REVIEW

Tocopheryl quinones and mitochondria

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In the past, the role of tocopherols and tocopheryl hydroquinones as antioxidants in mitochondria has been examined. However, structural properties of tocopherols and tocopheryl quinones (arrangement of polar/apolar moieties) have also been recognized as being crucial for the selective transport of RRR-α-congeners compared with other tocopherols in the cell, suggesting that these properties might be generally important for the binding of vitamin E-related compounds to proteins and enzymes in mitochondria. Therefore, direct modulation of mitochondrial activities, such as bioenergetics, production of reactive oxygen species and apoptosis, not exclusively related to the redox activity of these compounds is increasingly studied. This overview focuses on the influence of α-/γ-tocopheryl quinones and their parent α-/γ-tocopherols on mitochondrial functions, including formation of tocopheryl quinones, their analytical aspects, their potential as alternative substrates and their inhibitory activity for some mitochondrial functions. It is shown that the understanding of how tocopheryl quinones and tocopherols interfere with mitochondrial functions on the molecular level is still incomplete and that a better comprehension requires further research activities.

Keywords:
Inhibition / Mitochondria / Reactive oxygen species / Tocopherols / Tocopheryl quinones

1 Introduction

Although the basic biochemical and molecular biological principles for the essential role of vitamin E compounds in mammalian organisms have been elucidated [1, 2], there are still significant open issues, which include major differences between *in vitro* and *in vivo* benefits of vitamin E compounds [3].

In addition, since vitamin E is widely ingested as food supplement and a growing number of patients are taking prescribed drugs simultaneously, the issue of vitamin E–drug interactions becomes more and more important [4].

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Abbreviations: **α-TOS,** α-tocopheryl succinate; **CEHC,** 2(2′-carboxyethyl)-6-hydroxychroman; **CYP,** cytochrome P450; **cyt,** cytochrome; **DEPMPO,** 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline-*N*-oxide; **dUQ,** decylubiquinone, 2-decyl-5,6-dimethoxy-3-methyl-[1,4]benzoquinone; **dUQH$_2$,** decylubiquinol, reduced dUQ; **FAD,** flavin adenine dinucleotide; **IC$_{50}$,** concentration required for half inhibition; **NADH,** reduced nicotinamide adenine dinucleotide; **ROS,** reactive oxygen species; **SMP,** submitochondrial particles; **TAP,** tocopherol-associated protein; **Toc,** tocopherols (if no congener is specified the abbreviation refers to these compounds in general); **Toc$^{\bullet}$,** tocopheroxyl radicals; **TQ,** tocopheryl quinones (if no congener is specified the abbreviation refers to these compounds in general); **TQH$_2$,** tocopheryl hydroquinone, reduced TQ; **TQ$^{\bullet-}$,** tocopheryl semiquinone; **UQ$_n$,** ubiquinones, the index n indicates the number of isoprenic units in the side chain (without index it refers to ubiquinone in general); **UQH$_2$,** ubiquinol, reduced UQ; **UQ$^{\bullet-}$,** ubisemiquinone

A long known example is that vitamin E supplementation delays blood clotting in patients [5, 6] and could become a problem in combination with other potent anticlotting agents. This suggests interference of vitamin E and related compounds with drug actions and physiological functions independent of their antioxidant function.

It was hypothesized that some of these effects could not only be caused by vitamin E compounds (including tocopherols (Toc)) [6] but also by their metabolites: the tocopheryl quinones (TQ) [7, 8].

In contrast to the detailed knowledge of the pharmacological and toxicological properties of many approved drugs, the bioactivity of vitamin E metabolites has not been sufficiently elucidated yet. While enzymatic conversion of Toc leads to rather predictable products, during the antioxidative activity of Toc, which involves radical reactions, a variety of compounds are produced in addition to TQ, such as epoxides and adducts with lipids [9, 10]. The fact that many of these compounds occur in rather low concentrations *in vivo* and that it is difficult to synthesize such compounds delays research in this area. Even the intracellular formation, transport and bioactivity of the most simple oxidation products of vitamin E, TQ, is not yet completely known. Besides their hypothetic interference with the synthesis of coagulation factors [5], there are some clues that TQ depending on their substitution pattern might modulate mitochondrial functions, such as complex I–III activities, formation of reactive oxygen species (ROS) and the release of apoptotic signals.

In spite of the overwhelming research efforts on α-Toc due to its active transport in the mammalian organism [11], the human diet contains often more γ-Toc [12] and in vegetables α-TQ concentrations might reach about 50 mol% of α-Toc concentrations [13]. In addition, conversion of α-Toc to α-TQ was observed during food storage and processing [9, 14]. Therefore, it was suggested that besides α-Toc, γ-Toc and their related TQ in the diet might have impacts on physiological functions in mammals [13, 15].

This overview focuses on the bioactivity of α-/γ-TQ and their precursors α-/γ-Toc in relation to mitochondria. Since the concentration of TQ in mitochondria is influenced by several factors, analytical aspects, mechanism of formation, intracellular occurrence and metabolism will also be considered besides toxicological effects on mitochondria.

2 Formation and metabolism of TQ

In the case of vitamin E metabolism, the enzymatic conversion is superimposed by radical-induced oxidation of vitamin E [16]. Therefore, metabolism of Toc *via* TQ involves both nonenzymatic and enzymatic steps. While the enzymatic steps exhibit an inherent specificity, the nonenzymatic steps are complicated by numerous and rather unspecific radical reactions.

2.1 Nonenzymatic steps

The mechanism of formation of TQ is similar for all Toc congeners. Therefore, in Fig. 1 only the pathways of α-TQ formation are displayed, while similar steps are expected for γ-Toc oxidation.

2.1.1 One-electron oxidation of Toc

During the antioxidative activity, α-Toc is converted by radical attack to the tocopheroxyl radical (α-Toc$^{\bullet}$). This occurs for lipid peroxyl radicals (the most frequent natural co-reactants for Toc) in membranes most likely *via* single hydrogen atom transfer [17] and not *via* single electron transfer and subsequent proton transfer. The reaction rates of γ-Toc are about half that of α-Toc with respect to oxygen radicals [18].

Mechanistic studies have demonstrated that although oxygen-centered Toc$^{\bullet}$ can be converted to Toc dimers, this

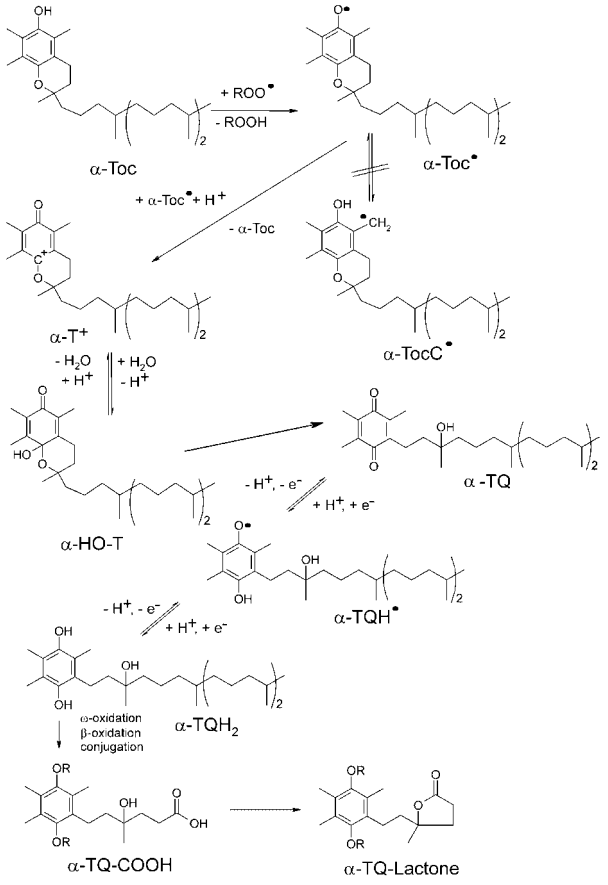

Figure 1. Formation and metabolism of TQ: α-Toc, α-tocopherol; α-Toc$^{\bullet}$, α-tocopheroxyl radical; α-TocC$^{\bullet}$, hypothetic α-Toc 5-C radical; α-T^{+}, α-tocopherone cation; α-HO-T, α-hydroxy-tocopherone; α-TQ, α-tocopheryl quinone; α-TQH$^{\bullet}$, α-tocopheryl semiquinone, α-TQ-COOH, conjugates of α-tocopheronic acid and α-TQ-lactone, α-tocopheronolactone.

does not occur *via* carbon-centered tocopherol radicals (α-TocC$^{\bullet}$) but *via* quinone methides [19, 20].

2.1.2 Disproportionation of Toc$^{\bullet}$

The subsequent formation of α-TQ from α-Toc$^{\bullet}$ proceeds *via* several intermediates (Fig. 1), such as tocopherone cations (α-T^{+}) and hydroxy- (or alkoxy-) tocopherones (α-HO-T) and requires the reaction of α-Toc$^{\bullet}$ with a second α-Toc$^{\bullet}$ or lipid peroxyl radicals [21, 22]. This multistep reaction is often referred to as disproportionation of two α-Toc$^{\bullet}$ radicals to one molecule α-Toc and one molecule α-TQ.

It has been shown *in vitro* that only 10–15% of α-Toc in mitochondria is converted to α-TQ under peroxide-induced lipid peroxidation [23]. Under these conditions, side products such as adducts with lipid radicals, epoxides and dimerization products of Toc are also formed [9, 21]. Besides these reactions of Toc with peroxyl radicals, α-TQ formation was also observed upon the oxidation of α-Toc by NO$^{\bullet}$. In addition to α-TQ in this reaction, other products, such as nitrate esters and spirodimers, were also observed [24].

Since mammalian cells are protected from oxidative stress by various mechanisms, which include the recycling of α-Toc$^{\bullet}$ back to α-Toc [25], the concentration of α-TQ *in vivo* is significantly lower than that of the parent Toc (Table 1).

2.1.3 Nonenzymatic modification of TQ

The lacking methyl group of γ-Toc/γ-TQ *versus* α-Toc/α-TQ has tremendous influence on the reactivities toward several biological reactants. It was shown that γ-Toc but not α-Toc reacts with NO_2 to 5-NO_2-γ-Toc (tocoyellow, (R)-2,7,8-trimethyl-5-nitro-2-((4R,8R)-4,8,12-trimethyl-tridecyl)-chroman-6-ol) and tocored ((R)-2,7,8-trimethyl-2-((4R,8R)-4,8,12-trimethyl-tridecyl)-3,4-dihydro-2H-chromene-5,6-dione) [26]. In addition, γ-TQ but not α-TQ is a strong electrophile, which is able to add to (arylate) sulfhydryl groups of low-molecular biomolecules (glutathione) and proteins leading to phenomena, such as ER stress [20, 27, 28]. On the other hand, the increase of cellular glutathione concentrations *via* an adaptive response mediated by an activating transcription factor 4 was also observed [29]. Therefore, nonenzymatic modification of γ-TQ leads to a variety of products thereby interfering with cellular functions.

2.2 Enzymatic steps

2.2.1 Reduction of TQ

TQ can be enzymatically reduced by microsomal and mitochondrial oxidoreductases with varying efficiency [30] forming antioxidant tocopheryl hydroquinones (TQH_2) *via* the corresponding semiquinone intermediate. Most effective in reducing α-TQ were mitochondrial complex I, microsomal cytochrome (cyt) b_5 reductase [30] and NAD(P)H:quinone oxidoreductase 1 (NQO1) [31]. Moreover, thioredoxin reductase-dependent reduction of α-TQ *via* ebselen was observed [32].

2.2.2 Degradation and conjugation

For α-Toc, enzymatic metabolism by consecutive ω- and β-oxidations leads to α-CEHC (2(2′-carboxyethyl)-6-hydroxychroman) [33]. By analogy, metabolic conversion of γ-Toc leading to γ-CEHC was observed [34]. In addition, it has been described that this metabolic route is shared with other lipophilic compounds, such as vitamin K and ubiquinones (UQ). The products obtained from labeled ^{14}C α-TQ suggest that TQ is also metabolized *via* this route, leading to α-tocopheronic acid and α-tocopheronolactone (α-TQ-lactone, Fig. 1) [35]. Although the artifactual formation α-TQ-lactone by acid hydrolysis of urinary α-CEHC conjugates was discussed previously [35, 36], a new LC/MS method, which directly detects the conjugates of α-TQ-lactone (glucuronide and sulfate), demonstrates its natural

Table 1. Concentrations of vitamin E-related compounds and UQ_9 in subcellular fractions of rat liver from Sprague–Dawley rats

	Mitochondria	Microsomes	Cytosol
α-Toc (nmol/mg protein)	2.12 ± 0.20	2.27 ± 0.15	0.23 ± 0.02
γ-Toc (nmol/mg protein)	0.055 ± 0.009	0.069 ± 0.004	0.010 ± 0.001
α-TQ (nmol/mg protein)	0.122 ± 0.030	0.025 ± 0.001	0.010 ± 0.002
UQ_9 (nmol/mg protein)	6.50 ± 2.18	0.33 ± 0.07	0.31 ± 0.04
Ratio α-TQ/α-Toc (mol%)	5.7	1.1	4.3
Ratio γ-Toc/α-Toc (mol%)	2.6	3.0	4.3
Ratio α-TQ/UQ_9 (mol%)	1.8	7.5	3.2
Ratio α-Toc/UQ_9 (mol%)	32	687	74

Data were obtained from subcellular fractions of five animals and represent mean ± SD (unpublished data from [98]). The ratios were calculated from the concentrations of the respective compounds and multiplied by 100 to obtain mol%. Subcellular fractions were obtained as described in [98] and analyzed after extraction with organic solvents by HPLC with precolumn reduction and electrochemical detection as described in [109].

presence in urine [37]. Therefore, TQH_2 is also obviously converted by side-chain oxidation such as Toc itself. Probably, the same cytochrome P450 subtypes (CYP 3A4 and CYP 4F2 in humans [38]) as for Toc are involved, however, experimental evidence is still lacking.

3 Analytical detection of TQ

3.1 Sample preparation

Bioquinones such as TQ and UQ exist in biological samples in two different states: the oxidized state (quinone, Q) and reduced state (hydroquinone). Therefore, the applied sample storage and extraction methods have to be adapted to the analytical aim of the respective experiments. A generally required precaution is the prevention of TQ formation from the excess Toc during sample work-up. This can be achieved by prevention of lipid peroxidation by addition of potent antioxidants (such as butylated hydroxytoluene) and iron chelators (such as diethylenetriaminepentaacetic acid or deferoxamine) during preparation of the biological samples [39]. Subsequent extraction of samples with organic solvents (*e.g.* hexane/ethanol) yields unbiased amounts of TQ. However, during the extraction procedure from mitochondrial fractions, most of the α-TQH_2 is oxidized by air oxygen or by the coextracted UQ resulting in the quantitative ($>90\%$) conversion to α-TQ and the formation of ubiquinol (UQH_2).

Both the oxidation of TQH_2 by oxygen and by UQ can be discussed on the base of one-electron redox potentials.

$$TQ + TQH_2 \rightarrow 2TQ^{\bullet -} + 2H^+ \quad (1)$$

$$TQ^{\bullet -} + UQ \rightarrow TQ + UQ^{\bullet -} \quad (2)$$

$$2UQ^{\bullet -} + 2H^+ \rightarrow UQ + UQH_2 \quad (3)$$

Compounds with more negative reduction mid-point potentials (E_{m7} $(TQ/TQ^{\bullet -}) = -254\,mV < E_{m7}$ $(UQ/UQ^{\bullet -}) = -110\,mV$) ($TQ^{\bullet -}$, tocopheryl semiquinone) are more powerful reductants. Therefore, the equilibrium of Eq. (2) is shifted to the right, indicating net reduction of UQ by TQH_2 [40, 41] *via* the semiquinone state. In addition, these reactions involve several prototropic equilibria, which modulate the thermodynamic potentials and the kinetics of these reactions [42]. Likewise, similar predictions can be made for the reduction of O_2 ($E(O_2(1M)/O_2^{\bullet -}) = -155\,mV$) by $TQ^{\bullet -}$ or $UQ^{\bullet -}$.

$$TQ^{\bullet -} + O_2 \rightarrow TQ + O_2^{\bullet -} \quad (4)$$

$$UQ^{\bullet -} + O_2 \rightarrow UQ + O_2^{\bullet -} \quad (5)$$

Therefore, reduction of O_2 by $TQ^{\bullet -}$ Eq. (4) is more effective than reduction by $UQ^{\bullet -}$ Eq. (5). This suggests that autoxidation of TQH_2 *via* semiquinones is a more effective source of $O_2^{\bullet -}$ than UQH_2. Most data presented in the literature refer exclusively to α-TQ and did not consider α-TQH_2. To obtain the approximate native concentration ratio of α-TQ and α-TQH_2, anaerobic extraction is required [43]. A possibility is rapid sample freezing and extraction of the biological material and storage of organic extracts under argon until chromatographic analysis. Also moderate acidification of organic extracts can slow down autoxidation of hydroquinones by preventing semiquinone formation from deprotonated hydroquinones. However, for most studies the analytical determination of TQ in relation to the amount of Toc is a sufficient parameter for oxidative stress [44]. In addition, the loss of γ-TQ by formation of adducts with glutathione and proteins during sample work-up was described previously [45].

3.2 Separation and detection methods

For α-Toc many different reversed-phase HPLC separation methods based on fluorimetric [45], and electrochemical detection [46–48] as well as GC/MS [49], and LC/MS [50] spectroscopic methods exist. These methods are widely used for Toc analysis in biological samples. Although MS detection using single ion detection modes with or without derivatization exhibits the highest specificity [51] for both Toc and TQ, it is not generally available yet and rather expensive. Simultaneous detection of Toc and TQ by fluorescence is not possible due to the lacking fluorescence emission of the respective quinones [45]. In contrast, electrochemical, and especially coulometric detection with its various possibilities for on-line derivatization provides a convenient and sensitive method to simultaneously detect Toc, TQ and other lipophilic quinones in extracts from biological matrices. Additional specificity for TQ is achieved by performing a pre or postcolumn reduction of quinones (TQ) to the respective hydroquinones (TQH_2) and subsequently Toc and TQH_2 can be detected by measuring the oxidation current. This technique was derived from a method developed for UQ derivatives [52], however, a more polar eluent composition to delay the elution of α-TQ and α-Toc from the HPLC column was used [53].

Depending on the chromatographic method and detection method applied different internal standards, which can be admixed to biological samples prior to extraction were used. For the extraction of lipophilic vitamin E-related compounds, the use of several compounds as internal standard was suggested, such as 2,2,5,7,8-pentamethylchroman-6-ol for fluorimetric detection [54], α-Toc-acetate for UV detection at 292 nm [55], and ubiquinone-6 (UQ_6) for electrochemical detection [56].

4 Distribution of TQ and Toc in subcellular fractions

The concentrations of α-TQ and the ratio and of α-TQ/α-Toc in blood plasma and tissues were extensively reviewed by Niki [44]. Lowest α-TQ/α-Toc values were reported in

plasma (around 0.1 mol%) and highest values in red blood cells (37 mol%) and cerebrospinal fluid (31-117 mol%) of patients [57, 58].

Toc and TQ share many pathways of uptake, distribution and metabolism with xenobiotics including drugs. Therefore, α-TQ and α-Toc potentially influence the pharmacodynamics and pharmacokinetics of drugs and *vice versa*.

Pharmacokinetic drug interactions are based on the α-Toc-mediated increased expression of *p*-glycoprotein responsible for biliary excretion and preferably CYP 3A, which is involved in metabolism [59] and activated *via* the pregnane X receptor [60]. Typical examples of drugs (among many others [61]) interacting with vitamin E-metabolizing CYP 3A enzymes are statins (metabolism *via* CYP 3A4, transport *via p*-glycoprotein) and the anticoagulant prodrug clopidogrel (activation *via* CYP 3A4). For drug distribution, the strong lipophilicity of α-TQ and α-Toc suggests a displacement of coadministered drugs from their binding to plasma proteins resulting in a higher portion of free drugs and potential adverse effects. α-Toc and α-TQ might also interfere in the pharmacodynamics of drugs. In this respect, the structural analogy of α-TQ with vitamin K and its antagonist warfarin suggests an interaction at the vitamin K epoxide reductase [8]. On the other hand, α-Toc was described to interfere in the metabolism of vitamin K_1 to more anticoagulant products, such as menaquinone-4, thus leading to increased bleeding under vitamin E supplementation [5]. These multiple interactions suggest that the subcellular concentration of α-TQ and α-Toc could also be biased by drugs and *vice versa*.

As summarized in Table 1, for rat liver subcellular fractions, the distribution of α-Toc in membranes is determined by the lipid content of membranes and is rather uniform. In contrast, the lipophilic antioxidant UQ (UQH_2) is highly accumulated in mitochondria and only minor amounts were found in microsomal membranes. Other studies reported slightly lower α-Toc concentrations (Table 1) in mitochondria ranging from 0.08 to 0.7 nmol/mg protein [30, 48, 62], suggesting a certain physiological variability. For mitochondria α-TQ concentrations from 5 to 30 pmol/mg protein were found [30, 42, 63]. The ratio of α-TQ to α-Toc was rather high in mitochondrial membranes and in cytosolic fractions (Table 1). The ratio of α-TQ to α-Toc for mitochondrial membranes in the experiments from Table 1 are higher than in a previous study (1–2 mol%) [30], however, at the lower end of the 5–50 mol% for hepatocyte homogenates reported by Hayashi *et al.* [64]. Previous studies have shown that within mitochondrial membranes the inner membrane contains a higher ratio of α-TQ to α-Toc suggesting that oxidative conversion of α-Toc to α-TQ is a major source for this oxidation product [30].

Likewise in the cytosol Toc seemed to be more prone to oxidation. Here, α-Toc is bound to transport proteins and probably more exposed to oxidants from the aqueous phase. The resulting α-TQ was shown in the cytosol to bind to certain proteins, such as glutathione-*S*-transferase [65], and to a supernatant protein factor (tocopherol-associated protein, TAP) of the SEC-14 family [66].

The ratio of α-TQ to α-Toc in liver microsomal membranes listed in Table 1 is rather small compared with the ratio in mitochondria. In a previous study [30], slightly higher α-TQ/α-Toc ratios in rat liver microsomes (2.73 mol%) than in rat liver mitochondria (1.1–1.71 mol%) were observed. This variation could arise from different isolation procedures (separate preparation of mitochondria and microsomes in [30], preparation of all fractions from the same liver homogenate in Table 1), or from physiological differences. Since microsomal monooxygenases can be sources of ROS as well [67], α-TQ formation is likely in these fractions. In addition, the question remains whether α-TQ and α-Toc are metabolized with the same rate in microsomes. Although side-chain oxidation is carried out by similar CYP subtypes, kinetic information concerning a preference of α-TQ is not yet available.

Traces of the oxidation product of γ-Toc were also detected in subcellular fractions of rat liver, but the concentration of γ-TQ was about ten times lower than that of α-TQ and too low for reliable quantification. In human plasma, it has been shown that α-Toc concentration was about 15 times higher than γ-Toc concentration [34]. In subcellular fractions from rat liver, the ratio of γ-Toc to α-Toc is decreasing from mitochondrial over microsomal to cytosolic fractions (Table 1). This fits to the observation during α- and γ-Toc supplementation experiments that, in general, α-Toc is better retained in membranes than γ-Toc [68] and suggests specific intracellular mechanisms for incorporation of α-Toc – but not of γ-Toc – into membranes.

The different distribution of α-TQ (α-Toc) *versus* UQ is based on biosynthesis of UQ in mitochondria and in the ER/Golgi system [69] while α-TQ is preferably derived from oxidation of α-Toc [30, 70]. The intracellular supply with α-Toc in turn is regulated by the α-tocopherol transfer protein [71], whereas α-TQ is preferably bound to the TAP [66], suggesting a transport independent of α-Toc. For UQ, rather less specific UQ-binding proteins [72] and a vesicle-mediated transport originating from the Golgi system [69] have been described.

5 Bioactivity of TQ and Toc in mitochondria

Besides the essential role of mitochondria in cellular bioenergetics, they are involved in biosynthesis, metabolism and signaling. It has been already known for a long time that the function of membrane-bound enzymes is modulated by their lipophilic environment. Often, this includes rather unspecific interactions with certain lipids and for some enzymes highly specific interactions with lipophilic substrates, such as UQ. Due to the similar reactivity of α-, γ-congeners with oxygen radicals [73], in general, a

certain portion of Toc in membranes and bound to soluble proteins is oxidized to TQ irrespective of their substitution patterns. In mitochondrial membranes, the concentrations of TQ are about 10–100 times lower than those of the parent Toc. In addition, the α-congener dominates over γ-TQ considerably. In comparison with UQ, α-TQ amounts constitute only to a few mol percent (Table 1). For γ-TQ, the ratio to UQ is another magnitude smaller.

5.1 Possible interactions

The bioactivity of TQ and Toc in mitochondria can be related to (i) their redox properties, (ii) their whole molecular structure for binding to proteins as signaling molecules or functional modifiers (iii) or a combination thereof.

Redox properties are important for the antioxidant activity of TQH_2/Toc in mitochondria, such as scavenging of peroxyl radicals. Studies using substituted phenoxyl radicals (*e.g.* galvinoxyl radicals) mimicking lipid peroxyl radicals have shown that α- and γ-TQH_2 react about three times faster than α-Toc and UQH_2 with these radicals (in ethanol) [73]. Theoretically, this would make α-TQH_2 a superior antioxidant [74], however, due to its low natural concentration in most tissues and mitochondria a significant contribution to the overall antioxidant defense seems questionable. In addition, the higher reducing power of hydroquinone-derived α-$TQ^{\bullet -}$ *versus* $UQ^{\bullet -}$ could increase $O_2^{\bullet -}$ formation (see Section 3.1 for details). On the other hand, α-TQ has also been suggested to possess prooxidative properties [75].

Interaction of TQ/Toc with enzymes is based on their structure and redox properties.

While Toc belongs to the group of chromanols, which in biological membranes undergo irreversible oxidation to TQ and other products, TQ can undergo highly reversible reduction to TQH_2 and again re-oxidation to TQ. TQ shares the ability to undergo reversible redox reactions with mitochondrial UQ, and the structures of UQ and TQ exhibit certain similarities. Both quinones belong to the chemical class of *p*-benzoquinones. However, aromatic methyl substitution of TQ in contrast to methoxy substituents in UQ results in slightly different redox properties (see redox potentials above). Furthermore, TQ possesses a hydroxy-substituted phytyl chain, whereas UQ has an isoprenic side chain. Due to the presence of π-electrons in the side chain of UQ, this implies interactions of UQ upon binding to proteins with other π-electron systems, such as aromatic amino acids. Therefore, physiological protein-binding sites for UQ might be in part suitable for the binding of TQ, however, with different affinities. The action of γ-TQ as proapoptotic agent [76] raises the question whether arylation of thiol groups of mitochondrial proteins is involved.

Other interactions could involve no redox activities and rely on the structure of the TQ/Toc head group and the lipophilic phytyl tail only. In this context, it is noteworthy that the chroman structure of Toc exhibits certain similarities to stigmatellin, a potent inhibitor of mitochondrial complex III of the electron transport chain.

5.2 Interference of TQ and Toc with mitochondrial functions

5.2.1 Mitochondrial respiratory chain

Mitochondrial electron transport enzymes, which use UQ as substrate and might also interact with Toc and TQ, include reduced nicotinamide adenine dinucleotide (NADH):UQ oxidoreductase (complex I, EC 1.6.5.3), succinate:UQ oxidoreductase (complex II, EC 1.3.5.1), UQH_2:cyt c^{3+} oxidoreductase (complex III, EC 1.10.2.2), electron-transferring-flavoprotein dehydrogenase (ETF, EC 1.5.5.1) and glycerol-3-phosphate:UQ oxidoreductase (EC 1.1.5.3). The suitability of TQ as substrate and Toc as functional modulator is only partially known for these enzymes.

For this type of enzymes two basic questions arise: (i) can TQ as alternative substrate be reduced to the corresponding antioxidant hydroquinones (Fig. 2B) and (ii) can TQ, TQH_2 and Toc modulate the function of mitochondrial electron transport enzymes (Fig. 2A).

Several publications have shown that mitochondrial electron transport complexes – and especially complex I – can reduce TQ to the corresponding antioxidative hydroquinones (TQH_2) [30]. However, the question how fast this reduction proceeds and whether this reduction interferes with the electron transport to UQ requires kinetic analysis. The analysis is complicated by the fact that native UQ often cannot be used as substrate for these measurements since it forms micelles, and substrate saturation, which is typical for enzyme kinetics, cannot be achieved [77]. Therefore, usually kinetic analysis is performed by using less lipophilic derivatives of UQ, such as decylubiquinone (dUQ). Likewise, the kinetic behavior of natural TQ (with lipophilic side chain) cannot be assessed directly and requires also low-molecular weight analogs, such as α-TQ_0 (5-(3-hydroxy-3-methyl-butyl)-2,3-dimethyl-[1,4]-benzoquinone) [30].

5.2.1.1 TQ as alternative substrates

It has been demonstrated that UQ-binding sites of complexes I and III are rather similar, however, different from binding sites at complex II [78]. Several studies using low-molecular weight analogs (α-TQ_0) have shown that α-TQ is preferably reduced by mitochondrial complex I > complex III, but not by mitochondrial complex II [30] (Fig. 2B). Since in the studies α-TQ analogs were compared with UQ analogs (dUQ, UQ_1) with similar lipophilicity, these results should also be valid for the native lipophilic TQ and UQ. These data suggest that for α-TQ complex I is the

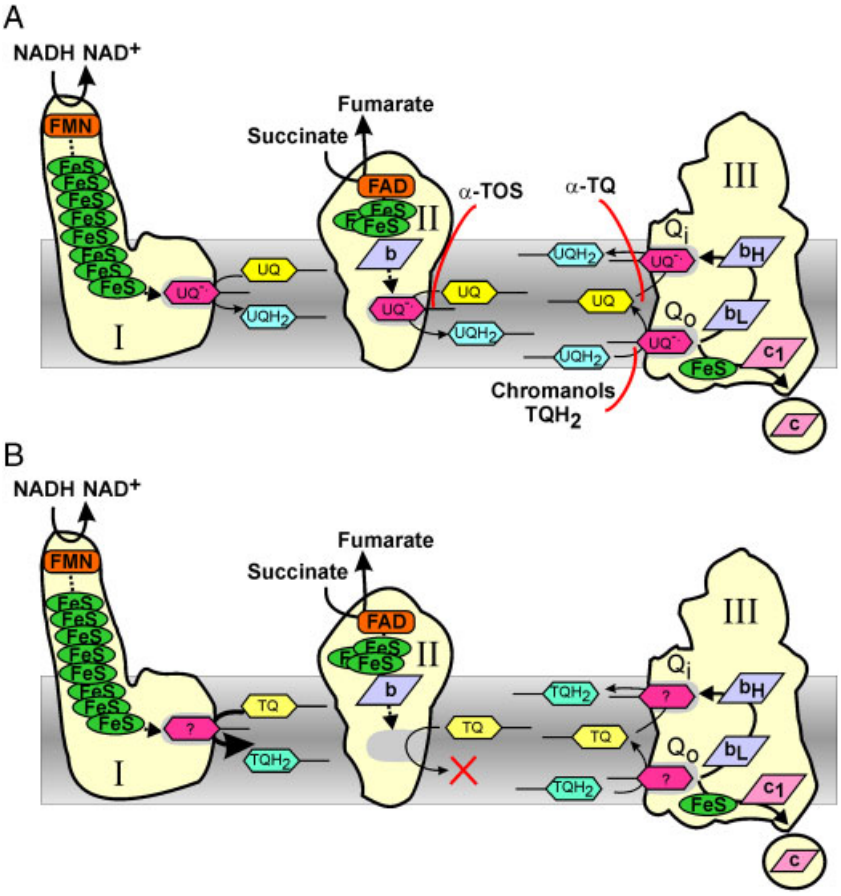

Figure 2. Scheme of the possible interference of TQ and Toc with the activity of UQ as mitochondrial electron carrier (A) and the role of α-TQ as alternative substrate for mitochondrial electron transfer complexes (B). In complex I, the reducing equivalents of NADH are transferred *via* flavin mononucleotide and several iron–sulfur clusters (probably 8–10 FeS) to the lipophilic substrate UQ under physiological conditions [110]. For complex II, electrons from succinate are transported *via* FAD and three FeS to a b-type cyt, which functions as electron donor for UQ [111]. Complex III requires both UQ and UQH_2 as substrate. The excess of UQH_2 (arising from complexes I–III) is oxidized at the Q_o pocket of the complex III donating one electron to the Rieske FeS and the second electron to the cyt b_L. This process called bifurcation is specifically linked to the thermodynamic and steric properties of UQ and the Q_o binding site and represents a major source of superoxide radicals produced by autoxidizing semiquinones. For all UQ-binding sites, the intermediate existence of ubisemiquinones ($UQ^{\bullet -}$) has been confirmed by EPR spectroscopy [112–115]. Whether α-TQ is reduced at complexes I and III directly *via* α-TQ semiquinones or by interaction with $UQ^{\bullet -}$ is not clear so far. However, it has been shown that α-Toc can modulate the superoxide radical formation at the Q_o site [93].

major reductant in mitochondria converting it into its antioxidative active form α-TQH_2. The latter, in turn, is a very poor substrate for mitochondrial complex III (Fig. 2B) and, therefore, it is not rapidly oxidized as long as enough UQH_2 is present in the mitochondrial respiratory chain to drive complex III. In addition, for mitochondrial complex III, a transhydrogenase activity was proposed [79], suggesting the reduction of TQ at the Q_i site of the cyt bc_1 complex to α-TQH_2.

5.2.1.2 TQ and Toc as modulators of mitochondrial electron transfer

On the other hand, the question arises whether Toc, being no substrate for mitochondrial respiratory complexes and TQ, which is a poor alternative substrate of the electron transfer complexes, can inhibit mitochondrial electron transfer in general. Already Yu and Yu [80] reported on the inhibition of succinate:cyt c^{3+} reductase (complex II+III) in the cholate-solubilized complex by α-Toc (more than 90% maximal inhibition) and α-TQ (about 70% maximal inhibition). The authors concluded that α-Toc changed the protein environment around cyt b of the detergent-solubilized complex III rather than displacing UQ from the enzyme. Similarly, Detwiler *et al.* [81] described an influence of vitamin E-related compounds on subfractionated respiratory chain components. Although they observed almost no effect of α-Toc and γ-Toc on NADH:cyt^{3+} and succinate:cyt c^{3+} oxidoreductase activity, they found for 340 μM α-TQ about 30% inhibition for both activities. The proapoptotic α-tocopheryl succinate (α-TOS, 340 μM) caused in these assays 85% inhibition of NADH:cytc^{3+} oxidoreductase activity but was without effect on succinate:cyt c^{3+} oxidoreductase activity. In contrast, Dong *et al.* [82] observed about 50% inhibition by 300 μM α-TOS on succinate:2,6-dichlorophenol-indophenol oxidoreductase activities but no inhibition of the NADH:dichlorophenol-indophenol oxidoreductase. In this context, it is important that reduction of dichlorophenol-indophenol rather assays the succinate dehydrogenase activity than the succinate: UQ oxidoreductase activity (complex II activity).

These conflicting data demonstrate that further information on inhibition of individual respiratory complexes is not only important for nutritional science but also for basic research in signaling and apoptosis fields. The assessment of the potential inhibition of individual complexes requires the use of adequate artificial substrates, such as dUQ and decylubiquinol ($dUQH_2$), similar to activity measurements and can be performed either with individual detergent

solubilized complexes or with inner mitochondrial membrane fragments. These experimental requirements complicate extrapolation of the results to physiological conditions with native substrates in intact mitochondria in cells. The native substrate UQ_{10} has a concentration of around 10 mM in mitochondrial membranes [77, 83]. In any case, the degree of inhibition will be determined by the ratio between substrate (irrespectively whether native or artificial) and inhibitor at the substrate-binding site of the respective membrane-bound enzyme within the membrane, which can be very different from the apparent concentration (calculated from the whole assay volume including aqueous phase).

The isolated cyt bc_1 complex in our experiments (Fig. 3) is solubilized in detergent (Triton X-100) during preparation. Although the amount of the detergent in final test is small, it is sufficient to solubilize the complex and up to a certain extent the added Toc or TQ compounds. This is the reason why the whole assay volume can be considered as one compartment and concentration required for half inhibition (IC_{50}) values measured in this system are supposed to be in the magnitude of membrane concentrations required for inhibition. For isolated complex III, up to millimolar concentrations of Toc and TQ are required for its half inhibition (IC_{50}) and for both a stronger inhibition by the γ- than the α-congeners is observed (Fig. 3). In addition, within one congener the TQ always exhibited a stronger inhibition than the corresponding Toc.

To extend the analysis to other mitochondrial complexes, submitochondrial particles (SMP) were used and α-TOS, which was supposed to mediate apoptosis *via* mitochondrial

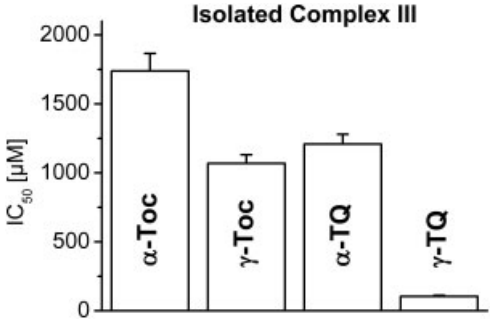

Figure 3. Inhibition of mitochondrial complex III by α-/γ-Toc and α-/γ-TQ. IC_{50} values for the $dUQH_2$:cyt c^{3+} oxidoreductase activity of isolated mitochondrial cyt bc_1 complex. Cyt bc_1 complex was prepared as described in [109]. The inhibition of $dUQH_2$:cyt c^{3+} oxidoreductase activity in isolated cyt bc_1 complex (6 nM) was determined in buffer (pH 7.2, 25°C) containing 250 mM sucrose, 50 mM KH_2PO_4, 0.2 mM EDTA, 2.5 mM KCN, 2 mM NaN_3 and 100 µM cyt c^{3+}. The reduction of cyt c^{3+} was determined photometrically as the difference of the absorptions at 550 nm (cyt c^{2+}) and 540 nm after addition of 75 µM $dUQH_2$ over 3 min using a Shimadzu Multispec 1501 diode array photometer. From the plots of activity *versus* Toc and TQ concentrations, the IC_{50} values were determined according to a four-parameter logistic model (4PL, Hill-Slope model).

complex II inhibition [82], was included as a reference compound. In the assays using SMP, a biphasic system (aqueous phase of buffer/lipid phase of SMP), the effective membrane concentrations of TQ and Toc are very different from the theoretical bulk-phase concentration (considering the admixed amount of TQ and Toc *per* buffer plus SMP volume). The following formula can be used to estimate membrane concentrations of lipophilic compounds ($C_{membrane}$, membrane concentration; C_{total}, bulk-phase concentration; α, relative volume of lipid phase in total assay volume; *P*, partition coefficient).

$$C_{membrane} = \frac{P \times C_{total}}{P \times \alpha + (1 - \alpha)} \quad (6)$$

Using this approximation for an experiment with 1 mg protein/mL of SMP that corresponds to 1 µL lipid phase in an assay volume of 1 mL and an average log *P* for TQ and Toc of 9, an IC_{50} value of 50 µM (= C_{total}) would correspond to membrane concentrations of about 50 mM. Although this example illustrates the difference between apparent and membrane concentrations of TQ and Toc, the value of this formula is limited since the lipid volume and the partition coefficients are very rough approximations. For example, Yoshida and Niki reported a log P_{OW} values for α-Toc of 3.36 [84], whereas all theoretical calculations predict values of 9 and above [85, 86].

Our measurements of complex III activities in SMP (Fig. 4B) in analogy to the isolated cyt bc_1 complex (Fig. 3) have shown that none of the compounds reaches half inhibition at 400 µM although the IC_{50} of γ-TQ in the isolated cyt bc_1 complex is below this concentration. However, the data obtained from SMP for complex III confirm the relative efficiency seen in the isolated complex: γ-congeners are more potent inhibitors than α-congeners and within a congener always the TQ inhibits more than the respective Toc. The strongest inhibition of complex III activity in SMP was obtained by α-TOS, which reaches about 50% at 400 µM. Furthermore, complex I inhibition was assayed by using dUQ as artificial substrate (NADH:dUQ oxidoreductase activity) (Fig. 4A). In general, with 400 µM Toc/TQ a higher inhibition of complex I than of complex III (Fig. 4B) was observed; however, a similar relative sequence of inhibition: γ-Toc > α-Toc, γ-TQ > α-TQ and α-/γ-TQ > α-/γ-Toc was obtained. This is in line with the observation that complexes I and III are inhibited by structurally similar inhibitors [87]. In these assays (Figs. 4A and B), TQ and Toc compete with the artificial substrates dUQ (complex I) and $dUQH_2$ (complex III). To analyze the inhibitory potential under more physiological conditions, UQ/UQH_2 would be required as substrates instead of $dUQ/dUQH_2$. Since UQ/UQH_2 are too lipophilic for kinetic experiments, the only way to circumvent this problem is the analysis of combined activities, *e.g.* complex I+III (Fig. 4C) and complex II+III (Fig. 4D) since the final acceptor in cyanide-blocked SMP is cyt c^{3+}, which is soluble in the aqueous phase. Under these conditions, TQ and Toc compete with native UQ_{10} for

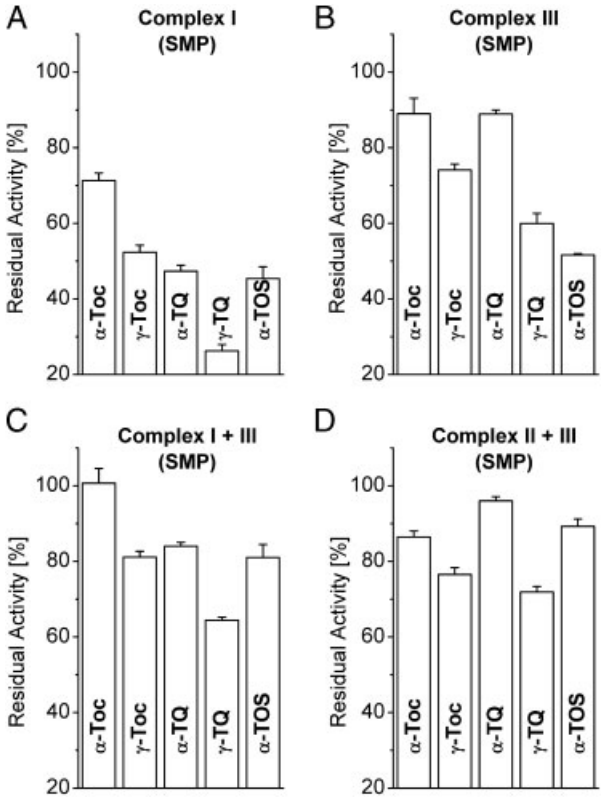

Figure 4. Inhibition of mitochondrial electron transfer complexes by 400 μM α-/γ-Toc, α-/γ-TQ and α-TOS in SMP. Residual activity expressed as % of control for (A) the NADH: dUQ oxidoreductase activity (complex I), (B) $dUQH_2$:cyt c^{3+} oxidoreductase activity (complex III), (C) NADH:cyt c^{3+} oxidoreductase activity (complex I+III) and (D) succinate:cyt c^{3+} oxidoreductase activity (complex II+III). The preparation of SMP was performed as described in [109]. All activities for SMP were measured in buffer containing 250 mM sucrose, 1 mM EDTA, 20 mM triethanolamine, 2.5 mM KCN and 2 mM NaN_3 (pH 7.4, 25°C). The α-/γ-TQ, α-/γ-Toc and α-TOS compounds were dissolved in ACN/ethanol 1:1 v/v and the control experiments (100%) contained the corresponding amounts of vehicle only. The $dUQH_2$:cyt c^{3+} oxidoreductase activity for SMP (0.008 mg/mL) was measured in analogy to Fig. 3. For the NADH:dUQ oxidoreductase activity, the oxidation of 100 μM NADH by SMP (0.183 mg/mL) was measured photometrically as the difference of the absorptions at 340 and 400 nm after addition of 75 μM dUQ over 3 min using a Shimadzu Multispec 1501 diode array photometer. The NADH:cyt c^{3+} oxidoreductase activity of SMP (0.057 mg/mL) was determined in analogy to the NADH:dUQ oxidoreductase activity (Fig. 4A) with the exception that dUQ was replaced by 100 μM cyt c^{3+} as acceptor. The succinate:cyt c^{3+} oxidoreductase activity was measured using 0.286 mg/mL SMP in analogy to the $dUQH_2$:cyt c^{3+} oxidoreductase activity with the exception that the donor $dUQH_2$ was replaced by 4 mM succinate. Data are mean values ± SE, $n = 3$.

binding to proteins. For this reason, the inhibition seen at 400 μM is in most cases smaller than the corresponding inhibition with respect to artificial substrates (Fig. 4C *versus* Figs. 4A and B). However, again the relative potency of the TQ and Toc compounds is identical, showing the strongest inhibition by γ-TQ. However, in this assay α-TOS was not as inhibitory as γ-TQ. For complex II+III activities, α-TQ was less inhibitory than α-Toc, however, again γ-TQ was the most effective compound, while α-TOS was less efficient for inhibition. In this context, it has to be considered that an increased superoxide radical production triggered by these compounds (as proposed for α-TOS) would mask a stronger inhibition if cyt c^{3+} is used as acceptor, which is easily reduced by superoxide radicals.

Although these data suggest rather high-effective IC_{50} values of γ-TQ in membranes, it cannot be excluded that complex I and complex III inhibition is involved in the disruption of the membrane potential by γ-TQ in cell cultures mediating apoptosis *via* mitochondria [76]. In contrast, results for α-TOS are less equivocal and at least in our assays a strong complex II inhibition was not seen.

5.2.2 Mitochondrial ROS formation

An even more complicated subject is the oxygen radical formation by the respiratory chain, since due to severe methodical differences no real consensus about the amount of superoxide radicals or H_2O_2 released from mitochondria exists in the literature. Values range from 0.003 nmol $O_2^{\bullet-}$/min/mg protein [88] to 6 nmol H_2O_2/min/mg protein [89] depending on the origin of mitochondria and the analytical method applied. However, about the origin of $O_2^{\bullet-}$ is the consensus that mitochondrial complex I and mitochondrial complex III are the major sites of superoxide production in mitochondria. While for complex I flavin adenine dinucleotide (FAD) and/or iron-sulfur clusters are held responsible for radical production [90], at mitochondrial complex III autoxidizing ubisemiquinones at the Q_o pocket are favored [91, 92] as electron donors to oxygen.

Therefore, if Toc, TQ or TQH_2 interfere in mitochondrial superoxide radical production, this action most likely occurs at the lipophilic binding sites of UQ and not at the rather hydrophilic environment of FAD/iron-sulfur clusters of complex I. Recently, Cuddihy *et al.* [93] demonstrated in brain mitochondria from vitamin E-deficient and tocopherol transfer protein (Ttp) −/− rats that addition of ethanolic solutions of α-Toc and α-TQ restores the ability of complex III to release superoxide radicals. Although the highly specific spin trapping technique using 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline-*N*-oxide (DEPMPO) was applied, not the native DEPMPO/$^{\bullet}$OOH spin adduct but only the DEPMPO/$^{\bullet}$OH adduct, which can arise from DEPMPO/$^{\bullet}$OOH and other precursors, was observed. The authors observed an increase of the DEPMPO/$^{\bullet}$OH up to 2–5 nmol α-Toc/mg protein and then again a decrease at higher α-Toc concentrations. Moreover, a similar action of α-TQ was suggested by the authors. In this context, it is noteworthy that it has been shown that ethanol, which was

used as vehicle in this study, stimulates ROS formation at mitochondrial complexes I and III [94].

In a previous study, TQ was added to freeze-dried SMP, which were subsequently rehydrated, and radical formation was detected by superoxide dismutase-sensitive reduction of cyt *c* [43]. In this experimental setup, enrichment of α-TQ (not α-TQH_2) in SMP decreases superoxide radical formation. However, in both studies the enrichment of Toc and TQ in membranes using ethanol as solvent [93], which is known to seriously damage mitochondrial function, and also freeze drying [43], which can change the structure of proteins and membranes are rather crude methods and may not reflect physiological conditions. Another approach considers the changes of α-TQ levels in relation to α-Toc in liver mitochondrial fractions under physiological and pathophysiological conditions. In rats that were treated with LPS, significant changes of some vitamin E-related compounds in liver mitochondrial fractions were observed. Endotoxic shock, or Gram-negative septic shock, can occur as a component of Gram-negative sepsis and is characterized by hypotension, poor tissue perfusion and multiple organ dysfunction [95]. A common model of shock is the infusion of LPS. Lung, liver, intestine, kidney and gut are the organs most susceptible to sepsis. It has been shown that endotoxin can modulate mitochondrial function but mechanisms underlying this effect are not understood yet [96, 97]. As shown in Fig. 5C, α-Toc levels in rat liver mitochondrial fractions were not changed in LPS-treated animals in comparison with control rats, which received saline only. However, for α-TQ (Fig. 5D) and the ratio α-TQ/α-Toc (Fig. 5B) a significant decrease was observed. On the other hand, mitochondria from LPS-treated rats were shown to generate more superoxide radicals than respective liver mitochondria of control animals by oxidation of the hydroxylamine CPH (1-hydroxy-2,2,5,5-tetramethyl-pyrrolidine-3-carboxylic acid) to the paramagnetic $CP^{\bullet}$ (2,2,5,5-tetramethyl-pyrrolidine-3-carboxylic acid-1-oxyl) and its subsequent detection by electron spin resonance spectroscopy (Fig. 5A). Since no change of mitochondrial cyt concentrations was observed [98] and the concentrations of Toc and UQ (Figs. 5C and E) were unchanged as well, the involvement of α-TQ or α-TQH_2 in the modulation of ROS could be possible. In addition, these data show that upon inflammatory response induced by LPS the concentration of α-TQ is independently regulated of the concentration of α-Toc. Although an increase of the α-TQ/α-Toc ratio can be easily argued by oxidative stress, a decrease suggests an independently stimulated transport (α-TQ: supernatant protein factor/TAP) or metabolism in such situations. In contrast, in liver of rats subjected to hemorrhagic shock and reperfusion, a condition supposed to be associated with increased oxidative stress, neither a decrease nor an increase of α-TQ in relation to α-Toc was observed [53]. This suggests that mechanism(s) regulating α-TQ and α-Toc levels *in vivo* are more complex than only responding to elevated oxidative stress.

5.2.3 Other mitochondrial functions

Since Toc and TQ are highly lipophilic molecules and occur preferably in membranes [99], the function of virtually every membrane-bound protein could be modulated by their presence. Most interesting is the poorly understood mechanism how certain Toc and TQ derivatives (such as γ-TQ [76], α-TOS [82]) induce apoptotic events *via* mitochondria. A key step in apoptosis is the oligomerization of proapoptotic members of the B-cell lymphoma (Bcl)-2 family, such as Bax or Bak, leading to an increased permeability of the outer mitochondrial membrane and the subsequent release of small portions of cyt *c* from the intramembrane space of mitochondria [100, 101]. Activation of apoptosis pathways liberates proapoptotic Bcl-2 members, which are normally engaged and inactivated by antiapoptotic

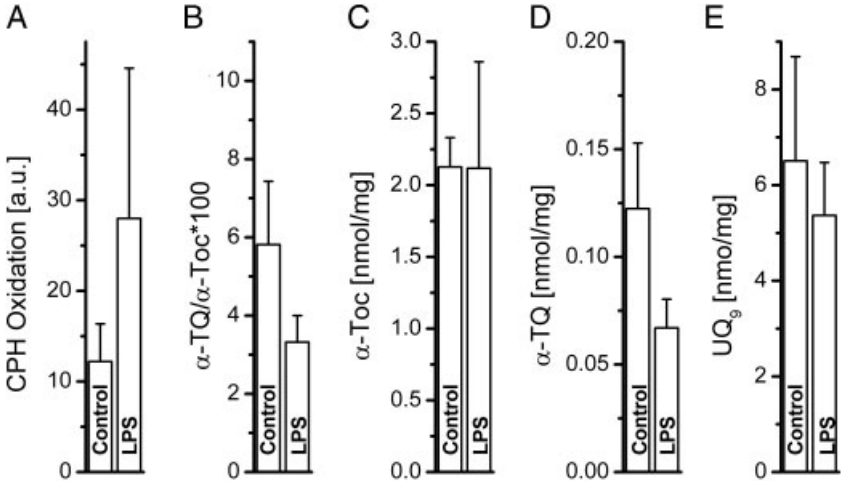

Figure 5. Changes of α-TQ levels and related parameters of liver mitochondria under pathophysiological conditions of LPS-induced septic shock in rats. (A) CPH-oxidation by glutamate/malate respiring rat liver mitochondria, (B) concentration ratio of α-TQ/α-Toc, concentrations of (C) α-Toc, (D) α-TQ and (E) ubiquinone-9 (UQ_9) in rat liver mitochondria. Treatment of rats with LPS, isolation of mitochondrial fractions and measurement of CPH oxidation were described in [116]. The content of α-TQ, α-Toc and UQ_9 was determined as described in Table 1. Data are mean values ± SD, $n = 4$.

members of the Bcl-2-family, such as Bcl-2 or Bcl-XL. Activated Bax translocates from cytosol to mitochondria and inserts with the *C*-terminus into the outer membrane forming channel-like oligomers [100]. Already a tetramer of Bax is sufficient to allow the release of cyt *c* through the outer membrane [101]. It has been shown that this process can occur independently of the permeability transition pore. Although α-TQ is rather well tolerated in cultured cells, γ-TQ is highly cytotoxic [28] and triggers apoptosis in concentrations of 20–50 μM [76, 102]. Mechanistic studies have shown that the toxicity of TQ, which is not fully substituted at the quinone ring [20], such as γ- and δ-TQ, is caused by their ability to arylate other biomolecules forming Michael adducts [28]. For example, the reaction of these quinones with glutathione was related to the development of ER stress and the development of an unfolded protein response in liver [103]. In mitochondria it was described that γ-TQ induces a drop of the membrane potential and release of mitochondrial *cyt* c with subsequent activation of caspase 9 [76]. Although the involvement of the mitochondrial permeability transition pore was suggested, the precise mechanism how γ-TQ causes these events in mitochondria is still unclear. Since the mitochondrial permeability transition pore is influenced by various quinones [104], a modulation by γ-TQ cannot be excluded. In this context, the inhibition of the mitochondrial electron transfer by Toc and TQ would be a plausible explanation for the observed drop of the mitochondrial membrane potential.

Another link between the apoptosis and the antioxidative activity of TQ and Toc is so far controversial. On the one hand, it is argued that mitochondrial ROS-induced cardiolipin oxidation, which decreases the close association of cardiolipin with cyt *c*, causes a higher mobility of cyt *c* facilitating its release from mitochondria [105]. In addition, it was shown that heterologous expression of Bax in yeast was associated with an increased amount of oxidized lipids [106]. This should be prevented by Toc and TQH_2 and fits to a general antioxidative plus physical stabilization of membranes by vitamin E derivatives [107]. On the other hand, it was observed that in human prostate cancer cells specifically γ-Toc-induced cyt *c* release and apoptosis by interrupting sphingolipid synthesis but no translocation of Bax to mitochondria was observed [108]. Furthermore, Toc derivatives such as α-TOS are believed to increase mitochondrial ROS formation thereby triggering apoptosis [82].

6 Concluding remarks

The mechanism by which vitamin E-related compounds influence mitochondrial bioenergetics and radical formation is, by far, not clear and requires further mechanistic studies. However, data obtained so far demonstrated that not only Toc but also their metabolites would interfere with metabolic and signaling processes mediated by mitochondria.

The authors have declared no conflict of interest.

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