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Effects of oxidative stress on the Dolichol content of isolated rat liver cells

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Abstract
Dolichol, a long-chain polyisoprenoid broadly distributed in all tissues and cellular membranes with unknown function(s), might have a role in free radical metabolism [it accumulates in older tissues and decreases after CCl_4 (in liver) or phenylhydrazine (in spleen and liver) administration]. The effects of the NADPH-ADP-Fe system on Dolichol levels in isolated hepatocytes were explored and the time-course of changes was compared with the release of MDA in the incubation medium and the decrease in CoQ 9 and 10 and Vitamin E levels. Results showed that the system increased lipid peroxidation and decreased Dolichol and CoQ levels in_parallel fashions and lowered Vitamin E levels with shorter latency. Meanwhile, no increase in dead cells and no Dolichol release in the medium were detected. In conclusion, an increase in oxidative stress possibly caused a rapid degradation of dolichol by the same (unknown) mechanism responsible for the breakdown of_Ubiquinone isoprenoid chains.

Keywords: *Dolichol, lipid peroxidation, NADPH-ADP-Fe, rat hepatocytes, Ubiquinone, Vitamin E*

Introduction

Dolichol is a long-chain polyisoprenoid containing 16–23 isoprene units, which may be in the form of a free alcohol or phosphorylated or esterified with a fatty acid. Dolichols are found in all eukaryotic organisms [1–3] and are an essential component of animal tissues [4] broadly distributed in all tissues and cellular membranes. The function of Dolichol is still obscure, but accumulation in older tissues [5,6] and the dramatic Vitamin-E-sensitive decrease in liver after the administration of carbon tetrachloride [7,8] and in spleen and liver after phenylhydrazine [9] may suggest that free-radical metabolism is somehow involved [10].

Lesion(s) and mechanism(s) underlying the accumulation and depletion of Dolichol in tissues have not been clarified yet, mainly because knowledge of the metabolism of Dolichol is quite limited. Synthesis *via* the mevalonate pathway yielding Cholesterol, Ubiquinone and Dolichol was demonstrated *in vitro* and *in vivo* in many tissues [11]; careful examination of Dolichol catabolism in rat liver revealed a half-life from 80–140 h for free Dolichol and $\sim$ 30 h for Dolichyl phosphate (by contrast dolichol turnover in the brain is extremely slow) [12]; in spite of the call for investigation [11], no enzyme pathway for Dolichol degradation has been discovered; identification of catabolism products has still not been completed [13]; and evidence was produced that Dolichol is not lost or exchanged between tissues or transferred to the liver *via* circulation [14].

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It was proposed that the molecules of Dolichol are susceptible to xenobiotic-induced oxidative degradation [8] and may be the preferred target of lipid peroxidation by CCl_4 [15]. In this *in vitro* study, the effects of the NADPH-ADP-Fe system on Dolichol levels were explored and the time-course was followed in detail. Results show that this model of lipid peroxidation (LPO) increased TBARS release and lowered Vitamin E and CoQ levels as already seen with a membrane system such as liposomes or microsomes [16,17] and mitochondria [18] and also with isolated hepatocytes [19,20]; and decreased Dolichol levels in less than 20 min without any increase in the number of dead cells or in the loss of dolichol in the incubation medium.

Materials and methods

Animals

Random groups of male 3-month-old Sprague-Dawley albino rats were maintained on standard laboratory food (Tekland, Harlan, Italy) and water *ad libitum*. Food was withdrawn 16 h before experimentation. Rats had free access to water. At the given age, rats were anaesthetized by an intraperitoneal injection of pentobarbital (50 mg/kg body weight) and liver was taken.

Experimental model

Liver parenchymal cells were isolated by the collagenase method [21]. Cell viability was tested by trypan blue exclusion and initially was at least 90% (Figure 1). Isolated liver cells were incubated with Krebs-Ringer-Hepes Buffer ($1{,}25 \times 10^6$ cells in 2 ml) in absence (control) or presence of a peroxidant solution containing 18 μM $FeSO_4$, 1,6 mM ADP and an NADPH-generating system consisting of 0.18 μM $NADP^+$, 4 mM Glucose-6-Phosphate and Glucose-6-Phosphate Dehydrogenase (0.19 unit/ml) [22] at 37°C for 5, 10 and 20 min. Presence of the peroxidant solution had no significant additional effect decreasing viability (see Figure 1). At the given time, cell suspension was centrifuged and supernatant and cells separated. Supernatant was used to assay the release of TBARS, MDA and Dolichol in the medium and cells were used to study the effects of the peroxidant solution on Dolichol, Ubiquinone and Vitamin E levels.

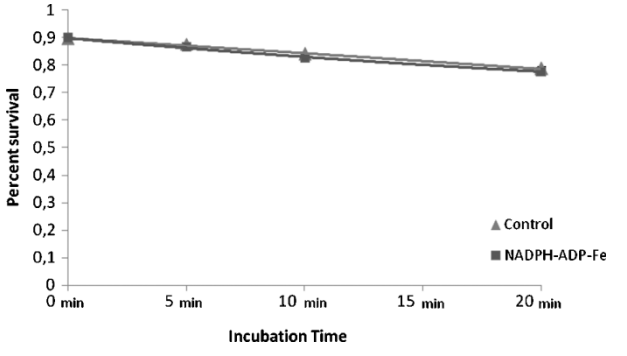

Figure 1. Effects of the peroxidant solution on the viability of liver cells freshly isolated from 3-month-old rats and incubated *in vitro*. On the ordinate: percentage survival; on the abscissa: time of incubation. Means ± SEM of at least six cases are given. The effects of the peroxidant solution on cell survival are not significant.

Quantification of lipid peroxidation

In order to compare effects on lipid peroxidation with published data on isolated liver cells obtained by the same incubation procedure [23], the formation of TBARS was measured from their release in the medium. However, since the reaction is unspecific, particularly in the case of a cellular system, the amount of released Malondialdehyde (MDA), one of the better-known secondary products of lipid peroxidation, was measured in the incubation fluid by the HPLC procedure [24].

Extraction of Dolichol, Ubiquinone and Vitamin E

Dolichol, Ubiquinone and Vitamin E were extracted simultaneously into hexane from a sodium dodecyl sulphate-treated homogenate [25]. Aliquots of the extract were taken for Dolichol, Ubiquinone and Vitamin E assay, dried under nitrogen and redissolved with isopropanol, methanol/reagent alcohol solution and methanol, respectively.

High-pressure liquid chromatography (HPLC) assay

Dolichol was assayed by an HPLC procedure [26]. Ubiquinone was assayed by an HPLC procedure and CoQ 9 and CoQ 10 peaks were fully separated by the use of a methanol/reagent alcohol 4/6 (v/v) solution [25]. Vitamin E was assayed as described by Tirmenstein et al. [27] and Ruperez et al. [28].

Statistical analysis

The analysis of variance (ANOVA) and Fisher test were used to evaluate differences between multiple conditions. Values of $p > 0.05$ were considered not significant.

Results

The incubation *in vitro* of control cells with the isoionic solution caused a small but significant decrease in viability (from 90% down to 80% in 20 min, Figure 1) and did not cause any significant change in the release of MDA in the medium and in the levels of Dolichol and fat-soluble antioxidants in the cells. Treatment with the peroxidant solution had no additional effect on cell viability (Figure 1)

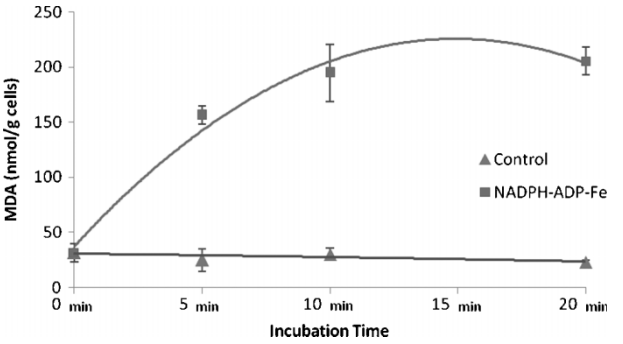

Figure 2. Effects of the peroxidant solution on the release of MDA from the isolated rat liver cells to the incubation medium. On the ordinate: amount of MDA (nmoles) in the incubation medium normalized per g of incubated cells; on the abscissa: time of incubation. Means ± SEM of at least six cases are given. The effects of the NADPH-ADP-Fe system and of the time of incubation were highly significant ($p<0.0001$, $p=0.0007$, respectively). The analysis of the residual variance shows that the interaction between treatments is significant ($p=0.0003$) [42].

Effects of the peroxidant solution on the release of MDA

Treatment with the peroxidant solution increased the release of MDA in the medium significantly (Figure 2). The increase was maximum during the first 5 min of incubation and almost linear for 10 min. Total TBARS release was assayed too and found to be very close to published data [23].

Effects of the peroxidant solution on Dolichol levels

The incubation of isolated liver cells with the peroxidant solution caused a decrease in Dolichol levels which was significant by 20 min (Figure 3). By the end of the incubation, the medium was separated and tested for its Dolichol content and no Dolichol was found.

Effects of the peroxidant solution on Ubiquinone levels

It is well known in the literature that oxidized Coenzyme Q is broken down during lipid peroxidation. The incubation of isolated liver cells with the peroxidant solution caused a decrease in ubiquinone levels. The time-course of the decrease in CoQ 9 and CoQ 10 after exposure to the peroxidant solution is shown in Figures 4 and 5, respectively: in both cases decrease was very small by 5 min and increased thereafter, following a linear pattern throughout incubation. Net changes were significant by 10 min and the temporal patterns were not significantly different than that of dolichol. The presence of the peroxidant solution had no significant effect on the ubiquinone/dolichol molar ratio during incubation (control: time 0 min: 6.4; time 5: 6.4; time 10: 6.1; time 20: 6.5. Treated: time 0: 6.1; time 5: 6.1; time 10: 5.7) with the possible exception of 20 min (molar ratio: 7.4).

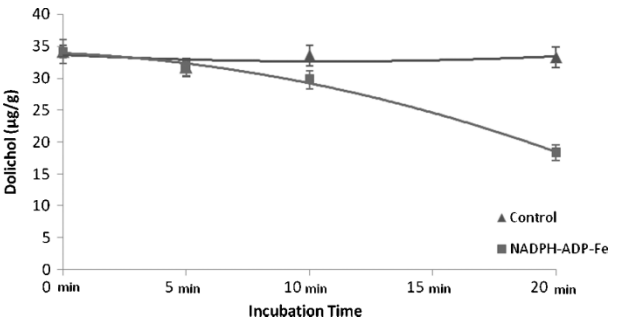

Figure 3. Effects of the peroxidant solution on Dolichol levels in isolated liver cells of 3-month-old rats incubated *in vitro*. On the ordinate: µg Dolichol/g packed cells; on the abscissa: time of incubation. Means ± SEM of at least six cases are given. The effects of the NADPH-ADP-Fe and of time of incubation were highly significant ($p<0.0002$). The analysis of the residual variance shows that the interaction between treatments is significant ($p<0.0002$) [42].

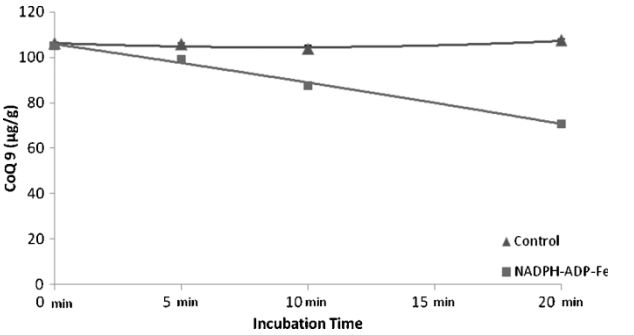

Figure 4. Effects of the peroxidant solution on CoQ 9 levels in isolated liver cells of 3-month-old rats incubated *in vitro*. On the ordinate: µg CoQ 9/g packed cells; on the abscissa: time of incubation. Means ± SEM of at least six cases are given. The effects of the NADPH-ADP-Fe and of time of incubation were highly significant ($p<0.0001$). The analysis of the residual variance shows that the interaction between treatments is significant ($p<0.0001$) [42].

Effects of the peroxidant solution on Vitamin E levels

Figure 6 shows that the incubation of isolated liver cells with the peroxidant solution caused an early,

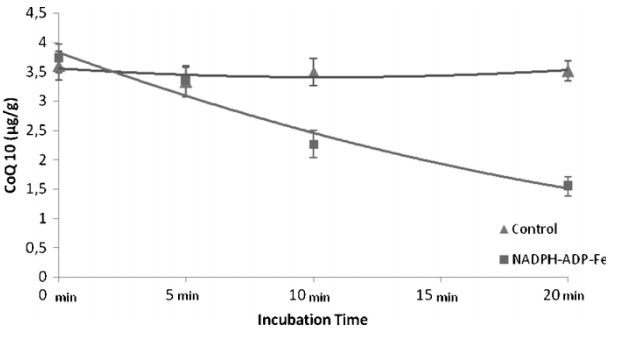

Figure 5. Effects of the peroxidant solution on CoQ 10 levels in isolated liver cells of 3-month-old rats incubated *in vitro*. On the ordinate: µg CoQ 10/g packed cells; on the abscissa: time of incubation. Means ± SEM of at least six cases are given. The effects of the NADPH-ADP-Fe and of time of incubation were highly significant ($p<0.0001$). The analysis of the residual variance shows that the interaction between treatments is significant ($p<0.0001$) [42].

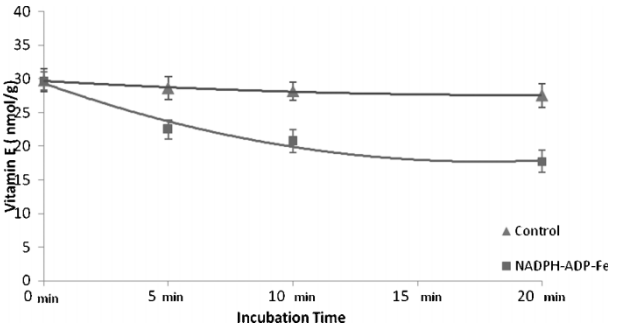

Figure 6. Effects of the peroxidant solution on the Vitamin E levels in isolated liver cells of 3-month-old rats incubated *in vitro*. On the ordinate: nmoles Vitamin E/g packed cells; on the abscissa: time of incubation. Means ± SEM of at least six cases are given. The effects of the NADPH-ADP-Fe system and of the time of incubation were significant ($p<0.0001$ and $p=0.0056$, respectively). The analysis of the residual variance shows that the interaction between treatments is not significant [42].

highly significant decrease in the content of vitamin E: ~ 24% of the α-tocopherol in liver cells was lost during the first 5 min of incubation with the peroxidant solution. Decrease was 30% by 10 min and 40% by 20 min.

Discussion

During evolution, exposure to free radicals made eucariotic cells develop a series of antioxidant mechanisms. Vitamin E and Ubiquinols are examples of relatively small-molecule natural fat-soluble antioxidants, which protect membrane lipids from oxidative damage [29]. The tocopherol molecule is a vitamin consisting of two function domains: a C_{16} hydrocarbon chain, which is responsible for the lipophilicity of the molecule [30], and a chromanol head group, which is responsible for the antioxidant activity [29]. In the latter capacity, the chromanol moiety functions as a chain-breaking antioxidant terminating radical chain by donating a hydrogen atom [31]. Ubiquinone is an endogenous isoprenoid lipid with a polar quinone ring and two major functions: an electron carrier in the mitochondrial respiratory chain and a lipid soluble antioxidant. Dolichol, a polyprenol molecule co-evoluted with ubiquinone, is said to have no known function or to have a role in the antioxidant machinery of cells [13].

In this research, attention was focused on the effects of oxidative stress on Dolichol. The temporal pattern of Dolichol consumption was monitored in detail during peroxidation as compared with those of known lipid soluble antioxidants. The ADP-Fe and NADPH-generating system was used to generate free radicals from the hydrophilic, extracellular environment, unlike the case of the CCl_4 model. The effects of treatment on lipid peroxidation were highly significant: TBARS and MDA release in the medium increased with the incubation time in a linear fashion for 10 min, in agreement with published data [19,23]. Data show that the increase in the oxidative stress by the NADPH-ADP-Fe system is associated with a highly significant (almost 50%) decrease in the Dolichol content of isolated liver cells in less than 20 min; an almost simultaneous, parallel loss of Coenzyme Q9 and Q10; and a loss of Vitamin E, which was seen earlier than the loss of the former lipids. The loss of Dolichol is very impressive but may not be surprising: an even bigger decrease in liver-cell Dolichol was observed with CCl_4 poisoning and latency was shorter (less than 5–10 min), suggesting that Dolichol could be a preferred target of oxidative stress [32].

The decrease in Dolichol, Coenzyme Q9 and Q10 and Vitamin E was not associated with an increase in cell death. By the end of incubation no detectable amount of Dolichol was found in the medium. It seems quite unlikely that such a dramatic and sudden depletion in this lipid could be justified by a decrease in the rate of Dolichol synthesis without an increase in the rate of degradation which should be at least 100-fold high (the half-life of Dolichol in the liver of young Sprague Dawley rats was measured and found to be ~ 100 h [33].

How Dolichol degradation occurs in tissues is still an unanswered question. Here, loss of Dolichol was similar and almost simultaneous with the loss of liver cell Ubiquinone. It is known in the literature that during lipid peroxidation oxidized Coenzyme Q is broken down. The half-life of rat liver ubiquinone was studied in detail under physiological conditions

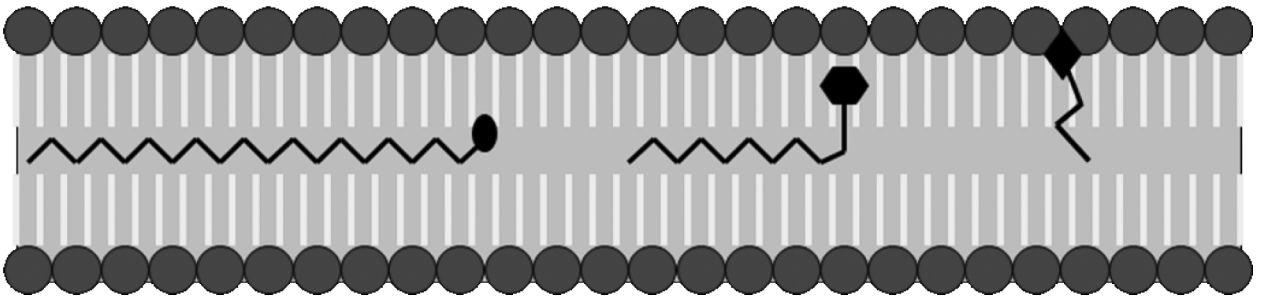

Figure 7. Proposed location of dolichol and lipophilic antioxidants in membranes. Locations of Dolichol (●), Ubiquinone (⬣) and Vitamin E (◆) [29,33].

Table I. Percentage decrease in fat-soluble antioxidants of isolated rat liver cells incubated either with NADPH-ADP-Fe (see materials and methods) or with 25 μl of CCl4 (as described by Parentini [6]) for 0, 5, 10 and 20 min (*$p<0.05$).

	Incubation time					
	5′		10′		20′	
	NADPH ADP-Fe	CCl4 (25 μl)	NADPH ADP-Fe	CCl4 (25 μl)	NADPH ADP-Fe	CCl4 (25 μl)
Vitamin E	24.2%*	0.9%	30.3%*	3.2%	40.3%*	3.2%
CoQ 9	5.7%*	–	16.7%*	–	32.7%*	–
CoQ 10	10.4%	–	39.3%*	–	68.6%*	–
Dolichol	8.0%	15.8%*	12.9%	36.5%*	46.4%*	59.5%*

and was found to be in the same range as Dolichol. Only two studies have been published on Ubiquinone catabolism and both have been performed with the assumption that exogenously supplied ubiquinone mimics endogenous Ubiquinone catabolism [34,35]. Two major compounds were identified in both urine and faeces, having an intact quinone ring (disulphated and glucuronidated) and a drastically shortened side chain. The sequence of the reactions leading to the breakdown of_Ubiquinone isoprenoid chains as well as the products of the decomposition are unknown [33]; an involvement of the same unknown mechanism(s) in the parallel, almost simultaneous disappearance of Dolichol's isoprenoid chains seems to be an attractive hypothesis which cannot be disproved at present.

Data show that the addition of the NADPH-ADP-Fe peroxidant system accelerated the rate of both Dolichol and Ubiquinone degradation almost simultaneously to a very similar extent. Ubiquinone is said to be involved in free radical metabolism being the only endogenously synthesized lipid with a redox function in mammals and a lipid-soluble antioxidant [36]. Dolichol was said not to be an antioxidant, based on the evidence that reconstitution of n-pentane-extracted rat liver microsome and mitochondria with dolichols of different length did not decrease lipid peroxidation [37]. However, no evidence was given that reconstituted Dolichols had been relocated inside the bilayer in the appropriate site and authors mentioned that the reconstituted Dolichol could negatively interact or not with the antioxidant activity of reconstituted Ubiquinol. More recently, an NMR study showed that Dolichol can slow down the rate of photosensitized peroxidation of methyl linoleate [38]. It has been shown that dolichol can undergo photodegradation very rapidly (in minutes) [39]. No enzymatic or other mechanism were found or identified in cells that are capable of reducing an 'oxidized form' of Dolichol. Without regeneration, antioxidant capacity is limited by the rate of antioxidant replacement, which in the case of Dolichol might be very low (but the rate of Dolichol synthesis might be enhanced by an increase in oxidative stress [40]). In conclusion, the question if Dolichol may act in free radical metabolism as an antioxidant is still an open matter. What is important, however, is that increasing evidence shows that any increase in oxidative stress both *in vitro* (by the addition of CCl_4 and xenobiotics, NADPH-ADP-Fe or by UV radiation) and *in vivo* (by the administration of CCl_4 or xenobiotics or phenylhydrazine) is coupled with a simultaneous dose and time dependent decrease in cell Dolichol. A new cellular function has been attributed to Dolichol, namely putative protection of cellular membranes against peroxidation. According to this suggestion the Dolichol chain might interact with PUFA and Ubiquinol (but not perhaps with Vitamin E) to form a highly-matched free-radical transfer chain from the lipophylic space in membranes to cytosol and constitute a shield against reactive oxygen species (ROS) generated either by UV light, chemicals or accumulated upon ageing [13]. Free radicals are very short lived and successful neutralization in membranes appears to be too good to be accounted for by a random diffusion of fat-soluble antioxidants. Available models (Figure 7); [29,33]) show that localization in membranes may enable each molecule of Dolichol to lie very close to (and possibly interact with) hundreds of phospholipid molecules (and twice as many fatty acid molecules) and with several molecules of CoQ (but not with Vitamin E); and might affect the exposure of the antioxidants to the free-radical attack in different ways, according to whether it is generated inside or outside of the membrane (see Table I). A possible protective role of Dolichol during lipid peroxidation in Parkinson's disease has also been postulated [41].

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