ANTIOXIDANTS & REDOX SIGNALING
Volume 14, Number 11, 2011

DOI: 10.1089/ars.2010.3247

COMPREHENSIVE INVITED REVIEW

# Trans-Plasma Membrane Electron Transport in Mammals: Functional Significance in Health and Disease

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**Abstract**

Trans-plasma membrane electron transport (t-PMET) has been established since the 1960s, but it has only been subject to more intensive research in the last decade. The discovery and characterization at the molecular level of its novel components has increased our understanding of how t-PMET regulates distinct cellular functions. This review will give an update on t-PMET, with particular emphasis on how its malfunction relates to some diseases, such as cancer, abnormal cell death, cardiovascular diseases, aging, obesity, neurodegenerative diseases, pulmonary fibrosis, asthma, and genetically linked pathologies. Understanding these relationships may provide novel therapeutic approaches for pathologies associated with unbalanced redox state. *Antioxid. Redox Signal.* 14, 2289–2318.



Reviewing Editors: *Michael Berridge, Rafael de Cabo, Philip Kuchel, Alfons Lawen, Walter Malorni, Miguel Ángel Medina, D. James Morré, Cecilia Prata, Asma Zaidi, and Davide Zannoni*

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## I. Introduction

As early as 1925, Voegtlin *et al.* first postulated the presence of a redox activity in the plasma membrane of normal and cancer cells (304). In 1964, Rossi and Zatti described a membrane-bound pyridine nucleotide oxidase responsible for the respiratory burst in polymorphonuclear leucocytes (246); the presence of this enzyme was subsequently confirmed by Hohn and Lehrer, who found that the oxidase was greatly reduced or absent in patients with chronic granulomatous disease (CGD), an inherited condition in which phagocytes are unable to express a functional respiratory burst (116). Later on, Crane and Low provided evidence for an NADH dehydrogenase activity in liver and fat cell plasma membranes (59), whereas Orringer and Roer reported a vitamin C-mediated nonenzymatic reduction of cell-impermeant anions in actively metabolizing human erythrocytes (224). Then, the discovery of transmembranous NADH dehydrogenase activities in several cell types opened a new perspective about the existence of an electron transport across the plasma membrane (102, 147). Starting from these pioneer works, ever growing evidence has firmly demonstrated that trans-plasma membrane electron transport (t-PMET) occurs in all types of organisms, including bacteria, yeast, plants, and animals (61, 247).

It is now generally agreed that t-PMET allows reduction of extracellular oxidants at the expense of intracellular reducing equivalents; in this way, cells can respond to changes in the redox microenvironment, regulating a variety of biological functions, including cell metabolism, proton pumping, activity of ion channels, growth, and death (110, 158, 177).

In this review, we will describe the individual entities involved in this electron flow and their physiological relevance, as well as the pathological conditions arising from perturbations of this potential plasma membrane redox controller.

## II. Measurements of Electron-Transfer Reactions

Different strategies can be employed for identifying the components responsible for electron transport in viable cells or in isolated plasma membranes. In general, they are aimed at (i) monitoring the disappearance of the electron donor [namely, NAD(P)H] (152), (ii) measuring the reduction of artificial electron acceptors (20, 28, 33, 281), (iii) measuring oxygen consumption (109, 112), (iv) determining the amount of superoxide formation (213, 264), (v) measuring ubiquinone reduction, or (vi) hydroquinone oxidation (152).

Ferricyanide, which is devoid to move across cell membranes because of its net negative charge, has been commonly used as electron acceptor in assays performed with intact cells (especially erythrocytes); ferricyanide is converted to ferrocyanide by one-electron reduction, and the rate of this reduction can be monitored spectrophotochemically or by $^{13}C$ nuclear magnetic resonance spectroscopy (NMR); in particular, the latter is a novel approach allowing real-time, noninvasive investigation of extra- and intracellular events simultaneously (115).

Different tetrazolium salts have also been employed as tools in cell biology; in particular, the second generation of tetrazolium dyes [2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide, 3-(4,5-dimethylthiazol-2-yl)-5-[3-(carboxymethoxy)phenyl]-3-(4,5-dimethyl-2-thiazolyl)-2-(4-sulfophenyl)-2H-tetrazolium and sodium 5-(2,4-disulfophenyl)-2-(4-iodophenyl)-3-(4-nitrophenyl)-2H-tetrazolium inner salt (WST-1)] (Fig. 1), carrying one or more sulfonate groups giving them neutral or net negative charge, are largely cell-impermeant and produce water-soluble, colored formazans (32). WST-1 has been widely used to measure superoxide formation during the respiratory burst in activated neutrophils (281), as well as to study cell surface and transmembrane NAD(P)H oxidase activities in whole cells (28, 30, 283, 284). WST-1 reduction is sensitive to ubiquinone redox inhibitors (such as capsaicin); moreover, this inhibition is greater in $\rho^0$ cells (devoid of mitochondrial DNA, thus being unable of mitochondrial respiration); these findings suggest that dye reduction occurs at the level of the plasma membrane and involves membrane ubiquinone redox cycling (29, 32). The intermediate electron carrier 1-methoxy-5-methylphenazinium methyl sulfate (Fig. 1) is usually used in conjunction with tetrazolium salts (especially WST-1), as it enables low and high potential electrons to be picked up from the cell surface oxidoreductases: without 1-methoxy-5-methyl-phenazinium methyl sulfate only superoxide, which has high potential electrons, is detected (32, 281). This intermediate electron carrier picks up electrons at the cell surface, forms a radical intermediate, and, finally, reduces the dye by two single-electron reduction steps (Fig. 2). Other frequently used cell-impermeant indicators are ferricytochrome c and dichloroindophenol (177, 203).

Finally, the use of specific inhibitors can allow discrimination between the different enzymatic activities responsible for t-PMET (see below).

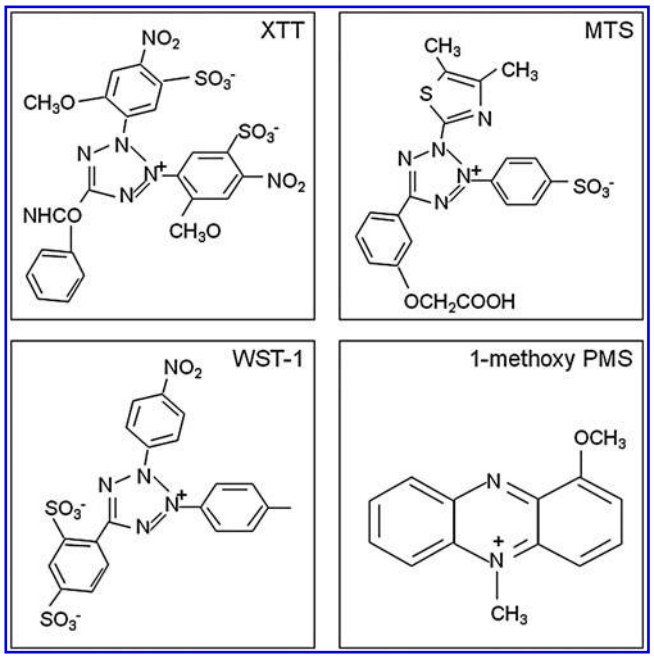


**FIG. 1. Chemical structures of the second generation of tetrazolium dyes (XTT, MTS, and WST-1) and the intermediate electron acceptor mPMS.** mPMS, 1-methoxy-5-methylphenazinium methyl sulfate; MTS, 5-[3-(carboxymethoxy) phenyl]-3-(4,5-dimethyl-2-thiazolyl)-2-(4-sulfophenyl)-2H-tetrazolium; XTT, sodium 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium inner salt; WST-1, sodium 5-(2,4-disulfophenyl)-2-(4-iodophenyl)-3-(4-nitrophenyl)-2H-tetrazolium inner salt.

## III. Components of the t-PMET

Due to the action of t-PMET, electrons coming from cytosolic donors flow outward to reduce extracellular molecules; this trans-plasma membrane flow may occur by enzyme-mediated and/or shuttle-based electron transfer (Fig. 3) (87, 157, 158).

Several components have been identified in the last two decades and some of them have been characterized at the molecular and biochemical level. Some are ubiquitously expressed and others are present only in certain cell types; some utilize only a subset of electron donors or acceptors, whereas others are even less specific (177). Unfortunately, enzymes and mechanisms involved remain still largely ill-defined and a better understanding of how the single entities work (alone or in concert) to regulate cellular functions is now needed.

### *A. Electron donors*

Intracellular reducing equivalents may derive from NADH or NADPH; the first coenzyme is produced during catabolic reactions, whereas the NADPH/$NADP^+$ system is involved in the synthesis of fatty acids and cholesterol, as well as in hydroxylation and detoxification reactions. As the $NAD^+$/NADH and $NADP^+$/NADPH ratios can affect numerous biological functions (121, 317), several methods (bioluminescence, chromatographic techniques, and cycling assays) have been developed to determine the changes of these ratios under both physiological and pathological conditions. However, technical problems may arise during extraction of pyridine nucleotides, as living cells contain enzymes able to hydrolyze them, and the different methods all have some limitations, especially concerning sensitivity, reproducibility, and interference with other reducing compounds present in the assay buffer (323).

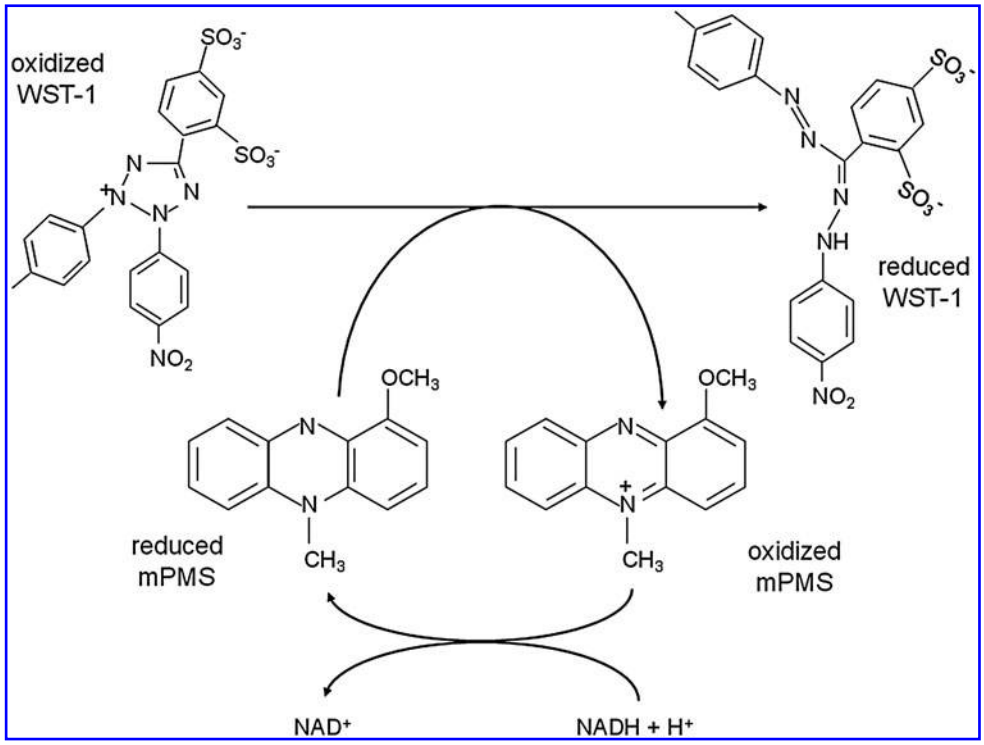


**FIG. 2. Reduction of WST-1 by the intermediate electron acceptor mPMS and formation of the reduced formazan.**

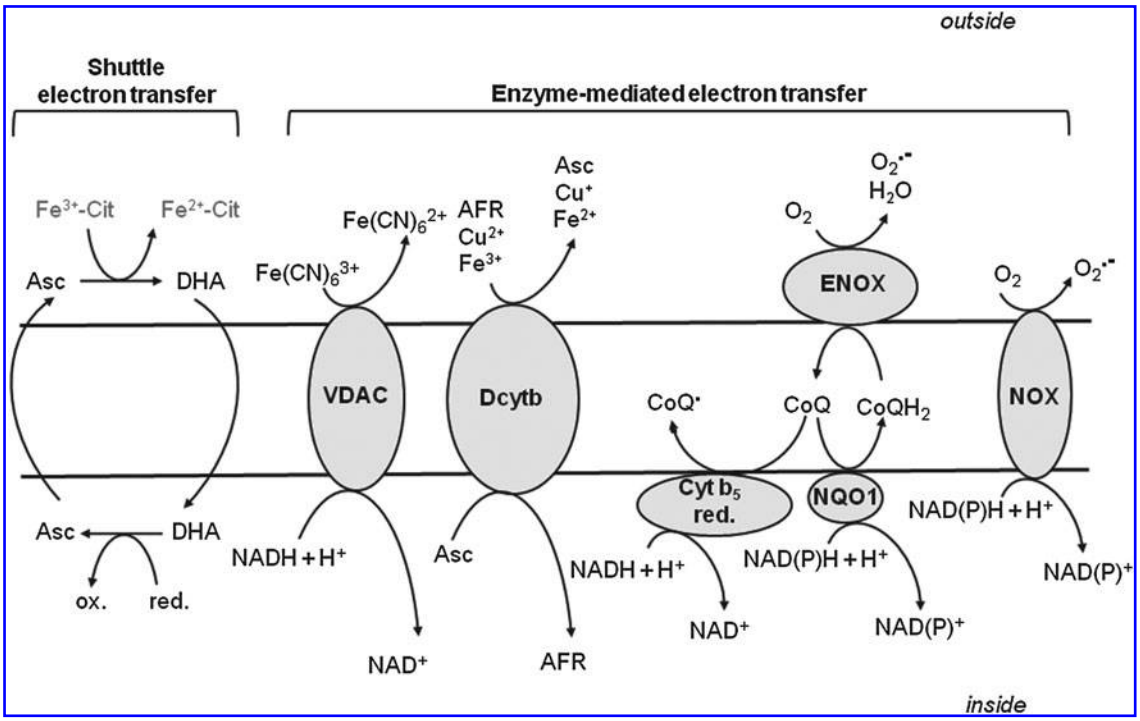


**FIG. 3. Key components of t-PMET.** Intracellular electron donors, provided by NAD(P)H or vitamin C (Asc), flow outward to reduce extracellular acceptors (oxygen, AFR, ferric, or cupric ions). This flow across the plasma membrane is allowed by either enzyme-mediated and/or shuttle-based electron transfer. Several enzymes contribute to t-PMET; some of them are located at the cytosolic side of the plasma membrane, including NQO1 and cyt $b_5$ red., others are transmembrane proteins (such as VDAC, Dcytb, and members of the Nox family), whereas ENOX proteins are enzymes located at the external side of the plasma membrane. CoQ is a mobile component, acting as electron shuttle located in the hydrophobic phospholipid bilayer. In Asc/DHA shuttling, Asc acts as a reducing agent in the extracellular space, forming DHA that is, subsequently, imported into the cell. Extracellular Asc is regenerated for further reduction events by intracellular reduction of DHA to Asc (at the expense of different redox couples ox./red.), followed by release of Asc. $Fe^{3+}$–Cit = ferric citrate. AFR, ascorbyl free radical; Asc, ascorbate; CoQ, coenzyme Q; cyt $b_5$ red., cytochrome $b_5$ reductase; Dcytb, duodenal cytochrome b; DHA, dehydroascorbate; ENOX, disulfide-thiol exchanger; Nox, NADPH oxidase; NQO1, NAD(P)H:quinone oxidoreductase 1; t-PMET, trans-plasma membrane electron transport; VDAC, voltage-dependent anion-selective channel.

Other known intracellular substrates are some flavonoids and ascorbate (Asc), which have been shown to protect cells from extracellular oxidant stressors. In particular, they appear to be crucial substrates for t-PMET activity of red blood cells, where they have been particularly well studied; indeed, in the bloodstream, erythrocytes encounter a variety of oxidants that may exert detrimental effects. Quercetin and myricetin, flavonoids abundantly present in fruits and vegetables, are taken up by erythrocytes and actively promote t-PMET activity; their ability to act as electron donors is strictly linked to their structure, as the catechol structure in the B ring appears to be necessary for the reducing activity of these molecules (86). The dependence of red blood cells on intracellular Asc levels arises from the observation that the treatment of erythrocytes with the nitroxide-free radical Tempol (2,2,6, 6,-tetramethyl-4-hydroxypiperidine-*N*-oxyl) leads to depletion of endogenous Asc (without affecting glutathione or α-tocopherol content) (191), as well as 80% inhibition of the basal rate of ferricyanide reduction observed in untreated cells (193, 194). A similar situation was recently shown for astrocytes, where the Asc-dependent t-PMET appears to be far more important than the NADH-dependent t-PMET (159). Asc contributes to t-PMET through two main mechanisms: (i) enzyme-mediated electron transport, where Asc is the electron donor for transmembrane oxidoreductases, and (ii) nonenzymatic electron transfer, where Asc is released from cells and directly acts as reducing agent, thus being oxidized to dehydroascorbate (DHA) *via* the intermediate ascorbyl free radical (AFR) (158, 160, 299). DHA is then imported and reduced back to Asc at the expense of reducing equivalents coming from cellular metabolism (Fig. 3) (114, 157, 158). This transmembrane metabolite shuttling/cycling mechanism seems to be a more generalized phenomenon involving other redox couples, including superoxide/dioxygen (98, 220), dihydrolipoic acid/α-lipoic acid (134, 192), and reduced glutathione/cysteine (81); nonetheless, the exact contribution of each system to t-PMET remains to be fully elucidated.

### *B. Electron acceptors*

The most important extracellular acceptor is molecular oxygen, which can be fully reduced to water or, otherwise, subjected to partial reduction with the generation of reactive oxygen species (ROS), including superoxide ($O_2^{\bullet -}$) and hydrogen peroxide ($H_2O_2$), which modulate specific cellular functions and signal transduction pathways (88).

Other putative physiological substrates are AFR, reduced back to Asc as described above (299), and ferric ion, which is reduced to ferrous ion (196). The latter reduction acquires particular importance in the proximal small intestinal epi-

thelium; here, iron is taken up by enterocytes, but it must be reduced before being transported across the membrane. Although the requirement for ferrireduction is well known, the mechanism of reduction is still unclear. Most models propose that intestinal iron absorption is facilitated by the presence of a membrane-bound ferric reductase on the brush border of duodenal mucosa (196), but Gunshin *et al.* found that loss of the putative ferrireductase in mice has no impact on body iron stores, even in the setting of iron deficiency (106). Further, a nonenzymatic ferrireduction by endogenous reductants (such as Asc) has been proposed by Lane and Lawen; indeed, they demonstrated that, in K562 cells, DHA uptake and release of Asc are sufficient for extracellular ferrireduction (160).

### C. Intermediate electron carriers

Several intermediate electron acceptors, including b cytochromes, flavin, and vitamin E, have been described so far. However, the most widely used electron shuttle is ubiquinone (or coenzyme Q [CoQ]) (Fig. 3). Indeed, being able to move between the membrane bilayers, it links the inside and outside of cells (12).

Ubiquinone is synthesized through a complex process involving the participation of at least 10 gene products (COQ1–COQ10) (290); nonetheless, the exact order of reactions remains to be fully elucidated. The enzymes involved in the last steps of the biosynthetic pathway are exclusively located in the inner mitochondrial membrane; then, ubiquinone uses the endo-exocytic pathway to distribute among cell membranes moving from and to mitochondria through mitochondria-associated membranes, and reaching the plasma membrane (85). Therefore, changes of ubiquinone concentration in plasma membranes may arise either from biosynthesis or translocation from intracellular reservoirs, such as the endoplasmic reticulum and mitochondria (257). Further, the amount of ubiquinone in membranes is also dependent on dietary vitamin E, selenium, and ubiquinone (162, 216).

Once translocated into the plasma membranes, ubiquinone cycles between reduced and oxidized states. In particular, it can accept one or two electrons, thus producing either the ubisemiquinone radical ($CoQ^{\bullet -}$) or hydroquinone ($CoQH_2$ or ubiquinol). The reduction of mitochondrial ubiquinone is accomplished by different oxido-reductases, located either in the cytosol (such as thioredoxin reductase and glutathione reductase) (35) or in the plasma membrane (such as cytochrome $b_5$ reductase and NAD(P)H:quinone oxidoreductase) (Fig. 4).

The ability of ubiquinone to accept either one or two electrons from internal donors may explain its dual function, since it exerts antioxidant or pro-oxidant activity, depending on the redox state of the cell. Indeed, it is located in proximity to unsaturated lipid chains; therefore, one of its main functions is to act as a scavenger of free radicals, thus protecting membranes from lipid peroxidation. Indeed, the amount of ubiquinone in many membranes is up to 30 times their tocopherol content (64). In addition, the reduced hydroquinone form regenerates α-tocopherol by reducing its phenoxyl radical (136) or Asc by reducing AFR (100). On the other hand, ubiquinone, through the formation of the semiquinone radical, has been shown to be associated with generation of superoxide anion and hydrogen peroxide in cellular membranes (64) (Fig. 4).

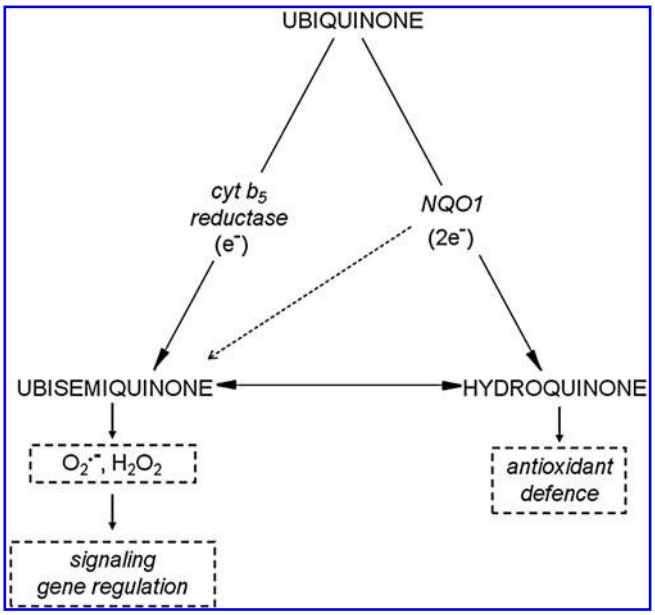


**FIG. 4. The redox quinone cycle.** Ubiquinone can be transformed to the ubisemiquinone radical or to hydroquinone, depending on the membrane enzyme involved (such as the NADH:cytochrome $b_5$ reductase or the NQO1) and electrons transferred. It should be recalled, however, that NQO1 can also generate radicals (*dashed arrow*) with some quinones, such as menadione (see text for details). The ubisemiquinone radical can lead to generation of reactive oxygen species, which are important signal transducers, whereas hydroquinone is responsible for antioxidant defense.

### D. Enzymes

1. NADPH oxidases. The family of superoxide-generating NADPH oxidases, also named Nox or phox (phagocytic oxidase), is the best characterized class of enzymes belonging to t-PMET and includes at least seven proteins (Nox1 to Nox5, Duox1, and Duox2). The first recognized and most extensively studied member is Nox2, expressed in mammalian professional phagocytes and responsible for superoxide production during engulfment of invading microbes (49, 279). All members of this family are now recognized to have specific cellular and sub-cellular localizations, thus serving a variety of biological functions, including signal transduction, host defense, development, angiogenesis, blood pressure regulation, and biosynthetic processes (96, 215). They catalyze the monoelectronic reduction of external oxygen using NADPH as an internal electron donor, thus producing superoxide anions; superoxide can then dismute (spontaneously or with superoxide dismutase [SOD]) to hydrogen peroxide and oxygen, the former of which acts as a second messenger molecule.

*a. Structure and mechanism of action.* On the basis of their structures, the Nox enzymes are classified into three functional groups: (i) Nox 1–4, (ii) Nox5, and (iii) Duox1 and Duox2 (Table 1). Below, an update of the current knowledge about the mechanism of action of all members of this family is given.

*(1) Nox 1–4.* The prototype of the first group is Nox2, as many molecular and biochemical data on its structure and

Table 1. Characteristics of human NADPH Oxidase Family Members

| | *Locus* | *Chromosome* | *Exons* | *Amino acids* | *References* |
|---|---|---|---|---|---|
| $p22^{phox}$ | *CYBA* | 16q24 | 5 | 195 | 232 |
| $gp91^{phox}$ | *CYBB* | Xp21.1 | 13 | 570 | 232 |
| Nox1 | *NOH-1* | Xq22 | 13 | 564 | 50, 97 |
| Nox3 | *NOX3* | 6q25.1 | 12 | 568 | 294 |
| Nox4 | *NOX4* | 11q14.2 | 18 | 578 | 265 |
| Nox5 | *NOX5* | 15q23 | — | 737 | 23, 24 |
| Duox1 | *DUOX1* | 15q15.3 | 35 | 1551 | 227 |
| Duox2 | *DUOX2* | 15q15.3 | 34 | 1548 | 227 |
| $p47^{phox}$ | *NCF1* | 7q11.23 | 11 | 390 | 278 |
| Nox organizer 1 | *NOXO1* | 16p13.3 | 8 | 371 | 50, 97, 48 |
| $p67^{phox}$ | *NCF2* | 1q25 | 16 | 526 | 292 |
| Nox activator 1 | *NOXA1* | 9q34.3 | 14 | 476 | 50, 97, 149 |
| $p40^{phox}$ | *NCF4* | 22q13.1 | 10 | 339 | 56, 322 |

Nox, NADPH oxidase.

function are now available. The catalytic part of Nox2 is cytochrome b558, a heterodimer composed of two subunits, namely, $p22^{phox}$ (light chain) and $gp91^{phox}$ (heavy chain) (Fig. 5) (232). The genes encoding both subunits have been localized and characterized (Table 1). Mutations in the *CYBA* gene for $p22^{phox}$ lead to an autosomal form of CGD, whereas mutations in the *CYBB* gene for $gp91^{phox}$ account for all cases of X-linked CGD (79, 80); enhanced susceptibility to microbial infections and recurrent inflammation are the main hallmarks of this pathology (273). The nonglycosylated integral membrane protein $p22^{phox}$ is required for the correct folding of the complex: its C-terminal domain contains a proline-rich region serving as an anchoring site, thereby juxtaposing $gp91^{phox}$ and its regulatory proteins (see below). The catalytic core is represented by $gp91^{phox}$, which is a membrane-bound glycoprotein with six α-helical transmembrane segments in the N-terminus and a cytoplasmic domain in the C-terminal portion. NADPH- and FAD-binding sites are located in the C-terminal domain, whereas the transmembrane region contains two hemes; the third and fifth helices each contain two histidine residues, which represent the axial and distal ligands for the two iron ions, so that one heme group is placed toward the cytoplasmic face and the other toward the outer face of the plasma membrane. The way this complex is formed, therefore, allows a two-step electron flow, first from NADPH to FAD and then to the heme group; finally, electrons move from heme to molecular oxygen with the generation of superoxide anions.

Nox2, usually inactive in resting cells, becomes activated during phagocytosis of invading microbes, as a result of membrane translocation of a ternary regulatory complex, formed by $p47^{phox}$, $p67^{phox}$, and $p40^{phox}$ subunits, as well as of the small GTPase Rac (Fig. 5) (56). The molecular and biochemical features of genes encoding the three subunits are shown in Table 1 (2, 322). The $p47^{phox}$ organizer protein contains two SH3 domains, which cooperatively interact with the proline-rich-domain of $p22^{phox}$, thus promoting membrane translocation and oxidase activation (278). In resting cells, the two SH3 domains are masked by intramolecular interaction with an auto-inhibitory region. Upon cell stimulation, serine residues located in this region are phosphorylated, thus unmasking the SH3 domains. The $p67^{phox}$ "activator" protein contains four important and conserved domains: (i) a C-terminal SH3 domain, which mediates membrane translocation through binding to the proline-rich region present in the $p47^{phox}$ tail, (ii) an N-terminal domain containing four tetratricopeptide repeat motifs interacting with the GTP-bound form of Rac, (iii) an activation domain able to act on $p91^{phox}$ and (iii) a PB1 domain interacting with $p40^{phox}$, the third protein of the ternary complex (292). Binding of Rac is essential for oxidase activation, as it induces a conformational change in $p67^{phox}$, thus allowing activation domain to interact with the catalytic core of Nox2. In contrast, the PB1 domain is dispensable for oxidase activation, but is necessary for oxidase assembly, as it enhances $p67^{phox}$ and $p67^{phox}$ recruitment to the membrane (especially to the phagosomal membrane).

Other members of this group are all homologs of the $gp91^{phox}$ subunit of Nox2 (Table 1 and Fig. 5) (146). Nox1 is primarily expressed in colon epithelial cells, but it has also been detected in vascular smooth muscle cells, uterus, and prostate (277). Like Nox2, Nox1 associates with $p22^{phox}$ and requires both organizer (namely, NoxO1) and activator (namely, NoxA1) proteins, as well as Rac1, for its superoxide-generating activity (50, 97). Nox1 enzyme activity is finely regulated through phosphorylation of organizer and activator proteins (37). Unlike $p47^{phox}$, NoxO1 lacks the internal auto-inhibitory region and, therefore, does not require the phosphorylation-dependent conformational changes for activation (48). On the other hand, NoxA1 appears to be regulated by cAMP-dependent protein kinase (PKA)-mediated phosphorylation of Ser172 and Ser461. This phosphorylation of NoxA1 facilitates its binding to 14-3-3 proteins, which sequester it from the plasma membrane, and thus prevent its interaction with NoxO1 and Rac1 (149). PKA itself is activated by low levels of ROS, but is inhibited by strong oxidizing conditions. Thus, we can imagine a self-limiting feedback mechanism in which Nox1 activity initially produces sufficient ROS to enhance PKA activity, which, in turn, will limit Nox1 activity and ROS output (37).

Nox3 mRNA has been detected in several foetal tissues, including kidney, liver, lung, and spleen (49, 146), but its particular localization is in the inner ear. Although it can associate with $p22^{phox}$, it does not necessarily require an organizer and/or activator protein for its enzymatic activity (294).

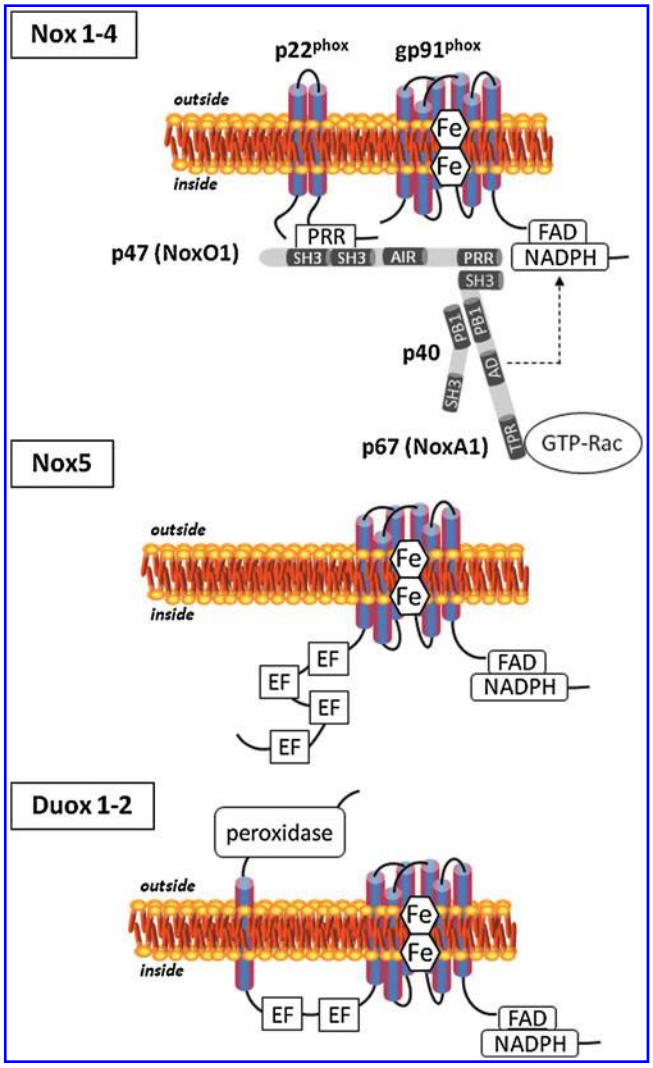


**FIG. 5. Schematic model of the structure of Nox 1–4, Nox5, and Duox enzymes.** Cylinders represent transmembrane domains. AD, activation domain; AIR, autoinhibitory region; EF, calcium-binding erectile function-hand domain; Fe, heme group; PB1, Phox/Bem1 domain; PRR, proline-rich region; SH3, Src homology 3 domain; TPR, tetratricopeptide repeat motifs. (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars).

Finally, Nox4 is highly expressed in kidney and vascular endothelial cells (5, 269). It is able to form a functional dimer with $p22^{phox}$, but, like its congener Nox3, it can also constitutively produce superoxide anions without the intervention of organizer and/or activator proteins (265).

*(2) Nox5.* Nox5 was originally found in testis, but it is also expressed in T- and B-lymphocytes (23). This oxidase is more distantly related to the other members of the family, as it shows an overall homology to $gp91^{phox}$ of only about 27% (23). In addition, it differs from other Nox isoforms in that its activity does not require the presence of accessory proteins and is fully dependent on elevation of the intracellular calcium concentration. The main structural characteristic of Nox5 is an N-terminal extension carrying three canonical and one noncanonical $Ca^{2+}$-binding EF-hands motifs (24) (Fig. 5); binding of $Ca^{2+}$ to these domains leads to conformational changes, thus promoting enzyme activation (24). Calcium sensitization is achieved *via* two main mechanisms: the first one involves the protein kinase C-dependent phosphorylation of Thr494 and Ser498 present in the FAD-binding domain (130), whereas the second one involves a calmodulin-binding site present in the NADPH-binding domain (286).

*(3) Duox 1–2.* Dual oxidases are mainly expressed in the membrane of thyroid glands (70). They are unusual, in that they produce $H_2O_2$ rather than $O_2^{\cdot -}$. Nonetheless, they have the basic structure of $gp91^{phox}$ enzymes (Table 1) (227); therefore, they are expected to produce superoxide anions, which are rapidly converted to $H_2O_2$ *via* intramolecular dismutation. According to the proposed mechanism, Duox1 and Duox2 possess an additional N-terminal peroxidase-like domain, located on the outer side of the membrane, where superoxide is usually released. This ecto-domain is separated from the $gp91^{phox}$-like portion by an additional trans-membrane segment and two EF-hands (Fig. 5). Therefore, Duox oxidases are also calcium responsive enzymes (10). Unlike Nox5, the two EF-hand motifs represent an auto-inhibitory domain: indeed, proteolytic removal of this region renders Duox enzymes fully $Ca^{2+}$-independent and irreversibly active. Thus, it is conceivable that, *in vivo*, calcium binding may induce reversible conformational changes that remove the auto-inhibitory domain from the active site (24).

*b. Biological functions.* Since distinct Nox isoforms show a tissue-specific distribution and cellular compartmentalization, they mediate distinct and specific functions. A detailed description of biological roles played by phox oxidases is given elsewhere and can be found in several recent reviews (42, 215); here, only a short summary of current knowledge is given.

Two main roles have been proposed for Nox1: immune defense and cell proliferation. Indeed, Nox1 appears to be upmodulated in guinea pig gastric pit cells in response to *Helicobacter pylori* lipopolysaccharides. Therefore, Nox1-mediated ROS release may serve for innate immune responses of gastric mucosa (139). Nox1 has also been implicated in hypertrophy, cell proliferation, and migration. In vascular smooth muscle cells, angiotensin II selectively activates Nox1 and, subsequently, the phosphatase SHP-2 and the kinase Akt, thus regulating vascular function (280). Migration in response to fibroblast growth factor (258) or platelet-derived growth factor (165) is impaired in Nox1 knock-out mice, thus providing evidence that Nox1 is a central mediator for smooth muscle cell migration.

Nox2 signaling has been extensively studied, given its physiological relevance in immune defense. Along with its well-established role in immune function, Nox2 has also been shown to be activated in endothelial cells by several stimuli, including vascular endothelial growth factor and thrombin, thus implicating Nox2 in new blood vessel formation (296, 297). Recently, Nox2 and other Nox isoforms have been shown to be involved in tumor cell proliferation (42, 137, 239).

Because of its typical expression in the inner ear, the most important function of Nox3 is to participate in normal vestibular functions. Indeed, mice with mutations in the gene coding for Nox3 show balance defects due to altered morphogenesis of otoconia, tiny biominerals required for the perception of motion and gravity (228).

By immunohistochemical and *in situ* hybridization, Nox4 has been found to localize primarily to the kidney, where it appears to play a role in oxygen sensing and regulation of erythropoietin synthesis. Further, by releasing ROS into the glomerular filtrate, it may act as an antimicrobial system or be involved in detoxification of urine wastes (167).

In testis, Nox5 may function to promote oxidative changes associated with sperm capacitation and acrosome reaction (17).

Finally, Duox1 and Duox2 are needed for thyroxine synthesis; indeed, loss of functional Duox2 is linked to the pathogenesis of congenital hypothyroidism (202). Duox1 and Duox2 supply $H_2O_2$ for the iodination step and cross-linking of thyroglobulin tyrosine residues, essential for synthesizing active thyroid hormones. Accordingly, Duox enzymes colocalize with thyroperoxidase in the plasma membranes of human thyrocytes, thus minimizing $H_2O_2$ leakage and optimizing working efficiency (272). In nonthyroid tissues, Duox proteins aid in defense and inflammation processes: in airways, they may supply $H_2O_2$ for lactoperoxidase-mediated generation of hypothiocyanite, a potent antimicrobial agent (87).

2. NAD(P)H:quinone oxidoreductase 1. The NAD(P)H: ubiquinone oxidoreductase (NQO1, EC 1.6.99.2), also named DT-diaphorase or QR1, is a cytosolic, homodimeric flavoprotein, which is overexpressed and translocated to the plasma membrane under oxidative conditions (127, 216).

*a. Structure and mechanism of action.* The human *NQO1* gene, located on chromosome 16, is ~20 kb in length and possesses six exons and five introns (131).

Crystallographic studies, performed on rat, mouse, and human NQO1, demonstrated that it consists of two identical subunits, each having a molecular weight of 30,000. The catalytic site forms a pocket at the dimer interface and the FAD prosthetic group is bound to each subunit (34). The shape of the catalytic site is such that it accomodates different ring-containing compounds, a characteristic shared by those enzymes that can metabolize a broad range of substrates. The electron donor (NADH or NADPH) and acceptor (quinone) share the same site, so that nicotinamides are bound and released during catalytic cycling, to allow substrate binding (34).

NQO1 catalyzes the obligatory two-electron reduction of quinones to hydroquinones, through a ping-pong mechanism. In particular, the reaction consists of a two-step hydride transfer, first from NAD(P)H to FAD and then from $FADH_2$ to the quinone (Fig. 6) (34, 170).

Kinetic analysis of NQO1 activity shows a substrate preference. Indeed, NQO1 has a similar $V_{max}$ for both NADH and NADPH, but the $K_m$ for NADH is twice that of NADPH (172). Nonetheless, in $\rho^0$ cells, where mitochondrial respiration is impaired and activity of t-PMET is enhanced (164, 255), the NADH/NADPH ratio is higher than that found in parental cells, thus raising the possibility that NADH is the major source of reducing cofactor for NQO1 (234).

NQO1 activity is efficiently inhibited by dicoumarol [3,3′-methylene-bis(4-hydroxycoumarin)] and dicoumarol analogs, which compete with NAD(P)H for binding to the enzyme (118). Being a selective inhibitor, dicoumarol has been widely used to measure specific NQO1 activity. These inhibitors are currently under investigation for their ability to suppress the malignant phenotype of pancreatic cancer cells, where NQO1 expression is upregulated (67, 219).

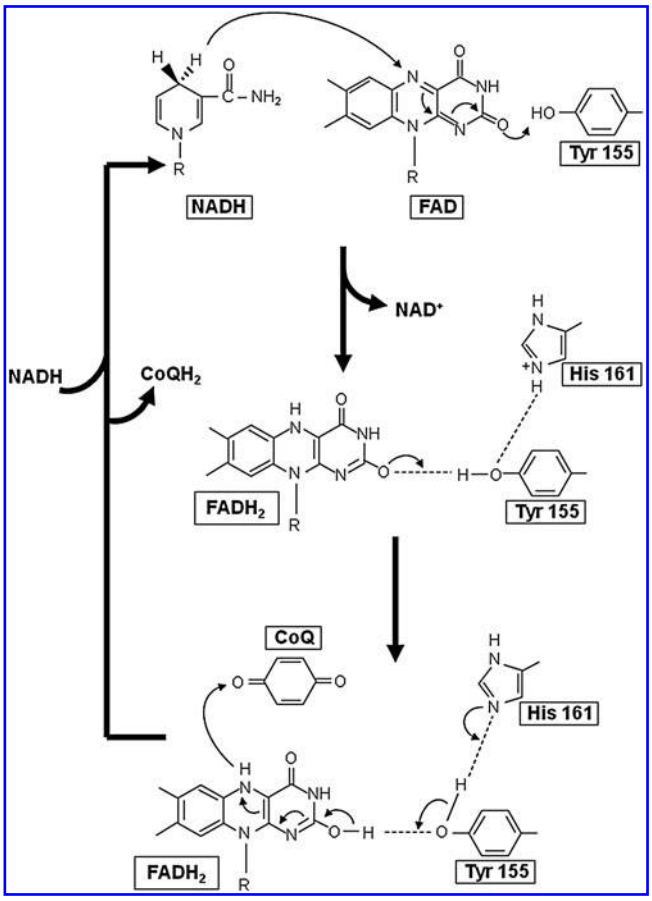


**FIG. 6. Schematic model for the reduction of CoQ by NQO1.**

*b. Biological functions.* NQO1 is a key enzyme involved in cellular defense against ROS, as the obligatory two-electron transfer avoids production of semiquinone, thus preventing the generation of superoxide and hydrogen peroxide (172). Further, it also possesses an NAD(P)H-dependent superoxide scavenging activity; thus, any potential radical produced by auto-oxidation of hydroquinones would be scavenged at its site of generation (261). In this way, NQO1 participates in t-PMET, facilitating the ubiquinone cycle and in-out transfer of electrons (301). It should be recalled, however, that, depending on the ring substituents of quinones, NQO1 activity may also result in production of redox-labile hydroquinones, which can undergo autoxidation to yield free radical species (284). For example, the NQO1-mediated reduction of menadione (2-methyl-l,4-naphthoquinone) has been shown to produce hydroxyl radicals and to induce single- and double-strand DNA breaks in human MCF-7 cells; this effect was reversed by the selective NQO1 inhibitor dicoumarol (221). Therefore, the protective functions of NQO1 cannot be viewed in a simplistic manner and its overall biological activity should be considered within complex metabolic pathways.

A second function ascribed to NQO1 is to act as a chemoprotective enzyme. Since it can reduce quinone-imines, nitro-, and azo-compounds, it participates in detoxification of

xenobiotics, which may otherwise promote cytotoxic and carcinogenic effects (170).

Third, NQO1 has the ability to modulate oncoprotein stability. Indeed, a regulatory role on the proteosomal degradation of proteins controlling cell growth, differentiation, and death (including p53, p73α and ornithine decarboxylase) has recently been demonstrated (13, 14). NQO1 knockdown with a specific siRNA or its inhibition with dicoumarol reduces p53 levels in human colon carcinoma cells. The observation that proteasome inhibitors prevent this effect suggests that p53 levels diminish because of increased degradation. Moreover, NQO1-overexpressing cells are resistant to dicoumarol-triggered degradation of p53, thus indicating a direct involvement of NQO1 in p53 stabilization. Hence, inhibition of NQO1 and induction of p53 degradation protect cells from apoptosis (14). These data may explain why individuals expressing a polymorphic inactive NQO1 are more susceptible to tumor development.

3. Disulfide-thiol exchangers. Disulfide-thiol exchangers (ENOXs) are a family of cell surface proteins exhibiting a putative time-keeping hydroquinone (NADH) oxidase activity and a protein disulfide-thiol interchange activity (206).

They are referred to as ENOX proteins because of their localization on the outer side of the plasma membrane (206) and to distinguish them from the phox-Nox proteins of host defense. This external location of ENOX proteins has been demonstrated both in plant and animal tissues. Much of the work on ENOXs was carried out by Morré and colleagues, who, in human cervical carcinoma (HeLa) cells, first described an NADH oxidase activity efficiently inhibited by the cell-impermeant antitumor agent, sulfonylurea, *N*-(4-methylphenylsulfonyl)-*N*′-(4-chlorophenyl)urea (211). Later on, they provided other experimental data supporting this external localization, including immuno-histochemistry (51), inhibition by specific antibodies (51), and direct enzymatic assays performed on intact plant and animal tissues (113, 148), as well as on right side-out plasma membrane vesicles isolated from HeLa cells (113, 148). These proteins are not exclusively associated with the plasma membrane, but instead can be released into the extracellular space. Indeed, soluble isoforms have been shown to be shed into culture medium conditioned by the growth of HeLa cells (311). They have also been detected in biological fluids, including sera (305), saliva (204), and urine (316).

*a. Structure and mechanism of action.* At least three members of this family have been described so far: (i) the constitutive NADH oxidase (ENOX1 or cNOX), expressed in normal cells and located on chromosome 13q14.11 (133), (ii) the tumor-associated NADH oxidase (also known as ENOX2 or tNOX), found on the surface of invasive cancer cells and located on chromosome Xq25-q26.2 (52), and (iii) the age-related NADH oxidase (also named arNOX), which seems to be related to the aging process, as it is present only in individuals after the age of 30, in late passage cultured cells and in senescent plants (213). This last activity has been detected in skin, the buffy coat fraction of blood, serum, saliva, sweat, and urine from aged patients. The first two isoforms have been cloned in the last decade (GenBank accession no. EF432052 and AF207881, for ENOX1 and ENOX2, respectively) and partially characterized, whereas the third one is an ENOX-like activity, whose sequence has not yet been identified (213). In particular, human ENOX1 shares 64% identity and 80% similarity with ENOX2; both proteins contain an NADH-binding site, a disulfide-thiol interchange site and potential copper-binding sites (133).

These enzymes can be distinguished on the basis of their responses to hormones, growth factors, and certain quinone-site inhibitors (210). Unlike ENOX2, the constitutive NADH oxidase is hormone and growth factor responsive. Indeed, rat liver plasma membranes incubated with growth factors (transferrin and epidermal growth factor) or hormones (insulin and pituitary extract) exhibit a 2–3-fold increase in NADH oxidase activity with respect to basal activity (41). Another difference between ENOX1 and ENOX2 is that the former is refractory to the quinone-site inhibitors, including capsaicin (8-methyl-*N*-vanillyl-6-noneamide), as the putative anticancer drug-binding site is absent (133). The NADH oxidase activity of rat liver plasma membranes is largely unaffected by capsaicin, whereas the NADH oxidase activity of HeLa plasma membranes is strongly inhibited (210). The results suggest a difference in response to capsaicin between the NADH oxidase activity of normal and transformed cells and tissues that correlates with the inhibition of growth and induction of apoptosis in transformed cells. Recently, we showed that, in human platelets, ENOX1 expression may be translationally modulated by capsaicin-evoked signaling: upregulation of ENOX1 expression appears to be achieved *via* activation of transient receptor potential cation channel, subfamily V, member 1 (TRPV1) (the natural receptor for capsaicin) and generation of ROS (253). The possibility that ENOX1 is a redox-sensitive protein may be of importance in terms of electron movement during platelet activation and cell-to-cell interactions (76).

ENOX proteins differ from all other NAD(P)H oxidases in that they are able to reduce pyridine nucleotides in the absence of bound flavin (53). ENOX1 and 2 lack iron or iron sulfur clusters, but sequence analysis reveals motifs that could be potential copper binding sites, necessary for electron transfer to external acceptors, such as molecular oxygen (133). Finally, ENOXs have been reported to have properties of prions, including stability, protease resistance, and ability to form amyloid (140).

Controversial suggestions about the mechanism of action of ENOXs have been proposed, based on results obtained with different assays. Morré *et al.* stated that the natural substrate is plasma membrane hydroquinone rather than NADH directly and the hydroquinone-derived electrons are used for reduction of external oxygen to water (Fig. 7) (152). They based their statement on the observation that (i) the NADH binding site is on the outside of the cell where there is usually little or no NADH, (ii) ENOX proteins in general do not carry out electron transfer to CoQ10 even in the presence of NADH, and (iii) the soluble form of ENOX2 seems to be a dimer containing four coppers, thus carrying out four electron transfers to molecular oxygen as required to form water (133, 152). On the other hand, others have linked NADH oxidation to WST-1 reduction at the cell surface (28), reaching different conclusions. Berridge's group suggested that ENOX is a single multifunctional enzyme, able to direct both trans and surface NADH oxidase activities (256). They stated that ENOXs accept reducing equivalents either from membrane-bound hydroquinone or extracellular NADH and that these electrons, depending on the surrounding milieu, can reduce external molecules, such as

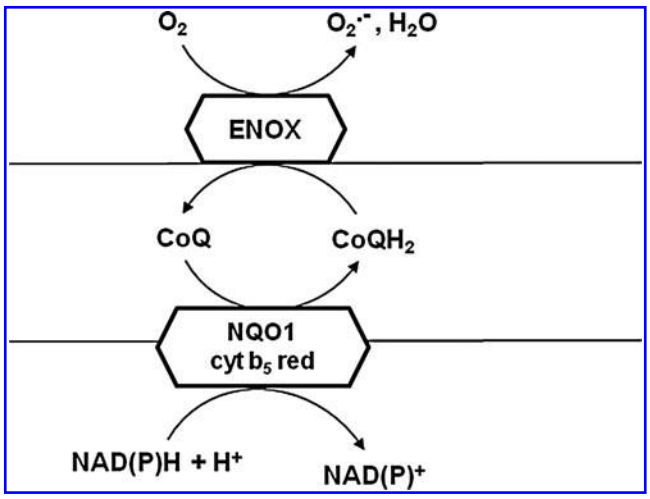


**FIG. 7. NAD(P)H oxidase activity of ENOX proteins.** Electrons coming from NAD(P)H are donated to external molecular oxygen; CoQ acts as a shuttle to transport electrons across the cell membrane. cyt $b_5$ red., NADH:cytochrome $b_5$ reductase.

oxygen, and/or plasma membrane molecules, such as ubiquinone. This hypothesis is corroborated by the observation that extracellular NADH can block cell surface oxygen consumption (109) and can increase reduction of a cell-permeant artificial electron acceptor (166). From these findings, it appears clear that more biochemical studies are needed to fully address the exact functions of ENOX enzymes.

ENOXs exhibit a second catalytic activity involving protein disulfide-thiol interchange (Fig. 8), although the CXXC motif (common to most members of the protein disulfide isomerase family) is missing. Although not universally accepted (256), the two activities have been claimed to alternate with a period length that is characteristic for each family member: ENOX2 exhibits oscillations with period lengths of about 22 min, 2 min shorter than those of ENOX1 and 4 min shorter than those of arNOX (205).

The arNOX protein seems to catalyze electron transfer, which ultimately leads to generation of superoxide. In aged cell cultures and plasma membranes prepared from aged plant tissues, this activity, measured by reduction of ferricytochrome c, is inhibited in the presence of SOD, thus corroborating the involvement of superoxide formation in the mechanism of action of arNOX (213). The electron donor for the superoxide-generating activity is plasma membrane ubiquinone, whereas the soluble form of the enzyme appears to use protein thiols as substrates (212). Despite the fact that this activity may resemble that of an NADH-cytochrome b5 reductase, it shares several features with known ENOX proteins: it is resistant to protease digestion (proteinase K) and heat shock (70°C–80°C); moreover, it shows the typical oscillation pattern of ENOXs (213). This activity is not inhibited by capsaicin or (−) epigallocatechin gallate, but can be inhibited by CoQ10 (214).

*b. Biological functions.* Several lines of evidence underline that ENOX1 is involved in at least two important cell functions: (i) it appears to drive the enlargement phase of cell growth and (ii) it appears to be component of a biological clock. The protein disulfide-thiol interchange activity may be involved in controlling cell growth. After division, the resultant cells must enlarge before dividing again; a variety of correlative and experimental data show that cell enlargement and ENOX activity are correlated. In particular, cell enlargement requires the formation of disulfide bonds in membrane proteins and, therefore, the disulfide–thiol interchange activity of ENOX proteins may be fundamental (308). The last biological function, mainly ascribed to ENOX1, is the regulation of circadian oscillatory system. The two enzymatic activities catalyzed by ENOX1 alternate within a 24-min period: the first activity rests after 12 min and the second one begins in a cycle that confers a time-keeping function to the protein. This oscillation pattern (which is also entrainable and temperature compensated) may be clock-related. Morré and colleagues found that cells transfected with mutated ENOX cDNAs, which have oscillation period lengths of 22, 36, or 42 min, exhibited circadian period lengths of 22, 36, or 42 h for circadian biochemical markers (53, 207). They also found that oscillations require bound copper and are recapitulated in solution by copper salts (209). The finding that mutations in a single protein (ENOX1) change the circadian period of an established cellular circadian marker (glyceraldehyde-3-phosphate dehydrogenase) from 24 to 22, 36, or 42 h suggests some relationship between ENOXs and the cellular biological clock. Yet, the relationship to the complex architecture of the

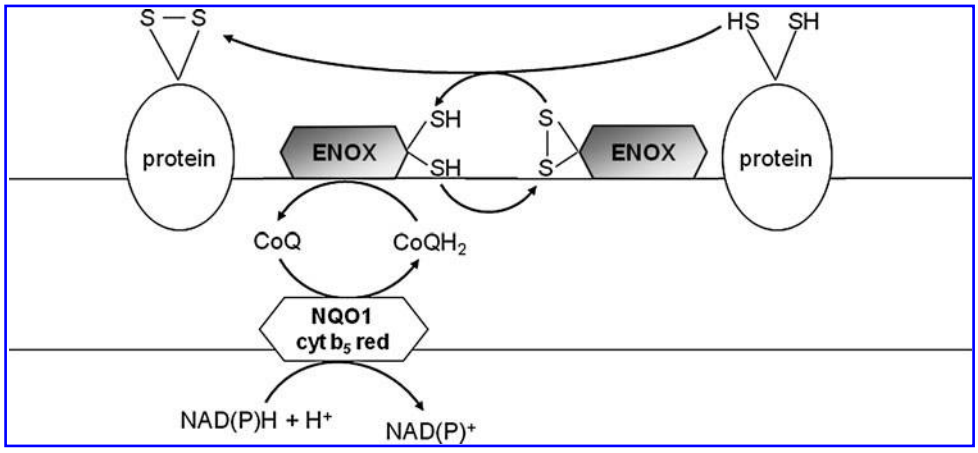


**FIG. 8. Protein disulfide-thiol interchange activity of ENOX proteins.** Electrons coming from CoQ are needed for reduction of thiol groups either of ENOX itself or of other membrane proteins.

circadian time-keeping system and attendant net-worked interactions remains to be taken into account (295).

4. Voltage-dependent anion-selective channels. Voltage-dependent anion-selective channels (VDACs, also called porins) represent a family of 30–35 kDa integral membrane proteins, first identified in the outer mitochondrial membrane (57). The recent finding that VDAC1, the best-characterized member of this family, possesses NADH-ferricyanide reductase activity (20) has led to including it among the components of t-PMET.

*a. Structure and mechanism of action.* Up to now, three highly conserved genes have been shown to encode distinct isoforms, named *VDAC1* (location: 5q31), *VDAC2* (location: 10q22), and *VDAC3* (location: 8p11.2).

The tertiary structure of human VDAC1 has recently been solved by a hybrid approach, integrating complementary information from NMR spectroscopy and X-ray crystallography (26). The protein is composed of 19 $\beta$-barrel, membrane-spanning strands with both the N and C termini oriented to the same side of the barrel. In particular, the N-terminus is a $\alpha$-helix folded horizontally midway within the pore. NMR studies also suggested that the protein may exist in monomer/dimer equilibrium with a predominance of the monomer in solution (26). Similar folding properties are likely shared by other VDAC proteins, as they have >80% sequence identity, at least in certain species, including mammals and fungi (26).

*b. Biological functions.* The major function of VDAC proteins is to control metabolite trafficking between the cytosol and mitochondria (27), by forming pores freely permeable to low molecular-weight molecules (including ADP, ATP, succinate, and citrate) (27). In addition, VDAC1 has been shown to play a role in the release of apoptogenic proteins from mammalian mitochondria (318). In the early stages of the apoptotic process, mitochondrial permeability is disrupted, because of the opening of the permeability transition pore (PTP), a channel localized at the inner/outer membrane contact site (40). As a result, loss of inner membrane potential, osmotic swelling of mitochondria, and release of intermembrane space proteins (such as cytochrome c and APAF-1) occur, thus driving the assembly of the apoptosome in the cytosol (4). The identity of proteins involved in PTP formation is still uncertain and controversial (31): although several articles pointed out the involvement of VDAC proteins in the formation of PTP (180, 266, 267, 291), VDAC-deficient mice have been shown to be indistinguishable from their wild-type counterparts in the ability to undergo permeability transition and cell death in response to different inducers of apoptosis (16, 154). Therefore, although being dispensable in mitochondrial cell death, VDAC could play a role in regulating PTP function.

Several experimental data have documented that VDAC1 is also present in the plasma membrane (20, 164, 285), thus opening new questions on its physiological role. Gonzalez-Gronow and colleagues (101) have demonstrated that VDAC1 is expressed on the plasma membrane of human endothelial cells, where it functions as a receptor for human plasminogen kringle 5, a structural domain (derived from the proteolytic cleavage of plasminogen) involved in regulation of endothelial cell proliferation. Indeed, binding of plasminogen kringle 5 to endothelial cells can be antagonized by anti-VDAC1 antibodies and, further, purified VDAC reconstituted into liposomes is able to directly bind this structural domain (101). Up to now, the most recognized function of VDAC1 in the plasma membrane is as a component of t-PMET. First, inspection of the amino acid sequence of human VDAC1 reveals two putative NADH binding motifs (residues 143–152 and 270–278), whose amino acid sequence is very similar to that of the NADH binding domain of alcohol dehydrogenase (20). Consistent with a function as a reductase, VDAC1 also possesses two cysteine residues, which appear to be essential for the catalytic activity, since addition of thiol chelators inhibits ferricyanide reduction (20). In addition, purified VDAC1 is able to reduce the cell-impermeant ferricyanide, by using NADH (but not NADPH) as the intracellular electron donor (20). Finally, the redox function of VDAC1 may also occur *in vivo*, as the overexpression of membrane-targeted VDAC1 leads to an enhanced rate of whole cell ferricyanide reduction (20). However, it should be recalled that other options may exist, including the possibility that VDAC acts as a channel directly releasing intracellular reducing equivalents into the medium (74). Although there is now no doubt that VDAC proteins are expressed at the plasma membrane and that their expression is associated with ferricyanide reduction, further research is needed to finally clarify the biological function of plasma membrane VDACs *in vivo*. Indeed, the effects attributed to VDAC may arise from VDAC itself or from its assembly with other proteins that drive specific functions (74).

How can a pore-forming protein (potentially lethal) be present in the plasma membrane? One possibility is that VDAC1 exerts two distinct functions on the basis of its cellular localization, namely, anion channel activity in the mitochondrion and NADH:ferricyanide reductase activity in the plasma membrane. In mice, this ability may rely on the finding that VDAC1 exists as two different proteins, generated by the use of alternative first exons (Fig. 9) (20, 44). Indeed, one isoform expresses a leader peptide in its N-terminus and, thus, is targeted to the secretory pathway, *via* the Golgi apparatus, whereas the second one, lacking this sequence, is directed into the mitochondrial membrane (44). However, the human homolog of such a leader peptide has not been found yet, although it may be not necessary for plasma membrane targeting (74). Other explanations for two distinct functions include (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. To date, the second option seems the more plausible, as VDAC1 purified from both plasma membrane and mitochondria possess reductase activity *in vitro* (20). The finding that VDAC1 expression shows a punctuate distribution pattern in the plasma membrane suggests that this protein may have a subdomain localization, thus preventing any harmful function (20, 74).

5. Duodenal cytochrome b. Duodenal cytochrome b (Dcytb, also known as Cybrd1), belonging to the widespread cytochrome b561 family, is a novel component of t-PMET. Both Dcytb mRNA and protein are induced in response to hypoxia and iron deficiency, indicating an important role in iron metabolism (196).

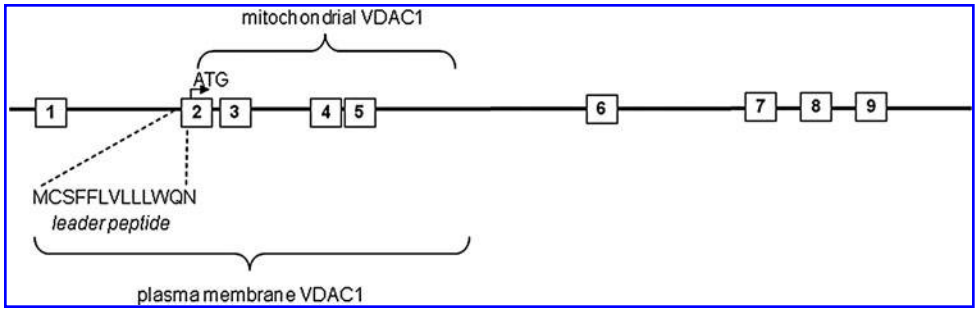


**FIG. 9. Alternative mRNAs encoding mitochondrial and plasma membrane mouse VDAC1.** Numbered squares indicate VDAC1 exons; mitochondrial-VDAC1 mRNA starts four bases into the second exon (*indicated as ATG arrow*), whereas plasma membrane-VDAC1 begins 39 bases 5′ adjacent to this point (leader peptide).

Dcytb was originally identified in duodenal enterocytes as a ferrireductase involved in dietary iron uptake (196). More recently, it has also been identified in the plasma membrane of other cell types, including astrocytes, erythrocytes, hepatocytes and epithelial cells (22, 196, 276, 293), indicating a more general role. One of the biological functions ascribed to Dcytb is to reduce extracellular AFR, thus maintaining ascorbic acid in the reduced state. In particular, the plasma membrane AFR reductase activity appears to be crucial for maintaining physiological Asc concentrations within biological fluids (*e.g.*, plasma, interstitial, and cerebrospinal fluid). This reduction is vital in quenching free radicals at the cell surface, and, given the role of extracellular AFR in apoptosis induction (71, 249), the regulation of extracellular AFR concentration may also play an important role in the control of cell growth.

*a. Structure and mechanism of action.* The tertiary structure of Dcytb has not yet been solved, presumably owing to difficulties related to purification of this integral membrane protein. Recently, however, an initial physical characterization of a recombinant form of the human Dcytb has been performed; electron paramagnetic resonance and magnetic CD confirmed that Dcytb is a monomeric protein binding two heme groups, as previously suggested by the phylogenetic relationship of this protein to the cytochrome b561 family (175).

The protein is composed of 286 amino acids (molecular weight: 32,000) and organized in six transmembrane domains; it has seven histidyl residues, four of which (His50, His86, His120, and His159) are highly conserved, thus likely representing heme ligand candidates. The protein possesses reductase activity, measured by both nitro-blue tetrazolium and ferric nitrilotriacetic acid reduction assays (196).

It is now widely accepted that reduction of extracellular AFR relies primarily on one of the t-PMET pathways, which starts from cytosolic Asc to extracellular acceptors. AFR reductase activity has been shown to be widely distributed phylogenetically, being found in numerous plants, animals, and protists (11). AFR reductase (EC 1.6.5.4) has been purified from plant sources (39, 68), as well as from *Neurospora crassa* (259), since there is a high concentration of the enzyme in these cells. Mammal AFR reductase has not been purified or fully characterized. Previously, its activity has been attributed to the cytochrome $b_5$/cytochrome $b_5$ reductase system (107) and, only recently, Dcytb has emerged as the most likely candidate for Asc-dependent transmembrane electron transfer (222, 275, 276).

Modeling studies have demonstrated that Dcytb shows partial conservation of Asc and AFR binding sites, first identified in cytochrome b561 and shown to be necessary for the Asc-to-AFR electron transfer in neuroendocrine secretory vesicles (89, 275). In addition, the midpoint reduction potentials of the two hemes, contained in the Dcytb protein, show that they can be reduced by Asc, thus establishing that the vitamin is a likely intracellular electron donor. The Asc binding site maps to the cytosolic side of the plasma membrane, whereas the AFR binding site is on the extracellular side, thus corroborating the hypothesis that Dcytb may act as a transplasma membrane Asc:AFR oxidoreductase (Fig. 10A) (275). In line with this observation, animals able to synthesize ascorbic acid do not express Dcytb on erythrocyte plasma membranes, suggestive of an adaptation toward Asc recycling, when the ability to endogenously produce the vitamin is missing (157, 276).

Alternative electron acceptors for Dcytb are extracellular ferric or cupric ions (Fig. 10B) (197, 314). The iron and copper reductase activities are enhanced when cells are loaded with DHA, which leads to increased levels of intracellular Asc, and this effect is reverted in the presence of phloretin, a glucose transporter inhibitor that blocks DHA uptake (314).

Finally, flavonoids, such as quercetin, have been shown to be alternate substrates to Asc for Dcytb. Therefore, at their physiological concentrations, they can modulate enzymatic activity of Dcytb, thus potentially enhancing iron uptake and also maintaining an optimal extracellular reducing potential.

*b. Biological functions.* Because of its expression on the brush border of duodenal enterocytes alongside the divalent

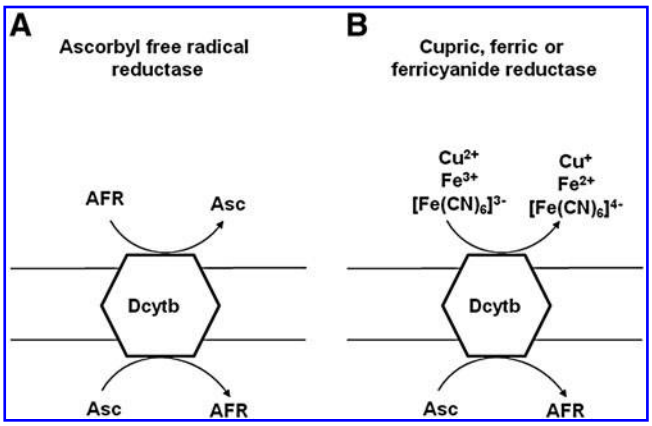


**FIG. 10. Ascorbyl free radical (A) and cupric, ferric, or ferricyanide (B) reductase activities displayed by Dcytb.**

metal ion transporter 1, Dcytb is believed to play a primary role in the uptake of dietary nonheme iron (196). Indeed, transfection experiments demonstrated that overexpression of Dcytb on plasma membrane is able to stimulate iron absorption, but the requirement for Dcytb does not appear to be essential, since Dcytb-knockout mice do not display iron deficiency (106). Overexpression of Dyctb also stimulates copper reduction, thus suggesting its involvement in intestinal copper uptake, as well.

6. Cytochrome $b_5$ reductase. Cytochrome $b_5$ reductase (EC 1.6.2.2), also named diaphorase-1 or methemoglobin reductase, catalyzes the one-electron reduction of ferricytochrome $b_5$ to ferrocytochrome $b_5$, by using NADH as the preferred physiological electron donor (125, 233, 274).

*a. Structure and mechanism of action.* Cytochrome $b_5$ reductase is encoded by the *CYB5R3* locus (previously known as *DIA1* gene), located on chromosome 22q 13-qter (287). This gene (32 kb in length), consisting of nine exons and eight introns, encodes for two isoforms of cytochrome $b_5$ reductase: a membrane-bound (~35 kDa) and a soluble (~32 kDa) variant, respectively. These two isoforms share the same catalytic domain and differ only in the N-terminal hydrophobic sequence. The N-terminal domain appears to be myristoylated, thus anchoring the reductase to cellular membranes (including endoplasmic reticulum, mitochondria, and nuclear and plasma membranes) (226). The soluble form may be generated either by alternative splicing or, alternatively, by post-translational processing of the membrane-bound form. Indeed, the second exon contains the junction between the membrane-binding domain and the catalytic domain, which might represent a site for proteolysis (287). However, an alternative first exon, which might be directly translated into the soluble form, has been identified in the human cytochrome $b_5$ reductase gene (82).

Defects in either transcription or translation of the human *CYB5R3* gene lead to recessive congenital methemoglobinemia, a disease that may occur in two forms (type I and type II methemoglobinemia) (235). Type I, caused by alterations in the erythrocytic, soluble isoform of cytochrome b5 reductase, is relatively well tolerated and primarily manifested as cyanosis. On the other hand, type II methemoglobinemia is caused by defects in the membrane-associated isoform of cytochrome b5 reductase, expressed in many somatic cells, and represents a more severe disease, characterized by cyanosis, neurological, and skeletal abnormalities and premature death (129, 260).

The tertiary structure of porcine, rat, and human cytochrome $b_5$ reductases (33, 218), determined by X-ray crystallography, shows that this enzyme is folded in a manner similar to other flavin-dependent oxidoreductases, such as ferredoxin: $NADP^+$ reductase and phthalate dioxygenase reductase (58, 138). Indeed, it is composed of two functional lobes: an amino-terminal domain (residues 33–147) containing the FAD-binding site, and a carboxyl-terminal domain (residues 171–300) containing the NADH binding site. The two domains are linked by a small flexible hinge region (residues 148–170), which is important for maintaining the critical protein architecture required for enzyme activity.

Cytochrome $b_5$ reductase catalyzes two coupled one-electron reduction reactions, by transferring an electron pair from NADH to the flavine ring of the FAD prosthetic group and, then, to two molecules of cytochrome $b_5$ (33, 150). In particular, two electrons are first transferred from NADH to FAD by hydride transfer and, subsequently, are donated, one by one, to cytochrome $b_5$, which represents the one-electron acceptor; the reduction of FAD appears to be the rate-limiting step in electron transfer (Fig. 11) (274).

*b. Biological functions.* The membrane associated enzyme, expressed in almost all mammalian cells, plays key roles in cell biology, being involved in fatty acid elongation and desaturation (145, 225), cholesterol synthesis (241), and hydroxylation of xenobiotics, such as hydroxylamine and amidoxime compounds (155). In erythrocytes, cytochrome $b_5$ and cytochrome $b_5$ reductase induce methemoglobin reduction, thus maintaining hemoglobin in its reduced state (125, 233). Finally, the enzyme also reduces AFR back to Asc (270).

In the plasma membrane, cytochrome $b_5$ reductase catalyzes the reduction of ubiquinone, since the levels of cytochrome $b_5$ are very low (302). Reduced CoQ directly or indirectly (by regenerating several antioxidant molecules, including Asc and α-tocopherol) prevents lipid peroxidation chain reactions and oxidative damage. In line with these findings, vitamin E deficiency and growth factor withdrawal increase both ubiquinone and its reductases in the plasma membrane, and this system is also involved in healthy aging triggered by caloric restriction and in apoptotic cell death (217).

Synaptic plasma membranes show a high rate of membrane trafficking during neuronal activity, and the elevated frequency of membrane fusion events between presynaptic vesicles and the neuronal plasma membrane could allow for an enrichment of cytochrome $b_5$ reductase in these cellular compartments. This would supply a rationale for the levels of Asc-dependent NADH oxidase activity observed in synaptic plasma membrane vesicles, which appear to be higher than those reported for the plasma membrane of other mammalian cells (187, 250). It is now accepted that cytochrome $b_5$ reductase accounts for most of this activity, as already demonstrated in non-neuronal mammalian cells (99, 251, 302).

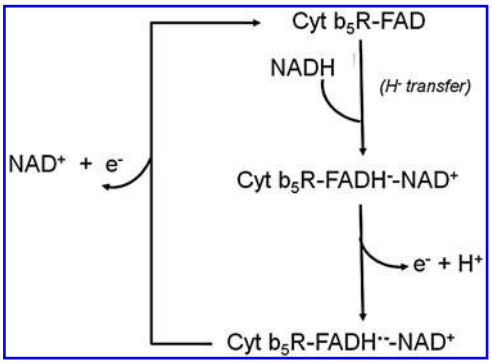


**FIG. 11. Schematic model for hydride transfer from NADH to FAD catalyzed by Cyt $b_5$R.** At first, two electrons are transferred from NADH to FAD by hydride ($H^-$) transfer; thereafter, the reduced enzyme $NAD^+$ complex (Cyt $b_5$R-$FADH^-NAD^+$) transfers two electrons to two one-electron acceptors one by one, *via* the anionic semiquinone form (E-$FAD^-NAD^+$), and the oxidized enzyme restarts the catalytic cycle. Cyt $b_5$R, cytochrome $b_5$ reductase.

Indeed, synaptic plasma membrane vesicles treated with cathepsin D, which releases the membrane-bound isoform of cytochrome $b_5$ reductase into the culture medium (38), show decreased Asc-dependent NADH oxidase activity, to the same extent as after treatment with anticytochrome $b_5$ reductase or anticytochrome $b_5$ antibodies (251). The reductase has been shown to be associated with specialized membrane microdomains, termed lipid rafts, and enriched at interneuronal contact sites in the neuronal soma. In the presence of anticytochrome $b_5$ reductase antibodies, binding to raft components is prevented. Conversely, anticaveolin-2 (a typical lipid raft marker) antibodies are able to inhibit NADH oxidase activity of neuronal plasma membranes. Therefore, membrane-bound cytochrome $b_5$ reductase forms redox centers associated with the neuronal plasma membrane and localized in the lipid rafts. The finding that this enzyme is largely present at the interneuronal contact sites raises the possibility that alterations of these redox centers may affect neuron–neuron interactions and cell survival (251). Finally, the activity of neuronal plasma membrane cytochrome $b_5$ reductase can be modulated by changes in the cytosolic content of cytochromes $b_5$ and c, although it is approximately threefold more sensitive to changes in the former. So, the reduction of oxidized cytochrome c (released from mitochondria) by cytochrome $b_5$ reductase may account for depletion of NADH observed at the early stages of apoptosis in cerebellar granule neurons (186).

## IV. Physiological Roles of t-PMET

One of the ubiquitous functions of t-PMET is to cooperate with intracellular redox pairs (like pyruvate and lactate) to maintain the cytoplasmic $NAD^+$/NADH ratio. The oxidation/reduction cycle of pyridine coenzymes (fluctuating in response to metabolic changes) plays a crucial role in regulating intracellular energy levels and redox homeostasis, as well as the redox state of the microenvironment surrounding cells (143, 171). Indeed, the $NAD^+$/NADH balance is required for ensuring ATP biosynthesis. Although cellular ATP levels are usually maintained by oxidative phosphorylation, in the presence of mitochondrial dysfunction, compensatory mechanisms (such as the pyruvate/lactate couple and enhanced t-PMET activity) are established to allow cell survival. Consistently, higher activity of t-PMET can be seen in mitochondrial gene knock-out $\rho^0$ cells (161, 255), as well as in lymphocytes derived from diabetes mellitus patients, which lack fully functional mitochondria (168).

Besides this general function, more specific roles can be proposed. Most of them have been described above, where we have tried to ascribe the specific enzyme responsible for the biological function considered. Other examples, not previously described in this review, are reported below.

### A. Proton pumping

t-PMET is correlated with modulation of internal pH and redox homeostasis, as it is able to activate proton release, a biological function that strictly requires the presence of ubiquinone in the plasma membrane (65). Proton movement is carried out by the $Na^+/H^+$ antiport, which allows $Na^+/H^+$ exchange across the membrane. This is an active process, since $Na^+$ ions have to be pumped out of the cell by $Na^+/K^+$ ATPase, which obtains energy from ATP hydrolysis. The involvement of t-PMET has been established by the finding that the $Na^+/H^+$ antiport activity increases when t-PMET is activated and, on the contrary, its activity is lowered by inhibitory CoQ analogs (62). Recently, the presence of a proton-driven ATP synthase on the surface of the plasma membrane of normal and tumoral cells has been reported; although yet debated, this enzyme may contribute for controlling intracellular pH changes. Indeed, Mangiullo *et al.* showed that $H^+$ efflux from the cytoplasm is responsible for ectopic ATP synthesis associated with alkalinization of the cytoplasmic space (181). Thus, t-PMET may cause internal and external pH alterations, as well as development of an inside negative membrane potential; both changes then drive many cellular functions, including cell-volume sensing and regulation, mitogenic/apoptogenic signaling, and transport across membranes (63).

### B. Sperm maturation and fertilization

Mammalian spermatozoa show an ROS-generating activity inversely related to their state of functional maturity. Indeed, lucigenin-dependent chemiluminescence and WST-1 reduction have been described in sperm suspensions; the observed activity is insensitive to mitochondrial chain inhibitors, but it is responsive to inhibition by SOD, capsaicin, and *N*-ethyl maleimide (6, 300). Although enzymes accounting for this redox activity are still unclear, cytochrome b5-reductase and cytochrome P450-reductase have been shown to be responsible, at least in part, for the NADH- and NADPH-dependent oxidase activities, respectively (18, 19). In addition, identification of the Nox2 complex in mouse spermatozoa (271), Duox in human spermatozoa (21), and Nox5 in equine spermatozoa (248) supports the involvement of t-PMET in sperm functions. In particular, t-PMET appears to be physiologically important for promoting the molecular events associated with sperm capacitation, sperm–zona interaction, and acrosome reaction (242). Further, since mitochondrial function is often defective in human spermatozoa, upregulation of t-PMET may occur to maintain an optimal $NAD^+$/NADH ratio.

Another biological function concerns the involvement of t-PMET in sperm-oocyte fusion during fertilization. Sea urchin eggs have been shown to express a homolog of Duox1 (named Udx1) at the cell surface of zygote (313). This dual oxidase generates hydrogen peroxide, necessary for crosslinking the fertilization envelope proteins, thus providing a structural block to polyspermy, after fertilization (313). It is tempting to speculate that a similar mechanism may also function in other species.

### C. Blood redox homeostasis

The activity of t-PMET is also critical for redox homeostasis in blood, since all cellular components are affected by changes in redox state. In particular, a close link between t-PMET and metabolic status of erythrocytes has been reported (141, 142). Indeed, glucose appears to be required for ferricyanide reduction and, moreover, the presence of ferricyanide causes small changes in the metabolic flux of erythrocytes, especially increasing the pentose phosphate pathway (115, 141, 183). In addition, the glycolytic enzymes phosphofructokinase, aldolase, and GAPDH have been shown to bind the membrane anion transport protein (band 3), thus being localized in the

plasma membrane as a multimeric complex (45, 198); the assembly appears to be a function of hemoglobin saturation, since a closer association can be found in oxygenated conditions (169). In deoxygenated erythrocytes, the enzymes lose their association with band 3 so that the glycolytic flux is enhanced; as a result, hypoxic red blood cells show reduced ability to recycle NADPH and GSH through the pentose phosphate pathway and, therefore, the membrane thiol pool is depleted, leading to oxidative stress (55, 245). In hypoxic conditions the activation of t-PMET may serve to compensate the impaired pentose phosphate pathway, thus ensuring a functional reducing capacity; in these conditions, t-PMET may use Asc as electron donor, since NADH derived from enhanced glycolysis is preferentially utilized by met-hemoglobin reductase (245).

Erythrocyte t-PMET has been shown to be affected in uremic patients, showing enhanced oxidative stress and impaired antioxidant defenses. In addition, a single hemodialysis session triggers a dual response: removal of toxic compounds exerts a positive action on protein-free sulfhydryl groups, but, at the same time, it transiently reduces erythrocyte t-PMET and glutathione levels, as well as plasma vitamin C content (190). Therefore, therapeutic strategies aimed at improving erythrocyte t-PMET efficiency may be useful to restore the reducing capacity in chronic uremia.

### D. Platelet physiology

t-PMET also appears to be fundamental in platelets, as activation of the coagulation pathways strictly depends on membrane activities, which are also required for the cross-talk between participating cells (platelets, monocytes, neutrophils, and endothelium). To date, the best characterized components of platelet t-PMET are Nox2 and ENOX1 (253, 264). Alterations in redox-state lead to dramatic changes in platelet functions, and thus play a key role in the pathogenesis of several diseases, including essential hypertension, sickle cell disease, preeclampsia, thalassemia, thrombotic vascular occlusion, and cardiovascular disorders (8, 9, 15). For instance, vitamin C has been shown to protect membrane components susceptible to free radical damage and to regulate ROS-mediated membrane signaling. Indeed, vitamin C down-regulates platelet CD40L, a transmembrane pro-inflammatory and pro-thrombotic protein implicated in the initiation and progression of atherosclerosis, by scavenging $O_2^{\cdot -}$ generated by Nox activation (236). Further, it has physiological relevance in aggregation and postclotting events. We found that intracellular Asc concentrations dose dependently modulate the redox state of surface thiols, essential for activation of $\alpha$IIb/$\beta$3 integrin in platelets, as well as platelet–fibrin clot strength and stability (254). Due to its relevance in platelet physiology, vitamin C uptake is controlled through translational modulation of its carrier; moreover, the plasma membrane AFR reductase activity prevents extracellular Asc autoxidation, thus contributing to stabilization of reduced vitamin C and, consequently, to modulation of the redox state in the microenvironment (15).

### E. Autophagy

Autophagy is an innate defense mechanism responsible for degradation of cytoplasmic components, as well as clearance of aberrant proteins and invading pathogens (200). Recently, Huang's group reported that toll-like receptors, which are membrane microbial sensing receptors, promote Nox2 assembly on phagosomes; ROS generated by Nox2 mediate recruitment of the autophagy machinery to phagosomes, thus promoting their maturation and microbial killing (124). The role played by Nox family members in regulating autophagy has been further suggested by the findings that (i) the autophagy targeting of phagosomes is impaired in Nox2-knockout neutrophils and (ii) ROS production by other Nox enzymes is necessary for autophagic targeting of *Salmonella Typhimurium* in nonphagocytic cells (123, 124). Since several autophagy-related genes have been found to be associated with inflammatory bowel disease and patients with CGD develop similar clinical and pathological features of inflammatory bowel disease, it should be attractive to study the pathogenesis of these two types of diseases, to identify probable common mechanisms.

### F. Antibody control

ROS generation by the plasma membrane enzymes is also involved in the oxidative unmasking of auto-antibodies in healthy individuals (195). Crane and Löw proposed that oxidative modification of antibodies can occur through the action of both NADH and NADPH plasma membrane oxidases able to generate superoxide and/or hydrogen peroxide; for example, the peroxidase activity of Duox proteins, being present on the outer surface of the cell, may be available to antibodies, thus playing a regulatory role in infections of the airway and intestinal epithelium (60). Further, redox imbalance may play a role in natural killer–cell-mediated cytotoxic activity and cytokine production, thus modulating immune system homeostasis (303).

The influence of t-PMET on immune responses should represent an important determinant in autoimmune diseases, such as rheumatoid arthritis and systemic lupus erythematosus, characterized by chronic overproduction of ROS, which in turn lead to generation of neo-antigenic determinants able to activate T and/or B cells (104, 182). Thus, understanding the role of t-PMET in autoimmune diseases is a highly attractive field of research, especially considering its therapeutic potential.

## V. Pathological Roles of t-PMET

Loss or overstimulation of t-PMET leads to distinct pathologies. In the next section, literature reports suggesting the involvement of t-PMET in various pathological conditions are described.

### A. Apoptosis

Apoptosis is a process involved in body homeostasis and tissue development. The process must be tightly regulated, since defective apoptotic progression has been implicated in several diseases: excessive apoptosis causes hypotrophy, whereas an insufficient amount results in uncontrolled cell proliferation, such as cancer (for recent reviews, see 120, 156, 305).

To date, ever increasing evidence stresses the involvement of t-PMET in regulating the apoptotic machinery. Inhibition of t-PMET causes ROS generation, which in turn leads to a

pro-oxidant environment at the plasma membrane and promotes apoptosis; cells overexpressing Bcl-2 are protected from this kind of cell death (312). In the same way, cells overexpressing VDAC1 on the plasma membrane are protected from apoptosis induced by anticancer drugs (*e.g.*, etoposide) or inhibitors of t-PMET (including capsaicin and resiniferatoxin) (164). Thus, plasma membrane VDAC1 may be targeted to (or stabilized at) the plasma membrane by the action of Bcl-2, as in the mitochondrial membrane. In line with the involvement of plasma membrane VDAC1 in cell death, it has recently been reported that both channel opening and NADH-ferricyanide reductase activities are activated in a hippocampal cell line undergoing apoptosis, thus suggesting that control of redox homeostasis and anion efflux may be essential during neuronal cell death (84). Further, activation of VDAC1 during apoptosis appears to be related to differentiation, since it can be observed in differentiated hippocampal neurons, but not in embryonic neural stem cells (7). Because of its involvement in regulating apoptosis, it is tempting to speculate that VDAC1 may be a pharmacological target, useful in treating apoptosis-related pathologies, such as cancer (188).

The vanilloids capsaicin and resiniferatoxin induce apoptosis in certain cell types by a variety of mechanisms, mainly depending on their vanillyl moiety (252). Indeed, they inhibit mitochondrial and plasma membrane electron transport (223, 312), induce ROS generation (178), depolarize mitochondria (75), and trigger apoptosis in transformed and activated cells (179). Some cellular effects depend on interaction with vanilloid receptors (namely, the transient receptor potential vanilloid subtype 1 or TRPV1), located on the inner side of the plasma membrane (135). Vanilloids/TRPV1 binding mostly occurs in cells with high expression levels of the receptor: killing of TRPV1-transfected cells by vanilloids is rapid and does not induce ROS generation and disruption of mitochondrial membrane potential, thus supporting the notion that it is likely to be mediated by a necrotic pathway. Conversely, in cells with low expression of TRPV1, vanilloids can trigger receptor-independent death, showing the typical hallmarks of apoptosis; in this situation, they act as ubiquinone analogues, thereby altering the functionality of t-PMET (179). The potential involvement of t-PMET in cell death is further suggested by the finding that $NAD^+$/NADH and $CoQ/CoQH_2$ ratios are important modulators of neutral sphingomyelinase, which catalyzes the generation of ceramide from sphingomyelin (73, 184, 185). Accumulated ceramide molecules self-associate, forming ceramide-enriched membrane platforms, which allow the clustering of death receptors and transmission of an efficient death signal into cells (Fig. 12); ubiquinone can inhibit neutral sphingomyelinase, thus avoiding ceramide accumulation and cell death (Fig. 12) (184). To date, few data about the mechanism of sphingomyelinase inhibition are available: the inhibition may be due to a direct effect of hydroquinone on the enzyme or to the inhibition of lipid peroxidation, a factor indirectly involved in sphingomyelinase activation (185).

### B. Cancer

Normal tissues derive most of their energy by metabolizing glucose to carbon dioxide and water, through mitochondrial

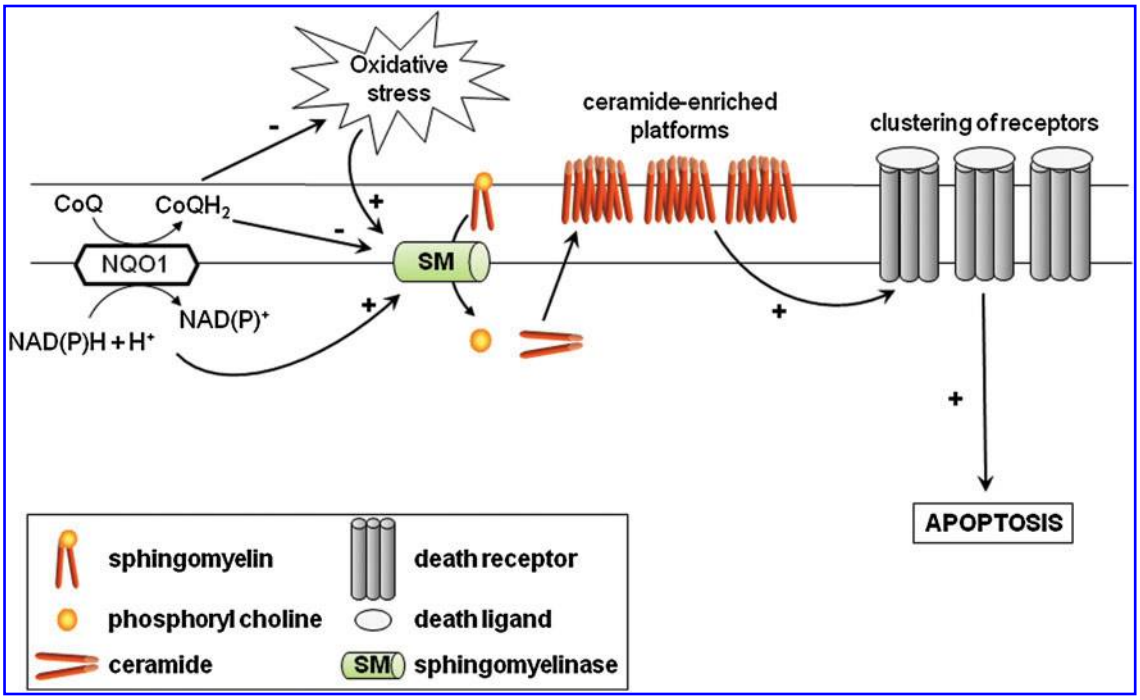


**FIG. 12. Model of ceramide-triggered clustering of death receptors and modulation of ceramide generation by t-PMET.** Different stimuli promote activation of SM, thus leading to production of ceramide plus phosphoryl choline; consequently, ceramide-enriched membrane microdomains are formed, thus promoting clustering of death receptors and signal transmission into the cell. $NAD^+$/NADH and $CoQ/CoQH_2$ ratios, together with redox balance, selectively modulate SM activity. +, positive regulation; −, negative regulation; SM, sphingomyelinase. (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars).

oxidative phosphorylation. Conversely, cancer cells (as well as normal tissues with a high proliferative rate) convert glucose to lactate regardless of whether oxygen is present. This phenomenon, described by Warburg in the early 1900s (known as the "Warburg effect") (309), emphasizes that cancer cells rely less on mitochondrial activity than their normal counterparts. More recently, it has been proposed that the Warburg effect is an adaptive mechanism: tumor cells reprogram themselves from oxidative to glycolytic metabolism, to use lactate as a fuel for ensuring survival, whereas nutrients are incorporated into the biomass (*e.g.*, nucleotides, amino acids, and lipids) needed to produce a new cell (263, 298). Up to now, it is not fully understood what triggers the switch from a mitochondrial to a glycolytic metabolism: inadequate oxygenation occurring within tumors and activation of the hypoxia-inducible transcription factor (HIF) have been implicated, as well as a more complex homeostatic system linking signaling pathways regulating both cell proliferation and metabolic control (262, 298).

The role of t-PMET in cancer biology relies on the observation that the metabolic changes described above may perturb key redox couples, including the NAD(P)H/NAD(P)$^+$ and $CoQH_2$/CoQ ratios, which play a pivotal role in supporting cell survival, function, and growth of fast proliferating cells. Indeed, a finely tuned control of the intracellular redox and bioenergetic state is critical for cell viability, and is guaranteed by both the inner mitochondrial membrane, containing the components of the respiratory chain, and the plasma membrane, containing the components belonging to t-PMET. Cancer cells usually exhibit elevated t-PMET activity, although a negative correlation between tumor malignancy and t-PMET has been described as well (78, 109). In some tumor cell lines, involvement of the plasma membrane in oxidation of NADH and cellular oxygen consumption has also been reported (109, 282); blocking this pathway compromises the viability of rapidly proliferating cells that almost exclusively rely on t-PMET.

It has been suggested that ENOX2 levels may be associated with the ability of tumor cells to acquire an aggressive phenotype. Mouse embryonic fibroblasts derived from transgenic mice overexpressing a truncated form of ENOX2 showed accelerated growth rates with respect to wild-type fibroblasts and enhanced invasiveness (315). In contrast, decreased cell growth and migration, as well as increased cell death, are observed when the function of ENOX2 is impaired, either by inhibiting the catalytic activity with (3)-epigallocatechin-3-gallate, capsaicin, phenoxodiol, or anti-ENOX2 antibodies (47, 72, 306, 315) or by down-modulating its expression by RNA interference (173). Morré and colleagues also viewed ENOX2 as both (i) a noninvasive, circulating tumor marker (based on their description of several cancer-type specific ENOX2 isoforms) (119, 307) and (ii) a potential antitumoral drug target, since this enzyme is blocked by quinone site inhibitors, which also exhibit anticancer activity (54, 119, 208). However, it should be recalled that many of these compounds are rather unspecific, and the primary cellular target(s) of their action remain largely unknown; for example, phenoxodiol is poorly water soluble and, thus, it is likely to act at the level of plasma membrane, but it may target sites other than t-PMET. In addition, Herst *et al.* refused the tumor-specific action of phenoxodiol, but instead they stated that sensitivity to the drug is related to dependency on t-PMET, regardless of the transformation status of the cell; for this reason, the authors claim that concentrations effective in compromising the proliferation of tumor cells can exert immunosuppressive effects, as the drug is also able to inhibit growth of primary immune cells (111).

NQO1 is also overexpressed in some tumors (such as colorectal, breast, liver, and nonsmall cell lung carcinomas) and, further, it can activate quinolic chemotherapeutic drugs (including mitomycins and anthracyclines), thus suggesting a potential exploitation of this enzyme as a target for developing antitumor compounds. As an example, $\beta$ lapachone is a nontoxic quinone that, once reduced and activated by NQO1, becomes toxic to several cells (237); a synthetic analogue of $\beta$ lapachone (ArQule 501) is currently in phase II clinical trials, as an anticancer drug (237).

Finally, some Nox isoforms have been implicated in early stages of carcinogenesis. For example, it has been shown that oncogenic transformation of mammalian cells by K-Ras is achieved *via* transcriptional upregulation of Nox1 (199); oxidase activity and ROS production are, then, functionally required for maintaining the transformed phenotype. In particular, Nox1-generated ROS oxidize the low-molecular-weight tyrosine phosphatase, thus impairing its activity; as a result, Rho activity is inhibited and thereby the signaling cascade regulating actin stress fiber and focal adhesion formation is disrupted (268). The finding that several tumors show enhanced expression of distinct Nox isoforms supports a potential role for these enzymes in cancer biology: up-modulation of Nox1 has been documented in human colon cancer (163), as well as in gastric neoplasms and transformed keratinocytes (46, 288), whereas a potential role for Nox4 in pancreatic cancer and for Nox5 in Barrett esophageal adenocarcinoma has recently been suggested (92, 201). Intriguingly, Duox1 and Duox2 seem to act according to a distinct mechanism, as their expression is silenced in lung cancer. Since Duox enzymes are involved in airway homeostasis and wound healing (310), their silencing may promote carcinogenesis by exacerbating pro-inflammatory responses and tissue injury (176).

The importance of t-PMET in cancer cell survival suggests that its targeting may be useful for anticancer drug development. Prata and colleagues (238) have recently reported that, in human leukemic cells, t-PMET is a primary site of action of new anticancer compounds [3-(2-chloro-5-methoxy-6-methyl-3-indolylmethylene)5-hydroxy-1,3-dihydroindol-2-one, 3-[(2,6-dimethylimidazo[2,1-b]-thiazol-5-yl)methylene]-5-methoxy-2-indolinone and guanylhydrazone of 2-chloro-6-(2,5-dimethoxy-4-nitrophenyl)imidazo[2,1-b]thiazole-5-carbaldehyde] with antiproliferative activity; therefore, specific targeting of t-PMET may be indicated for treatment of leukemia, perhaps in combination with standard chemotherapeutic drugs. However, it should be recalled that other molecules contributing to redox homeostasis and involved in cellular transport of chemotherapeutic drugs (such as RLIP76) can also be viewed as targets for cancer therapy.

### C. Cardiovascular diseases

A role for the t-PMET system, and in particular the central function of Nox-driven generation of superoxide anion, in cardiovascular diseases, including hypertension, atherosclerosis, and vascular complications of diabetes, is likely. Several studies carried out in experimental models, as well as in

human subjects, have addressed the involvement of vascular and phagocytic Nox in development and progression of hypertension. In a rat model of hypertension, obtained by chronic infusion of angiotensin II, a positive correlation between $p22^{phox}$ expression (and consequent Nox activity) and the hypertensive phenotype was found (93). The same association has been observed in the aorta of spontaneously hypertensive rats, with vascular wall hypertrophy and endothelial dysfunction (320). Hypertensive patients show increased Nox-dependent superoxide production in vascular smooth muscle cells, lymphoblasts, and mononuclear cells (90, 289). In addition, silencing of $p22^{phox}$ by antisense technology has been shown to powerfully reduce superoxide production and vascular smooth muscle cell hypertrophy induced by angiotensin II (294). All these findings suggest that modulation of $p22^{phox}$ expression by humoral and hemodynamic factors (83, 189), as well as the genetic background of individuals (321), may be critical determinants for the susceptibility of hypertensive patients to oxidative stress.

t-PMET may also regulate atherogenesis by controlling the redox state, and, therefore, the redox-dependent signaling pathways, in endothelial cells. Hyperhomocysteinemia has been proved to stimulate ferricyanide reductase activity and cytochrome b5 reductase expression, thus providing a potential link between t-PMET, oxidative stress, and endothelial dysfunction (244); likewise, Jessup's group found that enhanced t-PMET activity induces low-density lipoprotein oxidation (25), thus suggesting that upregulation of t-PMET may be numbered among atherogenic factors. Accordingly, a recent article (66) showed that the beneficial effects of statins on cardiovascular diseases may rely on their ability to reduce expression and activity of different members of the Nox family—Nox1, Nox2 and Nox4—thus opening new avenues for therapeutic interventions. Regular physical exercise training has also been shown to improve endothelium-dependent vasodilatation, by downregulating Nox subunits, especially $gp91^{phox}$, $p22^{phox}$, and Nox4 (3). Conversely, a potential protective role of t-PMET has been reported by Lee and colleagues, who provided evidence that oxidized phospholipids, accumulating in atherosclerotic lesions, activate t-PMET components (NQO1 and ENOX1), which, in turn, lead to NAD(P)H depletion and increased oxidative stress. As a result, the redox-sensitive transcription factor nuclear factor (erythroid-derived 2)-like 2 drives the transcription of heme oxygenase-1 gene, a cell-protective antioxidant enzyme (166).

### D. Aging

When ubiquinone-dependent electron transport is disrupted, t-PMET becomes a source of ROS, triggering oxidative stress and apoptosis; this phenomenon has been proposed to be one of the factors responsible for onset of age-related pathologies (217). At the same time, age-dependent increase in activity of erythrocyte t-PMET has been observed (243); although more work is needed, this finding suggests that t-PMET may exert a protective role by maintaining optimal levels of plasma antioxidants. In line with this hypothesis, caloric restriction, a common intervention able to delay age-related oxidative damage (319), increases the amounts of CoQ10 and $\alpha$-tocopherol, as well as the activity of several NAD(P)H oxidoreductases, in the plasma membrane of aged rats (69, 174). Hence, upregulation of t-PMET would be useful to decrease oxidative stress and confer an antiaging, stress-resistant phenotype, thus extending lifespan (128).

As well as being able to produce superoxide, arNOX activity may also be a crucial player in the onset of aged-related pathologies. Indeed, aged individuals expressing arNOX show increased production of ROS, which can propagate the aging cascade to adjacent cells and blood components. For example, arNOX-derived ROS can induce oxidative changes in circulating lipoproteins and cell membranes, thus leading to atherosclerosis and cell damage (144, 204). Superoxide-generating activity displaced by arNOX can be efficiently lowered by CoQ10 supplementation (180 mg/day for 28 days) and this inhibition correlated with subject age, being greatest between ages 60 and 65 (214). The mechanism whereby CoQ10 blocks arNOX-mediated generation of superoxide is unclear, although a specific effect on superoxide formation is suggestive. Indeed, CoQ10 does not inhibit either protein disulfide-thiol interchange activity or hydroquinone oxidation (212). The finding that arNOX activity is efficiently inhibited by CoQ10 provides a rational basis for developing new antiaging drugs, especially in highly susceptible tissues (144, 213).

### E. Obesity, metabolic syndrome, and diabetes

Obesity represents a worldwide nutritional problem, as it dramatically increased during the past 20 years and, moreover, it is often accompanied by an increased risk of mortality and morbidity. An unbalanced redox state has been implicated as one of the key factors leading to obesity-associated complications, such as the metabolic syndrome and diabetes. Thus, obesity may be viewed as a state of chronic oxidative stress, characterized by enhanced levels of ROS and impaired antioxidant defenses. t-PMET is likely to participate in the chronic oxidant/antioxidant unbalance during hyperglycemia and hyperinsulinemia observed in the context of metabolic syndrome (105). Indeed, Nox1, Nox2, and/or Nox4 have been shown to be implicated in pathways leading to steatosis and insulin resistance in the liver, as well as to pancreatic $\beta$-cell dysfunction, thereby allowing progression from the metabolic syndrome to type 2 diabetes (105). Accordingly, activity and expression of Nox2 increase in subjects with the metabolic syndrome, thus suggesting that this enzyme also contributes to inflammation leading to hepatic and pancreatic damage (91). In addition, hyperglycemia leads to accumulation of advanced glycation end products, which, in turn, drive intracellular signaling that activates Nox2, further enhancing ROS production (103). Studies carried out in obese mice demonstrated that inhibition of Nox activity reduces adipokine deregulation in adipose tissue, hepatic steatosis, and diabetes, thereby opening new avenues for therapeutic approaches (94).

NQO1 expression levels are also positively correlated with adiposity, insulin sensitivity, and markers of liver dysfunction. Indeed, a study conducted on 24 obese subjects showed that upregulation of NQO1 expression occurs in human adipocytes and that this modulation is correlated with the size of adipocytes (229). In addition, during diet-induced weight loss, NQO1 mRNA levels significantly decrease and parallel changes in anthropometric measurements of obesity, measurements of glucose tolerance, and markers of hepatic steatosis (229). These data are in line with studies carried out on NQO1 knockout mice that demonstrated a role for this enzyme in fat storage and metabolism (95).

In those tissues involved in energy metabolism (liver, muscle, and fat tissue), NQO1 expression levels are also related to ROS production, which usually occurs in obesity and diabetic conditions. The promoter of the NQO1 gene contains an antioxidant response element sequence, to which the transcription factor nuclear factor (erythroid-derived 2)-like 2 binds in response to ROS signaling (122). Studies performed in diet-induced obesity (DIO) and ob/ob mice showed that $\beta$-lapachone, a high-affinity substrate of NQO1, leads to higher $NAD^+$/NADH ratio and increased mitochondrial fatty acid oxidation (126).

Although attractive, a clear cause–effect relationship between t-PMET and obesity-related pathologies is lacking. Moreover, metabolic syndrome and diabetes are multifactorial pathologies strictly related to individual biological profiles, so that targeting of a single factor may be insufficient to reach a positive result.

### F. Neurodegenerative diseases

Progressive and irreversible loss of cognitive function is one of the most common manifestations characterizing neurodegenerative disorders in the elderly. The earliest clinical symptoms of disease onset are a mild cognitive impairment, usually associated with increased oxidative stress in affected brain regions.

Much experimental evidence suggests that some superoxide-generating Nox isoforms may be involved in the pathogenesis, as well as in clinical progression, of neurodegeneration (36). For instance, in the brain of patients affected by Alzheimer's disease membrane localization of $p47^{phox}$ is increased and, at the same time, amyloid-$\beta$ peptides can enhance Nox activity (132). Consistently, antisense knockdown of $p22^{phox}$ inhibits amyloid-$\beta$ peptides-induced neuronal apoptosis, whereas genetic deletion of $gp91^{phox}$ counteracts cognitive decline characterizing transgenic mice overexpressing the amyloid precursor protein (132, 231). Bruce-Keller and colleagues (43) have recently demonstrated that Nox expression and activity are specifically upregulated in vulnerable brain regions of subjects affected by mild cognitive impairment, a preclinical manifestation of Alzheimer's disease. Paradoxically, other studies reported that deficiency in Nox activity is associated with impaired cognitive function and memory. This phenomenon has been documented in patients and in mouse models of CGD (153, 230). This could be explained assuming that increased Nox activity may represent a compensatory mechanism to preserve cognitive functions. However, an abnormal activity of Nox may result in an opposite effect, leading to memory impairment and dementia (43).

### G. Pulmonary fibrosis

About 5 million people worldwide are affected by pulmonary fibrosis, a disease characterized by replacement of normal lung parenchyma with fibrotic tissue, as a result of failed tissue regeneration and epithelial repair. The incidence of the disease is usually age dependent as patients are commonly in their forties and fifties when diagnosed (240). It is a weakening disease, since thickening of tissue causes an irreversible decrease in oxygen diffusion capacity.

Recently, the Nox4 isoform has been shown to be involved in tissue repair functions and fibrogenesis. Indeed, transforming growth factor-$\beta$1 (TGF-$\beta$1), a typical pro-fibrogenic mediator, up-modulates Nox4 expression and activity in lung mesenchymal cells; Nox4 is also upregulated in injured lungs of mice and in humans affected by idiopathic pulmonary fibrosis (108). Nox4-mediated production of $H_2O_2$ is necessary for TGF-$\beta$1-induced myofibroblast differentiation, synthesis of extracellular matrix proteins (such as fibronectin and pro-collagen I) and contractility, as Nox4 knock-down by RNA interference is able to abrogate all TGF-$\beta$1-triggered effects (108). Finally, in murine models of lung injury, genetic, or pharmacologic targeting of Nox4 exerts a powerful antifibrotic effect (108). Therefore, this study proposed a central role for Nox4 during tissue fibrogenesis, so that targeting this enzyme may represent a therapeutic approach to treat human fibrotic disorders.

### H. Asthma

Asthma is a multifactorial disease characterized by inflammation of the airways that has been linked to increased ROS production by inflammatory cells, such as neutrophils, eosinophils, and macrophages, as well as by epithelial and endothelial cells. A large body of evidence suggests that Nox enzymes are the major source of ROS during inflammatory conditions, in airway and pulmonary artery smooth muscle cells (151). For example, Nox activity is necessary for eosinophil recruitment and infiltration in the lung, in experimental models of asthma (1). Moreover, a case–control study, consisting of 305 patients with clinically manifested asthma and 311 healthy subjects, showed a significant association between asthma and the specific *CYBA* haplotype for $p22^{phox}$, denoted $CYBA_3$ (−930G/242T/640A) (117). How genetic variability in the *CYBA* gene might be related to susceptibility to bronchial asthma (or its related phenotypes) remains to be elucidated.

## VI. Conclusions

Cell plasma membranes have complex signaling systems for regulating cellular metabolism. In particular, t-PMET can be seen as a crucial component supporting energy generation. Its upregulation maintains optimal levels of $NAD^+$, required for glycolytic ATP production under conditions of lowered mitochondrial respiratory activity (as it occurs during aging or in respiratory deficient $\rho^0$ cells) (161). In addition, increased activity of t-PMET may produce superoxide anions and/or hydrogen peroxide, which act as signaling molecules regulating cell growth. Many of the enzymes belonging to t-PMET are inducible systems, activated by a variety of extracellular effectors (such as growth factors, cytokines, and hormones), so that ROS production oscillates in response to the needs of different tissues. In addition, it is now recognized that many components of t-PMET are linked to cell metabolism. ENOX1 expression and activity are regulated by redox changes in human blood platelets (253), whereas the activity of erythrocyte t-PMET appears to be associated with the activity of glycolytic enzymes. Intracellular pH has also been shown to modulate t-PMET activity. Indeed, pharmacological blockade of $Na^+/H^+$ exchangers has been shown to inhibit the Asc-stimulated t-PMET activity in both erythrocytes and astrocytes (159).

Although many investigations have been carried out in the last decades, more information about specific signaling pathways remains to be elucidated. In particular, oxygen consumption at the cell surface raises some questions about

the molecular nature and the exact mechanism of action of the involved enzymes. As an example, cell surface superoxide production may not derive from the action of any of the Nox isoforms, at least in some cell types. In addition, nonmitochondrial oxygen consumption has been shown to be inhibited by extracellular NADH in several glycolytic cancer cell lines (109), whereas the oxygen burst observed in activated platelets and leukocytes was demonstrated to be stimulated by the presence of exogenous NADH (77). Superoxide production may occur *via* selective activation of different t-PMET components or another unrelated t-PMET pathway, as well as through one-electron leakage from a cell surface oxidase during surface oxygen consumption. Crane and Löw (60) have also suggested that ENOX proteins, possessing protein disulfide isomerase activity and copper binding motifs, may be involved in conversion of any superoxide formed by the transmembrane oxidases into hydrogen peroxide. Given involvement of t-PMET in several pathological conditions, it is mandatory to understand, in the future, the relative contribution of each oxidase system to ROS generation; this will be useful to design novel therapeutic approaches.

## References


1. Abdala-Valencia H, Earwood J, Bansal S, Jansen M, Babcock G, Garvy B, Wills-Karp M, and Cook-Mills JM. Nonhematopoietic NADPH oxidase regulation of lung eosinophilia and airway hyperresponsiveness in experimentally induced asthma. *Am J Physiol Lung Cell Mol Physiol* 292: L1111–L1125, 2007.
2. Abo A, Boyhan A, West I, Thrasher AJ, and Segal AW. Reconstitution of neutrophil NADPH oxidase activity in the cell-free system by four components: p67-phox, p47-phox, p21 racl and cytochrome b-245. *J Biol Chem* 267: 16767–16770, 1992.
3. Adams V, Linke A, Krankel N, Erbs S, Gielen S, Mobius-Winkler S, Gummert JF, Mohr FW, Schuler G, and Hambrecht R. Impact of regular physical activity on the NAD(P)H oxidase and angiotensin receptor system in patients with coronary artery disease. *Circulation* 111: 555–562, 2005.
4. Adrain C and Martin SJ. The mitochondrial apoptosome: a killer unleashed by the cytochrome seas. *Trends Biochem Sci* 26: 390–397, 2001.
5. Ago T, Kitazono T, Ooboshi H, Iyama T, Han YH, Takada J, Wakisaka M, Ibayashi S, Utsumi H, and Iida M. Nox4 as the major catalytic component of an endothelial NAD(P)H oxidase. *Circulation* 109: 227–233, 2004.
6. Aitken RJ and Baker MA. Oxidative stress and male reproductive biology. *Reprod Fert Dev* 16: 581–588, 2004.
7. Akanda N, Tofighi R, Brask J, Tamm C, Elinder F, and Ceccatelli S. Voltage-dependent anion channels (VDAC) in the plasma membrane play a critical role in apoptosis in differentiated hippocampal neurons but not in neural stem cells. *Cell Cycle* 7: 3225–3234, 2008.
8. Amer J and Fibach E. Oxidative status of platelets in normal and thalassemic blood. *Thromb Haemost* 92: 1052–1059, 2004.
9. Amer J, Ghoti H, Rachmilewitz E, Koren A, Levin C, and Fibach E. Red blood cells, platelets and polymorphonuclear neutrophils of patients with sickle cell disease exhibit oxidative stress that can be ameliorated by antioxidants. *Br J Haematol* 132: 108–113, 2006.
10. Ameziane-El-Hassani R, Morand S, Boucher JL, Frapart YM, Apostolou D, Agnandji D, Gnidehou S, Ohayon R, Noël-Hudson MS, Francon J, Lalaoui K, Virion A, and Dupuy C. Dual oxidase-2 has an intrinsic $Ca^{2+}$-dependent $H_2O_2$-generating activity. *J Biol Chem* 280: 30046–30054, 2005.
11. Arrigoni O, Dipierro S, and Borraccino G. Ascorbate free radical reductase, a key enzyme of the ascorbic acid system. *FEBS Lett* 125: 242–244, 1981.
12. Arroyo A, Kagan VE, Tyurin VA, Burgess JR, de Cabo R, Navas P, and Villalba JM. NADH and NADPH-dependent reduction of coenzyme Q at the plasma membrane. *Antioxid Redox Signal* 2: 251–262, 2000.
13. Asher G, Bercovich Z, Tsvetkov P, Shaul Y, and Kahana C. 20S proteasomal degradation of onithine decarboxylase is regulated by NQO1. *Mol Cell* 17: 645–655, 2005.
14. Asher G, Tsvetkov P, Kahana C, and Shaul Y. A mechanism of ubiquitin-independent proteasomal degradation of the tumour suppressor p53 and p73. *Genes Dev* 19: 316–321, 2005.
15. Avigliano L, Savini I, Catani MV, and Del Principe D. Trans-plasma membrane electron transport in human blood platelets. *Mini Rev Med Chem* 8: 555–563, 2008.
16. Baines CP, Kaiser RA, Sheiko T, Craigen WJ, and Molkentin JD. Voltage-dependent anion channels are dispensable for mitochondrial-dependent cell death. *Nat Cell Biol* 9: 550–555, 2007.
17. Baker MA and Aitken RJ. The importance of redox regulated pathways in sperm cell biology. *Mol Cell Endocrinol* 216: 47–54, 2004.
18. Baker MA, Krutskikh A, Curry BJ, Hetherington L, and Aitken RJ. Identification of cytochrome b5-reductase as the enzyme responsible for NADH-dependent lucigenin chemiluminescence in human spermatozoa. *Biol Reprod* 73: 334–342, 2005.
19. Baker MA, Krutskikh A, Curry BJ, McLaughlin EA, and Aitken RJ. Identification of cytochrome p450-reductase as the enzyme responsible for NADPH-dependent lucigenin and tetrazolium salt reduction in rat epididymal sperm preparations. *Biol Reprod* 71: 307–318, 2004.
20. Baker MA, Lane DJ, Ly JD, De Pinto V, and Lawen A. VDAC1 is a transplasma membrane NADH-ferricyanide reductase. *J Biol Chem* 279: 4811–4819, 2004.
21. Baker MA, Reeves G, Hetherington L, Müller J, Baur I, and Aitken RJ. Identification of gene products present in Triton X-100 soluble and insoluble fractions of human spermatozoa lysates using LC-MS/MS analysis. *Proteomics Clin Appl* 1: 524–532, 2007.
22. Balusikova K, Neubauerova J, Dostalikova-Cimburova M, Horak J, and Kovar J. Differing expression of genes involved in non-transferrin iron transport across plasma membrane in various cell types under iron deficiency and excess. *Mol Cel Biochem* 321: 123–133, 2009.
23. Banfi B, Molnar G, Maturana A, Steger K, Hegedus B, Demaurex N, and Krause KH. A Ca(2+)-activated NADPH oxidase in testis, spleen, and lymph nodes. *J Biol Chem* 276: 37594–37601, 2001.
24. Bánfi B, Tirone F, Durussel I, Knisz J, Moskwa P, Molnár GZ, Krause KH, and Cox JA. Mechanism of $Ca^{2+}$ activation of the NADPH oxidase 5 (NOX5). *J Biol Chem* 279: 18583–18591, 2004.
25. Baoutina A, Dean RT, and Jessup W. Trans-plasma membrane electron transport induces macrophage-mediated low density lipoprotein oxidation. *FASEB J* 15: 1580–1582, 2001.
26. Bayrhuber M, Meins T, Habeck M, Becker S, Giller K, Villinger S, Vonrhein C, Griesinger C, Zweckstetter M,

and Zeth K. Structure of the human voltage-dependent anion channel. *Proc Natl Acad Sci U S A* 105: 15370–15375, 2008.
27. Benz R. Permeation of hydrophilic solutes through mitochondrial outer membranes: Review on mitochondrial porins. *Biochim Biophys Acta* 1197: 167–196, 1994.
28. Berridge MV and Tan AS. Cell surface NAD(P)H-oxidase: relationship to transplasma membrane NADH-oxidoreductase and a potential source of circulating NADH-oxidase. *Antioxid Redox Signal* 2: 277–288, 2000.
29. Berridge MV and Tan AS. High-capacity redox control at the plasma membrane of mammalian cells: trans-membrane, cell surface, and serum NADH-oxidases. *Antioxid Redox Signal* 2: 231–242, 2000.
30. Berridge MV and Tan AS. Trans-plasma membrane electron transport: a cellular assay for NADH- and NADPH-oxidase based on extracellular, superoxide-mediated reduction of the sulfonated tetrazolium salt WST-1. *Protoplasma* 205: 74–82, 1998.
31. Berridge MV, Herst PM, and Lawen A. Targeting mitochondrial permeability in cancer drug development. *Mol Nutr Food Res* 53: 76–86, 2009.
32. Berridge MV, Herst PM, and Tan AS. Tetrazolium dyes as tools in cell biology: new insights into their cellular reduction. *Biotechnol Annu Rev* 11: 127–152, 2005.
33. Bewley MC, Marohnic CC, and Barber MJ. The structure and biochemistry of NADH-dependent cytochrome b5 reductase are now consistent. *Biochemistry* 40: 13574–13582, 2001.
34. Bianchet MA, Faig M, and Amzel LM. Structure and mechanism of NAD[P]H:quinone acceptor oxidoreductases (NQO). *Methods Enzymol* 382: 144–174, 2004.
35. Björnstedt M, Nordman T, and Olsson JM Extramitochondrial reduction of ubiquinone by flavoenzymes. *Methods Enzymol* 378: 131–138, 2004.
36. Block ML. NADPH oxidase as a therapeutic target in Alzheimer's disease. *BMC Neurosci* 9: S8–S15, 2008.
37. Bokoch GM, Diebold B, Kim JS, and Gianni D. Emerging evidence for the importance of phosphorylation in the regulation of NADPH oxidases. *Antioxid Redox Signal* 11: 2429–2441, 2009.
38. Borgese N, Macconi D, Parola L, and Pietrini G. Rat erythrocyte NADH-cytochrome b5 reductase. *J Biol Chem* 257: 13854–13861, 1982.
39. Borraccino G, Dipierro S, and Arrigoni O. Purification and properties of ascorbate free-radical reductase from potato tubers. *Planta* 167: 521–526, 1986.
40. Brenner C and Grimm S. The permeability transition pore complex in cancer cell death. *Oncogene* 25: 4744–4756, 2006.
41. Brightman AO, Wang J, Miu RK, Sun IL, Barr R, Crane FL, and Morré DJ. A growth factor- and hormone-stimulated NADH oxidase from rat liver plasma membrane. *Biochim Biophys Acta* 1105: 109–117, 1992.
42. Brown DI and Griendling KK. Nox proteins in signal transduction. *Free Radic Biol Med* 47: 1239–1253, 2009.
43. Bruce-Keller AJ, Gupta S, Parrino TE, Knight AG, Ebenezer PJ, Weidner AM, Levine H, Keller JN, and Markesbery WR. NOX activity is increased in mild cognitive impairment. *Antioxid Redox Signal* 12: 1371–1382, 2010.
44. Buettner R, Papoutsoglou G, Scemes E, Spray DC, and Dermietzel R. Evidence for secretory pathway localization of a voltage-dependent anion channel isoform. *Proc Natl Acad Sci U S A* 97: 3201–3206, 2000.
45. Campanella ME, Chu H, and Low PS. Assembly and regulation of a glycolytic enzyme complex on the human erythrocyte membrane. *Proc Natl Acad Sci U S A* 102: 2402–2407, 2005.
46. Chamulitrat W, Schmidt R, Tomakidi P, Stremmel W, Chunglok W, Kawahara T, and Rokutan K. Association of gp91phox homolog Nox1 with anchorage-independent growth and MAP kinase-activation of transformed human keratinocytes. *Oncogene* 22: 6045–6053, 2003.
47. Chen CF, Huang S, Liu SC, and Chueh PJ. Effect of polyclonal antisera to recombinant tNOX protein on the growth of transformed cells. *Biofactors* 28: 119–133, 2006.
48. Cheng G and Lambeth JD. NOXO1, regulation of lipid binding, localization, and activation of Nox1 by the Phox homology (PX) domain. *J Biol Chem* 279: 4737–4742, 2004.
49. Cheng G, Cao Z, Xu X, van Meir EG, and Lambeth JD. Homologs of gp91phox: cloning and tissue expression of Nox3, Nox4, and Nox5. *Gene* 269: 131–140, 2001.
50. Cheng G, Diebold BA, Hughes Y, and Lambeth JD. Nox1-dependent reactive oxygen generation is regulated by Rac1. *J Biol Chem* 281: 17718–17726, 2006.
51. Cho NM, Chueh P-J, Kim C, Caldwell S, Morré DM, and Morré DJ. Monoclonal antibody to a cancerspecific and drug-responsive hydroquinone (NADH) oxidase from the sera of cancer patients. *Cancer Immunol Immunother* 51: 121–129, 2002.
52. Chueh P-J, Kim C, Cho N, Morré DM, and Morré DJ. Molecular cloning and characterization of a tumor-associated, growth-related and time-keeping hydroquinone (NADH) oxidase (NOX) of the HeLa cell surface. *Biochemistry* 41: 3732–3741, 2002.
53. Chueh P-J, Morré DM, and Morré DJ. A site directed mutagenesis analysis of tNOX functional domains. *Biochim Biophys Acta* 1594: 74–83, 2002.
54. Chueh PJ, Wu LY, Morré DM, and Morré DJ. tNOX is both necessary and sufficient as a cellular target for the anticancer actions of capsaicin and the green tea catechin (-)-epigallocatechin-3-gallate. *Biofactors* 20: 235–249, 2004.
55. Cimen MY. Free radical metabolism in human erythrocytes. *Clin Chim Acta* 390: 1–11, 2008.
56. Clark RA, Volpp BD, Leidal KG, and Nauseef WM. Translocation of cytosolic components of neutrophil NADPH oxidase. *Trans Assoc Am Physicians* 102: 224–230, 1989.
57. Colombini M. Structure and mode of action of a voltage dependent anion-selective channel (VDAC) located in the outer mitochondrial membrane. *Ann N Y Acad Sci* 341: 552–563, 1980.
58. Correll CC, Batie CJ, Ballou DP, and Ludwig ML. Phthalate dioxygenase reductase: a modular structure for electron transfer from pyridine nucleotides to [2Fe-2S]. *Science* 258: 1604–1610, 1992.
59. Crane FL and Löw H. NADH oxidation in liver and fat cell plasma membranes. *FEBS Lett* 68: 153–156, 1976.
60. Crane FL and Löw H. Reactive oxygen species generation at the plasma membrane for antibody control. *Autoimmun Rev* 7: 518–522, 2008.
61. Crane FL, Sun IL, Clark MG, Grebing C, and Löw H. Transplasma-membrane redox systems in growth and development. *Biochim Biophys Acta* 811: 233–264, 1985.
62. Crane FL, Sun IL, Crowe R, and Löw H. Electron and proton transport across the plasma membrane. *J Bioenerg Biomemb* 23: 773–803, 1991.
63. Crane FL, Sun IL, Crowe, RA, and Löw H. Oxidase control of plasma membrane proton transport. In: *Principles of Medical Biology: 4, Cell Chemistry and Physiology, Part III,*

edited by Bittar EE and Bittar N. Greenwich, CT: JAI Press, 1996, pp. 169–186.
64. Crane FL. Biochemical functions of coenzyme Q10. *J Am Coll Nutr* 20: 591–598, 2001.
65. Crane FL. The evolution of coenzyme Q. *BioFactors* 32: 5–11, 2008.
66. Cui W, Matsuno K, Iwata K, Ibi M, Katsuyama M, Kakehi T, Sasaki M, Ikami K, Zhu K, and Yabe-Nishimura C. NADPH oxidase isoforms and anti-hypertensive effects of atorvastatin demonstrated in two animal models. *J Pharmacol Sci* 111: 260–268, 2009.
67. Cullen JJ, Hinkhouse MM, Grady M, Gaut AW, Liu J, Zhang YP, Darby Weydert CJ, Domann FE, and Oberley LW. Dicoumarol inhibition of NAD(P)H quinone oxidoreductase induces growth inhibition of pancreatic cancer via a superoxide mediated mechanism. *Cancer Res* 63: 5513–5520, 2003.
68. Dalton DA, Langeberg L, and Robbins M. Purification and characterization of monodehydroascorbate reductase from soybean root nodules. *Arch Biochem Biophys* 292: 281–286, 1992.
69. De Cabo R, Cabello R, Rios M, Lopez-Lluch G, Ingram DK, Lane MA, and Navas P. Calorie restriction attenuates age-related alterations in the plasma membrane antioxidant system in rat liver. *Exp Gerontol* 39: 297–304, 2004.
70. De Deken X, Wang D, Many MC, Costagliola S, Libert F, Vassart G, Dumont JE, and Miot F. Cloning of two human thyroid cDNAs encoding new members of the NADPH oxidase family. *J Biol Chem* 275: 23227–23233, 2000.
71. De Laurenzi V, Melino G, Savini I, Annichiarico-Petruzzelli M, Finazzi-Agrò A, and Avigliano L. Cell death by oxidative stress and ascorbic acid regeneration in human neuroectodermal cell lines. *Eur J Cancer* 31: 463–466, 1995.
72. De Luca T, Bosneaga E, Morré DM, and Morré DJ. Downstream targets of altered sphingolipid metabolism in response to inhibition of ENOX2 by phenoxodiol. *Biofactors* 34: 253–260, 2009.
73. De Luca T, Morré DM, Zhao H, and Morré DJ. NAD+/NADH and/or CoQ/CoQH2 ratios from plasma membrane electron transport may determine ceramide and sphingosine-1-phosphate levels accompanying G1 arrest and apoptosis. *Biofactors* 25: 43–60, 2005.
74. De Pinto V, Messina A, Lane DJ, and Lawen A. Voltage-dependent anion-selective channel (VDAC) in the plasma membrane. *FEBS Lett* 584: 1793–1799, 2010.
75. Dedov VN, Mandadi S, Armati PJ, and Verkhratski A. Capsaicin-induced depolarization of mitochondria in dorsal root ganglion neurons is enhanced by vanilloid receptors. *Neuroscience* 103: 219–226, 2001.
76. Del Principe D, Frega G, Savini I, Catani MV, Rossi A, and Avigliano L. The plasma membrane redox system in human platelet functions and platelet-leukocyte interactions. *Thromb Haemost* 101: 284–289, 2009.
77. Del Principe D, Mancuso G, Menichelli A, Gabriotti M, Cosmi EV, and Gherardi G. Production of hydrogen peroxide in phagocyting human platelets: an electron microscopic cytochemical demonstration. *Biol Cell* 38: 135–140, 1980.
78. del Valle AE, del Castillo-Olivares A, Sanchez-Jimenez F, Marquez J, Nunez de Castro I, and Medina MA. Plasma membrane redox system activity in tumour cell lines of mammary origins with different proliferation rates. *Protoplasma* 205: 169–173, 1998.
79. Dinauer MC, Orkin SH, Brown R, Jesaitis AJ, and Parkos CA. The glycoprotein encoded by the X-linked chronic granulomatous disease locus is a component of the neutrophil cytochrome b complex. *Nature* 327: 717–720, 1987.
80. Dinauer MC, Pierce EA, Bruns GAP, Cumutte JT, and Orkin SH. Human neutrophil cytocrome-b light chain (p22-phox): Gene structure, chromosomal location, and mutations in cytochrome negative autosomal recessive chronic granulomatous disease. *J Clin Invest* 86: 1729–1737, 1990.
81. Dringen R, Pfeiffer B, and Hamprecht, B. Synthesis of the antioxidant glutathione in neurons: supply by astrocytes of CysGly as precursor for neuronal glutathione. *J Neurosci* 19: 562–569, 1999.
82. Du M, Shirabe K, and Takeshita M. Identification of alternative first exons of NADH-cytochrome b5 reductase gene expressed ubiquitously in human cells. *Biochem Biophys Res Commun* 235: 779–783, 1997.
83. Dusi S, Donini M, Lissandrini D, Mazzi P, Bianca VD, and Rossi F. Mechanisms of expression of NADPH oxidase components in human cultured monocytes: role of cytokines and transcriptional regulators involved. *Eur J Immunol* 31: 929–938, 2001.
84. Elinder F, Akanda N, Tofighi R, Shimizu S, Tsujimoto Y, Orrenius S, and Ceccatelli S. Opening of plasma membrane voltage-dependent anion channels (VDAC) precedes caspase activation in neuronal apoptosis induced by toxic stimuli. *Cell Death Differ* 12: 1134–1140, 2005.
85. Fernández-Ayala DJ, Brea-Calvo G, López-Lluch G, and Navas P. Coenzyme Q distribution in HL-60 human cells depends on the endomembrane system. *Biochim Biophys Acta* 1713: 129–137, 2005.
86. Fiorani M and Accorsi A. Dietary flavonoids as intracellular substrates for an erythrocyte trans-plasma membrane oxidoreductase activity. *Br J Nutr* 94: 338–345, 2005.
87. Fischer H. Mechanisms and function of DUOX in epithelia of the lung. *Antioxid Redox Signal* 11: 2453–2465, 2009.
88. Fisher AB. Redox signaling across cell membranes. *Antioxid Redox Signal* 11: 1349–1356, 2009.
89. Fleming PJ and Kent UM. Cytochrome b561, ascorbic acid, and transmembrane electron transfer. *Am J Clin Nutr* 54: 1173S–1178S, 1991.
90. Fortuño A, Oliván S, Beloqui O, San Jose G, Moreno MU, Diez J, and Zalba G. Association of increased phagocytic NADPH oxidase-dependent superoxide production with diminished nitric oxide generation in essential hypertension. *J Hypertens* 22: 2169–2175, 2004.
91. Fortuño A, San José G, Moreno MU, Beloqui O, Díez J, and Zalba G. Phagocytic NADPH oxidase overactivity underlies oxidative stress in metabolic syndrome. *Diabetes* 55: 209–215, 2006.
92. Fu X, Beer DG, Behar J, Wands J, Lambeth D, and Cao W. cAMP-response element-binding protein mediates acid-induced NADPH oxidase NOX5-S expression in Barrett esophageal adenocarcinoma cells. *J Biol Chem* 281: 20368–20382, 2006.
93. Fukui T, Ishizaka N, Rajagopalan S, Laursen JB, Capers Q 4th, Taylor WR, Harrison DG, de Leon H, Wilcox JN, and Griendling KK. p22phox mRNA expression and NADPH oxidase activity are increased in aortas from hypertensive rats. *Circ Res* 80: 45–51, 1997.
94. Furukawa S, Fujita T, Shimabukuro M, Iwaki M, Yamada Y, Nakajima Y, Nakayama O, Makishima M, Matsuda M, and Shimomura I. Increased oxidative stress in obesity and its impact on metabolic syndrome. *J Clin Invest* 114: 1752–1761, 2004.

95. Gaikwad A, Long II DJ, Stringer JL, and Jaiswal AK. *In vivo* role of NAD(P)H: quinone oxidoreductase 1 (NQO1) in the regulation of intracellular redox state and accumulation of abdominal adipose tissue. *J Biol Chem* 276: 22559–22564, 2001.
96. Geiszt M and Leto TL. The Nox family of NAD(P)H oxidases: host defense and beyond. *J Biol Chem* 279: 51715–51718, 2004.
97. Geiszt M, Lekstrom K, Witta J, and Leto TL. Proteins homologous to p47phox and p67phox support superoxide production by NAD(P)H oxidase 1 in colon epithelial cells. *J Biol Chem* 278: 20006–20012, 2003.
98. Ghio AJ, Nozik-Grayck E, Turi J, Jaspers I, Mercatante DR, Kole R, and Piantadosi CA. Superoxide-dependent iron uptake: a new role for anion exchange protein 2. *Am J Respir Cell Mol Biol* 29: 653–660, 2003.
99. Gomez-Diaz C, Rodrıguez-Aguilera JC, Barroso MP, Villalba JM, Navarro F, Crane FL, and Navas P. Antioxidant ascorbate is stabilized by NADH-coenzyme Q10 reductase in the plasma membrane. *J Bioenerg Biomembr* 29: 251–257, 1997.
100. Gómez-Díaz C, Villalba JM, Pérez-Vicente R, Crane FL, and Navas P. Ascorbate stabilization is stimulated in $\rho^o$ HL-60 cells by CoQ10 increase at the plasma membrane. *Biochem Biophys Res Commun* 234: 79–81, 1997.
101. Gonzalez-Gronow M, Kalfa T, Johnson CE, Gawdi G, and Pizzo SV. The voltage-dependent anion channel is a receptor for plasminogen kringle 5 on human endothelial cells. *J Biol Chem* 278: 27312–27318, 2003.
102. Grebing C, Crane FL, Löw H, and Hall K. A transmembranous NADH-dehydrogenase in human erythrocyte membranes. *J Bioenerg Biomembr* 16: 517–533, 1984.
103. Griendling KK, Sorescu D, and Ushio-Fukai M. NAD(P)H oxidase: role in cardiovascular biology and disease. *Circ Res* 86: 494–501, 2000.
104. Griffiths HR. Is the generation of neo-antigenic determinants by free radicals central to the development of autoimmune rheumatoid disease? *Autoimmun Rev* 7: 544–549, 2008.
105. Guichard C, Moreau R, Pessayre D, Epperson TK, and Krause KH. NOX family NADPH oxidases in liver and in pancreatic islets: a role in the metabolic syndrome and diabetes? *Biochem Soc Trans* 36: 920–929, 2008.
106. Gunshin H, Starr CN, Direnzo C, Fleming MD, Jin J, Greer EL, Sellers VM, Galica SM, and Andrews NC. Cybrd1 (duodenal cytochrome b) is not necessary for dietary iron absorption in mice. *Blood* 106: 2879–2883, 2005.
107. Hara T and Minakami S. On functional role of cytochrome b5. *J Biochem* 69: 325–330, 1971.
108. Hecker L, Vittal R, Jones T, Jagirdar R, Luckhardt TR, Horowitz JC, Pennathur S, Martinez FJ, and Thannickal VJ. NADPH oxidase-4 mediates myofibroblast activation and fibrogenic responses to lung injury. *Nat Med* 15: 1077–1081, 2009.
109. Herst PM and Berridge MV. Cell surface oxygen consumption: a major contributor to cellular oxygen consumption in glycolytic cancer cell lines. *Biochim Biophys Acta* 1767: 170–177, 2007.
110. Herst PM and Berridge MV. Plasma membrane electron transport: a new target for cancer drug development. *Curr Mol Med* 6: 895–904, 2006.
111. Herst PM, Petersen T, Jerram P, Baty J, and Berridge MV. The antiproliferative effects of phenoxodiol are associated with inhibition of plasma membrane electron transport in tumour cell lines and primary immune cells. *Biochem Pharmacol* 74: 1587–1595, 2007.
112. Herst PM, Tan AS, Scarlett DJ, and Berridge MV. Cell surface oxygen consumption by mitochondrial gene knockout cells. *Biochim Biophys Acta* 1656: 79–87, 2004.
113. Hicks C and Morré DJ. Oxidation of NADH by intact segments of soybean hypocotyls and stimulation by 2,4-D. *Biochim Biophys Acta* 1375: 1–5, 1998.
114. Himmelreich U, Drew KN, Serianni AS, and Kuchel PW. 13C NMR studies of vitamin C transport and its redox cycling in human erythrocytes. *Biochemistry* 37: 7578–7588, 1998.
115. Himmelreich U and Kuchel PW. 13C-NMR studies of transmembrane electron transfer to extracellular ferricyanide in human erythrocytes. *Eur J Biochem* 246: 638–645, 1997.
116. Hohn DC and Lehrer RI. NADPH oxidase deficiency in X-linked chronic granulomatous disease. *J Clin Invest* 55: 707–713, 1975.
117. Holla IL, Kanková K, and Znojil V. Haplotype analysis of the NADPH oxidase p22 phox gene in patients with bronchial asthma. *Int Arch Allergy Immunol* 148: 73–80, 2009.
118. Hosoda S, Nakamura W, and Hayashi K. Properties and reaction mechanism of DT diaphorase from rat liver. *J Biol Chem* 249: 6416–6423, 1974.
119. Hostetler B, Weston N, Kim C, Morré DM, and Morré DJ. Cancer site-specific isoforms of ENOX2 (tNOX), a cancer-specific cell surface oxidase. *Clin Proteom* 5: 46–51, 2009.
120. Hotchkiss RS, Strasser A, McDunn JE, and Swanson PE. Cell death. *N Engl J Med* 361: 1570–1583, 2009.
121. Houtkooper RH, Cantó C, Wanders RJ, and Auwerx J. The secret life of $NAD^+$: an old metabolite controlling new metabolic signaling pathways. *Endocr Rev* 31: 194–223, 2010.
122. Hsieh TC, Lu X, Wang Z, and Wu JM. Induction of quinone reductase NQO1 by resveratrol in human K562 cells involves the antioxidant response element ARE and is accompanied by nuclear translocation of transcription factor Nrf2. *Med Chem* 2: 275–285, 2006.
123. Huang J and Brumell JH. NADPH oxidases contribute to autophagy regulation. *Autophagy* 5: 887–889, 2009.
124. Huang J, Canadien V, Lam GY, Steinberg BE, Dinauer MC, Magalhaes MA, Glogauer M, Grinstein S, and Brumell JH. Activation of antibacterial autophagy by NADPH oxidases. *Proc Natl Acad Sci U S A*; 106: 6226–6631, 2009.
125. Hultquist DE and Passon PG. Catalysis of methaemoglobin reduction by erythrocyte cytochrome B5 and cytochrome B5 reductase. *Nat New Biol* 229: 252–254, 1971.
126. Hwang JH, Kim DW, Jo EJ, Kim YK, Jo YS, Park JH, Yoo SK, Park MK, Kwak TH, Kho YL, Han J, Choi HS, Lee SH, Kim JM, Lee I, Kyung T, Jang C, Chung J, Kweon GR, and Shong M. Pharmacological stimulation of NADH oxidation ameliorates obesity and related phenotypes in mice. *Diabetes* 58: 965–974, 2009.
127. Hyun DH, Emerson SS, Jo DG, Mattson MP, and de Cabo R. Calorie restriction up-regulates the plasma membrane redox system in brain cells and suppresses oxidative stress during aging. *Proc Natl Acad Sci U S A* 103: 19908–19912, 2006.
128. Hyun DH, Hernandez JO, Mattson MP, and de Cabo R. The plasma membrane redox system in aging. *Ageing Res Rev* 5: 209–220, 2006.
129. Jaffè ER and Hsieh HS. DPNH-methemoglobin reductase deficiency and hereditary methemoglobinemia. *Semin Hematol* 8: 417–437, 1971.
130. Jagnandan D, Church JE, Banfi B, Stuehr DJ, Marrero MB, and Fulton DJ. Novel mechanism of activation of NADPH

oxidase 5. Calcium sensitization via phosphorylation. *J Biol Chem* 282: 6494–6507, 2007.
131. Jaiswal AK. Human NAD(P)H:quinone oxidoreductase (NQO1) gene structure and induction by dioxin. *Biochemistry* 30: 10647–10653, 1991.
132. Jana A and Pahan K. Fibrillar amyloid-beta peptides kill human primary neurons via NADPH oxidase-mediated activation of neutral sphingomyelinase. Implications for Alzheimer's disease. *J Biol Chem* 279: 51451–51459, 2004.
133. Jiang Z, Gorenstein NM, Morré DM, and Morré DJ. Molecular cloning and characterization of a candidate human growth-related and time-keeping constitutive cell surface hydroquinone (NADH) oxidase. *Biochemistry* 47: 14028–14038, 2008.
134. Jones W, Li X, Qu ZC, Perriott L, Whitesell RR, and May JM. Uptake, recycling, and antioxidant actions of α-lipoic acid in endothelial cells. *Free Radic Biol Med* 33: 83–93, 2002.
135. Jung J, Hwang SW, Kwak J, Lee SY, Kang CJ, Kim WB, Kim D, and Oh U. Capsaicin binds to the intracellular domain of the capsaicin-activated ion channel. *J Neurosci* 19: 529–538, 1999.
136. Kagan VE, Arroyo A, Tyurin VA, Tyurina YY, Villalba JM, and Navas P. Plasma membrane NADH-coenzyme Q0 reductase generates semiquinone radicals and recycles vitamin E homologue in a superoxide-dependent reaction. *FEBS Lett* 428: 43–46, 1998.
137. Kamata T. Roles of Nox1 and other Nox isoforms in cancer development. *Cancer Sci* 100: 1382–1388, 2009.
138. Karplus PA, Daniels MJ, and Herriott JR. Atomic structure of ferredoxin-NADP+reductase: prototype for a structurally novel flavoenzyme family. *Science* 251: 60–66, 1991.
139. Kawahara T, Teshima S, Oka A, Sugiyama T, Kishi K, and Rokutan K. Type I Helicobacter pylori lipopolysaccharide stimulates toll-like receptor 4 and activates mitogen oxidase 1 in gastric pit cells. *Infect Immun* 69: 4382–4389, 2001.
140. Kelker M, Kim C, Chueh P-J, Guimont R, Morré DM, and Morré DJ. Cancer isoform of a tumor-associated cell surfaceNADHoxidase (tNOX) has properties of a prion. *Biochemistry* 40: 7351–7354, 2001.
141. Kennett EC and Kuchel PW. Plasma membrane oxidoreductases: effects on erythrocyte metabolism and redox homeostasis. *Antioxid Redox Signal* 8: 1241–1247, 2006.
142. Kennett EC and Kuchel PW. Redox reactions and electron transfer across the red cell membrane. *IUBMB Life* 55: 375–385, 2003.
143. Kennett EC, Ogawa E, Agar NS, Godwin IR, Bubb WA, and Kuchel PW. Investigation of methaemoglobin reduction by extracellular NADH in mammalian erythrocytes. *Int J Biochem Cell Biol* 37: 1438–1445, 2005.
144. Kern DG, Draelos ZD, Meadows C, Morré DJ, and Morré DM. Controlling reactive oxygen species in skin at their source to reduce skin aging. *Rejuvenation Res* 13: 1–3, 2010.
145. Keyes SR and Cinti DL. Biochemical properties of cytochrome c-dependent microsomal fatty acid elongation and identification of products. *J Biol Chem* 255: 11357–11364, 1980.
146. Kikuchi H, Hikage M, Miyashita H, and Fukumoto M. NADPH oxidase subunit, gp91(phox) homologue, preferentially expressed in human colon epithelial cells. *Gene* 254: 237–243, 2000.
147. Kilberg MS and Christensen HN. Electron-transferring enzymes in the plasma membrane of the Ehrlich ascites tumor cell. *Biochemistry* 18: 1525–1530, 1979.
148. Kim C, MacKellar WC, Cho N, Byrn SR, and Morré DJ. Impermeant antitumor sulfonylurea conjugates that inhibit plasma membrane NADH oxidase and growth of HeLa cells in culture. Identification of binding proteins from sera of cancer patients. *Biochim Biophys Acta* 1324: 171–181, 1997.
149. Kim JS, Diebold BA, Babior BM, Knaus UG, and Bokoch GM. Regulation of Nox1 activity via protein kinase A-mediated phosphorylation of NoxA1 and 14-3-3 binding. *J Biol Chem* 282: 34787–34800, 2007.
150. Kimura S, Kawamura M, and Iyanagi T. Role of Thr(66) in porcine NADH-cytochrome b5 reductase in catalysis and control of the rate-limiting step in electron transfer. *J Biol Chem* 278: 3580–2589, 2003.
151. Kinnula VL. Production and degradation of oxygen metabolites during inflammatory states in the human lung. *Curr Drug Targets Inflamm Allergy* 2: 465–470, 2005.
152. Kishi T, Morré DM, and Morré DJ. The plasma membrane NADH oxidase of HeLa cells has hydroquinone oxidase activity. *Biochim Biophys Acta* 1412: 66–77, 1999.
153. Kishida KT, Hoeffer CA, Hu D, Pao M, Holland SM, and Klann E. Synaptic plasticity deficits and mild memory impairments in mouse models of chronic granulomatous disease. *Mol Cell Biol* 26: 5908–5920, 2006.
154. Krauskopf A, Eriksson O, CraigenWJ, Forte MA, and Bernardi P. Properties of the permeability transition in VDAC1(−/−) mitochondria. *Biochim Biophys Acta* 1757: 590–595, 2006.
155. Kurian JR, Bajad SU, Miller JL, Chin NA, and Trepanier LA. NADH cytochrome b5 reductase and cytochrome b5 catalyze the microsomal reduction of xenobiotic hydroxylamines and amidoximes in humans. *J Pharmacol Exp Ther* 311: 1171–1178, 2004.
156. Kurokawa M and Kornbluth S. Caspases and kinases in a death grip. *Cell* 138: 838–854, 2009.
157. Lane DJ and Lawen A. Ascorbate and plasma membrane electron transport—enzymes vs efflux. *Free Radic Biol Med* 47: 485–495, 2009.
158. Lane DJ and Lawen A. Transplasma membrane electron transport comes in two flavors. *Biofactors* 34: 191–200, 2009.
159. Lane DJ, Robinson SR, Czerwinska H, and Lawen A. A role for $Na^+/H^+$ exchangers and intracellular pH in regulating vitamin C-driven electron transport across the plasma membrane. *Biochem J* 428: 191–200, 2010.
160. Lane DJR and Lawen A. Non-transferrin iron reduction and uptake are regulated by transmembrane ascorbate cycling in K562 cells. J Biol Chem 283: 12701–12708, 2008.
161. Larm JA, Vaillant F, Linnane AW, and Lawen A. Up-regulation of the plasma membrane oxidoreductase as a prerequisite for the viability of human Namalwa rho 0 cells. *J Biol Chem* 269: 30097–30100, 1994.
162. Lass A, Forster MJ, and Sohal RS. Effects of coenzyme Q10 and alphatocopherol administration on their tissue levels in the mouse: elevation of mitochondrial alpha-tocopherol by coenzyme Q10. *Free Radical Biol Med* 26: 1375–1382, 1999.
163. Laurent E, McCoy JW 3rd, Macina RA, Liu W, Cheng G, Robine S, Papkoff J, and Lambeth JD. Nox1 is overexpressed in human colon cancers and correlates with activating mutations in K-Ras. *Int J Cancer* 123: 100–107, 2008.
164. Lawen A, Ly JD, Lane DJ, Zarschler K, Messina A, and 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* 37: 277–282, 2005.
165. Lee MY, San Martin A, Mehta PK, Dikalova AE, Garrido AM, Datla SR, Lyons E, Krause KH, Banfi B, Lambeth JD,

Lassègue B, and Griendling KK. Mechanisms of vascular smooth muscle NADPH oxidase 1 (Nox1) contribution to injury-induced neointimal formation. *Arterioscler Thromb Vasc Biol* 29: 480–487, 2009.
166. Lee S, Li R, Kim B, Palvolgyi R, Ho T, Yang Q-Z, Xu J, Szeto WL, Honda H, and Berliner JA. Ox-PAPC activation of PMET system increases expression of heme oxygenase-1 in human aortic endothelial cell. *J Lipid Res* 50: 265–274, 2009.
167. Lee YM, Kim BJ, Chun YS, So I, Choi H, Kim MS, and Park JW. NOX4 as an oxygen sensor to regulate TASK-1 activity. *Cell Signal* 18: 499–507, 2006.
168. Lenaz G, Paolucci U, Fato R, D'Aurelio M, Parenti Castelli G, Sgarbi G, Biagini G, Ragni L, Salardi S, and Cacciari E. Enhanced activity of the plasma membrane oxidoreductase in circulating lymphocytes from insulin-dependent diabetes mellitus patients. *Biochem Biophys Res Commun* 290: 1589–1592, 2002.
169. Lewis IA, Campanella ME, Markley JL, and Low PS. Role of band 3 in regulating metabolic flux of red blood cells. *Proc Natl Acad Sci U S A* 106: 18515–18520, 2009.
170. Li R, Bianchet MA, Talalay P, and 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 U S A* 92: 8846–8850, 1995.
171. Lin SJ and Guarente L. Nicotinamide adenine dinucleotide, a metabolic regulator of transcription, longevity and disease. *Curr Opin Cell Biol* 15: 241–246, 2003.
172. Lind C, Cadenas E, Hochstein P, and Ernster L. DT-diaphorase: purification, properties and function. *Methods Enzymol* 186: 287–301, 1990.
173. Liu SC, Yang JJ, Shao KN, and Chueh PJ. RNA interference targeting tNOX attenuates cell migration via a mechanism that involves membrane association of Rac. *Biochem Biophys Res Commun* 365: 672–677, 2008.
174. Lopez-Lluch G, Rios M, Lane MA, Navas P, and de Cabo R. Mouse liver plasma membrana redox system activity is altered by aging and modulated by calorie restriction. *AGE* 27: 153–160, 2005.
175. Ludwiczek S, Rosell FI, Ludwiczek ML, and Mauk AG. Recombinant expression and initial characterization of the putative human enteric ferric reductase Dcytb. *Biochemistry* 47: 753–761, 2008.
176. Luxen S, Belinsky SA, and Knaus UG. Silencing of DUOX NADPH oxidases by promoter hypermethylation in lung cancer. *Cancer Res* 68: 1037–1045, 2008.
177. Ly JD and Lawen A. Transplasma membrane electron transport: enzymes involved and biological function. *Redox Rep* 8: 3–21, 2003.
178. Macho A, Calzado MA, Munoz-Blanco J, Gomez-Diaz C, Gajate C, Mollinedo F, Navas P, and Muñoz E. Selective induction of apoptosis by capsaicin in transformed cells: the role of reactive oxygen species and calcium. *Cell Death Differ* 6: 155–165, 1999.
179. Macho A, Lucena C, Calzado MA, Blanco M, Donnay I, Appendino G, and Muñoz E. Phorboid 20-homovanillates induce apoptosis through a VR1-independent mechanism. *Chem Biol* 7: 483–492, 2000.
180. Madesh M and Hajnoczky G. VDAC-dependent permeabilization of the outer mitochondrial membrane by superoxide induces rapid and massive cytochrome c release. *J Cell Biol* 155: 1003–1016, 2001.
181. Mangiullo R, Gnoni A, Leone A, Gnoni GV, Papa S, and Zanotti F. Structural and functional characterization of F(o)F(1)-ATP synthase on the extracellular surface of rat hepatocytes. *Biochim Biophys Acta* 1777: 1326–1335, 2008.
182. Margutti P, Matarrese P, Conti F, Colasanti T, Delunardo F, Capozzi A, Garofalo T, Profumo E, Riganò R, Siracusano A, Alessandri C, Salvati B, Valesini G, Malorni W, Sorice M, and Ortona E. Autoantibodies to the C-terminal subunit of RLIP76 induce oxidative stress and endothelial cell apoptosis in immune-mediated vascular diseases and atherosclerosis. *Blood* 111: 4559–4570, 2008.
183. Marques F, Crespo ME, and Bicho M. Control of NADH ferricyanide reductase activity in the human erythrocyte by somatotrophin and insulin. *Redox Rep* 1: 113–117, 1995.
184. Martín SF, Gómez-Díaz C, Bello RI, Navas P, and Villalba JM. Inhibition of neutral Mg2+-dependent sphingomyelinase by ubiquinol-mediated plasma membrane electron transport. *Protoplasma* 221: 109–116, 2003.
185. Martín SF, Navarro F, Forthoffer N, Navas P, and Villalba JM. Neutral magnesium-dependent sphingomyelinase from liver plasma membrane: purification and inhibition by ubiquinol. *J Bioenerg Biomembr* 33: 143–153, 2001.
186. Martín-Romero FJ, García-Martín E, and Gutiérrez-Merino C. Inhibition of oxidative stress produced by plasma membrane NADH oxidase delays low-potassium-induced apoptosis of cerebellar granule cells. *J Neurochem* 82: 705–715, 2002.
187. Martın-Romero FJ, Gutierrez-Martın Y, Henao F, and Gutierrez-Merino C. The NADH oxidase activity of the plasma membrane of synaptosomes is a major source of superoxide anion and is inhibited by peroxynitrite. *J Neurochem* 82: 604–614, 2002.
188. Mathupala SP and Pedersen, PL. Voltage dependent anion channel-1 (VDAC-1) as an anti-cancer target. *Cancer Biol Ther* 9: 1053–1056, 2010.
189. Matsushita H, Lee K, and Tsao PS. Cyclic strain induces reactive oxygen species production via an endothelial NAD(P)H oxidase. *J Cell Biochem* 81: 99–106, 2001.
190. Matteucci E, Cupisti A, Caprioli R, Battipaglia E, Favilla S, Rindi P, Barsotti G, and Giampietro O. Erythrocyte transmembrane electron transfer in haemodialysis patients. *Nutr Metab Cardiovasc Dis* 17: 288–293, 2007.
191. May JM, Qu Z-C, and Mendiratta S. Protection and recycling of a-tocopherol in human erythrocytes by intracellular ascorbic acid. *Arch Biochem Biophys* 349: 281–289, 1998.
192. May JM, Qu ZC, and Nelson DJ. Uptake and reduction of α-lipoic acid by human erythrocytes. *Clin Biochem* 40: 1135–1142, 2007.
193. May JM, Qu Z-C, and Whitesell RR. Ascorbic acid recycling enhances the antioxidant reserve of human erythrocytes. *Biochemistry* 34: 12721–12728, 1995.
194. May JM. Is ascorbic acid an antioxidant for the plasma membrane? *FASEB J* 13: 995–1006, 1999.
195. McIntyre JA, Wagenknecht DR, and Faulk WP. Autoantibodies unmasked by redox reactions. *J Autoimmun* 24: 311–317, 2005.
196. 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, and Simpson RJ. An iron-regulated ferric reductase associated with the absorption of dietary iron. *Science* 291: 1755–1759, 2001.
197. McKie AT. The role of Dcytb in iron metabolism: an update. *Biochem Soc Trans* 36: 1239–1241, 2008.
198. Messana I, Orlando M, Cassiano L, Pennacchietti L, Zuppi C, Castagnola M, and Giardina B. Human erythrocyte

metabolism is modulated by the O2-linked transition of hemoglobin. *FEBS Lett* 390: 25–28, 1996.
199. Mitsushita J, Lambeth JD, and Kamata T. The superoxide-generating oxidase Nox1 is functionally required for Ras oncogene transformation. *Cancer Res* 64: 3580–3585, 2004.
200. Mizushima N, Levine B, Cuervo AM, and Klionsky DJ. Autophagy fights disease through cellular self-digestion. *Nature* 451: 1069–1075, 2008.
201. Mochizuki T, Furuta S, Mitsushita J, Shang WH, Ito M, Yokoo Y, Yamaura M, Ishizone S, Nakayama J, Konagai A, Hirose K, Kiyosawa K, and Kamata T. Inhibition of NADPH oxidase 4 activates apoptosis via the AKT/apoptosis signal-regulating kinase 1 pathway in pancreatic cancer PANC-1 cells. *Oncogene* 25: 3699–3707, 2006.
202. Moreno JC, Bikker H, Kempers MJ, van Trotsenburg AS, Baas F, de Vijlder JJ, Vulsma T, and Ris-Stalpers C. Inactivating mutations in the gene for thyroid oxidase 2 (THOX2) and congenital hypothyroidism. *N Engl J Med* 347: 95–102, 2002.
203. Morré DJ and Brightman AO. NADH oxidase of plasma membranes. *J Bioenerg Biomem* 23: 469–489, 1991.
204. Morré DJ and Morré DM. Aging-related cell surface ECTO-NOX protein, arNOX, a preventive target to reduce atherogenic risk in the elderly. *Rejuvenation Res* 9: 231–236, 2006.
205. Morré DJ and Morré DM. Cell surface NADH oxidases (ECTO-NOX proteins) with roles in cancer, cellular timekeeping, growth, aging and neurodegenerative diseases. *Free Radic Res* 37: 795–808, 2003.
206. Morré DJ, Chueh P-J, Lawler J, and Morré DM. The sulfonylurea-inhibited NADH oxidase activity of HeLa cell plasma membranes has properties of a protein disulfide–thiol oxidoreductase with protein disulfide–thiol interchange activity. *J Bioenerg Biomembr* 30: 477–487, 1998.
207. Morré DJ, Chueh P-J, Pletcher J, Tang X, Wu L-Y, and Morré DM. Biochemical basis for the biological clock. *Biochemistry* 41: 11941–11945, 2002.
208. Morré DJ, Hostetler B, Weston N, Kim C, and Morré DM. Cancer type-specific tNOX isoforms: a putative family of redox protein splice variants with cancer diagnostic and prognostic potential. *Biofactors* 34: 201–207, 2009.
209. Morré DJ, Orczyk J, Hignite H, and Kim C. Regular oscillatory behavior of aqueous solutions of CuII salts related to effects on equilibrium dynamics of ortho/para hydrogen spin isomers of water. *J Inorg Biochem* 102: 260–267, 2008.
210. Morré DJ, Sun E, Geilen C, Wu L-Y, de Cabo R, Krasagakis K, Orfanos CE, and Morré DM. Capsaicin inhibits plasma membrane NADH oxidase and growth of human and mouse melanoma lines. *Eur J Cancer* 32: 1995–2003, 1996.
211. Morré DJ. NADH oxidase activity of HeLa plasma membranes inhibited by the antitumor sulfonylurea N-(4-methylphenylsulfonyl)-N0-(4-chlorophenyl)urea (LY181984) at an external site. *Biochim Biophys Acta* 1240: 201–208, 1995.
212. Morré DM and Morré DJ. Specificity of coenzyme Q inhibition of an aging-related cell surface NADH oxidase (ECTO-NOX) that generates superoxide. *Biofactors* 18: 33–43, 2003.
213. Morré DM, Guo F, and Morré DJ. An aging-related cell surface NADH oxidase (arNOX) generates superoxide and is inhibited by coenzyme Q. *Mol Cell Biochem* 254: 101–109, 2003.
214. Morré DM, Morré DJ, Rehmus W, and Kern D. Supplementation with CoQ10 lowers age-related (ar) NOX levels in healthy subjects. *Biofactors* 32: 221–230, 2008.
215. Nauseef WM. Biological roles for the NOX family NADPH oxidases. *J Biol Chem* 283: 16961–16965, 2008.
216. Navarro F, Navas P, Burgess JR, Bello RI, De Cabo R, Arroyo A, and Villalba JM. Vitamin E and selenium deficiency induces expression of the ubiquinone-dependent antioxidant system at the plasma membrane. *FASEB J* 12: 1665–1673, 1998.
217. Navas P, Villalba JM, and de Cabo R. The importance of plasma membrane coenzyme Q in aging and stress responses. *Mitochondrion* 7: S34–S40, 2007.
218. Nishida H, Inaka K, Yamanaka M, Kaida S, Kobayashi K, and Miki K. Crystal structure of NADH-cytochrome b5 reductase from pig liver at 2.4 A resolution. *Biochemistry* 34: 2763–2767, 1995.
219. Nolan KA, Doncaster JR, Dunstan MS, Scott KA, Frenkel AD, Siegel D, Ross D, Barnes J, Levy C, Leys D, Whitehead RC, Stratford IJ, and Bryce RA. Synthesis and biological evaluation of coumarin-based inhibitors of NAD(P)H: quinone oxidoreductase-1 (NQO1). *J Med Chem* 52: 7142–7156, 2009.
220. Nozik-Grayck E, Piantadosi CA, van Adelsberg J, Alper SL, and Huang YCT. Protection of perfused lung from oxidant injury by inhibitors of anion exchange. *Am J Physiol Lung Cell Mol Physiol* 273: L296–L304, 1997.
221. Nutter LM, Ngo EO, Fisher GR, and Gutierrez PL. DNA strand scission and free radical production in menadione-treated cells. Correlation with cytotoxicity and role of NADPH quinone acceptor oxidoreductase. *J Biol Chem* 267: 2474–2479, 1992.
222. Oakhill JS, Marritt SJ, Gareta EG, Cammack R, and McKie AT. Functional characterization of human duodenal cytochrome b (Cybrd1): redox properties in relation to iron and ascorbate metabolism. *Biochim Biophys Acta* 1777: 260–268, 2008.
223. Okun JG, Lummen P, and Brandt U. Three classes of inhibitors share a common binding domain in mitochondrial complex I (NADH:ubiquinone oxidoreductase). *J Biol Chem* 274: 2625–2630, 1999.
224. Orringer EP and Roer ME. An ascorbate-mediated transmembrane-reducing system of the human erythrocyte. *J Clin Invest* 63: 53–58, 1979.
225. Oshino N, Imai Y, and Sato R. A function of cytochrome $b_5$ in fatty acid desaturation by rat liver microsomes. *J Biochem (Tokyo)* 69: 155–167, 1971.
226. Ozols J, Carr SA, and Strittmatter P. Identification of the NH2-terminal blocking group of NADH-cytochrome b5 reductase as myristic acid and the complete amino acid sequence of the membrane-binding domain. *J Biol Chem* 259: 13349–13354, 1984.
227. Pachucki J, Wang D, Christophe D, and Miot F. Structural and functional characterization of the two human ThOX/Duox genes and their 5′-flanking regions. *Mol Cell Endocrinol* 214: 53–62, 2004.
228. Paffenholz R, Bergstrom RA, Pasutto F, Wabnitz P, Munroe RJ, Jagla W, Heinzmann U, Marquardt A, Bareiss A, Laufs J, Russ A, Stumm G, Schimenti JC, and Bergstrom DE. Vestibular defects in head-tilt mice result from mutations in Nox3, encoding an NADPH oxidase. *Genes Dev* 18: 486–491, 2004.
229. Palming J, Sjoholm K, Jernas M, Lystig TC, Gummesson A, Romeo S, Lonn L, Lonn M, Carlsson B, and Carlsson LM. The expression of NAD(P)H:quinone oxidoreductase 1 is high in human adipose tissue, reduced by weight loss, and correlates with adiposity, insulin sensitivity, and markers

of liver dysfunction. *J Clin Endocrinol Metab* 92: 2346–2352, 2007.
230. Pao M, Wiggs EA, Anastacio MM, Hyun J, DeCarlo ES, Miller JT, Anderson VL, Malech HL, Gallin JI, and Holland. SM. Cognitive function in patients with chronic granulomatous disease: a preliminary report. *Psychosomatics* 45: 230–234, 2004.
231. Park L, Zhou P, Pitstick R, Capone C, Anrather J, Norris EH, Younkin L, Younkin S, Carlson G, McEwen BS, and Iadecola C. Nox2-derived radicals contribute to neurovascular and behavioral dysfunction in mice overexpressing the amyloid precursor protein. *Proc Natl Acad Sci U S A* 105: 1347–1352, 2008.
232. Parkos CA, Allen RA, Cochrane CG, and Jesaitis AJ. Purified cytochrome b from human granulocyte plasma membrane is comprised of two polypeptides with relative molecular weights of 91,000 and 22,000. *J Clin Invest* 80: 732–742, 1987.
233. Passon PG and Hultquist DE. Soluble cytochrome b 5 reductase from human erythrocytes. *Biochim Biophys Acta* 275: 62–73, 1972.
234. Pelicano H, Xu R, Du M, Feng L, Sasaki R, Carew JS, Hu Y, Ramdas L, Hu L, Keating MJ, Zhang W, Plunkett W, and Huang P. Mitochondrial respiration defects in cancer cells cause activation of Akt survival pathway through a redox mediated mechanism. *J Cell Biol* 175: 913–923, 2006.
235. Percy MJ and Lappin TR. Recessive congenital methaemoglobinaemia: cytochrome b(5) reductase deficiency. *Br J Haematol* 141: 298–308, 2008.
236. Pignatelli P, Sanguigni V, Paola SG, Lo Coco E, Lenti L, and Violi F. Vitamin C inhibits platelet expression of CD40 ligand. *Free Radic Biol Med* 38: 1662–1666, 2005.
237. Pink JJ, Planchon SM, Tagliarino C, Varnes ME, Siegel D, and Boothman DA. NAD(P)H:Quinone oxidoreductase activity is the principal determinant of beta-lapachone cytotoxicity. *J Biol Chem* 275: 5416–5424, 2000.
238. Prata C, Grasso C, Loizzo S, Sega FV, Caliceti C, Zambonin L, Fiorentini D, Hakim G, Berridge MV, and Landi L. Inhibition of trans-plasma membrane electron transport: a potential anti-leukemic strategy. *Leuk Res* doi:10.1016/j.leukres.2010.02.032, 2010.
239. Prata C, Maraldi T, Fiorentini D, Zambonin L, Hakim G, and Landi L. Nox-generated ROS modulate glucose uptake in a leukaemic cell line. *Free Radic Res* 42: 405–414, 2008.
240. Raghu G, Weycker D, Edelsberg J, Bradford WZ, and Oster G. Incidence and prevalence of idiopathic pulmonary fibrosis. *Am J Respir Crit Care Med* 174: 810–816, 2006.
241. Reddy VV, Kupfer D, and Capsi E. Mechanism of C-5 double bond introduction in the biosynthesis of cholesterol by rat liver microsomes. *J Biol Chem* 252: 2797–2801, 1977.
242. Rivlin J, Mendel J, Rubinstein S, Etkovitz N, and Breitbart H. Role of hydrogen peroxide in sperm capacitation and acrosome reaction. *Biol Reprod* 70: 518–522, 2004.
243. Rizvi SI, Jha R, and Maurya PK. Erythrocyte plasma membrane redox system in human aging. *Rejuvenation Res* 9: 470–474, 2006.
244. Rodríguez-Alonso J, Montañez R, Rodríguez-Caso L, and Medina MA. Homocysteine is a potent modulator of plasma membrane electron transport systems. *J Bioenerg Biomembr* 40: 45–51, 2008.
245. Rogers SC, Said A, Corcuera D, McLaughlin D, Kell P, and Doctor A. Hypoxia limits antioxidant capacity in red blood cells by altering glycolytic pathway dominance. *FASEB J* 23: 3159–3170, 2009.
246. Rossi F and Zatti M. Biochemical aspects of phagocytosis in polymorphonuclear leucocytes. NADH and NADPH oxidation by the granules of resting and phagocytizing cells. *Experientia* 20: 21–23, 1964.
247. Rubinstein B and Luster DG. Plasma membrane redox activity: components and role in plant processes. *Ann Rev Plant Physiol Plant Mol Biol* 44: 131–155, 1993.
248. Sabeur K and Ball BA. Characterization of NADPH oxidase 5 in equine testis and spermatozoa. *Reproduction* 134: 263–267, 2007.
249. Sakagami H, Satoh K, Ohata H, Takahashi H, Yoshida H, Iida M, Kuribayashy N, Sakagamy T, Momose K, and Takeda M. Relationship between ascorbyl radical intensity and apoptosis-inducing activity. *Anticancer Res* 16: 2981–2986, 1996.
250. Samhan-Arias AK, Duarte RO, Martín-Romero FJ, Moura JJ, and Gutiérrez-Merino C. Reduction of ascorbate free radical by the plasma membrane of synaptic terminals from rat brain. *Arch Biochem Biophys* 469: 243–254, 2008.
251. Samhan-Arias AK, Garcia-Bereguiain MA, Martin-Romero FJ, and Gutierrez-Merino C. Clustering of plasma membrane-bound cytochrome b5 reductase within "lipid raft" microdomains of the neuronal plasma membrane. *Mol Cell Neurosci* 40: 14–26, 2009.
252. Sánchez AM, Malagarie-Cazenave S, Olea N, Vara D, Chiloeches A, and Díaz-Laviada I. Apoptosis induced by capsaicin in prostate PC-3 cells involves ceramide accumulation, neutral sphingomyelinase, and JNK activation. *Apoptosis* 12: 2013–2024, 2007.
253. Savini I, Arnone R, Rossi A, Catani MV, Del Principe D, and Avigliano L. Redox modulation of Ecto-NOX1 in human platelets. *Mol Membr Biol* 27: 160–169, 2010.
254. Savini I, Catani MV, Arnone R, Rossi A, Frega G, Del Principe D, and Avigliano L. Translational control of the ascorbic acid transporter SVCT2 in human platelets. *Free Radic Biol Med* 42: 608–616, 2007.
255. Scarlett DJ, Herst P, Tan A, Prata C, and Berridge M. Mitochondrial gene-knockout (rho0) cells: a versatile model for exploring the secrets of trans-plasma membrane electron transport. *Biofactors* 20: 199–206, 2004.
256. Scarlett DJ, Herst PM, and Berridge MV. Multiple proteins with single activities or a single protein with multiple activities: the conundrum of cell surface NADH oxidoreductases. *Biochim Biophys Acta* 1708: 108–119, 2005.
257. Scholz RW, Minicucci LA, and Reddy CC. Effects of vitamin E and selenium on antioxidant defense in rat heart. Biochem. *Mol Biol Int* 42: 997–1006, 1997.
258. Schröder K, Helmcke I, Palfi K, Krause KH, Busse R, and Brandes RP. Nox1 mediates basic fibroblast growth factor-induced migration of vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol* 27: 1736–1743, 2007.
259. Schulze U, Schott H, and Staudinger H. Isolierung und charakterisierung einer NADH:semidehydroascorbinsaure-oxidoreduktase aus Neurospora crassa. *Hoppe-Seyler's Z Physiol Chem* 353: 1931–1942, 1972.
260. Scott EM and Griffith IV The enzymic defect of hereditary methemoglobinemia: diaphorase. *Biochim Biophys Acta* 34: 584–586, 1959.
261. Seigel D, Gustafson DL, Dehn DL, Han JY, Boonchoong P, Berliner LJ, and Ross D. NAD(P)H:quinone oxidoreductase 1: role as a superoxide scavenger. *Mol Pharmacol* 65: 1238–1247, 2004.
262. Semenza GL. Defining the role of hypoxia-inducible factor 1 in cancer biology and therapeutics. *Oncogene* 29: 625–634, 2010.

263. Semenza GL. Regulation of cancer cell metabolism by hypoxia-inducible factor 1. *Semin Cancer Biol* 19: 12–16, 2009.
264. 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, and Yokoyama M. Involvement of NADH/NADPH oxidase in human platelet ROS production. *Thromb Res* 103: 399–409, 2001.
265. Serrander L, Cartier L, Bedard K, Banfi B, Lardy B, Plastre O, Sienkiewicz A, Fórró L, Schlegel W, and Krause KH. NOX4 activity is determined by mRNA levels and reveals a unique pattern of ROS generation. *Biochem J* 406: 105–114, 2007.
266. Shimizu S, Matsuoka Y, Shinohara Y, Yoneda Y, and Tsujimoto Y. Essential role of voltage-dependent anion channel in various forms of apoptosis in mammalian cells. *J Cell Biol* 152: 237–250, 2001.
267. Shimizu S, Narita M, and Tsujimoto Y. BcL-2 family proteins regulate the release of apoptogenic cytochrome c by the mitochondrial channel VDAC. *Nature* 399: 483–487, 1999.
268. Shinohara M, Shang WH, Kubodera M, Harada S, Mitsushita J, Kato M, Miyazaki H, Sumimoto H, and Kamata T. Nox1 redox signaling mediates oncogenic Ras-induced disruption of stress fibers and focal adhesions by down-regulating Rho. *J Biol Chem* 282: 17640–17648, 2007.
269. Shiose A, Kuroda J, Tsuruya K, Hirai M, Hirakata H, Naito S, Hattori M, Sakaki Y, and Sumimoto H. A novel superoxide-producing NAD(P)H oxidase in kidney. *J Biol Chem* 276: 1417–1423, 2001.
270. Shirabe K, Landi MT, Takeshita M, Uziel G, Fedrizzi E, and Borgese N. A novel point mutation in a 3′ splice site of the NADH-cytochrome b5 reductase gene results in immunologically undetectable enzyme and impaired NADH-dependent ascorbate regeneration in cultured fibroblasts of a patient with type II hereditary methemoglobinemia. *Am J Hum Genet* 57: 302–310, 1995.
271. Shukla S, Jha RK, Laloraya M, and Kumar PG. Identification of nonmitochondrial NADPH oxidase and the spatiotemporal organization of its components in mouse spermatozoa. *Biochem Biophys Res Commun* 331: 476–483, 2005.
272. Song Y, Ruf J, Lothaire P, Dequanter D, Andry G, Willemse E, Dumont JE, Van Sande J, and De Deken X. Association of duoxes with thyroid peroxidase and its regulation in thyrocytes. *J Clin Endocrinol Metab* 95: 375–382, 2010.
273. Stasia MJ and Li XJ. Genetics and immunopathology of chronic granulomatous disease. *Semin Immunopathol* 30: 209–235, 2008.
274. Strittmatter P. The reaction sequence in electron transfer in the reduced nicotinamide adenine dinucleotide-cytochrome b5 reductase system. *J Biol Chem* 240: 4481–4487, 1965.
275. Su D and Asard H. Three mammalian cytochromes b561 are ascorbate-dependent ferrireductases. *FEBS J* 273: 3722–3734, 2006.
276. Su D, May JM, Koury MJ, and Asard H. Human erythrocyte membranes contain a cytochrome b561 that may be involved in extracellular ascorbate recycling. *J Biol Chem* 281: 39852–39859, 2006.
277. Suh YA, Arnold RS, Lassegue B, Shi J, Xu X, Sorescu D, Chung AB, Griendling KK, and Lambeth JD. Cell transformation by the superoxide-generating oxidase Mox1. *Nature* 401: 79–82, 1999.
278. Sumimoto H, Hata K, Mizuki K, Ito T, Kage Y, Sakaki Y, Fukumaki Y, Nakamura M, and Takeshige K. Assembly and activation of the phagocyte NADPH oxidase. Specific interaction of the N-terminal Src homology 3 domain of p47phox with p22phox is required for activation of the NADPH oxidase. *J Biol Chem* 271: 22152–22158, 1996.
279. Sumimoto H. Structure, regulation and evolution of Nox-family NADPH oxidases that produce reactive oxygen species. *FEBS J* 275: 3249–3277, 2008.
280. Tabet F, Schiffrin EL, Callera GE, He Y, Yao G, Ostman A, Kappert K, Tonks NK, and Touyz RM. Redox-sensitive signaling by angiotensin II involves oxidative inactivation and blunted phosphorylation of protein tyrosine phosphatase SHP-2 in vascular smooth muscle cells from SHR. *Circ Res* 103: 149–158, 2008.
281. Tan AS and Berridge MV. Superoxide produced by activated neutrophils efficiently reduces the tetrazolium salt WST-1 to produce a soluble formazan: a simple colorimetric assay for measuring respiratory burst activation and for screening anti-inflammatory agents. *J Immunol Methods* 238: 59–68, 2000.
282. Tan AS and Berridge MV. Distinct trans-plasma membrane redox pathways reduce cell-impermeable dyes in HeLa cells. *Redox Rep* 9: 302–306, 2004.
283. Tan AS and Berridge MV. Differential effects of redox-cycling and arylating quinones on trans-plasma membrane electron transport. *Biofactors* 34: 183–190, 2009.
284. Tan AS and Berridge MV. Evidence for NAD(P)H:quinone oxidoreductase 1 (NQO1)-mediated quinone-dependent redox cycling via plasma membrane electron transport: A sensitive cellular assay for NQO1. *Free Radic Biol Med* 48: 421–429, 2010.
285. Thinnes FP. Evidence for extra-mitochondrial localization of the VDAC/porin channel in eucaryotic cells. *J Bioenerg Biomembr* 24: 71–75, 1992.
286. Tirone F and Cox JA. NADPH oxidase 5 (NOX5) interacts with and is regulated by calmodulin. *FEBS Lett* 581: 1202–1208, 2007.
287. Tomatsu S, Kobayashi Y, Fukumaki Y, Yubisui T, Orii T, and Sakaki Y. The organization and the complete nucleotide sequence of the human NADH-cytochrome b5 reductase gene. *Gene* 80: 353–361, 1989.
288. Tominaga K, Kawahara T, Sano T, Toida K, Kuwano Y, Sasaki H, Kawai T, Teshima-Kondo S, and Rokutan K. Evidence for cancer-associated expression of NADPH oxidase 1 (Nox1)-based oxidase system in the human stomach. *Free Radic Biol Med* 43: 1627–1638, 2007.
289. Touyz RM and Schiffrin EL. Increased generation of superoxide by angiotensin II in smooth muscle cells from resistance arteries of hypertensive patients: role of phospholipase D-dependent NAD(P)H oxidase-sensitive pathways. *J Hypertens* 19: 1245–1254, 2001.
290. Tran UC and Clarke CF. Endogenous synthesis of coenzyme Q in eukaryotes. *Mitochondrion* 7: S62–S71, 2007.
291. Tsujimoto Y and Shimizu S. Role of the mitochondrial membrane permeability transition in cell death. *Apoptosis* 12: 835–840, 2007.
292. Tsunawaki S, Kagara S, Yoshikawa K, Yoshida LS, Kuratsuji T, and Namiki H. Involvement of p40phox in activation of phagocyte NADPH oxidase through association of its carboxyl-terminal, but not its amino-terminal, with p67phox. *J Exp Med* 184: 893–902, 1996.
293. Turi JL, Wang X, McKie AT, Nozik-Grayck E, Mamo LB, Crissman K, Piantadosi CA, and Ghio AJ. Duodenal cytochrome b: a novel ferrireductase in airway epithelial cells. *Am J Physiol Lung Cell Mol Physiol* 291: L272–L280, 2006.
294. Ueno N, Takeya R, Miyano K, Kikuchi H, and Sumimoto H. The NADPH oxidase Nox3 constitutively produces su-

peroxide in a p22phox-dependent manner: its regulation by oxidase organizers and activators. *J Biol Chem* 280: 23328–23339, 2005.
295. Ukai H and Ueda HR. Systems biology of mammalian circadian clocks. *Annu Rev Physiol* 72: 579–603, 2010.
296. Ushio-Fukai M. VEGF signaling through NADPH oxidase-derived ROS. *Antioxid Redox Signal* 9: 731–739, 2007.
297. Ushio-Fukai M, Zafari AM, Fukui T, Ishizaka N, and Griendling KK. p22phox is a critical component of the superoxide-generating NADH/NADPH oxidase system and regulates angiotensin II-induced hypertrophy in vascular smooth muscle cells. *J Biol Chem* 271: 23317–23321, 1996.
298. Vander Heiden MG, Cantley LC, and Thompson CB. Understanding the Warburg effect: the metabolic requirements of cell proliferation. *Science* 324: 1029–1033, 2009.
299. VanDuijn MM, Van der Zee J, and Van den Broek PJ. The ascorbate-driven reduction of extracellular ascorbate free radical by the erythrocyte is an electrogenic process. *FEBS Lett* 491: 67–70, 2001.
300. Vernet P, Fulton N, Wallace C, and Aitken RJ. Analysis of reactive oxygen species generating systems in rat epididymal spermatozoa. *Biol Reprod* 65: 1102–1113, 2001.
301. Villalba JM, Navarro F, Córdoba F, Serrano A, Arroyo A, Crane FL, and Navas P. Coenzyme Q reductase from liver plasma membrane: purification and role in trans-plasma-membrane electron transport. *Proc Natl Acad Sci U S A* 92: 4887–4891, 1995.
302. Villalba JM, Navarro F, Gomez-Dıaz C, Arroyo A, Bello RI, and Navas P. Role of cytochrome b5 reductase on the antioxidant function of coenzyme Q in the plasma membrane. *Mol Aspects Med* 18: S7–S13, 1997.
303. Viora M, Quaranta MG, Straface E, Vari R, Masella R, and Malorni W. Redox imbalance and immune functions: opposite effects of oxidized low-density lipoproteins and N-acetylcysteine. *Immunology* 104: 431–438, 2001.
304. Voegtlin C, Johnson JM, and Dyer HA Jr. Quantitative estimation of the reducing power of normal and cancer tissue. *J Pharmacol Exp Ther* 24: 305–334, 1925.
305. Wang C and Youle RJ. The role of mitochondria in apoptosis. *Annu Rev Genet* 43: 95–118, 2009.
306. Wang HM, Chueh PJ, Chang SP, Yang CL, and Shao KN. Effect of capsaicin on tNOX (ENOX2) protein expression in stomach cancer cells. *Biofactors* 34: 209–217, 2009.
307. Wang S, Morré DM, and Morré DJ. Sera from cancer patients contain two oscillating ECTO-NOX activities with different period lengths. *Cancer Lett* 190: 135–141, 2003.
308. Wang S, Pogue R, Morré DM, and Morré 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* 1539: 192–204, 2001.
309. Warburg O. (Eds). *The Metabolism of Tumors*. London: Constable & Co, 1930.
310. Wesley UV, Bove PF, Hristova M, McCarthy S, and van der Vliet A. Airway epithelial cell migration and wound repair by ATP-mediated activation of dual oxidase 1. *J Biol Chem* 282: 3213–3220, 2007.
311. Wilkinson F, Kim C, Cho N, Chueh P-J, Leslie S, Moya-Camarena S, Wu L-Y, Morré DJ, and Morré DM. Isolation and identification of a protein with capsaicin-inhibited NADHoxidase activity from culture media conditioned by growth of HeLa cells. *Arch Biochem Biophys* 336: 275–282, 1996.
312. Wolvetang EJ, Larm JA, Moutsoulas P, and Lawen A. Apoptosis induced by inhibitors of the plasma membrane NADH-oxidase involves Bcl-2 and calcineurin. *Cell Growth Differ* 7: 1315–1325, 1996.
313. Wong JL, Créton R, and Wessel GM. The oxidative burst at fertilization is dependent upon activation of the dual oxidase Udx1. *Dev Cell* 7: 801–814. 2004.
314. Wyman S, Simpson RJ, McKie AT, and Sharp PA. Dcytb (Cybrd1) functions as both a ferric and a cupric reductase *in vitro*. *FEBS Lett* 582: 1901–1906, 2008.
315. Yagiz K, Wu LY, Kuntz CP, James Morré D, and Morré DM. Mouse embryonic fibroblast cells from transgenic mice overexpressing tNOX exhibit an altered growth and drug response phenotype. *J Cell Biochem* 101: 295–306, 2007.
316. Yantiri F, Morré DJ, Yagiz K, Barogi S, Wang S, Chueh PJ, Cho N, Sedlak D, and Morré DM. Capsaicin-responsive NADH oxidase activities from urine of cancer patients. *Arch Biochem Biophys* 358: 336–342, 1998.
317. Ying W. NAD+/NADH and NADP+/NADPH in cellular functions and cell death: regulation and biological consequences. *Antioxid Redox Signal* 10: 179–206, 2008.
318. Zaid H, Abu-Hamad S, Israelson A, Nathan I, and Shoshan-Barmatz V. The voltage-dependent anion channel-1 modulates apoptotic cell death. *Cell Death Differ* 12: 751–760, 2005.
319. Zainal TA, Oberley TD, Allison DB, Szweda LI, and Weindruch R. Caloric restriction of rhesus monkeys lowers oxidative damage in skeletal muscle. *FASEB J* 14: 1825–1836, 2000.
320. Zalba G, Beaumont FJ, San Jose G, Fortuno A, Fortuno MA, Etayo JC, and Diez J. Vascular NADH/NADPH oxidase is involved in enhanced superoxide production in spontaneously hypertensive rats. *Hypertension* 35: 1055–1061, 2000.
321. Zalba G, San José G, Moreno MU, Fortuño A, and Díez J. NADPH Oxidase-mediated oxidative stress: genetic studies of the p22phox gene in hypertension. *Antioxid Redox Signal* 7: 1327–1336, 2005.
322. Zhan S, Vazquez N, Zhan S, Wientjes FB, Budarf ML, Schrock E, Ried T, Green ED, and Chanock SJ. Genomic structure, chromosomal localization, start of transcription, and tissue expression of the human p40-phox, a new component of the nicotinamide adenine dinucleotide phosphate-oxidase complex. *Blood* 88: 2714–2721, 1996.
323. Zhang Z, Yu J, and Stanton RC. A method for determination of pyridine nucleotides using a single extract. *Anal Biochem* 285: 163–167, 2000.

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Date of first submission to ARS Central, April 12, 2010; date of final revised submission, August 25, 2010; date of acceptance, September 2, 2010.

**Abbreviations Used**

AD = activation domain
AFR = ascorbyl free radical
AIR = auto-inhibitory region
arNOX = age-related NADH oxidase
Asc = ascorbate
CoQ = coenzyme Q
Dcytb = duodenal cytochrome b
DHA = dehydroascorbate
ENOX1 = disulfide-thiol exchanger 1
HeLa = human cervical carcinoma
mPMS = 1-methoxy-5-methyl-phenazinium methyl sulfate
MTS = 5-[3-(carboxymethoxy)phenyl]-3-(4,5-dimethyl-2-thiazolyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt
Nox = NADPH oxidase
NoxA1 = Nox activator 1
NoxO1 = Nox organizer 1
NQO1 = NAD(P)H:ubiquinone oxidoreductase
PKA = cAMP-dependent protein kinase
PRR = proline-rich region
PTP = permeability transition pore
ROS = reactive oxygen species
SOD = superoxide dismutase
Tempol = 2,2,6,6,-tetramethyl-4-hydroxypiperidine-*N*-oxyl
TGF = transforming growth factor
t-PMET = trans-plasma membrane electron transport
TPR = tetratricopeptide repeat motifs
TRPV1 = transient receptor potential cation channel, subfamily V, member 1
VDAC = voltage dependent anion selective channel
WST-1 = sodium 5-(2,4-disulfophenyl)-2-(4-iodophenyl)-3-(4-nitrophenyl)-2H-tetrazolium inner salt
XTT = sodium 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium inner salt

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2. Enrico Rinaldelli, Alessandra Panattoni, Andrea Luvisi, Enrico Triolo. 2012. Effect of mycophenolic acid on trans-plasma membrane electron transport and electric potential in virus-infected plant tissue. *Plant Physiology and Biochemistry* **60**, 137-140. [CrossRef]
3. Mei-Han Huang, Kuan-Hua Lin, Sheue-Jiun Chen, Ai-Yu Shen, Fang-Tzu Wu, Sheng-Nan Wu. 2012. Effects of ketamine and its metabolites on ion currents in differentiated hippocampal H19-7 neuronal cells and in HEK293T cells transfected with #-hslo subunit. *NeuroToxicology* . [CrossRef]
4. Emma Heart, Meridith Palo, Trayce Womack, Peter J.S. Smith, Joshua P. Gray. 2011. The level of menadione redox-cycling in pancreatic #-cells is proportional to the glucose concentration: Role of NADH and consequences for insulin secretion. *Toxicology and Applied Pharmacology* . [CrossRef]