

## Role of membrane redox in aging-related diseases

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**ABSTRACT** A number of different ECTO-NOX forms have been described as being connected with aging-related diseases. The constitutive form, CNOX, serves as a terminal oxidase of plasma membrane electron transport and functions in the growth process. tNOX is present in addition to CNOX on the surface of all cancer cells and contributes to the unregulated growth characteristic of cancer cells. An age-related ECTO-NOX, arNOX, generates superoxide and may contribute to age-related generation of reactive oxygen species. ECTO-NOX proteins and prions share properties in common as do amyloid-forming proteins of various neurodegenerative disorders. A better understanding of ECTO-NOX proteins may lead to new therapeutic strategies for these several age-related disorders.

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### KEY WORDS

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The most thoroughly studied involvement of an ECTO-NOX protein in disease has been that of tNOX and cancer (Morr   1998; Cho et al. 2002). More recently tNOX has been shown to exhibit properties of a prion and various amyloid generating proteins of neurodegenerative diseases (Alzheimer's  $\beta$ -amyloid, Parkinson's  $\alpha$ -synuclein). The mouse prion has properties in common with NOX proteins (Morr   and Morr   2003; Kim and Morr   2004; Markert et al. 2004). We also have described an aging-related ECTO-NOX potentially involved in the generation of reactive oxygen species at the cell surface.

### Methods

The oxidative activity of the ECTO-NOX proteins is most often measured from the decrease in  $A_{340}$  from the oxidation of NADH (or NADPH). With turbid membrane preparations, use of a dual beam instrument with the photo multiplier tube proximal to the sample, is essential to reduce light scattering error in continuous measurements. The Aminco SLM 2000 is a double beam, dual wavelength grating instrument designed for these types of measurements.

### Results and Discussion

#### tNOX and cancer

The cancer-associated, drug-responsive and constitutively-activated ECTO-NOX form (designated tNOX) is present at the cell surface of invasive human cancers (Morr   1998; Cho et al. 2002; Chueh et al. 2004). tNOX is shed into the circulation and, together with the cell surface form, provides a potential diagnostic drug and vaccine target (Cho et al. 2002). The normal constitutive NOX (CNOX) form is hormone- and

growth factor-regulated and responds to agents that control growth and development (Bruno et al. 1992). When a cell divides, it must reach some certain minimal size to divide again. When one or more NOX proteins are inhibited, cell enlargement is slowed or blocked. The resultant small cells fail to divide and ultimately undergo programmed cell death (apoptosis; Morr   and Morr   2003).

The ECTO-NOX form associated with human cancers, tNOX, differs from the constitutive form of normal cells (CNOX) primarily in that its activities are blocked by quinone-site inhibitors many of which have anticancer activity (Morr   1998). Examples include adriamycin, the antitumor sulfonyleureas, the antitumor vanilloids (capsaicin), the principal tea catechin, (-)-epigallocatechin-3-gallate, and several known differentiating agents including antitumor retinoids, sodium phenylacetate and calcitriol. These agents are largely without effect on the CNOX activity of normal cells. They inhibit both tNOX and growth of cancer cells at potentially therapeutic dosage levels without inhibiting CNOX or growth of non-cancer cells. Drug inhibition by tNOX has served as a defining tNOX characteristic used to guide its isolation and molecular characterization. The drug responsive tNOX appears to arise as a splice variant from a single tNOX gene different from that encoding CNOX and is delivered to the cell surface in a processed form.

NOX proteins are released from cells into the circulation. Sera of cancer patients contain both tNOX and CNOX proteins (Morr   et al. 1997; Morr   and Reust 1997; Cho et al. 2002). Sera of healthy volunteers or of patients with diseases other than cancer contain only the CNOX form. tNOX has been found in sera of patients with all major forms of cancer including leukemia and lymphomas (Morr   et al. 1997; Morr   and Reust 1997; Cho et al. 2002) and serves as the basis for a cancer diagnostic protocol under development.

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**Table 1.** Amyloid-forming proteins that exhibit periodic (copper-dependent) oscillations in NADH oxidation.

CNOX (CLOX) <sup>1</sup>	24 min	
tNOX <sup>2</sup>	22 min	ECTO-NOX proteins
arNOX <sup>3</sup>	26 min	
A $\beta$ 1-42	24 min	
Mouse prion	24 min	(Spongiform encephalopathies)
$\alpha$ -Synuclein	54 min	(Parkinson's disease)

<sup>1</sup>Constitutive NOX (normal cells and tissues)

<sup>2</sup>Tumor NOX (cancer specific)

<sup>3</sup>Age-related NOX (aged > 70 y individuals)

### ECTO-NOX proteins have characteristics of prions

ECTO-NOX proteins and prions share similar properties (Kelker et al. 2001). The ability to form insoluble aggregates and the presence of bound copper are ECTO-NOX protein characteristics (Morré and Morré 2003). Other unusual characteristics exhibited by tNOX and shown with other ECTO-NOX proteins include resistance to proteases, resistance to cyanogen bromide fragmentation, and an ability to form amyloid filaments closely resembling those of transmissible spongiform encephalopathies. Additionally, tNOX imparts protease resistance to a normally protease-susceptible protein as is characteristic of PrP<sup>Sc</sup> (PrP<sup>res</sup>), the presumed infective and proteinase K-resistant scrapie prion form. Recombinant mouse prion exhibited a copper dependent pattern of oscillating and alternating NADH oxidase and disulfide-thiol interchange with a 24 min period indistinguishable from that of CNOX (Kim and Morré 2004).

### Aging

An aging-related ECTO-NOX protein (arNOX) of human sera and buffy coat fractions of individuals > 60 y with a potential role in atherogenesis generates superoxide (Morré et al. 2000; 2003). Superoxide production capable of oxidizing circulating lipoproteins and other extracellular targets are measured by the reduction of ferricytochrome c. Activity is inhibited by both superoxide dismutase (SOD) and coenzyme Q<sub>10</sub>. Coenzyme Q<sub>0</sub>, Q<sub>2</sub> and Q<sub>6</sub> do not inhibit. The activity is reduced or absent from sera and/or buffy coat fractions of younger individuals (20 – 40 y) but appears to be widely distributed among other aged systems including late-passage cultured cells and senescing plant organs.

The superoxide generated is also active in the reduction of tetrazolium salts such as XTT (Na 3'-[(phenylamino)-carbonyl]-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene sulfonic acid) leading to colored formazan formation. Other NOX proteins lack this activity.

arNOX proteins (Morré 1998; Morré et al. 1999) and other ECTO-NOX proteins (de Grey 1999) have been postulated

to link the accumulation of lesions in mitochondrial DNA to cell surface accumulations of reactive oxygen species as one consequence of their role as a terminal oxidase in a plasma membrane electron transport chain. Cells with functionally deficient mitochondria become characterized by an anaerobic metabolism. NADH accumulated from the glycolytic production of ATP and an elevated plasma membrane electron transport activity are then necessary to maintain the NAD<sup>+</sup>/NADH homeostasis essential for survival.

### Neurodegenerative disorders

tNOX aggregates in the form of enzymatically inactive amyloid rods and open cylinders (rings) were observed by high resolution electron microscopy (Kelker et al. 2001). These structures were virtually indistinguishable from corresponding aggregates associated with neurodegenerative amyloid-forming proteins.

Observations were extended to Alzheimer's A $\beta$  1-43 peptide (Markert et al. 2004) and  $\alpha$ -synuclein of Parkinson's disease (Morré and Morré 2003). The Alzheimer's A $\beta$  peptide exhibits copper-dependent oscillations in NADH oxidase activity similar to that of tNOX and the mouse prion also with a 24 min period length (Morré and Morré 2003). The A $\beta$  peptide both lacks a cysteine and is unable to carry out disulfide-thiol interchange.  $\alpha$ -Synuclein also has a copper-dependent and oscillating NADH oxidase activity but the period length is 54 min. The  $\alpha$ -synuclein has not been evaluated for disulfide-thiol interchange activity. These various relationships are summarized in Table 1. Complete amino acid sequences are known for each of the proteins listed in Table 1. There is virtually no sequence similarity. Even though all bind copper, the copper binding strategies differ. For tNOX, the putative copper site is a HVH and a 3<sup>rd</sup> H downstream conserved with superoxide dismutase. The mouse prion utilizes the hexa repeats. For  $\alpha$ -synuclein and Alzheimer's A $\beta$ , a combination of tyrosines are utilized. It is unlikely that CNOX will exhibit substantial sequence similarity to tNOX based on data base searches and antisera specificity. Yet all examples in Table 1 bind adenine nucleotide (NADH) although the amino acid sequences within the putative binding regions differ.

Possibly the best source of a new set of tools to understand ECTO-NOX periodicity and oxidative function is now provided by the Alzheimer's A $\beta$  peptide of 43 amino acids in length. The copper site is known and a putative NADH site is found near the C-terminus as in tNOX in the vicinity of M-35. We are hopeful that a detailed analysis of various modifications within the A $\beta$  peptide will begin to shed light on how ECTO-NOX proteins keep time and carry out their oxidative functions.

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