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Coenzyme Q Cytoprotective Mechanisms for Mitochondrial Complex I Cytopathies Involves NAD(P)H: Quinone Oxidoreductase 1(NQO1)

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The commonest mitochondrial diseases are probably those impairing the function of complex I of the respiratory electron transport chain. Such complex I impairment may contribute to various neurodegenerative disorders e.g. Parkinson's disease. In the following, using hepatocytes as a model cell, we have shown for the first time that the cytotoxicity caused by complex I inhibition by rotenone but not that caused by complex III inhibition by antimycin can be prevented by coenzyme Q (CoQ_1) or menadione. Furthermore, complex I inhibitor cytotoxicity was associated with the collapse of the mitochondrial membrane potential and reactive oxygen species (ROS) formation. ROS scavengers or inhibitors of the mitochondrial permeability transition prevented cytotoxicity. The CoQ_1 cytoprotective mechanism required CoQ_1 reduction by DT-diaphorase (NQO_1). Furthermore, the mitochondrial membrane potential and ATP levels were restored at low CoQ_1 concentrations (5 μM). This suggests that the CoQ_1H_2 formed by NQO_1 reduced complex III and acted as an electron bypass of the rotenone block. However cytoprotection still occurred at higher CoQ_1 concentrations (>10 μM), which were less effective at restoring ATP levels but readily restored the cellular cytosolic redox potential (i.e. lactate: pyruvate ratio) and prevented ROS formation. This suggests that CoQ_1 or menadione cytoprotection also involves the NQO_1 catalysed reoxidation of NADH that accumulates as a result of complex I inhibition. The CoQ_1H_2 formed would then also act as a ROS scavenger.

Keywords: Coenzyme Q; Mitochondrial complex I; Parkinson's disease; Cytopathies

INTRODUCTION

Coenzyme Q (CoQ) serves as an electron donor and acceptor in mitochondrial energy-linked respiration[1] and has been proposed as a therapy for mitochondrial cytopathies.[2] CoQ in other intracellular membranes is likely reduced by rotenone resistant membrane NADH dehydrogenases/reductases in the plasma membrane[3,4] and lysosomal membrane[5] or by DT-diaphorase (NQO_1) in the cytosol.[6] The relative high levels of CoQ in most membranes are maintained principally as the reduced form in the mitochondria by inner membrane NADH dehydrogenase (complex I) and other dehydrogenases. In the fully reduced form membrane ubiquinol is believed to function as an antioxidant and protect membranes from prooxidative damage.[6]

Parkinson's disease (PD) is often associated with decreased complex I activity in brain/platelet mitochondria and can be induced by MPTP which inhibits complex I.[7] Recently, epidemiological evidence suggests that the use of rotenone, an organic pesticide and complex I inhibitor is also a risk factor for Parkinson's disease.[8] Alzheimer's disease is also associated with complex IV deficiency possibly as a result of an impaired assembly of cytochrome oxidase or the processing of its sub-

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units.[9] Numerous other mitochondrial cytopathies have also been reported involving deficiencies of complex I–IV resulting from mitochondrial DNA or nuclear DNA mutations.[10,11] As yet there is no effective therapy for these mitochondrial cytopathies. Recently CoQ therapy has shown some benefits for patients with mitochondrial cytopathies.[2] Furthermore CoQ_{10} may be beneficial for PD as it can prevent MPTP induced toxicity in mice.[12]

In an attempt to find novel therapies for different mitochondrial cytopathies, the molecular mechanism of cell death that occurs when complex I is inhibited with rotenone has been investigated. The cytoprotective mechanism of antidotes including CoQ_1 and menadione has also been investigated. Previously, we showed that the molecular cytotoxic mechanism when cyanide was used to inhibit hepatocyte complex IV under aerobic conditions was similar to hypoxia: reoxygenation injury in that it was more associated with reductive stress (an increased lactate: pyruvate ratio) and reactive oxygen species (ROS) formation than with cellular ATP depletion. Furthermore, glycolytic substrates prevented or enhanced complex IV inhibitor induced cytotoxicity depending on whether they restored redox potential or further increased cellular reductive stress.[13] In the following experiments it is shown that CoQ_1 or menadione prevented cytotoxicity induced by complex I inhibitors but not by complex IV inhibitors. The cytoprotective effect of CoQ_1 was attributed to its ability to act as an electron sink and/or an antioxidant rather than by acting as an electron bypass to restore ATP.

MATERIALS AND METHODS

Chemicals

1-Bromoheptane and menadione were obtained from Aldrich Chemical (Oakville, ON, Canada). Collagenase (from *Clostridium histolyicum*) was purchased from Worthington Biochemicals Corp. (Freehold, NJ). Bovine serum albumin (BSA) and HEPES were purchased from Boehringer–Mannheim (Montreal, Quebec, Canada). Trypan blue, rotenone, cyclosporin A, PK 11195, carnitine, catechin, quercetin and sodium pentobarbital and heparin were obtained from Sigma (Oakville, ON, Canada). 2′,7′-dichlorofluorescein diactetate was purchased from Fluka Chemic AG (Oakville, ON, Canada). All other chemicals were purchased from a local supplier and were of the highest commercial grade available.

Animals

Male Sprague–Dawley rats (280–300 g), fed a standard chow diet and given water ad libitum were used in all experiments.

Isolation and Incubation of Hepatocytes

Hepatocytes were obtained by collagenase perfusion of the liver as described by Moldeus *et al.*[14] Approximately 85–90% of the hepatocytes excluded trypan blue upon isolation (i.e. 85–90% cell viability). Cells were suspended at a density of 10^6 cells/ml in round bottom flasks, rotating in a water bath maintained at 37°C in Krebs–Hensleit buffer (pH 7.4), supplemented with 12.5 mM HEPES under an atmosphere of 10% O_2: 85% N_2: 5% CO_2. In all experiments rotenone was added last after the other chemicals. Glutathione (GSH) depleted hepatocytes were prepared by preincubating hepatocytes with 200 μM 1-bromoheptane for 30 min as described by Khan and O'Brien.[15] DT-diaphorase (NQO_1)-inactivated hepatocytes were prepared by preincubating hepatocytes with 15 μM dicumarol for 5 min. Hepatocyte DT diaphorase activity was inhibited 98±1%. It should be noted that dicumarol at much higher concentrations is cytotoxic (LD50 after 2 h is 50 μM) likely by uncoupling mitochondrial phosphorylation.[16]

Cell Viability

The viability of isolated hepatocytes was assessed from the intactness of the plasma membrane as determined by the trypan blue (0.2% w/v) exclusion assay. Aliquots of the hepatocyte incubate were taken at several time points during the 3 h incubation period. At least 80–90% of the control cells were still viable at the end of this period.

Determination of Reactive Oxygen Species

To quantify the generation of ROS in hepatocytes, 2′,7′-dichlorofluorescin diacetate was used as it penetrates the cells and becomes hydrolysed by an intracellular esterase to form dichlorofluorescein (DCFH).[17] The DCFH reacts with intracellular ROS to form the highly fluorescent 2′,7′-dichlorofluorescein, which exits the cell. Hepatocytes (10^6 cells/ml) were suspended in 10 mL modified Hank's balanced salt solution (adjusted to pH 7.4 with 50 mM Tris-HCl) and loaded with DCFH by incubating with 2′,7′-dichlorofluorescein diacetate for 2 min at 37°C. The fluorescence intensity of the 2′,7′-dichlorofluorescein formed was monitored at 500 (excitation) and 520 nm (emission). The results were expressed as fluorescent intensity per 10^6 cells.[17]

Determination of Hepatocyte ATP Levels and Reductive Stress

Intracellular ATP in hepatocytes was extracted using an alkaline extraction procedure and isolated for quantification using a C18 μ-Bondapak reverse-phase column (Waters) as previously described.[13] Reductive stress was measured by determining the hepatocyte lactate/pyruvate ratio using lactate dehydrogenase as previously described.[13]

Hepatocyte Mitochondrial Membrane Potential

The ability of hepatocyte mitochondria to take up the fluorescent dye rhodamine 123 was determined by using an adaptation of the method of Eamus *et al.*[18] as previously described.[19]

Hepatocyte Glutathione Efflux

Hepatocytes were removed at various time intervals from the incubation mixture by rapid centrifugation using a microcentrifuge. Supernatants (incubation media) were transferred to another set of tubes and then resuspeded in 0.8 ml incubation buffer. The total amount of GSH and GSSG in the cells as well as in the medium was measured in deproteinised samples (5% metaphosphoric acid) after derivatisation with iodoacetic acid and 1-fluoro-2,4-dinitrobenzene, by high performance liquid chromatography, using a μ-Bondapak NH_2 column (Waters Associates, Milford, MA). GSH and GSSG were used as external standards. A Waters 600A solvent delivery system, equipped with a model 660 solvent programmer, a Wisp 710A automatic injector and a Data Module were used for analysis. This method is a modification of that described by Reed *et al.*[20]

Statistical Analysis

The statistical significance of differences between the control and treatment groups in these studies was determined by *t*-test. Results represent the standard deviation (±SD) of triplicate samples. The minimal level of significance chosen was $p < 0.05$.

RESULTS

Effect of Rotenone on Hepatocyte Viability and Mitochondrial Membrane Potential

As shown in Table I, the complex I inhibitor rotenone when incubated with isolated hepatocytes decreased the mitochondrial transmembrane potential ($\Delta\psi_m$) before cell membrane lysis occurred. The loss of membrane potential was accompanied by a marked increase in hepatocyte ROS formation as determined by dichlorofluorescein oxidation. Furthermore the ROS scavengers catechin or quercetin prevented both rotenone-induced ROS formation and cytotoxicity but did not affect the decrease in mitochondrial membrane potential. By contrast cyclosporin A which blocks opening of the mitochondrial membrane permeability transition pore (MPT),[21] prevented the rotenone-induced decrease in mitochondrial membrane potential as well as ROS formation and cytotoxicity. Carnitine, a MPT pore-sealing agent[21] was similarly cytoprotective. In contrast, the isoquinoline carboxamide PK 11195, an agonist of the peripheral benzodiazepine receptor in the outer membrane that potentiates MPT pore opening[22] increased rotenone cytotoxicity and ROS formation. GSH depleted hepatocytes were more susceptible than control hepatocytes for rotenone-induced cytotoxicity, ROS formation and loss of membrane potential.

TABLE I Complex I induced hepatocyte cytotoxicity involves mitochondrial ψ collapse and "ROS" formation

Additions	Cytotoxicity % Trypan blue uptake				% Mitoch. membrane potential ψ_m	ROS formation fluorescence units
	30′	60′	120′	180′	30′	30′
Control hepatocytes	18±2	19±2	19±3	20±2	100	86±11
+Rotenone (50 μM)	31±4	43±6	53±6	67±5	28±4	188±21
Permeability transition modulators						
+Cyclosporin (2 μM)	29±2	32±3	38±4	39±3*	91±8	91±9
+Carnitine (2 mM)	28±2	34±4	33±4	38±3*	97±7	82±9
+PK 11195 (100 μM)	59±3	72±7	91±8	100*	14±3	311±30
ROS scavenging antioxidants						
+Catechin (200 μM)	28±3	32±2	34±3	35±3*	31±3	61±7
+Quercetin (50 μM)	23±2	29±4	31±3	34±4*	32±4	63±6
GSH-depleted hepatocytes	19±2	21±2	20±1	22±2	100	99±10
+Rotenone (50 μM)	65±6	79±7	90±8	100	17±2	283±31

*Significantly different from hepatocytes+rotenone ($p < 0.05$).
Hepatocytes (10^6 cells/ml) were incubated in Krebs–Henseleit buffer (pH 7.4) at 37°C. Cytotoxicity was determined as the percentage of cells taking up trypan blue. The hepatocyte mitochondrial membrane potential (ψ_m) was determined with rhodamine 123 as described.[18] ROS formation was expressed as fluorescent units (dichlorofluorescein) as described in Materials and Methods. Values are expressed as the means of at least three different experiments.

TABLE II Q_1 or menadione prevents complex I inhibitor-induced reductive stress cytotoxicity

Additions	Cytotox. (%Trypan blue uptake) 180′	ATP (nmoles/10^6 cells) 60′	% Mitoch. membrane potential (ψ_m) 60′	Redox potential lactate /pyruvate ratio 60′	ROS formation fluorescence units 60′
Control hepatocytes	22±2	27±3	100	8±2	78±9
+Rotenone (45 μM)	32±3	5±1	52±5	17±3	145±17
+Rotenone (60 μM)	71±7	2±1	28±4	88±11	222±25
+CoQ_1 (2 μM)	54±5	11±2	39±4	29±4*	101±11*
+CoQ_1 (5 μM)	33±3*	24±3*	81±8*	13±2*	85±9*
+CoQ_1 (10 μM)	34±4*	6±1	57±5*	11±1*	73±8*
+CoQ_1 (20 μM)	39±4*	2±1	35±3	6±1*	65±7*
+Menadione (5 μM)	34±3*	23±3*	79±7*	11±2*	79±7*
+Antimycin (6 μM)	63±7	6±1	24±3	81±9	257±21
+CoQ_1 (10 μM)	65±6	7±2	27±2	73±8	189±22
+Menadione (5 μM)	61±8	5±1	22±3	71±6	183±24
+NQO_1-inhibited hepatocytes	20±3	25±3	100	10±1	81±9
+Rotenone (45 μM)	67±7	3±1	34±3	83±9	235±21
+CoQ_1 (5 μM)	61±6	6±1	38±4	78±7	188±20
+Menadione (5 μM)	59±8	4±2	41±3	76±8	179±22

*Significantly different from hepatocytes+rotenone ($p < 0.05$).
Hepatocytes (10^6 cells/ml) were incubated in Krebs–Henseleit buffer (pH 7.4) at 37°C. Cytotoxicity, mitochondrial membrane potential and ROS formation were carried out as described in Table I. Hepatocyte ATP levels were determined by HPLC as described in Materials and methods. The hepatocyte lactate/pyruvate ratio was determined with lactate dehydrogenase as described previously.[13] Values are expressed as the means of at least three different experiments±SD

As shown in Table II the mitochondrial potential decrease and cytotoxicity induced by rotenone was dependent on the rotenone concentration and was accompanied by ATP depletion. Addition of CoQ_1 or menadione (5 μM) simultaneously with rotenone prevented the mitochondrial potential decrease, ATP depletion and cytotoxicity. At higher concentrations of CoQ_1 and menadione the mitochondrial potential decrease and cytotoxicity was still prevented but the ATP levels were not restored. Incubation of rotenone with hepatocytes also markedly increased the lactate/pyruvate ratio as well as ROS formation. This was prevented by the presence of CoQ_1 or menadione even at higher concentrations. However CoQ_1 or menadione did not protect NQO_1 inhibited hepatocytes (see Materials and Methods) against rotenone. By contrast with their effects on hepatocytes exposed to rotenone, CoQ_1 or menadione did not protect the hepatocytes against the cytotoxicity, mitochondrial potential decrease and ATP depletion induced by antimycin, a complex III inhibitor. As shown in Fig. 1 hepatocyte GSH efflux was markedly increased when the hepatocytes were incubated with rotenone. GSH efflux was further increased if the reductant dithiothreitol was present but was prevented if CoQ_1 was present at the time when rotenone was added.

DISCUSSION

Incubation of hepatocytes with rotenone, an inhibitor of NADH dehydrogenase (complex I) or antimycin A, an inhibitor of ubiquinone-cytochrome bc_1 reductase (complex III) caused a collapse of the mitochondrial potential and ROS formation before cytotoxicity ensued. Previously rotenone or antimycin A were shown to increase ROS formation by heart submitochondrial particles and complex I and III were suggested to be important mitochondrial sites of ROS generation.[23,24] Furthermore NAD^+-dependent H_2O_2 formation by intact brain or heart mitochondria was increased 6–7 fold by rotenone, which suggests that the site of this H_2O_2 generation is upstream from the rotenone block.[25] Whether backflow of electrons to this site from complex III contributes to antimycin A-induced mitochondrial H_2O_2 generation is not clear although complex III sites for antimycin-induced mitochondrial H_2O_2 generation have been implicated.[24]

The ROS scavenging catecholic flavonoids catechin and quercetin[26] also prevented ROS formation and rotenone-induced cytotoxicity without restoring the mitochondrial membrane potential. This suggests that ROS formation contributed to the cytotoxic process. A similar conclusion was reached for hepatocyte injury induced by hypoxia reoxygenation[27] and cyanide.[13] Cyclosporin A or other inhibitors of the mitochondrial permeability transition pore also prevented rotenone-induced cytotoxicity and prevented the loss of mitochondrial potential. Similar results for the cyclosporin A rescue of mitochondrial potential collapse in cultured hepatocytes induced by rotenone were recently reported. Cyclosporin A also prevented cell lysis and apoptosis 12 h later.[28] L-carnitine similarly prevented the rotenone-induced lysis of cultured hepatocytes and the permeability transition (large amplitude swelling) of isolated mitochondria.[21] PK 11195, an agonist ligand of the peripheral benzodiazepine receptor which potentiates the MPT pore, by contrast increased rotenone-induced

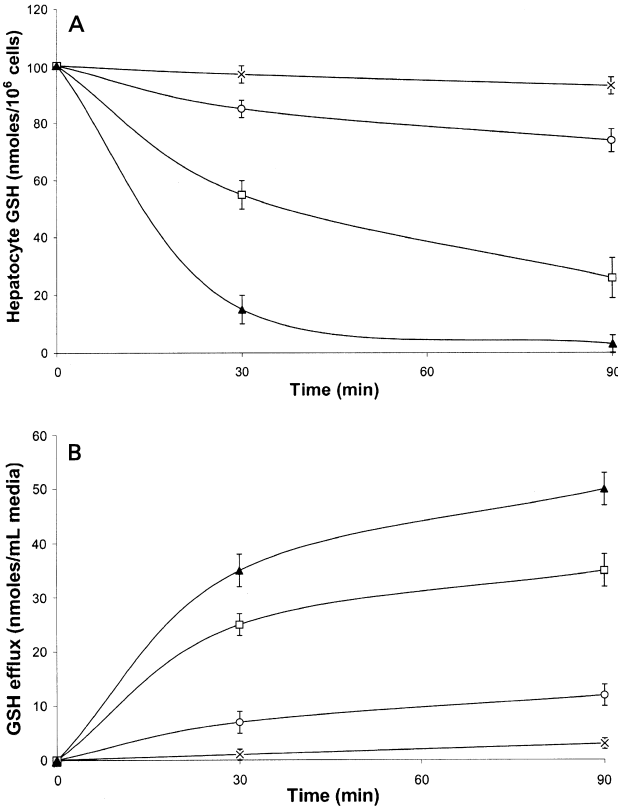


FIGURE 1 GSH efflux in complex I inhibited hepatocytes is prevented by CoQ_1. Depletion of GSH levels in the hepatocytes when the hepatocytes were incubated with rotenone (A) and GSH appearance in the media when the hepatocytes were incubated with rotenone (B). X control hepatocytes: ○+rotenone+CoQ_1 (5 μM); □+rotenone; ▲+rotenone+DTT (3 mM) GSH levels were determined by HPLC analysis as described under Materials and Methods. Values are expressed as means±S.E. of at least three separate experiments.

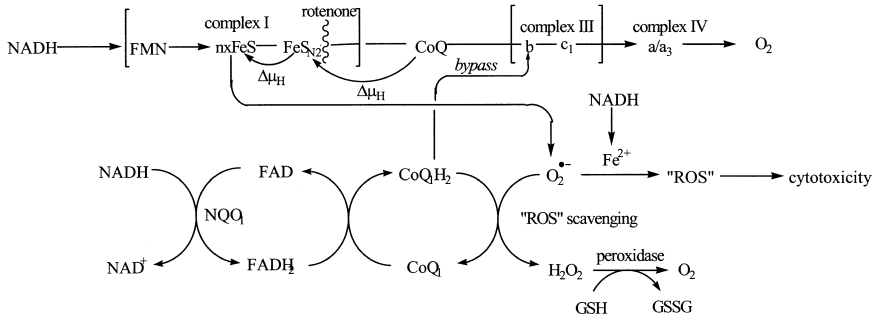
cytotoxicity, collapse of the mitochondrial membrane potential and ROS formation. PK 11195 also increased rotenone-induced cytotoxicity of cultured hepatocytes.[22]

CoQ_1 or menadione prevented rotenone-induced hepatocyte cytotoxicity over the concentration range (2–20 μM) investigated. However ATP levels and mitochondrial membrane potential were restored at an optimal concentration of 5 μM CoQ_1 or menadione. Hepatocyte respiration was also restored (results not shown). This suggested that the CoQ_1 or menadione restored mitochondrial electron transport. Menadione was also shown to restore mitochondrial respiration (an effect mediated by NQO_1) when the respiration of isolated rat-liver mitochondria was inhibited by rotenone.[29] DT-diaphorase (NQO_1) in the intact hepatocytes seems to play a role in cytoprotection by low concentrations of CoQ_1 or menadione because it catalyses their two electron reduction to ubiquinol or menadione, respectively. Consistent with this role, no cytoprotection occurred with NQO_1 inactivated hepatocytes. It should be noted that incorporation of CoQ_{10} into isolated rat hepatocytes provided protection from adriamycin-induced mitochondrial membrane damage and was also prevented by the co-incorporation of dicumarol to inhibit NQO_1.[6]

Ubiquinol (Q_1H_2) or menadiol formed by NQO_1 may be acting as an electron donor for complex III and thus NQO_1 and CoQ_1 could bypass the rotenone block. However, another cytoprotective mechanism seems to be involved at the higher CoQ_1 or menadione concentrations because the decreased cytotoxicity correlated with a restoration of the normal lactate/pyruvate ratio and decreased ROS formation but did not involve restoring ATP levels and mitochondrial membrane potential. This suggests that CoQ_1 or menadione oxidised NADH by a NQO_1-catalysed oxidation resulting in the restoration of the hepatocyte redox potential and the formation of Q_1H_2 or menadiol which scavenge ROS. Other investigators have shown that menadione at these concentrations normalise the lactate to pyruvate ratio that otherwise was increased when rotenone is incubated with confluent cultured skin fibroblasts. Furthermore ATP levels were only restored at low menadione concentrations (≤5 μM) but not at higher menadione concentrations.[30]

The marked increase in hepatocyte GSH efflux by rotenone before cytotoxicity ensued could decrease ROS detoxification and thus contribute to the increased ROS levels. The increased hepatocyte GSH efflux by dithiothreitol suggests the involvement of a redox regulated GSH transporter in the basolateral membrane. Previously we obtained similar results when hepatocytes were maintained under nitrogen. We also showed that GSH efflux was increased by NADH generators or dithiothreitol but was prevented by ROS scavengers or NADH-linked oxidants.[31] The prevention of cellular GSH efflux by CoQ_1 or menadione could therefore result from NADH oxidation or ROS scavenging by Q_1H_2 or menadiol. Recently cellular GSH extrusion has been suggested to be required for mitochondrial cytochrome c release and a key event in the mitochondrial pathway of apoptotic signalling.[32] These results suggest that clinical trials should be initiated to see if CoQ has beneficial effects in treating patients with complex I deficiency diseases.

A hypothesis explaining the above results is outlined in Scheme 1. Rotenone binds to the B/H domain of complex I and inhibits the proton-pumping NADH/ubiquinone oxidoreductase (complex I). This prevents site 1 ATP generation and NADH oxidation by the respiratory chain. The result is an increased hepatocyte NADH/NAD^+ ratio (i.e. lactate/pyruvate ratio). This is associated with the reduction and release of iron from intracellular stores[13,26] that likely contributes to the formation of cytotoxic ROS from H_2O_2 and $O_2^{\cdot-}$. H_2O_2 and $O_2^{\cdot-}$. are likely formed by a reduced iron–sulfur



SCHEME 1 CoQ_1 cytoprotective mechanisms towards complex I inhibitor induced cytotoxicity. Q_1 or K_3 prevents cytotoxicity of complex I deficient hepatocytes because (1) Q_1H_2 formed by NQO_1 bypasses complex I; (2) Q_1H_2 scavenges "ROS"; (3) CoQ_1 oxidises NADH catalysed by NQO_1 and restores cellular redox potential. $n \times$FeS: five ESR-defined iron–sulfur clusters. $\Delta\mu_H$: electrochemical H^+ potential driven reversed electron transfer in State 3.

centre or non-covalently bound FMN upstream of the rotenone block. The collapse of the mitochondrial membrane potential and opening of the MPT pore could release mitochondrial antioxidants such as GSH and ascorbate, along with cytochrome c, procaspases 3 and 9 and other proapoptosis factors. Mitochondrial ROS could then be released into the cell as a result of the loss of mitochondrial antioxidants and contribute to the cytotoxic process, particularly if cytosolic GSH and ascorbate exit the cell. The CoQ_1 or menadione cytoprotection may result from a NQO_1 catalysed two-electron reduction of CoQ_1 or menadione by NADH to form Q_1H_2 or menadiol. This reoxidation of NADH restores cellular redox potential and prevents cytotoxicity. The Q_1H_2 or menadiol formed could also act as an electron bypass and reduce complex III thereby restoring sites 2 and 3 of ATP formation. At the same time the superoxide radicals formed by complex I may be scavenged by Q_1H_2 or menadiol and the H_2O_2 formed may be detoxified by GSH peroxidase and catalase.[33]

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