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Dual Role of Plasma Membrane Electron Transport Systems in Defense

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ABSTRACT: Because oxidative stress is one of the main sources of severe cellular damage, cells have different defense weapons against reactive oxygen species. Ubiquitous plasma membrane redox systems play a role in defense against oxidative stress damage. On the other hand, a tightly controlled and localized production of reactive oxygen species by a plasma membrane NADPH oxidase can be used as a potent microbicidal weapon. This dual, prooxidant and antioxidant role of plasma membrane electron transport systems in defense is studied and discussed.

KEY WORDS: plasma membrane redox, NADPH oxidase, coenzyme Q, ascorbate, tocopherol, oxidative stress.

I. INTRODUCTION

Redox reactions are essential for the function of cell membranes. It should be stressed that every bioenergetically competent cell membrane does contain redox systems.[1] Surprisingly, the paradigm of the universal presence of redox systems at cell membranes is still not firmly established in the current biological literature, and the existence of membrane redox systems other than those of inner mitochondrial and thylacoid membranes is simply unknown by an important percentage of biologists, and it is underestimated by most of the rest. In fact, the wrong statement that electron transport is generally not found in the plasma membrane of eukaryotic cells can be found in the most up-to-date current literature.[2]

A plasma membrane electron transport system or plasma membrane redox system (PMRS) has been found in every living cell tested, either prokaryotic or eukaryotic, including bacteria, cyanobacteria, yeasts, algae, and all kind of plants and animal cells.[3-5] Preliminary observations can be traced back at least to the studies carried out

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Note: We would like to dedicate this article to Dr. Frederick L. Crane, an example of both scientific and human generosity.

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by Voeglin et al. in 1925 when they examined a relation between the redox state and cancer.[6] Despite other previous unconnected observations, the comprehensive study of PMRS has begun from the mid-1970s on, thanks mainly to the pioneering effort and contribution of Crane and collaborators.[7]

PMRS are not a simple curiosity, an evolutive relic. On the contrary, there is increasing, experimental evidence for their direct involvement in several vital functions, including bioenergetics, iron uptake and cell growth and proliferation.[3] Cell defense is another function in which PMRS are involved, and in this function reactive oxygen species play a double key role.

The term oxygen free radical (OFR) includes the superoxide anion-free radical, the hydroxyl radical, and lipid and other peroxy radicals. OFRs are part of the group of molecules called reactive oxygen species (ROS), all of them more strongly oxidizing than triplet molecular oxygen. ROS other than OFR include singlet oxygen, hydrogen peroxide, lipid peroxide, hypochlorous acid, and other *N*-chloramine compounds.[8] ROS are generated in cells by both enzymatic and nonenzymatic reactions.[9,10] There is an increasing consensus that ROS and free radicals are important biochemical intermediates involved in a large number of diseases.[9,11] In fact, ROS are potentially very toxic to cells due to their highly reactive nature. They can readily react with other molecules, either lipids, proteins, or nucleic acids, giving rise to cell damage. As membrane damage through lipid peroxidation is one of the most destructive effects of ROS,[9,12] systems to maintain adequate antioxidant levels into and around membranes are required. A constitutive PMRS, present in all cell types, mainly contribute to this function.[13-15]

On the other hand, ROS can be used — and, indeed, they are used — as a first defensive weapon against pathogens. To efficiently carry out this role, ROS should be generated at the cell surface, as a response against the stimulus caused by the presence of the pathogens. In fact, ROS are generated at the cell surface of certain lineages of cells by the inducible NADPH oxidase and by mieloperoxidase, two especialized PMRS.[3,4]

The aim of this review is to describe this dual behavior —prooxidant and antioxidant — of PMRS as two faces of the same coin, revealing the central role of PMRS in cell defense.

II. GENERATION OF ROS AT THE CELL SURFACE BY PMRS

The phagocytes of the immune system have the ability to produce reactive oxidants as microbicidal agents.[9,16,17] The precursor of these oxidants is superoxide anion. Because its production is associated with an abrupt rise in oxygen consumption, this process has been called respiratory burst, and the enzyme responsible for its production has been called respiratory burst oxidase.[17] Once it is produced, superoxide anion rapidly dismutes to hydrogen peroxide, which can be transformed into other more reactive ROS by other membrane enzyme systems. The most prominent among them is neutrophil myeloperoxidase, which generates hypochloric acid.[9,12]

The large burst in oxygen consumption by neutrophils during phagocytosis was first observed by Baldridge and Gerard.[18] This sudden increase in oxygen consumption was believed to provide the energy for phagocytosis. The term "respiratory burst" is somehow misleading because it can induce one to think in a process associated to mitochondrial respiratory chain; this is not the case, because respiratory burst is insensitive to inhibitors of the mitochondrial electron transport chain. It is currently accepted that this increase in oxygen

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consumption is due to the activation of an inducible, plasma membrane superoxide-generating NADPH oxidase.[17,19-22]

In resting cells, the oxidase is inactive, but it may be stimulated *in vitro* very rapidly by a wide variety of compounds. This respiratory burst is transient in nature, reaching a maximum rate in a few minutes after stimulation, and returning to the background levels in half an hour.[23]

The NADPH oxidase is a transplasma membrane heterodimeric cytochrome b, composed of a small α-subunit ($p22^{phox}$) and a larger β-subunit ($gp91^{phox}$), associated with two proteins located in the cytoplasma of unstimulated cells, called $p47^{phox}$ and $p67^{phox}$. All of the components have been purified, cloned, and sequenced.[20,21] After activation of the NADPH oxidase, there is a translocation of a small fraction of cytosolic $p47^{phox}$ and $p67^{phox}$ to the plasma membrane. In addition, at least another four components are required for complete NADPH oxidase activity: (1) Rac2, which is a cytosolic guanine nucleotide-binding protein required for oxidase activation; (2) $p40^{phox}$, a protein that enhances the activity of the purified recombinant cell-free system and bears a high degree of homology to $p47^{phox}$, including two SH3 domains; (3) an H^+-channel, which is essential for the activity of the oxidase; (4) Rap1A, which is a small membrane guanine nucleotide-binding protein.

A. Components of the NADPH Oxidase System

1. Cytochrome b_{558}

Segal et al.[24] first identified cytochrome b_{558} as a component of NADPH oxidase. It is a cytochrome with unusual properties.[25] Its redox potential, –245 mV, is atypically low for a cytochrome b, but this fact enables the reduction of oxygen to superoxide.[26] The rate of electron flow has been demonstrated to be matched by the rate of superoxide production.[27]

In neutrophils, cytochrome b_{558} is located in the plasma membrane and in the membrane of specific granules at a 3/7 or lower ratio.[28,29] Hence, in the resting neutrophil cytochrome b_{558} is mainly located in intracellular membranes. After activation, these granules fuse with the plasma membrane, transferring the cytochrome b_{558} to the cell surface. These data have led to the hypothesis that the granules behave as reservoirs of cytochrome b_{558}.[20]

As mentioned above, cytochrome b_{558} is a heterodimer. The β-subunit, $gp91^{phox}$, is a 570 amino acid polypeptide with three transmembrane helices, five N-linked glycosylation sites, and a C-terminus that interacts with cytosolic components during oxidase activation.[25,30] In mammals, $gp91^{phox}$ appears to be expressed exclusively in cells of the myeloid line. The regulation of this restricted expression has been shown to involve the 1.5-kb upstream region of $gp91^{phox}$ promoter, which contains a duplicated CCAAT box. This box can be recognized by the transcription activator CP1,[20] and by the repressor CDP.[31] In nonmyeloid cells, there are high CDP levels, excluding the binding of CP1 to the promoter CCAAT box and suppressing the expression of $gp91^{phox}$. In myeloid cells, there are much lower nuclear levels of CDP, and, thus, the repressor may be replaced on the $gp91^{phox}$ promoter by CP1 immediately after contact with activating stimuli.[17,20,32] Inflammatory cytokines can modulate $gp91^{phox}$ expression.[17,33]

The α-subunit, $p22^{phox}$, was cloned and sequenced by Parkos et al.[34] It is a transmembrane protein protruding on both faces of the plasma membrane.[25,30,35] Although it can be expressed in many cell types, the stable expression of the protein is restricted

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to myeloid cells.[14,17] The expression of p22*phox* mRNA is not regulated by these cytokines that modulate the activity of NADPH oxidase.[30]

2. Cytosolic Components

p47*phox* is a highly basic protein of 390 amino acids and p67*phox* contains 526 amino acids. Both proteins lack of regions of homology to known flavin or NADPH-binding sites, and their only known motifs are two SH3 domains, through which they can interact with other proteins.[21,36,37] Interestingly, in the cytosol of resting neutrophils, p47*phox* and p67*phox* are combined in a 250-kDa equimolar complex,[38,39] and there is also uncomplexed p47*phox*. The translocation of p47*phox* seems to precede and is necessary for the translocation of p67*phox*. Furthermore, the translocation of these cytosolic components after activation is only possible if functional cytochrome b_{558} is expressed.[40,41]

3. Rac2

Rac proteins are members of p21 protein family, a big group of small monomeric GTP-binding proteins that play important roles in cellular functions. Different experimental evidences strongly suggest that a prenylated Rac protein is required to achieve maximal NADPH oxidase activity.[42] In fact, it has been shown that Rac2 is the main p67*phox* interacting GTPase in human cells.[43] In the resting neutrophil, Rac2 seems to be complexed with Rho-GDI, in the GDP-bound cytosolic form. After activation of the oxidase, Rac2 dissociates from its GDI, and a small fraction translocates.[40,44] It has been proposed that Rac binds to p67*phox* through an "effector" region, binds to the membrane through its C-terminus, and interacts with cytochrome b_{558} through its "insert" region.[45]

4. p40*phox*

This is a protein with 339 amino acids, containing one SH3 domain and some degree of homology with p47*phox* in the so-called *phox* domain.[21,46] It can bind to both p67*phox* and p47*phox*, perhaps stabilizing the cytosolic equimolar complex. After activation, it only remains linked by its C terminus to p67*phox*.[21] Its actual function in respiratory burst is current object of controversy: some data suggest that p40*phox* is an inhibitory oxidase subunit; on the contrary, other experimental data suggest that it could be a stimulatory subunit.[47,48] This issue deserves further experimental effort.

5. H^+-channel

The generation of superoxide by NADPH oxidase is an electrogenic process leading to a rapid depolarization of plasma membrane potential.[20] However, this depolarization is complete within 1 min and reaches a steady state, indicating that the movement of a positive compensating charge must exactly balance that of the electron transferred. In fact, it has been shown that the activity of the oxidase is tightly coupled to the efflux of protons through a Zn^{2+}, Cd^{2+}-sensitive H^+-channel, which is activated by arachidonate.[49-52]

The H^+-channel activity has been shown to be vital for the activity of the oxidase. On the other hand, the channel is absent in cells that do not express any of the two cytochrome b_{558} subunits.[53] B lymphocytes lacking expression of one of the cytosolic factors express unaltered H^+-channel activity, provided that gp91*phox* is functional; on the

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contrary, lymphocytes that do not express gp91*phox* have not measurable proton translocating activity. These data strongly suggest that gp91*phox* is the arachidonate activable H^+-channel of human neutrophils.[54]

6. Rap1A

Rap1A is a member of the *ras* family of small GTP-binding proteins located at the plasma membrane of resting neutrophils. Like *ras*, it regulates cell proliferation, and its behavior is that of an antagonist of *ras*-dependent transformation.[55] RAp 1A tends to copurify with cytochrome b_{558}. Functional evidence for its participation in NADPH oxidase activation was obtained using a transfected Epstein-Barr virus transformed B-lymphocyte system; in such a system, two mutant Rap1A proteins, locked in the GDP-bound and the GTP-bound conformations, respectively, inhibited superoxide production induced by phorbol esters, but the wild-type protein had no effect.[56] These data suggest that Rap1A could contribute to carry NADPH oxidase from a "state 1" to a "state 2" and back, but nothing is known about the nature of these hypothetical states.

B. Activation of NADPH Oxidase

Both receptor-mediated and receptor-independent mechanisms have been shown to play a role in NADPH oxidase activation. In all cases, activation requires a continuous contact between the cell and the stimulus and NADPH oxidase activity depends on the inbalance toward activation of the equilibrium between activation and inactivation events.[57]

During oxidase activation, the two main cytosolic factors are phosphorylated by protein kinase C-dependent pathways.[58,59] Protein kinase C-independent pathways are also involved in p67*phox* phosphorylation.[59] However, phosphorylation of the cytosolic factor is not enough. They must be also translocated to the plasma membrane. The SH3 domains make possible binding between p22*phox* and p47*phox*, and between p47*phox* and p67*phox*. On the other hand, Rac protein allows a tight anchorage to plasma membrane by binding to three different sites, one in cytochorme b_{558}, another in the membrane itself, and the third one in p67*phox*. Finally, p40*phox* could interact with p67*phox* through its C-terminus.[17,60] The assembled activated complex contains the four basic components in equimolar amounts and a still undetermined number of small GTP-binding and p40*phox* proteins.[61] Figure 1 depicts the components of the NADPH oxidase system in both the resting and the activated states.

Although it is clear that NADPH oxidase activity should be associated to an electron transport through the plasma membrane, it is somehow surprising that only as late as 1998 did direct, definitive evidence for this electron transport shown.[2] On the basis of the amount of superoxide generated by granulocytes, it has been estimated that NADPH oxidase should transport 10^8 electrons per second per cell. Provided that this is an electrogenic pathway, currents of up to 10 to 20 pA are expected. To avoid interference with the proton channel activity, the authors inhibited proton current with 10 μM zinc ions and recorded electron fluxes by patch clamp in the whole cell configuration.[2]

C. Chronic Granulomatous Disease

Individuals who have lost the functional enzyme or components required to activate the oxidase suffer from chronic granulomatous disease, an inherited condition in which

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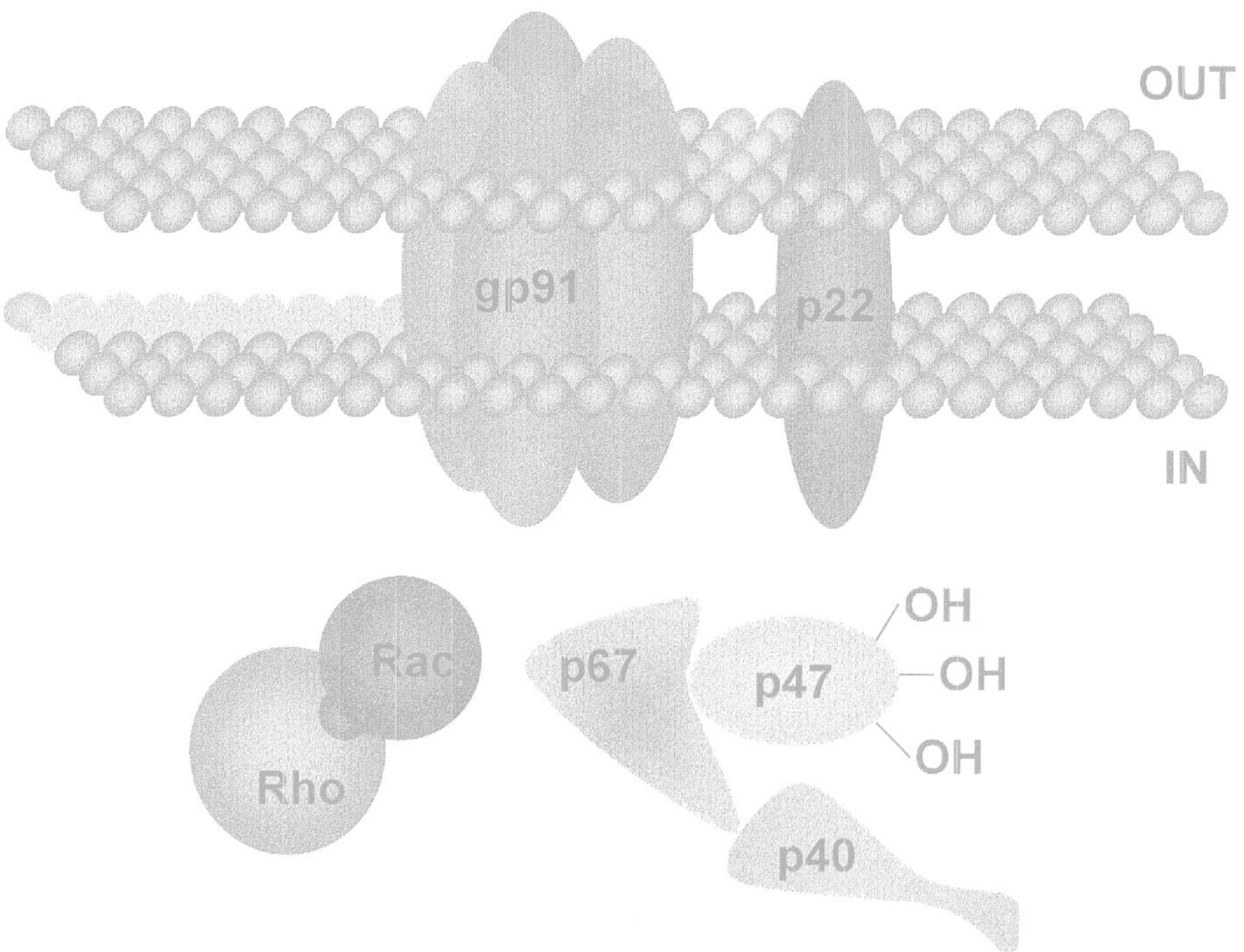

FIGURE 1. Scheme of the NADPH oxidase complex. A) Interactions among components in the resting state. B) Activation. This extremely simplified scheme shows the two main events linked to activation: phosphorylation and translocation of a fraction of the cytosolic components. It also shows the function of gp91^{phox} as a proton channel.

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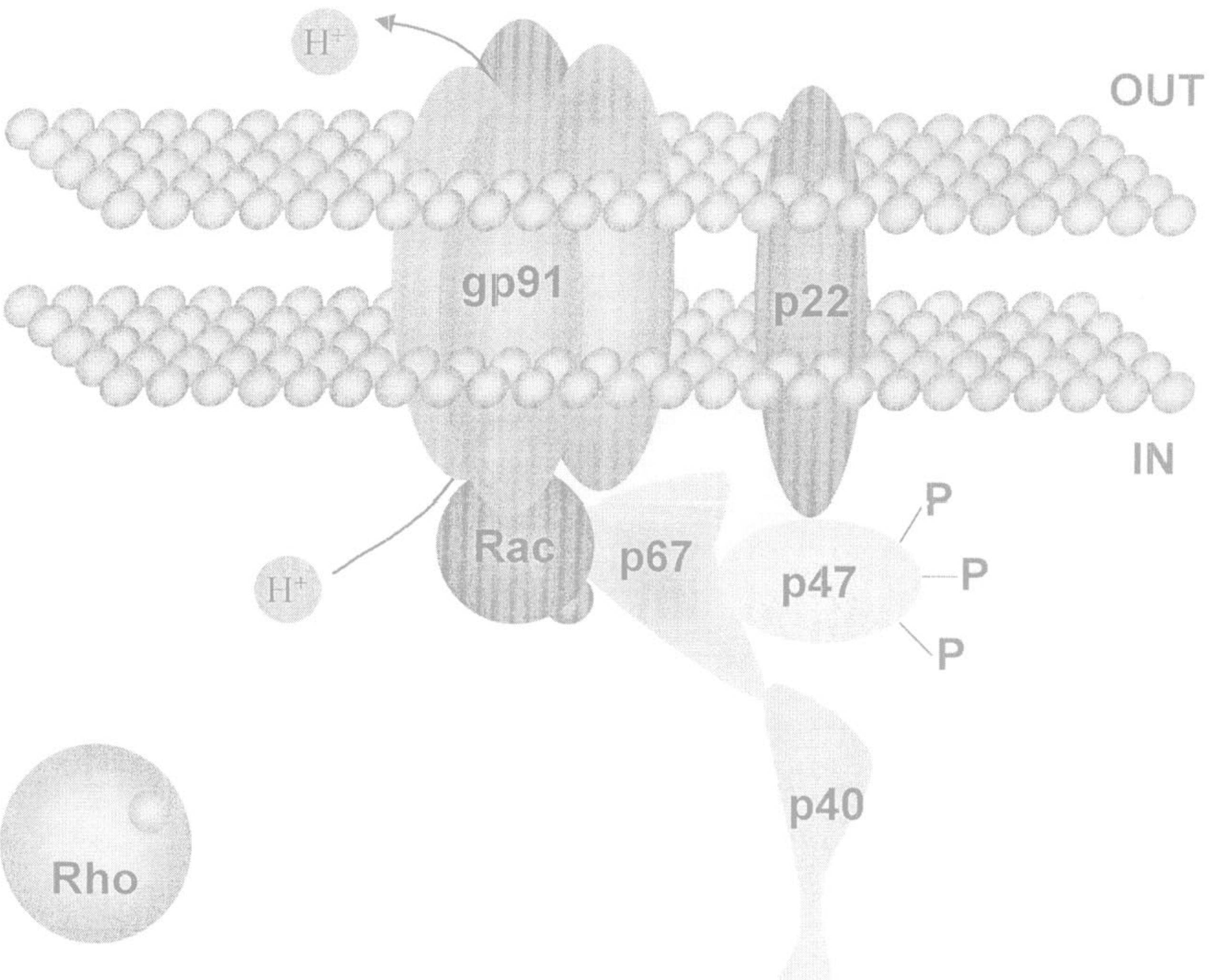

FIGURE 1b.

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there is an increased susceptibility to infections, especially in skin, lungs, liver, and bones, by some of those bacterial and fungal strains whose killing by neutrophils requires oxygen.[9] This disease has an incidence of 0.002 to 0.005% in the Western world. Prior to the discovery of antibiotics, it was practically lethal, leading to death following infection within the first year of life. The use of cells from chronic granulomatous disease patients has helped to study the different components of the NADPH oxidase.[20] Defects in the gene coding for gp91*phox* account for about two-thirds of the cases, and those located in the gene coding for p22*phox* account for 5% of autosomal recessively inherited chronic granulomatous disease.[21]

D. Other Cells and Other Functions

Although the best characterized NADPH oxidase is that of mammalian phagocytes, this is a host defense weapon not only restricted to mammals. Among animals, it has been found in fishes and insects,[17] and it is also present in other phyla. Thus, it is now well known that a NADPH oxidase actively participate in plant defense response to pathogens,[62,63] as recently described in excellent reviews.[4,64,65] A very special and specialized case is the respiratory burst oxidase of fertilization, involved in the alteration of the extracellular protein coats required to prevent the entry of supernumerary sperm.[66]

On the other hand, superoxide-generating NADPH oxidase may play different roles in cells not involved in host defense. Most of these cells are derived from the embryonic mesoderm. Different experimental results point to a role for superoxide and other ROS as biological signals produced in response to external factors and physical stress,[67] activating certain tyrosine kinases or the transcription factor NF-κB.[68,69] The carotid body and airway chemoreceptors may use a superoxide generating NADPH oxidase as an oxygen sensor.[70-72] A NADPH oxidase in liver and kidney plasma membrane also seems to have a role in the systemic sensing of hypoxia, generating H_2O_2 in the presence of oxygen and thus inhibiting the release of erythropoietin, a hormone synthesized in both organs under hypoxic conditions that stimulates the production of extra erythrocytes.[9,72] A calcium-dependent NADPH oxidase in thyroid plasma membrane generates the peroxides required for thyroid peroxidase.[9] In joint tissues, superoxide can be produced by types A and B synoviocytes and condrocytes.[73,74] Vascular endothelial cells generate ROS by both NADH and NADPH oxidases; although the physiological significance of the extracellular endothelial ROS production is unknown, it has been suggested that superoxide anions could antagonize the vasoregulatory action of nitric oxide.[75] ROS released by platelet plasma membranes might synergize with pro-aggregatory stimuli.[76] Finally, many other cell types have been described to contain NADPH oxidase components reseembling those in phagocytes; fibroblasts, B lymphocytes, erythrocytes, adipocytes or renal brush border epithelia are included in this list.[9]

In yeast, an inducible PMRS has been characterized at the molecular level. Although it has remarkable analogies with gp91*phox*, this is not an actual NADPH oxidase. In fact, it is a ferrireductase related to iron uptake, and it is analogous to the Turbo reductase described in plants at the enzyme level but not at the molecular or genetic level.[77-80]

III. PROTECTIVE ROLE OF PMRS AGAINST ROS-INDUCED DAMAGE

As mentioned above, cells are endowed with different enzyme systems and small

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molecules involved in redox reactions that play a central protective role for eliminating these reactive oxygen species. Cell membranes are mainly sensitive to oxidative stress damage caused by radical chain reactions leading to lipid peroxidation. The main mechanism of protection against reactive oxygen species at the plasma membrane is cutting off these radical chain reactions by small molecules, namely, ubiquinol/ubiquinone ($CoQH_2$/CoQ) redox pair and α-tocopherol inside the lipid bilayer and ascorbate in the interphase.[13,15] Ubiquitous multifunctional PMRS[3] mainly contribute to maintain a proper redox state of these molecules for their key, protective, antioxidant functions at the cell surface.

A. Role of Ascorbate in the Electron Transport Across the Plasma Membrane

The antioxidative properties of ascorbic acid are well known. Moreover, cytosolic ascorbate plays a significant role in cell defense against the toxic effects of free radicals and reactive oxygen species, although this protective roles is not yet fully understood.[81] On the other hand, ascorbate is also able to behave as a prooxidant compound. Thus, an ascorbate-dependent, iron-catalyzed peroxidation has been described.[82] Furthermore, ascorbate accelerates the release of iron from ferritin, stimulating its prooxidant effects.[83] However, it must be stressed that currently there is an intense, open discussion on whether ascorbate can act as a prooxidant under physiological conditions.[84-87]

Ascorbate incubated in buffered solution undergoes autooxidation in the presence of oxygen at 37°C,[88] giving rise to the intermediate free radical (AFR) as the first product of oxidation. AFR behaves both as one-electron oxidant and as one-electron reductant,[8,89] explaining both the antioxidative and prooxidant effects described for ascorbate. Although AFR is a relatively stable, non-hazardous biological free radical, ascorbate oxidation seems to contribute to the generation of other free radicals and reactive oxygen species, including hydroxyl or superoxide radicals and hydrogen peroxide.[82] The low levels of catalase and peroxidase activities in cancer cells render them potentially sensitive to ascorbate toxicity.[90,91] In fact, ascorbic acid has been reported to be cytotoxic for Ehrlich ascites tumor cells,[92] and to some human tumors,[93] including some leukemia and pediatric tumors.[94-96] It is interesting to mention that some authors have found survival effects even in the absence of cytotoxicity to the tumour.[97] On the other hand, several groups have found that ascorbate-treated animals have tumors that are less severe or more encapsulated than those of control, nontreated animals.[93,98-101]

Although the prooxidant role of ascorbate cannot be neglected, under normal, physiological conditions ascorbate mainly behaves as a first-order antioxidant that protects cellular components from free radical-induced damage by a direct quenching of soluble free radicals or by scavenging those radicals that can initiate lipid peroxidation.[84] It is noteworthy the fact that ascorbate can reduce membrane-bound tocopheroxyl radicals to tocopherol, thus preventing oxidative damage in membrane lipids. As different organisms, including human beings, cannot synthesize ascorbate, the mechanisms to stabilize this vitamin available in the diet are extremely important. The semioxidized form of ascorbate, AFR, has been proposed as a natural electron acceptor for the constitutive PMRS. In fact, stabilization of ascorbate by cells would be the consequence of the reduction of AFR by a NADH-AFR oxidoreductase activity of the PMRS.[102-108]

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Despite some criticism to its enzymatic nature,[109] evidence has accumulated supporting the participation of an enzyme system, at least for part of the activity.[110-112] This activity can be modulated by both extracellular and intracellular factors, including EGF, cAMP, and N-*myc* or *Ha-ras* oncogene expression.[113-116]

Alternatively, oxidized ascorbate can be recycled by its intracellular reduction, as occurs in neutrophils. This recycling has been shown to be induced in a 30-fold factor by the presence of pathogens.[117] In an era of emerging antibiotic resistance, ascorbate recycling can be a potential physiological means of enhancing host defense.

On the other hand, intracellular ascorbate has been proposed to be an important physiological electron donor for a PMRS involved in recycling of tocopherol.[118] According to data from the group of May, ascorbate could be — at least in erythrocytes — an electron donor more important than NADH for a trans-membrane protein containing sensitive sulfhydryl groups on both membrane faces. This observation does not rule out the possibility of a direct recycling mechanism.[119,120]

B. A Key Role for CoQ as a Very Low-Molecular-Weight Component of Antioxidant PMRS

CoQ is a lipophilic redox compound that is required in the electron transport of bioenergetically competent membranes, including plasma membranes.[1,3] The existence of a significant amount of CoQ in the quinol state in the plasma membrane indicates the necessity of a system for reduction of intramembrane CoQ. The specific participation of CoQ in trans-plasma membrane electron transport has been described. In fact, reduced CoQ acts as a carrier between an internal NADH dehydrogenase and an external side final acceptor. The internal dehydrogenase activity is a NADH-ubiquinone oxidoreductase that has been purifed to homogeneity from isolated liver plasma membranes.[121,122] It is a 34-kDa protein with an internal fragment sequence identical to cytochrome b_5 reductase. This system would use CoQ as an intermediate shuttle to provide electrons for different acceptors in the outer side of plasma membrane and in the extracellular medium. Thus, the relationship between ascorbate and CoQ at the plasma membrane interphase would be an integrated mechanism to maintain the antioxidant property of ascorbate using cytoplasmic NADH as unique electron source.[102]

After gentle extraction of quinones with heptane, AFR reductase activity strongly decreases, but it still remains a residual AFR reductase activity, probably due to a slow transfer of electrons from NADH via cytochrome b_5 reductase, throughout CoQ deeply buried into the lipid bilayer.[102] Similar residual ferricyanide reductase or NADH oxidase activities remain after gentle extraction with heptane.[121,122]

Long-term treatment of animal cells with ethidium bromide depletes cells of their mitochondrial DNA and renders them deficient in their mitochondrial electron transport.[123] Surviving ρ^0 cells require uridine and pyruvate to maintain growth.[124] CoQ can replace pyruvate to maintain growth of ρ^0 cells; furthermore, in ethidium bromide-induced ρ^0 cells the decreased mitochondrial electron transport rate parallels to an increase in both CoQ contents and CoQ-dependent NADH-AFR reductase activity in the plasma membrane, thus stimulating ascorbate stabilization.[125,126]

CoQ can also have a role as a free radical chain-breaking antioxidant, most likely due to its capacity to regenerate tocopherol and to scavenge peroxyl radicals in its hyd-

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roquinone form. Reduced CoQ also contributes indirectly to the regeneration of tocopherol, because CoQ-dependent NADH-AFR reductase regenerates ascorbate, which in turn can also reduce the tocopheroxyl radical. Figure 2 represents the relations among PMRS and the antioxidant molecules ascorbate, α-tocopherol, and coenzyme Q at the plasma membrane.

C. Plasma Membrane Cytochromes b_5 Reductase and Cytochromes b_5

Cytochrome b_5 reductase has not only been found in animal plasma membranes, but also in purified plasma membrane vesicles from corn roots.[127] The presence of a cytochrome b_5 reductase at the plasma membrane could suggest the presence of cytochrome b_5 in it. However, cytochrome b_5 reductase is by itself an active ferric citrate reductase, and its function may not necessarily involve cytochrome b_5. It is known that cytochrome b_5 exhibits a relatively low redox potential, within the range from –30 to –60 mV.[128,129] This fact fits well with the observation by redox titration analysis that has revealed a minor cytochrome component in different eukaryotes with redox potential within the range corresponding to cytochrome b_5.[130]

Apart from cytochrome b_5 and that included in NADPH oxidase, other plasma membrane cytochromes b could play a role as componentes of PMRS. The conversion of dopamine into noradrenaline takes place in the chromaffin cells of the adrenal medulla. The enzyme that catalyzes the reaction, dopamine β-hydroxylase, is located inside the chromaffin granules, and it uses ascorbate as the electron donor. The AFR generated in this reaction is, at least in part, reduced by a cytochrome b_{561} to regenerate ascorbate, as there is no transport of ascorbate through chromaffin vesicle membrane.[131-133] Recently, at least two genes have been sequenced from *Arabidopsis* showing a significant homology to mammalian cytochrome b_{561}.[134,135] This cytochrome b_{561} seems to have six-transmembrane domains, both N- and C-terminal domains in the cytosolic region, and two hemes related to six well conserved histidine residues.[136] Thus, it seems that this transmembrane cytochrome is a component of PMRS playing a central role in ascorbate recycling in those cells where it is present.

Very recently, a cytochrome P-30 has been described in rabbit peritoneal neutrophils.[137,138] It has been shown that this cytochrome P-30 is not a proteolytic product of gp91*phox*, but it rather seems to be related to cytochrome b_{561}.

D. Oxidative Stress-Related Apoptosis

Mild oxidative stress, such as that produced in cultured cells after serum or growth factor withdrawal, can induce apoptosis.[139] Ceramide accumulation appears a key step in this response, ceramide being able to induce apoptosis by activating proteases of the interleukin-1β-converting-enzyme/caspase-1 (ICE) family.[140,141] Antioxidant molecules can inhibit this kind of apoptosis through a mechanism involving Bcl-2 protein.[142,143] However, it has been shown that both lipid peroxidation and apoptosis triggered by serum withdrawal can be prevented by externally added ascorbate, tocopherol or CoQ, even in cells lacking Bcl-2 protein expression.[144,145] It is also interesting to note that ceramide accumulation after serum withdrawal can be prevented by externally added CoQ and that this accumulation does not occur in ρ^0 cells.[146] On the other hand,

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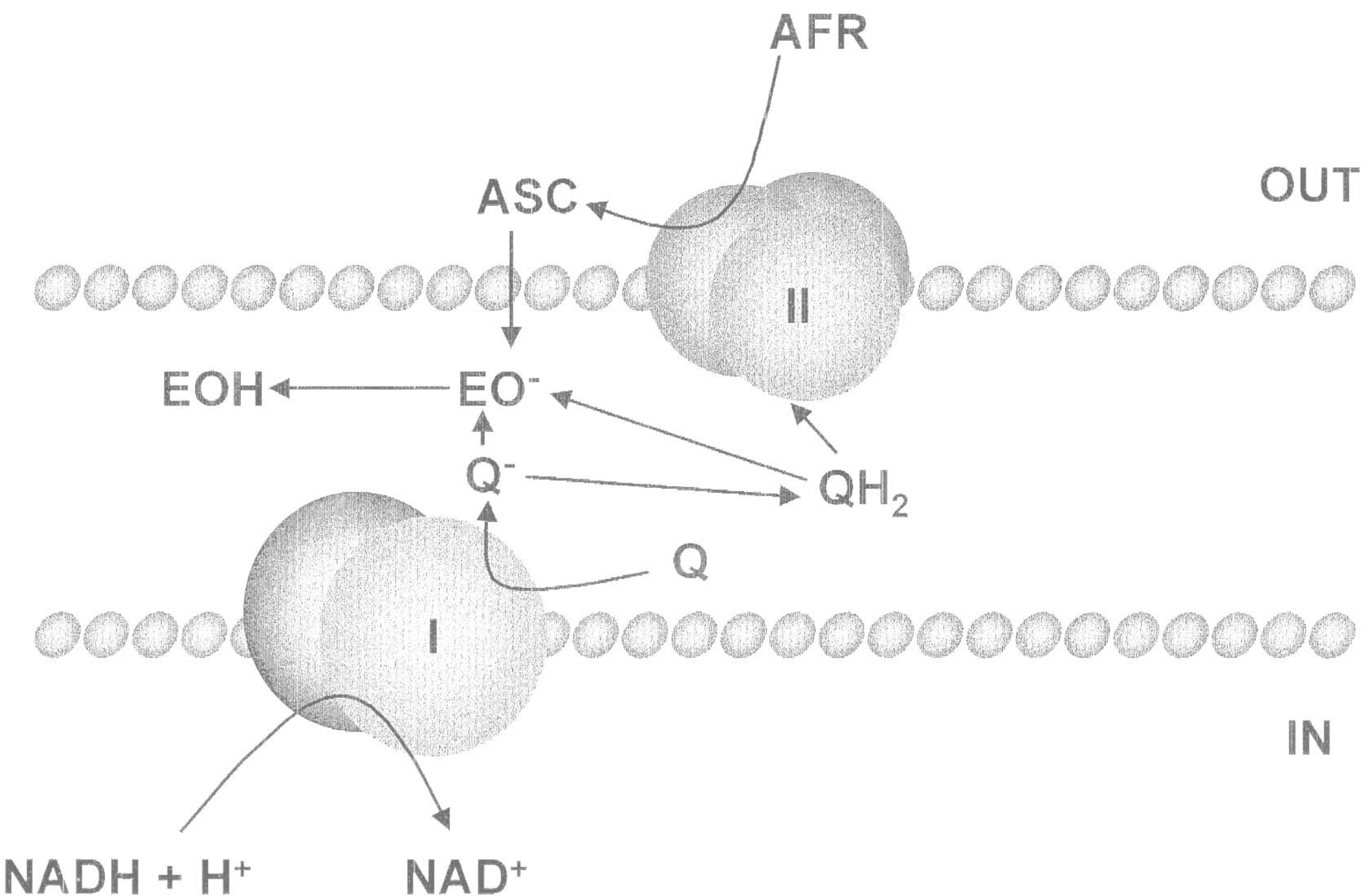

FIGURE 2. Role of coenzyme Q in antioxidant protection and transplasma membrane electron transport. The three possible redox states of coenzyme Q are depicted as Q, $Q^{\cdot}$ and QH_2. α-Tocopherol and α-tocopheroxyl radical are represented as E_{OH} and $E_{O^{\cdot}}$. ASC is ascorbic acid and AFR is ascorbate free radical. The complex I is plasma membrane NADH: coenzyme Q oxidoreductase, identified as a cytochrome b_5 reductase. Complex II is the final external electron donor and it could be the NADH oxidase described by Morre's group. See the text for more details.

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selective inhibition of PMRS activity induces apoptosis.[146] All these data clearly point to a central protective role for PMRS against mild oxidative stress.

This function is not present only in mammalian cells. A similar system has been described in yeasts, where extracellular ascorbate stabilization is mediated by CoQ_6, the ubiquinone present in yeast, instead of CoQ_{10}, present in animal cells.[147]

E. Inducibility of the Antioxidant PMRS

Although PMRS is constitutively expressed, some of its components show induced activities under conditions of α-tocopherol and selenium deficiency. It has been shown that this double deficiency induces a severe oxidative damage that results in adaptive responses during a time frame of several weeks.[148-151]

An α-tocopherol and selenium deficiency produces depletion of α-tocopherol at the plasma membrane and decreases in selenium-dependent enzyme activities, such as the membrane-bound phospholipid hydroperoxide glutathione peroxidase, as well as a dramatic increase in Ca^{2+}-independent phospholipase A_2 activity.[148,149] At the same time, an increase in CoQ associated to the plasma membrane is observed. This increase in plasma membrane CoQ contents is accompanied by increased rates of NADH-CoQ oxidoreductase, NADH-AFR oxidoreductase, and cytochrome b_5 reductase. Furthermore, under these conditions a significant translocation of DT-diaphorase to the plasma membrane is observed.[152]

Figure 3 depicts a model showing the changes in the Q-cycle at the plasma membrane under conditions of oxidative damage. A question remains to be elucidated: what is the nature of the external component of the PMRS in which coenzyme Q is involved? The final external component of PMRS that behaves as an acceptor of electrons coming from ubiquinol could be the NADH oxidase described by Morre's group.[153] A serious alternative to a putative enzyme with ubiquinol-AFR oxidoreductase activity at the external cell surface has been suggested very recently: the glycidic groups of glycocalix along with sulfhydryl groups at the cell surface could interact with negatively charged AFR, stabililizing it in the surroundings of plasma membrane and allowing it to accept electrons coming directly from coenzyme Q or tocopherol.[154]

F. NADH Oxidase

An ectoprotein described as a hormone-responsive external plasma membrane NADH oxidase was first studied in soybean and in rat liver.[153,155] Currently, four different forms of NADH oxidase are described, although only one of them, known as $tNOX_a$, has been cloned, sequenced, and expressed in bacteria.[156] The $tNOX_\alpha$ gene consists of ata least nine exons combined to yield an open reading frame of 1830 bp and a protein of 71.4 kDa comprised of 610 amino acids. The corresponding mRNA, identified by Northern blot analysis, is of 2.8 kb in size. The open reading frame of $tNOX_\alpha$ contains a putative signal sequence at its N-terminus; cleavage of the leader sequence would yield a polypeptide of 69.2 kDa. This cancer-specific NOX protein appears to be translated on polyribosomes associated with the rough endoplasmic reticulum and transferred to the Golgi apparatus, where it is N-glycosylated and processed to the 34-kDa mature protein for tis transport to the plasma membrane (Moore, personal communication). The protein presents a putative quinone binding site motif (EEMTE),

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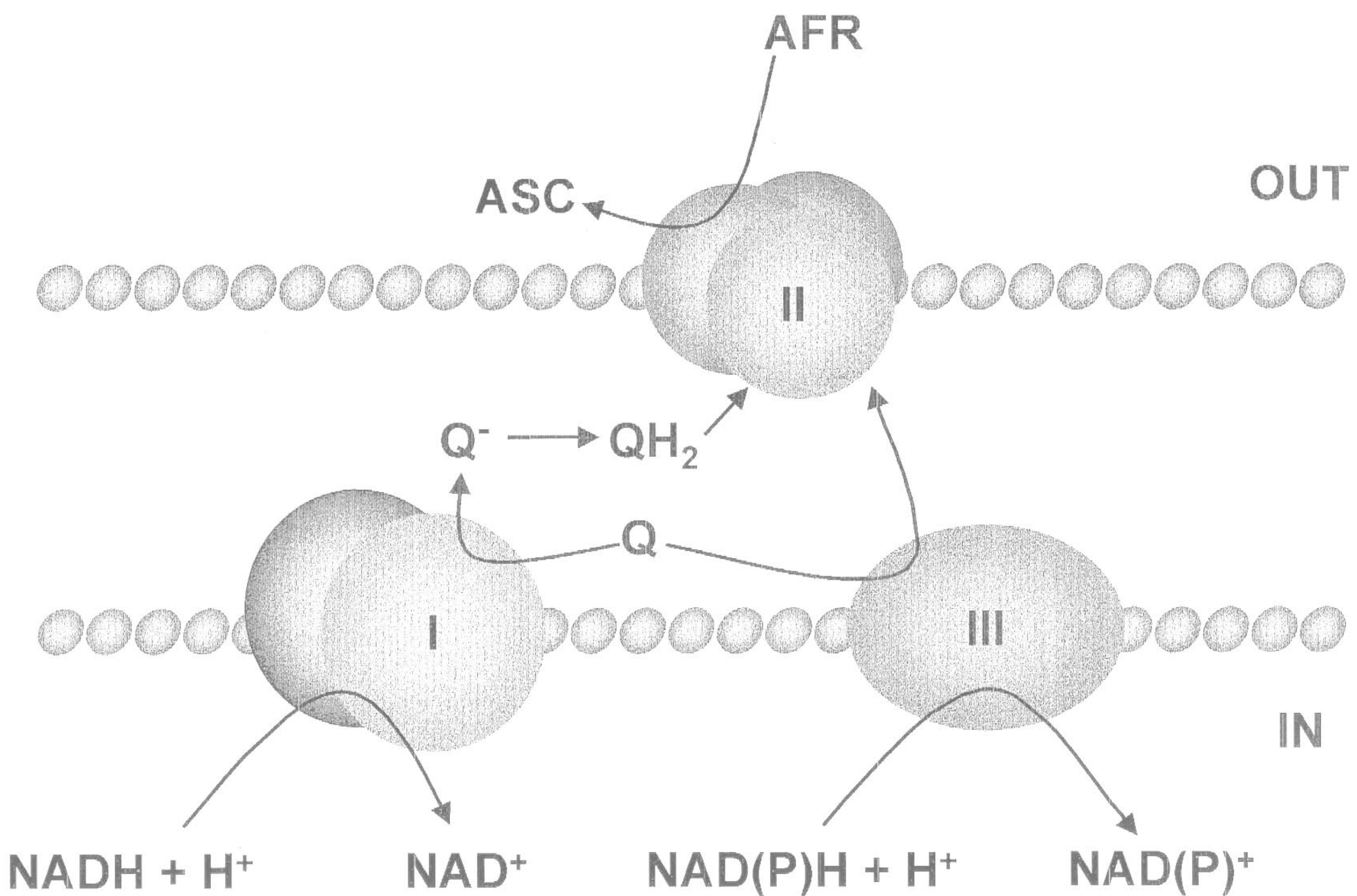

Figure 3. Q cycle at the plasma membrane of cells under mild oxidative conditions. The main difference with the situation described in figure 2 is the translocation to the plasma membrane of complex III (DT-diaphorase) after activation caused by mild oxidative stress. Under these conditions, coenzyme Q in the plasma membrane can assume a major role in antioxidant protection, either by itself or mediating ascorbate regeneration.

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an adenine nucleotide-binding site (TGVGASL), a putative C-XXXX-C protein thiol disulfide interchange motif, a HVHPFG copper binding motif, and eight cysteines.[156] Histidine-562, located 14 amino acids downstream of the copper binding motif, is the likely candidate for a third copper binding ligand in the NOX protein.

Because NADH oxidase is localized at the external cell surface, it should be deduced that NADH is not its physiological substrate. At least two major catalytic activities of NADH oxidase, both of them with putative functional relevance, can be considered. First, NADH oxidase could take part in plasma membrane protein disulfide thiol interchange, provided it contains a C-XXXX-C motif.[157-159] On the other hand, NADH oxidase could catalyze hydroquinone oxidation, behaving as a terminal oxidase for transplasma membrane redox system.[160] Furthermore, both functions could be connected, NADH oxidase transfering electrons from the donor $CoQH_2$ to an acceptor protein disulfide. Thus, NADH oxidase could be the putative terminal acceptor for the PMRS involved in defense against oxidative stress at the plasma membrane.

In the presence of an effector, such as iron or copper, capable of disrupting ordered two electron transport to terminal acceptors, ROS could be generated at the cell surface, as described for aging, eventually contributing to atherogenesis and other age-related disorders correlated with loss of mitochondrial function and the resultant enhanced PMRS activity.

Very recently, it has been shown that NADH oxidase activity oscillates with a period of about 24 min. Furthermore, both hydroquinone oxidase and protein disulfide thiol oxidase-thiol exchange activities of NADH oxidase show alternate periodicities.[153]

IV. CONCLUSIONS

This review has provided evidence for the original idea depicting NADPH oxidase as an special and inducible form of ubiquitous plasma membrane redox systems. Through NADPH oxidase, neutrophils and other cells of the immune system make a tightly controlled use of ROS as a potent weapon against pathogens. However, in usual situations ROS can induce severe damage in cells. To protect plasma membrane as the first target for oxidative stresss damage, cells are endowed with a constitutive PMRS actively involved in the maintenance of high levels of antioxidant molecules into and around the plasma membrane. A complete dissection of this constitutive system at the molecular level remains to be done.

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