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Role of mitochondria in neuronal cell death induced by oxidative stress; neuroprotection by Coenzyme Q_{10}

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Neuronal cells depend on mitochondrial oxidative phosphorylation for most of their energy needs and therefore are at a particular risk for oxidative stress. Mitochondria play an important role in energy production and oxidative stress-induced apoptosis. In the present study, we have demonstrated that external oxidative stress induces mitochondrial dysfunction leading to increased ROS generation and ultimately apoptotic cell death in neuronal cells. Furthermore, we have investigated the role of Coenzyme Q_{10} as a neuroprotective agent. Coenzyme Q_{10} is a component of the mitochondrial respiratory chain and a potent anti-oxidant. Our results indicate that total cellular ROS generation was inhibited by Coenzyme Q_{10}. Further, pre-treatment with Coenzyme Q_{10} maintained mitochondrial membrane potential during oxidative stress and reduced the amount of mitochondrial ROS generation. Our study suggests that water-soluble Coenzyme Q_{10} acts by stabilizing the mitochondrial membrane when neuronal cells are subjected to oxidative stress. Therefore, Coenzyme Q_{10} has the potential to be used as a therapeutic intervention for neurodegenerative diseases.

Keywords: Apoptosis; ROS; Oxidative stress; Neurodegeneration; Coenzyme Q_{10}

Introduction

Intracellular accumulation of reactive oxygen species (ROS) such as free radicals and peroxides occurs during normal metabolic processes and in response to various stimuli (Fleury et al., 2002; Frodovich, 1978). Mitochondria are the major location of ROS production in the cell. A small percentage of mitochondrial oxygen consumption results in the production of hydrogen peroxide (Chance et al., 1979). The ROS produced as by-products of the mitochondrial electron transport chain are quenched by anti-oxidants and converted to non-toxic compounds by free radical scavenging enzymes (Beal, 1999; Boveris and Chance, 1973; Sanders et al., 1993). However, accumulation of free radicals in tissues can result in cell dysfunction and death. Excessive cell death is a characteristic of many neurological disorders including stroke, ischemia, Parkinson's disease, and Alzheimer's disease.

Cell death can follow two distinct pathways, necrosis, or apoptosis (Chandra et al., 2000). Apoptosis, a form of programmed cell death, is a physiological process designed to maintain normal tissue development and homeostasis (Kerr et al., 1972). Further, apoptosis protects against growth of cells carrying cancerous mutations. Morphologically, apoptosis is characterized by cell shrinkage, chromatin condensation, and the formation of apoptotic bodies (McConkey et al., 1998; Wyllie et al., 1980). Necrosis is characterized by an increase in cell volume, rupture of the plasma membrane, and spilling of the cellular components into the intercellular milieu (Gores et al., 1990), causing further tissue damage by affecting neighboring cells and attracting pro-inflammatory cells to the lesion (Haslett, 1992).

Two apoptotic pathways are well understood. The extrinsic pathway employs ligand-induced activation of death receptors resulting in the recruitment and activation of initiator caspases that further activate effector caspases leading to apoptosis (Ashkenazi and Dixit, 1998; Pandey et al., 2003). In the intrinsic pathway, apoptotic signals affect the mitochondria resulting in the release of apoptogenic factors such as cytochrome *c*. Cytochrome *c* binds to pro-caspase-9 and adaptor protein apaf-1 to form the apoptosome. This process leads to the activation of effector caspases such as caspase-3, which activate downstream events ultimately leading to apoptosis.

Apoptotic mechanisms including production of ROS (Lotem et al., 1996; Tan et al., 1998; Um et al., 1996), release of apoptosis inducing factor (AIF) (Susin et al., 1999), activation of second mitochondria-derived activator of caspase (Smac) (Du et al., 2000; Verhagen et al., 2000), opening of permeability transition pores

Abbreviations: $\Delta\psi_m$, Mitochondrial membrane potential; ATP, Adenosine tri-phosphate; BDNF, Brain-derived neurotrophic Factor; CoQ_{10}, Coenzyme Q_{10}; PHPA, Para-hydroxyphenylacetate; PTP, Permeability transition Pore; ROS, Reactive oxygen species.

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(PTP) (Marzo et al., 1998; Zarotti and Szabo, 1995), and collapse of mitochondrial membrane potential ($\Delta\psi_m$) (Zamzami et al., 1995) all occur in the mitochondria. Past research has indicated that hydrogen peroxide induces apoptotic stimuli that depend on the mitochondrial respiratory chain (Chandra et al., 2002). Further, past research has also indicated that neuronal cells are highly sensitive to reactive oxygen species such as free radicals (Kim et al., 2002). It has been hypothesized that mitochondrial dysfunction and consequent production of ROS may induce neuronal cell death occurring in neurodegenerative disorders such as hypoxic-ischemia, Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis (Behl, 1999; Christen, 2000; Coyle and Puttafarcken, 1993; Halliwell, 1989; Kitazawa et al., 2003; Piantadosi and Zhang, 1996; Siesjo and Siesjo, 1996; Traystman et al., 1991).

Mitochondria produce ATP via the electron transport chain. Coenzyme Q_{10} (CoQ_{10}) is an important insoluble component of the mitochondrial respiratory chain, as well as an anti-oxidant. CoQ_{10} transports electrons between complexes I (NADH–ubiquinone oxidoreductase), II (succinate–ubiquinone oxidoreductase), and III (ubiquinone–cytochrome *c* oxidoreductase) (Papucci et al., 2003). Recently, a water-soluble formulation of Coenzyme Q_{10} was prepared according to US patent number 6,045, 826 (Borowy-Borowski et al., 2000). This formulation provides the means to investigate the mechanism of CoQ_{10} in cell culture studies.

In the present study, we have investigated the role of oxidative stress in neuronal cell death induced by hydrogen peroxide. In order to obtain a continuous supply of neuronal cells, we have used differentiated Human Teratocarcinoma Cells (NT2) and Human Neuroblastoma Cells (SH-SY5Y) as models. Further, we have investigated the neuroprotective potential of water-soluble Coenzyme Q_{10}. Our data indicate that treatment with hydrogen peroxide induces an increase in total cellular ROS generation and apoptotic cell death. We have demonstrated that pre-treatment with water-soluble Coenzyme Q_{10} inhibits ROS production and prevents cells from undergoing apoptosis. Furthermore, our results indicate that Coenzyme Q_{10} pre-treatment stabilized the mitochondrial membrane and prevented mitochondrial dysfunction. These results illustrate the potential of Coenzyme Q_{10} as a preventative therapeutic agent for the treatment of neurodegenerative diseases.

Materials and methods

Materials

Water-soluble CoQ_{10} was formulated at the National Research Council (NRC), Ottawa, Canada by a patented protocol developed by Dr. Marianna Sikorska and Dr. Henryk Borowy-Borowski.

Most of the chemicals including trypsin, hydrogen peroxide, collagen, para-hydroxyphenylacetic acid (PHPA), luciferin–luciferase, ATP, low melting point agarose, HEPES, succinate, Na_2EDTA, uridine, fluorodeoxyuridine, cytosine arabinoside, retinoic acid (RA), $MgCl_2$, bovine serum albumin, etc. were purchased from Sigma Chemical Company, Mississauga, ON, Canada. Ham's F12, DMEM, L-glutamine, gentamycin, and Triton X-100 were purchased from Gibco BRL, VWR, Mississauga, ON, Canada. DMSO, sucrose, NaOH, NaCl, and $NaHCO_3$ were purchased from BDH Inc., Toronto, Canada. Glycine and agarose were purchased from EM Sciences, NJ, USA. 2′ ,7′ -dichlorofluorescein diacetate (DCFDA), hoechst, Mitotracker CM-XH_2-Ros dye and JC-1 dye were obtained from Molecular Probes, OR, USA. Protein assay reagent was purchased from Bio Rad, Ontario, Canada. Fetal bovine serum (FBS) was purchased from Winsent Inc, USA. Matrigel matrix was purchased from Becton-Dickinson, CA, USA. Brain derived neurotrophic factor (BDNF) was purchased from Almone Labs, Israel.

Cell culture

Human teratocarcinoma cells

The cells (Stratagene cloning systems, La Jolla, CA, USA) were grown and differentiated using a slight modification of the manufacturer's protocol. NT2 cells were grown in D-MEM/F-12 growth medium supplemented with 10% by volume fetal bovine serum (FBS), 2 mM L-glutamine and 10 mg/ml gentamycin at 37°C and 5% CO_2. The cells were sub-cultured 1:5 twice a week. For differentiation, cells were seeded in a 25-cm^2 flask and treated with normal growth medium containing 10 μM retinoic acid (RA) twice a week for 5 weeks. The cells were sub-cultured 1:3 and replated in complete D-MEM/F-12 medium for 1 day. The following day, the cells were put in serum-free media for 5 min and differentiated neuronal cells were mechanically dislodged by gently striking the flasks. The cells were replated in 60 × 15 mm Petri plates (approximately 100,000 cells), which had been previously coated with matrigel matrix and maintained in normal growth media containing mitotic inhibitors (1 μM cytosine arabinoside (araC), 10 μM fluorodeoxyuridine (FrdU) and 10 μM uridine (Urd)) for at least 3 weeks. Over the 3 weeks, half of the medium was changed twice a week. Differentiated neurons (NT2N) were obtained 3–4 weeks after treatment with mitotic inhibitors.

Human neuroblastoma cells

SH-SY5Y cells were grown in Ham's F12 medium with 2 mM L-glutamine that had been modified to contain 1.5 g/l sodium carbonate, 10% fetal bovine serum and 10 mg/ml gentamycin at 37°C and 5% CO_2 (ATCC, Manassas, VA, USA). Differentiation of these cells was carried out by a slight modification of a previously published method (Encinas et al., 2000). Cells were plated in 60 × 15 mm Petri plates (approximately 300,000 cells), which had been pre-coated with 0.05% collagen. The following day, all-trans retinoic acid (RA) was added to a final concentration of 10 μM in F12 medium containing 15% fetal bovine serum. After 5 days in the presence of RA, the cells were washed twice with F12 medium and incubated with 50 ng/mL BDNF in serum-free media (F12 medium without FBS) for 7 days.

Experimental treatments

Oxidative stress

Differentiated NT2N and SH-SY5Y cells were either pre-treated with 30 μg/ml Coenzyme Q_{10} (CoQ_{10}) for 24 h prior to treatment or directly subjected to oxidative stress by incubation with 100 μM H_2O_2 for 1 h at 37°C. The media was replaced with fresh complete media, with or without 30 μg/ml CoQ_{10} and experiments were conducted at various time intervals as indicated in the figures.

Cellular staining and microscopy

In order to study the morphology, NT2N and SH-SY5Y cells were grown, differentiated and treated as previously explained and