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REVIEW

# SURFACE OXIDASE AND OXIDATIVE STRESS PROPAGATION IN AGING

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## Summary

**This report summarizes new evidence for a plasma-membrane-associated hydroquinone oxidase designated as CNOX (constitutive plasma membrane NADH oxidase) that functions as a terminal oxidase for a plasma membrane oxidoreductase (PMOR) electron transport chain to link the accumulation of lesions in mitochondrial DNA to cell-surface accumulations of reactive oxygen species. Previous considerations of plasma membrane redox changes during aging have lacked evidence for a specific terminal oxidase to catalyze a flow of electrons from cytosolic NADH to molecular oxygen (or to protein disulfides). Cells with functionally deficient mitochondria become characterized by an anaerobic metabolism. As a result, NADH accumulates from the glycolytic production of ATP. Elevated PMOR activity has been shown to be necessary to maintain the $NAD^+$/NADH homeostasis essential for survival. Our findings demonstrate that the hyperactivity of the PMOR system results in an NADH oxidase (NOX) activity capable of generating reactive oxygen species at the cell surface. This would serve to propagate the aging cascade both to adjacent cells and to circulating blood components. The generation of superoxide by NOX forms associated with aging is inhibited by coenzyme Q and provides a rational basis for the anti-aging activity of circulating coenzyme Q.**

Key words: hydroquinone (NADH) oxidase, mitochondria, ageing, cell surface, plasma membrane, electron transport, coenzyme Q, oxidative stress.

## A plasma membrane redox system essential to survival of mitochondrial-deficient cells during aging

A consistent characteristic of aging cells is the accumulation of somatic mutations of mitochondrial DNA (mtDNA) leading to defective oxidative phosphorylation through alterations that affect exclusively the four mitochondrial complexes involved in proton translocation (Harman, 1956, 1972; Miquel et al., 1980; Linnane et al., 1989; Arnheim and Cortopassi, 1992; Ozawa, 1995; de Grey, 1997, 1998; Lenaz et al., 1997, 1998). A major piece of the puzzle missing from our information is how mitochondrial lesions are propagated to adjacent cells and blood components during the aging cascade. Progress towards understanding how this might occur is provided by the studies of de Grey (1997, 1998), in which a largely hypothetical plasma membrane oxidoreductase (PMOR) system has been suggested to augment survival of mitochondrially deficient cells through the regeneration of oxidized pyridine nucleotide required to sustain glycolytic ATP production in the presence of diminished respiratory chain activity (Yoneda et al., 1995; Schon et al., 1996; Ozawa, 1997; Lenaz, 1998).

In this report, we describe a newly discovered cell-surface protein with hydroquinone (NADH) oxidase activity (designated NOX) (Kishi et al., 1999) that functions as a terminal oxidase of the PMOR system together with a complete electron transport chain involving a cytosolic hydroquinone reductase, plasma-membrane-located quinones and the NOX protein (Morré, 1998). This system, described in detail since the studies of de Grey (1997, 1998) appeared, provides a rational basis for the operation of the mitochondrial theory of aging and for the propagation of aging-related mitochondrial lesions, including a decline in mitochondrial ATP synthetic capacity (Boffoli et al., 1996) and other energy-dependent processes (Lenaz et al., 1998) during aging.

Alterations in mitochondrial DNA (mtDNA) are by far the most common sources of genetic lesions associated with cell aging and senescence. It has been widely noted that mtDNAs are located at the inner mitochondrial membrane near sites where highly reactive oxygen species and their products might be formed. Several subunits of the electron transport chain together with components of the ATP synthase and mitochondrial tRNAs and rRNAs are encoded by the mitochondrial genome. The flow of electrons through the

mitochondrial electron transport chain is not fully efficient, and up to 2–4 % of the oxygen metabolized by mitochondria has been estimated to be converted to oxygen radicals (Boveris et al., 1972; Richter et al., 1988). A major tenet of the mitochondrial theory of aging is that mtDNA may be unable to counteract the damage inflicted by oxygen radicals and their products because of a lack of excision and recombination repair mechanisms (Miquel, 1992). This has been demonstrated in cultured cells in which damage to mtDNA resulting from oxidase stress is not only greater but persists longer than does damage to nuclear DNA (Yakes and Van Houten, 1997). Using the amount of 8-oxo-2′-deoxyguanosine formed by the reaction of hydroxyl free radicals with guanine in mtDNA as a biomarker of oxidative DNA damage, the steady-state level of oxidative changes in mtDNA was found to be approximately 10–16 times greater than that of changes in nuclear DNA (Richter et al., 1988; Shigenaga et al., 1994). Even lipid peroxidation of mitochondrial membranes seems to lead to damage to mtDNA (Balcavage, 1982).

Despite this overwhelming mass of evidence, alterations to mtDNA *per se* and other forms of cellular and tissue changes related to aging have been difficult to link. Chief among these is the oxidation of low-density lipoproteins (LDLs) and its implications as causal to atherogenesis (Steinberg, 1997).

A model to link accumulation of lesions in mtDNA to an extracellular response such as the oxidation of lipids in LDLs and the attendant arterial changes was first proposed by de Grey (1997, 1998) on the basis of the observations of Larm et al. (1994) and Lawen et al. (1994) with Namalwa $\rho^0$ cells. These cells lack mtDNA and are unable to carry out oxidative phosphorylation. Larm et al. (1994) and Lawen et al. (1994) first demonstrated that the plasma membrane PMOR system actually functions to regenerate $NAD^+$ from NADH. In the absence of a functional mitochondrial respiratory chain, NADH accumulates as the result of glycolytic production of ATP (Fig. 1). The $\rho^0$ cells lacking functional mitochondria apparently survive through enhanced electron flow to molecular oxygen *via* PMOR. In addition, it may be possible that aging cells over-express PMOR when mitochondrial functions are depressed. Unpublished data from the laboratory of G. Lenaz (Table 1) has demonstrated that, in lymphocytes from insulin-dependent diabetic subjects, the mitochondrial membrane potential exhibits increased sensitivity to uncouplers as a result of decreased electron input from the respiratory chain. PMOR is accordingly over-expressed in these cells. Oxidative stress and LDL oxidation are common complicating features in diabetics (Kennedy and Lyons, 1998).

The capacity of cells to generate ATP is determined either by reoxidation of NADH by mitochondrial respiratory mechanisms (reduction of oxygen to water) or by cytosolic glycolytic mechanisms (reduction of pyruvate to lactate). If sufficient pyruvate and uridine are provided, cells can grow without a functional mitochondrial electron transport chain and oxidative phosphorylation. As shown by Vaillant et al. (1996), transformed human cells in culture provided with excess pyruvate grow anaerobically on a glucose medium because $NAD^+$ is regenerated from the NADH that is produced during glycolysis. This continual regeneration of $NAD^+$, including that generated by the PMOR, ensures that the glycolytic pathway will provide sufficient ATP to sustain cell growth and viability.

Table 1. *Bioenergetic parameters in peripheral lymphocytes from patients with insulin-dependent diabetes mellitus*

| | Site I respiration* | Site II respiration* | Sensitivity to FCCP‡ | PMOR activity§ (nmol $min^{-1}$ $10^{-6}$ $cells^{-1}$) |
|---|---|---|---|---|
| Patients | 0.12±0.05 | 0.08±0.08 | 19.7±7.5 | 2.7±0.6 |
| Controls | 0.26±0.12 | 0.21±0.07 | 5.2±1.9 | 1.9±0.5 |
| *P* | <0.02 | <0.01 | <0.001 | <0.005 |

Values are means ± s.e.m. (*N*=14 for patients and 13 for controls).

*$O_2$ uptake with glutamate/malate (site I) and with succinate/glycerol 3-phosphate (site II) are normalized to cytochrome *c* oxidase activity (the ratio of activity measured to cytochrome oxidase activity measured by ascorbate/N,N,N′,N′-tetramethyl-*p*-phenylenediamine (TMPD) oxidation). The measurements were performed in digitonin-permeabilized lymphocytes.

‡Slope of the green to red fluorescence ratio of 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC1) measured in flow cytometry after addition of increasing concentrations of the uncoupler 4-trifluoromethoxy-carbonylcyanidephenylhydrazone (FCCP) (expressed in arbitrary units).

§Dichlorophenolindophenol (DCIP) reduction by endogenous NADH in intact lymphocytes.

PMOR, plasma membrane oxidoreductase activity.

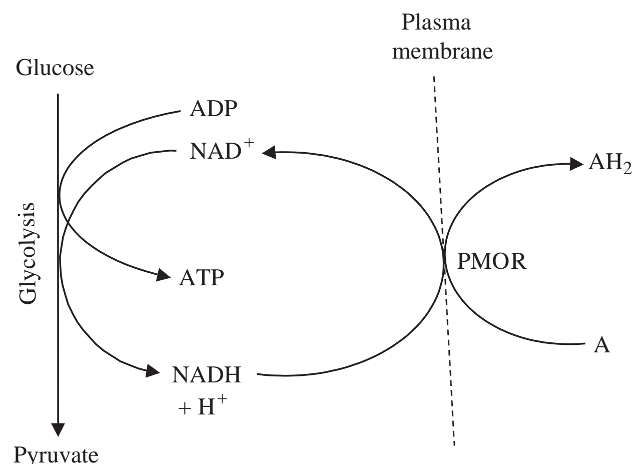


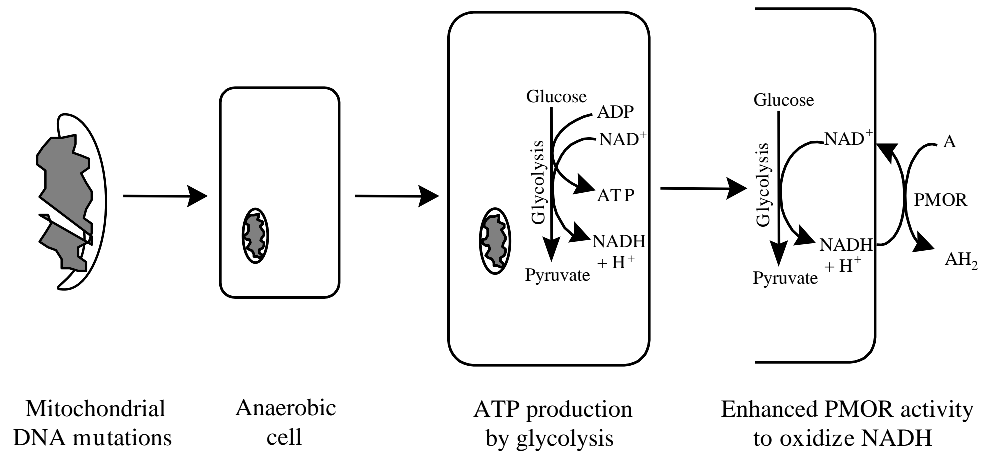

Fig. 1. Relationship between the plasma membrane oxidoreductase (PMOR) system and the regeneration of $NAD^+$ from NADH formed during glycolysis. A is the external acceptor.

*New evidence for a plasma membrane oxidoreductase chain important to aging*

As demonstrated with $\rho^0$ cells, a functional PMOR is essential to aging cells expressing mitochondrial lesions. Mitochondrial DNA encodes respiration and oxidative phosphorylation enzymes exclusively, so that cells with functionally deficient mitochondria become metabolically anaerobic. In such cells, the PMOR could regenerate sufficient reducing equivalents to maintain $NAD^+$/NADH homeostasis and ensure the survival even of cells completely deficient in aerobic respiratory capacity.

Our work demonstrates that, in cells in which the PMOR is over-expressed/activated, electrons are transferred from NADH to external acceptors *via* a recently defined electron transport chain in the plasma membrane (Kishi et al., 1999). The resultant transfer of electrons could result subsequently in the generation of superoxide and ultimately other reactive oxygen species (ROS) at the cell surface (Table 2). Such cell-surface-generated ROS would then be capable of propagating an aging cascade originating in mitochondria both to adjacent cells and to circulating blood components such as LDLs and to the vasculature (Fig. 2).

Work done in collaboration with Professor T. Kishi, Kobe-Gakuin University, Japan, has described a cell-surface NADH oxidase protein, designated NOX, capable of oxidizing hydroquinones (Kishi et al., 1999). This protein, which is located at the exterior of the cell (Morré, 1995; DeHahn et al., 1997), appears to be multifunctional but may have a major function as a terminal oxidase of the PMOR system. These findings make it possible, for the first time, to delineate a complete electron transfer chain in the plasma membrane capable of transferring electrons from NADH to an external electron acceptor *via* a reduced quinone intermediate. Mammalian plasma membranes are enriched in coenzyme Q (ubiquinone) (Table 3). The plasma membrane at the cytosolic surface contains a quinone reductase capable of oxidizing NADH and reducing coenzyme Q. The electron acceptor at the external cell surface is either molecular oxygen or, under certain conditions, both molecular oxygen and protein disulfides (Morré, 1994; Chueh et al., 1997; Morré et al., 1998). The enzyme can alternate between the two acceptors (Morré, 1998). Hormones and growth factors stimulate NADH oxidation and favor protein disulfide reduction at the expense of oxygen consumption (Brightman et al., 1992; Morré, 1994; Chueh et al., 1997). Chueh et al. (1997) demonstrated stoichiometric relationships among protein disulfide reduction, NADH oxidation and protein-thiol formation using isolated plasma membranes from a plant source stimulated by an auxin



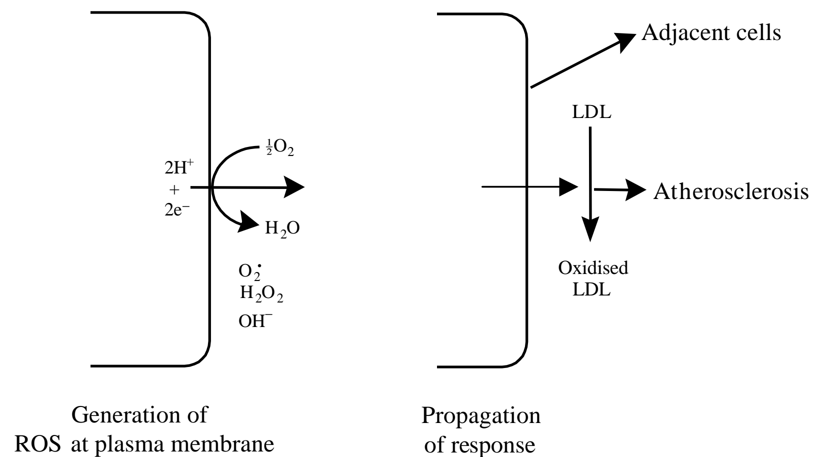




Fig. 2. Hypothesis to explain the mechanism whereby anaerobiosis resulting from mitochondrial lesions, the resultant stimulation of glycolysis and the enhancement of the plasma membrane oxidoreductase (PMOR) system result in the formation of reactive oxygen species (ROS) at the cell surface that can be propagated and affect both adjacent cells and circulating blood components. LDL, low-density lipoprotein.

plant growth factor 2,4-dichlorophenoxyacetic acid (2,4-D). A similar stoichiometry has been demonstrated for NADH oxidation in HeLa cells (Morré et al., 1998).

As a terminal oxidase of the PMOR electron transport chain, the NOX protein may be responsible not only for maintaining $NAD^+$/NADH homeostasis in metabolically anaerobic cells but may also play a role in the enhanced generation of ROS in aged cells expressing mitochondrial mutations that lead to impaired oxidative phosphorylation. Since oxygen appears to be the principal natural electron acceptor for the PMOR electron transport chain, a number of factors, including metals (iron or copper), could interrupt the orderly two-electron flow to molecular oxygen that ordinarily forms water and initiates a one-electron process producing superoxide ($O_2^{\bullet}$ or $O_2^-$) (Fig. 2). Superoxide then probably initiates a reaction that generates $H_2O_2$ and other aggressive oxidants such as the hydroxyl radical ($OH^{\bullet}$) (Papa and Skulachev, 1997). These ROS then would be released into the environment to react with neighboring cells and circulating molecules such as LDL (Steinberg, 1997).

## Plasma membrane hydroquinone (NADH) oxidase (NOX)

The plasma membrane NADH oxidase (NOX) is a unique cell-surface protein with hydroquinone (NADH) oxidase and protein-disulfide–thiol interchange activities that normally respond to hormones and growth factors (Brightman et al., 1992; Morré, 1994, 1998). A hormone-insensitive and drug-responsive form of the activity designated tNOX also has been described that is specific for cancer cells (Bruno et al., 1992; Morré and Morré, 1995; Morré et al., 1995a,b, 1996a, 1997c).

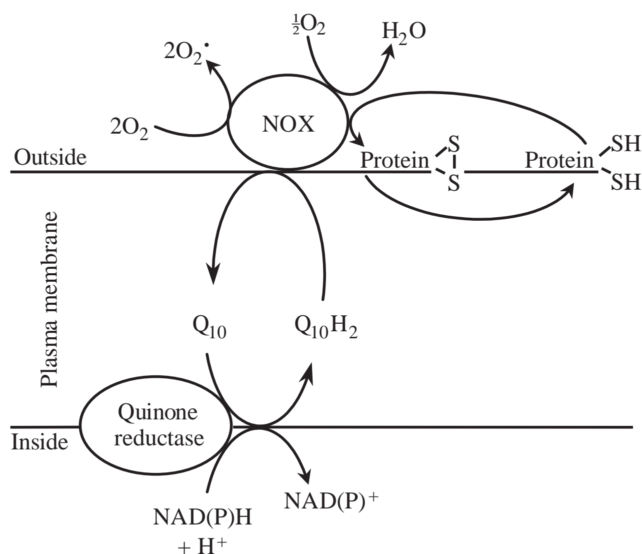



Fig. 3. Diagram showing the spatial relationships between the intracellular NAD(P)H:quinone reductase, the membrane pool of coenzyme Q ($Q_{10}$) and the external NADH oxidase (NOX) protein across the plasma membrane. In this manner, the NOX protein could function as a terminal oxidase of plasma membrane electron transport, donating electrons from cytosolic NADH either to molecular oxygen or to protein disulfides as electron acceptors.

Because the NOX protein is located at the external plasma membrane surface and is not a transmembrane protein (Morré, 1994; DeHahn et al., 1997), a functional role as an NADH oxidase is not considered likely (Morré, 1998). Although the oxidation of NADH provides a basis for a convenient method to assay the activity, the ultimate physiological electron donors are most probably hydroquinones (Kishi et al., 1999), as depicted in Fig. 3, with specific activities for hydroquinone oxidation being greater than or equal to those of NADH oxidation and/or protein-disulfide–thiol interchange.

The NOX protein partially purified from the surface of HeLa cells also exhibits ubiquinol oxidase activity (Kishi et al., 1999). These preparations completely lack NADH:ubiquinone reductase activity and oxidize the dihydroquinone $Q_{10}H_2$ at a rate of 3–6 $nmol\,min^{-1}\,mg^{-1}$ protein. The $K_m$ for $Q_{10}H_2$ is 30 $\mu mol\,l^{-1}$. Activities are inhibited competitively by the cancer-cell-specific NADH oxidase inhibitors capsaicin (8-methyl-*N*-vanillyl-6-noneamide) (Morré et al., 1995a, 1996a) and the antitumor sulfonylurea *N*-(4-methylphenylsulfonyl)-*N*′-(4-chlorophenyl)urea (LY181984) (Morré et al., 1995b). The oxidation of $Q_{10}H_2$ proceeds with what appears to be a normal two-electron transfer, in keeping with the participation of the plasma membrane NADH oxidase as a terminal oxidase of plasma membrane electron transport from cytosolic NAD(P)H *via* coenzyme Q to acceptors at the cell surface, as depicted in Fig. 3.

The NOX protein is distinguished from other oxidase activities by differential susceptibility of the activity to several common oxidoreductase inhibitors (Morré and Brightman, 1991) and to thiol reagents (Morré and Morré, 1994). In addition, the activity of tNOX correlates with the growth of transformed cells (Morré et al., 1995a, 1996a; Ozawa, 1995). When inhibited by tNOX-specific vanilloids or antitumor sulfonylureas, the cells initially divide normally, and DNA and protein synthesis is not inhibited, but the cells fail to enlarge (Morré and Morré, 1995; Morré et al., 1995a). The resultant small cells, however, fail to divide and, after a few days, begin to undergo apoptotic cell death (Morré and Morré, 1995; Morré et al., 1995a; DeHahn et al., 1997). Two monoclonal antibodies directed against tNOX, designated MAB 12.1 and MAB 12.5, were generated and were shown also to be inhibitory to cell enlargement in cancer cells but not in normal cells and to induce apoptotic cell death even more efficiently than did the drug inhibitors of tNOX (Morré, 1998).

In cancer cells, the tumor-associated (tNOX) activity was constitutively activated (Morré et al., 1995a) and inhibited by retinoids (Dai et al., 1997) and by other potential quinone-site-inhibitory drugs, such as the antitumor sulfonylurea LY181984 (Morré et al., 1995b) and capsaicin (Morré et al., 1995a), but was no longer hormone- or growth-factor responsive (Bruno et al., 1992; Morré et al., 1995a). The ability to oxidize NADH was reflected in the ability of the protein to function as an NADH:protein disulfide reductase or protein-disulfide–thiol oxidoreductase with protein-disulfide–thiol interchange

activity (Morré et al., 1997b). The latter may be related to low levels of a protein disulfide isomerase-like activity reported at the cell surface (Mandel et al., 1993). The protein is associated with the enlargement phase of cell growth (Morré, 1998). When NOX activity is inhibited, growth is also inhibited. In the presence of capsaicin (Morré et al., 1995a), the antitumor sulfonylurea LY181984 (Morré and Morré, 1995) and NOX-inhibitory retinoids (Dai et al., 1997), the inhibited cells fail to enlarge, division ceases and apoptotic cell death is the ultimate fate of the inhibited cells.

CNOX was originally defined as a drug-indifferent constitutive NADH oxidase activity associated with the plasma membrane of non-transformed cells that was the normal counterpart to tNOX. Indeed, a 36 kDa protein isolated from rat liver and from plants has NOX activity that is unresponsive to tNOX inhibitors.

While cancer cells exhibit both drug-responsive and hormone- and growth-factor-indifferent (tNOX) as well as drug-inhibited and hormone- and growth-factor-dependent (CNOX) activities, non-transformed cells exhibit only the drug-indifferent, hormone- and drug-responsive CNOX. Among the first descriptions of the so-called constitutive or CNOX activity of non-transformed cells and tissues was that in rat liver plasma membranes, in which the activity was stimulated by the growth factor diferric transferrin (Sun et al., 1987). Subsequent work demonstrated that this NADH oxidation was catalyzed by a unique enzyme exhibiting responsiveness to several hormones and growth factors (Bruno et al., 1992). Unlike mitochondrial oxidases, the hormone-stimulated NADH oxidase activity of rat liver plasma membranes was not inhibited by cyanide. The enzyme was also distinguished from other oxidase activities by its response to several common oxidoreductase inhibitors (i.e. catalase, azide and chloroquine) and to various detergents (i.e. sodium cholate, Triton X-100 and Chaps) (Morré and Brightman, 1991; Morré et al., 1997c). Like the tNOX of cancer cells, CNOX is a unique membrane-associated protein that is capable of oxidizing NADH, but its activity is modulated by hormones and growth factors.

Table 2 presents evidence that NOX proteins under certain conditions are capable of the production of ROS. We have used ultraviolet light as a source of oxidative stress in cultured cells to initiate superoxide generation (Morré et al., 1999). Such generation is presumably due to the NADH oxidase because in cell lines (HeLa, a human cervical carcinoma, and BT-20, a human mammary carcinoma) that contain a capsaicin-responsive NADH oxidase, the response to ultraviolet light is inhibited by capsaicin. In the MCF-10A cell line (a human mammary epithelium), lacking tNOX activity and not cancerous, the ultraviolet-light-induced generation of superoxide is unaffected by capsaicin and, presumably, the resultant effect of ultraviolet light on the plasma membrane CNOX (Table 2).

The switch whereby the oxidase may reduce oxygen by either a one-electron or a four-electron mechanism is not understood at present, but it may reside in a delicate redox balance between the carriers involved. Such a balance may be broken by oxidative stress or cell damage. Metal ions such as iron and copper, released by tissue damage (Hershko, 1992), may also play a role.

Table 2. *Reduction of cytochrome* c *as a measure of superoxide production by cell lines in response to ultraviolet irradiation and inhibition by superoxide dismutase and by capsaicin*

| | Rate of reduction of cytochrome *c* ($mol\ min^{-1}\ 10^6\ cells^{-1}$) | | | |
|---|---|---|---|---|
| | | After ultraviolet treatment[2] | | |
| Cell line[1] | Initial rate | No addition | +SOD[3] | +Capsaicin[4] |
| HeLa S | 0.8±0.16 | 4.0±1.0 | 1.1 | 0.8 |
| BT-20 | 0.7±0.2 | 5.1±2.1 | –0.1 | –3.7 |
| MCF-10A | 1.5±0.2 | 7.2±0.1 | –0.7 | 7.2 |

Values are means ± S.D., $N$=3.

[1]HeLa S, human cervical carcinoma; BT-20, human mammary adenocarcinoma; MCF-10A, human mammary epithelium (non-cancer).

[2]Treatment for 10 min with short-wavelength ultraviolet light.

[3]Treatment with 7.5 $\mu g\ ml^{-1}$ superoxide dismutase (SOD) (Sigma).

[4]Treatment with 2.5 $\mu mol\ l^{-1}$ capsaicin in dimethylsulfoxide (DMSO). Rates were corrected for a DMSO blank.

The generation of superoxide radical was determined by assaying the rate of SOD-inhibitable cytochrome *c* reduction (Mayo and Curnutte, 1990; McCord and Fridovich, 1968). The cytochrome *c* was from horse heart mitochondria (type VI, Sigma) and was dissolved in PBSG buffer (138 $mmol\ l^{-1}$ NaCl, 2.7 $mmol\ l^{-1}$ KC1, 8.1 $mmol\ l^{-1}$ $Na_2HPO_4$ and 1.47 $mmol\ l^{-1}$ $KH_2PO_4$, final pH 7.37–7.42, then supplemented with 0.9 $mmol\ l^{-1}$ $CaCl_2$, 0.5 $mmol\ l^{-1}$ $MgCl_2$ and 7.5 $mmol\ l^{-1}$ glucose) to make a solution with a final concentration of 1 $mg\ ml^{-1}$. Air-saturated reaction mixtures of 100 µl of cytochrome *c* stock solution and 50 µl of cell suspension (suspended in PBSG buffer) with a concentration of approximately $5\times10^6$ $cells\ ml^{-1}$ were added to 2 ml of PBSG buffer . The formation of reduced cytochrome *c* was measured in the presence and absence of 5 µl of SOD or capsaicin (in 1 $mmol\ l^{-1}$ in DMSO) by comparing the absorbance of the mixture at 550 and 540 nm. The SOD was obtained from Sigma, and a stock solution of 3 mg protein $ml^{-1}$ $H_2O$ was prepared and stored at 4 °C. Superoxide radical formation was stimulated by using a hand-held ultraviolet light (short-wavelength). Plastic cuvettes were used to allow the light to penetrate and reach the cells. The extent of cytochrome *c* reduction was monitored spectrophotometrically at 550 nm every 10 s, with gentle mixing between readings. Data were analyzed from the slope of the change in the difference in absorbance between 550 and 540 nm before and after ultraviolet treatment and then again after SOD or capsaicin treatment.

Results are expressed as nmol superoxide $10^6$ $cells^{-1}$, using a value of $E_{m550nm}$ of $2.1\times10^3\ l\ mol^{-1}\ cm^{-1}$ (Butler et al., 1982).

## Plasma membrane levels of coenzyme Q

In the model depicted in Fig. 3, plasma membrane ubiquinone or coenzyme Q is a major player in the PMOR system that we

Table 3. *The distribution of ubiquinone in subcellular fractions from rat liver*

| Fraction | [Ubiquinone-9] ($\mu g\, mg^{-1}$ protein) |
|---|---|
| Homogenate | 0.79±0.08 |
| Golgi apparatus | 2.62±0.15 |
| Lysosomes | 1.86±0.18 |
| Mitochondria | 1.40±0.16 |
| Inner mitochondrial membranes | 1.86±0.13 |
| Microsomes | 0.15±0.02 |
| Peroxisomes | 0.29±0.04 |
| Plasma membranes | 0.74±0.07 |
| Supernatant | 0.02±0.004 |

The values are means ± S.E.M. of seven experiments (see Kalén et al., 1987).

The predominant coenzyme Q species isolated from rat liver has nine isoprenoid units; hence, ubiquinone-9 or $CoQ_9$. The predominant coenzyme Q species from most other mammalian species has 10 isoprenoid units; hence, ubiquinone-10 or $CoQ_{10}$.

Table 4. *Reduction of cytochrome* c *as a measure of superoxide production and its inhibition by coenzyme Q in serum from young (21–46 years old) and aged (76–95 years old) individuals*

| Group | *N* | Rate of reduction of cytochrome *c* ($nmol\, min^{-1}\, ml^{-1}$ serum) | |
|---|---|---|---|
| | | No addition | +0.1 $mmol\, l^{-1}$ $Q_{10}$ |
| 21–46 years | 16 | 0.02±0.1 | – |
| 76–82 years | 15 | 1.5±0.9 | 0.6±0.2 |
| 83–95 years | 15 | 3.9±1.6 | 2.5±1.4 |

Values are means ± S.D.

$Q_{10}$, ubiquinone-10 ($CoQ_{10}$).

postulate as being responsible for the propagation of oxidative stress to the extracellular environment. Ubiquinone or coenzyme Q occurs ubiquitously among tissues. In rat liver, the highest levels are found in the Golgi apparatus (Crane and Morré, 1977), but it is also concentrated in the plasma membrane (Table 3) (Kalén et al., 1987). The ubiquinone content of plasma membrane is 2–5 times that of microsomes and only approximately half that of mitochondria.

Ubiquinone has long been considered to have both pro- and antioxidant roles (Ernster and Dallner, 1995) in addition to its more conventional role in mediating electron transport between NADH and succinic dehydrogenase and the cytochrome system of mitochondria (Crane and Barr, 1985). Both pro- and antioxidant and electron transport roles may now be considered for ubiquinone in the plasma membrane.

Coenzyme Q is normally a product of cellular biosynthesis (Andersson et al., 1994; Appelkvist et al., 1994) and provides a potentially important source of one-electron pro-oxidant oxygen reduction. In its reduced hydroquinone form (ubiquinol), it is a powerful antioxidant acting either directly on superoxide or indirectly on lipid radicals (Crane and Barr, 1985; Beyer and Ernster, 1990; Beyer, 1994) either alone or together with vitamin E (α-tocopherol) (Kagan et al., 1990; Ernster et al., 1992).

The antioxidant action of ubiquinol normally yields the ubisemiquinone radical. The latter is converted back to ubiquinol by re-reduction through the electron transfer chain in mitochondria or by various quinone reductases in various cellular compartments (Takahashi et al., 1995, 1996; Beyer et al., 1996, 1997) including the plasma membrane (Navarro et al., 1995; Villalba et al., 1995, 1997; Arroyo et al., 1999). Thus, ubiquinone may transform from a beneficial one- or two-electron carrier to a superoxide generator if the ubisemiquinone anion becomes protonated (Nohl et al., 1996).

In perfused rat liver (Valls et al., 1994) and in isolated rat hepatocytes (Beyer et al., 1996), the anti-cancer quinone glycoside adriamycin induced oxidative stress by enhancing ROS production. Exogenous addition of coenzyme Q prevented this ROS production and concomitantly protected the cells from oxidative damage. We have observed similar effects of exogenous coenzyme Q on NOX-mediated ROS production (Table 4). Such an antioxidant effect at the plasma membrane may very well ameliorate LDL oxidation by scavenging ROS through the PMOR produced at the cell surface (Fig. 2) (Thomas et al., 1997).

Some studies have shown that coenzyme Q levels decrease with age (Beyer et al., 1985; Kalén et al., 1990; Genova et al., 1995). However, this is not true for all tissues and especially for the brain, where high levels of coenzyme Q are maintained throughout aging (Söderberg et al., 1990; Battino et al., 1995). However, most important would be circulating levels of coenzyme Q that could come into contact with an overactive or aberrant cell surface PMOR system or with circulating NOX isoforms that may also play roles in aging related to oxidative stress.

## CNOX is shed by cells and circulates – preliminary evidence for an aging-related CNOX protein

The NOX protein is bound at the outer leaflet of the plasma membrane (Morré, 1995; DeHahn et al., 1997), and NOX activity has been shown to be shed in soluble form from the cell surface (Morré et al., 1996b). The presence of the activity in culture medium conditioned by the growth of cells prompted a search for a comparable shed activity in the serum of cancer patients. Serum from healthy volunteers or from cancer patients does contain NADH oxidase activities with properties similar, if not identical, to those of CNOX and tNOX, respectively, found at the cell surface (Morré and Reust, 1997; Morré et al., 1997a). The presence of the shed form in the circulation provides an opportunity to use serum from cancer patients as a source of the NOX protein for large-scale isolation and characterization studies and to examine NOX activity in the serum of subjects of advanced age in a simple and non-invasive procedure that permits side-by-side comparisons with serum from young adults. In this manner,

Table 5. *Reduction of cytochrome* c *as a measure of superoxide production by buffy coats from the blood of aged individuals and inhibition by coenzyme Q*

| Group | *N* | Rate of reduction of cytochrome *c* ($nmol\,min^{-1}\,10^6\,cells^{-1}$) | |
|---|---|---|---|
| | | No addition | $+0.1\,mmol\,l^{-1}$ $Q_{10}$ |
| 35–65 years | 5 | ND | ND |
| 80–89 years | 6 | 0.05±0.02 | −0.03±0.02 |
| 90–94 years | 6 | 0.36±0.07 | −0.07±0.07 |

Values are means ± S.D.; ND, not detected.
$Q_{10}$, ubiquinone-10 ($CoQ_{10}$).

we have identified a serum form of the CNOX activity that appears to be specific to serum from elderly subjects (76–95 years old) and absent from serum from younger subjects (21–46 years old). Results for elderly individuals 76–95 years of age are shown in Table 4. Not only is there a superoxide-generating and aging-related enzymatic activity present in the serum of the elderly subjects, but its activity is substantially reduced by the addition of $0.1\,mmol\,l^{-1}$ coenzyme Q.

The source of the circulating age-related form of the superoxide-generating activity is considered to be shedding from cells, as for other NOX forms. Consistent with this interpretation was the appearance of a coenzyme-Q-inhibitable age-related reduction of ferric cytochrome *c* in a buffy coat fraction (lymphocytes) comparing young and aged patients (Table 5).

On the basis of the presence of an age-related PMOR system capable of generating ROS at the cell surface, an approach to ablation of anaerobic cells in aged tissues may become feasible. Because only a small percentage of muscle fibers normally become anaerobic even in severely affected tissues, the elimination of these cells would not be expected to have deleterious side effects. In contrast, the benefits might be considerable in terms of lowering serum levels of oxidized lipoproteins and an overall reduction in the oxidative stress to surrounding healthy cells. While a direct approach to ablation of aging altered cells cannot yet be clearly outlined, cells in which the NOX protein is inhibited by drugs undergo apoptosis (Morré et al., 1995a; Vaillant et al., 1996; Dai et al., 1997). If aged cells express higher levels of a specific NOX form, drugs targeted to the aging NOX form might provide one approach. However, drugs or supplements designed to switch the NOX protein from oxygen reduction to protein disulfide reduction, as observed with plant cells in response to auxins (Chueh et al., 1997), may also be effective. In any event, until the aging form of the NOX molecule is better characterized and its structure is elucidated, it will be difficult to predict what additional options for ablation might be available on the basis of the properties of this unique family of proteins and the form specific to sera of elderly subjects.

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