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RELATIONS BETWEEN TOCOPHEROL DEPLETION AND COENZYME Q DURING LIPID PEROXIDATION IN RAT LIVER MITOCHONDRIA

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In order to evaluate different mitochondrial antioxidant systems, the depletion of alpha-tocopherol and the levels of the reduced and oxidized forms of CoQ were measured in rat liver mitochondria during Fe^{++}/ascorbate and NADPH/ADP/Fe^{++} induced lipid peroxidation. During the induction phase of malondialdehyde formation, alpha-tocopherol declined moderately to about 80% of initial contents, whereas the total CoQ pool remained nearly unchanged, but reduced CoQ9 continuously declined. At the start of massive malondialdehyde formation, CoQ9 reaches its fully oxidized state. At the same time alpha-tocopherol starts to decline steeply, but never becomes fully exhausted in both experimental systems. Evidently the oxidation of the CoQ9 pool constitutes a prerequisite for the onset of massive lipid peroxidation in mitochondria and for the subsequent depletion of alpha-tocopherol. Trapping of the GSH by addition of dinitrochlorbenzene (a substrate of the GSH transferase), results in a moderate acceleration of lipid peroxidation, but alpha-tocopherol and ubiquinol levels remained unchanged when compared with the controls. Addition of succinate to GSH depleted mitochondria effectively suppressed MDA formation as well as alpha-tocopherol and ubiquinol depletion. The data support the assumption that the protective effect of respiratory substrates against lipid peroxidation in the absence of mitochondrial GSH is mediated by the regeneration of the lipid soluble antioxidants CoQ and alpha-tocopherol.

KEY WORDS: Lipid Peroxidation, alpha-Tocopherol, Ubiquinone 9, antioxidative Systems, (rat liver mitochondria).

ABBREVIATIONS: MDA, malondialdehyde; CoQ, coenzyme Q; CoQ9, coenzyme Q9; $Q9H_2$, coenzyme Q9 reduced; Q9, coenzyme Q9 oxidized; GSH, reduced glutathione; DNCB, 2,2-dinitrochlorbenzene; DTNB, dithionitrobisbenzene.

INTRODUCTION

Mitochondria generate reactive oxygen species (superoxide, hydrogenperoxide) as side products of the electron transport in the respiratory chain (complex I and III).[1-3] Therefore they are prone to a functional impairment by oxidative stress, especially in the presence of metal ions. Because of the role of mitochondria in the cellular energy metabolism, impairments of mitochondrial functions are thought to be an important mechanism underlying the pathogenesis in conditions, such as ischemia,[4,5] hypoxia[6] or the drug induced damage of the heart muscle.[7] On the other hand mitochondria are strongly protected by water soluble (GSH-system)[8,9] and lipid soluble (alpha-tocopherol, QH_2) antioxidants.[10-14] The capacity of these systems depends on the regeneration of its oxidized species by reducing equivalents,

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which is dependent on the energetical state of the mitochondria. The GSH system depends on the delivery of reducing equivalents (NADPH) to enzymically reduce the GSSG,[15,16] whereas alpha-tocopherol can be regenerated by reduced constituents of the respiratory chain.[17-19] In a model system of isolated rat liver mitochondria which peroxidize in the presence of Fe^{++} and ascorbate, we have shown a progressive inhibition of the capacity of the respiratory chain during the lag phase of lipid peroxidation. This results in a collapse of the inner membrane potential, which occurs before the onset of massive lipid peroxidation.[20,21] This deenergization was found to be the reason for the rapid decline of the NADPH content and the concomitant GSH consumption which occurred around the start of massive lipid peroxidation.[20,9] In this context it is important to study the lipid soluble antioxidants alpha-tocopherol and CoQ9 to elucidate the effectiveness of lipid soluble antioxidants in the defence of mitochondria against oxidative stress in the presence and absence of an intact GSH system.

We report here a considerable protection of mitochondria against lipid peroxidation by the lipid soluble antioxidants as long as respiratory substrates are present. Our data strongly suggest that a nearly fully oxidized state of CoQ, an indicator for the exhaustion of endogenous substrates, predisposes to the onset of massive lipid peroxidation.

MATERIAL AND METHODS

Chemicals and Biochemicals

Chemicals were from Aldrich (Steinheim, F.R.G.) and biochemicals from Boehringer (Mannheim, F.R.G.) and Sigma Chemical Co. (Munich, F.R.G.)

Preparation of Rat Liver Mitochondria

Rat liver mitochondria from 12 h starved female Wistar strain rats were prepared essentially as described in[22]. Mitochondrial protein was determined by a modified biuret method using bovine serum albumin as standard.[23]

Incubation of Mitochondria and Initiation of Lipid Peroxidation

Mitochondria were incubated at 25°C at protein concentration of about 5 mg protein/ml in a medium of 0.1 M KCl and 10 mM TRIS/HCl adjusted to pH 7.4. To initiate lipid peroxidation either 0.5 mM ascorbate and 40 μM $FeSO_4$ were added or a NADPH generating system containing 6 mM glucose-6-phosphate, 0.4 U/ml glucose-6-phosphate dehydrogenase and 0.3 mM NADPH together with 3 mM ADP and 20 μM $FeSO_4$ was used.[20,24]

Assay of Lipid Peroxidation

The degree of lipid peroxidation was determined by the formation of thiobarbituric acid reactive substances (malonic dialdehyde test) according to.[25]

Extraction Procedure and HPLC Analysis of Alpha-tocopherol and Coenzyme Q

The extraction procedure of Burton[26] and the separation method of Ikenoya,[27] as described in[28] were used. Briefly, 1.0 ml mitochondrial suspension was added to 1.0 ml mitochondrial suspension was added to 1.0 ml 40 mM sodiumdodecylsulfate, adjusted to pH 4 by HCl, containing 2 mM desferrioxamine and 50 μl of a solution of 10 mg butylated hydroxyanisol/ml ethanol. The sample was rapidly shaken for 15 seconds and 2.0 ml reagent alcohol (ethanol/isopropylalcohol 95/5 v/v) was added. After 1 min of rigorous shaking 2.0 ml n-heptane were added and once more the sample was rigorously shaken for 1 min. The sample vial was centrifuged for 15 min at 4000 rpm in a T 23 centrifuge (Janetzki, Engelsdorf (Leipzig)) to achieve phase separation. 1.5 ml of the upper heptane phase was withdrawn and taken to dryness in a rotary evaporator, redissolved in 400 μl eluent and injected into the HPLC-apparatus. We tried to keep the sample in the dark during the extraction procedure. The HPLC-apparatus consisted of a linear pump HP 5001 (Laboratorny pristroje CSFR), a 20 μl Knauer injection valve, a 15 × 0.4 cm 5 μm ODS-cartridge column (Laboratorny Pristroje CSFR), an electrochemical detector set at + 0,8 V (Laboratorny Pristroje CSFR) and an UV-detector set at 289 nm (Laboratorny Pristroje CSFR). The eluent consisted of reagent alcohol/methanol/H_2O 700/300/50 v/v/v containing 50 mM $NaClO_4$ adjusted with $HClO_4$ to pH 4. Analysis of the chromatograms was performed on a microcomputer KC 85/3 (Mikroelektronik Mühlhausen).

RESULTS

The initial contents of alpha-tocopherol and the sum of ubiquinol and ubiquinone CoQ9 in the mitochondrial preparations of the female rats were 0.204 ± 0.03 (S.D.) nmol/mg protein and 1.49 ± 0.286 (S.D.) nmol/mg protein, respectively (n = 17). By extracting known amounts of reduced CoQ9 in the presence of mitochondrial protein, the recovery of $Q9H_2$ was determined to be about 85–90% of the initial content. Trapping of the iron ions during the extraction procedure by desferrioxamine was essential to avoid oxidation especially of the $Q9H_2$, which is more sensitive against the presence of iron ions than alpha-tocopherol. These extracts were stable for more then 5 hours. The initial degrees of reduction of CoQ9 with about 50–65% were slightly lower then those found by other authors[29].

Figure 1 depicts the lipid peroxidation and the alpha-tocopherol-, $Q9H_2$- and CoQ9-contents of isolated rat liver mitochondria which peroxidize in the presence of Fe^{++}/ascorbate.

The lipid peroxidation could be divided into two phases: a lag phase with slow malondialdehyde formation and a phase of exponential malondialdehyde formation. During the lag phase of malondialdehyde formation, alpha-tocopherol was drastically consumed by the lipid peroxidation processes (Figure 1). In the control incubations without Fe^{++} and ascorbate the alpha-tocopherol levels remained unchanged over the whole incubation time. In all incubations performed (n = 7), the extent to which alpha-tocopherol was depleted during the lag phase of malondialdehyde production was determined to about 20–25% of the initial content. The consumption of alpha-tocopherol during the exponential phase of malondialdehyde formation did, however, not result in its quantitative depletion. As indicated in Figure 1, in all incubations during the plateau-phase of malondialdehyde formation

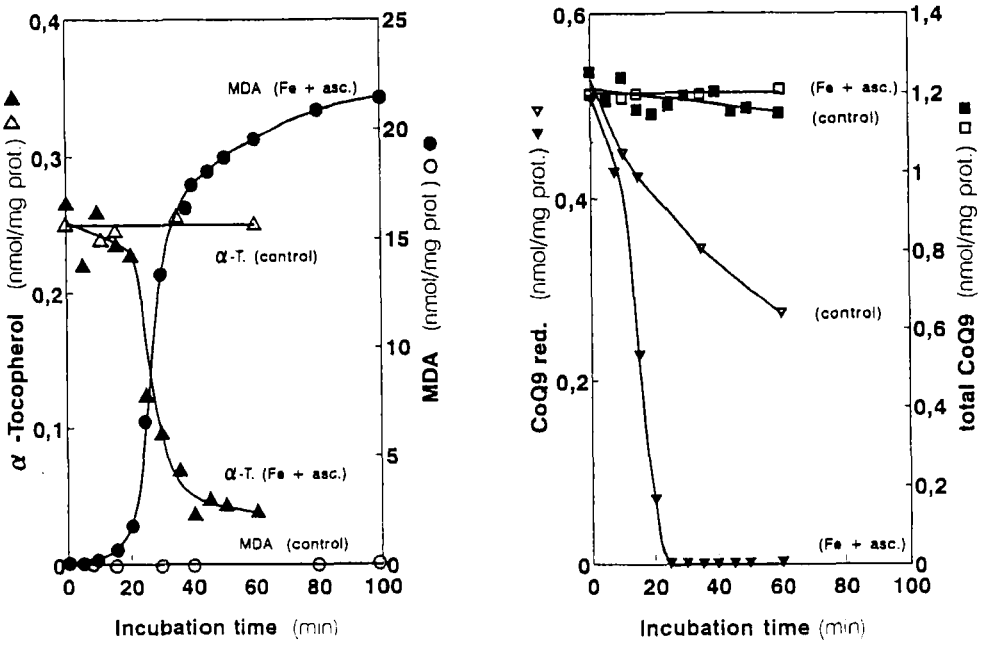

FIGURE 1 Time course of Fe^{++} and ascorbate-induced peroxidation in rat liver mitochondria. After the addition of Fe^{++} (40 μM) and ascorbate (0.5 mM) the changes in alpha-tocopherol malondialdehyde and CoQ9 were monitored at indicated times. Incubation conditions and determination of the compounds were detailed in materials and methods.

a portion of about 10–50% of the initial content of alpha-tocopherol appeared to be preserved.

A possible reason for this remaining part of alpha-tocopherol during the plateau phase of malondialdehyde formation could be the presence of ascorbate, which reduces the oxidized form of alpha-tocopherol in the Fe^{++}/ascorbate system. In order to exclude the ascorbate, the alpha-tocopherol- and CoQ9 contents were determined in an initiation system of NADPH/ADP/Fe^{++} (Figure 2). A significant portion of the alpha-tocopherol was also preserved in the NADPH/ADP/Fe^{++}-system during the plateau phase of lipid peroxidation. Therefore the remaining portion of alpha-tocopherol during the plateau phase of lipid peroxidation depends not on the presence of ascorbate.

In contrast to alpha-tocopherol, CoQ9 was not consumed during peroxidation. In the peroxidizing mitochondria as well as in the control incubations the sum of Q9 + $Q9H_2$ remained constant (Figure 1,2). The oxidative stress led, however, to a strong oxidation of the CoQ pool during the lag phase of peroxidation. In the Fe/ascorbate as well in the NADPH/ADP/Fe^{++} system a strong oxidation of the ubiquinol to the ubiquinone took place in comparison to the much slower oxidation rate in the control incubations. This may be explained by different load conditions in peroxidizing and control mitochondria.

Since the reduction of CoQ depends on the delivery of hydrogen for the respiratory chain, the oxidation of the CoQ pool obviously represents a progressive exhaustion of the endogenously present substrates in the mitochondria.

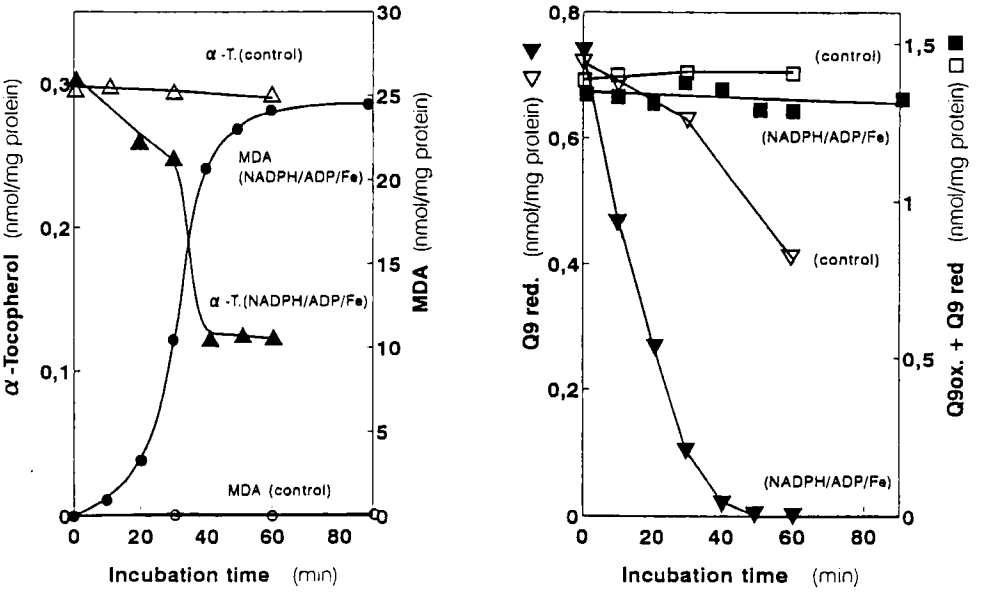

FIGURE 2 Time course of NADPH/ADP/Fe-induced mitochondrial peroxidation. The changes in alpha-tocopherol, malondialdehyde and CoQ9 were monitored at indicated times. Incubation conditions and determinations of the compounds as outlined in materials and methods.

In order to test the interrelationship between the loss of endogenous substrates and the oxidation of the CoQ under our conditions, the mitochondria were incubated in the presence of ADP (3 mM), glucose (2 mM) and hexokinase (0,4 u/ml) to deplete their endogenous substrates. In the presence of this ATP-trapping system, the CoQ in the mitochondria was oxidized from the initial $Q9/Q9H_2$ of 1.2 within 40 min to 3.2, whereas in the control incubations a constant degree of reduction of CoQ during this time period was found (initial $Q9/Q9H_2$ = 1,2; $Q9/Q9H_2$ after 40 min: 1.5).

Thus, the rapid oxidation of CoQ in the peroxidizing mitochondria indicates that the oxidative load is accompanied by a rapid consumption of the endogenous substrates.

Two approaches for analyzing the relationship between the consumption of alpha-tocopherol, the oxidation of the CoQ9 pool and the formation of malondialdehyde were applied: i, analysis of the contents of $Q9H_2$ and of alpha-tocopherol during the incubation; ii, determination of the relations between the time points of the start of massive MDA formation, massive alpha-tocopherol consumption and full oxidation of the CoQ9 pool.

The plot of the contents of $Q9H_2$ against that of alpha-tocopherol demonstrated that the initial alpha-tocopherol content was nearly unaffected as long as about 10–20% of the initial content of $Q9H_2$ was present (Figure 3). The phase of nearly unchanged alpha-tocopherol contents corresponds to the lag phase of peroxidation, when alpha-tocopherol was consumed to about 20–25%. This relation was found for the Fe^{++}/ascorbate and for the NADPH/ADP/Fe^{++}-system as well. Therefore the oxidation of the CoQ9 pool seems to represent a precondition for a massive

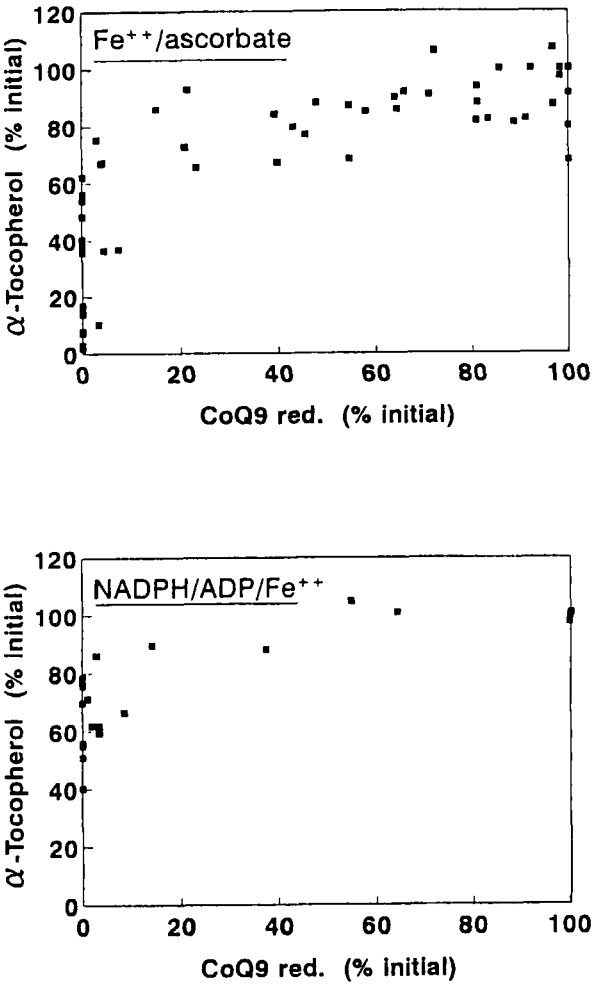

FIGURE 3 Correlation of the content of ubiquinol 9 and alpha-tocopherol in rat liver mitochondria during Fe/ascorbate and NADPH/ADP/Fe induced peroxidation. (Fe/ascorbate: n = 7; NADPH/ADP/Fe: n = 3). Values are expressed as the percentage of the intial content.

consumption of alpha-tocopherol, which does obviously not depend on the presence of exogenously added ascorbate in the system. Further support for the assumed causal relations between CoQ oxidation, alpha-tocopherol consumption and start of massive lipid peroxidation was obtained from the characteristic time points of these processes (Figure 4). These characteristic time points were graphically determined by drawing the tangents for the constant and declining phases of these compounds and taking the times of the crosspoints of these lines as the starting time of consumption resp. formation of these compounds. Since there is a strong coincidence between the times when massive MDA formation and alpha-tocopherol consumption starts and CoQ9 becomes fully oxidized, a causal chain between these events is strongly suggested. A longer preservation of a reduced state of CoQ9 is

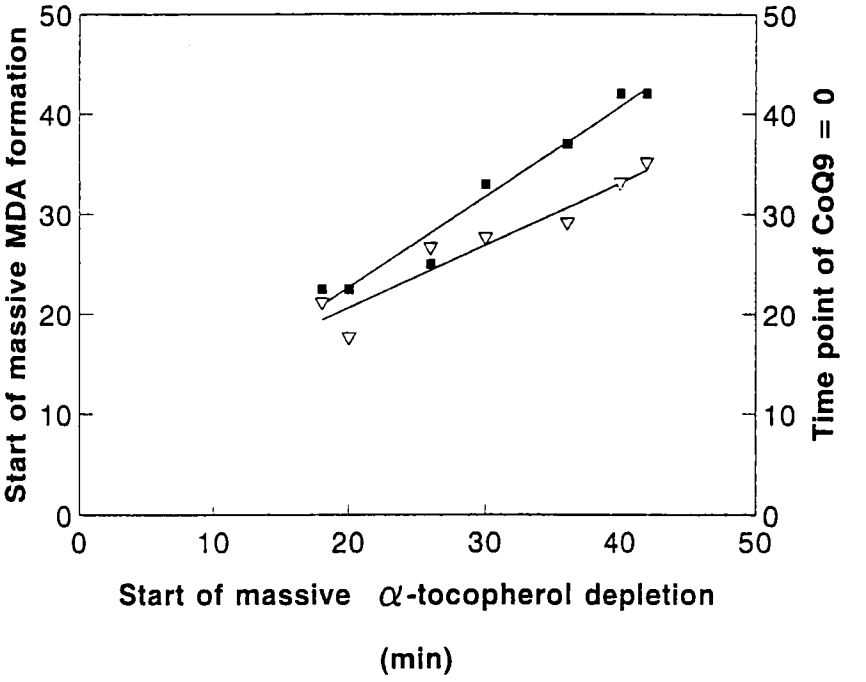

FIGURE 4 Relations between the time points of the start of rapid alpha-tocopherol consumption and the time point of the start of massive MDA formation (filled symbols) resp. complete oxidation of CoQ9 (open symbols). Each point represent one incubation experiment. Determination of the times is outlined in the text.

always connected with a longer duration of the lag phase of malondialdehyde formation and a parallel enlargement of the phase of nearly constant alpha-tocopherol contents.

To determine the capacity of the lipid soluble antioxidants without the possibility of interactions with the water soluble GSH system, incubations were done with GSH depleted mitochondria. To trap the mitochondrial GSH, we used dinitrochlorbenzene - a substrate of the GSH-transferase.[30] The addition of 50–75 μM dinitrochlorbenzene to a mitochondrial suspension of about 5 mg/ml protein resulted in a practically quantitative consumption of the mitochondrial GSH within 5 min. The initial value of 7.2 nmol/mg protein (±0.1 S.D.) declined to 0.17 nmol/mg protein (±0.1 S.D.) during this time period (n = 4). Because of the unspecific method of detection - dithionitrobisbenzene discovers generally SH-compounds, the small remaining part does apparently not constitute GSH, but likely may constitute Co A and other compounds with free SH groups. The depletion of the mitochondrial GSH resulted in an almost moderate acceleration of lipid peroxidation and alpha-tocopherol depletion. At the time point of half maximal MDA formation in the control incubation, the MDA levels in the GSH depleted mitochondria did rise to about 120% and the alpha-tocopherol content declined only to about 95% (n = 4).

This indicated a considerable protection of the mitochondria by the lipid soluble antioxidants alone. Since the decline of alpha-tocopherol during the incubation in

the GSH depleted mitochondria did practically not differ from that of the control mitochondria within the range of error any possible regeneration of the alpha-tocopherol seems not to depend on GSH under our conditions (Figure 5). This supports the view that alpha-tocopherol is regenerated by constituents of the respiratory chain in dependence on the supply of respiratory substrates. In order to test the interrelationship between improved hydrogen supply and improved protection against lipid peroxidation by the lipid soluble antioxidants incubations were done with GSH depleted mitochondria in the presence of succinate. As depicted in Figure 5, the addition of succinate resulted in a considerable suppression of malondialdehyde formation accompanied by a longer preserved ubiquinol and alpha-tocopherol content.

Therefore the data suggest an effective protection of mitochondria against oxidative stress in the absence of GSH as long as they are effectively supplied with respiratory substrates, mediating an effective recycling of lipid soluble antioxidants.

DISCUSSION

The aim of the present study was to determine the consumption of lipid soluble antioxidants in mitochondria during peroxidation. Both compounds, alpha-tocopherol

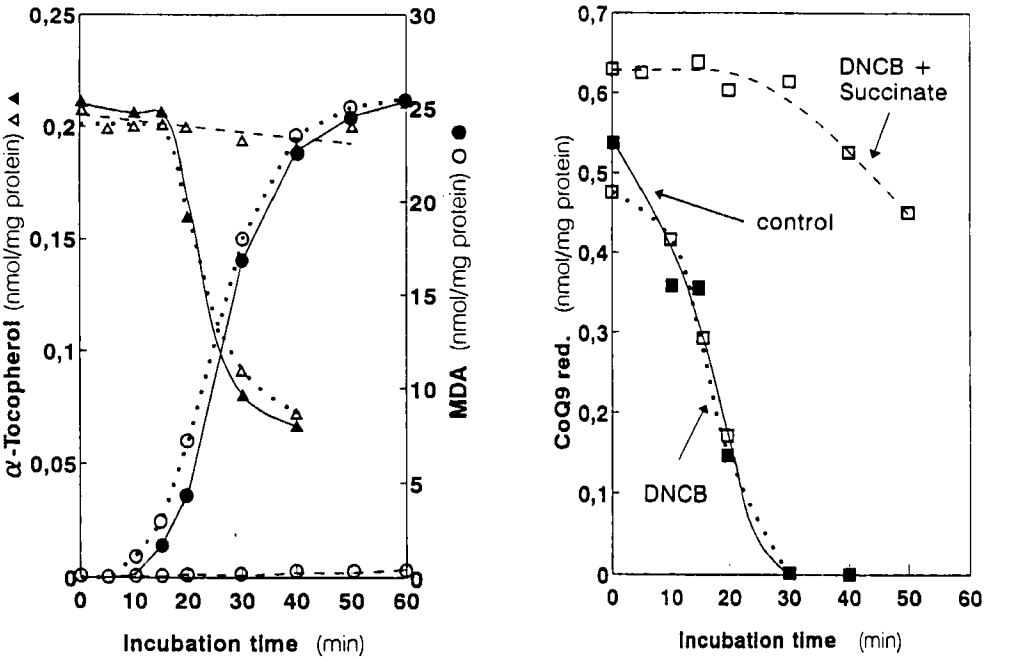

FIGURE 5 Effect of GSH-depletion and the addition of succinate on the contents of alpha-tocopherol, MDA and ubiquinol during Fe/ascorbate-induced peroxidation. Freshly prepared motochondria were suspended in the incubation medium and divided into three vessels. In two vessels the mitochondrial GSH was depleted by the addition of 0.07 mM DNCB. After addition of 100 mM succinate to one vessel of the GSH-depleted mitonchondria, lipid peroxidation was started in all vessels by the addition of Fe^{++} and ascorbate as outlined in material and methods. Solid line: mitochondria without DNCB and succinate, dotted line: mitochondria treated with 0.075 mM DNCB, dashed line: mitochondria treated with 0.075 mM DNCB and supplemented with 10 mM succinate.

and ubiquinols were demonstrated by different authors to be strong antioxidants.[12,13,31-34] In mitochondria the reduction of CoQ is connected to the respiratory chain, but the oxidized form of alpha-tocopherol could be recycled by constituents of the respiratory chain, too.[17-19] Therefore the protective effect of both should depend on the intactness of the respiratory chain, the availability of substrates resp. the energetical state of the mitochondria. The present results demonstrated that under our conditions the consumption of alpha-tocopherol is divided into two phases: i, A slow consumption down to about 75–80% of the initial content. This phase resembles almost exactly the lag phase of malondialdehyde formation. ii, A drastic, but non quantitative consumption. This phase resembles the exponential and plateau phase of malondialdehyde formation. This biphasic behavior was in general not influenced by the depletion of the mitochondrial GSH.

Thus, a complete depletion of alpha-tocopherol in the mitochondrial membranes is not a prerequisite for the start of massive lipid peroxidation. Similar reports were given for submitochondrial particles during irradiation induced lipid peroxidation and for liver mitochondria, peroxidizing with Fe/ascorbate.[11,36]

Furthermore, the recycling of oxidized alpha-tocopherol by ascorbate[35,42] in the Fe^{++}/ascorbate system seems to be of minor importance for the kinetics of alpha-tocopherol depletion under our conditions. Minor amounts of endogenously present ascorbate inside the mitochondria could not be excluded, which participate in the establishment of the lag phase of peroxidation, but the same tight link between oxidation of CoQ9 and alpha tocopherol depletion in the Fe/Ascorbate as well as in the NADPH/ADP/Fe-system indicates the importance of the presence of $Q9H_2$ in the protection of mitochondria against lipid peroxidation. In line with that the protective effects of QH_2 also in the absence of alpha-tocopherol in submitochondrial particles were reported.[37]

Further differences between the NADPH/ADP/Fe-system and the Fe/Ascorbate-system are obviously the additional energetical load introduced by the added ADP in the former system and the dependence on different enzyme activities for the generation of oxygen radicals e.g. Cyt.P450 in NADPH/ADP/Fe-induced lipid peroxidation.[38,39] Since also in the presence of ADP mitochondria are protected against massive lipid peroxidation as long as ubiquinols are present, this suggests direct or indirect antioxidative effects of ubiquinols by recycling of oxidized tocopherol.

As shown above (Figure 1,2) in both systems a portion of about 10–50% of the alpha-tocopherol remained even at a maximal degree of lipid peroxidation. Whether this remaining part is caused by a breakdown of the radical delivering enzymes and a stop of the initiation reactions or is the result of a localized peroxidation is difficult to decide. Destructions of the radical forming enzymes were shown[21] and investigations of the fatty acid pattern during Fe/ascorbate induced peroxidation in mitochondria revealed no quantitative loss of highly unsaturated fatty acids as the reason for the stop of lipid peroxidation during the plateau phase of malondialdehyde formation.[40]

The inverse relation between the kinetics of alpha-tocopherol depletion and lipid peroxidation with maximal rate of peroxidation in the presence of alpha-tocopherol and concomitant plateau formation of MDA- and alpha-tocopherol levels could also be explained by a mechanism proposed by BOWRY *et al.*, who concluded from the relation between the rate of lipid peroxidation and the alpha-tocopherol content in peroxidizing lipoproteins a strong prooxidant effect of the alpha-tocopheryl radical.[41] The abolishment of the regeneration of the alpha tocopheryl radical

induces the maximal rate of lipid peroxidation by elevated alpha tocopheryl radical levels and the lipid peroxidation stops after depletion of the alpha-tocopherol. This mechanism would be in favour of a localized peroxidation, since a considerable portion of alpha-tocopherol remained.

As shown above (Figure 3,4), the oxidation of the CoQ9 pool likely represents a prerequisite for the start of massive lipid peroxidation and the drastic alpha-tocopherol depletion as well. The protective effect of ubiquinols in mitochondria against oxidative stress has often been demonstrated.[12-14,33,34,37] The mechanism of protection was discussed as an antioxidant action by itself.[12-14,37] Alternatively it was discussed as a donation of electrons to the oxidized alpha-tocopherol.[17-19] In both cases it appears very likely that a reduced rate of regeneration of the mitochondrial antioxidants (alpha-tocopherol, QH_2, GSH) - due to a limitation in the substrate supply, as indicated by the oxidation of CoQ - is the reason for the onset of massive lipid peroxidation, rather than a critical value of 30% depleted alpha-tocopheral.

The presumed causal chain: oxidative load → exhaustion of endogenous substrates → no further regeneration of lipid soluble antioxidants is especially supported by the above shown strong protection of GSH depleted mitochondria under conditions of improved substrate supply.

We earlier demonstrated that the water soluble antioxidant GSH exhibits a biphasic consumption, similar to that shown above for alpha-tocopherol.[9] The dependency on the energetical state of the mitochondria was also established for the GSH system. In this case the heavy GSH consumption was accompanied by the drop of NADPH, which results from the growing inhibition of the respiratory chain during the lag phase and a breakdown of the inner membrane potential. GSH strongly protects the mitochondria against the oxidative stress, as documented by the shortening of the lag phase of MDA formation in the presence of N-ethyl maleimide - a strong SH trapping reagent.[9] Since this SH trapping compound reacts unspecifically with SH groups, which may give rise to an impairment of the respiratory chain,[43] a more specific method to deplete the mitochondrial GSH was applied by addition of dinitrochlorbenzene using the GSH transferase reaction.[30]

The considerable protection of the mitochondria by the lipid soluble antioxidants alone, e.g. in the absence of GSH, and the correlation between the oxidation of the CoQ9 and the start of drastic alpha-tocopherol consumption strongly supports that the lipid soluble antioxidants are effectively recycled by constituents of the respiratory chain. Generally the primary importance of the energetical state resp. supply of reducing equivalents in the defence against oxidative stress in mitochondria is documented.

Obviously the pronounced protective effect of succinate against lipid peroxidation in intact mitochondria is due to an improved recycling of lipid soluble antioxidants, favored by the high levels of ubiquinol, which is adjusted as consequence of the high hydrogen pressure excerted by succinate. It is evident that mitochondria are highly protected against lipid peroxidation in the presence of respiratory substrates, providing reducing equivalents to the respiratory chain, which may effectively regenerate lipid soluble antioxidants.

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