Trans-Plasma Membrane Electron Transport in Human Blood Platelets

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Abstract: The plasma membrane redox (PMR) system is important for cell metabolism and survival; it is also crucial for blood coagulation and thrombosis. This review will give an update on the PMR system, with a particular regard to platelets, and on the role of antioxidant vitamins belonging to this system.

Key Words: Plasma membrane redox system, platelets, antioxidants, free radicals, vitamin E, vitamin C, nicotinamide, ubiquinone.

PLASMA MEMBRANE REDOX SYSTEM

Enzymes involved in electron transport are usually associated to the respiratory chain, localized in the mitochondrial inner membrane and responsible for aerobic ATP production. However, the existence of a plasma membrane electron transport (PMET) or plasma membrane redox (PMR) system has been known since 1970, although its physiological relevance has only recently been investigated.

The primary biological function of the PMET system, which is ubiquitous in every living cell (including bacteria, yeast, plants and animals), is the maintenance of 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 PMET 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 [1], and in lymphocytes derived from diabetes mellitus patients, lacking functional mitochondria [2]. So, the PMR system guarantees glycolytic metabolism, thus ensuring survival of cells deficient in mitochondrial electron transport (e.g. aged or tumour cells).

Components of the PMR System

The basic components of the PMR system include electron donors and acceptors, antioxidants 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. NAD^+ is converted to NADH during the catabolism of carbohydrates, fats and proteins; on the contrary, the NADPH/$NADP^+$ system functions in anabolic reactions, including synthesis of fatty acids and cholesterol, as well as in hydroxylation and detoxification reactions. Oxidation/reduction cycle, fluctuating in response to metabolic changes, determines the piridine derivative ratio, which plays a crucial role in regulating the intracellular redox state [3].

The PMR system can also use intracellular vitamin C (named ascorbate in the reduced form) to reduce extracellular oxidants, with a mechanism that is dependent on the NADH content of the cell [4]. In the physiological pH range, ascorbate tends to donate a single hydrogen atom to different acceptors, being oxidized to ascorbyl free radical [5]. Other recently discovered, intracellular substrates are some flavonoids (quercetin, myricetin, fisetin); their ability to function as electron donors is linked to the presence of cathecol structure of the B ring (responsible for reducing activity) and the double bond and 4-oxo function of the C ring (responsible for cellular uptake) [6].

Although the membrane-impermeant ferricyanide has been routinely used to probe the activity of different enzymes, nonetheless several oxidants are physiologically reduced by the PMR system. Ubiquinone (or coenzyme Q; CoQ) is the direct acceptor of intracellular electrons in the plasma membrane. It can be transformed to the ubisemiquinone radical (CoQ^-) or to hydroquinone ($CoQH_2$), depending on the membrane enzyme involved, such as the NADH: cytochrome b5 reductase or the NAD(P)H: ubiquinone oxidoreductase [7,8]. Then, CoQ is a key membrane redox player, which can directly or indirectly (through the participation of intermediate carriers, including b cytochromes, flavin and vitamin E) transfer electron(s) to final acceptors. Membrane proteins also contribute to this "plasma membrane electron transport chain", since they may be responsible for the disulfide-thiol interchange activity ascribed to some enzymes belonging to the PMR system [9].

Oxygen is the final electron acceptor for membrane NAD(P)H oxidases [10], thus generating superoxide anions ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2). These reactive oxygen species (ROS) modulate specific bio-molecules involved in

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cellular functions and signal transduction. Other extracellular acceptors are the ascorbyl free radical, that is reduced to ascorbate, and the ferric iron, that is reduced to ferrous ion for absorption [11].

b. Antioxidants

Thanks to their ability to exist as stable radicals, some of the electron carriers described above (CoQ, vitamin E and vitamin C) also exert antioxidant effects.

The major lipophilic antioxidants are ubiquinone and alpha tocopherol, which protect cells against lipid peroxidation. Ubiquinone directly scavenges ROS which can damage unsaturated lipid chains; demonstrations of this phenomenon come from the findings that ubiquinone-deficient yeast mutants show high levels of lipid radicals [12]. In the quinol form, it is also necessary for regenerating tocopherol from tocopheryl radical and ascorbate outside the cell from ascorbyl radical [13]. Alpha-tocopherol (vitamin E) is also a lipophilic chain-breaking antioxidant; its radical can be reduced by ubiquinol, ascorbate and glutathione [8]. The antioxidant activity of alpha-tocopherol plays a key role in erythrocyte membranes, which are highly susceptible to peroxidation because of the presence of many polyunsaturated fatty acids, the continuous exposure to high concentrations of oxygen and the presence of a transition metal catalyst [14].

Although being water-soluble, vitamin C exerts some important antioxidant functions on the plasma membrane, since it is capable of neutralizing an array of ROS and regenerating alpha-tocopherol from its radical [15].

c. Enzymes

The PMR enzymes include the: (i) NADH:ferricyanide reductase, (ii) NAD(P)H:ubiquinone oxidoreductase, (iii) cytochrome b5 reductase, (iv) superoxide-generating NADPH oxidases, (v) NADH oxidases and (vi) NADH:ascorbate free radical oxidoreductase, see Fig. (**1**).

The NADH:ferricyanide reductase [known as Voltage-Dependent Anion-selective Channel (VDAC) or porin] was originally discovered in the outer mitochondrial membrane, but it is also present in the plasma membrane [16]. In mammals, three highly conserved genes have been shown to encode distinct isoforms, named VDAC1, VDAC2 and VDAC3, which mainly differ in their channel-forming activity. Structural modelling of the best-characterized member of this family, VDAC1, predicted in analogy to bacterial porins, suggested that it consists of several (13 or 16) β-barrel, membrane-spanning strands [17]. Furthermore, VDAC1 can exist as two different proteins, generated by the use of alternative first exons; one isoform, expressing a leader peptide in its N-terminus, is directed to the plasma membrane, while the second one, lacking the pre-sequence, is targeted to mitochondria. Thus, the same polypeptide may be localized in two membranes, where it exerts different functions; these could be explained assuming that (i) the protein changes its conformation when it is targeted to the plasma or mitochondrial membrane, or (ii) some not yet identified effector proteins control VDAC activity.

In the mitochondrion, VDAC1 forms pores freely permeable to low molecular-weight molecules (including ADP/ATP, succinate and citrate), thus controlling metabolite trafficking between the cytosol and mitochondria. It has also been suggested a role for this pore-forming protein in cytochrome c release during apoptosis, since VDAC can interact with the anti-apoptotic protein Bcl-2 [18]. Conversely, the protein exerts a NADH:ferricyanide reductase activity in the plasma membrane: indeed, the enzyme is capable of reducing the

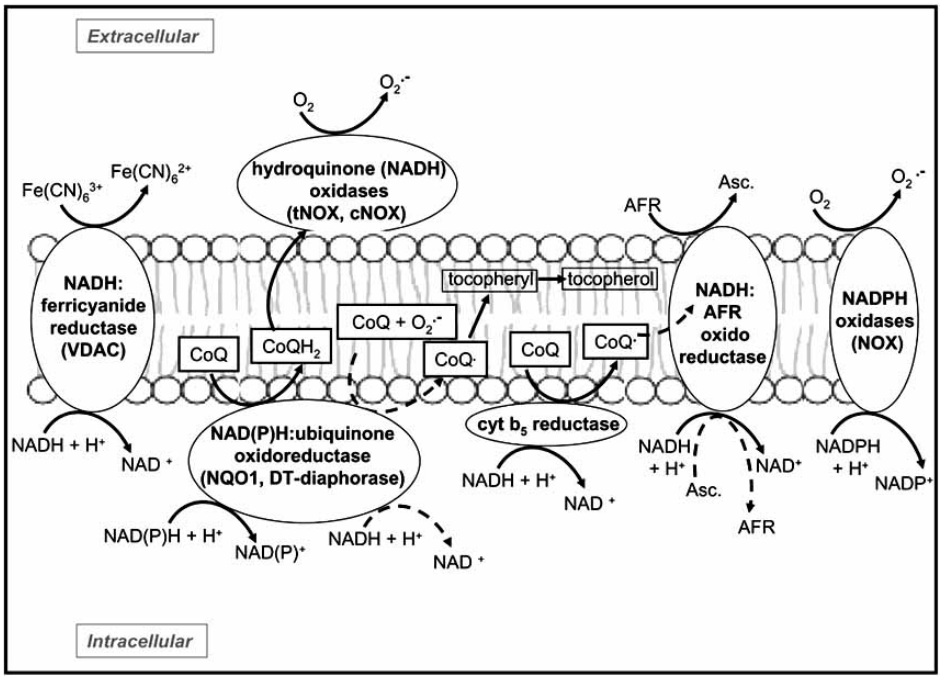

Fig. (1). Key enzymes of the PMR system. Membrane localisation and catalysed reactions are shown in the scheme. Alternative reactions are depicted with hatched lines. CoQ regenerates transplasma membrane tocopherol and extracellular ascorbate, thus protecting cells from lipid peroxidation and external oxidative insults. Asc: ascorbate. AFR: ascorbyl free radical.

cell-impermeant ferricyanide by using NADH as the intracellular electron donor [19]. The reductase activity is strictly dependent on NADH, as it could not be replaced by NADPH; in addition, it can not be inhibited by anion channel inhibitors, while it is sensitive to thiol chelators, suggesting a critical role for two cysteine residues, present in the molecule, in electron transfer. The natural substrate(s) of VDAC1 remains to be established, since ferricyanide is not a physiological molecule; however, recent data suggest that CoQ may act as an acceptor for the enzyme [19]. By this way, VDAC1 senses the cellular redox state by regulating the NAD^+/NADH ratio, thus enabling cell growth and apoptosis [18].

The NAD(P)H:ubiquinone oxidoreductase (NQO1, also named DT-diaphorase) is a largely cytosolic, homodimeric FAD-containing protein of 33 kDa, which is translocated into the plasma membrane under oxidative stress. It catalyzes obligatory NADH or NADPH-dependent two-electron reductions of quinones (in particular, it converts oxidised CoQ in the hydroquinone reduced form), but it also reduces quinoneimines, nitro and azo compounds [20]. These reactions prevent the cytotoxic and carcinogenic effects of xenobiotics, that can produce free-radicals leading to DNA and cell damage. An isoenzyme, lacking 43 residues at the C-terminus (called NQO2) and encoded by a different gene, has been found; unlike NQO1, it preferentially uses dihydronicotinamide riboside [instead of NAD(P)H], as a source of reducing equivalents [21], and it is not involved in PMET. Both genes are up-regulated in response to oxidative stress and are overexpressed in some tumours. The crystal structure of NQO1 reveals that the catalytic site is at the dimer interface and that the enzyme works through a ping-pong mechanism. Indeed, the FAD prosthetic group is always bound to each subunit, while NADH or NADPH are bound and released during catalytic cycling, in order to allow substrate binding: thus, the reaction consists of hydride transfer from NAD(P)H to FAD and, then, from $FADH_2$ to the quinone [22]. Conversely, NQO1 can also catalyzes a NADH-driven, superoxide-dependent, one-electron reduction of CoQ, thus generating the semiquinone radical, which in turn leads to regeneration of alpha-tocopherol [23].

The cytochrome b5 reductase is a FAD-containing monomer of 32 kDa, existing in two isoforms produced by alternative promoter usage. The first one is a membrane-bound enzyme catalyzing the one-electron transfer from NADH to CoQ, thus generating the radical $CoQ^{\cdot-}$ in the plasma membrane [8]. The second isoform produces the soluble form found in erythrocytes, which transfers electrons derived from NAD(P)H to the heme protein. In red blood cells, hemoglobin can be converted in methemoglobin, if the iron of the heme group is oxidized from the ferrous (Fe^{2+}) to the ferric (Fe^{3+}) state, thus impairing oxygen delivery. Cytochrome b5 reductase is the main endogenous enzymatic mechanism responsible for hemoglobin reduction; indeed, deficiency of this enzyme is the most common cause of congenital methemoglobinemia, characterized by increased levels of methemoglobin above its steady state [24].

The superoxide-generating NADPH oxidases represent a protein family, including at least seven proteins (NOX1 to NOX5, Duox1 and Duox2) [10], whose best characterized member is NOX2. This is an inducible, membrane-bound cytochrome b_{558}, composed of two subunits (named $p22^{phox}$ and $gp91^{phox}$) and mainly expressed in phagocytes of the immune system, as well as in platelets. The enzyme catalyzes the transfer of one electron from NADPH to molecular oxygen, thus generating $O_2^{\cdot-}$ anions. The Rac1 and Rac2 GTPases and three cytosolic factors ($p47^{phox}$, $p67^{phox}$, $p40^{phox}$) are needed for the catalytic activity; in particular, membrane translocation of these cytosolic proteins promote the production of superoxide. ROS generation by NOX2 represents the first mechanism of defence against pathogens: indeed, ROS act as microbiocidal agents in the phenomenon known as "respiratory burst" [25].

Other NOX family members are all homologs of the $gp91^{phox}$ subunit of NOX2. Like NOX2, they generate tightly controlled superoxide anions, but, showing a tissue-specific distribution, they mediate distinct and specific functions. NOX1 is primarily expressed in the colon, but it can be detected in vascular smooth muscle cells, where it has a role in angiotensin II- and growth factor-induced cell hypertrophy or proliferation [26]. Several findings also suggest that NOX1 could participate in the host defence of different cell types [27]. NOX3 is expressed in several foetal tissues, including kidney, liver, lung, and spleen [28]; it seems involved in tumour cell proliferation, but more studies are needed to provide better insight. Because of the peculiar expression in the inner ear, NOX3 has been proposed to play an essential role for normal vestibular function; indeed, impairment of NOX3 leads to balance defects due to absence of otoconia [29]. NOX4 is expressed at high levels in the kidney, where it appears to play a role in oxygen sensing and regulation of erythropoietin synthesis [30]. NOX5 was originally found in testis, but it is also present in T- and B-lymphocytes [31]. This oxidase, which is activated by intracellular calcium-dependent conformational changes, drives sperm capacitation, a process strictly dependent on ROS-mediated signal transduction; NOX5-induced ROS production is also important for acrosome reaction and sperm-oocyte fusion during fertilization. Finally, dual oxidases (Duox1 and Duox2) are peculiar, because they possess peroxidase and superoxide-generating NADPH oxidase activities. Furthermore, they are unusual, in that they produce H_2O_2 rather than $O_2^{\cdot-}$. They are expressed in the membrane of thyroid glands and serve in iodide oxidation during thyroxine synthesis [32].

The hydroquinone (NADH) oxidases represent a protein family, whose members exhibit a time-keeping $CoQH_2$ (NADH) oxidase activity and a protein disulfide-thiol interchange activity [33]. At least two members of this family have been described: the tumour-associated NADH oxidase (also known as tNOX) [34], which is present on the surface of invasive cancer cells and in the sera of cancer patients, and the constitutive NADH oxidase (cNOX), expressed in normal cells [35].

The only available informations about functional motifs and/or mechanism of action come from site-directed mutagenesis studies performed within the tNOX C-terminus: this region contains quinone, copper and adenine nucleotide putative binding domains, as well as two cysteine residues critical for catalytic activity [34]. In addition, tNOX is specifically inhibited by natural and synthetic quinone analogs

with anticancer activity (including capsaicin and adriamycin), whereas cNOX is not drug-responsive. The exact biological role of these two oxidases is not well understood, although they have been implicated in cell growth, aging and in neurodegenerative diseases [33].

The NADH:ascorbate free radical oxidoreductase is an enzymatic activity found in the plasma membrane [36], but not yet unequivocally ascribed to a specific protein; it uses NADH as an electron source for reduction of the ascorbyl radical. Two oxidoreductase activities have been described. The first one is an inner membrane activity, that binds NADH (donor) and ascorbyl radical (acceptor), both present in the cytosolyc side of the plasma membrane. The second one is a transmembrane activity, thus using intracellular NADH to reduce extracellular ascorbyl radical; CoQ can be an electron linker between electron donor and acceptor, since its removal from plasma membranes decreases the extent of the extracellular radical reduction. This activity is particularly important for enabling erythrocytes respond to changes in both intra- and extracellular redox environments [37].

Biological Functions of the PMR System

All the PMET pathways described so far have a general role in the control of redox homeostasis; thus, the intracellular redox state and, accordingly, the PMR system efficaciously modulate cell growth and survival.

Besides its above-mentioned role in the control of NAD^+/NADH ratio and bioenergetics, this system is also correlated with modulation of internal pH and redox homeostasis. Indeed, the activity of the PMR system is usually associated with a Na^+/H^+ antiport system, which pumps protons outside cells, thus buffering changes in intracellular pH and leading to acidification of the medium [13, 38]. Proton influx and efflux across the plasma membrane, not only contribute to maintain a constant intracellular pH, but also provide a mechanism modulating the polarization of biological membranes and regulating growth control of normal and transformed cells [39].

In addition, the PMR system exerts a modulating action on oxidative stress-induced apoptosis; indeed, CoQ sustains growth of cells in serum-limiting conditions characterized by mild oxidative stress and lipid peroxidation, whereas inhibitors of the PMR system induce cell growth arrest and apoptosis. The PMR system is constitutively activated in tumour cells and, thanks to its unique plasma membrane localisation, can be a novel target for the growth-inhibitory effects of several anti-cancer drugs (vanilloids, antracyclines, cis-platinum, bleomycin and phenoxodiol) [8, 40]; blocking its activity will compromise the cellular NADH/NAD^+ ratio, thus compromising the viability of transformed cells [41]. PMR, for example, is a primary site of action of phenoxodiol, since the drug is able to bind and inactivate the plasma membrane oxidoreductase tNOX, thus inducing G1 arrest and apoptosis [42, 43]. Nonetheless, phenoxodiol does not act specifically on tNOX or cancer cells, as it can inhibit proliferation and trigger cell death also in primary cells, including endothelial cells and activated primary T lymphocytes or peripheral blood mononuclear cells [44, 45]. These findings suggest a potential harmful role for some drugs, which alter the PMR system: pharmacological concentrations of phenoxodiol can induce immunosuppressive effects, as already reported in Phase I clinical trials where 19 out of 21 patients with solid cancer experienced lymphocytopenia at doses up to 30 mg/kg [46].

Parallel to the ubiquitous functions described above, some specific biological roles of the PMR system can be put in evidence, depending on the cell type considered. For shortness, only three significative examples are reported below.

a. Iron Uptake

Dietary iron uptake from the intestinal lumen is mediated by a divalent cation carrier (DCT1, Nramp2 or DMT1) [47] that transports ferrous iron. Thus, duodenal mucosa must express on its surface a ferrireductase driving the ferric to ferrous iron reduction; the enzyme, identified by McKie *et al*. [11], is a 31 kDa plasma membrane protein sharing 50% sequence similarity to the cytochrome b_{561} family of reductases. The mechanism of action is not completely known, but it seems to use ascorbate or glutathione as electron donors [11].

b. Cellular Defence

Because of its location, the PMR system represents a mechanism of cellular defence. It exerts a dual role, as it can be both pro-oxidant and anti-oxidant. Indeed, some PMR enzymes, including NADH:ascorbyl free radical and NADH: ubiquinone reductases, maintain adequate antioxidant levels into the plasma membrane to counteract oxidative stress. On the other hand, plasma membrane NADPH oxidases can produce localized ROS (such as superoxide, hydrogen peroxide and singlet oxygen), involved in death of invading microorganisms.

c. Fertilization

Another biological function concerns the involvement of the PMR system in fertilization. We have previously mentioned the presence of sperm-specific redox enzymes that control male fertility. Also oocytes possess a NADPH-specific oxidase needed for generating protein coats, which provide a structural block to polyspermy, after fertilization; this enzyme, homolog to the dual oxidase Duox1, accumulates at the cell surface of each zygote, generating hydrogen peroxide that is necessary for the physical block to polyspermy [48].

Finally, several components of the PMR system are modulated during the aging process, which is characterized by decreased levels of alpha-tocopherol and increased levels of lipid peroxidation. Lowered mitochondrial respiratory activity due to the aging process can be compensated by up-regulating the plasma membrane NADH oxidizing systems, to maintain NAD^+ levels and glycolytic activity [49]. Thereby, mechanisms enhancing the PMR system could delay aging processes; this is the case of calorie restriction, which up-regulates the plasma membrane redox activity, that otherwise declines with age [50].

From these findings, it appears clear that the PMR system plays a crucial role in body homeostasis. In particular,

PMR-derived ROS are necessary for normal cellular functions, metabolism and survival, but the upside-down of the medal is that compromising this system and overproducing ROS can contribute to pathological diseases, including neurotoxicity, endothelial dysfunction and cardiovascular diseases [51]. What determines the cellular biological response initiated by ROS? Different levels of ROS induce distinct responses within a cell and, moreover, there is considerable variation between cells in the concentration required to initiate a particular biological effect. For example, superoxide anion and hydrogen peroxide employed for the killing of bacteria are produced by macrophage plasma membrane NADPH oxidase in the mM range, while non-toxic nM concentrations are produced by the not-phagocytic NADPH oxidases. Furthermore, the ability of these Nox systems to be modulated by extracellular effectors (growth factors and hormones) may allow a tailored ROS production in order to respond to the needs of the various tissues [52]. All aerobic forms of life use intracellular H_2O_2 concentrations ranging from 0.001 to 0.5-0.7 μM for signaling purposes. As long as the intracellular H_2O_2 level is maintained below 0.7 μM cells will undergo proliferation, above 0.7 μM oxidative stress and apoptotic death will occur [53]. Recently, it has been also suggested that the local antioxidant capacity contributes to the susceptibility of a cellular target to oxidative damage or signaling [54].

PLASMA MEMBRANE REDOX SYSTEM IN PLATELETS

It has been shown, in the seventies, that platelets possess an enzymatic armamentarium linked to the plasma membrane. Early papers [55, 56] suggested the presence of superoxide in the medium of platelet suspensions, based on the ability of resting and stimulated cells to reduce the nitroblue tetrazolium salt (NBT); indeed, the product of NBT reduction (formazan), being insoluble and unable to cross membranes, can give indications about $O_2^{\cdot-}$ localization. Later on, Marcus *et al*. [57] demonstrated the presence of two reducing activities in platelets. The first one led to production of extracellular superoxide, with a mechanism independent from the aggregation process; the second one was a superoxide-independent reducing activity, which increased with aggregation and seemed to be membrane-bound. Then, the burst in oxygen consumption, observed in stimulated platelets, was shown to be dependent on NADH or NADPH [58]. A plasma membrane activity was hypothesized, since pyridine nucleotides are membrane-impermeant and, moreover, the O_2 consumption was cyanide insensitive. In addition, the presence of multiple plasma membrane-associated activities was postulated, based on different results obtained with NADH or NADPH [58]. The platelet membrane redox activity, as well as its involvement in the aggregation response, is now widely recognized, thanks to the use of last generation tetrazolium salts, which are membrane-impermeant [59].

The first evidence of a NADH oxidase activity producing hydrogen peroxide (H_2O_2), in platelet plasma membrane, derived from an ultrastructural-cytochemical demonstration [60]; after that, several platelet agonists (thrombin, collagen, immunological stimuli) have been shown to stimulate H_2O_2 production [61, 62]. More recently, hydrogen peroxide produced by platelets has been implicated in specific signal transduction pathways, such as tyrosine phosphorylation [63]. Finally, the presence of an enzyme similar to tNOX or cNOX has been suggested from the study of Peter *et al*. [64], which demonstrated a periodic and light responsive oxidation of NADH by human buffy coats (a mixture of white cells and platelets).

Platelets also possess a NADPH oxidase complex, similar to that identified in phagocytic cells. The cytosolic ($p47^{phox}$, $p67^{phox}$) and membrane-bound ($p22^{phox}$ and $gp91^{phox}$) components of this enzymatic complex have been identified in platelets [65-67]. On the other hand, platelets are considered "covercytes" and not true phagocytic cells and, most importantly, they do not kill bacteria [68]; thus the function of NADPH oxidase in platelets is different. NADPH oxidase plays a key role in intracellular signaling leading to α_{IIb}/β_3-integrin activation [69]; furthermore, $O_2^{\cdot-}$ (or the derivative H_2O_2) enhances the ADP release, resulting in increased platelet recruitment [67]. In addition, in host defence response, platelet NADPH oxidase causes the release of thromboxane A2, which, in turn, enhances ROS production by neutrophils and their cytotoxic action [70].

It is noteworthy that hydrogen peroxide has been disclosed as the primary second messenger in several cell types, affecting the activity of protein kinases and phosphatases [71]. Thus, the NADPH oxidase-derived $O_2^{\cdot-}$ is likely converted to H_2O_2 which can diffuse inside cells. The temporal and spatial organization of ROS production appears to be important for activation of specific redox signaling pathways, as recently reported [72]. Since ROS generation by platelets may occur either inside the cells [69, 73, 74] or on the external surface, the platelet PMR activity may induce autocrine and paracrine effects, as shown in Fig. (**2**).

Although the NAD(P)H oxidases are the main enzymes of the PMR system in platelets, nonetheless other components has been identified, including NADH-diaphorase, cytochrome b5 reductase [75] and, more importantly, at least three thiol-related enzymes. In particular, two thiol isomerases and a glutathione reductase activity appear crucial for the rearrangement of disulfide bonds [76, 77]. Platelet membrane proteins contain redox-sensitive sulfhydryl groups needed for platelet aggregation, secretion and post-aggregation events through the activation of integrin receptors. The thiol/disulfide balance is fine-tunely regulated by low molecular weight thiols (glutathione or homocysteine) and by nitric oxide (NO), derived from S-nitrosothiols, which can change in several diseases [78]. 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 β3 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 [76] suggested the involvement of external reducing compound(s) (such as glutathione), maintained in its active form by either NADPH oxidase or glutahione reductase. On the other hand, the presence, on platelet surface, 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 rela-

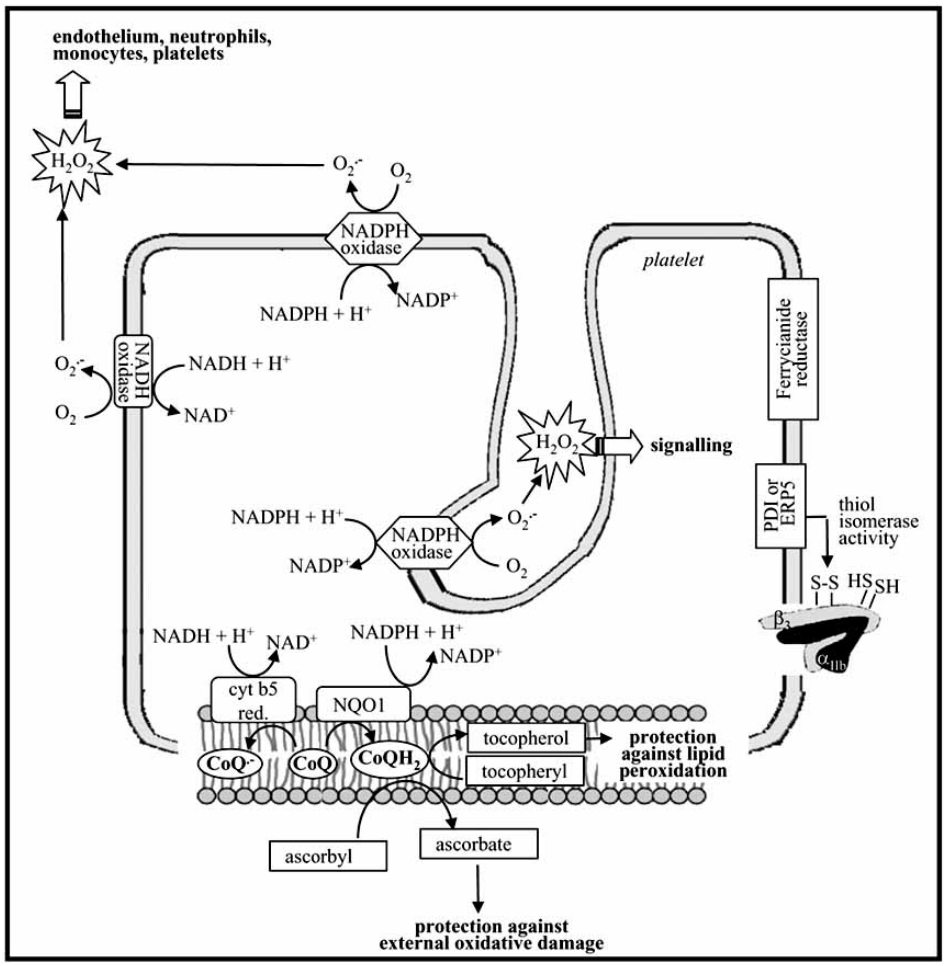

Fig. (2). The PMR system in platelets. Plasma membrane enzymes are present on membranes, looking outside or inside (channels of the open canalicolar system) the cell. The main components are NADH- and NADPH-related enzymes (NADH and NADPH oxidases), which maintain the levels of NAD(P)H/$NAD(P)^+$ and reduced coenzyme Q (CoQ), while generating reactive oxygen species ($O_2^{\cdot-}$ and H_2O_2).

ROS may act intra- and extra-cellularly, inducing either autocrine or paracrine effects. The reducing activity may also contribute to thiol/disulfide balance (through PDI or ERP5 activity), thus affecting platelet activation and aggregation.

tionship between PDI and NADPH oxidases has been recently discovered: in this case, PDI regulates the activity of NADPH oxidase [79], so that a bi-directional mode of action may lead to reciprocal regulation of these two enzymes.

MEDICINAL EFFECTS ON PLATELET PMR SYSTEM

From the literature data reported above, it is conceivable that increased levels of ROS play a key role in the pathogenesis of several diseases, including diabetes, essential hypertension, sickle cell disease, preeclampsia, thalassemia and cardiovascular disorders. In blood, alteration on redox-state leads to dramatic changes in platelet functions, such as the activation/aggregation process; thus, platelets are involved in thrombotic vascular occlusion, and chronic platelet activation may play a role in thromboembolic complications.

Vitamin C has physiological relevance in platelets, because it can modulate the redox state of sulfhydryl groups. Indeed, we have recently demonstrated that intracellular ascorbate concentrations dose-dependently increased the amounts of surface thiols, while vitamin depletion decreased them. This effect appears to be specific for surface sulphydryl groups, since intracellular thiols and glutathione content (the main non-protein thiol in platelets) are unaffected by ascorbate supplementation or depletion [80]. Beside its role during aggregation, vitamin C seems to have also important effects on platelet–fibrin clot strength and stability, that is crucial in pathophysiological conditions. Thrombus strength generated during post-clotting events is significantly reduced in ascorbate-depleted platelets with respect to control platelets and this parameter is in part restored if depleted platelets are supplemented with ascorbate [80]. Due to its relevance in platelet physiology, vitamin C uptake is strictly controlled: platelets compensate for fluctuations in ascorbate levels by modulating (at translational level) the expression of the Na^+-dependent transporter SVCT2. The control of ascorbate uptake, through regulation of its carrier, also occurs during platelet activation, characterized by vitamin C deprivation and alteration in redox state [80]. The platelet PMR system is capable 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; indeed, changes on

redox potential of blood may regulate activation of α_{IIb}/β_3 integrin in platelets. 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 [81].

Accumulating evidences show that vitamin C is also indirectly involved in platelet functions, by protecting membrane components susceptible to free radical damage and by regulating membrane systems sensitive to ROS-mediated signalling. Vitamin C reduces platelet CD40L expression, a transmembrane pro-inflammatory and pro-thrombotic protein implicated in the initiation and progression of atherosclerotic disease through scavenging the $O_2^{\cdot-}$ generated by NADPH oxidase activation [82].

By these data, it appears clear that a rational use of vitamin C supplementation may have beneficial effects on platelet-related diseases. It can decrease levels of platelet-derived microparticles, exerting a protective effect during myocardial infarction in patients with high thrombotic risk [83], and ameliorate the oxidative stress-mediated hypercoagulable state of patients with sickle cell disease and thalassaemia [84, 85]. Vitamin C, whose levels (together with those of vitamins E) are lowered in preeclamptic women, can counteract platelet activation and oxidative stress, involved in early pregnancy and in the pathogenesis of preeclampsia [86]. In chronic smokers, oral vitamin C administration reverted the NADPH oxidase-mediated oxidative stress of platelets; this is achieved by restoring nitric oxide release, intraplatelet cGMP levels and platelet aggregability [87]. Finally, ascorbate supplementation suppresses platelet nitric oxide and $O_2^{\cdot-}$ production, as well as NADH oxidase activity, often associated with nitrate tolerance observed after continuous administration of organic nitrates [88].

Vitamin E supplementation has been shown to play a protective effect against several cardiovascular disorders, through its ability to influence platelet activation and aggregation. Platelet vitamin E, whose levels strictly depend on dietary intake, has a dual role, exerting antioxidant as well as signalling effects unrelated with its antioxidant function [89]. Indeed, vitamin E supplementation reverts biochemical abnormalities (such as lipid peroxidation) observed in platelets from haemodialysis patients [90]; it also inhibits prostaglandin production and platelet aggregation, thus reducing vascular complications in diabetic patients [91], and, like vitamin C, reduces the platelet reactivity of thalassemic patients or individuals with sickle cell disease [86, 92]. Among the antioxidant-unrelated mechanisms of action, there is the vitamin E-dependent modulation of protein kinase C (PKC) activity. Indeed, the vitamin, by interfering with the PKC signalling pathway(s), inhibits platelet adhesion, activation and aggregation [93, 94]. In addition, mixed tocopherols have been shown to affect platelet aggregation by increasing NO release and decreasing superoxide production, through regulation of NOS and SOD activity [95]. Finally, the finding that vitamin E can impair NADPH-oxidase activation, in human subjects and animal models, can suggest an additional mechanism by which tocopherols prevent coronary diseases [96].

The importance of nicotinamide supplementation in the maintenance of NAD^+ intracellular levels (thus influencing cellular life span) has been recently demonstrated [97, 98]. Beside its action as $NAD^+/NADP^+$ precursor, nicotinamide is also a substrate for three classes of enzymes: mono-ADP-ribosyltransferases, poly-ADP-ribose polymerase and ADP-ribosyl cyclase. In platelets, the multifunctional CD38 membrane enzyme has been shown to possess ADP-ribosyl cyclase and NAD^+ hydrolase activities [99]. The resulting cyclic ADP-ribose leads to calcium mobilization from intracellular stores, thus playing a crucial role during platelet aggregation [99]; this is further supported by the findings that thrombin stimulation leads to α_{IIb}/β_3 integrin-dependent association of the enzymatic activity with cytoskeleton [100]. Also mono ADP ribosylation of proteins has been demonstrated in platelets and a functional role in platelet activation has been suggested [101]. So, ADP ribosylation and NAD^+ glycohydrolase activity [99, 102], leading to NAD^+ depletion, influence the activity of platelet PMR system. Since exogenous nicotinamide has been shown to be effective when an unbalance in the NAD^+/NADH ratio occurs (at least in some cell types) [97, 98], further studies on the effects of supplementation on platelet functions should be hoped.

In conclusion, the PMR system is crucial in platelets, keeping in mind that the activation of coagulation pathways strictly depends on membrane activities, which are also required for the cross-talk among cells (platelets, monocytes, neutrophils, endothelium). Further knowledge in regards to the role of the PMR system in platelet functions should promote the development of therapeutic approaches against several diseases, including diabetes, inflammation and thromboembolic pathologies.

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ABBREVIATIONS

PMR	=	Plasma membrane redox system
PMET	=	Plasma membrane electron transport
CoQ	=	Ubiquinone coenzyme Q
ROS	=	Reactive oxygen species
NQO	=	NAD(P)H:ubiquinone oxidoreductase
VDAC	=	Voltage-Dependent Anion-selective Channel
NOX (1-5)	=	NADPH oxidases
tNOX	=	Tumour-associated NADH oxidase
cNOX	=	Constitutive NADH oxidase
PDI	=	Protein disulphide isomerase
ERP5	=	Endoplasmic reticulum protein.

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