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Rapid Letter

Preferential Inhibition of the Plasma Membrane NADH Oxidase (NOX) Activity by Diphenyleneiodonium Chloride with NADPH as Donor

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ABSTRACT

The cell-surface NADH oxidase (NOX) protein of plant and animal cells will utilize both NADH and NADPH as reduced electron donors for activity. The two activities are distinguished by a differential inhibition by the redox inhibitor diphenyleneiodonium chloride (DPI). Using both plasma membranes and cells, activity with NADPH as donor was markedly inhibited by DPI at submicromolar concentrations, whereas with NADH as donor, DPI was much less effective or had no effect on the activity. The possibility of the inhibition being the result of two different enzymes was eliminated by the use of a recombinant NOX protein. The findings support the concept that NOX proteins serve as terminal oxidases for plasma membrane electron transport involving cytosolic reduced pyridine nucleotides as the natural electron donors and with molecular oxygen as the electron acceptor. Antioxid. Redox Signal. 4, 207–212.

INTRODUCTION

THE DESIGNATION NADH OXIDASE is used for a broad spectrum of often widely divergent enzymes that oxidize reduced pyridine nucleotides [NAD(P)H] with transfer of protons and electrons to molecular oxygen to generate water. Our laboratory has described a novel family of cell-surface hydroquinone oxidases that use external NADH as an alternative artificial substrate that we suggest may function as terminal oxidases of an electron transport chain from cytosolic NAD(P)H to molecular oxygen via transmembrane ubiquinone as an electron shuttle (12). These enzymes of the external plasma membrane surface differ from other NOX (for NADH oxidase) proteins in that quinone oxidation alternates with protein disulfide–thiol interchange (protein disulfide isomerase) to generate a persistent pattern of oscillations with a temperature-compensated period length of 24 min (16). To distinguish these NOX proteins as a family from other NOX proteins, we suggest the designation CLOX for cycling oxidase. One member of the CLOX family, a tumor- (cancer-) specific isoform called tNOX, has been cloned (accession number AF207881). CLOX proteins appear to be involved both in the enlargement phase of cell growth (22) and in cellular time-keeping (7).

There are little or no reduced pyridine nucleotides at the external cell surface. Also, as pyridine nucleotides are generally unable to cross the plasma membrane, we regard externally supplied NAD(P)H as an artificial

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electron donor for the NOX proteins where electrons and protons are normally supplied by plasma membrane electron transport during the oxidative part of the CLOX cycle (16).

In the past, a number of artificial electron acceptors have been used to monitor plasma membrane electron transport. These include ferricyanide (9, 10), tetrazolium salts (1), and thiazine dyes such as methylene blue (4, 5). Natural electron acceptors for plasma membrane electron transport in addition to molecular oxygen include ascorbate free radical (21) and protein disulfides (6). Ascorbate also can function as an intracellular electron donor for ferricyanide and dye reduction (15). A common characteristic of many of these transfers is inhibition by diphenyleneiodonium chloride (DPI). DPI is a general flavoprotein inhibitor, commonly used to inhibit NAD(P)H oxidases (11). DPI is reported to sensitize cells to Fas-mediated apoptosis (8), providing a link between plasma membrane redox activities and resistance to apoptosis.

The question is often raised or the assumption is made that the above artificial electron acceptors all serve as measures of the same or similar NOX proteins. In this report, we have examined the effect of DPI on CLOX activities comparing both NADH and NADPH as electron donors for the electron transport measured. Using cells and plasma membranes from both plant and animal sources, as well as recombinant NOX protein, we show that with NADH as electron donor, DPI has little or no effect on NOX activity. However, with NADPH as donor, DPI inhibits in the micromolar range of concentrations. The findings provide an important correlation to implicate the cell-surface NOX protein as the principal terminal oxidase at the cell surface for plasma membrane electron transport.

MATERIALS AND METHODS

Materials

Unless described otherwise, all chemicals were from Sigma Chemical Company.

Plasma membranes/tissue/cell culture

HeLa cells (human cervical carcinoma) were grown and assayed as described (19). Etiolated seedlings of soybean (6) were used as a source of plasma membranes isolated as described (23). The plasma membrane preparations used were predominantly right side-out (cytoplasmic side-in) and have been characterized extensively (18). A recombinant truncated NOX was expressed in *E. coli* (7) and purified from French Press extracts by ammonium sulfate fractionation.

Assay of NAD(P)H oxidase

NAD(P)H oxidase activities were measured using paired Hitachi U3210 spectrophotometers over successive 5-min intervals (two to four or more at low inhibitory concentrations) at 37°C. The activity was determined as the disappearance of NAD(P)H measured at 340 nm. The reaction mixture included sample, 50 m*M* Tris-MES, pH 7.0, 2 m*M* KCN, and 150 μ*M* NAD(P)H in a total volume of 2.5 ml at 37°C with stirring. A millimolar extinction coefficient of 6.22 was used to determine NAD(P)H disappearance.

DPI (Sigma) was dissolved and diluted in ethanol. Blanks received only ethanol.

RESULTS AND DISCUSSION

NOX activity was estimated using the decrease in absorbance at 340 nm as a measure of NADH or NADPH oxidation. In general, the rate with NADH was at least twice that of the rate with NADPH with plants, but with HeLa cells the rates with the two electron donors were nearly equal, and for the recombinant tNOX protein, the rate was greatest with NADPH.

The response to DPI, a flavin inhibitor (11), was determined from the dose-response over the range 10^{-4} to 10^{-8} *M* (Figs. 1 and 2). Rates were averaged over several successive 5-min intervals to normalize variations due to the periodic oscillations that characterize the cell-surface NOX proteins (16). Comparisons were with NADH or NADPH as substrate. As the NAD(P)H site of the protein is on the ex-

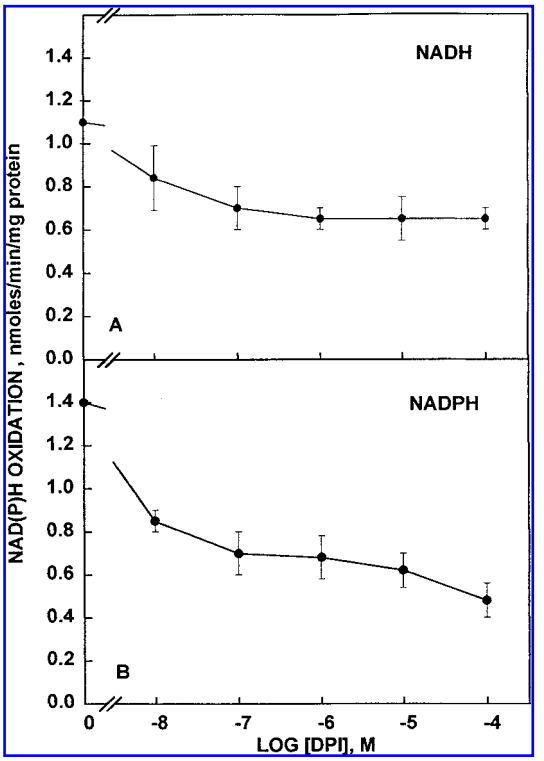

FIG. 1. Cell surface-associated reduced pyridine nucleotide oxidase (NOX) activity of HeLa cells in culture comparing NADH (A) and NADPH (B) as substrate and inhibition by DPI. Results are averages of five (NADH) or three (NADPH) experiments ± standard deviations.

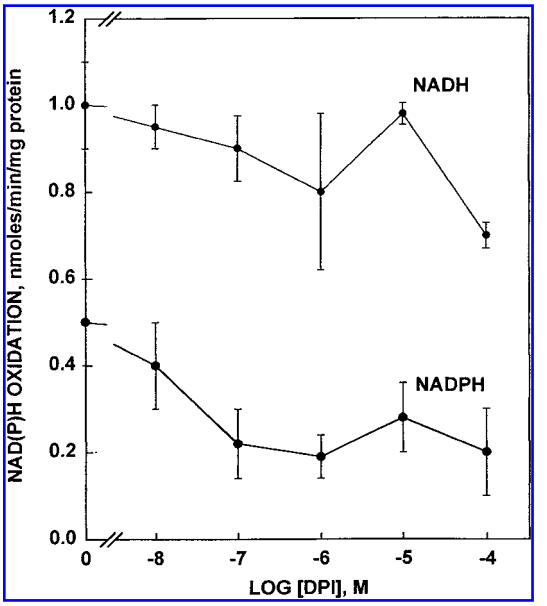

FIG. 2. Reduced pyridine nucleotide oxidase activity of plasma membrane vesicles isolated from sections cut from the zone of cell elongation of etiolated seedlings of soybean (*Glycine max*) and inhibition by DPI. Results are averages of six different experiments ± standard deviations.

ternal cell surface (18), it is possible to estimate the activity using whole cells, as well as with isolated preparations of plasma membranes, solubilized and partially purified preparations of the activity, and the recombinant protein.

With HeLa cells, a preferential inhibition by DPI of NADPH oxidation versus NADH oxidation was observed despite the fact that both substrates were utilized with nearly equal efficiency (Fig. 1) The EC_{50} for DPI inhibition of NADPH oxidation by HeLa cells was 0.1 μM, whereas an EC_{50} for NADH oxidation was never reached. Similarly, the EC_{50} for DPI inhibition of NADPH oxidation by solubilized NADH oxidase released from the surface of HeLa cells by a low pH extraction (Table 1) was 0.1 μM compared with $\sim$1 μM for NADH.

With plasma membranes prepared from 1-cm sections of dark-grown soybeans, DPI was largely without effect on rates of NADH oxidation (Fig. 2). In contrast, with NADPH as the electron donor, the inhibition was much stronger (Fig. 2). The EC_{50} was 0.1 μM. With the plasma membranes from soybean, NADH was the preferred substrate with the activity being two times that with NADPH as substrate.

To demonstrate that the DPI effect was on the plasma membrane NADH oxidase, a recombinant plasma membrane NADH oxidase (tNOX) where the cDNA was cloned from HeLa cells was tested as well (Table 1). Both NADH and NADPH served as substrates, but only with the NADPH was the DPI effective as an inhibitor (Table 1).

Interpretation of measurements of plasma membrane electron transport with different electron acceptors would be aided greatly by availability of one or more common inhibitors. Plasma membrane electron transport often has been estimated using tetrazolium dyes (1) such as the cell-impermeant tetrazolium salt WST-1 [2-(4-indophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium], with the cell-impermeant thiazine redox indicators (4, 5, 15) or with ferricyanide (10) as acceptors. Although oxygen

TABLE 1. INHIBITION OF NOX ACTIVITY BY DPI ($C_{12}H_8Cl$)

Source of NOX activity	*nmol/min/mg of protein*		*EC_{50} for DPI inhibition*	
	NADH	*NADPH*	*NADH*	*NADHPH*
HeLa cells	1.1 ± 0.2	1.4 ± 0.3	Not reached	0.1 μ*M*
Released from HeLa cells by low pH	45 ± 10	30 ± 5	1 μ*M*	0.1 μ*M*
Soybean plasma membrane	1.0 ± 0.2	0.5 ± 0.05	Not reached	0.1 μ*M*
Recombinant tNOX	450	1,350	No response to DPI	0.1 μ*M*

is often assumed to be the natural electron acceptor (9), a mechanism whereby electrons coming from NADH or NADPH could be transferred to oxygen at the cell surface has been missing from our information. The discovery of the external plasma membrane-associated NADH oxidase provides such a mechanism. By functioning as an external oxidase catalyzing electron transfer from hydroquinones to oxygen (12), the plasma membrane-associated NADH oxidase might represent a key component of the long-sought physiological electron transport chain of the plasma membrane.

A means to test the operation of the plasma membrane-associated NADH oxidase (NOX proteins) as terminal oxidases of plasma membrane electron transport is afforded by the observation that plasma membrane electron transport can be inhibited by DPI at micromolar concentrations, but only in the presence of NADPH as electron donor. With HeLa cells, the activities with NADH and NADPH were nearly equal, more so than with plants, for example, where NADH has been shown previously to be the preferred substrate (17). With tetrazolium dyes, the ratio of activity with NADPH and NADH as substrate varies from 0.95 to 5.22 depending on cell type. This could represent an argument for two different enzymes or two different nucleotide-combining sites within a single enzyme. However, recombinant tNOX with only a single apparent adenine nucleotide binding site exhibited both activities, only one of which, that with NADPH as donor, was inhibited by DPI.

One physiological role for plasma membrane electron transport with NADH oxidase in the terminal enzyme is to regenerate pyridine nucleotide for glycolytic ATP production. Proliferating cells apparently use glycolytic metabolism to generate ATP for growth (2, 13, 14). According to Lawen and collaborators (13, 14), the KCN-insensitive oxygen consumption of cultured cells is 10% of the total (2×10^8 electrons/cell). Plasma membrane electron transport, as well as the plasma membrane-associated NADH oxidase, is cyanide-insensitive (17). If only superoxide were generated, the cells would be killed. A four-electron transfer to oxygen to reduce oxygen to water is required. Therefore, the plasma membrane emerges as a major respiratory organ, perhaps even more important than mitochondria. Cells apparently do not require mitochondria to proliferate. The latter may be involved only when there is an acute demand for energy as in muscle contraction. Cell proliferation apparently does not require mitochondrial ATP *per se*.

Although the DPI findings suggest that electron transport using tetrazolium salts may be catalyzed by one or more CLOX proteins, a number of observations remain inconsistent with these findings. With Jurkat cells, the reduction of the tetrazolium dye WST-1 was blocked by superoxide dismutase to suggest that WST-1 was reduced extracellularly by superoxide (1). Under normal circumstances, CLOX proteins generate little or no superoxide (20). An equivalence of ferricyanide reduction and tetrazolium salt reduction is considered doubtful on the basis that human erythrocyte ghosts that actively reduce ferricyanide (24) fail to reduce WST-1 (1). Ferricyanide reduction and NADH oxidation have been shown from protein purifica-

tion studies with rat liver plasma membranes to be catalyzed by different proteins (17).

To account for the rapid and superoxide dismutase-sensitive reduction of WST-1 (1, 3), a second enzyme might be operative (A. de Grey, personal communication). However, if such an enzyme exists, it has not been identified. Direct transfer of one electron from reduced ubiquinone, not protein catalyzed, in the presence of dyes or ferricyanide is an alternative possibility.

DPI is a flavin inhibitor. Most NADH oxidases carry bound flavin. Thus, it might be assumed that the DPI target in plasma membrane redox would be a flavoprotein. However, the cloned plasma membrane cell-surface NOX, tNOX, does not require flavin for activity nor does it contain sequence homology to known flavin binding domains (6), yet it is markedly inhibited by DPI with NADPH as donor, but not with NADH as donor.

ACKNOWLEDGMENTS

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ABBREVIATIONS

CLOX, cycling oxidase; DPI, diphenyleneiodonium chloride; NADH, reduced nicotinamide adenine dinucleotide; NADPH, reduced nicotinamide adenine dinucleotide phosphate; NOX, cell-surface hydroquinone (NADH) oxidase with protein disulfide-thiol interchange activity; tNOX, tumor-associated NOX; WST-1, 2-(4-indophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium.

REFERENCES

1. Berridge MW 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.
2. Berridge MW and Tan AS. High capacity redox control at the plasma membrane of mammalian cells, transmembrane, cell surface and serum NADH oxidases. *Antioxid Redox Signal* 2: 231–243, 2000.
3. Berridge MW and Tan AS. Cell-surface NAD(P)H-oxidase: relationship to trans-plasma membrane NADH-oxidoreductase and a potential source of circulating NADH oxidase. *Antioxid Redox Signal* 2: 277–288, 2000.
4. Bongard RD, Krenz GS, Linehan JH, Roerig DL, Merker MP, Widell JL, and Dawson CA. Reduction and accumulation of methylene blue by the lung. *Am J Physiol* 77: 1480–1491, 1994.
5. Bongard RD, Merker MP, Shundo R, Okamoto Y, Roerig DL, Linehan JH, and Dawson CA. Reduction of thiazine dyes by bovine pulmonary arterial endothelial cells in culture. *Am J Physiol* 13: L78–L84, 1995.
6. Chueh P-J, Morré DM, Penel C, DeHahn T, and Morré DJ. The hormone-responsive NADH oxidase of the plant plasma membrane has properties of a NADH:protein disulfide reductase. *J Biol Chem* 272: 11221–11227, 1997.
7. Chueh P-J, Morré DM, and Morré DJ. A site-directed mutagenesis analysis of tNOX functional domains. *Biochim Biophys Acta* 1594:74–83, 2002.
8. Clement MV and Stamenkovic I. Superoxide anion is a natural inhibitor of FAS-mediated cell death. *EMBO J* 15: 216–225, 1996.
9. Crane FL, Sun IL, Clark MG, Grebing C, and Low H. Transplasma-membrane redox systems in growth and development. *Biochim Biophys Acta* 811: 233–264, 1985.
10. Crane FL, Sun IL, Barr R, and Low H. Electron and proton transport across the plasma membrane. *J Bioenerg Biomembr* 23: 773–803, 1991.
11. Hampton MB and Winterbourn CC. Modification of neutrophil oxidant production with diphenyleneiodonium and its effect on bacterial killing. *Free Radic Biol Med* 18: 633–639, 1995.
12. 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.
13. 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.
14. Lawen A, Martinus RD, McMullen GL, Nagley P, Vaillant F, Wolvetang EJ, and Linnane AW. The universality of bioenergetic disease: the role of mitochondrial mutation and the putative inter-relationship between mitochondria and plasma membrane NADH oxidoreductase. *Mol Aspects Med* 15: s13–s27, 1994.
15. Merker MP, Olson LE, Bongard RD, Patel MK, Linehan JH, and Dawson CA. Ascorbate-mediated transplasma membrane electron transport in pulmonary endothelial cells. *Am J Physiol* 274: L685–L693, 1998.
16. Morré DJ. NADH oxidase: a multifunctional ectoprotein of the eukaryotic cell surface. In: *Plasma Membrane Redox Systems and Their Role in Biological Stress and Disease*, edited by Asard H, Bérczi A, and Caubergs R. Dordrecht, The Netherlands: Kluwer Academic Publishers, 1998, pp. 121–156.

17. Morré DJ and Brightman AO. NADH oxidase of plasma membranes. *J Bioenerg Biomembr* 23: 469–489, 1991.
18. Morré DJ and Morré DM. Applications of aqueous two-phase partition to isolation of membranes from plants: a periodic NADH oxidase activity as a marker for right side-out plasma membrane vesicles. *J Chromatogr B* 743: 377–387, 2000.
19. Morré DJ, Wu L-Y, and Morré DM. Inhibition of NADH oxidase activity and growth of HeLa cells by the antitumor sulfonylurea, *N*-(4-methylphenylsulfonyl)-N′-(4-chlorophenyl)urea and response to epidermal growth factor. *Biochim Biophys Acta* 1355: 114–120, 1997.
20. Morré DJ, Pogue R, and Morré DM. A multifunctional ubiquinol oxidase of the external cell surface and sera. *Biofactors* 9: 179–187, 1999.
21. Navas P, Alcain FJ, Buron I, Rodriquez-Aguilera J-C, Villalba JM, Morré DM, and Morré DJ. Growth factor-stimulated trans plasma membrane electron transport in HL-60 cells. *FEBS Lett* 299: 223–226, 1992.
22. Pogue R, Morré DM, and Morré DJ. CHO cell enlargement oscillates with a temperature-compensated period of 24 min. *Biochim Biophys Acta* 1498: 44–51, 2000.
23. Sandelius AS, Barr R, Crane FL, and Morré DJ. Redox reactions of plasma membranes isolated from soybean hypocotyls by phase partition. *Plant Sci* 48: 1–10, 1986.
24. Sun IL, Sun EE, Crane FL, Morré DJ, Lingren A, and Low H. Requirement for coenzyme Q in plasma membrane electron transport. *Proc Natl Acad Sci USA* 89: 11126–11130, 1992.

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This article has been cited by:

1. Hans Löw, Frederick L. Crane, D. James Morré. 2012. Putting together a plasma membrane NADH oxidase: A tale of three laboratories. *The International Journal of Biochemistry & Cell Biology* **44**:11, 1834-1838. [CrossRef]
2. Marta R. M. Lima, Alberto C. P. Dias. 2011. Phaeomoniella chlamydospora-induced Oxidative Burst in Vitis vinifera Cell Suspensions: Role of NADPH Oxidase and Ca2+. *Journal of Phytopathology* no-no. [CrossRef]
3. Yongchun Shi, Yunpeng Fu, Weiqun Liu. 2011. NADPH oxidase in plasma membrane is involved in stomatal closure induced by dehydroascorbate. *Plant Physiology and Biochemistry* . [CrossRef]
4. Pritish Sarkar, G. K. Suraishkumar. 2011. pH and Temperature Stresses in Bioreactor Cultures: Intracellular Superoxide Levels. *Industrial & Engineering Chemistry Research* 110621093542056. [CrossRef]
5. Jana Frahm, Mats-Olof Mattsson, Myrtill Simkó. 2010. Exposure to ELF magnetic fields modulate redox related protein expression in mouse macrophages. *Toxicology Letters* **192**:3, 330-336. [CrossRef]
6. Rajesh Kumar Tewari, Praveen Kumar, Soohyun Kim, Eun-Joo Hahn, Kee-Yoeup Paek. 2009. Nitric oxide retards xanthine oxidase-mediated superoxide anion generation in Phalaenopsis flower: an implication of NO in the senescence and oxidative stress regulation. *Plant Cell Reports* **28**:2, 267. [CrossRef]
7. Smita S. Matkar, Lisa A. Wrischnik, Utha Hellmann-Blumberg. 2008. Production of hydrogen peroxide and redox cycling can explain how sanguinarine and chelerythrine induce rapid apoptosis. *Archives of Biochemistry and Biophysics* **477**:1, 43-52. [CrossRef]
8. Claudia Piccoli, Annamaria D'Aprile, Maria Ripoli, Rosella Scrima, Domenico Boffoli, Antonio Tabilio, Nazzareno Capitanio. 2007. The hypoxia-inducible factor is stabilized in circulating hematopoietic stem cells under normoxic conditions. *FEBS Letters* **581**:16, 3111-3119. [CrossRef]
9. Roman A. Volkov, Irina I. Panchuk, Phillip M. Mullineaux, Friedrich Schöffl. 2006. Heat stress-induced H2O2 is required for effective expression of heat shock genes in Arabidopsis. *Plant Molecular Biology* **61**:4-5, 733-746. [CrossRef]
10. Susmita Sahoo, K. Krishnamurthy Rao, G.K. Suraishkumar. 2006. Reactive oxygen species induced by shear stress mediate cell death inBacillus subtilis. *Biotechnology and Bioengineering* **94**:1, 118-127. [CrossRef]
11. Akira Murakami, Hajime OhigashiMolecular Targets for Antiinflammation and Dietary Component—Drug Synergy 287-317. [CrossRef]
12. Marcin Kami?ski, Edyta Niemczyk, Makoto Masaoka, Mariusz Karbowski, Anna Hallmann, Jakub K? dzior, Anna Majczak, Dorota Knap, Yuji Nishizawa, Jiro Usukura, Micha? Wo?niak, Jerzy Klimek, Takashi Wakabayashi. 2004. The switch mechanism of the cell death mode from apoptosis to necrosis in menadione-treated human osteosarcoma cell line 143B cells. *Microscopy Research and Technique* **64**:3, 255-268. [CrossRef]
13. Dorothy M. Morré, D. James Morré. 2003. Specificity of Coenzyme Q inhibition of an aging-related cell surface NADH oxidase (ECTO-NOX) that generates superoxide. *BioFactors* **18**:1-4, 33-43. [CrossRef]
14. M. V. Wright, T. B. Kuhn. 2002. CNS neurons express two distinct plasma membrane electron transport systems implicated in neuronal viability. *Journal of Neurochemistry* **83**:3, 655-664. [CrossRef]