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MODULATION OF PLATELET FUNCTIONALITY BY DEHYDROEPIANDROSTERONE AND DEHYDROEPIANDROSTERONE-SULFATE

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1. INTRODUCTION

1.1 Platelets

Platelets are small, discoid, anucleated cellular fragments, that in human circulate at a concentration of 250000-350000 cells/µl of blood and are essential components of primary haemostasis (1, 2).

Platelets are formed from megakaryocytes, large bone marrow cells (3), and they have a life span of 7 to 10 days; after that period they are removed from blood circulation (4).

Resting platelets circulate as discoid anucleated cells. The platelet is surrounded by a typical bilamellar plasma membrane that extends through the multiple channels of the surface-connected open canalicular system, greatly increasing the surface area of the cell (4). The surface-connected open canalicular system provides access to the interior for plasma-borne substances and an egress route for products of the release reaction (5). On the cytoplasmic side of the plasma membrane, just under the platelet surface, it is organized a regular system of filamentous elements that create the membrane skeleton. The sub-membrane filaments may cooperate with cytoskeleton to maintain platelet discoid shape, play a role in the extrusion and stabilization of pseudopodia, and interact with other elements of the platelet contractile mechanism to affect platelet cohesion and clot retraction (6). Dispersed in the platelet cytoplasm there are numerous organelles, including mitochondria, glycogen particles, lysosomes and peroxisomes. α-granules and dense bodies are platelet-specific storage granules. The α-granules contain coagulation factors, such as fibrinogen, factor V, high molecular weight kininogen, vWF, antiheparins, such as platelet factor 4, β-thromboglobulin, platelet basic protein, growth-promoting factors and mitogens, such as PDGF, thrombospondin. Other proteins including IgG, albumin and fibrinogen are taken up from plasma by a novel endocytotic mechanism (1). 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. In human platelets ADP is the predominant nucleotide, and Ca^{2+} the predominant divalent cation (7).

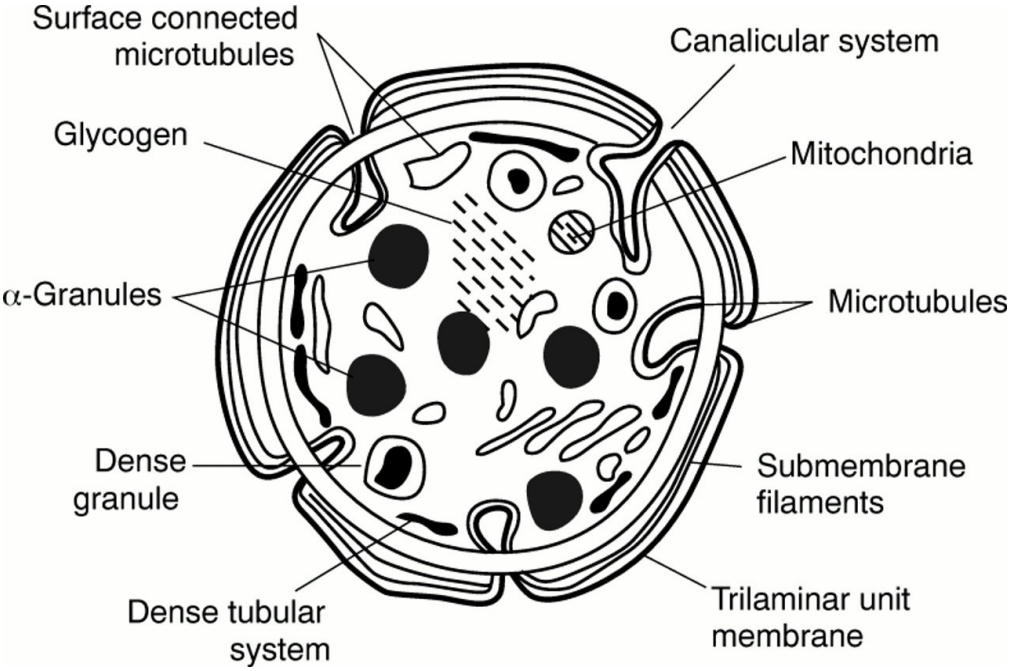

Fig. 1.1.1. Longitudinal section of a resting platelet (8).

1.2 Platelet activation

Platelets play an essential role in the first phases of the haemostatic process. Whenever a blood vessel is damaged at its luminal side, subendothelial elements come into contact with the blood elements. In particular, when platelets are exposed to non-endothelial surface, they rapidly change their shape from a disc to a spiny sphere with long and fine filopodia, adhere, flatten and spread on the surface. Blood platelets become activated by subendothelial collagen, microfibrils and vWF: secrete granule content and regulate the expression and binding affinity of some adhesion receptors. That processes contribute to the formation of the so-called “white thrombus”, which closes the gap in the injured blood vessel. Subsequent to platelet activation, the coagulation cascade will form fibrin fibrils, that strengthen the primary thrombus and transform it into a “red thrombus” (5).

*Fig. 1.2.2. Resting and Activated platelets. (*www.ouhsc.edu/platelets)

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 experimentally by several potential physiological agonist, the most important of which are ADP and thrombin; other potential agonists include epinephrine, TxA_2 and Platelet Activating Factor (PAF) (1). Many physiological agonists contribute to platelet activation and consequently to signal transduction pathways that trigger biochemical, morphological and functional changes which culminate in irreversible aggregation. Thrombin, TxA_2 and ADP bind to receptors coupled to the $G\alpha_q$ protein. $G\alpha_q$ activates the phospholipase Cβ (PLCβ) that increases the levels of IP_3 and Diacylglycerol (DAG) by hydrolysis of PIP_2. IP_3 enhances the concentration of intracellular Ca^{2+} by liberating it from the intracellular stocks (9). DAG activates protein kinase C (PKC) that contributes to protein phosphorylation, granule secretion and fibrinogen receptor exposure (10).

Thrombin is one of the most important agonists for platelet recruitment and aggregation which is mediated by the binding of fibrinogen to its adhesive receptor. This "inside-out" signaling would lead to some changes in the extracellular domain of integrin $\alpha_{IIb}\beta_3$ (integrin activation) increasing the binding affinity for fibrinogen. Fibrinogen binding to integrin $\alpha_{IIb}\beta_3$ mediates the "outside-in" signaling inducing a series of intracellular events that lead to the release of procoagulant membrane vesicles, the cytoskeletal reorganization and the fibrin clot retraction (11).

Thrombin is the most potent activator of platelets *in vivo*. Thrombin provokes platelet shape change and release of the contents of platelet granules, which contain ADP and serotonin as well as chemokines and growth factors. Thrombin also triggers the synthesis and release of TxA_2, mobilization of P-selectin and CD40 ligand to the platelet surface and activation of the integrin $\alpha_{IIb}\beta_3$. Thrombin causes expression of procoagulant activity on the platelet surface, which supports additional thrombin generation (12). Thrombin activates platelets proteolitically acting on PAR

receptors and this enzymatic action is essential for signal transduction into the cell. Thrombin cleaves a specific portion of 40 aminoacids at the N-terminal exodomain of PAR receptors. This cleavage event unmasks a new N-terminus that then serves as a tethered ligand, binding intramolecularly to the body of the receptor to effect transmembrane signaling. Synthetic peptides containing aminoacidic residues complementary to the new exposed sequence, are able to mimic the tethered ligand and activate the receptor independent of protease and receptor cleavage. PAR receptors are formed by a single polypeptide with an extracellular N-terminus, an intracellular C-terminus and seven membrane spanning domains and interact in their cytoplasmic sites with heterotrimeric G-proteins. Four PARs are known: PAR1 is expressed on human platelets, endothelial cells, vascular smooth muscle cells, fibroblasts and neurons, PAR2 is expressed on human endothelial cells, keratinocytes, vascular smooth muscle cells and gastrointestinal epithelium, but not on human platelets, PAR3 is present on mouse and rat platelets, and PAR4 is expressed in both human and murine platelets. PAR1, PAR3, and PAR4 can be activated by thrombin. PAR2 can be activated by trypsin and tryptase as well as by coagulation factors VIIa and Xa, but not by thrombin (12). Human platelets express PAR1 and PAR4, and activation of either is sufficient to trigger platelet secretion and aggregation.

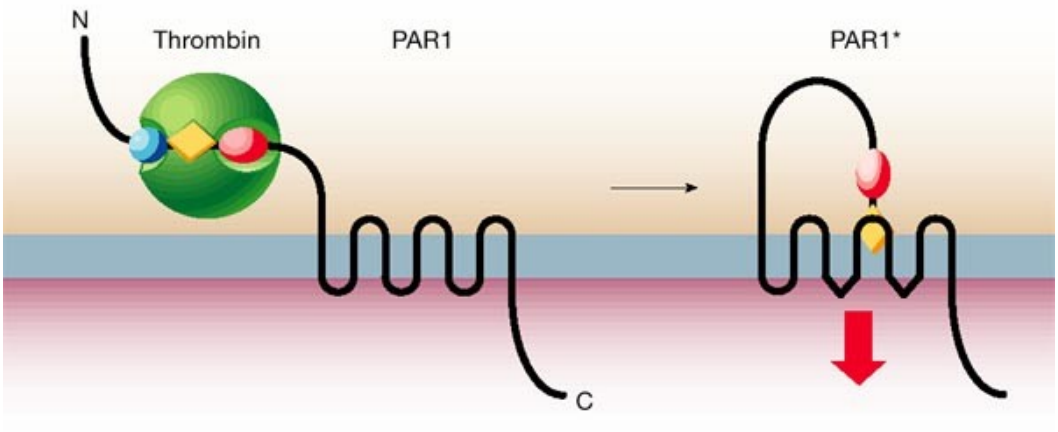

Fig. 1.2.3. Thrombin receptor. (13)

In addition to interacting with PARs, thrombin also binds to glycoprotein Ibα (GP Ibα) on the surface of human platelets without cleaving it. GP Ibα is part of a multifunctional protein complex that also binds vWF and P-selectin. Studies support the model that GP Ibα may serve as a cofactor for thrombin cleavage of PARs on the platelet surface (12).

1.3 Protein kinases and their substrates

The level of protein tyrosine phosphorylation within the cell is tightly regulated by both tyrosine kinases and tyrosine phosphatases. In general, protein tyrosine kinases are classified into two broad groups: the transmembrane receptor family and the cytosolic non-receptor group. Transmembrane tyrosine kinases include the receptors for a variety of soluble growth factors and hormones, while the cytosolic non-receptor protein tyrosine kinases consist of members of the Src, Btk, Csk, Syk and FAK families. The majority of tyrosine kinases identified in platelets are non-receptor forms. The tyrosine phosphorylation of platelet proteins, by Src and other kinases, are important for platelet activation. In fact, protein tyrosine phosphorylation is a general feature of platelet activation induced by a range of platelet agonists and the time course of these events correlates well with specific platelet functional responses (14). In resting platelets, only a small number of proteins are tyrosine phosphorylated. The tyrosine phosphorylation of cellular proteins is a dynamic, reversible process, regulated by both kinases and phosphatases. Moreover, these enzymes appear to play a major role in maintaining the low level of phosphotyrosine within the resting cell and may prevent premature platelet activation (14).

The tyrosine kinase Src. The most abundant tyrosine kinase in platelets is $pp60^{c\text{-}src}$, constituting 0.2-0.4% of total platelet protein (15). Other members of the Src family expressed in platelets are Fyn, Lyn, Yes, Hck. Src contains an N-terminal myristoylation signal, SH2 and SH3 domains, a catalytic site and a C-terminal autoregulatory tail. SH2 and SH3 domains play critical roles in regulating intra- and inter-molecular protein-protein interactions. SH2 domains are highly conserved regions, which recognize specific consensus sequences encompassing tyrosine phosphorylated residues, whereas SH3 domains bind specific proline-rich sequences. The N-terminal glycine residue undergoes myristoylation and 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 in the activating loop of the kinase domain, is unphosphorylated, while Tyr527 is phosphorylated, binds the SH2 domain and stabilizes a repressed conformation of the enzyme's catalytic site. Binding of Src partner proteins to either the SH2 or SH3 domain of Src can release Src from its inhibited conformation into an unfolded position. Under these circumstances, Tyr527 is dephosphorylated and Tyr416 is phosphorylated, and changes in the phosphorylation status of the two regulatory tyrosines lead to Src activation. Src is rapidly activated in thrombin-stimulated platelets and associates with the actin-rich cytoskeleton, where phosphorylates a number of cytoskeletal proteins. The association of Src with the platelet cytoskeleton is regulated by integrin $\alpha_{IIb}\beta_3$-mediated platelet aggregation, and is considered to play an important role in the formation of integrin-rich cytoskeletal signaling complexes. The N-terminal myristoylation sequence, SH2 and SH3 domains of Src are essential for

its ability to associate with focal adhesions and promote cell spreading (14). Moreover, it was demonstrated that Src and its regulatory kinase, Csk, are constitutively associated with integrin $\alpha_{IIb}\beta_3$ in resting platelets. Upon soluble fibrinogen binding to $\alpha_{IIb}\beta_3$ or platelet adhesion to immobilized fibrinogen, Csk dissociates from $\alpha_{IIb}\beta_3$ and Src to become activated and independent of actin polymerization. Activated Src localizes to the periphery of spreading platelets, including filopodia (16).

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 (17). The MAP kinases are activated by a double phosphorylation on conserved serine-threonine residues into a specific consensus sequence TXY (T, threonine, X, ever aminoacid, Y, tyrosine), mediated by their direct activators, the MAP kinase kinases. 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 and the subsequent phosphorylation of the receptor on one or more tyrosine residues. This creates sites on the receptor for binding the Grb2/Sos complex. Sos is an exchange factor for Ras, causing it to exchange GDP for GTP and bind to the protein kinase Raf-1. Raf-1 in turn phosphorylates MEK, causing it to phosphorylated the p42 and p44 forms of MAPK.

p42/p44 MAP kinases. In platelets the hypothesized model for ERK1 (p44)/ERK2 (p42) activation suggests that receptor activation, mediated by either GTP-binding proteins or tyrosine kinases, leads to the activation of the low molecular weight GTP-binding protein Ras which recruits the serine/threonine kinase Raf to plasma membrane. Raf, in turn, phosphorylates and activates the MAP kinase kinases MEK1 and MEK2, which subsequently activate ERK1 and ERK2 by threonine/tyrosine phosphorylation into the consensus sequence TEY (Thr-Glu-Tyr) (18). ERK1 and ERK2 are translocated to the cytoskeleton following platelet activation by thrombin or TxA2. This translocation is associated with both phosphorylation and activation of the enzymes. The cytoskeletal incorporation of the MAP kinases occurs in a manner similar to other proteins such as myosin, actin, actin-binding protein, α-actinin, tropomyosin and cortactin. Potentially, any of these proteins may be the substrate for, and be regulated by, ERK1 and ERK2. The cytoskeleton may act as a frame, which spatially aligns the MAP kinases with substrates in a highly integrated platelet signal transduction pathway (18).

1.4 Platelet inhibition

Activation of platelets is counterregulated by biochemical processes which attenuate or prevent agonist-induced responses. Platelet activation is inhibited by factors released from endothelial cells that constitute the inner cell layer of the vessel wall. The most important inhibitory endothelium-derived factors are nitric oxide (NO) and prostaglandin I2 (PG-I2), which inhibit platelets by increasing the level of intracellular cyclic nucleotides (19, 20). PGE1 and PGI2 bind to prostaglandin $G\alpha_s$ coupled receptors. The $G\alpha_s$ subunit activates the adenylate cyclase (AC) and increases the levels of cyclic AMP (cAMP) in the platelets. NO and other NO-donors stimulate directly the intracellular guanylate cyclase (GC) enhancing the intracellular concentration of cyclic GMP (cGMP). Cyclic nucleotides induce platelet inhibition via the activation of cAMP- and cGMP-dependent protein kinases (PKA and PKG respectively) (20-22). It is not yet clear how the substrates of PKA and PKG regulate the inhibition of platelets, however several proteins phosphorylated by PKA are involved in the regulation of actin cytoskeleton.

The activity of adenylate cyclase is regulated by G-proteins: it is stimulated by G_s and inhibited by G_i. G_s is coupled to the receptors for the inhibitory prostaglandins PGI2/PGE2 and PGD2 and to the adenosine receptor. Some aggregating agents inhibit adenylate cyclase via their action on G_i and levels of cAMP are reduced in platelets with elevated cAMP concentrations. Aggregating agents increase levels of cGMP in platelets, but this is the effect, rather than the cause of platelet aggregation. Indeed, cGMP is inhibitory in platelets, acting as a feedback inhibitor of platelet activation. The synthesis of cGMP is stimulated by NO, formed by normal endothelium through a nitric oxide synthase (23). NO inhibits platelet activation and can revert the aggregation. cGMP and cAMP also activate phosphodiesterases (PDE) that control and turn off the inhibitory signals by degradation of cyclic nucleotides. In human platelets three different PDE subtypes were identified: PDE2 acts on both cGMP and cAMP and is stimulated by c-GMP; PDE3 is known as cGMP that inhibit cAMP phosphodiesterase, because it has high affinity for both cAMP and cGMP, but a much lower efficacy of hydrolysis for cGMP; PDE5 degrades cGMP and is activated by cGMP (24).

VASP. Vasodilator-stimulated phosphoprotein (VASP) is the founding member of the Ena/VASP family (25,26) that includes Drosophila Enabled (ena), the mammalian and the avian Ena orthologs (Mena and Avena, respectively (25) and the Ena-VASP-like protein (Evl). All the proteins of the Ena/VASP family share the same domain organization that consists of highly conserved N-terminal and C terminal regions (Ena-VASP homology domain (EVH) 1 and 2, respectively), separated by more variable low complexity (LCR) and proline rich regions (PRR).

In response to the cyclic nucleotide-regulating platelet antagonists NO and PGI2, VASP is phosphorylated by both PKA and PKG. VASP phosphorylation closely correlates with platelet

inhibition and is accompanied by inhibition of the platelet fibrinogen receptor $\alpha_{IIb}\beta_3$ activation. Three distinct phosphorylation sites were biochemically identified in VASP (serine 157, serine 239, and threonine 278) (27, 28). Phosphorylation of serine 157, the site preferred by the PKA, leads to a marked shift in apparent molecular mass of VASP in SDS-PAGE from 46 kDa to 50 kDa (28, 29). In experiments with well characterized protein kinase activators, analysis of the shift in the apparent molecular mass of VASP from 46 to 50 kDa in SDS-PAGE has been helpful in analyzing the preferential activation of both PKA and PKG in intact cells (30-32). VASP is believed to play an important role in controlling the cytoskeletal organization because it binds filamentous actin and regulates actin dynamics (33-35). With specific antibodies, VASP phosphorylation provides a sensitive monitor of defective NO/cGMP signaling, and reduced NO bioavailability in several pathophysiological states correlates with reduced VASP phosphorylation. Thus, decreased VASP phosphorylation after NOS inhibition could directly contribute to the observed changes in platelet activation (36). 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 (37,38).

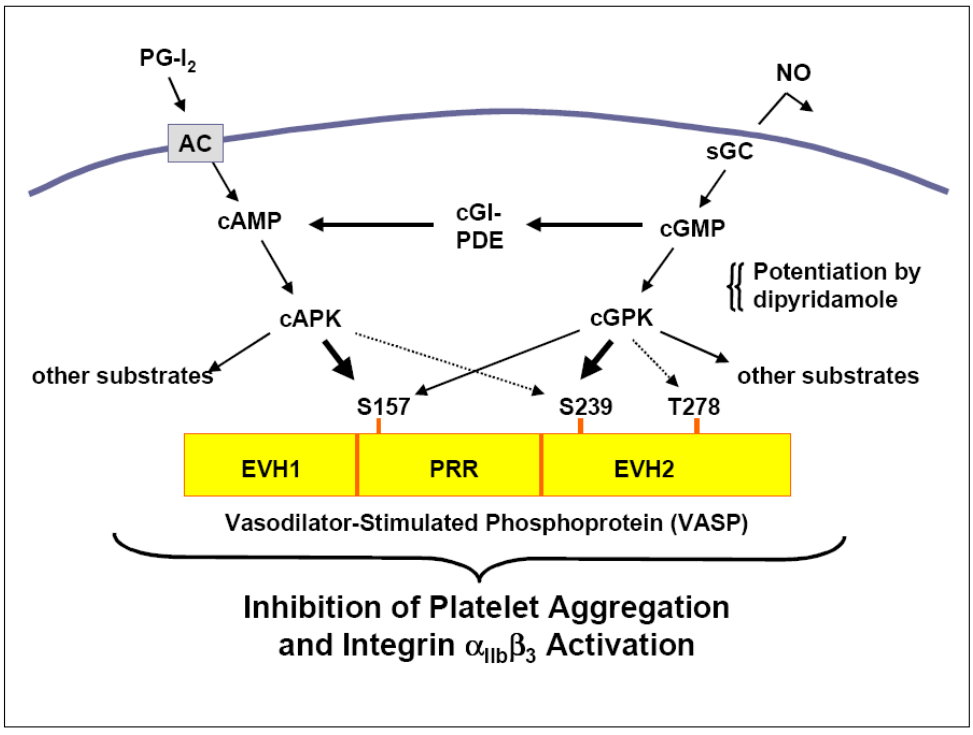

Fig. 1.3.4. VASP phosphorylation induced by cyclic nucleotides in platelets (38).

1.5 Dehydroepiandrosterone

Dehydroepiandrosterone (DHEA) and its sulfated form, DHEA-S, are the most abundant steroids circulating in human blood (39). DHEA is a critical metabolic intermediate in the syntesis of androgen and estrogen, whereas DHEA-S is thought to be an end-product of metabolism that can be desulfated back to DHEA. The circulating and intracellular levels of the hormones are related to the quantities secreted by the human adrenal, differential renal clearence of the two hormones and the tissue-specific expression of sulfatase and sulfotransfeerase enzymes. Althought with wide variations, the plasmatic concentrations of these steroids progressively decline with age (40), suggesting that DHEA may be implicated in the aging process. In human, levels of DHEA-S peak around ages 20 to 25 years and then decline to values of 20% to 30% at approximately 70 to 80 years of age. The fact that the concentration of DHEA/DHEA-S progressively declines with aging and that there are many pathological states the incidence of which increases with age, has lead to a number of hypotheses about the possible causal role of DHEA/DHEA-S in pathophysiology of many diseases, especially coronary heart disease, atherosclerosis, obesity, diabetes mellitus, cancer (41). Indeed a considerable number of data reports beneficial roles of DHEA administration in both animals and humans (42-45). In humans oral administration of DHEA stimulated immune responses, enhanced cognitive ability and exerted antidepressant effect (46-48).

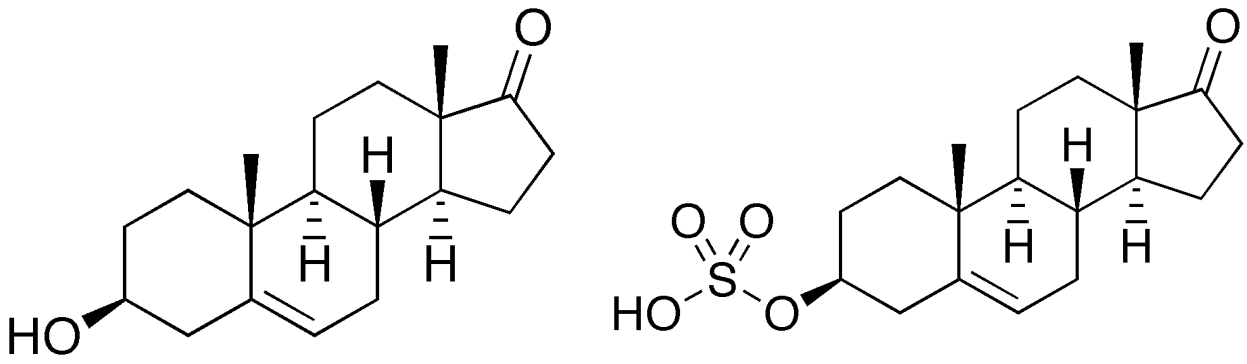

Fig. 1.4.5. DHEA and DHEA-S

Moreover, clinical and population-based studies suggested that DHEA/DHEA-S could play a protective role against atherosclerosis and coronary artery disease (49,50). Furthermore, in various animal models of atherosclerosis and vascular injury DHEA had anti-atherogenic and vasculotonic effects. *In vitro* studies showed beneficial effects of DHEA on vascular endothelium and smooth muscle and on other key mediators of atherogenesis (51). In fact, DHEA was able to induce

vasodilatation (52), to inhibit smooth muscle cells proliferation (53), PAI-1 production (54) and platelet aggregation (55). Beneficial effect of chronic DHEA administration on lipid profile in humans have also been reported (56), though the anti-atherogenic effect of DHEA was shown to be independent of its action on serum lipids, at least in animal studies (40).

DHEA is an intermediary metabolite in the androgenic pathway leading to the production of sex steroid hormones including estrogens, hence some of the positive effects of DHEA may be related to the effects of metabolically active downstream steroids (57). However, analogs of DHEA, not metabolized to androgens or estrogens, also improved cardiovascular risk profiles in humans (51). Although the mechanisms through which DHEA functions in the vasculature are still largely unknown, recent evidence have shown that in endothelial cells DHEA stimulates NO production by a cell surface-initiated G-protein coupled mechanism (58).

DHEA circulates in blood mainly in its sulfated form, DHEA-S, which is primarily synthesized in adrenal cortex and liver from DHEA by DHEA-sulfotransferase 2A1 (SULT2A1) (59,60). Although in human blood the concentration of DHEA-S is in the micromolar range, one thousand-fold higher than that of DHEA (61), DHEA-S did not participate in endothelial cell stimulation, and the biological role of this abundant blood component, if any, is totally unknown. At present it is generally accepted that the sulfated form of DHEA represents a soluble, rapidly available reservoir of DHEA (61). Whilst DHEA is highly lipophylic and can freely pass through cell membranes, DHEA-S is hydrophilic and to enter the cell requires active transport, probably mediated by members of the organic anion transporter family (62). Once in the cell, DHEA-S can be converted to DHEA after hydrolysis of the sulfate moiety by the microsomal steroid sulfatase (STS) (63) and then DHEA can be metabolized to estrogens, androgens or it can signal through a specific receptor. It appears that the direction of conversion of DHEA depends on the tissue-specific expression of steroidogenic enzymes, which define the biological action of DHEA as steroid precursor in a specific target tissue. Although sulfotransferase and sulfatase activities have been detected in various tissues and cell types (63,64), including platelets (65), and administration of different doses of DHEA for different periods of time increased *in vivo* both DHEA and DHEA-S concentration in plasma of men and women, the impact of intracellular DHEA/DHEA-S metabolization on rapid non-genomic DHEA-dependent signaling in endothelial and other cell types is still totally unknown. It is a consolidate knowledge that endothelium-derived NO activates guanylyl cyclase in platelets and decreases the responsiveness of these cells to physiological agonists (23). Since endothelium-derived NO is one of the most effective tools against undesirable platelet aggregation and thrombus formation, actually DHEA could take a relevant part in the molecular mechanisms that physiologically act on platelets maintaining them in a resting state and avoiding thrombosis in circulation (66).

2. AIMS

Despite the evidence that *in vivo* the positive cardiovascular effects of DHEA seem to lead back to inhibition of platelet function by the endothelium-derived NO, little is known of a possible direct effect of this steroid hormone on human platelets. It has been reported that DHEA-S is able to inhibit human platelet aggregation promoted by arachidonic acid in samples of platelet rich plasma (67). However, the impact of DHEA and DHEA-S on platelet function and their possible role in modulating the response of human platelets to physiological agonists were not investigated.

The aim of my PhD project has been to investigate *in vitro* the effects of DHEA and DHEA-S, the most abundant steroid hormones in circulating blood (39), on platelet function, with particular attention to the signal transduction pathways involved. The main goals of the project were as follows:

1. To understand if in gel-filtered platelets DHEA or DHEA-S were able to inhibit platelet secretion and aggregation thrombin-induced.
2. To characterize the signal transduction pathways activated after treatment with DHEA or DHEA-S

3. MATERIALS and METHODS

3.1 Materials

Sodium nitroprusside and U46,619 were from Alexis Biochemicals (Lausen, Switzerland). cAMP Biotrak Enzymeimmunoassay systemc, hybond-P polyvinylidene difluoride (PVDF) transfer membrane were from Amersham GE Healthcare (Milan, Italy) cGMP complete EIA kit was from Assay Design (Vinci, FI Italy). Monoclonal antibody against phospho-Vasodilator-stimulated Phosphoprotein VASP (pSer 239), monoclonal antibody against phospho-Vasodilator-stimulated Phosphoprotein VASP (pSer 157), polyclonal antibody against VASP, N^G-Monomethyl-L-arginine, Monoacetate Salt (L-NMMA), N^G-Nitro-L-arginine Methyl Ester (L-NAME), were from Calbiochem (San Diego, CA, USA). Polyclonal antibody against phospho-pAkt (S473), polyclonal antibody against phospho-p44/42 MAP Kinase (Thr202/Tyr204), polyclonal antibody against phospho-p38 MAPK were from Cell Signaling Technology (Beverly, MA, USA). CHRONO-LUME reagent was from CHRONO-LOG Corporation (Havertown, PA, USA). FITC-PAC-1 and PE-CD62P were from BD Bioscience (Milan, Italy). Collagen was from Mascia Brunelli (Milano, Italy). Enhanced chemiluminesccence reagent was from Perkin Elmer Life Science (Boston, MA, USA). Monoclonal antibody against integrin β-3, monoclonal antibody against actin were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Polyclonal antibodies against phospho-Tyr418-Src, Adenosine diphosphate, Thrombin, Methanol, Apyrase, dehydroepiandrosterone-sulfate, dehydroepiandrosterone, EMATE, and forskolin were from Sigma-Aldrich (St Louis, MO, USA). ODQ, Indomethacin, and ICI 182,780 were from Tocris Bioscience (Bristol, UK).

Methods

3.2 Platelets preparation

Blood was withdrawn from healthy donors, who had not taken any drugs for at least 2 weeks before venipuncture, using ACD (130 mM citric acid, 152 mM Na-citrate, 112 mM D-glucose) 1:10 as anticoagulants. The blood was centrifuged at 120 x g for 15 minutes at room temperature. The platelet rich plasma was removed and centrifuged at 300 x g for 10 minutes to sediment the platelets. Then the platelet pellets were resuspended in a little volume of ACD and gel-filtered on Sepharose CL- 2B column. The platelet count was adjusted with Hepes buffer (10 mM Hepes, 137

mM NaCl, 2.9 mM KCl, 12 mM NaHCO3, pH 7.4) and cell suspension was rendered 2 mM $CaCl_2$, 2 mM $MgCl_2$ and 5 mM glucose.

3.3 Evaluation of platelet aggregation and ATP secretion

Samples of gel-filtered platelets (0.4 mL, $3x10^8$ cells/mL) were prewarmed at 37° C in a Chronolog lumiaggregometer for 5 minutes under constant stirring (1000 rpm), incubated with 10 nM DHEA, 0.075-0.15-0.3 mM DHEA-S or MetOH 0.3 % for 1 minute and then stimulated with different doses of thrombin (0.025 U/ml to 0.1 U/mL). Aggregation was monitored continuously for at least 5 minutes. In some experiments inhibitors were added before DHEA-S. The L-NMMA and L-NAME were used at 0.3 mM for 5 minutes; ODQ at 2 μM for 5 minutes; ICI 182,780 at 10 μM for 5 minutes; EMATE at 10 μM for 1 minute; for all the inhibitors control samples were treated with vehicle for the same times. Measurement of platelet secretion was carried out by adding the luciferine-luciferase CHRONO-LUME reagent 5 minutes after thrombin.

3.4 Electrophoresis and Immunoblotting analysis

Samples of gel-filtered platelets ($1x10^9$ cells/mL) were preincubated with 0.3 mM DHEA-S from 30 seconds to 10 minutes at 37° C and then stimulated with 0.05 U/mL thombin for different times, as indicated. In some experiments inhibitors were added before addition of DHEA-S. Stimulation was stopped by adding 0.5 volumes of 3X Laemmli 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 instruments and analyzed with QuantityOne software. In some experiments membranes were stripped and reprobed. Antibodies used: anti-P-Ser239-VASP, anti-P-Ser157-VASP, anti-VASP, anti-phospho-pAkt (S473), anti-phospho-ERK1/2 (Thr^{202}/Tyr^{204}), anti-phospho-p38 MAPK, anti-$pTyr^{418}$ Src kinases, anti-actin and anti-integrin β-3.

3.5 Analysis of cyclic nucleotides contents

Samples of gel-filtered platelets (500 μL, $4x10^8$ cells/mL for cGMP and 200 μL, $1x10^8$ cells/mL for cAMP assay) were rendered 0.5% BSA and 1 mM $CaCl_2$ and incubated for 1 minute with 0.3 mM DHEA-S, 10 μM forskolin, as positive control for cAMP, or 100 μM sodium

nitroprussiate, as positive control for cGMP. The reaction was stopped by adding 2 vol of ice-cold 96% ethanol or 0.1 M HCl, for cAMP and cGMP assay, respectively. Lysates were centrifuged at 15500xg for 10 minutes at 4° C, supernatants were recovered. The supernatants were pulled and lyophilized for the cAMP analysis or stored at -80 for cGMP analysis. The lyophilized samples were dissolved in bidistilled water and analyzed for their content in cyclic nucleotides using cAMP Biotrak Enzymeimmunoassay System or cGMP complete EIA kit, following the manufacturer's instruction for the acetylation protocol.

3.6 Flow Cytometry.

Washed human platelets ($1x10^6$/samples) stimulated with buffer, 0.3 mM DHEA-S or 10 nM DHEA for 1 minute and then with 0.05 U/ml thrombin, incubated with FITC-PAC-1, PE-CD62P or an isotype match control for 20 minutes at room temperature in the dark. The reaction was stopped by adding 1 ml of PBS. The cells were analyzed using a BD Bioscience FACSCalibur flow cytometer by acquiring 10000 events of the gated platelet population.

3.7 Statistical analysis

Data reported are the means ± SD or SEM of at least three independent experiments. In platelet experiments statistical analysis was performed by the Student T test. Significant differences were accepted at $p<0.05$ (*) or $p<0.01$ (**).

4. RESULTS

4.1 DHEA-S, and not DHEA, inhibited platelet aggregation caused by thrombin

With the aim to investigate the role of DHEA and DHEA-S in platelet function, samples of gel-filtered platelets were stimulated with 0.3 mM DHEA-S or 10 nM DHEA. Neither DHEA nor DHEA-S were able to induce any appreciable aggregation of human platelets (Figure 4.1.1A) as indicated by the absence of variation in the transmittance of platelet samples for at least 5 minutes after the addition of both steroid molecules. Thus, platelets were preincubated for 1 minute with 0.3 mM DHEA-S, 10 nM DHEA or vehicle and then stimulated with 0.05 U/ml thrombin, a concentration that caused about 70% of platelet aggregation. Surprisingly, while DHEA, the active form of the hormone, did not exert any appreciable effect, DHEA-S inhibited the platelet response to thrombin (Figure 4.1.1A). To confirm this data, platelets were stimulated also with different agonists such as collagen (Figure 4.1.1B) and U46,619 (Figure 4.1.1C), a stable analogue of tromboxane A_2 (TxA_2). Even with these agonists was possible to obtain a significant inhibition of platelet aggregation with DHEA-S, but not with DHEA. Considering the similarity of the results obtained with different agonists, the study has been continued using only thrombin.

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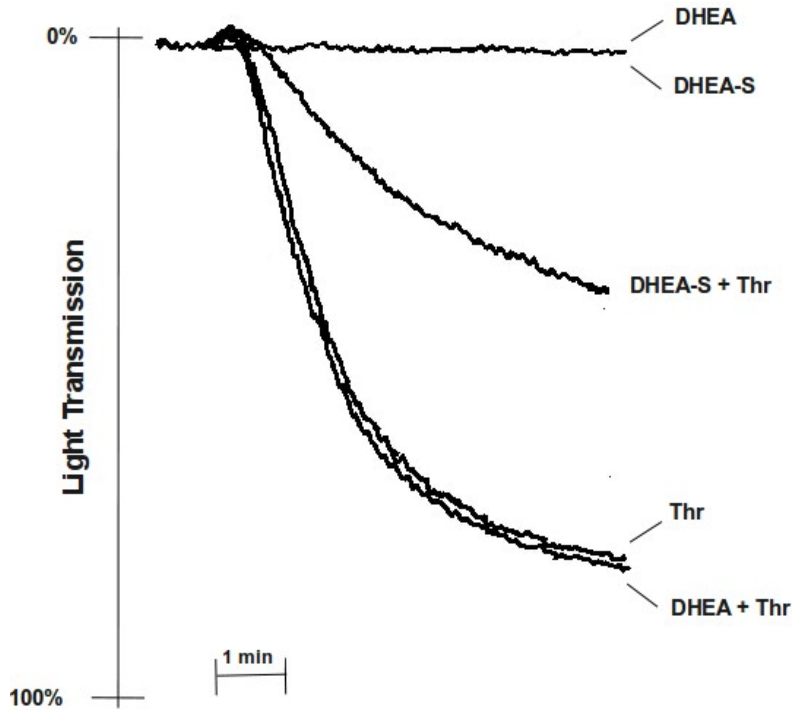

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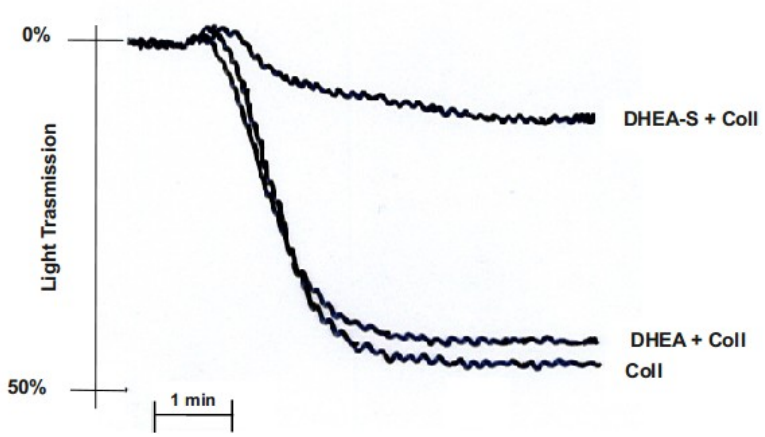

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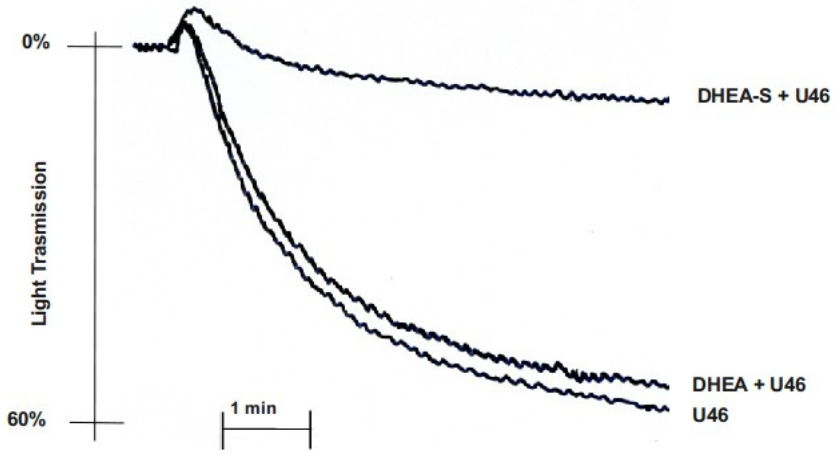

Figure 4.1.1: DHEA-S, and not DHEA, inhibits platelet aggregation.

Platelets were pretreated with 0.3 mM DHEA-S, 10 nM DHEA or vehicle for 1 minute and then stimulated with **(A)** 0.05 U/ml thrombin (Thr), with **(B)** 2 μg/ml collagen (Coll), with **(C)** 500 nM U46,619 (U46), as indicated. Aggregation was measured in a lumiaggregometer for 5 minutes, at 37° C, under stirring conditions. Results were expressed as increase in light transmission. Aggregation traces are from a representative experiment out of 3 performed with platelets obtained from different donors.

The different effects of DHEA and DHEA-S on thrombin-dependent platelet aggregation could be a consequence of diverse platelet membrane permeability to these compounds. It has been shown that DHEA-S can be actively transported across the plasma membrane by the organic anion transporting polypeptide 2B1 (OATP2B1) (68), which has also been described in platelets (67). Once in the cell DHEA-S can be metabolized by the platelet estrone/DHEA sulfatase to DHEA and/or to active downstream steroids. Thus, platelets were preincubated with 10 μM EMATE,

inhibitor of the estrone/DHEA sulfatase (69), and then DHEA-S or vehicle were added before stimulation with thrombin. Figure 4.1.2 shows that either in the presence or absence of EMATE, DHEA-S inhibited thrombin-dependent aggregation in the same manner. Hence, we concluded that neither DHEA nor DHEA-S-derived steroids but only DHEA-S was actually able to negatively affect thrombin-triggered signals leading to platelet aggregation. At present little is known of the pathways through which DHEA/DHEA-S signals in different cell types. In DHEA-treated endothelial cells estrogen receptors were engaged by both DHEA and DHEA-derived estrogens (57). Estrogen receptors are also expressed in human platelets, and the β isoform is the main estrogen receptor in these cells (67). We have recently demonstrated that estrogen receptor β is localized in the plasma membrane and in 17β-estradiol-treated platelets it translocated to the lipid raft fraction and formed a signaling complex with the active kinases Src and Pyk2 (70). To investigate the role of estrogen receptors in DHEA-S signaling in human platelets, samples of gel-filtered platelets were incubated with 10 μM ICI 182,780 and then treated with DHEA-S before stimulation with 0.05 U/ml thrombin. ICI 182,780 binds to estrogen receptors and inhibits the effect of 17β-estradiol in many cell types including platelets (71). ICI 182,780 treatment did not modify the extent of thrombin-dependent platelet aggregation in presence of DHEA-S (Figure 4.1.2). Thus, the platelet estrogen receptors are not recruited by DHEA-S, DHEA or DHEA(S)-derived estrogens in this process.

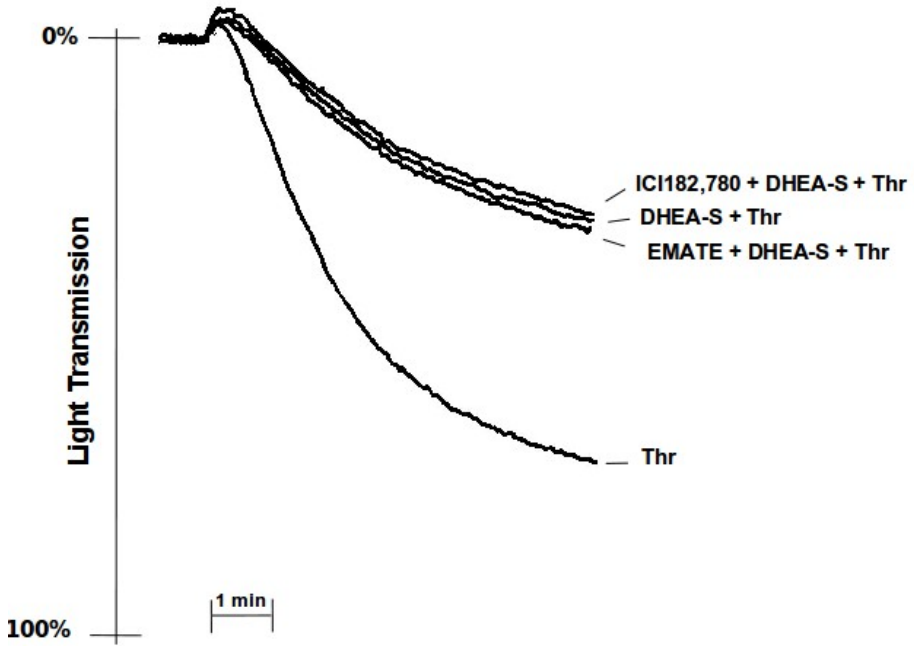

Figure 4.1.2: DHEA-S, and not DHEA, inhibits thrombin-induced platelet aggregation without signaling through the estrogen receptors.

Samples of gel-filtered platelets were stimulated with 0.05 U/ml of thrombin (Thr) alone or in association with 0.3 mM DHEA-S for 1 minute. Where indicated 10 μM ICI182,780, antagonist of estrogen receptor, or 10 μM EMATE, estrone sulfatase inhibitor, were added 5 minutes before stimulation with DHEA-S. Aggregation was measured in a lumiaggregometer for 5 minutes, at 37° C, under stirring conditions. Results were expressed as increase in light transmission. Aggregation traces are from a representative experiment out of 3 performed with platelets obtained from different donors.

In order to better characterize the kinetics of DHEA-S inhibition of thrombin-induced platelet aggregation, platelets were preincubated with different concentrations of DHEA-S (0.075 to 0.3 mM) and then stimulated with either high or low doses of thrombin (0.025, 0.05, 0.1 U/ml). These concentrations of thrombin elicited in platelets signal transduction pathways resulting in different extents of platelet aggregation. Figure 4.1.3 shows that 0.3 mM DHEA-S was able to completely prevent the aggregation caused by the lower dose of thrombin, it inhibited by 47.9±2.60% the aggregation induced by 0.05 U/ml thrombin, while it failed to cause any significant inhibition of platelet aggregation induced by the higher dose of agonist. In addition, the concentration near at fisiological range, 0.075 mM DHEA-S, was able to induce a robust inhibition of platelet aggregation elicited by a low dose of thrombin (0.025 U/ml) but it was totally ineffective

when platelets were stimulated with higher doses of agonist. The extent of platelet aggregation induced by low doses of thrombin such as 0.025 U/ml displayed high individual variability. In order to minimize the impact of this behaviour, in the following experiments we used 0.05 U/ml thrombin to stimulate platelets, and consequently we increased the concentration of DHEA-S to 0.3 mM. These experimental conditions ensured about 50% inhibition of the aggregation induced by 0.05 U/ml thrombin.

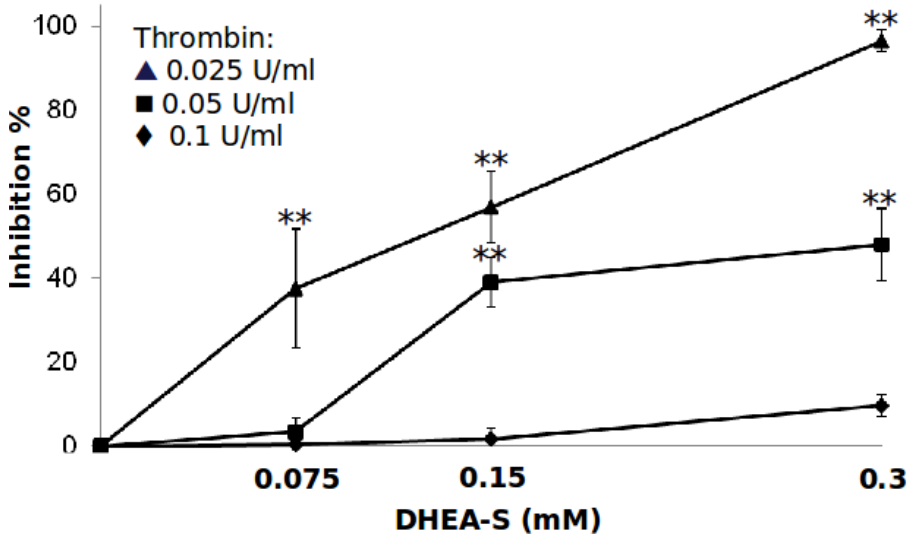

Figure 4.1.3: DHEA-S inhibits thrombin-induced platelet aggregation in a dose-dependent manner.
Platelets were incubated for 1 minute with increasing concentration of DHEA-S (0.075-0.15-0.3 mM) and then stimulated with different doses of thrombin (▲ 0.025 U/ml; ■ 0.05 U/ml; ♦ 0.1 U/ml). Results are expressed as percentage of inhibition after normalization to aggregation induced by the correspondent thrombin concentration in each experiments. Data are means ± SD of 3 independent experiments. The asterisks represent statistically significant differences from the correspondent thrombin-stimulated samples, determined by Student's t-test (**$p<0.01$).

4.2 DHEA-S and DHEA activate the NOS/cGMP/PKG pathway in human platelets

One of the best characterized inhibitory pathway in platelets is activated by the endothelium-derived NO and leads to intraplatelet cGMP increase, activation of PKG and phosphorylation of many substrates, among which is VASP, vasodilator-stimulated phospho-protein. VASP is associated with actin microfilaments and is localized to lamellipodia, actin stress fibres, filopodia and to focal adhesions (72). Phosphorylated VASP has reduced ability to bind to cytoskeletal proteins, thus leading to inhibition of cytoskeleton reorganization, event essential for irreversible platelet aggregation (73). VASP is a major substrate of PKA and PKG, which phosphorylate VASP

on Ser157 and Ser239, respectively. Noteworthy, phosphorylation on Ser157 produces a characteristic electrophoretic mobility shift of VASP from 46 to 50 kDa (73).

Furthermore, because in endothelial cell only DHEA and not DHEA-S activated NOS (58), we also evaluated the effect of DHEA on the activation of NOS/cGMP/PKG signaling pathway. Thus, platelets were incubated for increasing times with DHEA-S or DHEA and analyzed by immunoblotting with anti-P-Ser239- or anti-P-Ser157-VASP antibody. In contrast with the effects of the hormones on platelet aggregation, we found that in human platelets not only DHEA-S but also DHEA (Figure 4.2.4) promoted a rapid and sustained phosphorylation of VASP on both Ser239 and Ser157. Although VASP was phosphorylated by both hormones also on Ser157, the low extent of the phosphorylation on Ser157 and the absence of a shifted band in the p-Ser239 blot lead us to speculate that the kinase upstream of VASP phosphorylation was PKG and not PKA, suggesting that both DHEA-S and DHEA could activate in platelets the NO/cGMP/PKG pathway. Indeed, a similar mechanism has already been described downstream of DHEA in endothelial cells (48).

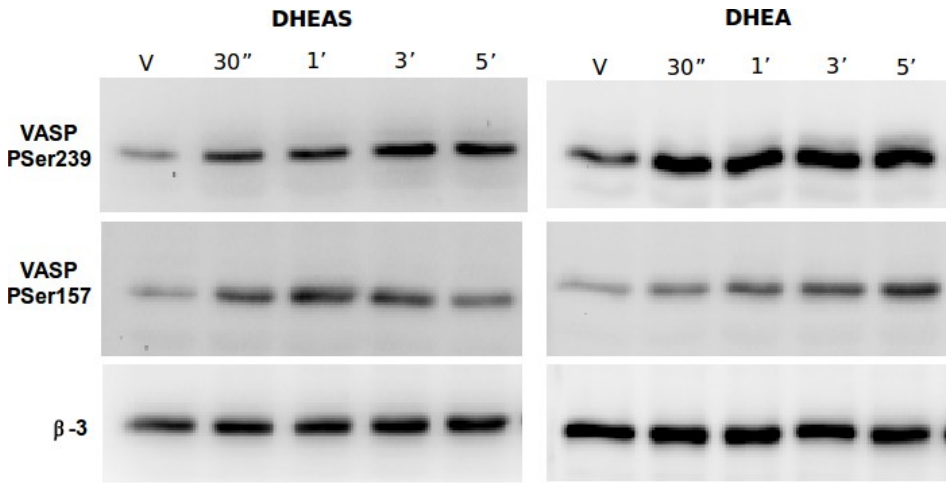

Figure 4.2.4: DHEA-S and DHEA lead to VASP phosphorylation.

Platelets were incubated at 37° C with vehicle (V), 0.3 mM DHEA-S or 10 nM DHEA for the indicated times. Aliquots containing the same amount proteins were analyzed by immunoblotting with anti-P-Ser239-VASP, anti-P-Ser157-VASP and anti-integrin β3 antibodies. The immunoblotting experiment shown is representative of 3 identical experiments performed with platelets obtained from different donors.

Endothelium-derived NO plays a role of primary importance in avoiding undesidered platelet activation and NO is the main positive modulator of guanylyl cyclase in many cell types, including platelets (74). Platelet heme-containing soluble guanylyl cyclase is the intracellular receptor of endothelial-derived NO, which is continuously released by the endothelium and acts both to limit platelet adhesion to extracellular matrix and to inhibit platelet aggregation in response

to a number of physiological agonists in circulation (66). Although in platelets the presence of NOS and the endogenous synthesis of NO are still matter of debate (23), we decided to analyze the contribution to DHEA and DHEA-S signaling of some component of the NO/cGMP/PKG pathway.
To begin we investigated the cyclic nucleotides cGMP and cAMP content in platelets treated with DHEA or DHEA-S and with sodium nitroprussiate (SNP) or forskolin as positive controls. As shown in Figure 4.2.5A, 0.3 mM DHEA-S and 10 nM DHEA was not able to induce any detectable modification of the cAMP content with respect to the basal level. By contrast, both DHEA-S and DHEA caused a significant increase in the cGMP level (Figure 4.2.5B) that was blunted by inhibition of either guanylyl cyclase with ODQ or NOS activity with L-NAME/L-NMMA. Where necessary platelets were preincubated for 5 minutes with vehicle, 2 μM ODQ or 0.3 mM L-NMMA/L-NAME and than all samples treated with 0.3 mM DHEA-S, 10 nM DHEA or 100 μM SNP for 1 minute. These results confirm that both DHEA-S and DHEA activate the pathway NO/cGMP/PKG in human platelets.

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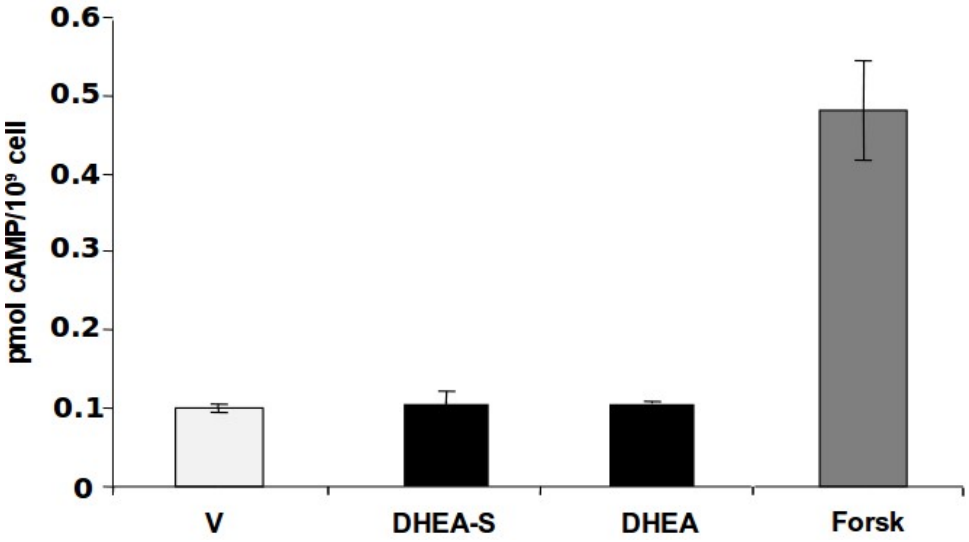

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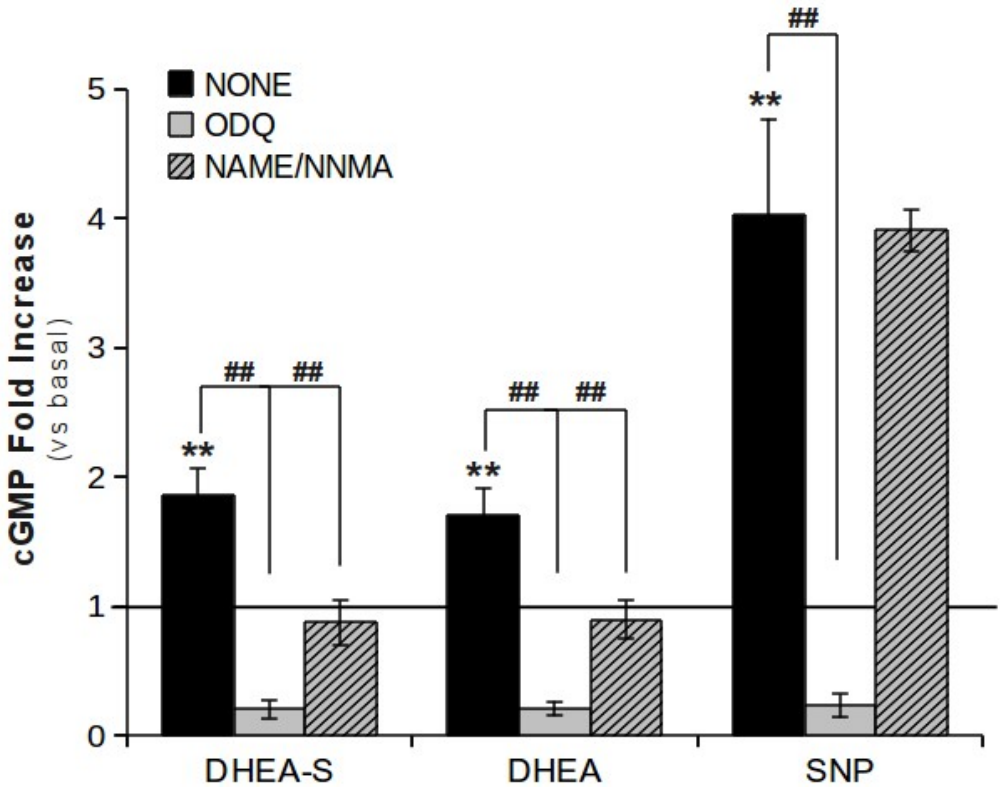
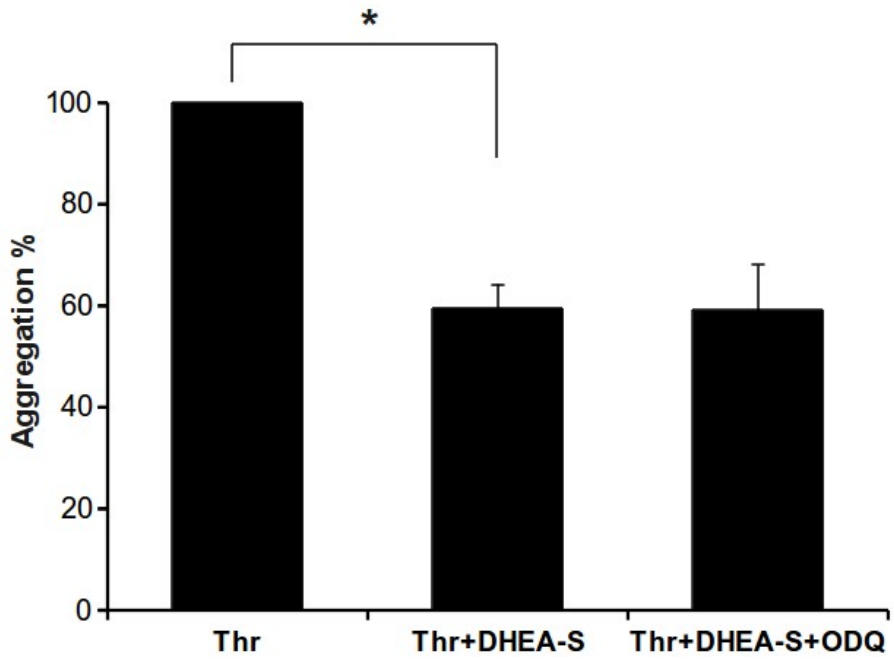


Figure 4.2.5: DHEA-S and DHEA increased cGMP but not cAMP levels in human platelets. **(A)** Gel-filtered platelets were incubated with vehicle (V), 0.3 mM DHEA-S or 10 nM DHEA, 10 μM forskolin (Forsk) for 1 minute at 37° C. cAMP levels were determined by the Amersham Bioscience Biotrack Enzymeimmunoassay Graphics represent the means ± S.D. of 3 independent experiments. **(B)** Platelets were incubated with vehicle (NONE), 2 μM ODQ or 0.3 mM L-NAME and L-NMMA (NAME/NNMA) for 5 minute and then stimulated with 0.3 mM DHEA-S, 10 nM DHEA or 100 μM SNP for 1 minute at 37° C. cGMP levels were determined by the cGMP complete EIA Kit. Results are expressed as Fold increase over basal level set arbitrarily to 1 (solid line). Graphics represent the means ± SEM of at least 3 independent experiments. The ** represent statistical significance ($p<0.01$) compared to basal cGMP levels, while ## represent statistical significance ($p<0.01$) compared to the correspondent DHEA-S-, DHEA- or SNP-treated sample.

DHEA-S, and not DHEA, inhibited thrombin-induced platelet aggregation, whereas both hormones were able to promote the activation of the NOS/cGMP/PKG pathway, suggesting that this pathway is not directly involved in DHEA-S action on platelet aggregation. In order to verify whether the inhibition of thrombin-induced aggregation was dependent on VASP phosphorylation induced by DHEA-S treatment, platelets were treated for 5 minutes with 2 μM ODQ, then with DHEA-S for 1 minute and finally stimulated with thrombin. We found that, while the inhibition of platelet aggregation caused by DHEA-S was not reverted by the inhibition of guanylyl cyclase activity (Figure 4.2.6A), the inhibition of soluble guanylyl cyclase with ODQ was able to completely block VASP phosphorylation on both Ser239 and Ser157 (Figure 4.2.6B). Therefore, the NO/cGMP/PKG pathway is activated in human platelets by DHEA-S and DHEA, but it is not the unique pathway involved in DHEA-S-mediated inhibition of platelet function.

A)



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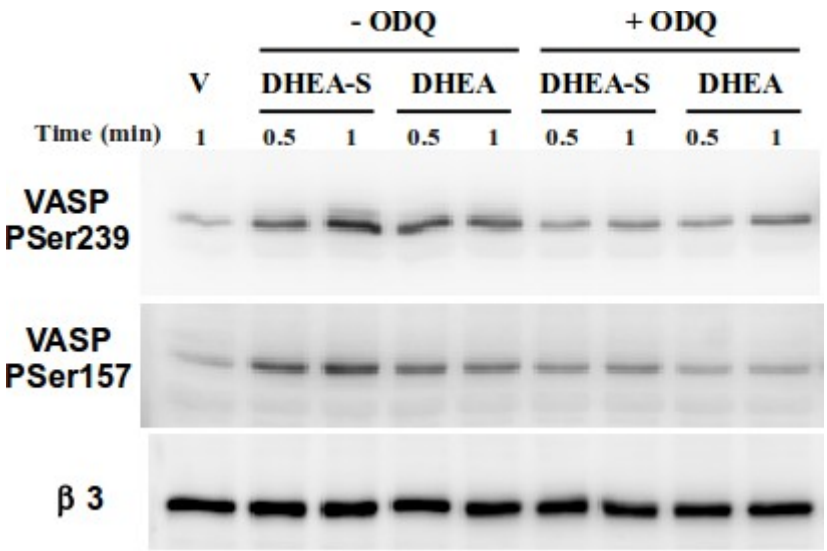

Figure 4.2.6: DHEA-S inhibits platelet aggregation independently of NO/cGMP/PKG pathway.

(A) Samples of gel-filtered platelets incubated with vehicle or 2 μM ODQ for 5 minutes at 37° C followed by treatment with 0.3 mM DHEA-S were then aggregated with 0.05 U/ml of thrombin. Aggregation was measured in a lumiaggregometer for 5 minutes at 37° C under stirring conditions. Data are means ± SD from 3 independent experiments normalized to the aggregation induced by 0.05 U/ml thrombin, set arbitrarily at 100%. The * represent statistically significant differences from the correspondent thrombin-stimulated samples, determined by Student's t-test (*$p<0.05$). **(B)** Platelets were incubated with vehicle (- ODQ) or 2 μM ODQ for 5 minutes at 37° C and then stimulated with vehicle (V), 0.3 mM DHEA-S or 10 nM DHEA for the indicated times. Aliquots containing the same amount proteins were analyzed by immunoblotting with anti-P-Ser239-VASP, anti-P-Ser157-VASP and anti-integrin β3 antibodies. The immunoblotting is representative of 3 identical experiments performed with platelets obtained from different donors.

4.3 DHEA-S potentiates NO-dependent inhibition of platelet aggregation

The main effect of DHEA on the vasculature is to cause endothelium-derived NO increase, which leads to platelet cGMP increase and inhibition of these cells in the circulation. In this study we showed that DHEA-S action on platelet aggregation is not totally dependent of the activation of the NO/cGMP/PKG. In conclusion, to better understand the contribution of this pathway to the inhibition of platelet aggregation, we analyzed the effect of DHEA-S and DHEA in platelets treated with the NO-donor SNP. We chose a concentration of SNP (100 nM) that was previously shown (data not shown) to halve platelet aggregation induced by 0.1 U/ml Thr. To note that when platelets are stimulated by this high concentration of thrombin also the highest concentration of DHEA-S is not able to inhibit platelet aggregation.

As shown in Figure 4.3.7 when platelets were incubated with 10 nM DHEA and 100 nM SNP prior to thrombin stimulation, platelet aggregation was inhibited at the same extent as SNP alone. However, when platelets are treated with 0.3 mM DHEA-S along with SNP thrombin-induced aggregation is completely inhibited. These date show that DHEA-S can act in synergism with endothelium-derived NO in inhibiting platelet aggregation.

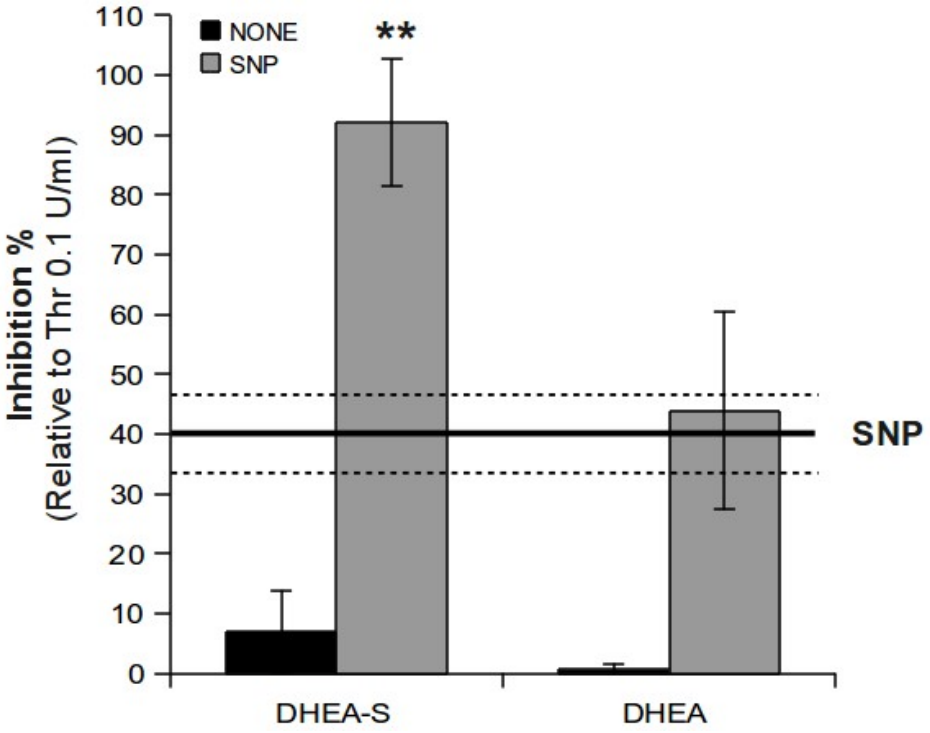

Figure 4.3.7: DHEA-S potentiates NO-dependent inhibition of platelet aggregation.

Platelets were treated with 0.3 mM DHEA-S or 10 nM DHEA in the presence (SNP) or not (NONE) of 100 nM SNP for 1 minute and then aggregated with 0.1 U/ml of thrombin. Aggregation was measured in a lumiaggregometer for 5 minutes at 37° C under stirring conditions. Data are means ± SD from 3 independent experiments expressed as percentage of inhibition after normalization to the aggregation induced by 0.1 U/ml thrombin, set arbitrarily at 100%. The average inhibition caused by SNP alone is indicated by the solid line, while the dotted lines represents the ± SD interval. The ** represent statistically significant differences from the correspondent DHEA-S-stimulated samples, determined by Student's t-test (**$p<0.01$).

4.4 DHEA-S selectively inhibited dense granule secretion

Platelet aggregation requires a coordinated series of events that lead to secretion of granule content (e.g. fibrinogen and ADP) and culminate in the activation of integrin $\alpha_{IIb}\beta_3$ through a process known as "inside-out" signaling (75). To promote platelet secretion and activation of integrin $\alpha_{IIb}\beta_3$, thrombin induces activation of various protein kinases including Src, the MAP kinases p38 MAPK and ERK1/2, and PI3K. Full activation of MAP Kinases, PI3K and integrin $\alpha_{IIb}\beta_3$ requires the recruitment of the P2Y12 receptor by the ADP released from dense granules upon thrombin stimulation (76). In addition, the PKC/MEK1/2 pathway and the tyrosine kinase Src have been implicated in ERK1/2 phosphorylation. Furthermore, in collagen-stimulated platelets VASP phosphorylation was linked to the inhibition of platelet aggregation but not to inhibition of granule

secretion (77). This led us to hypothesize that DHEA-S was modulating some processes important for platelet aggregation that were not dependent on VASP.

Thus, to better characterize the inhibition of platelet function by DHEA-S, we investigate granule secretion and integrin activation in thrombin-stimulated platelets.

To evaluate the effect of DHEA-S and DHEA on dense granule secretion, platelets preincubated with 0.3 mM DHEA-S or 10 nM DHEA for 1 minute, were stimulated with 0.05 U/ml thrombin and then allowed to aggregate. After 5 minutes the amount of secreted ATP was measured by adding luciferine-luciferase. Similarly at platelet aggregation DHEA-S, but not DHEA, inhibited dense granule secretion in thrombin-stimulated cells (Figure 4.4.8A). To verify if the inhibition of secretion was dependent on the activation by DHEA-S of the NO/cGMP/PKG pathway, ATP release was measured in presence of ODQ (2 μM for 5 minutes) to inhibit cGMP increase and VASP phosphorylation. As shown in Figure 4.4.8B ODQ treatment did not modify the ability of DHEA-S to inhibit thrombin-induced ATP release.

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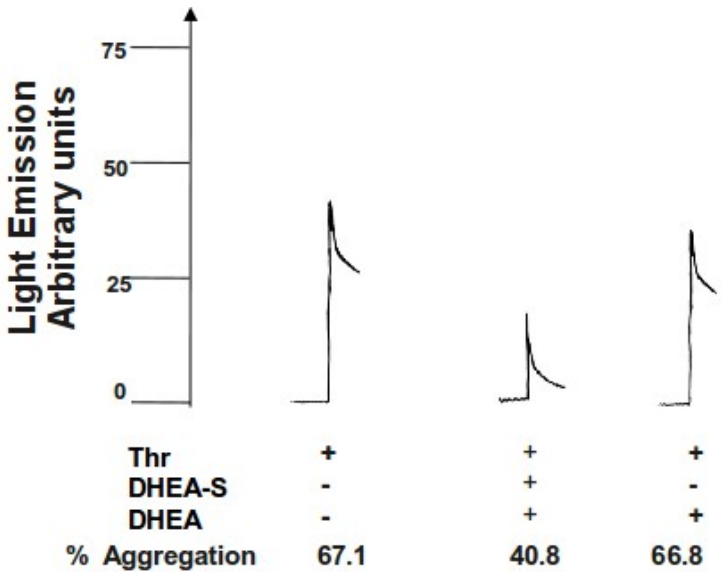

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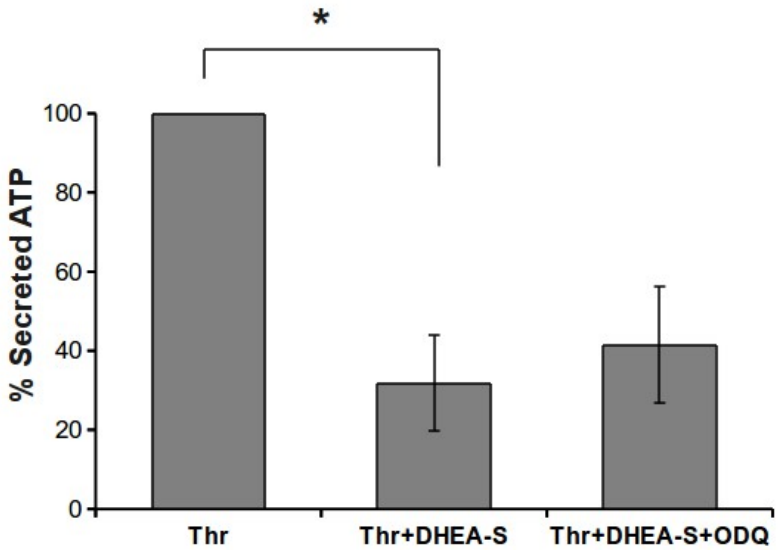
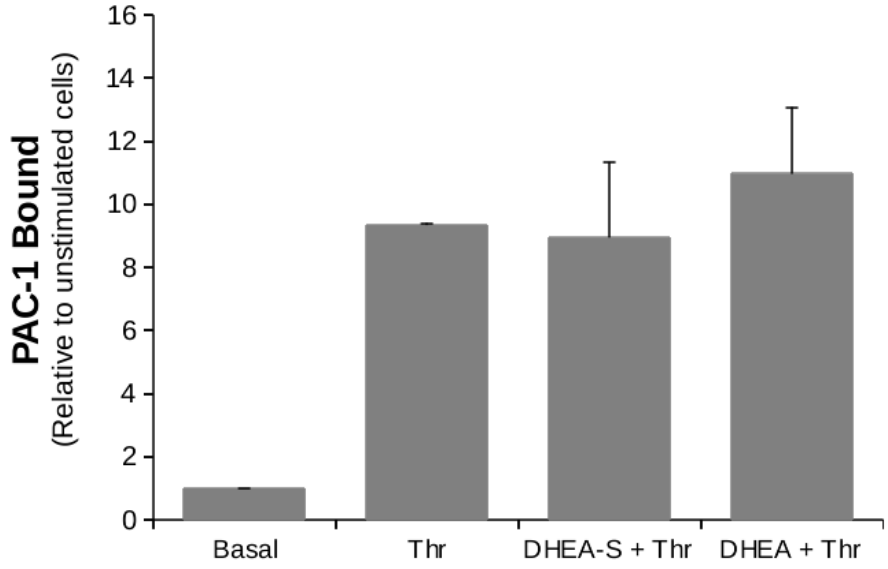


Figure 4.4.8: DHEA-S inhibits thrombin-induced dense granule secretion independently of cGMP/PKG signaling.

Measurement of platelet secretion was carried out by evaluating the ATP released after 5 minutes of stimulation with thrombin using the CHRONO-LUME reagent. The activation of platelets was performed in a lumiaggregometer at 37°C with stirring. **(A)** Samples of gel-filtered platelets were treated with vehicle, 0.3 mM DHEA-S or 10 nM DHEA for 1 minute at 37° C and then stimulated with 0.05 U/ml thrombin. The results are expressed in arbitrary light emission units. The correspondent percentage of aggregation are also reported. Traces are from a representative experiment out of 3 performed. **(B)** Samples of gel-filtered platelets incubated with vehicle or 2 μM ODQ for 5 minutes at 37° C followed by treatment with 0.3 mM DHEA-S were then aggregated with 0.05 U/ml thrombin. Data are means ± SEM from 3 independent experiments normalized to the aggregation induced by thrombin, set arbitrarily at 100%.

To evaluate the activation state of integrin $\alpha_{IIb}\beta_3$ we examined by flow citometry PAC-1 binding (an antibody specific for the activated conformation of αIIbβ3). Platelets were preincubated with 0.3 mM DHEA-S or 10 nM DHEA and then activated with 0.05 U/ml thrombin. As expected thrombin induced PAC-1 binding to platelets, but neither DHEA-S nor DHEA were able to affect integrin activation by thrombin (Figure 4.4.9A). α-granule secretion was analyzed as surface expression of P-selectin (CD62P), P-selectin expression increased by 12.58 ± 0.44-fold in thrombin-stimulated in respect to basal platelets and this was unaffected by pre-treatment of the cells with either DHEA-S or DHEA (12.78±0.54 and 13.53±0.50 respectively) (Figure 4.4.9B).
These results suggest that DHEA-S specifically modulated dense granule secretion independently of the NO/cGMP/PKG pathway.

A)



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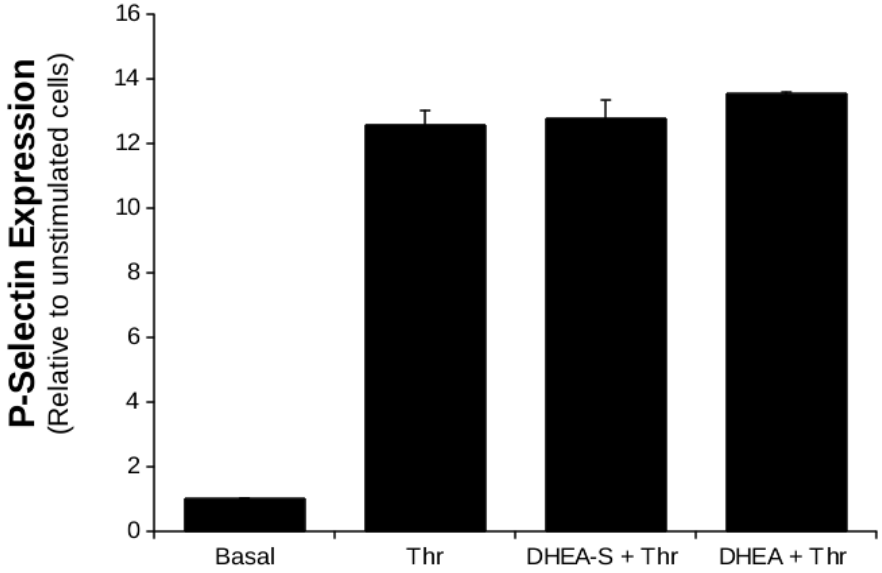


Figure 4.4.9: DHEA-S and DHEA do not affect αIIbβ3 activation and α-granule secretion

Samples of gel-filtered platelets were pretreated with 0.3 mM DHEA-S or 10 nM DHEA for 1 minute and then stimulated with 0.05 U/ml of thrombin (Thr). Incubation with **(A)** FITC-conjugated PAC-1 and **(B)** PE-conjugated P-selectin antibody were carried out in the presence of Thr for 20 minutes at room temperature in the dark. Data are mean ± SEM of 3 independent experiments expressed as Fold increase relative to unstimulated cells (Basal).

These finding allowed us to hypothesize that in the presence of DHEA-S the phosphorylation of the protein kinase, previously reported, caused by thrombin could be impaired as a consequence of a decreased ADP secretion and/or a decrease in Src activation. Therefore, samples of platelets preincubated with vehicle, 0.3 mM DHEA-S, 10 nM DHEA or 1 μM SNP for 1 minute were stimulated with 0.05 U/ml thrombin for 3 minutes. The lysates were analyzed in immunoblotting with antibodies specific to the phosphorylated forms of Src, Akt, p38MAPK and ERK1/2. Figure 4.4.10 shows that, in the presence of DHEA-S, but not with DHEA, the phosphorylation of Akt and ERK1/2 caused by thrombin was significantly decreased; while the phosphorylation of Src and p38MAPK remains comparable to thrombin. These results indicate that is damaged the signaling that leads to dense granule secretion.

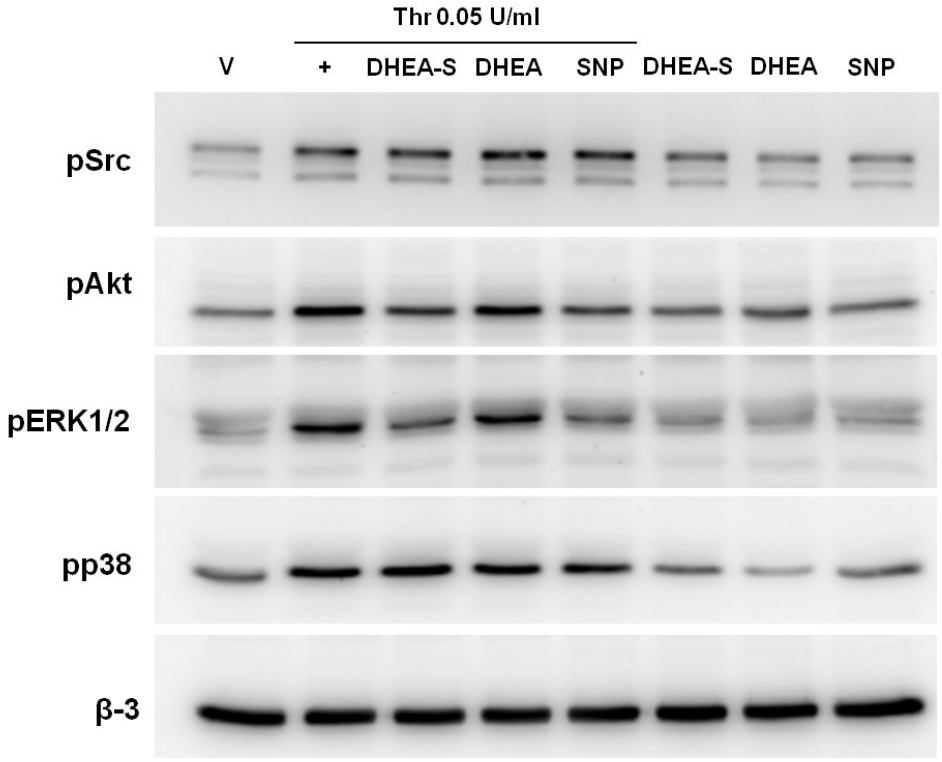
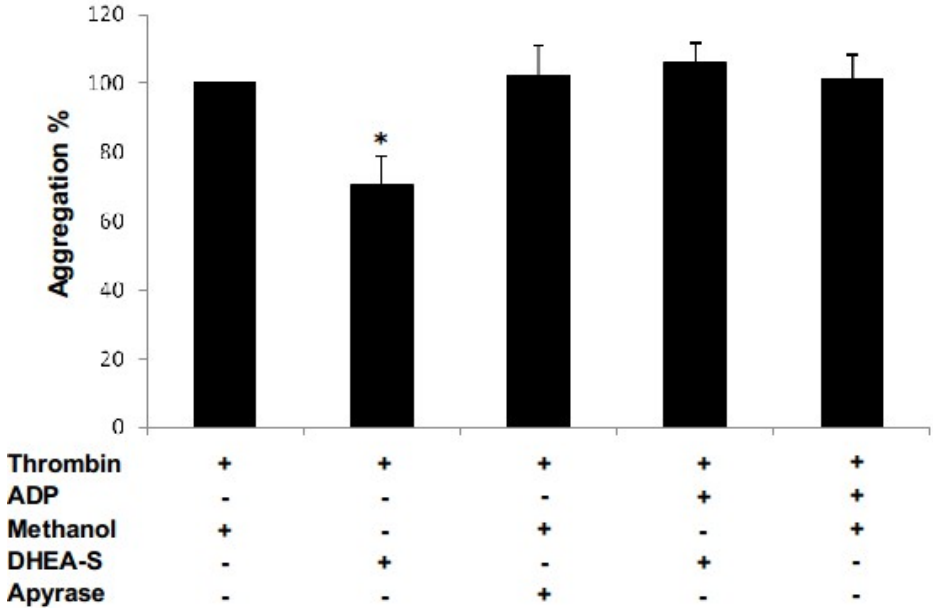


Figure 4.4.10: DHEA-S, but not DHEA, inhibits protein phosphorylation.
Platelets were treated with vehicle (V), 0.3 mM DHEA-S, 10 nM DHEA o 1 μM SNP for 1minute and then stimulated with 0.05 U/ml thrombin for 3 minutes. Aliquots from each sample were analyzed by immunoblotting with anti-pTyr418-Src, anti-p-Akt, anti-p-p38 MAPK, anti-p-ERK1/2 and anti-integrin β-3 antibodies. Integrin β-3 was used to verify the equal loading. The image reported is from a representative experiment out of the 3 performed with platelets obtained from different donors.

At this point, with the purpose of complete these evidences we have conduced the aggregations giving back ADP to samples treated with DHEA-S. If DHEA-S prevents a correct secretion, the extracellular ADP can replace the released ADP. Platelets were treated with vehicle or 0.3 mM DHEA-S for 1 minute and than stimulated with 0.05 U/ml thrombin alone or in association with 10 μM ADP. We found that the inhibition of platelet aggregation caused by DHEA-S was reverted by the administration of ADP (figure 4.4.11A). Part of the platelet suspension was than stimulated without stirring condition, the samples were lysated and analyzed in immunoblotting with antibodies specific to the phosphorylated forms of Akt and ERK1/2 (figure 4.4.11B). DHEA-S was able to inhibit protein phosphorylation also in presence of ADP. This evidence validates our hypothesis: DHEA-S inhibites protein activation across granule secretion reduction; moreover platelet functionality was recovered miming secretion.

In conclusion, our results indicate that in the presence of DHEA-S the granule secretion and the resulting protein phosphorylation caused by thrombin were delayed but not completely suppressed, suggesting that inhibition pathways are presumably elicited in DHEA-S-treated platelets in order to explain the strong and long lasting inhibition of aggregation.

A)



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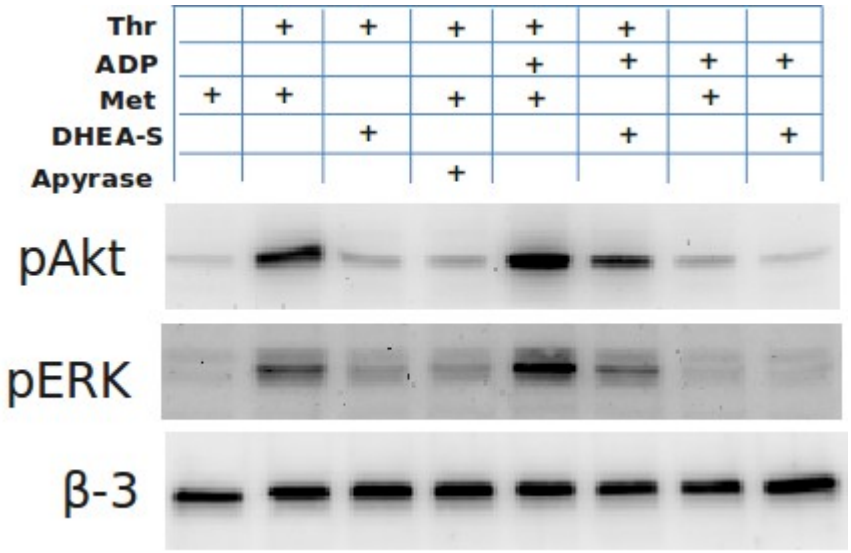

Figure 4.4.11: DHEA-S inhibits thrombin-induced dense granule secretion inhibiting the phosphorylation of protein as Akt and ERK1/2.
(A) Platelets were treated with vehicle or 0.3 mM DHEA-S for 1 minute and than stimulated with 0.05 U/ml thrombin alone or in association with 10 μM ADP. Some samples were treated with 1 U/ml apyrase for 10 minutes before of the stimulus with thrombin. Aggregation was measured in a lumiaggregometer for 5 minutes at 37° C under stirring conditions. Data are means ± SEM from 3 independent experiments expressed as percentage of inhibition after normalization to the aggregation induced by 0.05 U/ml thrombin, set arbitrarily at 100%. **(B)** Samples of platelets stimulated at the same manner of "A" were analyzed by immunoblotting with anti-Akt, anti-p-ERK1/2 and anti-integrin β-3 antibodies. The image reported is from a representative experiment out of the 3 performed with platelets obtained from different donors.

Subsequently, we have used indomethacin, a non-selective inhibitor of cyclooxygenase (COX) 1 and 2, and apyrase, that catalyses the hydrolysis of ATP and ADP to yield AMP: concurrent use of both can block the effect of the dense granule secretion on protein phosphorylation. The platelets were treated with veichle, 0.3 mM DHEA-S, 1 μM SNP for 1 minute or 1 μM indomethacin and 0.2 U/ml apyrase for 10 minutes before of the stimulus with 0.05 U/ml thrombin for 1 and 3 minutes. The lysates were analyzed in immunoblotting with antibodies specific to the phosphorylated forms of $pTyr^{418}$-Src, ERK 1/2 e p38MAPK (figure 4.4.12). This data shows that treatment with DHEA-S reduce protein phosphorylation like as indomethacin/apyrase, having an inhibitory effect on granule dense secretion. This result together with the previous reported indicate that DHEA-S exerts its inhibitory effect by reducing the release of ADP from the granules.

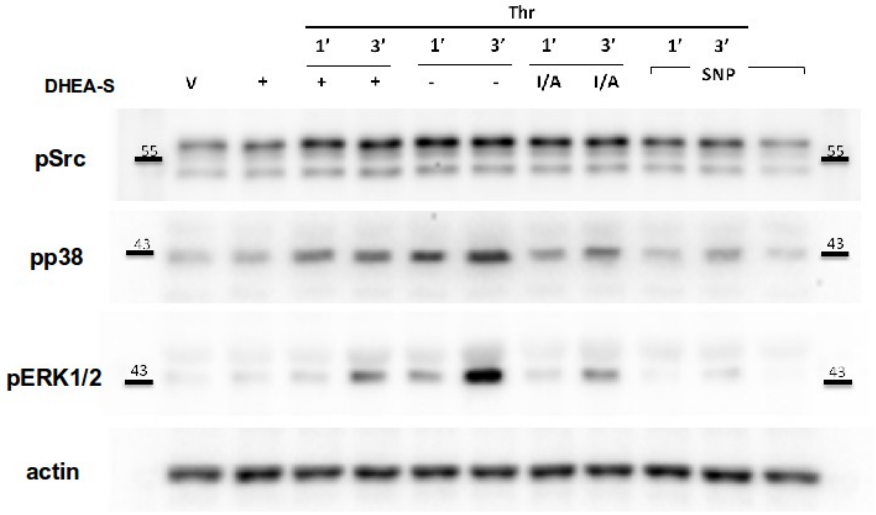

Figure 4.4.12: DHEA-S inhibits protein phosphorylation.
Samples of platelets were treated with veichle (V), 0.3 mM DHEA-S, 1 μM SNP for 1 minute or 1 μM indomethacin and 0.2 U/ml apyrase for 10 minutes before of the stimulus with 0.05 U/ml thrombin for 1 and 3 minutes. Aliquots from each sample were analyzed by immunoblotting with anti-pTyr418-Src, anti-p-p38 MAPK, anti-p-ERK1/2 and anti-actin antibodies. Actin was used to verify the equal loading. The image reported is from a representative experiment out of the 3 performed with platelets obtained from different donors.

5. DISCUSSION

The aim of this study was to investigate *in vitro* the effect on platelet function of DHEA and DHEA-S, the most abundant steroid hormones in circulating blood (39). Although many reports showed evidence that DHEA stimulates endothelial cells to produce higher amount of NO in the circulation leading to platelet inhibition (52), the effect of this hormone on platelet function have not yet been investigated in some detail. We found that DHEA-S was able to inhibit platelet function and in particular the aggregation of platelets stimulated with thrombin as well as with other platelet agonists like collagen and U46,619 (Figure 4.1.1). The ability of DHEA-S to counteract the effect of thrombin was dependent on the dose of agonist used to stimulate platelets. When thrombin was 0.025 or 0.05 U/ml DHEA-S inhibited platelet aggregation with a dose dependent mechanism, while it was uneffective on 0.1 U/mL or higher thrombin treated platelets (Figure 4.1.3).

It is generally accepted that DHEA-S is formed by action of steroid sulphotransferases that are expressed in different cell types (63), including platelets (65) and serves as reservoir of DHEA, which in turn can modulate cell function by either genomic or non genomic mechanisms (78). However, we found that DHEA did not inhibit platelet aggregation caused by thrombin (neither at 10 nM, figure 4.1.1, nor a higher concentration, data not shown) while DHEA-S did. Moreover, in the presence of EMATE, which inhibits both estrone and dehydroepiandrosterone sulphatase (69), the effect of DHEA-S on thrombin-dependent aggregation was not decreased, supporting the evidence that DHEA-S, and not DHEA, inhibited thrombin-dependent platelet aggregation (Figure 4.1.2).

Data from literature reported a direct interaction of DHEA with both estrogen and androgen receptors, with agonistic and antagonistic effect, respectively (61). Human megakaryocytes and platelets contain estrogen receptor β and androgen receptor (79). Recently we have investigated the

effect of 17β-estradiol on human platelet function and demonstrated that 17β-estradiol *in vitro* exerted a priming effect and potentiated the aggregation caused by low doses of thrombin (71).
Here we demonstrated that the inhibition of platelet aggregation caused by DHEA-S does not require the recruitment of estrogen receptors (Figure 4.1.2). Hence the evidence that 17β-estradiol and DHEA-S generate signals with opposite effects on platelet function lead to conclude that 17β-estradiol and DHEA-S trigger different signals in the platelet. However, the inhibition of aggregation caused by DHEA-S in thrombin-stimulated platelets showed a dose-dependent behaviour (Figure 4.1.3), suggesting that DHEA-S action is mediated by receptors on the platelet surface.

The first series of results shows that in human platelets both DHEA-S and DHEA caused the increase of cGMP (but not cAMP) level in the platelet cytoplasm (Figure 4.2.5), thus activating PKG and leading to VASP phosphorylation (Figure 4.2.4). This finding was dependent on NO production by platelets and activation of platelet guanylyl cyclase (Figure 4.2.5). However, the inhibition of platelet guanylyl cyclase with ODQ did not prevent the inhibition of either aggregation or dense granule secretion induced by thrombin (Figure 4.2.6A and 4.4.8B). These data suggest that the inhibition of platelet secretion and aggregation mediated by DHEA-S does not rely on the activation of the platelet NO/cGMP/PKG pathway, and indeed DHEA activated the same pathway but was not able to inhibit neither secretion nor aggregation in thrombin-stimulated platelets. In fact, in human platelets DHEA-S, but not DHEA, was able to negatively interfere with the activation signal transduction pathways (Figure 4.4.10) and in particular with dense granule secretion and aggregation induced by thrombin in human platelets. Our data also indicate that the inhibitory effect of DHEA-S on platelet secretion and aggregation is reverted miming the granule secretion by addition of ADP (Figure 4.4.11). This findings suggest that DHEA-S plays a specific inhibitory action on the release of ADP from dense granule. In addition the reduction in the secretion of ADP caused by DHEA-S leads to a decreased levels of protein phosphorylation and, consequently, reduction of platelet aggregation.

Anyway, these evidences could be relevant to determine the biological effect of DHEA-S that was undetectable in resting platelets but became dramatically evident in platelets stimulated with low doses of thrombin or when the blood NO is elevated. Many reports in literature support the hypothesis that the main effect of DHEA in the vasculature is to cause endothelium-derived NO increase, which leads to platelet cGMP increase and inhibition of these cells in the circulation. Our results suggest that DHEA-S might also play some important role in this context. Indeed, DHEA-S at non-inhibiting concentration synergied with endothelium-derived NO in blocking platelet aggregation (Figure 4.3.7).

In conclusion, in the light of the results obtained we can assume that, though both DHEA and DHEA-S activate platelet NO/cGMP/PKG pathway, only DHEA-S can inhibit the aggregation of platelet stimulated with low doses of thrombin by modulating dense granule secretion. Although the molecular mechanisms involved are not yet completely characterized, it is possible to conclude that DHEA-S function with multiple mild mechanisms that did not hamper completely the functionality of platelets, allowing them to be fully active when stimulated by high doses of thrombin. However, since DHEA-S was able to counteract efficiently platelet activation caused by a mild stimulus, it may be hypothesized that DHEA-S participates in maintaining platelets in a resting state, mainly when the functionality of vascular endothelium is compromised, for instance by the development of the atherosclerotic plaque (40,57).

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7. PhD PAPERS

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