface connected to the open canalicular system. Contractile forces within platelets needed for granules centralization and secretion are generated by the interaction of the actin filaments with myosin heads and by contraction of the band of microtubules around the cell circumference.

Most of the platelets that accumulate at the sites of injury do not adhere directly to subendothelial structures, but rather to each other. This process of platelet-platelet interaction is termed aggregation. Platelet aggregation can be triggered by several potential physiological agonist, the most important of which are ADP and thrombin; other potential agonists include epinephrine, TxA2, and Platelet Activating Factor (PAF)1. Weak aggregating agents or low concentration of stronger ones cause reversible ("primary") aggregation, whereas stronger stimuli cause an irreversible ("secondary") aggregation which is associated with prostaglandin synthesis and the release reaction. The aggregation reaction itself causes TxA2 synthesis (*Blockmans et al*, 1995).

Moreover platelets participate to clot retraction, mechanism preventing an excessive blood vessels stenosis that could cause thrombotic and ischaemic events. The contractile actomyosinic apparatus of platelets is the mechanic strength that contributes to the retraction of the integrin αIIbβ3, that is internally bound to actin and externally to the fibrin among aggregated platelets. Thanks to the interaction between actin filaments and myosin heads, platelets retract and with them the whole thrombus.

Platelet signaling

Platelet activation

Many physiological agonists contribute to platelet activation and to the consequent multiple signaling pathways that trigger biochemical, morphological, and functional changes which culminate in an irreversible aggregation. These pathways involve the metabolism of membrane phospholipids, the mobilization of intracellular calcium, the phosphorylation of specific platelet proteins on serine, threonine, and tyrosine residues, the translocation of some proteins to actin cytoskeleton and the modulation of integrin avidity (*Jackson et al,* 1996).

Some cellular messengers, such as heterotrimeric G proteins, the phospholipases C (PLC) and A2 (PLA_2), and the phosphatidylinositol 3-kinase (PI3-K), play a pivotal role in all platelet signal transduction pathways.

The heterotrimeric G proteins are coupled to seven membrane-spanning receptors and are composed of three subunits, α, β, γ (*Blockmans et al,* 1995). Platelets express 10 different G_{α} proteins: the G_i family ($G_{i1\alpha}$, $G_{i2\alpha}$, $G_{i3\alpha}$, and $G_{z\alpha}$), the G_q family ($G_{q\alpha}$, $G_{16\alpha}$), the G_{12} family ($G_{12\alpha}$, $G_{13\alpha}$), and G_s (*Gilman*, 1987). The members of G_i family are coupled to adenylyl cyclase and their activation triggers the decrease of cAMP levels. The members of G_q family are strong activators of PLC.

PLC activation is one of the earliest responses of platelets to many agonists and it is implicated in secretion. In particular different PLC isoforms exist: PLCβ is activated by βγ dimers of heterotrimeric G proteins, whereas the PLCγ is activated by phosphorylation of ITAM sequences mediated by tyrosine kinases Src, Syk, Fyn, Lck. The phospholipases are primarily responsible for the rapid hydrolysis of membrane lipid phoshatidylinositol 4,5-bisphosphate (PIP2) into second messengers inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG). IP3 is a soluble molecule that allows the release of sequestered Ca^{2+} and the influx of Ca^{2+} from extracellular environment, increasing the intracellular levels of

this ion to almost 10 μM. Calcium mobilization triggers granule secretion and ADP and serotonin release, in order to potentiate platelet activation. On the other hand DAG activates protein kinase C (PKC) that contributes to protein phosphorylation, granule secretion, and fibrinogen receptor exposure (*Blockmans et al,* 1995).

One of the most important pathways for signaling in platelets is the arachidonate pathway. During platelet activation, arachidonate is released from membrane phospholipids by PLA_2. After deacylation, arachidonate is rapidly metabolized to diverse biologically active products through the cycloxygenase and lipoxygenase pathways. In human platelets, cycloxygenase oxygenates arachidonic acid to the prostaglandin endoperoxides PGG_2 and PGH_2 (*Kroll & Schafer,* 1989), that are converted to TxA2. These are strong platelet agonists which bind to specific platelet receptors and directly activate PLC, through a G-protein signal transducer (*Blockmans et al,* 1995).

Phosphatidylinositol 3-kinases are a family of kinases which phosphorylate phosphatidylinositol, phosphatidylinositol 4-phosphate, phosphatidylinositol 4,5-bisphosphate to produce phosphatidylinositol 3-phosphate, phosphatidylinositol 3,4-bisphosphate, phosphatidylinositol 3,4,5-trisphosphate. Human platelets express two PI3-K isoforms, called Ia and Ib, which consist of a regulatory, adaptor subunit (p85) and a catalytic subunit (p110) (*Zhang et al*, 1998). The PI3-K activity is stimulated by different platelet agonists, such as thrombin, ADP, TxA2, through the FcγIIA receptor. Its activation is essential for different physiologic changes, such as proliferative responses to growth factors, differentiation, anti-apoptotic events, cytoskeletal rearrangements, integrin activation, integrin-mediated cell motility and carcinoma invasion (*Zhang et al*, 1998). These responses are due to the presence of PI3-K products in the inner leaflet of the plasma membrane, that could activate PLCγ or PKB/Akt through the protein PDK, that directly binds the phosphoinositides 3-phosphate. Akt activation plays diverse roles in platelet activation.

Protein tyrosine kinases

Tyrosine kinases and tyrosine phosphatases play an important role in the regulation of protein tyrosine phosphorylation level within the cell. Regarding protein tyrosine kinases, they are classified into two groups: the transmembrane receptor family, that include the receptors for a variety of soluble growth factors and hormones and the cytosolic non-receptor group, such as Src, Btk, Csk, Syk, and FAK families. These last kinases are the most abundant in platelets where they play essential roles in platelet activation. Indeed, it has been reported that inhibitors of tyrosine kinases are able to down-regulate a number of platelet responses, while tyrosine phosphatase inhibitors enhance the level of protein tyrosine phosphorylation within the cell and promote platelet activation (*Jackson et al,* 1996). In resting platelets, only a small number of proteins are tyrosine phosphorylated, while platelets stimulated with different agonists and in response to adhesion molecules show an increase of tyrosine phosphorylation in multiple platelet proteins, suggesting that these events represent a general feature of platelet activation.

As previously mentioned, the tyrosine phosphorylation of cellular proteins is a dynamic and reversible process regulated by both kinases and phosphatases. These last enzymes appear to play a major role in maintaining low levels of phosphotyrosine within the resting cell and may prevent premature platelet activation.

*The tyrosine kinase Src***.** Members of the Src family expressed in platelets are Fyn, Lyn, Yes, Hck, and pp60 c-Src, the most abundant tyrosine kinase in platelets constituting 0.2-0.4% of total platelet proteins (*Golden et al*, 1986).

Src contains an N-terminal myristoylation site, a catalytic site, a C-terminal autoregulatory tail, and two domains SH2 and SH3, important in the regulation of intra- and inter-molecular protein-protein interactions. SH2 domain is a highly conserved region, which recognizes specific consensus sequences encompassing tyrosine phosphorylated residues, whereas SH3

domain binds specific proline rich sequences. The N-terminal glycine residue undergoes myristoylation and it is responsible for localizing Src to cellular membranes. In addition, at the C-terminal site Src possesses two important regulatory tyrosine phosphorylation sites, Tyr416 and Tyr527. Under basal conditions, Tyr416 is not phosphorylated, while Tyr527 is phosphorylated, and binds the SH2 domain stabilizing a close and inactive conformation of the enzyme catalytic site.

When Src partner proteins bind SH2 or SH3 domain, Src structure is driven into an unfolded conformation, that permits Tyr527 dephosphorylation, Tyr416 phosphorylation and the subsequent Src activation. Activated Src associates with the actin-rich cytoskeleton, where is able to phosphorylate a number of cytoskeletal proteins. Moreover the N-terminal myristoylation sequence, SH2 and SH3 domains of Src are essential for its ability to associate with focal adhesions and to promote cell spreading (*Jackson et al,* 1996).

*The tyrosine kinase Pyk2***.** Pyk2 (Proline-rich Tyrosine Kinase) is a cytoplasmic tyrosine kinase related to FAK (Focal Adhesion Kinase). It is composed by a kinase domain, a large N-terminal, and a C-terminal domain that contains a proline-rich stretch of residues essential for the interaction between Pyk2 and other proteins containing SH3 domains. Pyk2 presents at least two tyrosine residues that become phosphorylated: Tyr882, which appears to be a Grb2 binding site, and Tyr402, that binds members of the Src family (*Avraham et al*, 1995). It has been reported that stimulation of platelets by several agonists such as thrombin, collagen, ADP, and epinephrine, induces Pyk2 tyrosine phosphorylation during the early phase of platelet activation, independently from integrin $\alpha IIb\beta 3$ (*Raja et al*, 1997). Moreover, Pyk2 plays a role in the signal transduction events activated by the binding of vWF to GP Ib-IX-V (*Canobbio et al*, 2002). It has been proposed a model in which Pyk2 is associated with PI3-K in resting platelets and upon activation, an increase in tyrosine phosphorylation and activation of Pyk2 occurs, increasing Pyk2/PI3-K interaction and PI3-K activation. These events

develop in the early phase of platelet activation and are independent of aggregation (*Sayed et al*, 2000).

The MAP kinases. Stimulation of a variety of tyrosine kinase receptors leads to a rapid elevation of the enzymatic activity of a family of closely related serine-threonine kinases, known as MAP kinases. These kinases are able to convert extracellular stimuli to intracellular signals that control gene expression, cell proliferation, and differentiation. MAP kinases have been classified into three subfamilies: ERKs (Extracellular signal-Regulated Kinases), JNK/SAPK (c-Jun N-terminal or Stress-Activated Kinase), and p38 MAP kinase (*Lopez-Ilasaca*, 1998). The MAP kinases are activated by the MAP kinase kinases through a double phosphorylation on conserved serine-threonine residues into a specific consensus sequence TXY (T, threonine, X, any aminoacid, Y, tyrosine). Both tyrosine kinase receptors and G protein-coupled receptors share common biochemical components that lead to the activation of MAP kinase cascades. A well-known pathway involves the binding of a growth factor to its tyrosine kinase receptor, causing the subsequent phosphorylation of the receptor on tyrosine residues. This event permits the binding of Grb2/Sos complex. Sos is an exchange factor that promotes the removal of GDP from Ras and the subsequent binding of GTP. Activated Ras can bind the protein kinase Raf-1, that in turn can phosphorylate MEK, causing it to phosphorylate the p42 and p44 forms of MAPK. Three different pathways that may lead to MAP kinases activation have been described in platelets. Two are the immediate consequence of G-protein-coupled receptor activation: the first arising from the Ca^{2+}-dependent activation of Pyk2, the second from the PI3-Kγ-dependent activation of Shc. The third pathway is an indirect consequence of receptor activation because it results from outside-in signaling after integrin engagement (*Lopez-Ilasaca*, 1998).

p42/p44 MAP kinases. As previously mentioned, it has been hypothesized that in platelets receptor activation leads to the activation of the low molecular weight GTP-binding protein Ras which recruits the serine/threonine kinase Raf to the plasma membrane. Raf phosphorylates and activates the MAP kinase kinases MEK1 and MEK2, which subsequently activate ERK1 (p44) and ERK2 (p42) by threonine/tyrosine phosphorylation into the consensus sequence TEY (Thr-Glu-Tyr) (*McNicol et al*, 2001). Subsequently, ERK1 and ERK2 are incorporated into the cytoskeleton in a manner similar to other proteins such as myosin, actin, actin-binding protein, α-actinin, and tropomyosin. It has been proposed that the cytoskeleton may act as a frame, which spatially aligns the MAP kinases with substrates in a highly integrated platelet signal transduction pathway.

Platelet inhibition

The premature platelet activation is prevented by biochemical processes which attenuate agonist-induced responses. Elevation of intracellular cyclic AMP (cAMP) and cyclic GMP (cGMP) is the most potent endogenous mechanism of platelet inhibition. Cyclic nucleotide levels are up-regulated by synthesis through adenylyl cyclases (ACs) and guanylyl cyclases (GCs) and down-regulated by degradation through phosphodiesterases (PDEs). ACs are integral membrane glycoproteins that catalyze the synthesis of cAMP from ATP. Platelet AC is activated by the α subunit of the stimulatory G-protein ($G_{\alpha s}$), and strongly inhibited by the α subunit of the inhibitory G-protein ($G_{\alpha i}$) (*Offermanns*, 2000). Binding of prostaglandins to their receptor, which is coupled to G_s, therefore leads to stimulation of cAMP formation. Receptors for adenosine and α-adrenergic agents are also coupled to G_s and activate AC. On the other hand, some platelet activators such as thrombin, epinephrine or ADP induce the release of $G_{\alpha i}$, thereby down-regulating cAMP levels.

GCs exist in membrane-bound and soluble forms. It has been reported that only the soluble form of GCs, located in the cytoplasm, exists in platelets. Soluble GCs contain heme as a prosthetic group and is activated by nitric oxide (NO), nitrovasodilators, and other NO generating agents.

Cyclic nucleotide-dependent protein kinases, PKA and PKG, are the major effector molecules mediating physiological effects initiated by cyclic nucleotide formation. Compared to other cell types, human platelets contain particularly high concentrations of both PKA and PKG (*Eigenthaler et al*, 1992). Activation of these kinases in intact platelets leads to the phosphorylation of a large number of proteins. Some of these are targets for both PKA and PKG; others may be differentially regulated by the cAMP and cGMP pathways.

One of the major substrates of PKA and PKG is the cytoskeleton-associated vasodilator-stimulated phosphoprotein (VASP) that was found to be located in focal adhesions, stress fibers, cell-cell contacts and highly dynamic membrane regions in various cell types (*Reinhard et al*, 2001) and that is present in particularly high concentrations in platelets (*Eigenthaler et al*, 1992). VASP is believed to play an important role in controlling the cytoskeletal organization because it regulates actin dynamics (*Laurent et al*, 1999). Moreover it has been reported that its phosphorylation causes a decrease in the ability of VASP to bind F-actin. VASP contains three phosphorylation sites: Ser157, that is preferentially phosphorylated by PKA, Ser239, that is preferentially phosphorylated by PKG and Thr278 (*Butt et al*, 1994) (**Fig. 5**).

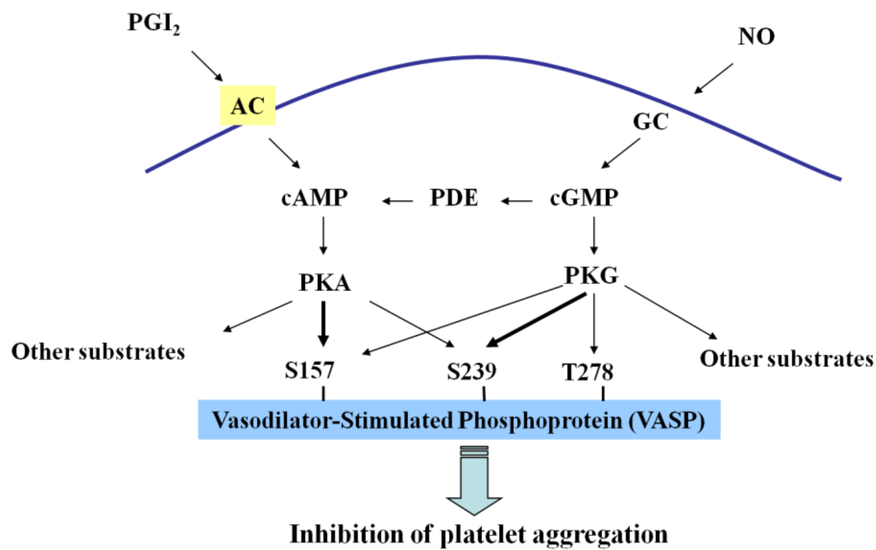

Fig. 5: *Mechanism of VASP phosphorylation*

In platelets, the phosphorylation of VASP at Ser157, which causes a mobility shift in SDS-polyacrylamide gels changing the apparent molecular mass of VASP from 46 to 50 kDa, has been shown to closely correlate to inhibition of the fibrinogen receptor (*Horstrup et al*, 1994). Moreover it has been shown that VASP-deficient mice present enhanced collagen- and thrombin-induced platelet activation and impaired cyclic nucleotide-mediated inhibition (*Aszodi et al*, 1999). The participation of VASP in dynamic cytoskeletal processes and its phosphorylation induced by platelet antagonists are hints to consider it as an important player in the regulation of platelet inhibition, even if its exact role is not fully understood.

VASP phosphorylation is not the only PKA and PKG substrate in platelet cytoskeletal system. Indeed it has been reported that these kinases also act on proteins involved in microfilament regulation such as the actin-binding protein (*Chen & Stracher*, 1989), caldesmon (*Hettasch & Sellers*, 1991), the glycoprotein Ibβ (*Wardell et al*, 1989), the heat shock protein 27 (*Butt et al*, 2001), the myosin light chain kinase (*Hathaway et al*, 1981), and the small GTPase Rap1b (*Siess et al*, 1990).

Different studies have also reported that cAMP and cGMP increase cause an inhibition of cytosolic calcium release and platelet secretion. As previously mentioned, elevation of cytosolic Ca^{2+} levels plays a key role during platelet activation, since it regulates multiple Ca^{2+}-dependent enzymes. It has been observed that after an increase of intracellular cAMP or cGMP, the activity of PLC is inhibited (*Ryningen et al*, 1998). Moreover, in human platelets PKA and PKG may directly phosphorylate IP3 receptors, which mediate the release of Ca^{2+} from the dense tubular system (*Cavallini et al*, 1996).

In addition, it has been demonstrated that the incubation of human platelets with vasodilators affects dense granule, α-granule, and lysosomal secretion, thereby inhibiting the release of procoagulatory factors into the blood, and the translocation of adhesive glycoproteins, like integrin αIIbβ3, P-selectin (CD62P), and CD40 ligand, from intracellular stores to the platelet surface membrane (*Michelson et al*, 1996).

FLAVONOIDS

Flavonoids have been known as plant pigments for over a century. The first observation regarding their biological activities was published in 1936 (*Rusznyak & Szent-Gyorgyi*, 1936), where they were been proposed as vitamins.

Flavonoids belong to a vast group of polyphenolic compounds that are widely distributed in all foods of plant origin. Plant polyphenols have been of interest to scientists for decades, originally because of their importance in plant pigmentation and flavor. It has been reported that polyphenols are produced as the result of the secondary metabolism of plants and that they are involved in plant growth and reproduction, provide resistance to pathogens and predators, and protect crops from disease and preharvest seed germination (*Bravo*, 1998). Flavonoids are the largest class of polyphenols, with a common structure of diphenylpropanes, consisting of two aromatic rings linked by three carbons (*Croft*, 1998). They can be further subdivided into six major subclasses, based upon variations in the heterocyclic C-ring including flavones, flavonols, flavanones, catechins, anthocyanidins, and isoflavones (*Bravo*, 1998).

Flavonoids have been of interest because of their observed biological effects *in vitro*. Indeed it has been reported that they possess antioxidant, antiestrogenic, and antiproliferative activities, which are commonly ascribed to explain their potential benefit in reducing the occurrence of cardiovascular diseases and cancer.

Reactive oxygen species are formed *in vivo* during normal aerobic metabolism and can cause damage to DNA, proteins, and lipids, despite natural antioxidant defense systems. The accumulation of unrepaired damaged products may be critical to the development of cancer, atherosclerosis, diabetes, and chronic inflammation (*Halliwell*, 1994). Flavonoids, in conjunction with other antioxidants, including vitamins C and E, are thought to inhibit lipid

peroxidation in the phospholipid bilayer caused by reactive oxygen species. In contrast to vitamins C and E, which are concentrated in the aqueous phase and phospholipid bilayer, respectively, flavonoids are localized between the two phases because of their hydrophilicity. Flavonoids may trap chain-initiating radicals at the interface of the membranes, preventing the progression of the radical chain reaction (*Duthie & Crozier*, 2000). Moreover it has been demonstrated that dietary flavonoids may protect free-radical-induced damage to DNA by a mechanism other than solely direct free-radical scavenging (*Anderson et al*, 2000): some flavonoids can chelate transition metal ions responsible for the generation of reactive oxygen species and therefore inhibit the initiation of the lipoxygenase reaction. Flavonoids may also exert antioxidant abilities through protection or enhancement of endogenous antioxidants. Indeed numerous flavonoids have been shown to alleviate oxidative stress by inducing glutathione S-transferase (GST), an enzyme proposed to protect cells against free-radical damage (*Fiander & Schneider*, 2000).

Several in vitro studies have investigated flavonoids as inhibitors of cellular transformation and proliferation. Franke and colleagues have demonstrated that flavonoids are able to inhibit neoplastic transformation in mouse fibroblasts induced by 3-methylcholanthrene (*Franke et al*, 1998). In addition it has been shown that flavonoids present antiproliferative activity in the absence of cell cytotoxicity in two human colon cancer cell lines (*Kuntz et al*, 1999). It has been reported that flavones are chemoprotective agents. Indeed they cause dramatic changes in the expression of cell-cycle and apoptosis-related genes, such as cycloxygenase-2, nuclear transcription factor kappaB, and bcl-X (*Wenzel et al*, 2000). Moreover several flavonoids can inhibit DNA topoisomerase II, an enzyme that catalyzes the double-strand breakage and rejoining of DNA, by stabilizing the cleavage complex, thereby facilitating apopotosis (*Strick et al*, 2000).

It has also been reported that many flavonoids show a potent estrogenic activity. Phytoestrogens are plant-derived compounds that structurally or functionally mimic mammalian estrogens (*Cos et al*, 2003). Indeed similarly to the numerous effects of estrogens on the human body, phytoestrogens are known to exert beneficial effects toward human health, especially against cancer, osteoporosis, irregular menopause syndrome, cardiovascular disease, and possibly neurodegenerative disease (*Knight & Eden*, 1996). In fact, Asian populations consuming diets rich in soy flavonoids have a lower incidence of hormone-dependent tumors, such as breast, ovarian, and endometrial cancers, compared to western populations (*Messina & Barnes*, 1991). Moreover, the prevalence of osteoporosis, menopausal syndrome and coronary heart disease is less frequent (*Knight & Eden*, 1995). The way how phytoestrogens protect may vary depending on the cancer type and the individual compound: some phytoestrogens act by competing with the endogenous estrogen for receptor sites, while others act by inhibiting signal transduction pathways that lead to aberrant growth.

8-prenylnaringenin

The plant of *Humulus lupulus L.* is well-known throughout the world as the raw material in the brewing industry. The female inflorescences (hop cones), rich in polyphenolic compounds and acyl phloroglucides, are widely used as preservative and flavoring agents in beer. Moreover hop cones have long been used for medicinal purposes. In particular, hop preparations were mainly recommended for the treatment of sleeping disorders, as a mild sedative, and for the activation of gastric function (*Dixon*, 2004).

Starting from the second half of the 20th century, several phytochemical studies were performed to investigate the composition of hop cones and other parts of the plant, leading to the isolation and identification of pharmacologically relevant compounds such as flavanones, chalcones, and phloroglucinol derivatives. During the past decade, many pharmacological *in*

vitro and *in vivo* investigations tried to produce scientific evidence of the reported traditional uses. Recently the estrogenic properties as well as the potential cancer chemopreventive activities of hops have been investigated and some active compounds from hop have received much attention. Among these, 8-prenylnaringenin (8-PN) has raised a growing interest (**Fig. 6**). This molecule belongs to the prenylated flavanones group, which also includes isoxanthohumol, 6-prenylnaringenin, and can be found in beer in a concentration of 0.24 mg/l (*Stevens et al*, 1999; *Rong et al*, 2001).

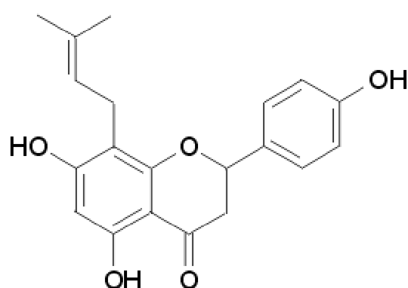

Fig. 6: *8-PN structure*

Many *in vitro* studies have identified 8-PN as one the most potent phytoestrogens in hops, with a potency greater than that of other well established phytoestrogens such as genistein or coumestrol (*Kitaoka et al*, 1998; *Milligan et al*, 2000; *Zierau et al*, 2002). It should be noted that 8-PN and estradiol show structural similarities, while the presence of a prenyl side chain at the C-8 position in 8-PN apparently results in an enhanced affinity towards the estrogen receptor with respect to naringenin, that exhibits only weak estrogenic properties (*Milligan et al*, 1999; *van Lipzig et al*, 2004).

To evaluate its estrogenic activity, Diel and coworkers tested the effect of 8-PN *in vivo*: a dose of 10 mg/kg given once or for 3 days caused uterotrophic response and a longer application of two weeks had positive effects on bone density and urinary bone turnover

markers (*Diel et al*, 2004). It has been also reported that oral administration of hop extract is able to positively influence postmenopausal hot flushes. In particular, a prospective, randomized, double-blind, placebo-controlled study has been performed. The daily administration of a hop extract standardized on 8-PN (100 or 250 μg) to these women decreased the incidence of hot flushes and other discomforts associated to estrogen deficiency (*Heyerick et al*, 2006).

Furthermore different studies have observed that 8-PN possesses anti-angiogenic effects (*Pepper et al*, 2004) and is able to upregulate the function of the E-cadherin/catenin complex in human mammary carcinoma cells (*Rong et al*, 2001). In addition 8-PN has been evaluated as an inhibitor of cytochrome P450 isozymes (*Henderson et al*, 2000).

However despite its potential application to the treatment and prevention of a wide range of diseases, at present human exposure to 8-PN occurs primarily only through the consumption of hop-derived products, such as beer and an increasing number of dietary supplements containing hop extracts to reduce the discomforts associated to menopause (*Nikolic et al*, 2004) and inducing “breast enhancement” (*Milligan et al*, 1999).

Flavonoids and platelets

Certain dietary flavonoids have been shown *ex vivo* to inhibit platelet function (*Janssen et al*, 1998; *Pearson et al*, 2002)., Different mechanisms of inhibition of platelet signaling pathways have been hypothesized *in vitro*, and some studies have related the flavonoid structure to their inhibitory potential. Thus, flavonoids have been shown to impair enzymes activity involved in cellular signaling such as cycloxygenases and lypoxygenases (*Polette et al*, 1996), phosphodiesterases (*Landolfi et al*, 1984), tyrosine kinases (*Akiyama et al*, 1987) and phospholipases (*Lindahl et al*, 1997; *Lee et al*, 1997), and to affect calcium mobilization and subsequent platelet secretion (*Navarro-Núñez et al*, 2009). Moreover studies in rats have

demonstrated that genistein and raloxifene could affect platelet aggregation through their direct effect on vascular tissue by increasing nitric oxide synthesis, enhancing phospholipase A2 and prostacyclin release in an estrogen receptor-dependent manner (*Polini et al*, 2007).
Moreover it has been shown that genistein displays a strong inhibitory effect on both inside-out signaling and outside-in signaling events on human platelets, as assessed by abrogation of platelet adhesion to collagen, and spreading on fibrinogen, and that the antiplatelet activity of quercetin may be due, at least in part, to the inhibition of multiple platelet kinases (*Hubbard et al*, 2003; *Navarro-Núñez et al*, 2010)

AIM

Phytoestrogens are plant-derived compounds that structurally and functionally mimic mammalian estrogens (*Cos et al*, 2003). Indeed, similarly to the numerous effects of estrogens on the human body, phytoestrogens are known to exert beneficial effects toward human health. Moreover it has been reported that a diet rich in flavonoids with phytoestrogenic activity may decrease the incidence of hormone-dependent tumors (*Messina & Barnes*, 1991), osteoporosis, menopausal syndrome and coronary heart disease (*Knight & Eden*, 1995). Regarding cardiovascular diseases it has been extensively demonstrated that certain dietary flavonoids are able to inhibit platelet function (*Janssen et al*, 1998; *Pearson et al*, 2002). Different mechanisms of inhibition of platelet signaling pathway have been hypothesized, and some *in vitro* studies have related the flavonoid structure to their inhibitory potential. Thus, flavonoids have been shown to impair the activity of enzymes involved in platelet signaling and to affect calcium mobilization and subsequent platelet secretion (*Navarro-Núñez* et al, 2009).

On the other hand, previous studies in our laboratory have reported that in platelets stimulated with low concentrations of thrombin, 17β-estradiol caused a strong potentiation of integrin αIIbβ3 activation and platelet aggregation, through a mechanism depending on ERβ engagement and Src kinase activation (*Moro et al*, 2005). This estrogen signaling pathway is coordinated by membrane lipid rafts. Indeed ERβ reversibly translocated to the lipid raft fractions in a hormone-dependent manner, and promoted the rapid and transient recruitment and activation of the tyrosine kinases Src and Pyk2 within the membrane raft domains (*Reineri et al*, 2007).

Among flavonoids, we have focalized our attention on 8-prenylnaringenin, a prenylated flavanone with a potent estrogenic activity. This molecule is extracted from the lupulin glands

of the hop flowers and can be found in beer in a concentration of 0.24 mg/l (*Stevens et al*, 1999; *Rong et al*, 2001) and in an increasing number of dietary supplements containing hop extracts to reduce the discomforts associated to menopause (*Nikolic et al*, 2004) and to induce "breast enhancement" (*Milligan et al*, 1999). Due to its estrogenic potency, safety questions arise concerning unrestricted long-term use of freely accessible herbal preparations, and there is a need to study long term applications. However, no investigations of 8-PN role in modulating platelet function have been undertaken to date. On the basis of these considerations, in the present report we sought to evaluate whether this phytoestrogen could influence platelet activation and aggregation with the objective to define the mechanisms of platelet function modulated by 8-PN.

METHODS

PLATELET PREPARATION

Blood was withdrawn from healthy volunteers, who had not taken any drugs for at least ten days before venipuncture, using ACD (130 mM citric acid; 152 mM sodium citrate; 112 mM glucose) as anticoagulant. Platelets were isolated by gel-filtration on Sepharose CL-2B and eluted with Tyrode buffer (10 mM HEPES, 137 mM NaCl, 2.9 mM KCl, 12 mM NaHCO3, pH 7.4). Platelets were counted and their concentration was adjusted in Tyrode buffer containing 2 mM $CaCl_2$, 2 mM $MgCl_2$ and 2 mM glucose.

MEASUREMENT OF PLATELET AGGREGATION

Samples of gel-filtered platelets ($3x10^8$ cells/ml) were pre-warmed at 37°C under constant stirring (1000 rpm), stimulated with different concentration of 8-PN or vehicle (CH_3OH 0.125%) for 5 minutes and then induced to aggregate with 5 µg/ml of collagen. Transmittance of platelet samples was monitored continuously up to at least 5 min in a Chronolog lumi-aggregometer. In some experiments inhibitors were added before 8-PN. L-NMMA and L-NAME were used at 300 µM for 5 min; ODQ at 2 µM for 5 min; for all the inhibitors control samples were treated with vehicle for the same times.

ELECTROPHORESIS AND IMMUNOBLOTTING ANALYSIS

Samples of gel-filtered platelets ($5x10^8$ cells/ml) were preincubated with 30 µM 8-PN or methanol 0.125% from 30 seconds to 15 minutes at 37°C and stimulated with 5 µg/ml collagen for different times, where indicated. In some experiments inhibitors were added before 8-PN stimulation. The reaction was stopped by adding of 3x Laemmly buffer (6%

SDS, 1.5% DTT, 30% glycerol, 0.03% bromophenol blue, 3x protease inhibitors mix, 3 mM phosphate glycerol, 20 mM sodium pyrophosphate, 1 mM Na_3VO_4, 10 mM NaF, 1 mM EDTA). Proteins were separated by SDS-PAGE, transferred to PVDF membranes, and probed with specific antibodies. Immunoreactive bands were detected by an ECL reaction using a Versadoc instrument and analyzed with QuantityOne software (Bio-Rad, Milan, Italy).

PLATELET cAMP DETERMINATION

Gel-filtered human platelets (1 x 10^8 cells/ml) were stimulated with 8-PN 30 µM after 1 minute with 5 µM forskoline to elevate intracellular cAMP concentration. The reaction was stopped with 96% ice-cold ethanol after 5 minutes. Then samples were centrifuged at 15000 x g for 10 minutes at 4°C. The supernatants were dried under vacuum and platelet cAMP levels were measured using the cAMP Biotrak Enzyme Immunoassay (EIA) system (Amersham GE Healthcare) following the manufacturer's instructions.

PLATELET cGMP DETERMINATION

To prepare platelet sample to evaluate cGMP levels we have followed the protocol previously described by Dunkern and Hatzelmann (*Dunkern & Hatzelmann,* 2005). Briefly, gel-filtered platelets (4x10^8 cells/ml) were rendered in 5.5 mM glucose and incubated for 5 minutes with 30 µM 8-PN or 0.1 mmol/L sodium nitroprussiate, as positive control. Sometimes different inhibitors were added, as indicated. The reaction was stopped using 0.1 M HCl and incubated for 10 minutes at RT. Lysates were centrifuged at 1000 x g for 10 min at 4°C. The supernatants were recovered and stored at -20°C. Samples were then analyzed for their cGMP content using the cyclic GMP Complete (Assay Designs, Vinci, FI Italy) following the manufacturer's instructions for the acetylation protocol.

ADHESION ASSAY

Glass coverslips (12 mm Ø) were coated for 2 hours at 37°C with 100 µg/ml of collagen type I or 0.5% BSA, all diluted in PBS1X. Coverslips were then washed 3 times with PBS, blocked with 5% BSA in PBS for 2 hours at 37°C, and then washed with PBS 3 more times. Human gel-filtred platelets were diluted to 3 x 10^7 platelets/ml in Tyrode buffer containing 2 mM $MgCl_2$, 2 mM $CaCl_2$ e 5.5 mM glucose and stimulated with 30 µM 8-PN or 0.125% CH_3OH for 5 minutes. Aliquots of stimulated platelet (0.2 ml) were added to the coated coverslips and incubated for 30 or 60 minutes at 37°C. Non-adherent cells were removed and dishes were washed 3 times with PBS. Adherent platelets were fixed for 10 minutes at room temperature with 3% paraformaldehyde-4% sucrose in PBS, permeabilized with 0.2% Triton-X100 for 5 minutes, and stained with Alexa488-phalloidin. Platelets were viewed on a confocal microscope (Leica TCS SP2) and digital images (63X) were acquired. The number of adherent cells as well as the average cell area (index of platelet spreading) were determined. At least 10 different fields were analyzed for each specimen.

STATISTICAL ANALYSIS

Data reported are the means ± SD or SEM of at least three independent experiments. Statistical analysis was performed by the Student's t test or one-way Anova followed by Bonferroni post-hoc test. Significant differences were accepted at $p<0.05$ at least. Data were analyzed using StatMate software, version 4.0 (GraphPad, San Diego, CA, USA).

RESULTS

8-PN INHIBITS PLATELET AGGREGATION

8-prenylnaringenin is one of the most potent phytoestrogen *(Milligan et al,* 1999*)*. Previous studies in our laboratory have demonstrated that estrogens are able to potentiate platelet aggregation induced by thrombin (*Moro et al,* 2005). On the other hand it has been shown that many flavonoids are able to inhibit platelet functionality. However, to date no investigations on 8-PN role in modulating platelet activity have been undertaken. For these reasons we have focalized our attention on the effects of 8-PN on platelet activation and aggregation.

8-PN was not able to elicit platelet aggregation *per se* (data not shown), thus we investigated if pre-treatment of platelets with 8-PN was able to modulate platelet aggregation induced by collagen.

In order to do that, samples of gel-filtered platelets (3×10^{8} /ml) were incubated with increasing doses of 8-PN from 0.5 µM to 40 µM of 8-PN, or vehicle, for 5 minutes prior stimulation with 5 µg/ml of collagen.

Our results have shown that 8-PN was able to inhibit platelet aggregation induced by collagen in a concentration-dependent manner, with an IC50 of 12.8 µM. Besides maximum inhibitory effect was achieved at a concentration of 30 µM, that caused a 76.17±3.72% inhibition of platelet aggregation (**Fig. 7**). Thus, we have decided to used 30 µM 8-PN in all the subsequent experiments.

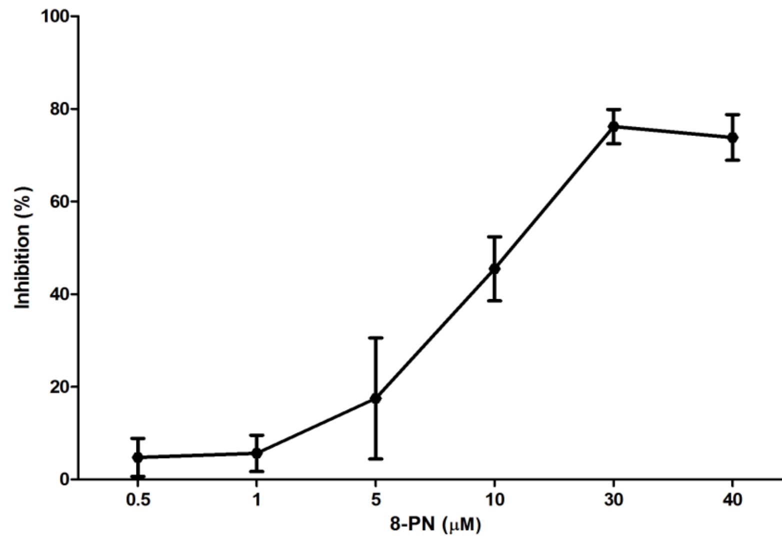

Fig. 7:

8-PN inhibits platelet aggregation in a dose-dependent manner*. Gel-filtred platelets were incubated with different concentrations of 8-PN and then induced to aggregate with collagen (5 µg/ml). Results are expressed as the % of inhibition of platelet aggregation with respect to control (100% of aggregation). Data are the means ± SD of three independent experiments.*

To better understand the dynamic of the inhibitory activity of 8-PN on platelet aggregation, a time course was performed. Gel-filtered platelets were stimulated with 30 µM 8-PN or vehicle (CH_3OH 0.125%) for increasing periods of time (from 1 minute to 2 hours) and then induced to aggregate with 5 µg/ml of collagen. All data have been normalized to an aggregation with collagen alone, arbitrarily set to 100%. Our results have shown that 8-PN was able to inhibit constantly platelet aggregation (**Fig. 8**).

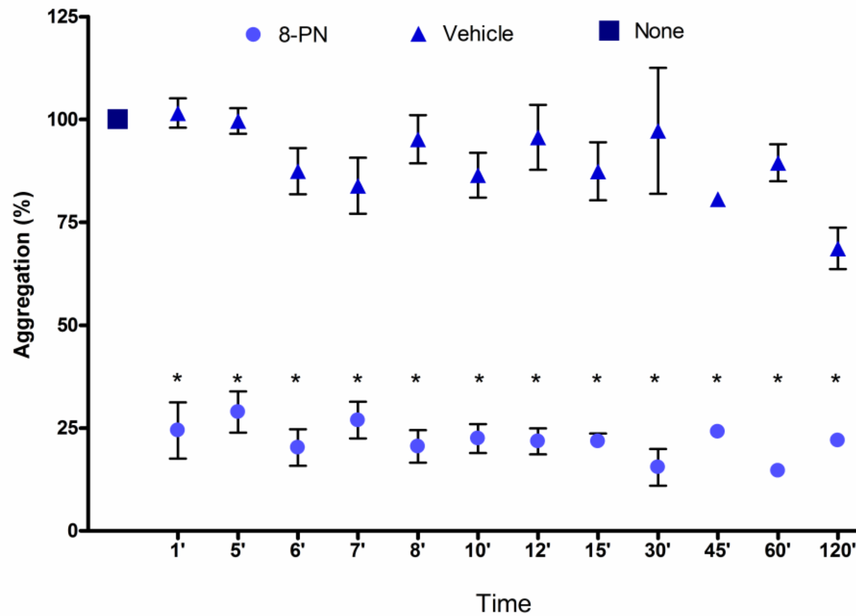

Fig. 8:

***8-PN irreversibly inhibits platelet aggregation**. Gel-filtered platelets were incubated with 30 µM 8-PN or vehicle (CH_3OH 0.125%) for increasing periods of time and then induced to aggregate with collagen (5 µg/ml). Results are expressed as the % of platelet aggregation with respect to untreated cells (None). Data are the means ± SD of three independent experiments. Asterisks represent significant differences determined by Student's T test (*$p<0.01$).*

Subsequently, to evaluate if 8-PN ability to inhibit platelet aggregation was dependent by its presence in the medium, a wash out assay was performed. Briefly, gel-filtered platelets were stimulated with 30 µM 8-PN or vehicle for 5 minutes and then centrifugated 300 x g for 10 minutes. Pellets were resuspended in freshly prepared Tyrode with glucose, $CaCl_2$ and $MgCl_2$. Platelets were then induced to aggregate with 5 µg/ml collagen. Our data have shown that 8-PN was able to inhibit platelet aggregation even if it was no longer present in the medium (data not shown).

8-PN PROMOTES VASP PHOSPHORYLATION IN HUMAN PLATELETS

The best characterized inhibitory pathways in platelets lead to intraplatelet cGMP and cAMP increase, activation of PKG and PKA respectively, and phosphorylation of many substrates, among which VASP. When phosphorylated, VASP shows a reduced ability to bind cytoskeletal proteins, inhibiting the cytoskeleton reorganization, essential event for irreversible platelet aggregation (*Reinhard et al*, 2001). Therefore we decided to analyze the contribution of 8-PN in the activation of cGMP/PKG and cAMP/PKA pathways.

First of all, we investigated the phosphorylation state of VASP. VASP is the major substrate for PKG and also PKA. PKA and PKG preferentially phosphorylate VASP on Ser157 and Ser239, respectively. Noteworthy, phosphorylation on Ser157 produces a characteristic electrophoretic mobility shift of VASP from 46 to 50 kDa (*Butt et al*, 1994).

Thus, gel-filtred platelets (5 x 10^8/ml) were incubated for increasing periods of time from 10 seconds to 15 minutes with 30 µM 8-PN or vehicle and analyzed by immunoblotting with an anti-P-Ser239- or an anti P-Ser-157-VASP antibody (both from Calbiochem, San Diego, CA, USA). Our data have shown that 8-PN was able to phosphorylate VASP on both Ser239 and Ser157 in a time-dependent manner (**Fig. 9**), suggesting that 8-PN may promote VASP phosphorylation by activating both PKG and PKA.

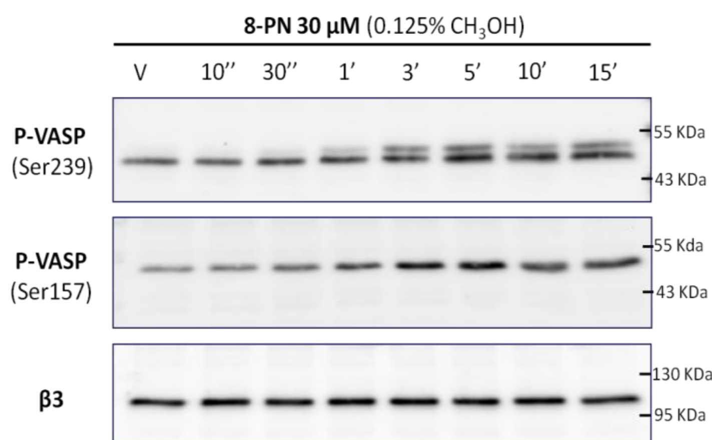

Fig. 9:

8-PN phosphorylated VASP in a time-dependent manner. *Platelets were incubated at 37°C with vehicle (V), or 30 µM 8-PN for the indicated times. Aliquots containing the same amount of proteins were analyzed by immunoblotting with anti-P-Ser239-VASP, anti-P-Ser157-VASP and anti-β3 antibodies. The immunoblotting experiment shown is representative of 3 identical experiments performed with platelets obtained from different donors.*

It has been reported that 8-PN, as well as other phytoestrogens, is able to signal through the estrogen receptors (*Milligan et al*, 2000). Previous studies in our laboratory have demonstrated that estrogens have a proaggregatory activity in human platelets (*Moro et al*, 2005), while 8-PN acts as an anti-platelet agent. To evaluate the involvement of the estrogen receptors in the inhibitory action of 8-PN, we have analyzed the effect of ICI 182,780, an antagonist of estrogen receptors, on VASP phosphorylation induced by 8-PN. Gel-filtered platelets (5 x 10^8/ml) were incubated with vehicle (Methanol 0.125%) or 30 µM 8-PN in absence or presence of 10 µM ICI 182,780 and analyzed by immunoblotting with anti-P-Ser239-VASP antibody (Calbiochem, San Diego, CA, USA). Our data showed that ICI 182,780 had no effects on VASP phosphorylation induced by 8-PN (**Fig. 10**). This result suggest that estrogen receptors were not involved in 8-PN signaling in platelets.

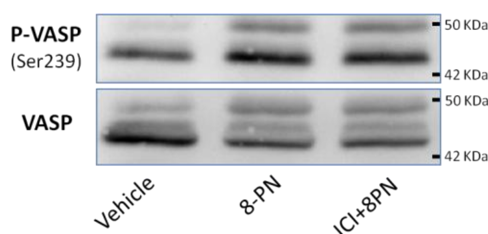

Fig. 10:

ICI 182,780 does not prevent 8-PN-mediated VASP phosphorylation. *Platelets were incubated at 37°C with 10 µM ICI 182,780 for 5 minutes, where indicated, and then stimulated with vehicle, or 30 µM 8-PN for 5 minutes. Aliquots containing the same amount of proteins were analyzed by immunoblotting with anti-P-Ser239-VASP and anti-VASP antibodies. The immunoblotting experiment shown is representative of 3 identical experiments performed with platelets obtained from different donors.*

8-PN ACTIVATES THE NOS/CGMP/PKG PATHAWAY IN HUMAN PLATELETS

To confirm the role of PKA and PKG downstream of 8-PN signaling, we have measured the intracellular content of cyclic nucleotides, because the classical pathways that activate PKG and PKA depend on the availability of cGMP and cAMP.

To study the elevation in cAMP content by 8-PN, the basal cAMP concentration was increased by treating cells with 5 µM forskolin for 1 minute prior to stimulation. In this condition 30 µM 8-PN was able to triple forskolin-induced cAMP levels (from 22.55±0.55 with forskolin alone, to 61.43±0.40 fmol/10^6 platelets) (**Fig. 11**), whereas 2 µM PGE2 for 1 minutes increased the cAMP content of 27.50±0.46 folds over basal (from 2.7±0.06 (basal) to 74.26±0.46 fmol/10^6 platelets), indicating that 8-PN increased the concentration of cAMP,

however this was significantly lower than the physiological concentration known to inhibit platelets.

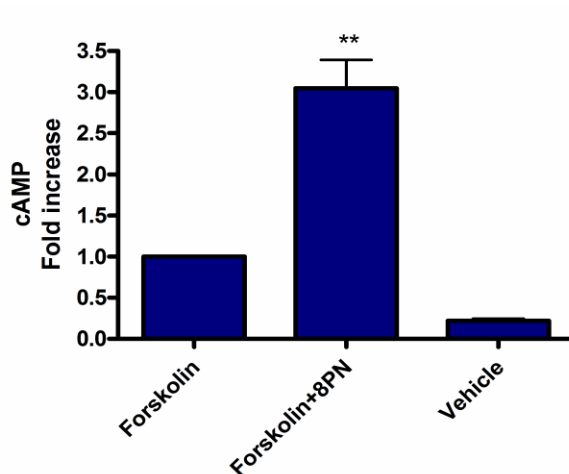

Fig 11:

Effects of 8-PN on cAMP levels. *Gel-filtered platelets (1 x 10^8 /ml) were treated with methanol 0.125% (Vehicle) or with 5 µM forskolin for 1 minute to induce intracellular cAMP generation, and then incubated with 30 µM 8-PN, where indicated. Platelet cAMP levels were measured using a kit from Amersham GE Healthcare. Histograms represent the means ± SEM of at least 3 independent experiments. Asterisks represent significant differences from 5 µM forskolin-stimulated sample determined by Student's T test (**P<0.01 vs 5 µM forskolin-stimulated sample).*

Subsequently we evaluated cGMP levels. Our data demonstrated that 30 µM 8-PN induced a 2.34 ± 0.03 fold increase in cGMP respect to non-treated cells (from 4.82 ± 1,18 pmol/10^9 platelets (basal level) to 9.05 ± 2.3 pmol/10^9 platelets) while the well-characterized NO donor, sodium nitroprusside (100 µM SNP for 1 minute) increased the cGMP concentration of 5.14±0.06 folds over basal (data not shown) (**Fig. 12**). It is known that cGMP levels are increased in response to established platelet inhibitors such as NO which directly activates the soluble guanylyl cyclase (sGC) (*Wollny et al*, 1999). Therefore we have evaluated the

activation of NOS and sGC. Platelets were treated with 30 μM 8-PN or 0.125% methanol for 5 minutes after preincubation with L-NAME (NG-Nitro-L-arginine Methyl Ester), a non selective inhibitor of all the NOS isoforms, and L-NMMA (NG-Monomethyl-L-arginine, Monoacetate Salt), the eNOS and iNOS inhibitor, or with 2 μM ODQ (1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one), a competitive inhibitor of GC, for 5 minutes. The measurement of intracellular cGMP contents revealed that, ODQ and the two NOS inhibitors were able to prevent the increase of cGMP levels induced by 8-PN (**Fig. 12**).

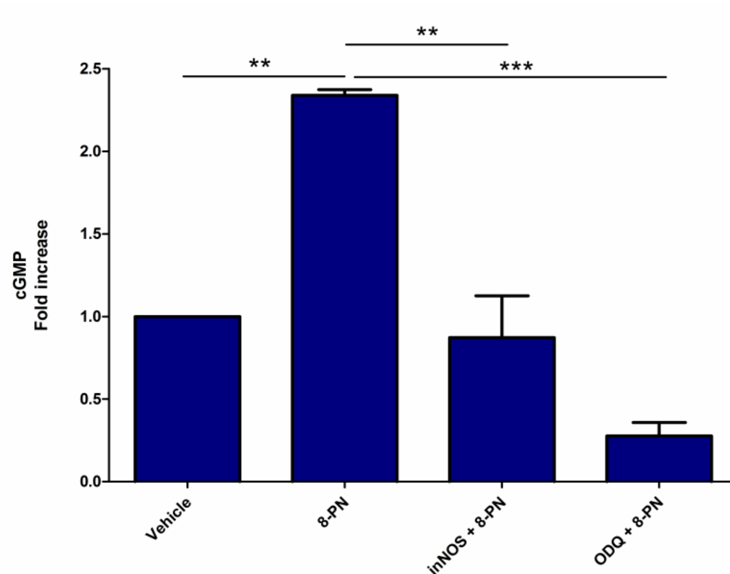

Fig 12:

***Effects of 8-PN on cGMP levels**. Gel-filtered platelets (4 x 10^8 /ml) were treated with ODQ or L-NAME and L-NMMA (inNOS) where indicated, prior stimulation with methanol 0.125% (Vehicle) or 30 μM 8-PN. cGMP levels were determined by the cGMP complete EIA Kit. Histograms represent the means ± SEM of at least 3 independent experiments. Asterisks represent statistical significance compared to 8-PN cGMP levels determined by one-way Anova followed by Bonferroni post-hoc test. (**p<0.01 and *** p<0.001 vs 8-PN-stimulated sample).*

To better characterize the involvement of sGC and NOS in 8-PN signaling, we have evaluated VASP phosphorylation in platelets treated with ODQ or L-NAME and L-NMMA prior incubation with 8-PN or vehicle.

Our data suggest that ODQ, but not the NOS inhibitors, was able to prevent 8-PN-mediated VASP phosphorylation on both Ser157 and Ser239 (**Fig. 13**).

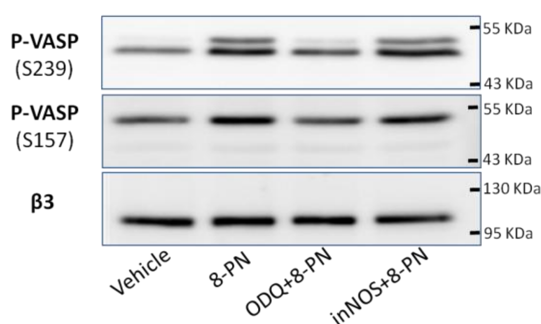

Fig. 13:

Effects of sGC and NOS inhibitors on VASP phosphorylation induced by 8-PN. *Platelets were incubated with 2 µM ODQ or L-NAME and L-NMMA 300 µM each (inNOS) for 5 minutes at 37°C, where indicated, prior stimulation with methanol 0.125% (Vehicle) or 30 µM 8-PN for 5 minutes. Aliquots containing the same amount of proteins were analyzed by immunoblotting with anti-P-Ser239-VASP, anti-P-Ser157-VASP and anti-β3 antibodies. The immunoblotting experiment shown is representative of 3 identical experiments performed with platelets obtained from different donors.*

ACTIVATION OF NOS/CGMP/PKG PATHAWAY IS NOT INVOLVED IN 8-PN INHIBITION OF PLATELET AGGREGATION

In order to verify whether the inhibition of platelet aggregation was dependent on VASP phosphorylation induced by 8-PN stimulation, platelets were treated with ODQ or the combination of L-NAME and L-NMMA for 5 minutes. Subsequently, platelets were stimulated with 8-PN for 5 minutes and induced to aggregate with collagen.

Surprisingly, the inhibition of platelet aggregation caused by 8-PN was reverted neither by inhibition of the guanylyl cyclase activity nor by inhibition of NOS (**Fig. 14**).

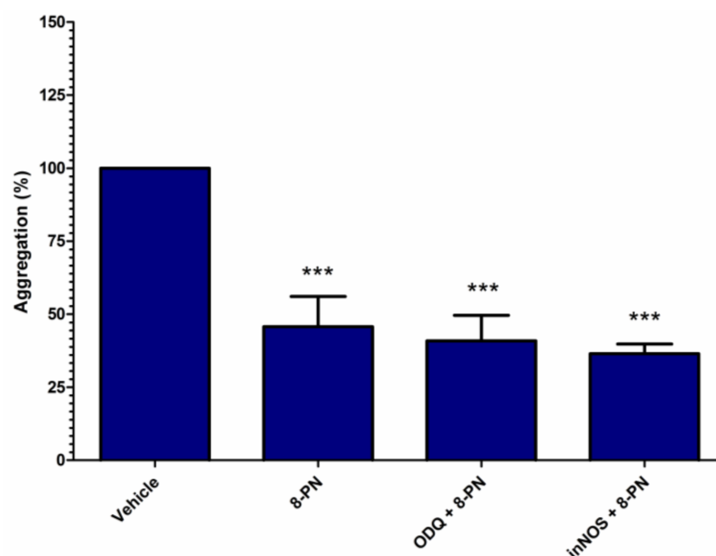

Fig. 14:

***sGC inhibitor and NOS inhibitors do not prevent 8-PN-mediated blockade of platelet aggregation**.*

*Gel-filtered platelets were incubated with 2 µM ODQ or 300 µM L-NAME and 300 µM L-NMMA (inNOS) for 5 minutes, where indicated, prior stimulation with methanol 0.125% (Vehicle) or 30 µM 8-PN for 5 minutes and then induced to aggregate with collagen (5 µg/ml). Results are expressed as the % of platelet aggregation with respect to cells treated with vehicle alone (100%). Data are the means ± SD of at least three independent experiments. Asterisks represent statistical significance determined by one-way Anova followed by Bonferroni post-hoc test. (*** p<0.001).*

Taken together these results suggest that the NO/cGMP/PKG pathway, although activated in human platelets by 8-PN, was not involved in 8-PN-mediated inhibition of platelet function.

8-PN INHIBITS PROTEIN PHOSPHORYLATION

Tyrosine phosphorylation plays an important role in regulating different cell functions. An increasing number of studies regarding tyrosine phosphorylation indicate that platelets present a high tyrosine kinase activity. The precise role of protein tyrosine phosphorylation in signal transduction in platelets is not fully understood. However, it occurs in response to many stimuli, involves multiple pathways and has functional implications. Diaz-Ricart and coworkers have demonstrated that tyrosine phosphorylation of proteins is crucial in the development of platelet activation and functional activity. When challenged with two of the strongest physiological agonists, thrombin or collagen, platelets underwent a series of morphological and biochemical transformations including shape change, secretion, aggregation and expression of procoagulant activity. It can be inferred that all these events are orchestrated by tyrosine phosphorylation of proteins (*Diaz-Ricart et al*, 2008).

Therefore, we analyzed if 8-PN was able to inhibit collagen-induced tyrosine phosphorylation by immunoblotting with anti-phosphotyrosine antibody.

8-PN treatment did not have any effect on the basal state of protein phosphorylation. As expected platelet stimulation with collagen for different times (90 seconds, 3 and 5 minutes) induced tyrosine phosphorylation of several proteins with different molecular weights. Moreover, pretreatment with 30 μM 8-PN resulted in an inhibition of protein tyrosine phosphorylation induced by 5 μg/ml collagen (**Fig. 15**).

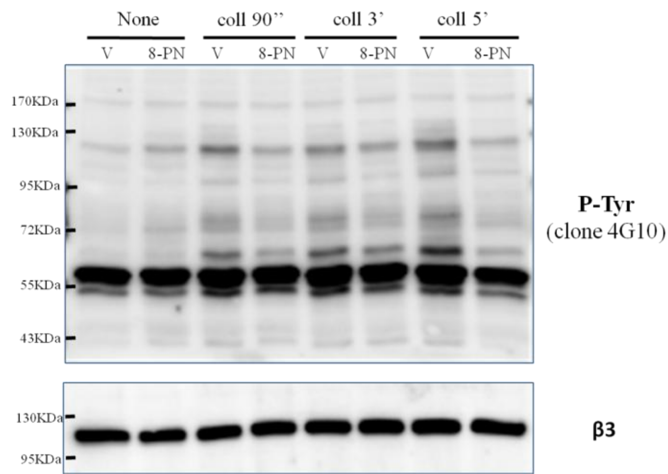

Fig. 15:

Effects of 8-PN on collagen-induced protein tyrosine phosphorylation. *Samples of gel-filtered platelets (5 x 10^8 plt/ml) were incubated at 37°C with vehicle (V), or 30 µM 8-PN for 5 minutes and then stimulated with collagen (5 µg/ml) for 90 seconds, 3 and 5 minutes. Aliquots containing the same amount of proteins were analyzed by immunoblotting with anti-phosphotyrosine (4G10) and anti-β3 antibodies. The immunoblotting experiment shown is representative of 3 identical experiments performed with platelets obtained from different donors.*

It has been extensively demonstrated that tyrosine phosphorylation levels within the cell are regulated by tyrosine kinases and tyrosine phosphatases and that tyrosine kinases inhibitors are able to down-regulate a large number of platelet responses (*Jackson et al,* 1996). Therefore we analyzed the phosphorylation pattern of several kinases: Pyk2 tyrosine kinase, Akt serine/threonine kinase and Erk 1/2 MAP kinases. Gel-filtered platelets were incubated with 30 µM 8-PN for 5 minutes prior stimulation with collagen for increasing periods of time. Identical aliquots of each sample were analyzed by immunoblotting with a phospho-specific antibody recognizing the active forms of Pyk2 (P-Pyk2 Tyr402), ERK1/2 (P-ERK 1/2 Thr202/Tyr204) (**Fig. 16A**) and Akt (P-Akt Ser473) (**Fig. 16B**).

As shown in Figure 16, pretreatment of platelets with 8-PN was able to inhibit collagen-induced phosphorylation of all the analyzed proteins.

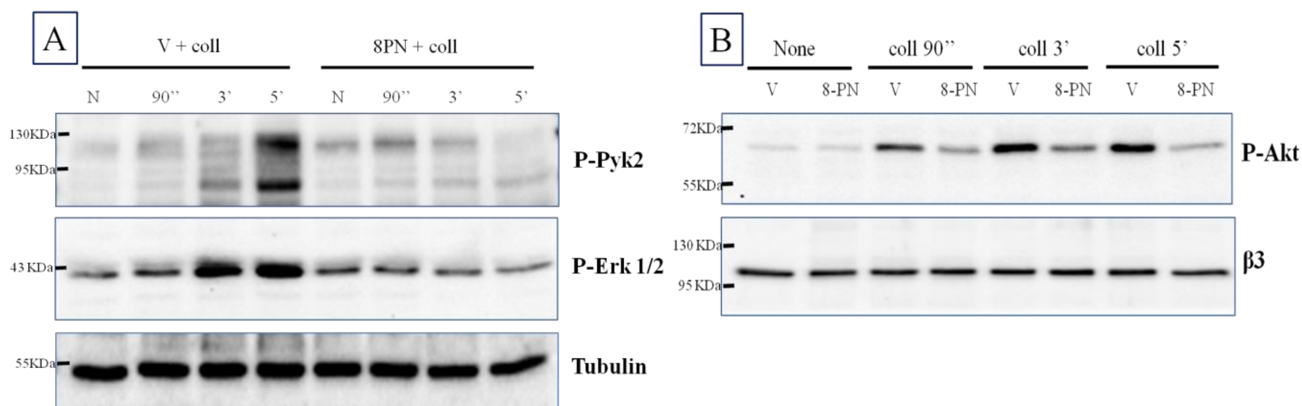

Fig. 16:

Effects of 8-PN on collagen-induced protein phosphorylation. *Samples of gel-filtered platelets (5 x 10^8 plt/ml) were incubated at 37°C with vehicle (V), or 30 μM 8-PN for 5 minutes and then stimulated with collagen for 90 seconds, 3 and 5 minutes. Aliquots containing the same amount of proteins were analyzed by immunoblotting with anti-P-Pyk2, anti-P-Erk 1/2, anti-Tubulin (**A**), anti-P-Akt and anti-β3 (**B**) antibodies. The immunoblotting experiment shown is representative of 3 identical experiments performed with platelets obtained from different donors.*

EFFECTS OF 8-PN ON PLATELET ADHESION

Previous studies have reported that platelet activation by collagen requires a functional cytoskeleton to trigger signaling through tyrosine phosphorylation (*Diaz-Ricart et al*, 2002). Therefore we have evaluated the effects of 8-prenylnaringenin on platelet adhesion to collagen matrix. Gel-filtered platelets were incubated with 30 μM 8-PN or vehicle for 5 minutes. Subsequently, platelets were let adhere to coated coverslips for 30 or 60 minutes at 37°C. Adherent platelets were fixed, permeabilized, and stained by Alexa488-phalloidin. Platelets were observed on a confocal microscope (Leica TCS SP2) and digital images (63X)

were acquired. Our results have shown that 8-PN was able to modify the number (**Fig. 17A**), the dimensions (**Fig. 17B**) and the morphology (**Fig. 17C and 17D**) of adherent platelets. These effects were more appreciable after 60 minutes of adhesion.

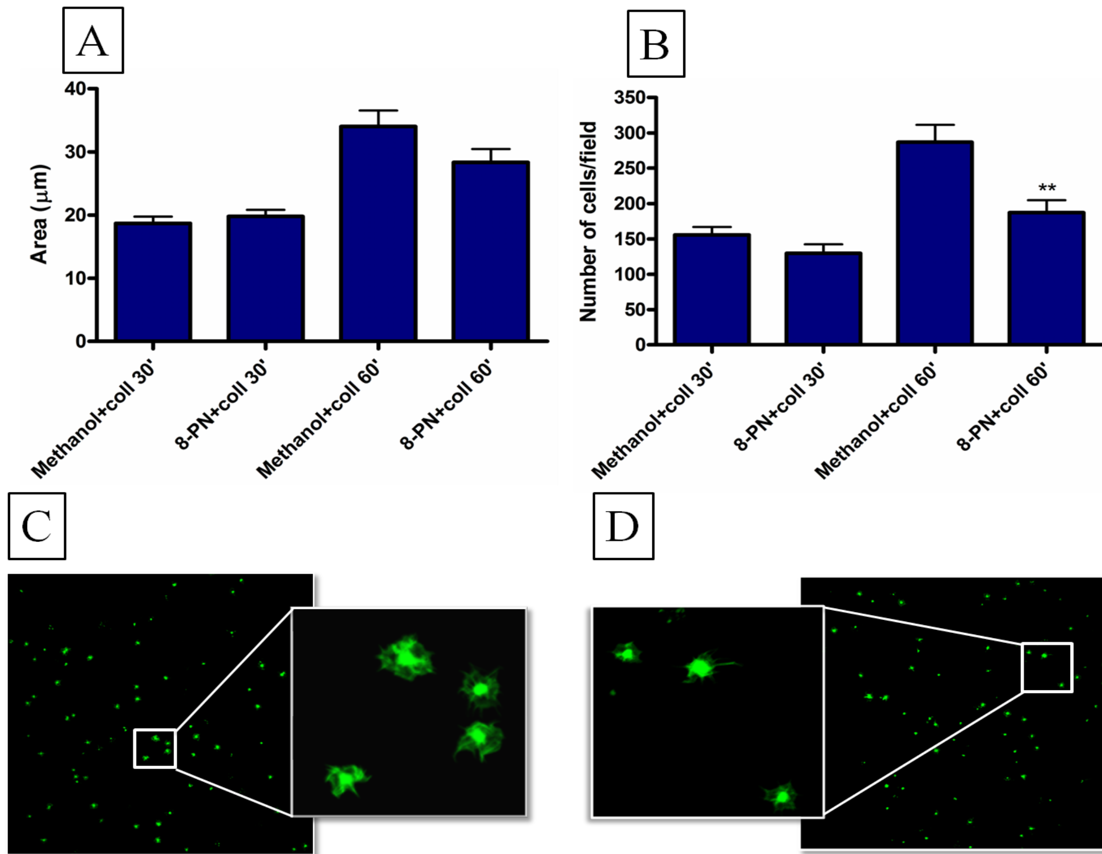

Fig. 17:

Effects of 8-PN on platelet adhesion. *Samples of gel-filtered platelets (3 x 10^7 plt/ml) were stimulated with 30 µM 8-PN or methanol 0.125% for 5 minutes and then let adhere to coated coverslips with 100 µg/ml collagen for 30 or 60 minutes at 37°C. The number and the morphology of adherent platelets were viewed on a confocal microscope (Leica TCS SP2) and digital images (63X) were acquired. Image* ***A*** *shows the number of adherent platelets in each field, image* ***B*** *shows the area of adherent platelets in each field. Images* ***C*** *and* ***D*** *show the morphology of adherent cells treated with methanol (**C**) or 8-PN (**D**). Data are the means ± SEM of at least three independent experiments. Asterisks represent statistical significance determined by Student's t test (** p=0.003).*

CONCLUSIONS

Phytoestrogens are plant-derived polyphenolic compounds that structurally and functionally mimic mammalian estrogens (*Cos et al*, 2003). These molecules are known to exert long-term beneficial effects on human and animal health. In particular it has been reported a strong correlation between flavonoids intake and a decrease risk of cardiovascular deseases, probably caused by their direct action on platelet functionality (*Janssen et al*, 1998; *Hubbard et al*, 2003).

In the last decade the prenylated flavanone 8-prenylnaringenin was focused as a novel phytoestrogen, unique with respect to receptor specificity and potency, which is much more higher than that of other phytoestrogens so far investigated (*Milligan et al*, 2000). This molecule is extracted from the lupulin glands of the hop flowers and can be found in beer at the concentration of 0.24 mg/l (*Stevens et al*, 1999; *Rong et al*, 2001) and in an increasing number of dietary supplements containing hop extracts to reduce the discomforts associated to menopause (*Nikolic et al*, 2004) and to induce "breast enhancement" (*Milligan et al*, 1999). However, to date no investigations of 8-PN role in modulating platelet function has been undertaken. Given these considerations, in the present study we have evaluated whether this phytoestrogen could influence platelet functionality with the objective to define the mechanisms of platelet function modulated by 8-PN.

Our results show for the first time that 8-prenylnaringenin was able to irreversibly inhibit platelet function and in particular the aggregation, spreading, and adhesion of platelets stimulated with collagen (**Fig. 8** and **17**). This effect was irreversible (**Fig. 8**) and was exerted in a dose-dependent manner, with an IC50 of 12.8 μM and a maximum inhibitory activity at 30 μM (**Fig. 7**).

8-PN is one of the most potent phytoestrogens and is known to signal through the estrogen

receptors (*Milligan et al*, 2000). Recently we have demonstrated that 17β-estradiol is able to potentiate the aggregation caused by low doses of thrombin *in vitro* (*Moro et al*, 2005); on the other hand our data suggest that 8-PN acts as an anti-platelet agent. In order to clarify this aspect we have used ICI 182,780, an antagonist of estrogen receptors. Our results have demonstrated that the inhibitory action of 8-PN on platelet function is not related to its ability to bind to the estrogen receptor, indeed ICI 182,780 was completely ineffective in counteracting 8-PN effects (**Fig. 10**).

This evidence leads to conclude that 17β-estradiol and 8-PN trigger different signals in platelets.

It has been extensively demonstrated that VASP phosphorylation, caused by cGMP and cAMP elevation, plays an essential role in platelet inhibition. Indeed, when phosphorylated, VASP shows a reduced ability to bind cytoskeletal proteins, thus leading to the inhibition of cytoskeleton reorganization, essential event for irreversible platelet aggregation (*Reinhard et al*, 2001).

Treatment of platelets with 8-PN promoted VASP phosphorylation on both Ser157 and Ser239 (**Fig. 9**) in a time-dependent manner, suggesting that both cAMP and cGMP elevation and the subsequent PKA and PKG activation were involved. Indeed it has been demonstrated that in a purified system, PKA and PKG preferentially phosphorylate VASP on Ser157 and Ser239, respectively. However, in intact cells each kinase can equally phosphosphorylate both Ser residues. Hence we directly measured the increase of both cAMP and cGMP upon 8-PN treatment.

Our results have shown that 8-PN was able to increase both cyclic nucleotide concentration in intact platelets (**Fig. 11** and **12**).

Cyclic nucleotides levels in cells may increase because of either active synthesis by guanylyl and adenylyl cyclases or inhibition of phosphodiesterases (PDE). 8-PN induced a very low

increase in cAMP levels compared to PGE1, a well known platelet antagonist acting through a Gs-protein coupled receptor. This cAMP elevation was more consistent with an inhibition of the PDE3 than with activation of adenylyl cyclase. In fact it is known that cGMP competes with cAMP at the catalytic site to inhibit PDE3, at cGMP concentrations similar to those activating PKG (*Nolte et al*, 1994), and thus increases cAMP.

To date the classical pathway leading to the increase in the cGMP levels in platelets entails stimulation of NOS to produce NO, which activates soluble guanylyl cyclases, even if the presence of NOS and the role of platelet-derived NO is still controversial. In any case we tested the involvement of both NOS and guanylyl cyclase through pharmacological inhibition. Our data showed that both sGC inhibitor (ODQ) and NOS inhibitors (L-NAME and L-NMMA) were able to prevent 8-PN-induced elevation of cGMP (**Fig. 12**). However only ODQ was able to prevent VASP phosphorylation in platelets treated with 8-PN (**Fig. 13**). Moreover neither ODQ nor NOS inhibitors blocked the effects of 8-PN on platelet aggregation (**Fig. 14**).

Taken together these data suggest that the inhibition of platelet aggregation mediated by 8-PN does not rely on the activation of the platelet NO/cGMP/PKG pathway.

Therefore we focused our attention on protein phosphorylation. Indeed it has been extensively demonstrated that tyrosine phosphorylation is crucial for platelet activation and functional activity and that phosphorylation levels within the cell are regulated by tyrosine kinases. Our results showed that 8-PN treatment did not have any effect on the basal state of protein phosphorylation. Nevertheless 8-PN inhibited the collagen-induced phosphorylation in tyrosine (**Fig. 15**) and the activation of several kinases: Pyk2, Akt, and Erk 1/2 (**Fig. 16**).

In conclusion in the present study we have demonstrated for the first time that 8-PN exerts antiaggregatory and antiadhesive effects on human platelets, independently of estrogen receptors. However the molecular mechanisms involved are not completely characterized. It is

possible that 8-PN functions with multiple mechanisms. Indeed it may be hypothesized that 8-PN acts as an activator of phosphatases or an inhibitor of multiple proteins essential for morphological and biochemical transformations that occur during platelet activation and aggregation. Further studies will be necessary to better clarify the mechanisms by which 8-PN works. Anyway we can conclude that 8-PN may represent a useful tool in the therapy and prevention of vascular diseases associated with platelet aggregation, such as atherosclerosis, myocardial infarction, coronary artery disease, and thrombosis. This phytoestrogen may be an effective negative regulator of platelet aggregation and it could be a valid alternative to estrogen therapy because it is able to mimic its beneficial effects and it may selectively block the unwanted effects as thrombosis.

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PhD Papers:

1. **Di Vito C**, Bergante S, Balduini A, Rastoldo A, Bagarotti A, Surico N, Bertoni A, Sinigaglia F. *The oestrogen receptor GPER is expressed in human haematopoietic stem cells but not in mature megakaryocytes.* Br J Haematol. 2010; 149:150-2.

2. Bertoni A, Rastoldo A, Sarasso C, **Di Vito C**, Bagarotti A, Sampietro S, Sinigaglia F. *Inhibition of thrombin-dependent platelet aggregation by dehydroepiandrosterone-sulfate. New foundations for dehydroepiandrosterone-based anti-platelet therapies.* Submitted.

Other papers:

1. **Di Vito C**, Emanuele E, Lista S. *Demineralized bone matrix enriched with human recombinant interleukin-11: a novel therapeutic option in treatment of delayed unions and nonunions?* Med Hypotheses. 2007; 69:954.

2. Minoretti P, Bruno A, **Di Vito C**, Emanuele E. *Leflunomide as an antiatherogenic drug.* Med Hypotheses. 2007; 68:1175-6.

3. **Di Vito C**, Bruno A, Emanuele E. *Potential usefulness of sevelamer hydrochloride to improve cardiac outcome in patients with coronary disease and normal renal function*. Med Hypotheses. 2007; 68:1182-3.

4. Minoretti P, Politi P, Coen E, **Di Vito C**, Bertona M, Bianchi M, Emanuele E. *The T393C polymorphism of the GNAS1 gene is associated with deficit schizophrenia in an Italian population sample.* Neurosci Lett. 2006, 397:159-63.

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The oestrogen receptor GPER is expressed in human haematopoietic stem cells but not in mature megakaryocytes

Megakaryocytes are bone-marrow precursor cells that differentiate to produce blood platelets via intermediate cytoplasmic extensions known as proplatelets (Patel *et al*, 2005). Recent advances in the understanding of megakaryocyte differentiation and platelet formation have been mostly obtained by biological studies of cultured cells. Although platelet formation from megakaryocytes has been actively studied, the molecular mechanisms of this process are still incompletely understood. Growing evidence has accrued that sex hormones may play a crucial role during megakaryopoiesis (Kostyak & Naik, 2007). Accordingly, it has been shown that high levels of oestrogens and conventional hormone replacement therapies increase the number of megakaryocytes in mice (Perry *et al*,2000) and in postmenopausal women (Bord *et al*, 2000). Nagata *et al* (2003) have also shown that oestradiol can be synthesised by murine megakaryocytes and that oestradiol positively affects proplatelet formation. In addition, Bord *et al* (2004) reported that oestrogens can promote megakaryocyte proliferation and maturation, thereby modulating the expression of the classical oestrogen receptors (ER)α and ERβ. We have previously demonstrated that oestrogens can potentiate platelet aggregation through a rapid and reversible ERβ-mediated signalling (Moro *et al*, 2005) and that membrane lipid rafts coordinate this pathway (Reineri *et al*, 2007).

Megakaryocytes and platelets are known to express ERβ (Khetawat *et al*, 2000). Genomic effects of oestrogens in megakaryocytes have been suggested to contribute to gender differences in platelet function. However, oestrogens can also induce rapid, non-genomic effects through interaction with classical ERs, localised on the plasma membrane, and through a member of the 7-transmembrane G protein-coupled receptor family, GPR30 (Cheskis *et al*, 2007), now known as G protein-coupled oestrogen receptor 1 (GPER). Despite the potential importance of GPER in megakaryocyte differentiation and maturation, its exact role in this process has not yet been determined. In this context, we sought to evaluate whether the expression of GPER could change during megakaryocytic differentiation.

$CD34^+$ cells were isolated from human cord blood and cultured in StemSpan® Serum-Free Expansion Medium medium (Stem-Cell Technologies, Vancouver, BC, Canada) supplemented with 10 ng/ml thrombopoietin, interleukin (IL)6 and IL11 (all from PeproTech EC Ltd, London, UK) at 37°C in 5% CO_2 to induce megakaryocyte differentiation.

After 13 days of culture cells were harvested, cytospun on glass coverslips, and stained with goat polyclonal anti-CD61 (Santa Cruz, Heidelberg, Germany) and secondary antibody conjugated with Alexa Fluor-488 (Invitrogen, Milan, Italy). Nuclear counterstaining was performed with Hoechst 33258. Specimens were mounted in Mowiol-488. Conventional fluorescence microscopy was performed through an Axioscope 2 Plus microscope (Carl Zeiss, Göttingen, Germany), using a 63/1·25 or a 100/1·30 Plan Neofluar oil-immersion objective. For each specimen, at least 100 cells were evaluated. The percentage of mature megakaryocytes positive for CD61 staining and with polyploid nuclei at the end of culture was 70 ± 15% (Fig 1).

To evaluate GPER expression during megakaryocyte differentiation three independent experiments were performed, each using a different pool of three cord blood samples (a total of nine cord blood samples was used), to eliminate any biological variability.

GPER expression was assessed in cells on days 0 ($CD34^+$), 3, 7 of differentiation, as well as in mature megakaryocytes isolated at day 13 by CD61 immunomagnetic beads technique (Miltenyi-Biotec, Bergisch Gladbach, Germany). Total RNA was extracted with the RNAqueous kit (Ambion Inc, Foster City, CA, USA) and reverse transcription reactions were

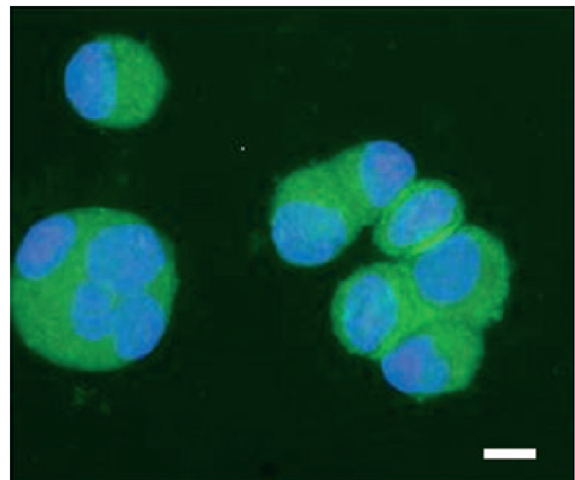

Fig 1. Representative immunofluorescence image of mature megakaryocytes derived from $CD34^+$ cells, as revealed by staining for CD61 (scale bar = 20 μm).

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doi:10.1111/j.1365-2141.2009.08028.x

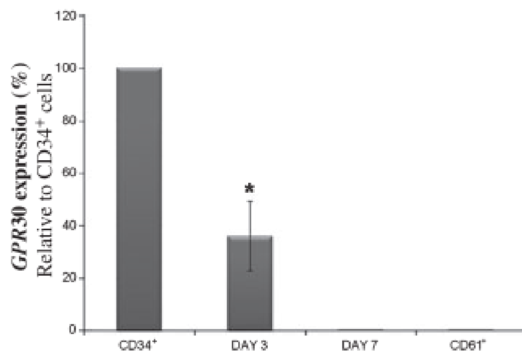

Fig 2. Differential expression of *GPER* during megakaryocyte differentiation. Results are expressed as percentage of *GPER* expression compared with expression levels in CD34$^+$ (100%). Each sample was analysed in triplicate. We performed gene expression analysis using the comparative ΔΔCT method. Values are mean ± SEM of three independent experiments.

performed using QuantiTect Reverse Transcription kit (Qiagen, Hilden, Germany). Real-time polymerase chain reaction was carried out on an ABI 7000 thermal cycler (Applied Biosystems, Foster City, CA 94404, USA) using the TaqMan chemistry. *GAPDH* was used as endogenous control, and CD34$^+$ cells at day 0 of culture were used as calibrator. Each sample was analysed in triplicate and the results were consistent. *GPER* expression in CD34$^+$ cells was arbitrarily set to 100. *GPER* expression decreased dramatically at day 3 (35·83 ± 13·25% with respect to calibrator; $^*P < 0{\cdot}01$). Moreover, *GPER* was no longer detectable both at day 7 and in mature megakaryocytes (CD61$^+$) (Fig 2). The finding of a rapid decrease of *GPER* expression during the first stages of megakaryocytic differentiation leads to the conclusion that it is not involved in megakaryocyte maturation and proplatelet formation. However, we cannot firmly exclude that this receptor can eventually play a role in later stages of megakaryocytic differentiation. Future studies are also needed to evaluate the expression of GPER during haematopoietic stem cells differentiation in other cell lineages. Further research on the role of oestrogen-dependent signalling mediated by ERα and ERβ in megakaryocyte differentiation and platelet development is also warranted.

Acknowledgements

We thank Malara Alessandro, Badalucco Stefania and Pallotta Isabella (University of Pavia, Italy) for providing the necessary training regarding the isolation and culture of human cord blood CD34$^+$ cells, and Drs Galli Licia and Perego Beatrice (University of Piemonte Orientale A. Avogadro) for umbilical cord blood collection.

This work was supported by grants from Ministero dell'Università e della Ricerca-PRIN 2007 (to FS), from Regione Piemonte -Ricerca Sanitaria Finalizzata 2006, 2007 and -Ricerca Scientifica Applicata 2004 and from Università del Piemonte Orientale-Ricerca Locale.

Author contributions

CDV performed experiments, analysed data and wrote the paper. SB performed experiments and analysed data. A Balduini performed experiments and analysed data. AR performed experiments and analysed data. A Bagarotti performed experiments and analysed data. NS provided human cord blood samples. A Bertoni designed the project, analysed data and wrote the paper. FS financed the project and wrote the paper.

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Keywords: megakaryocyte differentiation, G protein-coupled receptor 30, G protein-coupled oestrogen receptor, oestrogen receptor, platelet formation.