



**UNIVERSITÀ DEGLI STUDI DI ROMA
"TOR VERGATA"**

FACOLTA' di MEDICINA e CHIRURGIA

**DOTTORATO di RICERCA in
BIOCHIMICA e BIOLOGIA MOLECOLARE**

XXI ciclo
2009-2010

***“INTRACELLULAR AND SURFACE REDOX
SYSTEMS IN HUMAN PLATELETS”***

Dott. ROSARIA ARNONE

Docente Guida: ***Prof. Luciana Avigliano***

Coordinatore: ***Prof. Alessandro Finazzi Agrò***

DIPARTIMENTO DI MEDICINA SPERIMENTALE & SCIENZE BIOCHIMICHE
VIA MONTPELLIER 1 – 00133 ROMA

ABSTRACT

Redox balance is important for platelet physiology, indeed it is crucial for blood coagulation and thrombosis. This thesis will give an update on intracellular and plasma membrane systems involved on redox homeostasis in human platelets, with particular regard to vitamin C and plasma membrane NAD(P)H oxidases.

The redox balance is linked to the presence of intracellular antioxidants; in particular, vitamin C appears to be a key modulator of platelet oxidative state, since these cells physiologically accumulate ascorbic acid. Here, we showed, for the first time, that platelets could compensate for fluctuations in ascorbate levels by modulating the expression of the Na⁺-dependent transporter SVCT2 at translational level. Moreover, we showed that changes in intracellular ascorbic acid content had physiological relevance, since they modulate the surface sulfhydryl content and the thrombus viscoelastic properties.

Intracellular reducing equivalents can be propagated to the plasma membrane and electrons transferred to external acceptors, thus affecting both adjacent cells and circulating blood components. In platelets, the plasma membrane redox (PMR) system has not yet been fully characterized and the molecular identities of most components are unknown. Here we described the presence of at least one member of the plasma membrane hydroquinone-(NADH) oxidase family (namely Ecto-NOX1). We found that Ecto-NOX1 is sensitive to capsaicin: indeed, it is up-modulated through a mechanism requiring binding of capsaicin to its receptor, namely the transient receptor potential vanilloid subtype 1 (TRPV1). Ligand-receptor interaction triggers a signalling cascade leading to reactive oxygen species (ROS) production, which in turn enhances the expression and activity of Ecto-NOX1. Redox regulation of Ecto-NOX1 may be important for platelet recruitment and activation during inflammatory diseases.

RIASSUNTO

Lo stato redox è importante per la corretta fisiologia delle piastrine, dal momento che esso risulta cruciale durante la coagulazione del sangue e la trombosi. Questa tesi intende descrivere i sistemi intracellulari e di membrana coinvolti nel mantenimento dell'omeostasi dello stato redox nelle piastrine umane, con particolare riguardo alla vitamina C ed alle NAD(P)H ossidasi presenti sulla membrana plasmatica.

L'equilibrio redox è chiaramente correlato alla presenza di antiossidanti cellulari: in particolare, la vitamina C è un modulatore importante dello stato redox piastrinico, dal momento che le piastrine stesse accumulano l'acido ascorbico in condizioni fisiologiche. In questo lavoro, abbiamo evidenziato per la prima volta che le piastrine possono compensare le fluttuazioni nei livelli di ascorbato, modulando, a livello tradizionale, l'espressione del trasportatore Na^+ -dipendente SVCT2. Inoltre, abbiamo dimostrato che cambiamenti nel contenuto intracellulare di acido ascorbico hanno una rilevanza fisiologica, dal momento che questi possono modulare il contenuto di gruppi sulfidrilici nella membrana plasmatica e le proprietà viscoelastiche del trombo.

Gli equivalenti riducenti intracellulari possono essere trasferiti alla membrana plasmatica e gli elettroni trasferiti ad accettori esterni, influenzando così sia le cellule adiacenti che i diversi componenti del sangue. Nelle piastrine il sistema redox di membrana non è stato ancora completamente caratterizzato e l'identità molecolare di molti componenti di questo sistema rimangono sconosciuti. Abbiamo evidenziato la presenza di almeno un membro della famiglia delle idrochinone-(NADH) ossidasi localizzate sulla membrana plasmatica; tale membro è designato Ecto-NOX1. Abbiamo anche dimostrato che Ecto-NOX1 è sensibile alla capsaicina; infatti, tale composto è in grado di aumentare l'espressione di Ecto-NOX1 mediante un meccanismo che richiede il legame della capsaicina al suo recettore naturale (designato recettore transiente per il vanilloide di tipo 1 o TRPV1). L'interazione ligando-recettore innesca una cascata del segnale che porta alla produzione di specie reattive dell'ossigeno, le quali, a loro volta, aumentano l'espressione e l'attività di Ecto-NOX1. La regolazione redox di questo enzima potrebbe essere importante per il reclutamento e l'attivazione delle piastrine durante i processi infiammatori.

INDICE

| | |
|---|-----------|
| ABSTRACT..... | I |
| 1. PLATELETS..... | 1 |
| 1.1 Platelet morphology..... | 1 |
| 1.2 Platelet and protein synthesis..... | 5 |
| 1.3 Platelet activation..... | 6 |
| 1.4 Dynamics of thrombus formation..... | 6 |
| 2. REDOX SYSTEMS IN HUMAN PLATELETS..... | 11 |
| 2.1 Reactive oxygen/nitrogen species..... | 11 |
| 2.2 Antioxidant defence mechanisms..... | 13 |
| 2.3 The plasma membrane redox system..... | 14 |
| 2.4 Vitamin C..... | 19 |
| 2.4.1 General aspects..... | 19 |
| 2.4.2 Metabolism..... | 21 |
| 2.4.3 Biochemical Functions..... | 25 |
| 3. OBJECTIVES OF RESEARCH..... | 27 |
| 4. RESULTS..... | 29 |
| 4.1 Translational control of ascorbic acid transporter SVCT2..... | 29 |
| 4.1.1 Ascorbic acid is accumulated through the SVCT2 carrier..... | 29 |
| 4.1.2 Expression of SVCT2 is regulated by intracellular AA levels..... | 30 |
| 4.1.3 Vitamin C is maintained in the reduced form inside cells..... | 33 |
| 4.1.4 SVCT2 expression is enhanced during platelet activation..... | 33 |
| 4.1.5 AA modulates surface sulfhydryl groups..... | 36 |
| 4.1.6 AA modulates thrombus rigidity..... | 38 |
| 4.2 Modulation of surface NADH-oxidase enzyme by capsaicin | 38 |
| 4.2.1 Ecto-NOX1 is present in human platelets..... | 38 |
| 4.2.2 Ecto-NOX1 expression is up-regulated by capsaicin | 44 |
| 4.2.3 The capsaicin-mediated regulation of ecto-NOX1 requires binding to vanilloid receptor..... | 44 |
| 5. DISCUSSION..... | 49 |
| 6. CONCLUSIONS..... | 53 |
| 7. EXPERIMENTAL PROCEDURES..... | 55 |

| | |
|--------------------------------------|-----------|
| 8. REFERENCES..... | 61 |
| 9. CURRICULUM VITAE..... | 73 |
| 10. LIST OF PUBLICATIONS..... | 75 |

LIST OF ABBREVIATIONS

| | |
|------------------------------|--|
| 4E-BP1..... | 4E-binding protein1 |
| 5'-IRTX..... | 5'-iodo-resiniferatoxin |
| AA..... | ascorbic acid |
| AA-2P..... | ascorbic acid-2 phosphate |
| AFR..... | ascorbyl free radical |
| arNOX..... | age-related NADH oxidase |
| Caps..... | capsaicin |
| CM-H ₂ DCFDA..... | 5(and-6)chloromethyl-2',7'-dichlorodihydro- fluorescein diacetate, acetyl ester |
| cNOX or Ecto-NOX1... | constitutive NADH oxidase |
| CoQ..... | ubiquinone; coenzyme Q; |
| CoQ [•] | semiquinone radical |
| CoQH ₂ | hydroquinone |
| Cpz..... | capsazepine |
| DHA..... | dehydroascorbic acid |
| DTNB..... | 5-5'dithiobis(2-nitrobenzoic acid) |
| DOG..... | deoxyglucose |
| DTT..... | dithiotreitol |
| ERP5..... | endoplasmic reticulum protein. |
| FXI..... | factor XI |
| FXII..... | factor XII |
| GP..... | glycoprotein |
| GPx..... | glutathione peroxidase |
| GSH..... | reduced glutathione |
| GSSG..... | oxidized glutathione |
| NAC..... | N-acetyl-L-cysteine |
| NOX (1-5)..... | NADPH oxidases; |
| NQO..... | NAD(P)H:ubiquinone oxidoreductase |
| PBS..... | phosphate buffered saline |
| PDI..... | protein disulphide isomerase |
| PMA..... | phorbol 12-myristate 13-acetate |
| PMR..... | plasma membrane redox system |
| <i>m</i> PMS..... | 1-methoxy-5-methyl-phenazinium methyl sulphate |

| | |
|-----------------------|--|
| RO/NS..... | reactive oxygen and nitrogen species |
| ROS..... | reactive oxygen species; |
| RTX..... | resiniferatoxin |
| SOD..... | superoxide dismutase |
| SVCT..... | Na ⁺ -dependent transporters |
| TF..... | tissue factor |
| tNOX or Ecto-NOX2.... | tumour-associated NADH oxidase |
| TRPV1..... | vanilloid receptor type 1 |
| Trx..... | thioredoxin |
| TrxR..... | thioredoxin reductase |
| TX..... | thromboxane |
| VDAC..... | voltage-Dependent anion-selective channel |
| vWF..... | van Willebrand Factor |
| WST-1..... | 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt |

RINGRAZIAMENTI

Desidero esprimere la mia gratitudine alla Prof.ssa Luciana Avigliano, riferimento essenziale ed eccellente supervisore, per gli insegnamenti ricevuti durante questo percorso di studio e per avermi guidato con la sua grande esperienza in questi anni di lavoro.

Ringrazio inoltre il Collegio dei Docenti del Dottorato di Ricerca in Biochimica e Biologia Molecolare ed, in particolar modo, il Coordinatore Prof. Alessandro Finazzi Agrò.

Un ringraziamento particolare alle Prof.sse Isabella Savini e Maria Valeria Catani per i loro preziosi insegnamenti e per l'infinita pazienza e disponibilità.

Infine, ringrazio sentitamente tutte le persone con cui ho condiviso la vita di laboratorio in questi anni e che mi hanno supportato durante la stesura di questa tesi.

Un pensiero particolare va a tutti i colleghi del corso di Dottorato di Ricerca in Biochimica e Biologia Molecolare per i numerosi piacevoli momenti trascorsi durante questo periodo di lavoro.

1. PLATELETS

Platelets, discovered by G. Bizzozero in 1882, have been “rediscovered” in the 1960s after many decades of oblivion. Their role was initially more clearly associated with thrombosis than with haemostasis (de Gaetano, 2001). Platelets are anucleated fragments of cytoplasm originating from megakaryocytes, haematopoietic cells residing in the bone marrow, and released into circulating blood to function as sentinels of the integrity of vascular system (Figure 1.1) (Chang et al., 2007). They circulate in blood for approximately 10 days, normally in a concentration of $150-400 \times 10^9/L$ (inferring that approximately 1.5 million platelets are formed every second) (Dale, 1997). Platelets react with any type of vessel wall injury, that alters the endothelial cell lining, whether accompanied or not by exposure of subendothelial matrix components. This “defence” mechanism is elicited whenever there is a deviation from normal condition of vasculature. The most common situation inducing a platelet response results from traumatic injuries to tissue, where the continuity of the vascular tree is interrupted and blood begins to pour outside.

Haemostasis is a complex set of regulated events leading to the arrest of post-traumatic hemorrhage and preventing death from exsanguinations. Platelets cannot distinguish vessel damage caused by traumatic wounds from damage arising as a complication of pathologic vascular alterations. Under the latter circumstances, the beneficial role that prevents excessive blood loss may become a life-threatening disease mechanism. Indeed, platelets may participate in the formation of occlusive thrombi, that may be the cause of sudden death or serious pathological conditions, such as ischemic syndromes of heart and brain.

1.1 Platelet morphology

Platelets are the smallest corpuscular components of human blood: their mean diameter is $<1.5-2.5 \mu\text{m}$, from one-third to one-fourth that of erythrocytes. Platelets are no true cells, as they are not provided with a nucleus. Their origin is the bone marrow, where megakaryocytes liberate platelets as the end product of protrusions of their membrane and cytoplasm. The typical shape of resting platelets is discoid, but upon activation they undergo a shape change to a spiny sphere with long, thin filopodia extending up to $5 \mu\text{m}$ out from platelet and ending in points that are as small as $0.1 \mu\text{m}$.

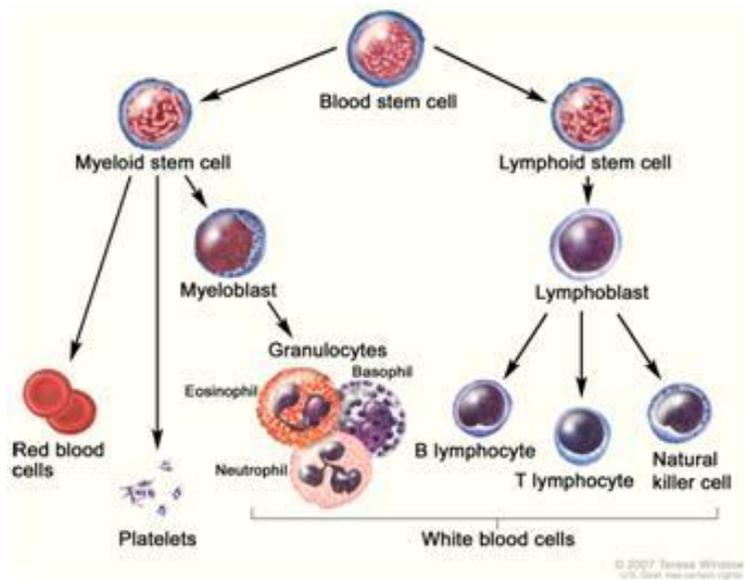


Figure 1.1. Origin of blood cells (from 2007 Terese Winslow).

Electron microscopy reveals that a resting platelet is divided into three zones (Figure 1.2):

- *Peripheral zone*: responsible for adhesion and aggregation. It consists of membrane glycoproteins, glycolipids, mucopolysaccharides, adsorbed plasma proteins and integrins.

Integrins, ($\alpha_{2b}\beta_3$, $\alpha_v\beta_3$, $\alpha_2\beta_1$) are transmembrane heterodimeric proteins consisting of two noncovalently associated glycoproteins, α and β . The $\alpha_2\beta_1$ integrin, commonly referred to as GPIa/IIa, plays a role in the adhesion of platelets to collagen (Corral et al., 1999; Kritzik et al., 1998). GPIb/IX/V complex is the major platelet receptor mediating interaction with von Willebrand Factor (vWF) (Rivera et al., 2000; Luo et al., 2007); it also binds to other adhesive proteins (collagen, thrombospondin-1), α -thrombin and coagulation factors (kininogen, FXI, FXII). It also plays a substantial role in platelet interaction with activated endothelial cells and with leukocytes (Gregg et al., 2004).

Immediately below the plasma membrane, a network of short actin filaments makes up a membrane cytoskeleton that stabilizes the membrane's discoid shape and supports platelet spreading after adhesion. (Authi et al., 1993; Rendu et al., 2001).

- *Sol-Gel zone*: responsible for contraction and constitutes the support microtubule system. Contains the connecting system, called the open canalicular system, and the dense tubular system located near the membrane in the vicinity of microtubule. This intracellular membrane organelle contains phospholipid-modifying enzymes that catabolize arachidonic acid towards thromboxane, namely cyclooxygenase and thromboxane (TX) synthetase activities.

- *Organelle zone*: contains different types of cytoplasmic organelles, that contain biologically active molecules crucial for platelet functions (Rendu & Brohard-Bohn, 2001). They include: the dense granules (containing high concentrations of adenine nucleotides), alpha granules (containing growth factors and cytokine-like proteins), lysosomal granules (containing digestive enzymes, such as glycosidases, proteases, cationic proteins with bactericidal activity), mitochondria and glycogen granules.

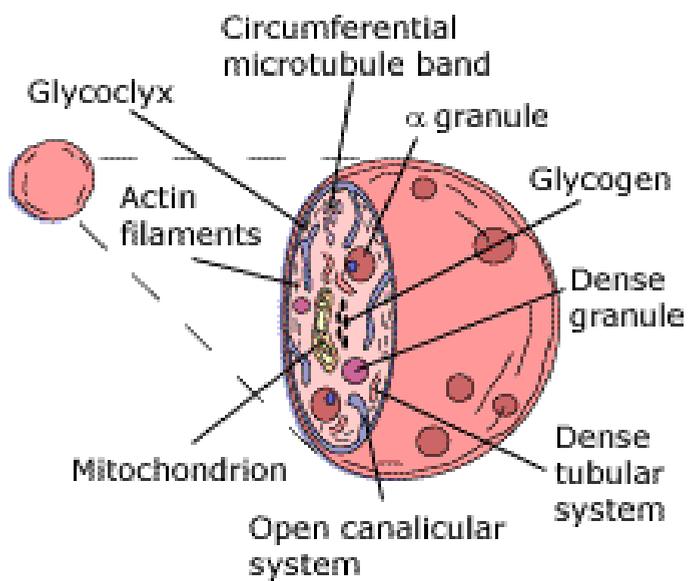


Figure 1.2. Platelet anatomical structure. Cartoon depicting all the essential features of a human platelet.

1.2 Platelet and protein synthesis

Booyse et al. showed, already in 1967, that platelets retain megakaryocyte-derived, translationally active mRNAs (Booyse & Rafelson, 1967a; Booyse & Rafelson, 1967b); it was not until the late 1990s that the interest of this mRNA was reviewed. Electron microscopy studies revealed the presence of rough endoplasmic reticulum and polyribosomes and they retain the ability for protein biosynthesis from cytoplasmic mRNA. (Belloc et al., 1987; Kieffer et al., 1987; Newman et al., 1988).

Quiescent platelets generally display minimal translational activity, but quiescent platelets stimulated by agonists such as α -thrombin increases protein synthesis of various platelet proteins (Gnatenko et al., 2003).

In 1998 Weyrich et al. demonstrated that resting platelets contain mRNA for Bcl-3, and that, upon activation, they translate the message into protein via a signalling pathway that involves the phosphorylation of the eukaryotic initiation factor 4E-binding protein 1 (4E-BP1); the rapid and sustained accumulation was unexpected because platelets were considered incapable of regulated protein synthesis.

During the last few years platelet mRNA have been extensively studied by quantitative PCR, microarray (Bugert et al., 2003; Gnatenko et al., 2003; McRedmond et al., 2004) and SAGE (serial analysis of gene expression) (Gnatenko et al., 2003; Dittrich et al., 2006). These studies have revealed a platelet specific transcriptome with 2500 different transcripts. Furthermore, recent studies documented the synthesis of a number of specific proteins, some of which with a highly sophisticated regulation. (Brogren et al., 2004; Denis et al., 2005; Evangelista et al., 2006; Lindemann et al., 2001).

To date, proteins synthesized by freshly platelets include actin, thrombosthenin, membrane glycoprotein (GP)Ib, GPIIb, and GPIIIa, fibrinogen, thrombospondin and vWF, all originating from mRNAs abundantly expressed (Thon & Devine, 2007). Moreover, Panes et al. (2007) demonstrated that circulating platelets contain tissue factor (TF) mRNA, the initiator of clotting, which is spliced and made readily functional to participate in haemostatic or thrombotic processes; this observation suggests that regulated synthesis of proteins is a signal-dependent response of human platelets.

1.3 Platelet activation

The activation of platelets involves a series of coordinated, tightly regulated events, to maintain haemostasis. Endothelial cells are the main regulators of blood flow and in their basal state provide a non-thrombogenic surface, that helps to prevent undesired thrombosis (Cines et al., 1998).

After blood vessel injury, the endothelium rapidly becomes an adhesive surface with intercellular gaps that allow the passage of soluble plasma and inflammatory cells from the vascular lumen to the underlying tissue (Michiels, 2003).

Platelets get activated following vascular injury and subsequently lead to the formation of a platelet plug. This process involves shape change, secretion of granule contents, aggregation and generation of lipid mediators and platelet activating factor. Immediate platelet adherence and aggregation with concomitant release of their granule contents, is followed by local activation of blood coagulation. Together, these events prevent life-threatening blood loss and invasion of microorganisms and ensure wound healing. Upon contact with plasma proteins, tissue factor (expressed in deeper layers of the vessel wall or in association with activated platelets) induces the formation of minute amounts of thrombin, that in turn will amplify its further production through direct activation of coagulation factor XI (a pro-form of a serine protease) and the non-enzymatic protein cofactors VIII and V (Ruf et al., 1999; Mann et al., 2003; Müller et al., 2003). Together with anionic glycerophospholipids, that become exposed after platelet activation or cellular disruption, the subsequent calcium-dependent assembly of multicomponent protease complexes comprises the central aspect of the exploding coagulation enzyme cascade (MacFarlane, 1964; Dahlbäck, 2000), that allows spatio-temporal fibrin formation for tight sealing of the wound, necessary for tissue repair.

1.4 Dynamics of thrombus formation

Haemostasis and pathological thrombus formation are dynamic processes that require a coordinated series of events involving platelet membrane receptors, bidirectional intracellular signals, and release of platelet proteins and inflammatory substances.

Initial tethering and firm adhesion of platelets to the exposed

subendothelium is mediated by GPIb/IX/V complex and collagen receptors, GPVI and $\alpha_2\beta_1$ integrin, in the platelet surface, and by vWF and collagen in the vascular site. Interactions between these elements are largely influenced by flow and trigger signalling events that reinforce adhesion and promote platelet activation. Thereafter, soluble agonists (ADP, thrombin and TxA₂, produced/released at the site of vascular injury) act in autocrine and paracrine mode, to amplify platelet activation and to recruit circulating platelets to the developing thrombus. Specific interactions of these agonists with their G-protein coupled receptors generate inside-out signalling leading to conformational activation of integrins, in particular $\alpha_{IIb}\beta_3$, increasing their ligand affinity. Binding of $\alpha_{IIb}\beta_3$ to its ligands, mainly fibrinogen, supports processes such as clot retraction and platelet aggregation. Stabilization of thrombi is supported by the late wave of signalling events promoted by close contact between aggregated platelets (Rivera et al., 2009).

Several steps can be identified during formation of platelet plugs at sites of vascular damage (Figure 1.3):

- *initiation phase*: consists of platelet arrest onto the exposed subendothelium creating a monolayer of activated cells, that interact with the extracellular matrix components exposed to blood.

- *extension phase*: consists of recruitment of additional platelets, which upon activation acquire the ability to stick to each other in a process commonly referred to as platelet aggregation.

- *stabilization phase*: consists of thrombus formation that arrests blood loss at the site of vascular injury. Essential events for thrombus growth and stabilization are cytoskeletal reorganization, formation and stabilization of large platelet aggregates, development of a procoagulant surface and a clot retraction that helps to narrow the gaps between platelets and to increase the local concentration of soluble platelet agonists (Watson et al., 2005; Woulfe et al., 2004).

- *thrombus dissolution*: the fibrinolytic system regulates the resolution of a thrombus, by degrading the fibrin into soluble fibrin degradation products. The fibrinolytic system consists of plasminogen activators (tissue plasminogen activator and urokinase-type plasminogen activator), that convert inactive plasminogen into active plasmin, which is responsible for

fibrin degradation. Plasmin activity is regulated by its inhibitor α_2 -antiplasmin.

Thiol groups or the rearrangement of disulfide bonds are strictly required for platelet responses, including aggregation and secretion (Essex, 2004). It has long been known that the poorly membrane-permeant sulfhydryl blocking reagent p-chloromercuribenzenesulfonate (pCMBS) inhibits platelet aggregation (Aledort et al., 1968) and that reduction of disulfide levels in the fibrinogen receptor by reducing agents induces platelet aggregation (MacIntyre & Gordon, 1975). In addition, a protein disulphide isomerase, present on platelet plasma membrane, mediates activation of α IIb β 3 and α 2 β 1 collagen receptor (Lahav et al., 2000, 2003); glutathione at concentrations normally found in blood potentiates platelet aggregation, by increasing sulfhydryls in the β 3 subunit (Essex et al., 2001).

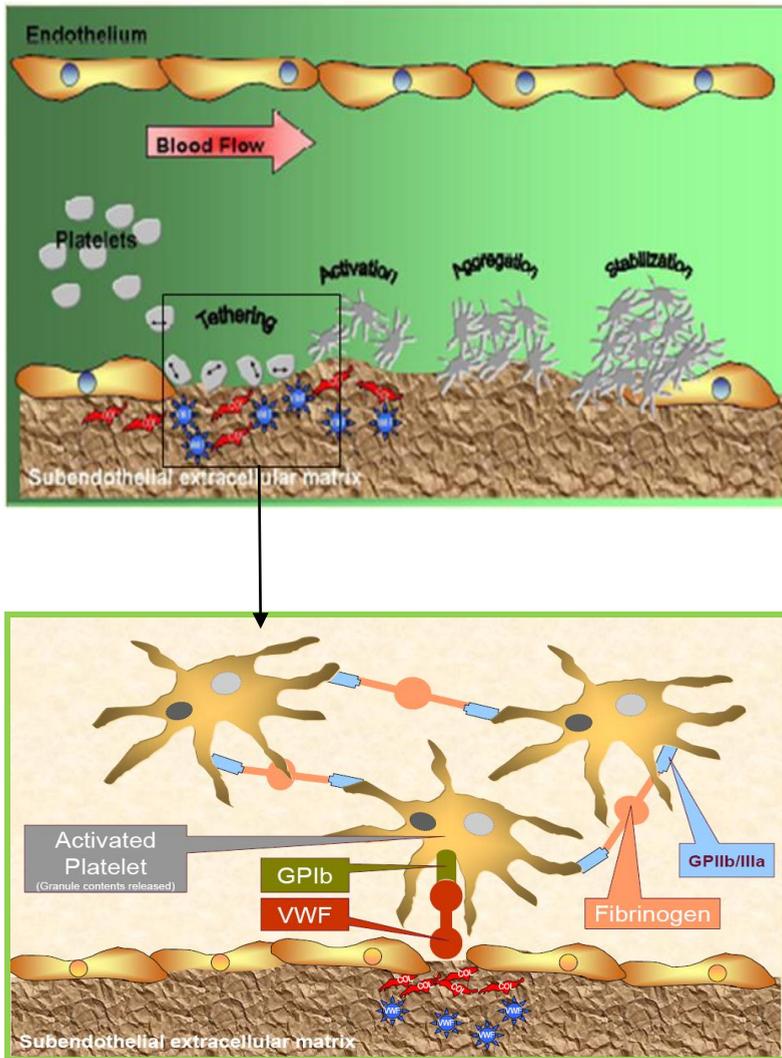


Figure 1.3. Formation of platelet plugs at sites of vascular damage (from www.clot-ed.com). See text for details.

2. REDOX SYSTEMS IN HUMAN PLATELETS

Platelet interaction with the vessel wall serves physiological and pathophysiological functions; this is reflected by the fact that platelets release growth factors (English et al., 2001), lipid mediators (Bolz, 2003; Kaul et al., 1994) and cytokines (Boehlen, 2001). Consequently, regulation of platelet activity plays a role not only for thrombus formation and regulation of the vascular tone (Agha et al., 1992), but also for the vascular pathophysiology of angiogenesis and inflammation. Recently, several papers have suggested that the redox (oxidation-reduction) state represents a new modulator of platelet activity.

The intracellular redox state is a dynamic condition, reflecting the amounts of oxidant and antioxidant species present inside cells. The relative abundance of different species, instead the absolute concentration, appear to be important, as the oxidant/antioxidant balance plays a crucial role in cell signalling pathways. Indeed, low levels of pro-oxidant agents, which include reactive oxygen/nitrogen species (RO/NS), act as second messenger molecules promoting cell growth and differentiation; but if RO/NS concentrations overwhelm the antioxidant capacity, oxidative stress will arise (Freeman et al., 1982; Halliwell et al., 1990a). For this reason, ROS levels have to be modulated by a variety of antioxidant systems (including enzymes and small molecules), that are able to quench most of the ROS (Duval et al., 2002).

2.1 Reactive oxygen/nitrogen species

The most common free radicals present in our organism are superoxide anion ($O_2^{\bullet-}$), hydroxyl radical (OH^{\bullet}) and nitric oxide (NO). Other reactive oxygen species, such as hydrogen peroxide (H_2O_2) and hypochlorous acid (HClO), are usually produced, but they are not radicals, as they do not possess unpaired electrons; nonetheless, they can diffuse across biological membranes and equally produce oxidative damage. Mechanisms and sites of RO/NS production are schematically shown in Figure 2.1 and Table I.

Lipids, proteins and DNA are targets for RO/NS injury (Reznick et al., 1998; McArdle et al., 2001, 2004; O'Neill et al., 1996; Silveira et al., 2003). Polyunsaturated fatty acids, possessing weak C-H bonds, are susceptible to radical attack, thus forming C-centered radicals; in this way, RO/NS initiate

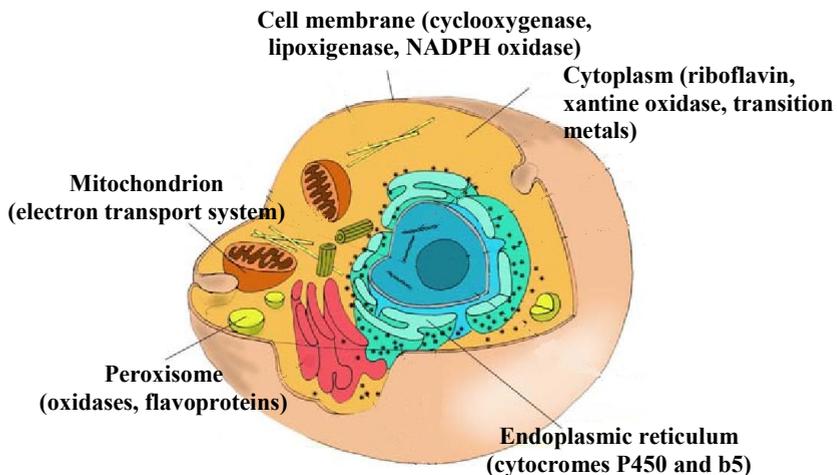


Figure 2.1. Intracellular sources of RO/NS.

Table I. Main reactive oxygen/nitrogen species

| RADICAL | TYPICAL BIOLOGICAL TARGET |
|---|----------------------------------|
| Superoxide $O_2 + e \rightarrow O_2^-$ | enzymes |
| Hydrogen peroxide $O_2^- + e + 2H^+ \rightarrow H_2O_2$ | unsaturated fatty acid |
| R-oxyl $RO \cdot + H^+ + e \rightarrow ROH$ | unsaturated fatty acid |
| Hydroxyl $OH \cdot + e \rightarrow OH^-$ | all biomolecules |
| Nitroxyl $2NO + H_2O + 2e \rightarrow N_2O + 2OH^-$ | several |
| Hypochlorous acid $ClO^- + H_2O + 2e \rightarrow Cl^- + 2OH^-$ | aminoacids, amines, hemoprotein |

radical chain reactions leading to lipid peroxidation and membrane damage (Davies et al., 1982; Alessio et al., 1988; Ji et al., 1988; Venditti et al., 1996; Venditti et al., 1977; Rajguru et al., 1993). RO/NS may attack proteins, modifying aminoacid structures and altering the functional properties of enzymes (Pacifici et al., 1990; Leeuwenburgh et al., 1997a-c; Leeuwenburgh et al., 1999). Genetic RO/NS targets are poly-ribonucleotides and poly-deoxyribonucleotides (Floyd, 1991; Halliwell et al., 1991; Shigenaga et al., 1990): ROS may induce single and double DNA breaks, modify purinic and pyrimidinic bases and create deletions or thymine dimers. DNA damage is mainly induced by OH• (McBride et al., 1991) and H₂O₂; the latter may diffuse into the nucleus and react with iron or copper ions forming the hydroxyl radical (Halliwell et al., 1990a-b). Genetic alterations lead to pathological diseases, including degenerative processes, carcinogenesis and immunodeficiency.

Within the vessel wall, there is a constant, low-quantity flux of ROS. In the endothelium, NAD(P)H-oxidase, cyclooxygenase isoforms 1 and 2, cytochrome P450 epoxygenase isoform 2C9 (CYP2C9), xanthine oxidase, uncoupled endothelial NO synthase and mitochondrial respiration are the main ROS-generating systems (Krotz, 2004). It is already established that enhanced ROS release from the vascular wall can affect platelet activity by scavenging NO, thereby decreasing the anti-platelet properties of the endothelium (Loscalzo, 2003). Furthermore, changes on redox potential of blood may regulate activation of α_{IIb}/β_3 integrin in platelets.

In addition to their exposure to ROS derived from the vascular wall, platelets themselves can generate oxidant species; the release of ROS has been reported both from resting platelets and after platelet stimulation with agonists, such as collagen or thrombin (Caccese et al., 2000; Finazzi-Agro et al., 1982; Wachowicz et al., 2002). This endogenous formation of ROS suggests that they have autocrine or paracrine roles in platelet activation, similar to those described for exogenous ROS (Krötz, 2004).

2.2 Antioxidant defence mechanisms

To neutralize the oxidative effects of ROS, aerobic organisms are endowed with several system, including:

1. *cellular compartmentalization*, which minimally reduces diffusion of ROS from the site of production to other cellular districts;
2. *chelating proteins*, which bind and sequester transition metals;
3. *non-enzymatic systems*, such as ubiquinone, vitamins, polyphenols, glutathione and thioredoxin, which scavenge ROS.

As other cells, platelets possess an antioxidant machinery. Among the enzymatic systems, it should be recalled (i) mitochondrial (Mn-SOD) and cytosolic (Cu-Zn SOD) superoxide dismutase, which catalyzes the dismutation of superoxide anion to hydrogen peroxide, (ii) catalase, which converts hydrogen peroxide to water, and (iii) glutathione peroxidase, which neutralize peroxides utilizing the reducing equivalents of glutathione. Other enzymatic systems are thioredoxin reductase and glutathione reductase that act to regenerate antioxidant molecules, such as thioredoxin, glutathione and ubiquinone.

Among the non-enzymatic systems, it should be recalled the low molecular weight scavengers of ROS, which include tocopherols, carotenoids, ubiquinone, urate, glutathione, thioredoxin and ascorbate.

Different antioxidant molecules are shown in Table II.

2.3 The plasma membrane redox system

Being ubiquitous in every living cell (including bacteria, yeast, plants and animals), the plasma membrane redox (PMR) system has the primary function of maintaining the cytoplasmic NAD^+/NADH ratio, thus regulating energy levels and redox homeostasis. Cellular ATP usually comes from oxidative phosphorylation, but when mitochondrial activity is depressed (e.g. in the presence of mitochondrial dysfunction or strenuous physical activity) cells can survive by using alternative pathways, including cytoplasmic glycolysis. In such conditions, the NAD^+/NADH balance, needed for sustaining ATP levels, is maintained by compensatory mechanisms, such as the pyruvate/lactate couple and enhanced PMR activity. Indeed, higher activity of the PMR system has been observed in human ρ^0 cells (devoid of mitochondrial DNA), where mitochondrial respiration is impaired (Scarlett et al., 2004), and in lymphocytes derived from diabetes mellitus patients, lacking functional mitochondria (Lenaz et al., 2002). So, the PMR system guarantees glycolytic metabolism, thus ensuring survival of cells deficient in mitochondrial electron transport (e.g. aged or tumour cells).

Table II. Main antioxidant species.

| | |
|--------------------------|---|
| <u>Enzymes</u> | Mn-superoxide dismutase (mitochondria) Cu,Zn-superoxide dismutase (cytosol) DT diaphorase (cytosol) GSH-peroxidase (cytosol) GSH-reductase (cytosol) Thioredoxin-reductase (cytosol and plasma membrane) Catalase (peroxisomes) |
| <u>Scavengers</u> | Tocopherols Carotenoids Ubiquinone Ascorbate Glutathione Urate |
| <u>Proteins</u> | Lactoferrin Transferrin Ferritin Caeruloplasmin |

The basic components of the PMR system include electron donors and acceptors and reductases. These systems may have operational flexibility, since electrons can be transferred from different donors to several acceptors.

a) Electron donors and acceptors

The intracellular sources of reducing equivalents are mainly represented by the pyridine coenzymes NADH and NADPH derived from the hydrophilic vitamin niacin, although other components (such as intracellular vitamin C) may act as electron donors in specific cell types (VanDuijn et al., 2001). Intracellular electrons are then used for reducing extracellular molecules, thus modulating the redox state of the microenvironment surrounding the cell. Oxygen is the most common final electron acceptor of membrane NADH/NADPH oxidases (Geiszt & Leto, 2004); partial reduction of molecular oxygen leads to generation of superoxide anions and hydrogen peroxide. Beside oxygen, other natural acceptors have been described, including ferri-heme and ascorbyl free radical; in this way, heme delivery to cells is facilitated by reduction of iron ions, while reduction of ascorbyl free radical maintain the tone of vitamin C in extracellular fluids (McKie et al., 2001; Goldenberg et al., 2000;).

Electrons flow outwards thanks to their sequential transfer to plasma membrane carriers: although several acceptors exist (including b cytochromes, flavin, vitamin E and membrane proteins), the most widely used is ubiquinone (or coenzyme Q; CoQ). It is widely distributed in cell membranes and, depending on the enzyme involved, it can be transformed to the semiquinone radical (CoQ[•]) or to hydroquinone (CoQH₂) (Villalba et al., 1997; Villalba & Navas, 2000).

b) Enzymes

Specific enzymes localized in the plasma membrane allow this “in-outside” electron flux. Briefly, the PMR enzymes in human cells include (Figure 2.3):

1. the NADH:ferricyanide reductase, known as Voltage-Dependent Anion-selective Channel (VDAC) or porin, is present on both plasma and mitochondrial membranes (Lawen, 2005);
2. the NAD(P)H:ubiquinone oxidoreductase (NQO1, also named DT-diaphorase) is a flavoprotein, which is translocated into the plasma membrane under oxidative stress (Li, 1995). Its primary physiological role is to act as an antioxidant enzyme, thus preventing the harmful effects of xenobiotics;

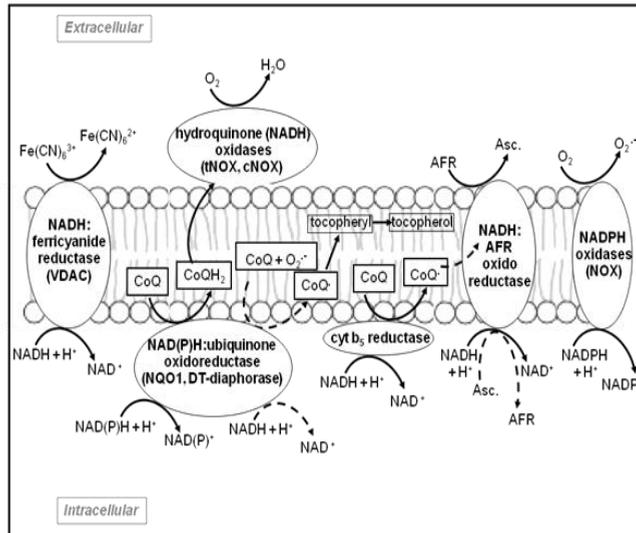


Figure 2.3. Key enzymes of the PMR system. Membrane localisation and catalysed reactions are shown in the scheme. Alternative reactions are depicted with hatched lines. CoQ regenerates transplasma membrane tocopherol and extracellular ascorbate, thus protecting platelets from lipid peroxidation and external oxidative insults. Asc: ascorbate. AFR: ascorbyl free radical.

3. the cytochrome b_5 reductase is a membrane-bound flavoprotein, whose function in erythrocytes is to be responsible for haemoglobin reduction (Prchal et al., 2005);
4. the NADH:ascorbate free radical oxidoreductase is an enzymatic activity found in the plasma membrane, but not yet unequivocally ascribed to a specific protein, which uses NADH to reduce the ascorbyl free radical (Savini et al., 1999);
5. the superoxide-generating NADPH oxidases represent a protein family, including at least seven proteins (NOX1 to NOX5, Duox1 and Duox2), whose best characterized member is NOX2 (Geiszt & Leto, 2004). They are composed of catalytic (p22^{phox}, gp91^{phox}) and regulatory (Rac1, Rac2, p47^{phox}, p67^{phox} and p40^{phox}) subunits and generate tightly controlled superoxide anions, in a tissue-and function-specific manner (Geiszt & Leto, 2004).
6. the NADH oxidases represent a protein family, whose members exhibit a time-keeping NADH:CoQ oxidase activity and a protein disulfide-thiol interchange activity (Morré et al., 2003). They are designated as Ecto-NOX proteins, to distinguish them from other NAD(P)H oxidases and to underline their feature to be located on the outside surface of the cell. At least three members of this family have been described so far: the tumour-associated NADH oxidase (also known as tNOX or Ecto-NOX2) (Chueh et al., 2002), which is present on the surface of invasive cancer cells and in the sera of cancer patients, the constitutive NADH oxidase (cNOX or Ecto-NOX1), expressed in normal cells (Wang, 2001), and the age-related NADH oxidase (arNOX), expressed exclusively in aged cells (Morré et al., 2003).

Human platelets (Figure 2.4) possess a NADPH oxidase complex, similar to NOX2 (Seno et al., 2001; Pignatelli et al., 2004; Krotz et al., 2002). In these cells, the NADPH oxidase plays a key role in intracellular signalling leading to α_{IIb}/β_3 -integrin activation (Begonja et al., 2005); furthermore, $O_2^{\cdot-}$ (or the derivative H_2O_2) enhances the ADP release, resulting in increased platelet recruitment (Krotz et al., 2002). In addition, in host defence response, platelet NADPH oxidase causes the release of thromboxane A_2 , which, in turn, enhances ROS production by neutrophils and their cytotoxic action (Chlopicki et al., 2004). Platelets may also possess a NADH oxidase activity, whose presence has firstly been suggested by Del Principe et al. (1980); After that, the presence of an enzyme similar to Ecto-NOX1 has been suggested from the study of Peter et al. (2000), which demonstrated a

periodic and light responsive oxidation of NADH by human buffy coats (a mixture of white cells and platelets). However, the exact molecular identity of NADH oxidase remains unknown.

Other components have been identified, such as NADH-diaphorase, cytochrome b₅ reductase (Takeshita et al., 1982) and, more importantly, at least three thiol-related enzymes. In particular, two thiol isomerases and a glutathione reductase activity are responsible for the rearrangement of disulfide bonds (Figure 2.4) (Essex et al., 2004; Essex, 2004); many platelet membrane proteins contain redox-sensitive sulfhydryl groups, so that the thiol/disulfide balance appears crucial for platelet aggregation, secretion and post-aggregation events. Among the thiol isomerase family, protein disulphide isomerase (PDI) and endoplasmic reticulum protein 5 (ERP5) have been identified on the platelet surface; PDI interacts with $\alpha_2\beta_1$ and $\alpha_{IIb}\beta_3$ integrins, whereas ERP5 is associated only with the β_3 integrin subunit. Importantly, PDI itself is activated by changes in the sulfhydryl state of its active site. So far, it is not clear which mechanism mediates PDI activation; Essex et al. (2004) suggested the involvement of external reducing compound(s) (such as glutathione), maintained in the active form by either NADPH oxidase or glutathione reductase. On the other hand, the presence of NADH oxidases with protein disulfide-thiol interchange activity can raise the possibility of an alternative mechanism of action, which directly modulates PDI (or integrins). Finally, a relationship between PDI and NADPH oxidases has been discovered: in this case, PDI regulates the activity of NADPH oxidase (Janiszewski, 2005), so that a bi-directional mode of action may lead to reciprocal regulation of these two enzymes.

2.4 VITAMIN C

2.4.1 General aspects

Vitamin C, also referred to as ascorbic acid (AA) or ascorbate, belongs to the water-soluble class of vitamins. It exists in two active forms: the reduced form, known as AA, and the oxidized form dehydroascorbic acid (DHA). Physiologically, AA provides electrons for enzymes, oxidants or other electron acceptors. Under physiological pH, the predominant form is the ascorbate anion, that undergoes the oxidative process, leading to an intermediate compound, named ascorbyl free radical (AFR) (Frei, 1994; Jacob, 1999). AFR is a radicalic, not dangerous compound, as it is not a

strong oxidizing agent and, moreover, its reactivity with oxygen (and thus the possibility to generate the superoxide anion) is low (Figure 2.5).

Vitamin C is required for many reactions of hydroxylation, involved in the synthesis of collagen (Bremer, 1983), carnitine (Nelson et al., 1981), norepinephrine and serotonin; all of them are catalyzed by mono- and di-oxygenases, which contain iron or copper as prosthetic group (Dhariwal et al., 1989; Murthy et al., 1987; Padh, 1990; Jacob, 1994). Vitamin C may also have vasodilatory and anticlotting effects within the body, by stimulating nitric oxide release. Furthermore, vitamin C can act as antioxidant by sequestering the singlet oxygen radical, stabilizing the hydroxyl radical and regenerating reduced vitamin E back to the active state. These reactions work together to halt peroxidation of cellular lipid membranes (Sen, 2001; Padh, 1991; England et al., 1986).

2.4.2 Metabolism

a) Transport

All members of the animal kingdom (except men, guinea pig, primates and some fishes and birds) are able to synthesize AA by the glucuronic acid pathway, in liver or kidney (Nishikimi et al., 1994). Since human cells cannot *de novo* synthesize the vitamin, they must import it from extracellular fluids and store it inside cells at high concentrations (Peake, 2003).

Vitamin C is imported inside cells by two different systems (Figure 2.6); specific Na^+ dependent co-transporters for AA and facilitative glucose transporters for DHA. There are two Na^+ -dependent transporters, namely SVCT1 and SVCT2 (Tsukaguchi et al., 1999). SVCT1 and SVCT2 are highly homologous to each other; however, they have different functional characteristics, with SVCT2 exhibiting a higher affinity/lower capacity for AA than SVCT1. Furthermore, the two isoforms show different tissue distribution: *in situ* hybridization experiments and northern analysis revealed that SVCT1 is largely confined to bulk transporting epithelia (e.g., small intestine, kidney) (Tsukaguchi et al., 1999; Wang et al., 2000), as well as in other epithelial and endocrine tissues (e.g. skin, liver, lung, prostate, ovary, pancreas, thymus) (Wang et al., 2000; Savini et al., 2002). The distribution and kinetic parameters suggest that the primary role of SVCT1 transporter is the absorption of dietary AA and renal reabsorption, thus maintaining the

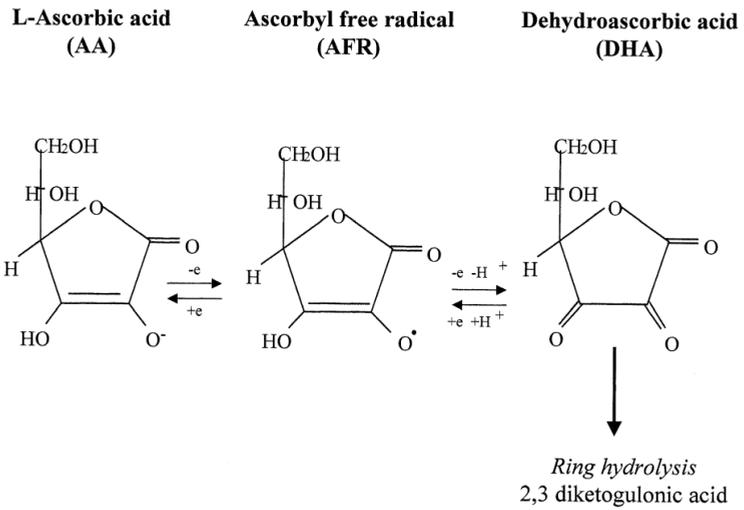


Figure 2.5 Vitamin C redox state.

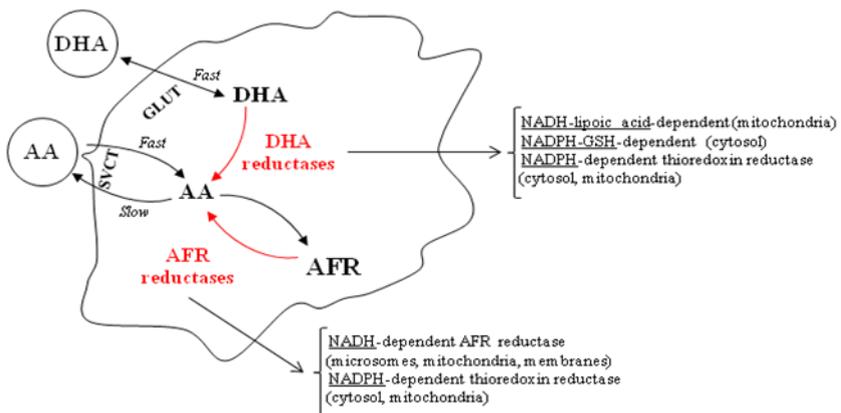


Figure 2.6 Import of AA and DHA inside cells and mechanisms of vitamin C recycling.

whole-body homeostasis. SVCT2 is widely expressed, and its mRNA has been detected in several metabolically active and specialized cells and tissues (neurons, brain, eye, placenta, osteoblasts, chondrocytes) (Tsukaguchi et al., 1999; Castro et al., 2001; Rajan et al., 1999; Kannan et al., 2001; Wu et al., 2004; Clark et al., 2002); the possible role for this transporter may be the widespread uptake of AA required to protect metabolically active cells from oxidative stress. It has been recently demonstrated that mice lacking SVCT2 die within minutes of birth, supporting the important role of this transporter in development (Sotiriou et al., 2002).

Cells, can also import DHA by the means of hexose GLUT transporters (Korcok et al., 2003) (Figure 2.4). These transporters are unlikely to play a major role in vitamin C uptake under physiological conditions, as the high concentrations of glucose will block the influx; however, this pathway may be crucial during oxidative stress, when the oxidized vitamin is locally concentrated.

b) Recycling

Playing its physiological role, AA is oxidized to AFR, which then rapidly disproportionates to AA and DHA. Under neutral or alkaline conditions, DHA undergoes an irreversible hydrolysis, with opening of the ring and loss of vitamin activity; the product of this reaction (2,3-dioxo-L-gulonic acid) is splitted in oxalic acid and L-threonic acid.

The active, reduced form of vitamin C can be regenerated both from AFR and DHA. In those organisms unable to synthesize AA, the efficiency of systems involved in vitamin C recycling is essential to lower dietary requirements (Figure 2.5).

AFR reduction is mediated by several enzymatic systems, localized in almost all cellular compartements. AFR can be reduced by (i) the plasma membrane NADH-dependent system, (ii) the microsomal cytochrome b₅-cytochrome b₅ reductase system (Hara et al., 1971) and (iii) the cytosolic and mitochondrial NADPH-dependent thioredoxin reductase (May et al., 1998).

DHA can be reduced to AA in an enzymatic or non-enzymatic manner, through a direct chemical reaction with reduced glutathione (Basu et al., 1979). The exact nature of the enzymatic systems involved in DHA reduction, in human cells, is still under debate, though a tissue-specific difference appears to be implicated. Many studies indicate that different

enzymatic systems are involved in DHA reductase activity, though current experimental evidence does not specify the extent to which this occurs *in vivo*. Enzymatic systems of mammalian tissues include: (i) NADPH-glutathione-dependent DHA reductases, such as glutaredoxin, protein disulfide isomerase (Wells et al., 1990) and glutathione peroxidase (Washburn et al., 1999); (ii) NADPH-dependent DHA reductases, such as thioredoxin reductase (May et al., 1997; Mendiratta et al., 1998) and 3 α -hydroxysteroid dehydrogenase (Del Bello et al., 1994); (iii) NADH-lipoic-acid-dependent lipoamide dehydrogenase (Xu et al., 1996).

The reduction of DHA to AA by intracellular enzymes keeps the cytosolic concentration of DHA low, thus contributing to a gradient favoring DHA uptake across the plasma membrane (Himmelreich et al., 1998).

2.4.3 Biochemical functions

Vitamin C has physiological relevance in platelets, because it can modulate the platelet oxidative state. Indeed, the vitamin inhibits lipid peroxidation and ROS production in blood platelets, thus protecting membrane components susceptible to free radical damage and regulating membrane systems sensitive to ROS-mediated signalling (Olas et al., 2002). Vitamin C also reduces the expression of platelet CD40L, a transmembrane protein with pro-inflammatory and pro-thrombotic properties (Pignatelli et al., 2005); this protein has been implicated in the initiation and progression of atherosclerotic disease, through scavenging the O₂ generated by NADPH oxidase activation (Ushio-Fukai et al., 2006). Other functions of vitamin C include regulation of platelet-derived NO production (Takajo et al., 2001), inhibition of thromboxane B₂ formation (Ho et al., 1976), protection against nickel-induced inhibition of aggregation (Chen & Lin, 2001), and stimulation of prostaglandin E1 production (Srivastava, 1985). Finally, the platelet PMR system is able to prevent extracellular ascorbate autoxidation (our unpublished data), thus contributing to stabilization of reduced vitamin C and, consequently, to modulation of the redox state in the micro-environment, which, in turn, modulates platelet functions. The ascorbate/dehydroascorbate redox couple (instead of ascorbate *per se*) seems to be important in those events accompanying platelet aggregation, as dehydroascorbate can be a substrate for PDI present on platelet surface (Rhee et al., 2000).

3. OBJECTIVE OF RESEARCH

Maintenance of a correct redox balance inside and outside the cell is a crucial factor in platelet biology; indeed, the oxidant/antioxidant balance plays a central role during activation of the coagulation pathway. This equilibrium is achieved through different mechanisms, including modulation of intracellular and surface antioxidants, as well as of the plasma membrane redox (PMR) system. Aim of this research was to characterize intracellular and surface systems in freshly human platelets, especially focusing on vitamin C homeostasis and plasma membrane NAD(P)H oxidases. Development of this research has been planned according to five main goals:

First, we characterized the ascorbic acid (AA) uptake in human platelets, as these cells are able to accumulate millimolar concentrations of this vitamin. To this end, we performed Western blot analysis, to document the presence of the two specific Na⁺-dependent AA transporters (SVCT1 and SVCT2), and kinetic assays, in order to investigate the transport efficiency of platelets. All studies have been carried out in resting and activated platelets (with PMA or thrombin), to assess the potential modulation of vitamin C accumulation during platelet activation and aggregation.

Second, we investigated the activity of trapping systems responsible for maintaining the vitamin in the reduced form inside cells; in particular we evaluated dehydroascorbate (DHA) and ascorbyl free radical (AFR) reductase activities.

Third, we investigated if vitamin C, beside its role during aggregation, has some biological functions also in post-aggregatory events. We assessed if intracellular and surface sulfhydryls groups (with particular regard to glutathione content) were modified in relation to changes in vitamin C content. We also assessed the potential role played by AA in dynamics of platelet thrombus formation, calculating the time of thrombus formation and thrombus elasticity.

Fourth, we characterized the NAD(P)H-oxidase proteins expressed on platelet plasma membrane. In particular, we focused on Ecto-NOX proteins, which are expressed on the external side of plasma membrane. We performed activity assays, evaluating cofactor specificity (NADH or NADPH), membrane localization of the enzymes and drug-specific inhibition (capsaicin).

Fifth, we investigated the potential modulation of NAD(P)H enzymes. To this aim, we evaluated ROS content, surface and intracellular sulfhydryl groups and GSSG/GSH ratio in relation to enzyme expression and activity.

4. RESULTS

4.1 Translational control of ascorbic acid transporter SVCT2

4.1.1 Ascorbic acid is accumulated through the SVCT2 carrier

Freshly isolated platelets contain intracellular ascorbate as much as 24 fold higher than that found in plasma (1.7 ± 0.1 vs 0.07 ± 0.03 mM) and the vitamin is all present in the reduced form (data not shown). Thus, resting platelets physiologically accumulate AA, although the mechanism of such accumulation has never been investigated. In order to go further insight on the role played by vitamin C, we first evaluated the presence of the two specific Na^+ -dependent AA transporters (SVCT1 and SVCT2) in human platelets. Both messengers were detected by RT-PCR, but with different levels of expression: exponential amplification showed huge amounts of SVCT2 mRNA, while the amplified product for SVCT1 messenger was less evident (Figure 4.1A). Accordingly, only protein corresponding to the SVCT2 transporter could be found in resting platelets, while western blot for SVCT1 protein showed a band exclusively in CaCo-2 cells, used as positive control (Figure 4.1B). Western blot analysis showed one reactive band with an apparent molecular weight of about 45-50 kDa. Competition studies performed with a blocking peptide to which the antibody was made confirmed the specificity of labelling (Figure 4.1B). Interestingly, our experiments showed that, in human platelets, the apparent molecular weight of SVCT2 was lower than that found in other species. Indeed, almost all data present in the literature referred to mouse or rat cells, where the available anti-SVCT2 antibodies recognize two major bands at 70–80 and 66 kDa, possibly correlated to the glycosylated and nonglycosylated forms of the protein (Wu et al., 2003; Garcia et al., 2005; Mun et al., 2006). In human platelets, as well as in other human cells (Li et al., 2003; Godoy et al., 2007; our unpublished data on epidermal, neuronal, intestinal, lymphoid cells), only a specific 50-kDa reactive band could be seen. Altogether, these findings suggested that a species-specific (and not a tissue-specific) processing could determine the molecular weight of SVCT2 observed in human platelets.

To confirm the involvement of SVCT2, as the unique carrier responsible for AA accumulation, we carried out uptake experiments. The time course of

AA transport was linear for at least 15 minutes with the maximum accumulation time at 30 min (range 5–100 μM) (data not shown). Concentration dependence experiments showed that AA was accumulated with a saturable mechanism (Figure 4.2); calculated kinetic parameters (apparent $K_m = 29 \pm 1 \mu\text{M}$ and $V_{\text{max}} = 130 \pm 8 \text{ pmol/min/mg protein}$; see Figure 2, panel inside) were in agreement with values just reported for the SVCT2 transporter in other cell types (Rajan et al., 1999; Daruwala et al., 1999). By supplementing platelets with exogenous AA (100 μM for 15 min), we were able to increase the intracellular concentration from 1.7 ± 0.1 to $1.9 \pm 0.1 \text{ mM}$.

Confirmatory results were obtained by the usage of specific SVCTs inhibitors. AA uptake was significantly inhibited by sulfapyrazone (an anion transport inhibitor) and, to a lesser extent, by ouabain (an inhibitor of Na^+/K^+ -ATPase) (Table I). The incomplete inhibition of AA transport by ouabain was probably due to incomplete collapse of the Na^+ -membrane gradient (Pales et al., 1988); complete removal of sodium ions was not allowed in this experimental model, since replacement of NaCl with choline chloride caused lysis of platelets, as already described by Bergsten et al. (1995) for lymphocytes.

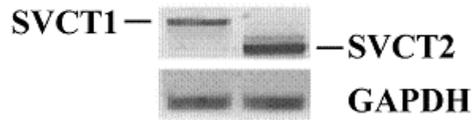
AA uptake was not affected by the GLUTs inhibitors phloretin (200 μM) and cytochalasin B (10 μM), which otherwise inhibited DHA import (Table I).

Usage of GLUT inhibitors was also employed to investigate AA efflux from cells. Vitamin C leakage could occur after AA oxidation and DHA exit through GLUT transporters (Vera et al., 1995). In platelets, intracellular AA fell down to $0.52 \pm 0.03 \text{ mM}$, corresponding to 30% of the basal value, after 3 washings with 10 ml PBS for 40 min. The loss was almost completely inhibited (90%) after incubation with cytochalasin B, indicating that the major vitamin efflux was mediated by exose transporters (data not shown).

4.1.2 Expression of SVCT2 is regulated by intracellular AA levels

As demonstrated for other cell types (Dixon et al., 1992; Wilson et al., 1990; MacDonald et al., 2002), AA uptake was self-regulated. Kinetic analysis of vitamin C-depleted platelets (washed with PBS as described above and containing only 30% of the basal vitamin C content) showed that V_{max} was increased to $440 \pm 21 \text{ pmol/min/mg protein}$, whereas apparent K_m value was unaffected ($28 \pm 1 \mu\text{M}$).

A



B

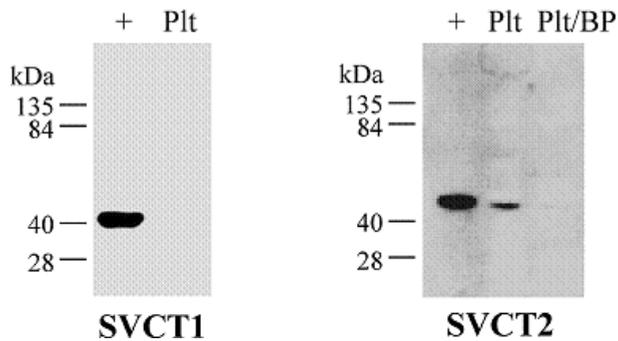


Figure 4.1 Expression of the Na⁺-dependent AA transporters in resting platelets. **(A)** Analysis by RT-PCR of SVCT1 and SVCT2 expression. Total RNA was purified from freshly isolated platelets, reverse-transcribed and amplified with primers specific for SVCT1 or SVCT2. The blot is representative of three independent experiments. Amplification of the housekeeping GAPDH messenger is shown as loading control. **(B)** Western blot analysis of AA transporters. Platelet extracts were purified and immunoblotted with either anti-SVCT1 or anti-SVCT2 antibodies. The radiograph is representative of five similar experiments. Positive controls (+) were cellular extracts from CaCo-2 and SH-SY5Y cells, for SVCT1 and SVCT2 respectively. Plt: freshly isolated platelets. Plt/BP: competition study with SVCT2 blocking peptide.

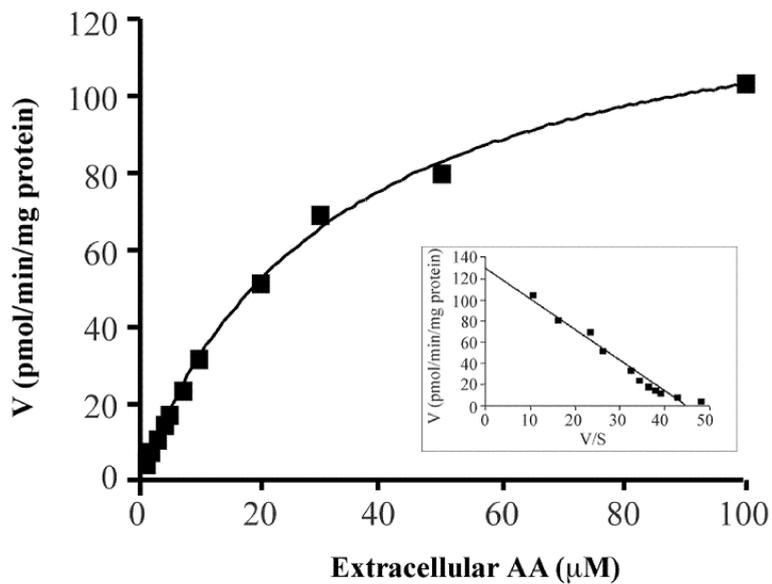


Figure 4.2 Concentration dependence of ascorbic acid transport. Platelets were incubated with [^{14}C]AA and adequate concentrations of the respective unlabeled compound for 15 minutes; then, AA uptake was determined by scintillation spectrometry. **Panel inside:** Eadie-Hofstee plot of AA transport. Values are the mean of four independent experiments. S.D. $\leq 5\%$.

The increased transport ability of depleted cells suggested a modulation of transporter activity, which in turn could be the result of increased expression. To test this hypothesis, we checked for protein levels by Western blot: we found that SVCT2 expression in AA-depleted cells was higher with respect to native platelets (Figure 4.3). Conversely, native platelets supplemented with 500 μ M AA for 30 min showed a slight decrease in SVCT2 levels (Figure 4.3), in agreement with the potential regulation of SVCT2 protein by AA and/or redox state.

4.1.3 Vitamin C is maintained in the reduced form inside cells

During oxidative conditions, intracellular AA is oxidized to AFR, which then rapidly disproportionates to AA and DHA. The active reduced form of vitamin C can be regenerated both from AFR and DHA by distinct reductases (Wells et al., 1990; Washburn et al., 1999; Del Bello et al., 1994; May et al., 1997; Xu et al., 1996).

In order to assess the reduction ability, we first loaded platelets with the oxidised form of vitamin C. Kinetic parameters (apparent $K_m = 1.1 \pm 0.05$ mM and $V_{max} = 39 \pm 1.5$ nmol/min/mg protein) for DHA import were in agreement with the presence of GLUT3, the only transporter present in platelets (Craik et al., 1995). HPLC analysis revealed that platelets incubated with 0.1 mM extracellular DHA for 10 min accumulated 8 ± 0.5 mM AA; thus, all the imported DHA was immediately converted to AA, demonstrating the presence of efficient reductase systems. Indeed, we found intracellular DHA and AFR reductase activities of 0.1 ± 0.01 nmol/min/mg protein and 8.7 ± 0.6 nmol/min/mg protein, respectively; intra-platelet AA removal unaffected these enzymatic activities (data not shown).

Thus, platelets possess an efficient AFR reductase activity and low, although measurable, DHA reduction ability. Therefore, AFR reductase appeared to be the most relevant system in maintaining vitamin C in the reduced state.

4.1.4 SVCT2 expression is enhanced during platelet activation

We wondered if platelet activation had some effects on vitamin C handling. To this end, we treated platelets for 15 min with two activators acting on different pathways: PMA, a non-selective protein kinase-C

Table I
Effect of inhibitors on AA and DHA transport

| Inhibitor | AA transport (% of control) | DHA transport (% of control) |
|----------------------|--|---|
| 5 mM sulfinpyrazone | 20 ± 1 | 100 ± 3 |
| 0.5 mM ouabain | 70 ± 3 | 93 ± 4 |
| 10 μM cytochalasin B | 98 ± 4 | 30 ± 1 |
| 200 μM phloretin | 100 ± 4 | 2 ± 0.1 |

The AA content was determined by scintillation. In control platelets, AA transport was 105 ± 5 pmol/min/mg of protein (after incubation with 0.1 mM [¹⁴C]AA), whereas DHA transport was 5 ± 0.2 nmol/min/mg of protein (after incubation with 0.1 mM [¹⁴C]DHA). Values are the mean of three independent experiments.

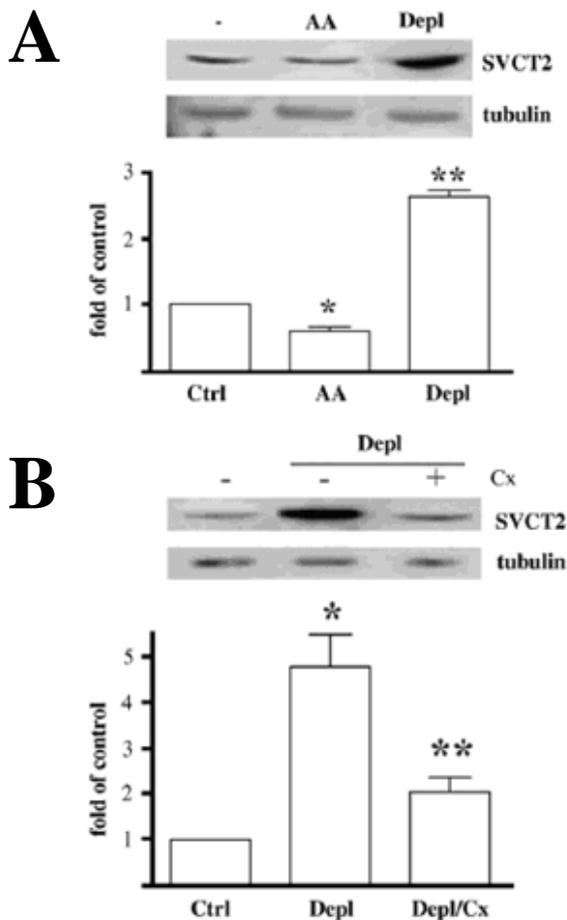


Figure 4.3 Modulation of SVCT2 expression by AA levels. **(A)** Resting platelets were left untreated (-) or supplemented with 500 μ M AA for 30 min (AA) or depleted by exhaustive washing (30% of the basal vitamin C content; Depl). Cell extracts were purified and immunoblotted with either anti-SVCT2 or tubulin antibodies. The histogram represents the densitometric analysis of autoradiography. Values are the means \pm SEM of three independent experiments. * $p=0.02$ vs Ctrl; ** $p=0.003$ vs Ctrl. **(B)** Resting platelets were left untreated (-) or depleted by exhaustive washing, in the absence (Depl) or presence (Depl/Cx) of 1 mM cycloheximide. Cell extracts were purified and immunoblotted with either anti-SVCT2 or tubulin antibodies. The histogram represents the densitometric analysis of autoradiography. Values are the means \pm SEM of three independent experiments. * $p=0.02$ vs Ctrl; ** $p=0.01$ vs Depl. In both histograms, values are reported as fold of control, arbitrarily set to 1, after normalization with tubulin.

activator, and thrombin, a receptor mediated agonist.

As shown in Figure 4.4A, both 100 nM PMA and 0.5 U/ml thrombin increased SVCT2 expression, resulting in a two-fold increase in AA transport. Thus, we investigated if enhanced SVCT2 protein reflected a translational control of expression; to this end, we pre-treated platelets with cycloheximide, an inhibitor of 60S peptidyl transferase and translational elongation. Our results demonstrated, for the first time, that SVCT2 can be modulated at the translational level, as protein amounts, increased after incubation with PMA, were significantly reduced when cells were incubated with cycloheximide (Figure 4.4B).

Parallel to enhanced AA import, DHA reduction decreased in activated platelets. Stimulation of platelets with both thrombin and PMA caused a 2.5-fold increase in radiolabelled 2-DOG uptake (data not shown), indicating a higher GLUT activity. These results were in agreement with the reported translocation of GLUT-3 to the cell surface after platelet stimulation (Heijnen et al., 1997). Nonetheless, DHA uptake was halved with respect to resting platelets (Figure 4.5). These results could be explained by a decrease in reduction ability, with subsequent leakage of imported DHA; indeed, we found that DHA reduction activity was decreased of about 50% (Figure 4.5). Accordingly, glutathione, which is an important factor for reducing DHA, was lowered in activated platelets with respect to resting cells (1.4 ± 0.1 vs. 2.0 ± 0.2 nmol/mg of protein).

4.1.5 AA modulates sulfhydryls groups

Thiols and disulfides on platelet surface play an important role in platelet responses, including aggregation, secretion and activation of integrins (Essex, 2004). Thus, we evaluated if intracellular AA levels could modulate these surface sulfhydryls groups.

We found that intracellular AA changed the surface sulfhydryl content, as demonstrated by studies performed in vitamin C-depleted or supplemented cells. As shown in Figure 4.6A, exogenous supplementation dose-dependently increased the amounts of surface thiols, while vitamin depletion decreased them. Furthermore, depleted platelets incubated with AA (Figure 4.6A, last column) reverted the effects of vitamin depletion.

To evaluate if AA specifically preserved surface thiols or its effect was a more generalized phenomenon (thus preserving total cellular thiols), we

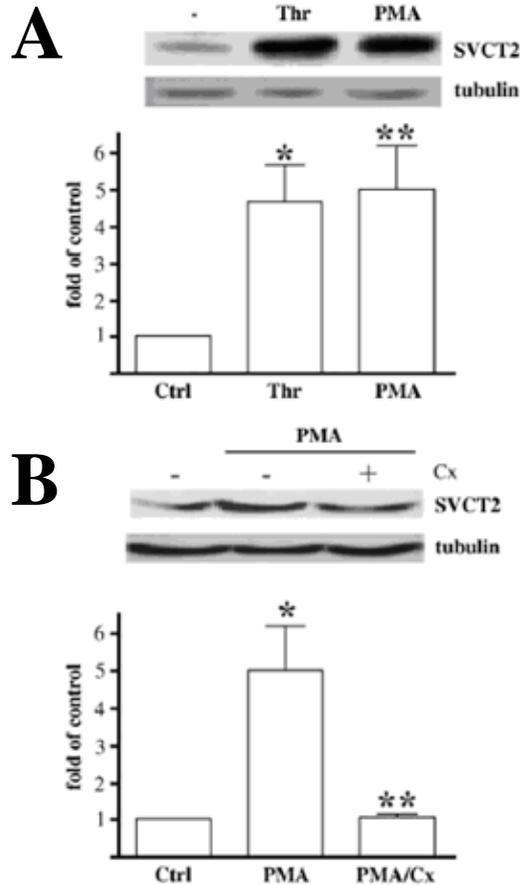


Figure 4.4 SVCT2 expression in stimulated platelets. **(A)** Platelets were left untreated (-) or stimulated either with 100 nM PMA or 0.5 U/ml thrombin (Thr), at 25°C for 15 min. Cell extracts were purified and immunoblotted with either anti-SVCT2 or tubulin antibodies. The histogram represents the densitometric analysis of autoradiography. Values are the means \pm SEM of three independent experiments. * $p=0.03$ vs Ctrl; ** $p=0.04$ vs Ctrl. **(B)** Inhibition of SVCT2 up-regulation by cycloheximide. Platelets were left untreated (lane 1) or stimulated with 100 nM PMA for 15 min, in the absence (lane 2) or in the presence (lane 3) of 5 μ g/ml cycloheximide. Cell extracts were purified and immunoblotted with either anti-SVCT2 or tubulin antibodies. The histogram represents the densitometric analysis of autoradiography. Values are the means \pm SEM of three independent experiments. * $p=0.03$ vs Ctrl; ** $p=0.03$ vs PMA.

In both histograms, values are reported as fold of control, arbitrarily set to 1, after normalization with tubulin.

measured intracellular sulfhydryl groups and glutathione content. As shown in Fig. 4.6B, no significant differences in the intracellular thiol content were observed after AA supplementation or depletion. Also the glutathione content (the main nonprotein thiol in platelets) was unaffected by intracellular AA levels: glutathione amounts were unchanged after vitamin C supplementation (2.02 ± 0.2 nmol/mg of protein) or depletion (2.2 ± 0.2 nmol/mg of protein), with respect to untreated platelets (2.0 ± 0.2 nmol/mg of protein).

4.1.6 AA modulates thrombus rigidity

Typical rheometer tracings showed logarithmic damping and frequency shift as a function of time; by these tracings, time of thrombus formation and thrombus elasticity were calculated.

Thrombus formation for fresh platelet suspensions stimulated with SFLLRN occurred in 12-15 min; in the presence or absence of AA, platelets did not show significant differences. At about 50 min after thrombus formation, an increase either in frequency or in logarithmic damping, indicating thrombus contraction, was recorded; platelets with or without AA did not show any difference in the onset of these changes. The “elastic” or “storage” modulus (G') is the measurement of energy stored during deformation and related to the elastic portion of the thrombus, thus reflecting its resistance to elastic deformation. Figure 4.7 showed the maximal values of G' (G'_{max}) of platelets in relation to the presence or absence of ascorbic acid. As shown in the figure, thrombus strength generated during postclotting events was significantly reduced in AA depleted platelets with respect to freshly isolated platelets; the strength was in part restored if platelets were incubated with 0.5 mM AA.

4.2. Modulation of surface NADH-oxidase enzyme by capsaicin

4.2.1 Ecto-NOX1 is present in human platelets.

Since oxidant/antioxidant balance plays a crucial role during platelet activation and/or aggregation, we focused on the PMR system, as it has been shown to regulate the extra/intracellular electron flow in several cell types (del Castillo-Olivares et al., 2000). In human platelets this system has not yet

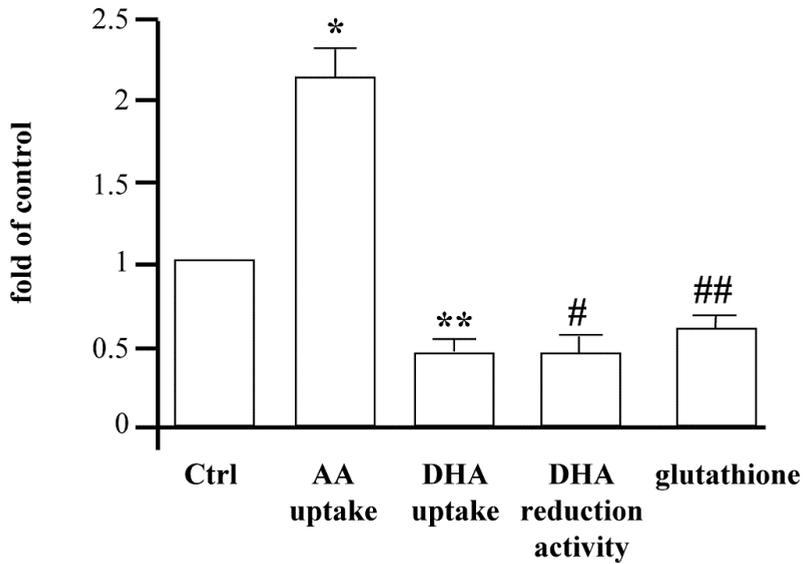


Figure 4.5 Vitamin C uptake and reduction, and glutathione content after platelet activation. Platelets were left untreated or stimulated with 100 nM PMA at 25°C for 15 min. Vitamin C uptake was evaluated by HPLC analysis after incubation with 0.1 mM AA or DHA for 15 min. Data are the means \pm SEM of four independent experiments, each performed in duplicate. Values are reported as fold of control, arbitrarily set to 1. Control absolute values were: AA uptake = 1.6 ± 0.07 nmol/mg protein; DHA uptake = 78 ± 4 nmol/mg protein; DHA reduction activity = 0.13 ± 0.01 nmol/min/mg protein; glutathione content = 2.0 ± 0.2 nmol/mg of protein. * $p=0.003$ vs Ctrl; ** $p=0.001$ vs Ctrl; # $p=0.001$ vs Ctrl; ## $p=0.002$ vs Ctrl.

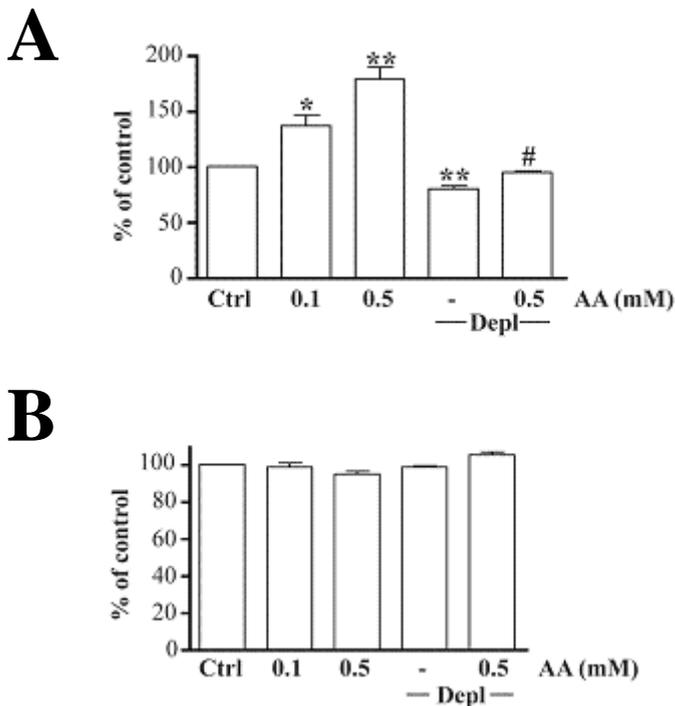


Figure 4.6 Modulation of sulfhydryl groups by intracellular AA. Platelets were left untreated (Ctrl), supplemented for 15 min with 0.1 or 0.5 mM AA or depleted (Depl) by 3 washings with 10 ml PBS for 40 min; the last column represents depleted platelets supplemented again with 0.5 mM AA for 15 min. **(A)** Surface sulfhydryl groups. Values are reported as percent of control, arbitrarily set to 100% (100% = ΔOD_{412} nm 0.41 ± 0.11 /mg protein). * $p < 0.05$ vs Ctrl; ** $p < 0.001$ vs Ctrl; # $p < 0.01$ vs Depl. **(B)** Intracellular sulfhydryl groups. Values are reported as percent of control, arbitrarily set to 100 (100% = ΔOD_{412} nm 13.4 ± 3.3 /mg protein).

Data are the means \pm SEM of three independent experiments, each performed in triplicate. Intracellular AA concentrations were: 1.7 ± 0.1 mM (Ctrl), 1.9 ± 0.1 mM (Ctrl + 0.1 mM AA), 2.2 ± 0.1 mM (Ctrl + 0.5 mM AA), 0.52 ± 0.03 mM (depleted), 1.4 ± 0.1 mM (depleted + 0.5 mM AA).

fully characterized. Thus, our first attempt was to better understand the enzyme(s) potentially linked to the PMR system; in particular, we investigated the hydroquinone NAD(P)H oxidase system, by evaluating cofactor specificity (NADH or NADPH) and membrane localization of the enzymes.

Platelets showed an electron donor preference: indeed, oxidation of exogenous NADH appeared to be more efficient than NADPH oxidation (Figure 4.8). Similar results were obtained when we monitored the reduction of the exogenous acceptor WST-1, in the presence of pyridine nucleotides (Figure 4.8). These findings indicated that platelets have a NAD(P)H oxidase activity able to direct an electron flow on the external side of the plasma membrane, although electron leakage into the cytoplasm could not be excluded.

Trans-membrane oxidase activity, evaluated by measuring the WST-1 reduction in the presence of the electron carrier *mPMS*, was very low (0.12 ± 0.01 nmol/min/mg of protein). The ratio between surface and trans-membrane activity was much higher than that reported by Berridge & Tan (2000) for other cell types. The latter observation, together with the NADH preference, suggested that, in human platelets, surface NADH oxidases could be important components of the PMR system.

In order to define the enzyme(s) present on the platelet membrane, we assessed the NADH-dependent WST-1 reduction, by directly adding to the assay mixture 100 μ M capsaicin, which it has been recognized as an inhibitor of some NAD(P)H oxidases (Vaillant et al., 1996; Scarlett et al., 2005; Joung et al., 2007). Both external NADH oxidation and WST-1 reduction resulted partially inhibited (20 ± 3 %) in the presence of capsaicin, thus suggesting that at least one capsaicin-sensitive NADH oxidase should be on the platelet surface. In the meanwhile, the finding that only 20% inhibition was seen led us to speculate about the presence of a second, capsaicin-insensitive NADH oxidase.

As members of the Ecto-NOX family can account for the biochemical features arising from our experiments, we tried to establish if Ecto-NOX proteins were expressed in platelets. In agreement with biochemical results showing two NADH oxidases with distinct sensitivity to capsaicin, protein bands likely corresponding to both Ecto-NOX1 and Ecto-NOX2 could be seen in the membrane fractions (Figure 4.9). The Ecto-NOX1 antibody recognized a major band of about 70 kDa, which is close to the molecular weight of full length Ecto-NOX1 protein (Jiang et al., 2008). On the other hand, the Ecto-NOX2 antibody recognized four main bands; however, the

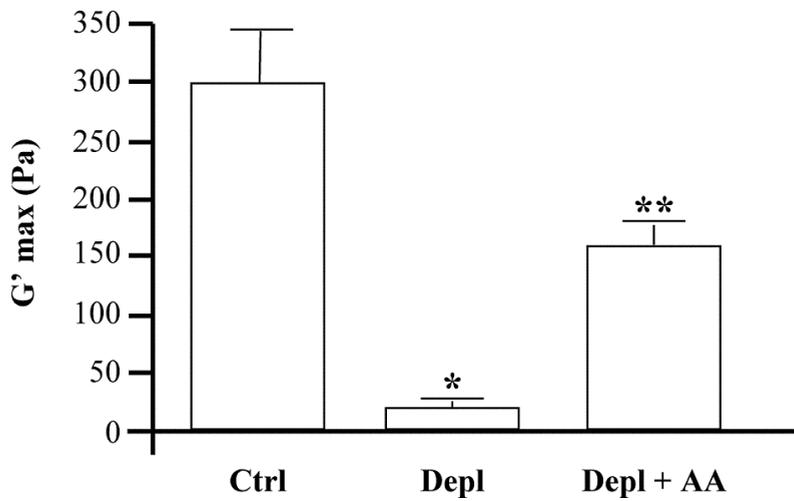


Figure 4.7 Modulation of thrombus rigidity by intracellular AA. Ctrl: freshly isolated platelets. Depl: platelets depleted by 3 washings with 10 ml PBS for 40 min (30% of the basal vitamin C content). Depl + AA: depleted platelets supplemented again with 0.5 mM AA for 15 min. Each sample contained $3-4 \times 10^8$ platelets in 1 ml PBS. Data are the means \pm SEM of three independent experiments. * $p < 0.01$ vs Ctrl; ** $p < 0.05$ vs Depl and Ctrl.

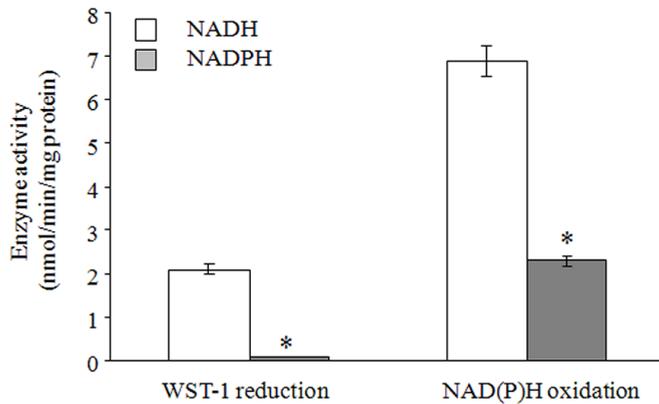


Figure 4.8 Cofactor specificity of the platelet NAD(P)H oxidase system. Enzyme activities were measured spectrophotometrically, as NAD(P)H oxidase activity and NAD(P)H-dependent WST-1 reduction. Values are the means of five independent experiments, each performed in triplicate. * $p < 0.001$ vs NADH-containing assays.

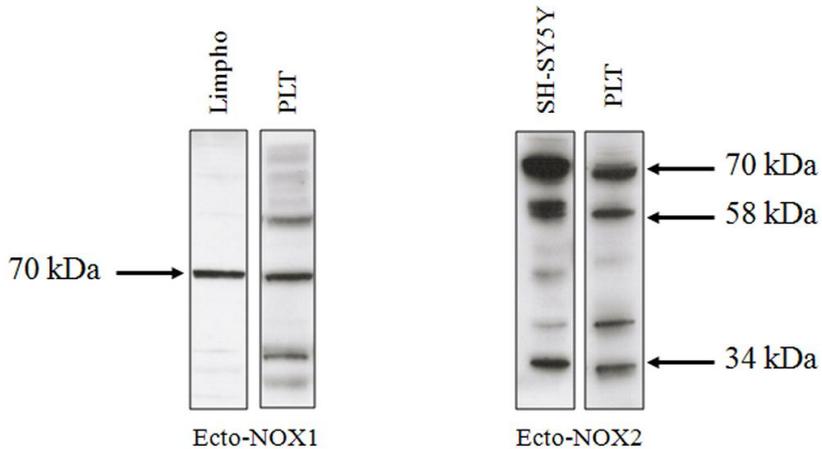


Figure 4.9 Identity of the platelet NAD(P)H oxidase system. Western blot analysis of Ecto-NOX proteins was performed on platelet membranes (PLT) with either anti-Ecto-NOX1 or anti-Ecto-NOX2 antibodies. Positive controls were cellular extracts from lymphocytes (Lympho) for Ecto-NOX1, and SH-SY5Y cells for Ecto-NOX2. The arrows point to Ecto-NOX bands. The radiograph is representative of four similar experiments.

antibody employed in this study appeared to be largely unspecific. The lack of commercially available specific antibodies did not allowed us to give irrefutable evidence for the presence of Ecto-NOX2 on platelet surface; this is a crucial point, as many data in the literature stated the cancer-specificity expression of this protein. Further studies are needed to get unequivocal proofs of Ecto-NOX2 expression on normal human platelets.

4.2.2 Ecto-NOX1 expression is up-regulated by capsaicin

As capsaicin is a quinone analogue and, therefore, perturbs the electron flow through the plasma membrane, we investigated the platelet response to prolonged exposure to this drug. To this end, we incubated human platelets with capsaicin for 45 minutes. When we looked at NADH oxidase activity, we found an increase in enzymatic activity: both NADH oxidation (data not shown) and NADH-dependent WST-1 reduction (Figure 4.10A) significantly increased in capsaicin-treated platelets. This enhancing effect seemed to be related to alteration in redox balance, as N-acetyl-L-cysteine (NAC) prevented the capsaicin-triggered increase (Figure 4.10A).

Therefore, we considered whether this effect was due to modulation of protein levels. The drug induced Ecto-NOX1 expression and this up-regulation could be prevented by the presence of NAC (Figure 4.10B). In addition, experiments performed with 1 mM cycloheximide suggested that Ecto-NOX1 can be modulated at the translational level (Figure 4.10B). Thus, Ecto-NOX1 was capsaicin-responsive.

4.2.3 The capsaicin-mediated regulation of Ecto-NOX1 requires binding to vanilloid receptor

In platelets, capsaicin induced a significant increase in ROS production. Time course experiments (Figure 4.11) showed that ROS amounts, apart from a brief spike at 2 minutes, began to rise after 15 minutes incubation, reaching a 4-fold increase, if compared to basal levels. The capsaicin-evoked ROS increase was completely abrogated by NAC (data not shown).

Accordingly, capsaicin resulted in a 30% decrease in total glutathione content and a 2-fold increase in the GSSG/GSH ratio, as well as in a 40% decrease in the levels of ascorbic acid (Table II). We also found that capsaicin reduced AFR reductase activity and both surface and intracellular

sulfhydryl groups, although the effects were significant only on the former (Table II). Depletion of sulfhydryls could explain the decrease in AFR activity, since enzymes accounting for AFR reduction (including thioredoxin reductase and cytochrome b₅ reductase) have been shown to be redox-sensitive (Nordberg et al., 2001; Shirabe et al., 1991).

As capsaicin could bind to the TRPV1 receptor, recently shown to be expressed in human platelets (Harper et al., 2009), we investigated whether capsaicin effects on the redox state and Ecto-NOX1 modulation were exerted through a vanilloid receptor-dependent pathway. First, we investigated the possible presence of TRPV1 protein in platelet samples, as our purification protocol differed from that adopted by Harper et al. (2009). Western blot analysis showed two reactive bands with an apparent molecular weight of about 70 and 100 kDa: only the immunoreactive band with the highest molecular weight corresponded to the TRPV1 receptor, as confirmed by competition studies performed with the blocking peptide (Figure 4.12A).

To investigate the involvement of TRPV1 in capsaicin-induced ROS production, an ultra potent agonist (resiniferatoxin) (Szolcsanyi et al., 1990) and two specific antagonists (5'-IRTX and capsazepine) (Wahl et al., 2001; Dickenson & Dray, 1991) of the vanilloid receptor were used. Figure 4.12B showed that activation of the TRPV1 receptor by either capsaicin or resiniferatoxin resulted in a strong induction of ROS generation. Accordingly, receptor antagonists exerted the opposite effects; in fact, the 4-fold increase elicited by capsaicin alone was halved when platelets were pre-treated with either 20 μ M 5'-IRTX or 50 μ M capsazepine. In line with our hypothesis, pre-incubation of platelets with capsazepine prevented the capsaicin-triggered increase of Ecto-NOX1 expression and activity (Figure 4.12C).

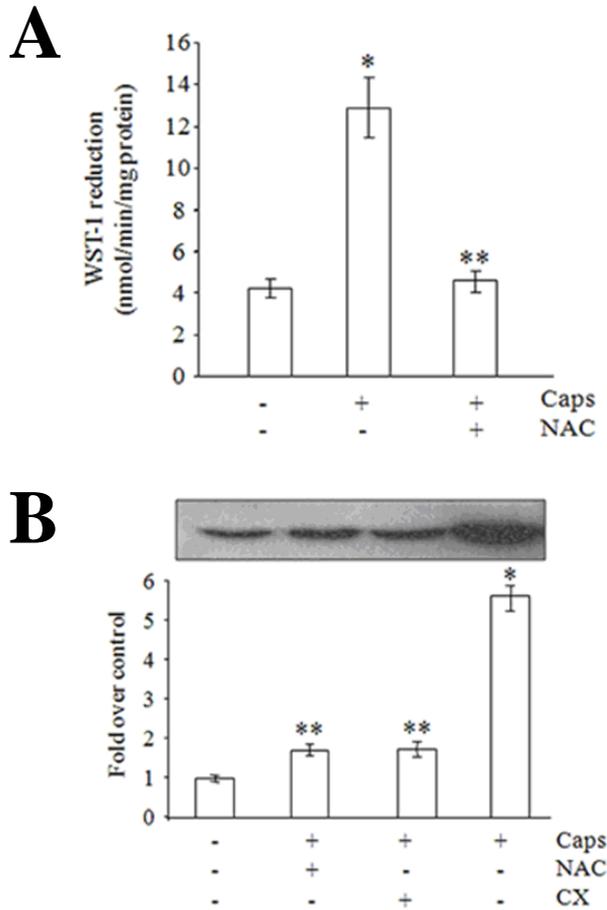


Figure 4.10 NADH-dependent WST-1 reduction and expression of Ecto-NOX1. **(A)** NADH-dependent WST-1 reduction. Platelets were left untreated or treated with capsaicin (Caps); some samples were also pre-treated with NAC for 15 minutes, before incubation with capsaicin. **(B)** Western blot analysis of Ecto-NOX1. Platelets were treated as in A and immunoblotted with anti-Ecto-NOX1 antibody; the third bar represents platelets incubated with capsaicin in the presence of 1 mM cycloheximide (CX). The radiograph is representative of four similar experiments. The histogram represents the densitometric analysis of autoradiography. Values are the means \pm S.E. of three independent experiments, values are reported as fold over control, arbitrarily set to 1, after normalization with tubulin. * $p < 0.001$ vs untreated platelets; ** $p < 0.05$ vs capsaicin.

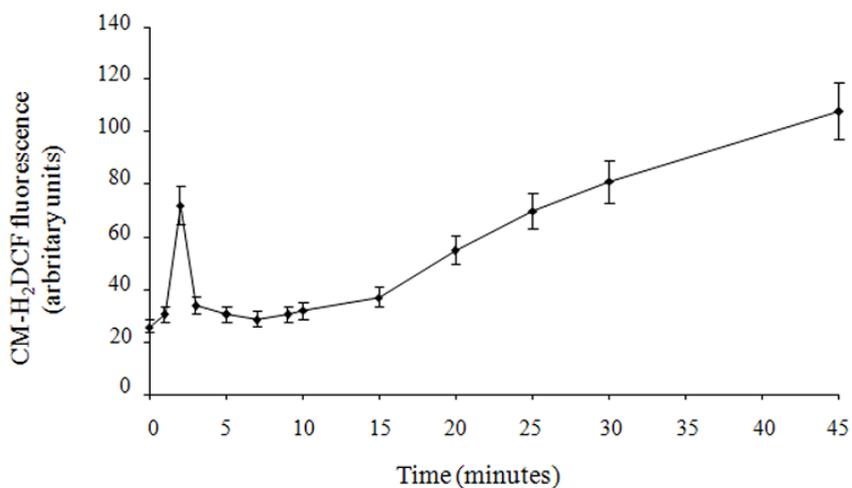


Figure 4.11 Time course experiment of ROS production by capsaicin. Platelets were incubated with 100 μ M capsaicin for the indicated times, before assessing radical formation by flow cytometry. Values are the means \pm S.E. of five independent experiments.

Table II
Effect of capsaicin on platelet redox state

| | CTRL | CAPSAICIN |
|--|---------------|----------------|
| Total glutathione (nmol/mg protein) | 2.7 \pm 0.3 | 2.0 \pm 0.2* |
| GSSG/GSH * 100 | 2.6 \pm 0.3 | 5.0 \pm 0.4* |
| AA (mM) | 1.6 \pm 0.1 | 1.0 \pm 0.1* |
| Surface sulfhydryl groups (nmol DTNB/mg protein) | 27 \pm 2.6 | 15 \pm 2.0 |
| Intracellular sulfhydryl groups (nmol DTNB/mg protein) | 860 \pm 90 | 690 \pm 70 |

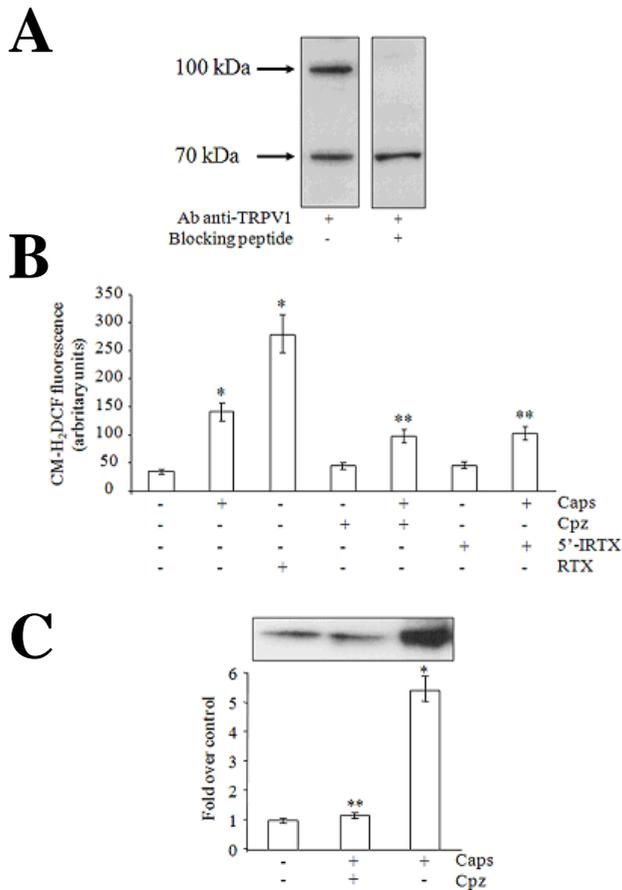


Figure 4.12 TRPV1 involvement in Ecto-NOX1 modulation. **(A)** Western blot analysis of TRPV1 receptor. Platelet extracts were immunoblotted with anti-TRPV1 antibody, alone or with TRPV1-specific blocking peptide. The radiographs are representative of four similar experiments. **(B)** ROS production with TRPV1 agonists and antagonists. Platelets were left untreated or treated with capsaicin (Caps, 100 μ M), capsazepine (Cpz, 50 μ M), 5'-iodo-resiniferatoxin (5'-IRTX, 20 μ M) and resiniferatoxin (RTX, 10 μ M); some samples were also pre-treated either with capsazepine or 5'-iodo-resiniferatoxin for 10 minutes, before incubation with capsaicin. Data are the means \pm S.E. of three independent experiments, each performed in triplicate. * $p < 0.001$ vs untreated platelets; ** $p < 0.05$ vs capsaicin. **(C)** Western blot analysis of Ecto-NOX1 with TRPV1 agonists and antagonists. Platelets were left untreated or treated with capsaicin; some samples were also pre-treated with capsazepine for 10 minutes, before incubation with capsaicin. The radiograph is representative of four similar experiments. The histogram represents the densitometric analysis of autoradiography. Values are the means \pm S.E. of three independent experiments, values are reported as fold over control, arbitrarily set to 1, after normalization with tubulin. * $p < 0.001$ vs untreated platelets; ** $p < 0.05$ vs capsaicin.

5. DISCUSSION

Platelet activation and aggregation are modulated by ROS (Iuliano et al., 1997; Del Principe et al., 2009), which control both outside-in and inside-out signalling, by modulating the activity of key molecules through thiol/disulfide exchanges. The downstream effect of ROS is oxidation of proteins, which can be reversed by intracellular reducing agents; an imbalance between ROS production and antioxidant defences may play a role in cardiovascular pathologies (Touyz, 2003, 2004). Intracellular antioxidants (such as vitamin C) and the PMR system may be of importance in terms of electron movement during platelet activation and cell-to-cell interactions (platelet-platelet, platelet-leukocyte, platelet-endothelial cells) (Hidalgo et al., 2009; Looney & Matthay, 2009).

Here, we found that platelets are able to compensate for fluctuations in vitamin C levels by regulating the specific AA transporter SVCT2. In AA-depleted platelets, SVCT2 protein levels were up-regulated, in order to restore intracellular vitamin concentration; conversely, high intracellular AA levels lowered the SVCT2 protein. Substrate regulation of Na⁺-dependent transporters seems to be a more generalized phenomenon, not restricted to platelets, since it has already been described in other cell types. Downregulation of SVCT2 activity has been demonstrated in osteoblasts (Dixon & Wilson, 1992) and astrocytes (Wilson et al., 1990), where V_{max} changed as a function of AA levels. More recently, decreased mRNA levels for SVCT1 after AA supplementation have been demonstrated in enterocytes (MacDonald et al., 2002). The molecular mechanisms explaining such changes have not yet been identified; several key points should control SVCT activity, acting at transcriptional or post-translational levels, or modulating protein turnover. In particular, a translational control mechanism for SVCT2 emerged from our study; indeed, platelets are anucleated cells, thus ruling out the involvement of transcription factors in regulation of expression. We found that translation of the SVCT2 messenger was modulated at very short times; as suggested by others, the rapid response may result from localization of transcripts in polysomes in resting platelets (Lindemann et al., 2001). RNA secondary structure prediction of SVCT2 mRNA by the program GeneBee-Net v.2.0 (www.genebee.msu.ru) shows that the 3'-untranslated region could fold in conformations stable enough at 37°C. Therefore, it is possible that the 3'-untranslated region of SVCT2 mRNA is important to keep it stable enough in order to allow its regulation.

The ability to compensate for fluctuations in ascorbate levels should have

biological relevance in conditions where AA dramatically falls, such as in scurvy or during platelet activation (Zucker et al., 1990). Nonetheless, by our findings, it is not clear if vitamin C acts on its own carrier directly or indirectly by altering the redox state. Sensitivity of the SVCT2 carrier to changes in cellular redox state has already been reported in muscle cells (Savini et al., 2005). Furthermore, during platelet activation both vitamin C deprivation and induction of oxidative stress occur (Finazzi-Agrò et al., 1982).

Also Ecto-NOX1, a component of the PMR system, whose presence in platelets has been described for the first time in this study, is under translational control. Our data showed that resting platelets had both transmembrane and surface NAD(P)H oxidase activities. Western blot analysis allowed us to combine the observed enzymatic activity with the expression of Ecto-NOX1. We have also shown that the ubiquinone analogue capsaicin inhibited the NADH oxidase activity of about 20%. However, after prolonged exposure to capsaicin, platelets showed enhanced Ecto-NOX1 protein expression and activity. The translational modulation of Ecto-NOX1 appeared to be dependent on activation of TRPV1 (the natural receptor for capsaicin) and generation of ROS. It should be recalled that TRPV1 activation led to biphasic ROS production: in capsaicin-treated platelets, we found a spike in ROS concentration as early as 2 minutes followed by a gradual rise after 15 minutes. Thus, ligand-receptor binding activated the signalling cascade responsible for the early events, as already reported (Harper et al., 2009), but the TRPV1-dependent Ecto-NOX1 activation appeared to rely on prolonged activation of the receptor, since it is a later event.

As noted above, the redox state controls platelet functions, through protein folding and disulfide bond formation. Functional sulfhydryl groups are present in platelet membrane proteins and provide redox-sensitive sites stimulating platelet aggregation, secretion, and postaggregation events through the activation of platelet integrin receptors (Essex, 2004; Kashiwagi et al., 1999; Yan & Smith, 2000). What is emerging from our study is that the redox state of sulfur atoms exposed on platelet surface may be modulated by intracellular antioxidants, as well as by the PMR system. In our model, the surface sulfhydryl content appeared to be related to the intracellular vitamin C content; on the other hand, the particular localization of Ecto-NOX1, and its disulfide/thiol interchange activity, may be of importance in terms of protein folding during platelet activation.

Under specific conditions, such as during inflammation and tissue injury,

Ecto-NOX1 activity may be enhanced, as a result of increased protein translation and substrate availability. The release of several mediators, including histamine, anandamide, prostaglandins and cytokines (collectively termed endovanilloids), activates TRPV1 and promotes ROS increase, which in turn induces Ecto-NOX1 expression; in the meanwhile, cell damage may promote NADH release into extracellular compartments.

Beside the importance in aggregating events, the redox state of sulfhydryl groups may also play a key role after aggregation. Here, we found a strict relationship between intracellular AA and dynamics of platelet thrombus formation. Thus, intracellular antioxidants can regulate the thrombus viscoelastic properties, thus modulating clot stiffness and platelet contacts occurring during postaggregation events (Carr, 2003).

6. CONCLUSIONS

This study characterizes intracellular and surface systems in freshly human platelets, especially focusing on vitamin C homeostasis and plasma membrane NAD(P)H oxidases.

Our conclusions are supported by several findings: (i) platelets express the Na⁺-dependent AA transporter SVCT2; (ii) SVCT2 expression is under translational control, related to substrate availability and platelet activation; (iii) platelets express Ecto-NOX1 protein, located on the external side of plasma membrane and responsible for NADH oxidation; (iv) Ecto-NOX1 expression is under translational control, with a mechanism depending on TRPV1 activation and ROS generation.

Intracellular antioxidants (such as vitamin C) and the PMR system are involved in the modulation of sulfhydryl groups present in platelet membrane proteins, crucial for platelet aggregation, secretion, and post-aggregation events.

7. EXPERIMENTAL PROCEDURES

Reagents

[¹⁴C]-AA was purchased from Amersham (Arlington Heights, IL). DHA was obtained from ICN (Aurora, OH). Ascorbate oxidase was obtained from Boehringer Mannheim (Mannheim, Germany); 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST-1) was obtained from Cayman Chemical Company (Ann Arbor, Michigan, USA). 5'-iodo-resiniferatoxin (5'-IRTX) was obtained from Tocris (Bristol, UK); capsaicin, NADH, NADPH, were obtained from Sigma Chemical (St. Louis, MO, USA).

Platelet isolation and drug treatments

After informed consent, platelets were isolated from blood samples obtained from 12 healthy donors (20 to 40 years old) free of medication for at least the preceding 30 days. Blood was collected in plastic tubes and diluted 1:5 with ACD buffer (80 mM glucose, 25 mM citric acid 45 mM sodium citrate). The platelet pellet was prepared by centrifuging plasma through a Ficoll layer (23% w/v) at 80 x g for 20 min, as previously described (Washburn et al., 1999); after washings, platelets were resuspended in tyrode's buffer (100 mM HEPES, 1.3 M NaCl, 29 mM KCl, 10 mM NaHCO₃).

Platelet stimulation was achieved by preincubating samples with 100 nM phorbol 12-myristate 13-acetate (PMA) or 0.5 U/ml thrombin at 25°C for 15 min. Platelet count and mean volume were measured by Beckman Coulter HMX.

Capsaicin treatments were performed by adding capsaicin dissolved in ethanol just before use; 100 μM capsaicin corresponded to 0.004% ethanol final concentration. Where indicated, platelets were pre-treated for 10 min either with 50 μM capsazepine or 20 μM 5'-IRTX, or for 15 min with 5 mM N-acetyl-L-cysteine (NAC), before incubation with capsaicin. After treatments, platelets were rinsed with tyrode's buffer.

Western blot

Platelets were resuspended in lysis buffer (25 mM Tris-HCl pH 8.0, 1 mM EDTA, 0.5% SDS) and disrupted by three freeze-thawing cycles. Plasma membranes were separated on a sucrose step gradient, as already reported (MacDonald et al., 2002). Membrane fractions (10 µg) were subjected to SDS-PAGE on a 10% polyacrylamide gel and then electroblotted onto a PVDF membrane. Blots were blocked with 5% non fat dry milk (Biorad, Hercules, CA, USA) and then incubated with anti-SVCT1 (sc-9924), anti-SVCT2 (sc-9926), anti-TRPV1 (sc-12498), anti-tubulin (sc-9104) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-Ecto-NOX1 (H00055068-B01; Abnova, Taipei City, Taiwan) and anti-Ecto-NOX2 (HPA000514; Sigma) primary antibodies. After washings and incubation with the horseradish peroxidase-conjugated secondary antibody, detection was carried out with ECL (Amersham, Arlington Heights, IL, USA).

Competition experiments were carried out by incubating the antibody solution with a 100-fold molar excess of specific blocking peptides for 3 hours at room temperature, before performing Western blot analysis.

RT-PCR

Total RNA was isolated by Trizol (Invitrogen Corporation, Carlsbad, CA), reverse-transcribed using the Superscript preamplification system (Invitrogen Corporation) and, finally, PCR-amplified. Control reactions were performed to ensure complete removal of DNA and exponential amplification of mRNA. Twenty microliters of the reaction were electrophoresed on a 1.2% (w/v) agarose gel and the amplified products were visualized by ethidium bromide staining.

The primers used were as follows: hSVCT1-forward 5'-ATTTGGCACCCACGGATACG-3' and hSVCT1-reverse 5'-TCAAGGTCAGGACATAGCAGAGC-3'; hSVCT2-forward 5'-AGAAGGCAAATACGAAGACGAGG-3' and hSVCT2-reverse 5'-GCTCTGCTGTTCCATTGGCAAC-3'. The amplification parameters were as follows: 94°C 30 s, 58°C 30 s and 72°C 1 min for 30 cycles.

Amplification of GAPDH mRNA was performed as loading control. The primers were as follows: hGAPDH-forward 5'-TCGGAGTCAACGGATTTGGTTCG-3' and hGAPDH-reverse 5'-GCTCTCCAGAACATCATCCCTGCCT-3'. The amplification parameters

were as follows: 94°C 30 s, 60°C 30 s and 72°C 40 s for 22 cycles.

Vitamin C uptake

Intra-platelet AA amount was measured by using HPLC with UV detection at 265 nm, as previously described (Savini et al., 1999). Briefly, $0.5-1 \times 10^8$ platelets were dissolved in 50 μ l of 0.1 M phosphate buffered saline (PBS) and directly extracted with 116 μ l ice-cold methanol containing 1 mM EDTA. Following sonication at 4°C, the precipitated proteins were removed by centrifugation at 16,000 rpm for 5 min and 20 μ l of the supernatant was injected onto the column for HPLC analysis. Since DHA does not adsorb at 265 nm, it was quantified as AA by treating samples with 10 mM dithiothreitol (DTT) for 10 min. Intracellular AA molar amounts were calculated by measuring platelet mean volume for each experiment.

Because endogenous AA concentrations would produce a high background with HPLC analysis, transport experiments were performed by scintillation spectrometry. In time-dependent experiments, $0.5-1 \times 10^8$ platelets were incubated in 1 ml of transport medium (5 mM KCl, 1.9 mM KH_2PO_4 , 5.5 mM glucose, 0.3 mM MgSO_4 , 1 mM MgCl_2 , 0.3 mM CaCl_2 , 10 mM HEPES, 147 mM NaCl, 1.1 mM Na_2HPO_4 pH 7.4), containing 0.1 mM [^{14}C]AA (specific activity 1 mCi/mmol) and 1 mM DTT (to prevent AA oxidation) or 0.1 mM [^{14}C]DHA (1 mCi/mmol) or 0.2 mM [$1,2\text{-}^3\text{H}$]-2-deoxyglucose (2-DOG; 1 mCi/mmol). DHA was prepared by incubating [^{14}C]AA with 2 units of ascorbate oxidase immediately before being added to the incubation medium; complete oxidation was verified by HPLC. After incubation at 37°C, platelet samples were washed twice with ice-cold 0.1 M PBS and resuspended in 100 μ l of 0.1 M PBS. Then, 10 μ l of samples were analysed for protein content (Bradford, 1976) and the remaining 90 μ l were dissolved in 500 μ l of ice-cold methanol and counted in a liquid scintillation counter. For concentration-dependence experiments, platelets were incubated with 1-100 μM [^{14}C]AA for 15 min or with 1-5000 μM [^{14}C]DHA for 5 min.

For transport inhibition studies, platelets were pre-incubated with cytochalasin B (10 μM), ouabain (0.5 mM), sulfapyrazone (5 mM) and phloretin (200 μM) for 15 min at 37°C before the addition of labeled compounds.

Vitamin C recycling

DHA-reductase activity was assayed as previously described (Savini et al., 2000). Platelets were resuspended in 50 mM Tris-HCl pH 7.4, 1 mM EDTA, 30 mg/ml catalase, 1 mM PMSF and sonicated. Samples (0.2-0.3 mg of protein) were incubated at 37°C for 20 min in 50 mM Tris-HCl pH 7.5 containing 1 mM EDTA and 1 mM DHA. After incubation and precipitation of proteins by methanol, the ascorbate content was evaluated by HPLC.

Ascorbyl free radical (AFR) reductase activity was assessed spectrophotometrically through the rate of ascorbate free radical-dependent oxidation of NADH, by monitoring the decrease in 340 nm absorbance ($E = 6.2 \text{ mM}^{-1} \text{ cm}^{-1}$) at 25°C (Savini et al., 1999). Corrections have been made for direct oxidation of NADH by homogenates. The assay mixture contained 0.05 mM Tris-HCl pH 7.8, 1 mM EDTA, 0.1 mM NADH, 1 mM ascorbate and 0.3 mg of proteins. The reaction was started by adding 0.28 U of ascorbate oxidase to generate the ascorbate free radical.

Free Oscillation Rheometry

Four-channel FORs (Reorox4) was obtained from the Global Haemostasis Institute Linköping Sweden. The method is based on the fact that the rheological properties of sample change during coagulation (Ramstrom et al., 2003). The time of thrombus formation was recorded immediately after addition of 1 mg/ml fibrinogen and 10.5 µg/ml SFLLRN (a thrombin-receptor-agonist peptide; kindly provided by the Global Haemostasis Institute). Stimulation with SFLLRN is particularly useful for studies of platelet rheological properties, since the peptide, unlike thrombin, does not allow the conversion of fibrinogen to fibrin (Kinlough-Rathbone et al., 1993; Coughlin, 1999).

To allow determination of thrombus elasticity, gold-plated reaction chamber was used. The reaction chamber included a cylindrical sample cup and an inner cylinder, a golden bob, attached to a hollow shaft and immersed into the centre of the sample cup. Frequency and amplitude of the free oscillation are functions of elastic and viscous properties of the thrombus. For evaluation of the FOR curves, the elasticity modulus G' was plotted as a function of time in the ReoRox4 Viewer software, according to the

manufacturer's instructions. The maximal G' was determined from curves and expressed as Pa.

Surface NAD(P)H oxidase activity

NADH and NADPH oxidase activities were determined by monitoring the decrease in absorbance (340 nm) of exogenously added nucleotides. Briefly, platelets (1×10^8) were incubated in 1 ml of tyrode's buffer containing 0.2 mM NADH or 0.2 mM NADPH at 37 °C with continuous stirring, and rates of NADH or NADPH oxidation were calculated using the extinction coefficient $6.2 \text{ mM}^{-1} \text{ cm}^{-1}$.

NAD(P)H oxidase activity was also measured by monitoring the reduction of WST-1, a cell-impermeable artificial electron acceptor, usually employed to measure surface Ecto-NOX activity (Bergsten et al., 1995; Wells et al., 1990; Washburn et al., 1999; Del Bello et al., 1994). Briefly, platelets (1×10^8) were incubated with 0.5 mM WST-1 and 0.2 mM NADH or 0.2 mM NADPH. All experiments were performed at 37°C, with continuous stirring, and WST-1 formazan production was monitored in real time by the increase of absorption at 450 nm. Rates of WST-1 reduction were expressed as nmol WST-1 reduced/min/mg protein (extinction coefficient = $37 \text{ mM}^{-1} \text{ cm}^{-1}$).

Trans-PMR activity

Trans-PMR activity was evaluated by measuring WST-1-reduction in the presence of 1-methoxy-5-methyl-phenazinium methyl sulphate (*mPMS*), which is a stable electron-transport mediator between NAD(P)H and WST-1 (May et al., 1997). Washed platelets (1×10^9) were incubated in 1 ml of tyrode's buffer containing 0.5 mM WST-1 and 20 μM *mPMS* and WST-1 reduction was monitored in real time at 450 nm.

Intracellular ROS

Intracellular ROS generation was measured by using the fluorescent probe 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H₂DCFDA; Molecular Probes Inc., Eugene, OR). After treatment, platelets were incubated with 10 μM CM-H₂DCFDA for 20 min at 37°C in the dark. Radical formation was assessed by flow cytometry in a

FACSCalibur Flow Cytometer (Becton Dickinson, CA, USA). CM-H₂DCFDA mean fluorescence was registered at 530 nm (bandwidth 30 nm), exciting at 488 nm using a 15 mW Argon laser. One hundred thousand events were evaluated for each analysis.

Sulfhydryl groups and glutathione content

Surface sulfhydryl groups were evaluated by using the membrane-impermeant reagent 5-5'-dithiobis(2-nitrobenzoic acid) (DTNB). Briefly, 1×10^9 platelets in 1 ml tyrode's buffer were incubated with 0.2 mM DTNB for 15 min at 37°C. Platelets were pelleted (12,000 rpm for 5 min) and 2-nitro-5-thiobenzoic acid was measured in the supernatant at 412 nm (extinction coefficient = $14.150 \text{ mM}^{-1} \text{ cm}^{-1}$). Total sulfhydryl groups were evaluated in lysed platelets by the same assay, and the intracellular amounts were obtained by difference (Xu et al., 1996).

Intracellular glutathione content was quantified by a DTNB-glutathione reductase recycling assay as described (Craik et al., 1995).

Kinetics calculations and statistics

Kinetics for all substrates were determined when transport was linear. The transport kinetic parameters were calculated by Michaelis-Menten equation and linear transformation of Eadie-Hofstee. Statistical analysis was conducted with the program GraphPad Prism 4.00 (GraphPad Software Inc., La Jolla, CA, USA). Either paired t-test or analysis of variance (ANOVA) followed by Bonferroni post hoc comparisons were performed to evaluate differences between samples. Significant differences were accepted at $p < 0.05$.

8. REFERENCES

- Agha A, Schluter H, Konig S, Biel K, Tepel M, Zidek W. A novel platelet-derived renal vasoconstrictor agent in normotensives and essential hypertensives. *J Vasc Res.* 1992. 29:281–289.
- Aledort LM, Troup SB, Weed RI. Inhibition of sulfhydryl-dependent platelet functions by penetrating and non-penetrating analogues of parachloromercuribenzene. *Blood* 1968. 31:471-479.
- Alessio HM, Goldfarb AH. Lipid peroxidation and scavenger enzymes during exercise: adaptive response to training. *J Appl Physiol.* 1988. 64(4):1333-1336.
- Authi KS, Bokkala S, Patel Y, Kakkar VV, Munkonge F. Ca²⁺ release from platelet intracellular stores by thapsigargin and 2,5-di-(t-butyl)-1,4-benzohydroquinone: relationship to Ca²⁺ pools and relevance in platelet activation. *Biochem J.* 1993. 294:119-126.
- Basu S, Som S, Deb S, Mukherjee D, Chatterjee IB. Dehydroascorbic acid reduction in human erythrocytes. *Biochem Biophys Res Commun.* 1979. 90:1335-1340.
- Begonja AJ, Gambaryan S, Geiger J, Aktas B, Pozgajova M, Nieswandt B, Walter U. Platelet NAD(P)H-oxidase-generated ROS production regulates alphaIIb beta3-integrin activation independent of the NO/cGMP pathway. *Blood* 2005. 106: 2757-2760.
- Belloc F, Heilmann E, Combrie R, Boisseau MR, Nurden AT. Protein synthesis and storage in human platelets: a defective storage of fibrinogen in platelets in Glanzmann's thrombasthenia. *Biochim Biophys Acta.* 1987. 925:218-225.
- Bizzozero G. Sur les petites claque du sang des mammiferes, deuxième note. *Archives Italiennes de Biologie* 1882b. 1: 1-4.
- Boehlen F, Clemetson KJ. Platelet chemokines and their receptors: what is their relevance to platelet storage and transfusion practice? *Transfus Med.* 2001. 11:403– 417.
- Bolz SS, Pohl U. Highly effective non-viral gene transfer into vascular smooth muscle cells of cultured resistance arteries demonstrated by genetic inhibition of sphingosine-1-phosphate-induced vasoconstriction. *J Vasc Res.* 2003. 40: 399–405.
- Booyse F, Rafelson ME Jr. In vitro incorporation of amino-acids into the contractile protein of human blood platelets. *Nature.* 1967a. 215:283-244.

- Booyse F, Rafelson ME Jr. Stable messenger RNA in the synthesis of contractile protein in human platelets. *Biochim Biophys Acta*. 1967b 145:188-190.
- Bremer J. Carnitine: metabolism and functions. *Physiol. Rev.* 1983. 63:1420-1480.
- Brogren H, Karlsson L, Andersson M, Wang L, Erlinge D, Jern S. Platelets synthesize large amounts of active plasminogen activator inhibitor 1. *Blood* 2004. 104: 3943-3958.
- Bugert P, Dugrillon A, Günaydin A, Eichler H, Klüter H. Messenger RNA profiling of human platelets by microarray hybridization. *Thromb Haemost.* 2003. 90(4):738-48.
- Caccese D, Pratico D, Ghiselli A, Natoli S, Pignatelli P, Sanguigni V, Iuliano L, Violi F. Superoxide anion and hydroxyl radical release by collagen-induced platelet aggregation—role of arachidonic acid metabolism.
- Carr ME Jr. Development of platelet contractile force as a research and clinical measure of platelet function. *Cell Biochem. Biophys.* 2003. 38:55-78.
- Castro M, Caprile T, Astuya A, Millan C, Reinicke K, Vera JC, Vasquez O, Aguayo LG, Nualart F. High-affinity sodium-vitamin C co-transporters (SVCT) expression in embryonic mouse neurons. *J Neurochem* 2001. 78:815-823.
- Chang Y, Bluteau D, Debili N, Vainchenker W. From hematopoietic stem cells to platelets. *J Thromb Haemost.* 2007. 1:318-327.
- Chen CY, Lin T. H. Effects of nickel chloride on human platelets: enhancement of lipid peroxidation, inhibition of aggregation and interaction with ascorbic acid. *J Toxicol Environ Health A.* 2001. 62:431-438.
- Chlopicki S; Olszanecki R; Janiszewski M; Laurindo FR; Panz T, Miedzobrodzki J. Functional role of NADPH oxidase in activation of platelets. *Antioxid Redox Signal.* 2004. 6:691-698.
- Chueh PJ; Kim C, Cho N, Morr  DM, Morr  DJ. Molecular cloning and characterization of a tumor-associated, growth-related, and time-keeping hydroquinone (NADH) oxidase (tNOX) of the HeLa cell surface. *Biochemistry.* 2002. 41:3732-3741.
- Cines DB. Glycoprotein IIb/IIIa antagonists: potential induction and detection of drug-dependent antiplatelet antibodies. *Am Heart J.* 1998. 135:S152-S159.

- Clark AG, Rohrbaugh AL, Otterness I, Kraus VB. The effects of ascorbic acid on cartilage metabolism in guinea pig articular cartilage explants. *Matrix Biol.* 2002. 21:175-184.
- Corral J, González-Conejero R, Rivera J, Ortuño F, Aparicio P, Vicente V. Role of the 807 C/T polymorphism of the alpha2 gene in platelet GP Ia collagen receptor expression and function-effect in thromboembolic diseases. *Thromb Haemost* 1999. 81:951-956.
- Dahlbäck B, Stenflo J. The Protein C Anticoagulant System. In: *The Molecular Basis of Blood Disease*. 3rd ed. W.B. Saunders Company, Philadelphia, 2000:614–656.
- Dale GL. Platelet kinetics. *Curr Opin Hematol.* 1997. 4:330-334.
- Davies KJA, Quintanilla AT, Brooks GA, Packer L. Free radicals and tissue damage produced by exercise. *Biochem. Biophys. Res. Commun.* 1982. 107:1198-1205.
- De Gaetano G. Historical overview of the role of platelets in hemostasis and thrombosis. *Haematologica* 2001. 86:349-356.
- Del Bello B, Maellaro E, Sugherini L, Santucci A, Comporti M, Casini AF. Purification of NADPH-dependent dehydroascorbate reductase from rat liver and its identification with 3 alpha-hydroxysteroid dehydrogenase. *Biochem J.* 1994. 304:385-390.
- Del Principe D, Frega G, Savini I, Catani MV, Rossi A, Avigliano L. The plasma membrane redox system in human platelet functions and platelet-leukocyte interactions. *Thromb Haemost.* 2009.101:284-289.
- Del Principe D; Mancuso G; Menichelli A; Gabbiotti M; Cosmi EV; Ghepardi G. Production of hydrogen peroxide in phagocytizing human platelets: an electron microscopic cytochemical demonstration. *Biol Cell* 1980. 38:135-140.
- Denis MM, Tolley ND, Bunting M, Schwertz H, Jiang H, Lindemann S, Yost CC, Rubner FJ, Albertine KH, Swoboda KJ, Fratto CM, Tolley E, Kraiss LW, McIntyre TM, Zimmerman GA, Weyrich AS. Escaping the nuclear confines: signal-dependent pre-mRNA splicing in anucleate platelets. *Cell.* 2005. 122:379-391.
- Dhariwal K, Washko P, Hartzell W, Levine M, Ascorbic acid within chromaffin granules. In situ kinetics of norepinephrine biosynthesis. *J Biol Chem.* 1989. 264:15404-15409.
- Dittrich M, Birschmann I, Pfrang J, Herterich S, Smolenski A, Walter U, Dandekar T. Analysis of SAGE data in human platelets: features of the transcriptome in anucleate cell. *Thromb Haemost.* 2006. 95:643-651.

- Dixon SJ, Wilson JX. Adaptive regulation of ascorbate transport in osteoblastic cells. *J Bone Miner Res.* 1992. 7:675-681.
- Duval C, Augé N, Frisach MF, Casteilla L, Salvayre R, Nègre-Salvayre A. Mitochondrial oxidative stress is modulated by oleic acid via an epidermal growth factor receptor-dependent activation of glutathione peroxidase. *Biochem J.* 2002. 367:889-894.
- Englard S, Seifter S. The biochemical functions of ascorbic acid. *Annu Rev Nutr.* 1986. 6:365-406.
- English D, Garcia JG, Brindley DN. Platelet-released phospholipids link haemostasis and angiogenesis. *Cardiovasc Res.* 2001. 49:588 -599.
- Essex DW, Li M, Feinman RD, Miller A. Platelet surface glutathione reductase-like activity. *Blood* 2004. 104:1383-1385.
- Essex DW, Li M, Miller A, and Feinman RD. Protein disulfide isomerase and sulfhydryl-dependent pathways in platelet activation. *Biochemistry* 2001. 40: 6070–6075.
- Evangelista V, Manarini S, Di Santo A, Capone ML, Ricciotti E, Di Francesco L, Tacconelli S, Sacchetti A, D'Angelo S, Scilimati A, Sciulli MG, Patrignani P. De novo synthesis of cyclooxygenase-1 counteracts the suppression of platelet thromboxane biosynthesis by aspirin. *Circ Res.* 2006. 98:593-595.
- Finazzi-Agro' A; Menichelli A; Persiani M, Biancini G; Del Principe D. Hydrogen peroxide release from human blood platelets. *Biochim Biophys Acta.* 1982. 18:21-25.
- Floyd RA. Oxidative damage and repair: Chemical, biological and medical aspects (Davies KJA ed.) Pergamon Press Oxford. 1991.
- Freeman BC, Crapo JD. Biology of disease. Free radicals and tissue injury. *Lab Invest.* 1982. 47:412-426.
- Frei B. Reactive oxygen species and antioxidant vitamins mechanism of action. *Am J Med.* 1994. 97:3A-13S.
- Geiszt M; Leto TL. The Nox family of NAD(P)H oxidases: host defense and beyond. *J Biol Chem.* 2004. 279: 51715-51718.
- Gnatenko D V, Dunn JJ, McCorkle SR, Weissmann D, Perrotta PL, Bahou WF. Transcript profiling of human platelets using microarray and serial analysis of gene expression BLOOD. 2003.101: 2285.
- Goldenberg H, Landertshamer H, Laggner H. Functions of vitamin C as a mediator of transmembrane electron transport in blood cells and related cell culture models. *Antioxid Redox Signal.* 2000 2:189-196.

- Gregg D, de Carvalho DD, Kovacic H. Integrins and coagulation: a role for ROS/redox signaling? *Antioxid Redox Signal* 2004. 6:757-764.
- Halliwell B, Aruoma OI. DNA damage by oxygen-derived species. Its mechanism and measurement in mammalian system. *FEBS Lett.* 1991. 281:9-19.
- Halliwell B, Gutteridge JMC. Role of free radicals and catalytic metal ions in human disease: An overview. *Methods Enzymol.* 1990a. 186:1-85
- Halliwell B, Gutteridge JMC. The antioxidants of human extracellular fluids. *Arch Biochem Biophys.* 1990b. 280:1-8.
- Harper AG, Brownlow SL, Sage SO. A role for TRPV1 in agonist-evoked activation of human platelets. *J Thromb Haemost.* 2009. 7:330-338.
- Hidalgo A, Chang J, Jang JE, Peired AJ, Chiang EY, Frenette PS. Heterotypic interactions enabled by polarized neutrophil microdomains mediate thromboinflammatory injury. *Nat Med.* 2009. 15:384-391.
- Himmelreich U, Drew KN, Serianni AS, Kuchel PW. ¹³C NMR studies of vitamin C transport and its redox cycling in human erythrocytes. *Biochemistry* 1998. 37:7578-7588.
- Ho PP; Walters CP, Sullivan HR. Biosynthesis of thromboxane B₂: assay, isolation, and properties of the enzyme system in human platelets. *Prostaglandins* 1976. 12:951-970.
- Iuliano L, Colavita AR, Leo R, Pratico D, Violi F. Oxygen free radicals and platelet activation. *Free Radic Biol Med.* 1997. 22:999-1006.
- Jacob R.A., Vitamin C. In: *Modern Nutrition in Health and Disease*. Ninth Edition. Edited by Maurice Shils, James Olson, Moshe Shike, and A. Catharine Ross. Baltimore: Williams & Wilkins, 1999, 467-482.
- Janiszewski M, Lopes LR, Carmo AO, Pedro MA, Brandes RP, Santos CX, Laurindo FR. Regulation of NAD(P)H oxidase by associated protein disulfide isomerase in vascular smooth muscle cells. *J Biol Chem.* 2005 280:40813-40819.
- Ji LL, Stratman FW, Lardy HA. Antioxidant enzyme system in rat liver and skeletal muscle: influences of selenium deficiency, acute exercise and chronic training. *Arch Biochem Biophys.* 1988. 263:150-160.
- Kannan R, Stolz A, Ji Q, Prasad PD, Ganapathy V. Vitamin C transport in human lens epithelial cells: evidence for the presence of SVCT2. *Exp Eye Res.* 2001. 73:159-165.
- Kashiwagi H, Tomiyama Y, Tadokoro S, Honda S, Shiraga M, Mizutani H, Handa M, Kurata Y, Matsuzawa Y, Shattil SJ. A mutation in the extracellular cysteine-rich repeat region of the beta3 subunit activates integrins alphaIIb beta3 and alphaV beta3. *Blood* 1999. 93:2559-2568.

- Kaul S, Padgett RC, Heistad DD. Role of platelets and leukocytes in modulation of vascular tone. *Ann N Y Acad Sci.* 1994. 714:122–135.
- Kieffer N, Guichard J, Farcet JP, Vainchenker W, Breton-Gorius J. Biosynthesis of major platelet proteins in human blood platelets. *Eur J Biochem.* 1987. 164:189-95.
- Korcok J; Dixon SJ; Lo TC; Wilson JX. Differential effects of glucose on dehydroascorbic acid transport and intracellular ascorbate accumulation in astrocytes and skeletal myocytes. *Brain Res.* 2003. 993:201-207.
- Kritzik M, Savage B, Nugent DJ, Santoso S, Ruggeri ZM, Kunicki TJ. Nucleotide polymorphisms in the $\alpha 2$ gene define multiple alleles that are associated with differences in platelet $\alpha 2$ b1 density. *Blood* 1998. 92:2382-2388.
- Krotz F, Sohn HY, Gloe T, Zahler S, Riexinger T, Schiele TM, Becker BF, Theisen K, Klauss V, Pohl U. NAD(P)H oxidase-dependent platelet superoxide anion release increases platelet recruitment. *Blood.* 2002. 100: 917-924.
- Krötz F, Sohn HY, Pohl U. Reactive oxygen species: players in the platelet game. *Arterioscler Thromb Vasc Biol* 2004. 24: 1988-1996.
- Lahav J, Gofer-Dadosh N, Luboshitz J, Hess O, Shaklai M. Protein disulfide isomerase mediates integrin-dependent adhesion. *FEBS Lett.* 2000. 475:89-92.
- Lahav J, Wijnen E, Hess O, Griiffiths D, Makris M, Knight CG, Essex DW, Farndale RW. Enzymatically catalyzed disulfide exchange is required for platelet adhesion to collagen via integrin $\alpha 2$ beta1. *Blood* 2003. 102:2085-2092.
- Lawen A, Ly JD, Lane DJ, Zarschler K, Messina A, De Pinto V. Voltage-dependent anion-selective channel 1 (VDAC1) a mitochondrial protein, rediscovered as a novel enzyme in the plasma membrane. *Int J Biochem Cell Biol.* 2005.37:277-282.
- Leeuwenburgh C, Hansen PA, Holloszy JO, Heinecke JW. Hydroxyl radical generation during exercise increases mitochondrial protein oxidation and levels of urinary dityrosine. *Free Rad Biol Med.* 1999. 27:186-192.
- Leeuwenburgh C, Hardy MM, Hazen SL, Wagner P, Ohishi S, Steinbrecher UP, Heinecke JW. Reactive nitrogen intermediates promote low density lipoprotein oxidation in human atherosclerotic intima. *J Biol Chem.* 1997b. 272:1433-1436.
- Leeuwenburgh C, Rasmussen JE, Hsu FF, Mueller DM, Pennathur S, Heinecke JW. Mass spectrometric quantification of markers for protein oxidation by tyrosyl radical, copper, and hydroxyl radical in low density

- lipoprotein isolated from human atherosclerotic plaques. *J Biol Chem.* 1997a. 272:3520-3526.
- Leeuwenburgh C, Wagner P, Holloszy JO, Sohal RS, Heinecke JW. Caloric restriction attenuates dityrosine cross-linking of cardiac and skeletal muscle proteins in aging mice. *Arch Biochem Biophys.* 1997c. 346:74-80.
- Lenaz G, Paolucci U, Fato R, D'Aurelio M, Parenti Castelli G, Sgarbi G, Bigini G, Ragni L, Salardi S, Cacciari E. Enhanced activity of the plasma membrane oxidoreductase in circulating lymphocytes from insulin-dependent diabetes mellitus patients. *Biochem Biophys Res Commun.* 2002. 290:1589-92.
- Leoncini G, Maresca M, Colao C, Piana A, Armani U. Increased hydrogen peroxide formation in platelets of patients affected with essential thrombocythaemia (ET). *Blood Coagul Fibrinolysis.* 1992. 3:271-277.
- Li R, Bianchet MA, Talalay P, Amzel LM. The three-dimensional structure of NAD(P)H:quinone reductase, a flavoprotein involved in cancer chemoprotection and chemotherapy: mechanism of the two-electron reduction. *Proc Natl Acad Sci.* 1995. 92:8846-8850.
- Lindemann S, Tolley ND, Dixon DA, McIntyre TM, Prescott SM, Zimmerman GA, Weyrich AS. Activated platelets mediate inflammatory signaling by regulated interleukin 1beta synthesis. *J Cell Biol.* 2001. 154(3):485-490.
- Lindemann S, Tolley ND, Dixon DA, McIntyre TM, Prescott SM, Zimmerman GA, Weyrich AS. Activated platelets mediate inflammatory signaling by regulated interleukin 1beta synthesis. *J Cell Biol.* 2001. 154:485-490.
- Looney MR, Matthay MA. Neutrophil sandwiches injure the microcirculation. *Nat. Med.* 2009. 15:364-366.
- Loscalzo J. Oxidant stress: a key determinant of atherothrombosis. *Biochem Soc Trans.* 2003. 31:1059-1061.
- Luo SZ, Mo X, Afshar-Kharghan V, Srinivasan S, Lopez JA, Li R. Glycoprotein Ibalph forms disulfide bonds with 2 glycoproteins Ibbeta subunits in the resting platelets. *Blood* 2007. 109:603-609.
- MacDonald L, Thumser AE, Sharp P. Decreased expression of the vitamin C transporter SVCT1 by ascorbic acid in a human intestinal epithelial cell line. *Br. J. Nutr.* 2002. 87:97-100;
- MacFarlane RG. *Nature* 1964. 202:498-499.
- MacIntyre DE, Gordon JL. Proceedings: Factors influencing the binding of radiolabelled collagen to blood platelets. *Thromb Diath Haemorrh.* 1975. 34:332.

- Mann KG, Orfeo T, Butenas S, Undas A, Brummel-Ziedins K. Blood coagulation dynamics in haemostasis. *Hamostaseologie*. 2009. 29(1):7-16.
- May JM, Mendiratta S, Hill KE, Burk RF. Reduction of dehydroascorbate to ascorbate by the selenoenzyme thioredoxin reductase. *J Biol Chem*. 1997. 272:22607-22610.
- McArdle A, Pattwell D, Vasilaki A, Griffiths RD, Jackson MJ. Contractile activity-induced oxidative stress: cellular origin and adaptative responses. *Am J Physiol Cell Physiol*. 2001. 280:C621-C627.
- McArdle A, Van der Meulen J, Pattwell D, Van Remmen H, Huang TT, Richardson AG, Epstein CJ, Faulkner JA, Jackson MJ. The role of mitochondrial superoxide dismutase in contraction-induced generation of reactive oxygen species in skeletal muscle extracellular space. *Am J Physiol. (Cell Physiol)* 2004. 286:1152-1158.
- McBride TJ, Preston BD, Loeb LA. Mutagenic spectrum resulting from DNA damage by oxygen radicals *Biochemistry*. 1991. 30:207-213.
- McKie AT, Barrow D, Latunde-Dada GO, Rolfs A, Sager G, Mudaly E, Mudaly M, Richardson C, Barlow D, Bomford A, Peters TJ, Raja KB, Shirali S, Hediger MA, Farzaneh F, Simpson RJ. An iron-regulated ferric reductase associated with the absorption of dietary iron. *Science* 2001. 291: 1755.
- McRedmond JP, Park SD, Reilly DF, Coppinger JA, Maguire PB, Shields DC, Fitzgerald DJ. Integration of proteomics and genomics in platelets: a profile of platelet proteins and platelet-specific genes. *Mol Cell Proteomics*. 2004.3(2):133-144.
- Mendiratta S, Qu ZC, May JM. Erythrocyte ascorbate recycling: antioxidant effects in blood. *Free Radic Biol Med*. 1998. 24:789-797.
- Michiels C. Endothelial cell functions. *J Cell Physiol*. 2003. 196:430–443.
- Morré DM, Guo F, Morré D J. An aging-related cell surface NADH oxidase (arNOX) generates superoxide and is inhibited by coenzyme Q . *Molecular and Cellular Biochemistry*. 2003. 254:101–109.
- Morré DJ, Morré DM. Cell surface NADH oxidases (ECTO-NOX proteins) with roles in cancer, cellular time-keeping, growth, aging and neurodegenerative diseases. *Free Radic Res*. 2003. 37:795-808.
- Müller I, Besta F, Schulz C, Li Z, Massberg S, Gawaz M. Effects of statins on platelet inhibition by a high loading dose of clopidogrel. *Circulation*. 2003. 108:2195-2197.

- Murthy AS, Keutmann HT, Eipper BA. Further characterization of peptidylglycine alpha-amidating monooxygenase from bovine neurointermediate pituitary. *Mol Endocrinol*. 1987. 1:290-299.
- Nelson PJ, Pruitt RE, Henderson LL, Jenness R, Henderson LM. Effect of ascorbic acid deficiency on the in vivo synthesis of carnitine. *Biochim Biophys Acta*. 1981. 672:123-127.
- Newman PJ, Gorski J, White GC 2nd, Gidwitz S, Cretney CJ, Aster RH. Enzymatic amplification of platelet-specific messenger RNA using the polymerase chain reaction. *J Clin Invest*. 1988. 82:739-743.
- Nishikimi M, Fukuyama R, Minoshima S, Shimizu N, Yagi K. Cloning and chromosomal mapping of the human nonfunctional gene for L-gulonolactone oxidase, the enzyme for L-ascorbic acid biosynthesis missing in man. *J Biol Chem*. 1994. 269:13685-13688.
- Olas B, Wachowicz B. Resveratrol and vitamin C as antioxidants in blood platelets. *Thromb Res*. 2002. 106:143-148.
- O'Neill C, Stebbens CL, Bonigut S, Halliwell B, Longhurst JC. Production of hydroxyl radicals in contracting skeletal muscle of cats. *J Appl Physiol*. 1996. 81:1197-1206.
- Pacifici RE, Davies KJA. Protein degradation as an index of oxidative stress. *Methods Enzymol*. 1990. 186:485-502.
- Padh H. Cellular functions of ascorbic acid. *Biochem Cell Biol*. 1990. 68:1166-1173.
- Padt H. Vitamin C: newer insights into its biochemical functions. *Nutr Rev*. 1991. 49:65-70.
- physiological role. *J Physiol Biochem* 2000. 56:355-365.
- Pignatelli P, Sanguigni V, Lenti L, Ferro D, Finocchi A, Rossi P, Violi F. gp91phox-dependent expression of platelet CD40 ligand. *Circulation*. 2004. 110: 1326-1329.
- Pignatelli P, Sanguigni V, Paola SG, Lo Coco E; Lenti L, Violi F. Vitamin C inhibits platelet expression of CD40 ligand. *Free Radic Biol Med*. 2005. 38:1662-1666.
- Prchal JT, Gregg XT *Hematology Am Soc Hematol Educ Progr*. 2005. 1, 19.
- Rajan DP, Huang W, Dutta B, Devoe LD, Leibach FH, Ganapathy V, Prasad PD. Human placental sodium-dependent vitamin C transporter (SVCT2): molecular cloning and transport function. *Biochem Biophys Res Commun*. 1999. 262:762-768.
- Rajguru SU, Yeargans GS, Seidler NW. Exercise causes oxidative damage to rat skeletal muscle microsomes while increasing cellular sulfhydryls. *Life Sci*. 1993. 54:149-157.

- Rendu F, Brohard-Bohn B. Platelets. 2001. 12:261-73.
- Reznick AZ, Packer L, Sen CK, Holloszy JO, Jackson MJ. (Eds.) Oxidative stress in skeletal muscle. Basel: BirkHauser Verlag: 1998.
- Rivera J, Lozano ML, Corral J, González-Conejero R, Martínez C, Vicente V. Platelet GP Ib/IX/V complex:
- Rivera J, Lozano ML, Navarro-Núñez L, Vicente V. Platelet receptors and signaling in the dynamics of thrombus formation. *Haematologica*. 2009. 94:700-711.
- Ruf JC. Wine and polyphenols related to platelet aggregation and atherothrombosis. *Drugs Exp Clin Res*. 1999. 25:125-131.
- Savini I, Catani MV, Duranti G, Ceci R, Sabatini S, Avigliano L. Vitamin C homeostasis in skeletal muscle cells. *Free Radic Biol Med*. 2005. 38:898-907.
- Savini I, Catani MV, Rossi A, Duranti G, Melino G, Avigliano L. Characterization of keratinocyte differentiation induced by ascorbic acid: protein kinase C involvement and vitamin C homeostasis. *J Invest Dermatol*. 2002. 118:372-379.
- Savini I, D'Angelo I, Ranalli M, Melino G, Avigliano L. Ascorbic acid maintenance in HaCaT cells prevents radical formation and apoptosis by UV-B. *Free Radic Biol Med*. 1999. 26:1172-1180.
- Scarlett DJ, Herst P, Tan A, Prata C, Berridge M. Mitochondrial gene-knockout (rho0) cells: a versatile model for exploring the secrets of transplasma membrane electron transport. *Biofactors*. 2004. 20:199-206.
- Sen C.K. Antioxidants in exercise nutrition. *Sports Med*. 2001. 31:891-908.
- Seno T, Inoue N, Gao D, Okuda M, Sumi Y, Matsui K, Yamada S, Hirata KI, Kawashima S, Tawa R, Imajoh-Ohmi S, Sakurai H, Yokoyama M. Involvement of NADH/NADPH Oxidase in Human Platelet ROS Production. *Thromb Res*. 2001. 103: 399-409.
- Shigenaga MK, Park JW, Kundy KC, Gimeno CJ, Ames BN. In vivo oxidative DNA damage: measurement of 8-hydroxy-2'-deoxyguanosine in DNA and urine by high-performance liquid chromatography with electrochemical detection. *Methods Enzymol*. 1990. 186:521-530.
- Silveira LR, Pereira-Da-Silva L, Juel C, Hellsten Y. Formation of hydrogen peroxide and nitric oxide in rat skeletal muscle cells during contractions. *Free Radic Biol. Med*. 2003. 35:455-464.
- Sotiriou S, Gispert S, Cheng J, Wang Y, Chen A, Hoogstraten-Miller S, Miller GF, Kwon O, Levine M, Guttentag SH, Nussbaum RL. Ascorbic-acid transporter Slc23a1 is essential for vitamin C transport into the brain and for perinatal survival. *Nat Med*. 2002. 8:514-517.

- Srivastava KC. Ascorbic acid enhances the formation of prostaglandin E1 in washed human platelets and prostacyclin in rat aortic rings. *Prostaglandins Leukot Med.* 1985. 18:227-233.
- Takajo Y, Ikeda H, Haramaki N, Murohara T, Imaizumi T. Augmented oxidative stress of platelets in chronic smokers. Mechanisms of impaired platelet-derived nitric oxide bioactivity and augmented platelet aggregability. *Am Coll Cardiol.* 2001. 38:1320-1327.
- Takehita M, Matsuki T, Tanishima K, Yubisui T, Yoneyama Y, Kurata K, Hara N, Igarashi TJ. Alteration of NADH-diaphorase and cytochrome b5 reductase activities of erythrocytes, platelets, and leucocytes in hereditary methaemoglobinaemia with and without mental retardation. *Med Genet.* 1982. 19:204-209.
- Thon JN, Devine DV. Translation of glycoprotein IIIa in stored blood platelets. *Transfusion* 2007. 47:2260-2270.
- Thromb Haemost.* 2000. 83:485– 490.
- Touyz RM. Reactive oxygen species in vascular biology: role in arterial hypertension. *Expert Rev Cardiovasc Ther.* 2003. 1:91-106.
- Touyz RM. Reactive oxygen species, vascular oxidative stress, and redox signaling in hypertension: what is the clinical significance? *Hypertension.* 2004. 44:248-252.
- Tsukaguchi H, Tokui T, Mackenzie B, Berger UV, Chen XZ, Wang Y, Brubaker RF, Hediger MA. A family of mammalian Na⁺-dependent L-ascorbic acid transporters. *Nature* 1999. 399:70-75.
- Ushio-Fukai M. Localizing NADPH oxidase-derived ROS. *Sci STKE.* 2006. 349. Review.
- VanDuijn MM, Van der Zee J, Van den Broek PJ. The ascorbate-driven reduction of extracellular ascorbate free radical by the erythrocyte is an electrogenic process. *FEBS Lett.* 2001. 491:67-70.
- Venditti P, Di Meo S. Antioxidants, tissue damage, and endurance in trained and untrained young male rats. *Arch Biochem Biophys.* 1996. 331:63-68.
- Venditti P, Di Meo S. Effect of training on antioxidant capacity, tissue damage, and endurance of adult male rats. *Int J Sports Med.* 1977. 18:497-502.
- Villalba JM, Navarro F, Gómez-Díaz C, Arroyo A, Bello RI, Navas P. Role of cytochrome b5 reductase on the antioxidant function of coenzyme Q in the plasma membrane. *Mol Aspects Med.* 1997;18 Suppl:S7-13.
- Villalba JM, Navas P. Plasma membrane redox system in the control of stress-induced apoptosis. *Antioxid Redox Signal.* 2000. 2: 213-230.

- Wachowicz B, Olas B, Zbikowska HM, Buczynski A. Generation of reactive oxygen species in blood platelets. *Platelets*. 2002. 13:175–182.
- Wang S, Pogue R, Morr  DM, Morr  DJ. NADH oxidase activity (NOX) and enlargement of HeLa cells oscillate with two different temperature-compensated period lengths of 22 and 24 minutes corresponding to different NOX forms. *Biochim Biophys Acta*. 2001. 1539:192-204.
- Washburn MP, Wells WW. Identification of the dehydroascorbic acid reductase and thioltransferase (Glutaredoxin) activities of bovine erythrocyte glutathione peroxidase. *Biochem. Biophys. Res. Commun*. 1999. 257:567-571.
- Watson SP, Auger JM, McCarty OJ, Pearce AC. GPVI and integrin $\alpha\text{IIb}\beta\text{3}$ signaling in platelets. *J Thromb Haemost*. 2005. 3:1752-1762.
- Wells WW, Xu DP, Yang Y, Rocque PA. Mammalian thioltransferase (glutaredoxin) and protein disulfide isomerase have dehydroascorbate reductase activity. *J Biol Chem*. 1990. 265:15361-15364.
- White JG. Platelets are coverocytes, not phagocytes: uptake of bacteria involves channels of the open canalicular system. *Platelets*. 2005. 16: 121-131.
- Wilson JX, Jaworski EM, Kulaga A, Dixon SJ. Substrate regulation of ascorbate transport activity in astrocytes. *Neurochem Res*. 1990. 15:1037-1043.
- Woulfe D, Yang J, Prevost N, O'Brien P, Fortna R, Tognolini M, et al. Signaling receptors on platelets and megakaryocytes. *Methods Mol Biol*. 2004. 273:3-31.
- Wu K, Knox R, Sun X, Joseph P, Jaiswal A, Zhang D, Deng P, Chen S. Catalytic properties of NAD(P)H:quinone oxidoreductase-2 (NQO2), a dihydronicotinamide riboside dependent oxidoreductase. *Arch Biochem Biophys*. 1997. 347:221-228.
- Xu DP, Wells W. α -Lipoic acid dependent regeneration of ascorbic acid from dehydroascorbic acid in rat liver mitochondria. *J. Bioenerg. Biomembr*. 1996. 28:77-85.
- Yan B, Smith JW. A redox site involved in integrin activation. *J Biol Chem*. 2000. 275:39964-39972.
- Zucker MB, Wu YS, Mauss EA. Decrease in platelet ascorbic acid produced by aggregation. *Thromb. Haemost*. 1990. 64:179.

9. CURRICULUM VITAE

Name: Rosaria Arnone

Birth: 29-05-1975, Caltanissetta

Address: Via Campi D'Annibale 211, Rocca di Papa, 00040 Rome

Telephone: 349-2636923

e-mail: rosarn@neomedia.it

EDUCATION

- 2003** Degree in Biology, Bio-molecular field, final score 110/110 cum laude. Experimental thesis at the University of Palermo, on "Differentiative effects of 3-aminobenzamide in human osteosarcoma MG63cells".
- 2003** Professional ability in Biology.
- 2008** Registration to National Council of Biologists.
- 2005-09** PhD student in Biochemistry and Molecular Biology, at the University of Rome Tor Vergata.

FORMATION

- 2001-02** Teaching activity at the Course "Laboratory on Experimental Biology", Biology Degree, University of Palermo.
- 2002-03** Tutor at the University of Palermo.
- 2003** Chemical-clinical training at the Albano Laziale Laboratory, Rome.
- 2004-05** Employment at the Water Research Institute (IRSA), CNR, Rome. Teaching activity at the Course "General and Inorganic Chemistry", additional degree classes, Rome.
- 2005** Teaching activity at the Course "Chemical and Microbiological Analyses of Water", G. Falcone Professional Institute for Laboratory Technicians, Rome.
Teaching activity at the Course "Biochemistry", Motor Sciences Degree, University of Rome Tor Vergata.
- 2006** Teaching activity at the Course "Biochemistry", Vocational Training for Sport Instructors, Motor Sciences Degree, University of Rome Tor Vergata.
- 2007** Teaching activity at the Course "Applied Dietary Sciences and Techniques" Degree on Sciences and Techniques of Preventive and

Adaptive Motor Activities and Degree on Dietician, University of Rome Tor Vergata.

TECHNICAL EXPERTISE

Cell cultures, blood cells isolation

Protein extraction and Western blotting

Nucleic acid extraction and reverse transcriptase-polymerase chain reaction (RT-PCR)

High pressure liquid chromatography (HPLC)

Spectrofluorimetric determination of kinetic parameters

Determination of vitamin C uptake and recycling systems

Qualitative and quantitative determination of water-contaminating bacteria by FISH and DAPI techniques.

Chemical, physical and bacteriological analyses of urines. Antibigrams (Kyrbi Bauer method), Gram staining, optical microscopy of biological samples.

Analysis of coagulation factors (TP, PTT, anti-thrombin III, fibrinogen)

INFORMATIC EXPERTISE

Microsoft Office, Adobe Photoshop, Internet Explorer

LANGUAGE ABILITY

English (good)

10. LIST OF PUBLICATIONS

1. Savini I., Catani M.V., **Arnone R.**, Rossi A., Frega G., Del Principe D., Avigliano L.
Translational control of the ascorbic acid transporter SVCT2 in human platelets.
Free Radic Biol Med. 2007;42:608-616.
2. Savini I., **Arnone R.**, Catani M.V., Avigliano L.
Origanum vulgare induces apoptosis in human colon cancer Caco2 cells.
Nutr Cancer. 2009;61:381-389.
3. Savini I., Carbonelli M.G., **Arnone R.**, Catani M.V.
Redox balance in obesity.
Biochemical Aspects of Human Nutrition. Research Signpost, 2009, in press.
4. Savini I., **Arnone R.**, Rossi A., Catani M.V., Del Principe D., Avigliano L.
Ecto-NOX1 and Ecto-NOX2 are expressed in human platelets.
Molecular Membrane Biology. 2009 submitted.