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Mitochondria-derived reactive oxygen species mediate caspase-dependent and -independent neuronal deaths

Meagan J. McManus [b], Michael P. Murphy [c], James L. Franklin [a,*]

[a] Department of Pharmaceutical and Biomedical Sciences, The University of Georgia College of Pharmacy, 357 Wilson Pharmacy, Athens, GA 30602, USA
[b] Center of Mitochondrial and Epigenomic Medicine, Children's Hospital of Philadelphia, Colket Translational Research Building, 3501 Civic Center Blvd, Room 6100, Philadelphia, PA 19104, USA
[c] MRC Mitochondrial Biology Unit, Wellcome Trust/MRC Building, Hills Road, Cambridge CB2 0XY, UK

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ABSTRACT

Mitochondrial dysfunction and oxidative stress are implicated in many neurodegenerative diseases. Mitochondria-targeted drugs that effectively decrease oxidative stress, protect mitochondrial energetics, and prevent neuronal loss may therefore lend therapeutic benefit to these currently incurable diseases. To investigate the efficacy of such drugs, we examined the effects of mitochondria-targeted antioxidants $MitoQ_{10}$ and $MitoE_2$ on neuronal death induced by neurotrophin deficiency. Our results indicate that $MitoQ_{10}$ blocked apoptosis by preventing increased mitochondria-derived reactive oxygen species (ROS) and subsequent cytochrome *c* release, caspase activation, and mitochondrial damage in nerve growth factor (NGF)-deprived sympathetic neurons, while $MitoE_2$ was largely ineffective. In this paradigm, the most proximal point of divergence was the ability of $MitoQ_{10}$ to scavenge mitochondrial superoxide ($O_2^{\bullet-}$). $MitoQ_{10}$ also prevented caspase-independent neuronal death in these cells demonstrating that the mitochondrial redox state significantly influences both apoptotic and nonapoptotic pathways leading to neuronal death. We suggest that mitochondria-targeted antioxidants may provide tools for delineating the role and significance of mitochondrial ROS in neuronal death and provide a new therapeutic approach for neurodegenerative conditions involving trophic factor deficits and multiple modes of cell death.

1. Introduction

Mitochondrial dysfunction and oxidative stress are prominent features in both acute neuropathologies and progressive neurodegenerative conditions (Green and Kroemer, 2004). When mitochondrial energetics are impaired, electron leakage from respiratory complexes increases resulting in elevated production of the free radical superoxide ($O_2^{\bullet-}$) via a one electron reduction of O_2 (Murphy, 2009). Mitochondria are the primary source of $O_2^{\bullet-}$ within mammalian cells and, thus, are also the most likely organelle to suffer damage from $O_2^{\bullet-}$ or downstream reactive oxygen species (ROS) resulting from dismutation of $O_2^{\bullet-}$ (Turrens, 2003). Protection against oxidative damage is achieved by a series of interdependent mitochondrial antioxidant systems (Kaplowitz et al., 1985; Kowaltowski et al., 2001; Sheeran et al., 2010). A significant imbalance in either system will eventually lead to mitochondrial dysfunction, oxidative stress, and cellular demise (Wallace, 2010). If these events significantly contribute to the etiology of neurodegenerative diseases, therapeutics that protect and preserve mitochondrial energetics may prevent the progression of neuronal death and its consequential symptoms.

Although the factors that trigger programmed cell death (PCD) in neurons differ between pathological settings, most of the subsequent signaling events involve two major pathways and appear highly conserved. Perhaps the most extensively characterized model of PCD in neurons is that of sympathetic neurons deprived of nerve growth factor (NGF) (Davies, 1996; Putcha and Johnson, 2004). Similar to vulnerable neurons in the aging brain, decreased neurotrophic supply in cultured sympathetic neurons reduces their size and number, suppresses neurite outgrowth and decreases cellular glucose uptake (Chang et al., 2003; Weissmiller and Wu, 2012). Within 6 h of deprivation, the proapoptotic protein Bax associates with the outer mitochondrial membrane (OMM) and orchestrates the release of cytochrome *c* and production of mitochondrial-derived $O_2^{\bullet-}$ (Kirkland et al., 2002a, 2002b, 2007, 2010). In the first pathway to cell death, the apoptogenic

Abbreviations: BAF, boc-aspartyl fluoromethyl ketone; FCCP, p-trifluromethoxyphenylhydrazone; IMM, inner mitochondrial membrane; NGF, nerve growth factor; OMM, outer mitochondrial membrane; ROS, reactive oxygen species; $O_2^{\bullet-}$, superoxide; TPP^+, triphenylphosphonium; $MitoQ_{10}$, [10-(4,5-dimethoxy-2-methyl-3,6-dioxo-1,4-cycloheexadienl-yl)decyl] triphenylphosphonium methanesulfonate; $MitoE_2$, [2-(3,4-dihydro-6-hydroxy-2,5,7,8-tetramethyl-2H-1-benzopyran-2-yl)ethyl] triphenylphosphonium chloride; CM-H2DCFDA, chloromethyl-2′,7′-dicholorodihydrofluorescein diacetate; L-15, Leibovitz's 15 medium; DIV, days in vitro; PBS, phosphate buffered saline; $\Delta\psi m$, mitochondrial membrane potential; $TMRM^+$, tetramethylrhodamine methyl ester; TBS, tris-buffered saline; SEM, standard error of the mean; (dTPP), n-decyl triphenylphosphonium; (mPTP), mitochondrial permeability transition pore.

* Corresponding author.
E-mail addresses: megangm@gmail.com (M.J. McManus), michael.murphy@mrc-mbu.cam.ac.uk (M.P. Murphy), jfrankli@rx.uga.edu (J.L. Franklin).

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cytochrome *c* molecules released into the cytosol activate caspases via the formation of the apoptosome (Vaughn and Deshmukh, 2008; Wright et al., 2006). If the executioner caspases 3/7 are inhibited, cell death is delayed until mitochondrial integrity and membrane potential are lost (Chang and Johnson, 2002; Chang et al., 2002; Deshmukh et al., 2000), at which point cell death occurs through a second, caspase-independent pathway. The established temporal relationship of these events in sympathetic cultures provided an ideal model to evaluate the mechanistic basis of neuroprotective therapeutics.

Mitochondria-targeted antioxidants are a new class of drugs designed to protect the cell from ROS damage at the source. The high potential across the inner mitochondrial membrane (IMM) allows the lipophilic cation, triphenylphosphonium (TPP^+) to selectively accumulate in the mitochondrial matrix (James et al., 2007). TPP^+ can be covalently conjugated to antioxidants, such as ubiquinone or vitamin E (Fig. 1), enabling these compounds to easily traverse the plasma membrane and the outer mitochondrial membrane (OMM) and selectively accumulate within the matrix and adsorb to the IMM of treated cells (James et al., 2007). In the current study, we examined the effects of two such compounds, $MitoQ_{10}$ and $MitoE_2$ (Fig. 1) on the events that dictate the commitment to neuronal death (Chang and Johnson, 2002; Chang et al., 2002). Our findings indicate that $MitoQ_{10}$ but not $MitoE_2$ prevented the progression of both caspase-dependent and -independent pathways to death in cultured sympathetic neurons by protecting the organelle from excessive mitochondrial $O_2^{\bullet-}$ and downstream ROS.

2. Results

2.1. $MitoQ_{10}$ prevented death of NGF-deprived sympathetic neurons

NGF deprivation induces apoptosis in mouse sympathetic neurons. After 24 h of deprivation, approximately half of the neurons are unable to be rescued by NGF re-addition and are thus committed to die (Deshmukh and Johnson, 1997). To assess the ability of $MitoQ_{10}$ and $MitoE_2$ to prevent neuronal apoptosis, mouse sympathetic neurons in cell culture were deprived of NGF for 48 h alone or in the presence of $MitoQ_{10}$ or $MitoE_2$ (250 nM–1 μM), or a combination of both (250 nM each). Approximately 70% of neurons treated with $MitoQ_{10}$ (250 nM–1 μM) from the time of deprivation were capable of rescue by NGF-readdition 48 h later. Concentrations of $MitoQ_{10}$ much lower than 250 nM produced no protective effect (e.g., 100 nM from visual screening experiments, not shown) and those above 1 μM were toxic, as demonstrated by the sharp decline in survival with 5 μM $MitoQ_{10}$ (Fig. 2A). dTPP consists of the mitochondria-targeting moiety (TPP^+) attached to a ten-carbon chain and was used to control for nonspecific effects of the lipophilic cation. dTPP produced no detectable changes in the survival rate of NGF-deprived neurons, thus the ubiquinone moiety of $MitoQ_{10}$ was responsible for its antiapoptotic effect (Fig. 2A, C).

$MitoE_2$ was ineffective in preventing neuronal death at all concentrations studied. Ubiquinol enhances the efficacy of vitamin E in membranes and cells by reducing the tocopheroxyl radical back to the active antioxidant after its oxidation by a reactive species (Burton and Traber, 1990; James et al., 2004; Kagan and Tyurina, 1998). We hypothesized that the mitochondria-targeted analogs may perform similarly. To investigate the ability of $MitoQ_{10}$ to increase the efficacy of $MitoE_2$, cultures were treated with a combination of $MitoE_2$ and $MitoQ_{10}$ (250 nM each) from the time of deprivation and the effect on the cell death was determined. There was a significant increase in survival over that of untreated NGF-deprived neurons ($p < 0.001$) but not as great as that observed with 250 nM MitoQ alone ($p < 0.001$, $MitoQ_{10}$ 250 nM versus $MitoE_2 + MitoQ_{10}$) (Fig. 2A, C).

To determine the effect of $MitoQ_{10}$ and $MitoE_2$ on caspase-independent death, neuronal cultures were deprived of NGF for 72 h in the presence of the caspase inhibitor, BAF, alone or with $MitoQ_{10}$ or $MitoE_2$. In this rescue paradigm, ~60% of BAF-treated neurons can be rescued by NGF readdition up to 48 h after NGF-withdrawal, but by 72 h death prevails by a caspase-independent mechanism (Deshmukh et al., 2000). Accordingly, BAF-treatment alone was not sufficient to prevent death after this length of deprivation (Fig. 2B, C). $MitoQ_{10}$-treated neurons escaped impending death and recovered upon NGF readdition after 72 h of deprivation. Although $MitoQ_{10}$ treatment did not prevent the commitment to die at later time points (96 h, data not shown), these results indicate that $MitoQ_{10}$ significantly delayed the commitment to die beyond that of caspase inhibition by BAF.

2.2. $MitoQ_{10}$ prevented cytochrome c redistribution and caspase activation after NGF deprivation (Commitment 1)

The two most critical check-points that dictate a sympathetic neuron's commitment to die involve the redistribution of mitochondrial

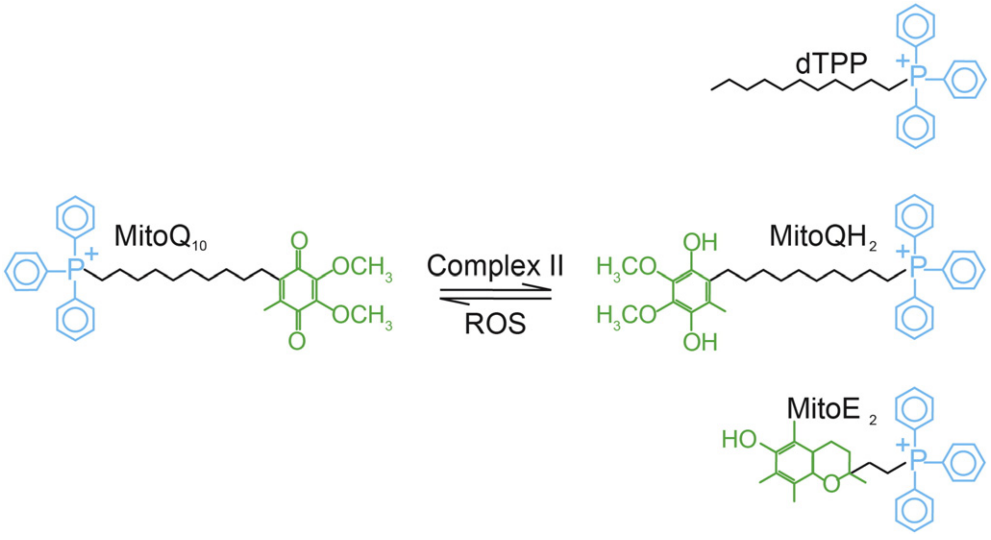

Fig. 1. Mitochondria-targeted compounds. These compounds contain a lipophilic triphenylphosphonium cation (TPP^+) and can pass directly through lipid membranes to accumulate within the mitochondria driven by the high $\Delta\psi m$ of the across the IMM. DecylTPP (dTPP) is similar to $MitoQ_{10}$ (Asin-Cayuela et al., 2004), but lacks the antioxidant moiety; ubiquinone is attached to the ten-carbon chain to produce $MitoQ_{10}$ (James et al., 2007), while a two-carbon bridge connects the α-tocopherol moiety of vitamin E to TPP^+ in $MitoE_2$ (Smith et al., 1999). In these compounds, the length of the hydrophobic alkyl bridge determines the extent of accumulation and penetration in the mitochondrial inner membrane (Asin-Cayuela et al., 2004) and therefore influences their potential to suppress ROS derived from the mitochondrial electron transport chain. Within the mitochondria, $MitoQ_{10}$ is continuously reduced to the ubiquinol form ($MitoQH_2$) by respiratory complex II after detoxifying a ROS.