Evaluation of α -tocopherol antioxidant activity in microsomal lipid peroxidation as detected by low-level chemiluminescence

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The significance of microsomal vitamin E in protecting against the free-radical process of lipid peroxidation was evaluated with the low-level-chemiluminescence technique in microsomal fractions from vitamin E-deficient and control rats. The induction period that normally precedes the ascorbate/ADP/Fe³⁺-induced lipid peroxidation was taken as reflecting the microsomal vitamin E content and was found to be 5–6-fold decreased in microsomal fractions from vitamin E-deficient rats. Supplementation of microsomal fractions from vitamin E-deficient rats with exogenous vitamin E partially restores the induction period observed in that from control rats. The decrease in chemiluminescence intensity and the increase in the induction period both correlate linearly with the amount of vitamin E added. However, the efficiency of exogenous vitamin E is about 50-fold lower than that exerted by the naturally occurring vitamin E in microsomal membranes. These observations are discussed in terms of the process of re-incorporation of vitamin E into membranes, the experimental model for lipid peroxidation selected, and the method to evaluate lipid peroxidation, namely low-level chemiluminescence.

The fundamental biological role of vitamin E (α -tocopherol) has been discussed recently (Burton *et al.*, 1983a; Diplock, 1983; Willson, 1983, 1984), and it seems to depend primarily on its reactivity towards peroxy radicals. Chromanoxo radicals are formed, as expected from mechanistic considerations of vitamin-E-free-radical interactions (Simic, 1981; Forni & Willson, 1983); vitamin C could play an important biological role by participating in the recovery of vitamin E by reducing the chromanoxo radicals (Packer *et al.*, 1978). In addition to the antioxidant activity displayed by vitamin E in model systems *in vitro*, there is ample evidence for its antioxidant activity *in vivo* (Tappel, 1980). Recently it was demonstrated that vitamin E functions as the major lipid-soluble chain-breaking antioxidant in human blood (Burton *et al.*, 1983a,b).

The aim of the present study is to evaluate the significance of microsomal vitamin E in protecting against the free-radical process of lipid peroxidation. This protection might be expressed by the occurrence of the induction period that normally precedes lipid peroxidation. We have used the low-level-chemiluminescence method to estimate this induction period; the method is particularly useful because, as far as this experimental model is

concerned, it (a) closely reflects lipid peroxidation (Cadenas *et al.*, 1984) and (b) provides a continuous monitoring of oxidative reactions (Boveris *et al.*, 1981). This approach was taken with microsomal fractions (referred to below simply as microsomes) from vitamin E-deficient rats (as such and supplemented with external vitamin E) and from control rats.

Materials and methods

Chemicals

Vitamin E was purchased from Sigma Chemie G.m.b.H. (Taufkirchen, West Germany). Biochemicals were from Boehringer (Mannheim, West Germany) and chemicals from Merck (Darmstadt, West Germany).

Biological materials

Rat liver microsomes from control and vitamin E-deficient rats were prepared as previously described (Cadenas & Sies, 1982). Vitamin E-deficient rats were fed on a vitamin E-deficient diet (Altromin G.m.b.H., Lage, West Germany) for 14 weeks. Vitamin E contents of microsomes from control and vitamin E-deficient rats were

0.38 ± 0.08 and 0.07 ± 0.03 nmol/mg of protein, respectively. Values for control microsomes are in agreement with previous reports (Taylor *et al.*, 1976; Kornbrust & Mavis, 1980).

Addition of vitamin E to microsomes from vitamin E-deficient rats was carried out as follows: 5 μ l of a vitamin E solution in ethanol (different concentrations) was added to the microsomal suspension (approx. 10.5 mg of protein) and stirred with a vortex mixer for 60 s; longer mixing periods did not increase significantly the incorporation of vitamin E. This treatment was followed by dilution of the microsomal suspension with 0.1 M-potassium phosphate buffer, pH 7.4, to a final concentration of 1.5 mg of protein/ml. Measurement of vitamin E in the 105 000 g pellet from the above suspension showed that the vitamin was totally associated with the membranes; added vitamin E was not detectable in the 105 000 g supernatant.

Spectrophotometric assays

Vitamin E concentration of the ethanol suspensions was determined by measuring the absorption difference $A_{292} - A_{323}$ ($\Delta\epsilon = 3.47 \text{ mm}^{-1} \cdot \text{cm}^{-1}$ in ethanol; Weast, 1975). A dual-wavelength spectrophotometer (Sigma Instruments model ZW-11; Biochem Co., Munich, West Germany) was used for this purpose.

Analysis of vitamin E in microsomes was performed by the method of Taylor *et al.* (1976), consisting of saponification of the microsomal suspension in the presence of antioxidants and lipid extraction with hexane, followed by spectrofluorimetric measurement of the tocopherol at 326 nm with an Aminco-Bowman spectrofluorimeter (excitation wavelength 289 nm); fluorescence interference by vitamin A was eliminated by H_2SO_4 treatment of the samples (see Taylor *et al.*, 1976). The concentration of vitamin E was calculated from the fluorescence intensity against a standard curve ranging from 2 μM - to 20 μM -vitamin E. Extracted samples were also assayed spectrophotometrically at $A_{292} - A_{323}$ with the dual-wavelength spectrophotometer described above.

Low-level chemiluminescence

Low-level chemiluminescence was measured with a single-photon counter as described by Boveris *et al.* (1981) and Cadenas & Sies (1984), with a red-sensitive photomultiplier (EMI 9658; EMI-Gencom, Plainview, NY, U.S.A.). Chemiluminescence measurements were carried out in a 35 mm $\times$ 5 mm $\times$ 56 mm glass cuvette provided with a lid with ports for constant oxygenation and a motor-driven magnetic stirrer, which assured a homogeneous microsomal suspension and mixing.

Assay conditions

Lipid-peroxidation measurements were carried out in a system consisting of 0.1 M-potassium phosphate buffer, pH 7.4, containing 2 mM-ADP, 16 μM - FeSO_4 and 1.5 mg of microsomal protein/ml. The reaction was initiated by addition of 0.5 mM-ascorbate to the assay mixture. The temperature was kept constant at 37°C.

Results and discussion

The $\text{O}_2/\text{Fe}^{3+}$ -dependent peroxidation in microsomes is accompanied by the formation of excited states, some of which happen to be chemiluminescent. The generation of these excited states seems to be a consequence of free-radical interactions, e.g. the self-reaction of secondary lipid peroxy radicals (Sugioka & Nakano, 1976). The rise in chemiluminescence intensity from peroxidizing microsomes is preceded by a lag phase or induction period (τ) (Bartoli *et al.*, 1983), which is thought to reflect the presence of antioxidant defences. These antioxidant defences have to be overcome in order to observe lipid peroxidation, and thereby chemiluminescence. This lag period was previously observed in tissue homogenates (Cadenas *et al.*, 1981a) and isolated hepatocytes (Cadenas *et al.*, 1981b), and its duration differs in different tissues (Cadenas *et al.*, 1981a), presumably depending on the vitamin E/polyunsaturated fatty acids ratio.

Since the presence of the induction period of lipid peroxidation could be related to the occurrence of endogenous antioxidants such as vitamin E, it was decided to determine its characteristics in microsomes from control and vitamin E-deficient rats. Fig. 1(a) shows that the induction period (τ_c) of microsomes from control rats is much longer than that of microsomes from vitamin E-deficient rats (τ_E); the τ_c/τ_E -ratio with several microsomal preparations is about 5.7 ± 0.2 . These different values of τ are in close agreement with the vitamin E content of both types of microsomes, 0.38 ± 0.08 and 0.07 ± 0.03 nmol/mg of protein for those from control and vitamin E-deficient rats respectively. In addition, both the rate of increase of chemiluminescence intensity and the maximal chemiluminescence intensity are higher in microsomes from vitamin E-deficient rats (6700 ± 1200 counts/s per min and $105\,000 \pm 5500$ counts/s respectively) than in microsomes from control rats (4050 ± 450 counts/s per min and $76\,500 \pm 4200$ counts/s respectively). These observations suggest a relationship between the resistance of microsomes to lipid peroxidation (determined by the duration of their induction period) and the occurrence of vitamin E. The induction period of microsomes from vitamin E-deficient rats can be

Fig. 1. Low-level chemiluminescence and induction period from ascorbate/Fe-induced lipid peroxidation by microsomes from control and vitamin E-deficient rats

(a) Liver microsomes from control and vitamin E-deficient rats were incubated as described in the Materials and methods section. The chemiluminescence reaction was started by addition of 0.5 mM-ascorbate; the incubation mixture was gassed with O_2 during the reaction period. τ_E and τ_C are the induction periods for microsomes from vitamin E-deficient and control rats respectively. (b) As in (a), but microsomes from vitamin E-deficient rats and vitamin E-deficient rats supplemented with exogenous vitamin E are compared. τ_E is the induction period for microsomes from vitamin E-deficient rats; τ is the induction period of vitamin E-supplemented microsomes from vitamin E-deficient rats.

prolonged by supplementing the microsomes with exogenous vitamin E (Fig. 1b).

The dependence of chemiluminescence intensity and induction period on vitamin E content is shown in Fig. 2. The induction period is expressed as τ/τ_E , a relative value that consists of the ratio between the induction period in the presence of vitamin E and the induction period in the absence of the added antioxidant. τ/τ_E would reflect the antioxidant efficiency of externally added vitamin E in the frame of microsomal membranes against free radicals generated during the ascorbate-supported reduction of Fe^{3+} . Assuming that the occurrence of the induction period is a direct consequence of vitamin E concentration [as suggested for a different experimental model by Burton *et al.* (1983b)], the τ/τ_E ratio illustrated in Fig. 2 would be expressed by the following equation:

$$\tau/\tau_E = 1 + K[\text{vitamin E}]$$

the slope of which is $0.19 \text{ (nmol of vitamin E/mg of protein)}^{-1}$, resulting in a calculated K value of about $0.13 \times 10^6 M^{-1}$. The K value might be of

Fig. 2. Effect of added vitamin E on chemiluminescence intensity and τ/τ_E ratio for microsomes from vitamin E-deficient rats

Assay conditions were as described in the Materials and methods section and for (b) in Fig. 1 legend. Chemiluminescence intensity (●) is the peak height (highest chemiluminescence) observed during the time course of the reaction. ○, τ/τ_E .

interest for comparison of the antioxidant efficiency of vitamin E in preventing or 'delaying' lipid peroxidation in experimental models other than the non-enzymic (ascorbate-dependent) lipid peroxidation studied here.

The evaluation of these observations requires consideration of (a) the technique utilized to incorporate vitamin E into microsomal membranes, (b) the selected experimental model to induce lipid peroxidation, and (c) the method utilized to assess the extent of lipid peroxidation, in this case low-level chemiluminescence.

Although added vitamin E associates totally with the microsomal membrane (see the Materials and methods section), this incorporation does not seem to occur exclusively at specific places that would make the tocopherol a more efficient antioxidant. The occurrence of specific binding sites for vitamin E in membranes of erythrocytes (Wimalasena *et al.*, 1982) and adrenocortical-cell membranes (Kitabchi *et al.*, 1980) suggests that a physiologically relevant activity of vitamin E must be associated with its binding to specific places in the membrane. However, positive effects, as judged by prolonged induction periods, were already observed in the low range of a few nmol of vitamin E/mg of protein. The content of naturally occurring tocopherol in microsomal membranes (0.38 ± 0.08 nmol/mg of protein) can induce a 5–6-fold increase of the induction period over that from microsomes from vitamin E-deficient rats (Fig. 1a). In order to observe a similar increase of the value with externally added vitamin E, a 50-fold larger amount of vitamin E is needed (calculated from extrapolation from data of Fig. 2). This lower antioxidant efficiency of exogenous vitamin E is illustrated in Fig. 1(b), where added tocopherol at a concentration of 3.6 nmol/mg of protein (9.5-fold higher concentration than in control microsomes) gives a 2.4-fold increase of the induction period. The lower efficiency of exogenous vitamin E might imply either that the added vitamin is not properly incorporated into specific sites where it could develop its activity, or that a fraction of tocopherol might be oxidized by the Fe^{3+} contained in the buffer under the experimental conditions. The last possibility, however, does not seem to apply because no oxidation of vitamin E was observed upon its incubation with Fe^{3+} , both reactants being present in similar molar concentrations.

The antioxidant efficiency exerted by vitamin E *in vitro* (Figs. 1 and 2) applies to the selected method of inducing lipid peroxidation, i.e. the non-enzymically induced lipid peroxidation. Since the free-radical protection exerted by some antioxidants is known to change with different models of lipid peroxidation (Willson, 1983), it is to be expected that the *K* value described in the above

equation will also be dependent on the experimental model used. It might be of interest to evaluate the antioxidant activity of vitamin E during NADPH-induced lipid peroxidation (enzymically mediated), free-radical metabolism of toxic chemicals such as CCl_4 , and cytochrome *P*-450-mediated oxidative breakdown of organic hydroperoxides.

Finally, the method utilized in the present work to evaluate lipid peroxidation, namely low-level chemiluminescence, is particularly suitable, owing to its characteristic of continuous monitoring, for measurement of the induction periods that precede the propagation of lipid peroxidation.

The detection of low-level chemiluminescence was regarded in the present context as reflecting lipid peroxidation, and no special attention was paid to the excited species formed during the process [study carried out by Sugioka & Nakano (1976)]. Also, the possibility of the reaction of α -tocopherol with singlet molecular oxygen, which is known to proceed with a rate constant of about $10^8 \text{ M}^{-1} \cdot \text{s}^{-1}$ (Grams & Eskins, 1972; Foote, 1976), was not considered either. However, the generation of excited species during lipid peroxidation might be associated with membrane damage, which only occurs at a later stage, probably when vitamin E is already exhausted in the quenching of free-radical reactions.

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At the time this manuscript was accepted, a paper appeared (Hill & Burk, 1984) also providing evidence on the dependence of the lag phase of lipid peroxidation on vitamin E content of microsomes.

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