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Coenzyme Q blocks biochemical but not receptor-mediated apoptosis by increasing mitochondrial antioxidant protection

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Abstract Generation of free radicals is often associated with the induction and progression of apoptosis. Therefore, antioxidants can prove anti-apoptotic, and can help to elucidate specific apoptotic pathways. Here we studied whether coenzyme Q, present in membranes in reduced (ubiquinol) or oxidised (ubiquinone) forms, can affect apoptosis induced by various stimuli. Exposure of Jurkat cells to α-tocopheryl succinate (α-TOS), hydrogen peroxide, anti-Fas IgM or TRAIL led to induction of apoptosis. Cell death due to the chemical agents was suppressed in cells enriched with the reduced form of coenzyme Q. However, coenzyme Q did not block cell death induced by the immunological agents. Ubiquinol-10 inhibited reactive oxygen species (ROS) generation in cells exposed to α-TOS, and a mitochondrially targeted coenzyme Q analogue also blocked apoptosis triggered by α-TOS or hydrogen peroxide. Therefore, it is plausible that ubiquinol-10 protects cells from chemically-induced apoptosis by acting as an antioxidant in mitochondria. Our results also indicate that generation of free radicals may not be a critical step in induction of apoptosis by immunological agents.

Key words: Apoptosis; Signalling; Coenzyme Q; Reactive oxygen species; Lymphoma cell

1. Introduction

Apoptosis is a way of cell self-destruction characterised by a series of tightly controlled steps so that the neighbouring cells are not exposed to inflammatory stimuli, and is an inherent feature of development and disease defence [1,2]. The individual steps of apoptotic pathways have been elucidated to a certain extent and many of them are shared in various models of apoptosis [3].

Of the subcellular organelles, mitochondria are thought to play a central role in apoptosis triggered by chemical and, possibly, immunological stimuli, although the precise mechanisms are not known [4]. The general understanding is that, following exposure of cells to an apoptogenic agent, the mitochondrial outer membrane becomes destabilised. This leads to the release into the cytoplasm of pro-apoptotic factors including cytochrome *c* (cyt *c*) [5,6], Smac/Diablo [7] and AIF [8]. Cyt *c* forms an apoptosome complex in the cytosol with pro-caspase-9 and apaf-1 [9], with subsequent cascade-like activation of the effector caspases [10] and caspase-dependent nucleases [11].

As mitochondrial destabilisation plays a key role in apoptosis, it is important to understand this process and how it can be modulated. Reactive oxygen species (ROS) are involved in the early stages of some forms of apoptosis [12], and are produced by the mitochondrial respiratory chain [13]. Thus, ROS may be involved in destabilisation of the mitochondrial [14] and other subcellular membranes [15] with ensuing release of pro-apoptotic factors, thereby mediating or exacerbating some forms of cell death [16].

A correlation between ROS production and apoptosis has been shown, e.g. for cells overexpressing the mitochondrial anti-apoptotic protein bcl-2, which featured a lower extent of ROS generation and were more resistant to apoptosis induction [17]. Furthermore, antioxidants such as α-tocopherol or ascorbate inhibited apoptosis induced by different stimuli [18,19], suggesting that pharmacological agents can modulate apoptosis on the level of ROS generation; however, the role of mitochondrial ROS in this process is uncertain.

Ubiquinol-10, the reduced form of coenzyme Q_{10}, is a redox-active component of the inner mitochondrial membrane [20]. Although its role as an antioxidant has been studied extensively, little is known about its potential to modulate apoptosis. In vivo, its level can be manipulated by dietary means, and the ratio of the reduced and oxidised forms of coenzyme Q is tightly controlled [21,22]. Prompted by the finding that coenzyme Q protects cells exposed to hydrogen peroxide from DNA strand breaks [23] and the notion that ROS are likely mediators of apoptosis, we investigated the effects of coenzyme Q on induction of apoptosis in Jurkat T

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Abbreviations: CoQ_{10}, oxidised coenzyme Q_{10}; $CoQ_{10}H_2$, reduced coenzyme Q_{10}; DCFH-DA, 2′,7′-dichlorodihydrofluorescein diacetate; FACS, fluorescence-assisted cell sorting; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benzimidoazoyl-carbocyanino iodide; PS, phosphatidyl serine; ROS, reactive oxygen species; α-TOS, α-tocopheryl succinate; TRAIL, TNF-related apoptosis-inducing ligand; $\Delta\Psi_m$, mitochondrial inner membrane potential

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lymphoma cells by a range of pro-apoptotic stimuli. Here we show that the reduced, but not the oxidised, form of coenzyme Q is protective against apoptosis induced by biochemical but not immunological agents.

2. Materials and methods

2.1. Cell culture and treatment

Jurkat T lymphoma cells were maintained in RPMI-1640 supplemented with 10% FCS and antibiotics. The cells were split when reaching about 1.5×10^6 per ml, and used for experiments at 0.5×10^6 per ml. Apoptosis was induced by addition of 50 μM α-tocopheryl succinate (α-TOS) (Sigma) in DMSO, 100 μM hydrogen peroxide, 20 ng/ml anti-Fas IgM (Coulter Immunochemicals) or 40 ng/ml human recombinant TNF-related apoptosis-inducing ligand (hrTRAIL) prepared as follows. The extracellular part of human TRAIL (amino acids 95–281) obtained by PCR from the HPB T cell line cDNA library was subcloned into pBSK, sequenced and further subcloned into the His-tagged reading frame of pET15b. The protein was expressed in *Escherichia coli* and purified using the TALON (Clontech) and SP-Sepharose columns. In some cases, cells were enriched with reduced ($CoQ_{10}H_2$) or oxidised coenzyme Q (CoQ_{10}) (see below) prior to apoptosis induction, or pre-treated with a mitochondrially targeted coenzyme Q (mito-Q) [24] or methyltriphenylphosphonium bromide (TPMP; Aldrich).

2.2. Assessment of apoptotic markers

Phosphatidyl serine (PS) externalisation was assessed using the annexin V-FITC kit (PharMingen) as described elsewhere [25]. Caspase activity was assessed as described [26], using Ac-DEVD-pNA and Ac-VEID-pNA, as substrates for caspase-3 and -6, respectively. Activation of caspase-3 was estimated by incubating cells with an anti-caspase-3 IgG (PharMingen) recognising its activated form, followed by incubation with secondary antibody, and the fluorescence intensity of the cells was assessed by fluorescence-assisted cell sorting (FACS; Becton Dickinson) [25]. Mitochondrial inner membrane potential ($\Delta\Psi_m$) was estimated using 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidoazoyl-carbocyanino iodide (JC-1) [27]. Cells were washed, incubated with 10 μM JC-1 for 15 min at 37°C, washed with PBS and analysed by FACS. Formation of ROS was assessed by flow cytometric analysis of cells labelled with 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA; Molecular Probes) as described [28]. Briefly, following treatments, cells were incubated for 30 min at 37°C with 5 μM DCFH-DA, washed, resuspended in PBS and fluorescence intensity assessed by FACS.

2.3. Enrichment of cells with coenzyme Q and its analysis

CoQ_{10} (a generous gift from Eisai, Japan) at 1 mg/ml in EtOH was reduced to $CoQ_{10}H_2$ in 3 ml of 10 mM carbonate buffer with 25 mM sucrose (pH 7.4) by addition of 10 mg sodium dithionite. The suspension was mixed until it appeared white-opalescent, and $CoQ_{10}H_2$ extracted with three times 2 ml of cyclohexane. The organic phase was washed with 6 ml of carbonate buffer and dried under nitrogen.

Multilamellar liposomes containing CoQ_{10} or $CoQ_{10}H_2$ were prepared by dissolving 16 mg of dimyristyl-phosphatidylcholine (DMPC; Sigma) in 1 ml of EtOH containing either form of coenzyme Q (1 mg/ml) at a coenzyme Q/DMPC molar ratio of 1:20. The solution was evaporated under a stream of nitrogen. The resulting film was dispersed in 10 ml of degassed PBS by placing the container on a vortex mixer and sonicated for 20 min. The suspension was centrifuged ($100\,000\times g$, 1 h) and the supernatant containing small multilamellar vesicles collected.

Jurkat cells were enriched with CoQ_{10} or $CoQ_{10}H_2$ by incubation for 30 min at 37°C with liposomes containing the respective form of coenzyme Q (final concentration, 100 μM). After incubation, cells were washed with PBS and the cellular content of CoQ_{10} and $CoQ_{10}H_2$ determined as follows. Cells (3×10^6) were pelleted and precipitated with 250 μl of iso-propanol, stirred for 1 min and centrifuged for 3 min at $1000\times g$. 100 μl of the supernatant were subjected to HPLC using a C8 column (250×4.6 mm C8, 5 μm particle size; Supelco) and two C8-ABZ pre-columns (50×4.6 mm, 5 μm; Supelco). The mobile phase was prepared by dissolving $LiClO_4$ (10 mM) in MeOH:iso-propanol:EtOH (72:8:20, v/v/v), and used at a flow rate of 0.8 ml/min. Eluting compounds were detected using a Coulochem electrochemical detector with three serial electrodes set at −0.6, −0.15 and +0.6 V, respectively. Quantification of the antioxidants was performed by area comparison with the solution of authentic standards at known concentrations determined spectroscopically. The extinction coefficients used were $\varepsilon_{275}=14\ M^{-1}\ cm^{-1}$ for $CoQ_{10}H_2$ and $\varepsilon_{290}=4.1\ M^{-1}\ cm^{-1}$ for $CoQ_{10}H_2$.

2.4. Cell fractionation and Western blotting

Jurkat cells were fractionated into mitochondrial and cytosolic fractions as follows. Cells (10^7) were pelleted ($200\times g$, 5 min), washed and resuspended in 500 μl of buffer comprising 250 mM sucrose, 20 mM HEPES–KOH (pH 7.4), 10 mM KCl, 1.5 mM Na–EGTA, 1.5 mM Na–EDTA, 1 mM $MgCl_2$, 1 mM DTT and a protease inhibitor cocktail (Boehringer Mannheim). After a 30-min incubation on ice, cells were broken in a glass homogeniser, and the nuclei, unbroken cells and debris removed by centrifugation ($800\times g$, 10 min, 4°C). Supernatants containing mitochondria were further centrifuged at $22\,000\times g$ for 15 min at 4°C. The resulting supernatant representing the cytosolic fraction was stored at −70°C until use. The mitochondrial pellet was lysed by a 20-min incubation at 4°C in 100 μl of buffer containing 50 mM HEPES (pH 7.4), 1% (v/v) Nonidet P-40, 10% (v/v) glycerol, 1 mM EDTA, 2 mM DTT and the protease inhibitor cocktail. Cellular debris was removed by centrifugation ($22\,000\times g$, 15 min, 4°C), and the supernatant containing mitochondrial proteins stored at −70°C.

For Western blotting, proteins of the cytosolic and mitochondrial fractions were separated by 15% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE), and transferred to a nitrocellulose membrane. The membrane was blocked overnight at 4°C in skim milk and incubated with anti-cytochrome *c* IgG (Pharmingen) for 1 h at room temperature followed by a 1-h incubation with peroxidase-labelled secondary IgG. Blots were developed using the ECL kit (Amersham), and exposed to X-Omat AR film.

3. Results and discussion

As apoptosis induced by various stimuli involves generation of ROS, increased intracellular levels of redox-active substances can prove anti-apoptotic. $CoQ_{10}H_2$, the reduced form of coenzyme Q, exerts a potent antioxidant activity in membranes and lipoproteins, and its level can be enhanced by supplementation [20–22]. Recently, coenzyme Q has been shown to suppress DNA strand breaks in lymphocytes challenged with oxidative stress [23]. Its effect on apoptosis, however, has not yet been studied in detail. We therefore inves-

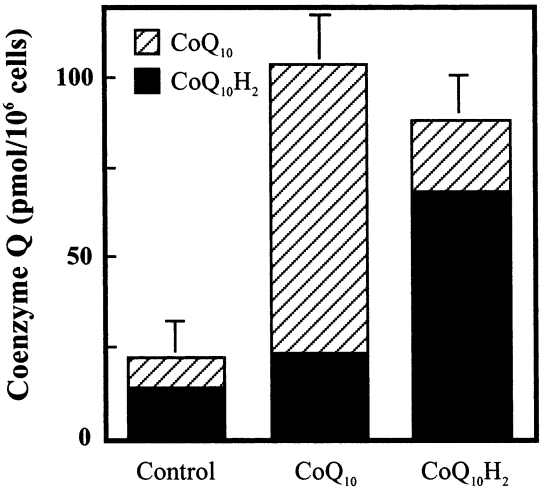

Fig. 1. Jurkat cells can be enriched with coenzyme Q. Jurkat cells were incubated with liposomes containing CoQ_{10} or $CoQ_{10}H_2$ or with liposomes only (control), and cell extracts assessed for the two coenzyme Q forms.

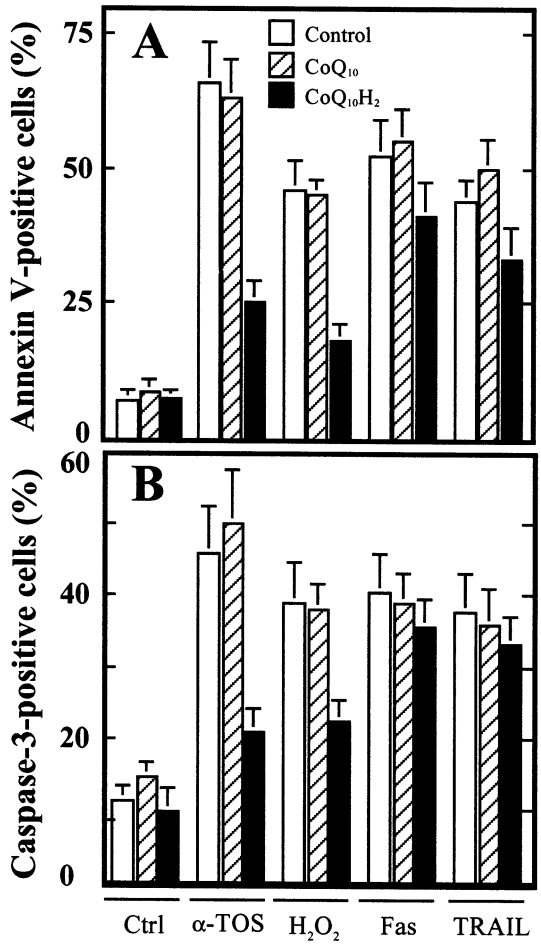

Fig. 2. Reduced coenzyme Q inhibits chemical- but not receptor-induced apoptosis in Jurkat cells. Cells were enriched with the vehicle (control) or with CoQ_{10} or $CoQ_{10}H_2$, exposed to α-TOS (50 μM, 12 h), hydrogen peroxide (100 mM, 6 h), anti-Fas IgM (20 ng/ml, 12 h) or rhTRAIL (40 ng/ml, 12 h), and assessed for FACS estimation of PS externalisation (A) and caspase-3 activation (B).

tigated whether enrichment of Jurkat T lymphoma cells with $CoQ_{10}H_2$ or CoQ_{10} can protect them from apoptosis induced by chemical and immunological apoptogens. Incubation of cells with $CoQ_{10}H_2$- or CoQ_{10}-enriched liposomes led to an approximate 5-fold increase in the intracellular level of the respective redox forms of coenzyme Q_{10} (Fig. 1).

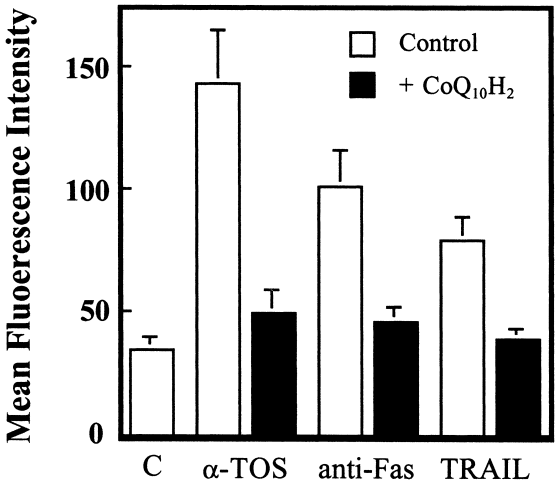

Fig. 3. Formation of ROS in chemical- and receptor-induced apoptosis is inhibited by reduced coenzyme Q. Control or $CoQ_{10}H_2$-enriched cells were exposed to α-TOS, anti-Fas IgM or rhTRAIL (conditions as in legend to Fig. 2), and the level of ROS assessed by FACS.

Exposure of Jurkat cells to both chemical (α-TOS and hydrogen peroxide) and immunological inducers of apoptosis (anti-Fas IgM and hrTRAIL) resulted in typical apoptotic features, including PS externalisation, caspase-3 and -6 activation and in a drop in $\Delta\Psi_m$ (Fig. 2, Table 1). Although these markers of apoptosis were observed with all the apoptogens used, the sequence of events leading to cell death may be different for the various stimuli. To investigate the role of ROS in the induction of apoptosis, we studied the effect of coenzyme Q supplementation on the process of cell death. When $CoQ_{10}H_2$-supplemented cells were exposed to α-TOS and H_2O_2, apoptosis was suppressed, as documented by changes of the above apoptotic markers (Fig. 2, Table 1). In contrast, $CoQ_{10}H_2$ enrichment exerted only a modest effect on apoptosis induced by the immunological agents anti-Fas IgM and hrTRAIL (Fig. 2, Table 1).

To clarify the mechanism by which $CoQ_{10}H_2$ may suppress apoptosis induced by chemical and not by immunological agents, we studied the effect of coenzyme Q_{10} supplementation on cellular production of ROS. As shown in Fig. 3, exposure of Jurkat cells to α-TOS, anti-Fas IgM and hrTRAIL led to ROS formation, and this was suppressed in $CoQ_{10}H_2$- but not CoQ_{10}-enriched cells. These findings suggest that ROS are

Table 1
Ubiquinol-10 protects Jurkat cells from chemical- but not immunological-induced apoptosis

Treatment		Caspase-3 (A_{405})	Caspase-6 (A_{405})	$\Delta\Psi_m$, low cells (% of total)
Control		0.03 ± 0.01	0.04 ± 0.012	14.5 ± 2.5
α-TOS	–	0.13 ± 0.021	0.16 ± 0.035	45.3 ± 6.2
	CoQ_{10}	0.11 ± 0.02	0.13 ± 0.034	43.2 ± 5.1
	$CoQ_{10}H_2$	0.036 ± 0.01	0.055 ± 0.012	22.1 ± 3.2
H_2O_2	–	0.1 ± 0.029	0.12 ± 0.03	47.9 ± 6.6
	CoQ_{10}	0.12 ± 0.032	0.14 ± 0.034	43.3 ± 5.7
	$CoQ_{10}H_2$	0.042 ± 0.012	0.052 ± 0.008	25.9 ± 3.4
anti-Fas	–	0.15 ± 0.034	0.1 ± 0.025	41.2 ± 4.7
	CoQ_{10}	0.13 ± 0.04	0.12 ± 0.012	44.5 ± 5.5
	$CoQ_{10}H_2$	0.095 ± 0.025	0.08 ± 0.02	38.7 ± 3.3
TRAIL	–	0.11 ± 0.02	0.09 ± 0.014	38.9 ± 3.9
	CoQ_{10}	0.13 ± 0.03	0.11 ± 0.023	39.8 ± 4.5
	$CoQ_{10}H_2$	0.1 ± 0.035	0.075 ± 0.017	36.7 ± 3.1

playing a major role in apoptosis induced by α-TOS rather than by the two immunological apoptogens, and that the redox function of $CoQ_{10}H_2$ can modulate apoptosis induced by chemical stimuli. Thus, $CoQ_{10}H_2$ may act as an antioxidant, blocking the actions of the oxidants implicated in the induction of apoptosis. This is supported by the finding that protection from apoptosis was not observed in cells enriched with CoQ_{10}, confirming that the antioxidant potential of ubiquinol-10 is essential for its anti-apoptotic activity. This notion is consistent with reports showing coenzyme Q-dependent inhibition of mitochondrial production of ROS, such as the superoxide anion radical [29,30].

While mitochondria are central to apoptosis induced by chemical triggers, the signalling pathways may bypass mitochondria in receptor-induced apoptosis. However, in this type of cell death, mitochondria may amplify the pro-apoptotic signalling even if they are not involved in the initial recruitment of the initiator caspase to the death domain on the cognate receptor. To investigate the potential site of the anti-apoptotic action of $CoQ_{10}H_2$, we used a recently synthesised mitochondrially targeted analogue of coenzyme Q_{10}, mito-Q [24]. This compound contains at the end of its aliphatic side chain a positively charged triphenylphosphonium group, which transports it to mitochondria, so that the charged group resides within the mitochondrial matrix while its quinone function is on the surface of the mitochondrial inner membrane [31]. Moreover, the oxidised form of mito-Q is reduced and continuously recycled to its active antioxidant form by the mitochondrial respiratory chain [24]. Consistent with the effect of $CoQ_{10}H_2$ on apoptosis, we found that mito-Q-enriched cells were protected from chemically-, but not immunologically-, induced apoptosis at the level of PS externalisation and cytochrome *c* re-localisation (Fig. 4).

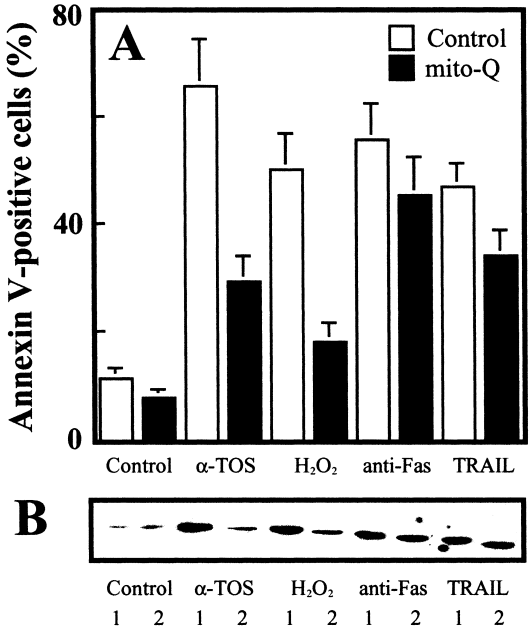

Fig. 4. Mitochondrially targeted coenzyme Q protects from chemical- but not receptor-induced apoptosis. Jurkat cells were pre-incubated with mito-Q (1 μM, 1 h), exposed to α-TOS, hydrogen peroxide, anti-Fas IgM or rhTRAIL, and assessed for PS externalisation (A) and cytosolic cyt *c* re-localisation (B: lanes 1, control; lanes 2, mito-Q-enriched cells).

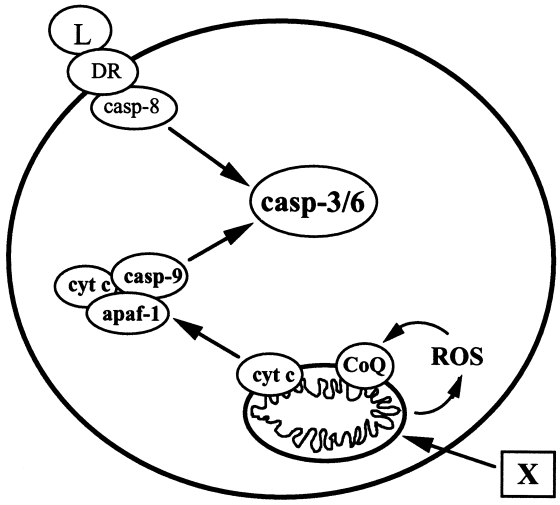

Fig. 5. Suggested role for coenzyme Q as an inhibitor of apoptosis. A chemical agent (X) induces generation of reactive oxygen species (ROS) via mitochondrial-dependent processes, and this results in destabilisation of the mitochondrial outer membrane with ensuing release of the pro-apoptotic cytochrome *c* (cyt *c*). The reduced form of coenzyme Q, $CoQ_{10}H_2$, mediates dissipation of ROS, thereby suppressing cyt *c* release and the downstream events. In the proximal apoptosis signalling pathway, a ligand (L) interacts with its cognate death receptor (DR), which leads to the recruitment of the initiator caspase-8 followed by activation of the effector caspase-3 and -6, largely bypassing the distal, mitochondrial pathway where coenzyme Q acts.

TPMP itself exerted no effect (data not shown). This finding suggests mitochondria as the site of the anti-apoptotic action of $CoQ_{10}H_2$.

On the basis of these results, we suggest that ubiquinol-10, but not ubiquinone-10, inhibits apoptosis induced by chemical apoptogens, which largely use the distal, mitochondrial signalling pathways. On the other hand, apoptosis triggered by immunological agents, such as anti-Fas IgM or TRAIL, which rely on their cognate receptors in apoptosis induction, appears largely independent of the coenzyme Q status of the cell (Fig. 5). This further supports the idea of ROS participation in apoptosis induction and progression, and is consistent with differences in the pro-apoptotic signalling pathways used by chemical and immunological agents. Our data thus link the antioxidant capacity of redox-active compounds like coenzyme Q and its analogues [32–35] to their anti-apoptotic potential. That our findings may be of (patho)physiological relevance is also supported by reports showing disruption of the redox state of antioxidants including coenzyme Q in pathological situations [36].

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